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Mkh1, a MEK Kinase Required for Cell Wall Integrity and Proper Response to Osmotic and Temperature Stress in *Schizosaccharomyces pombe*

AMEET S. SENGAR, NANCY A. MARKLEY, NICHOLAS J. MARINI, AND DALLAN YOUNG*

Department of Medical Biochemistry, University of Calgary Health Science Centre, Calgary, Alberta T2N 4N1, Canada

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We have identified a *Schizosaccharomyces pombe* gene, *mkh1*, that encodes a MEK kinase (MEKK) homolog. The coding region of *mkh1* is contained within a single exon encoding a 1,116-amino-acid protein. The putative catalytic domain of Mkh1 is 54% identical to the catalytic domain of *S. cerevisiae* Bck1, the most closely related protein. Deletion of *mkh1* did not significantly affect cell growth or division under standard conditions. However, *mkh1*Δ cell growth was inhibited by high KCl or NaCl concentrations. *mkh1*Δ cells required a longer time to reenter the cell cycle after prolonged stationary-phase arrest. Also, *mkh1*Δ cells exhibited a round cell shape, while overexpression of Mkh1 resulted in an elongated cell shape. *mkh1*Δ cells exhibited a more dramatic phenotype when grown in nutrient-limiting conditions at high temperature or in hyperosmotic medium. In such conditions, completion of cytokinesis was inhibited, resulting in the growth of pseudohyphal filaments with multiple septa and nuclei. Also, *mkh1*Δ cells were hypersensitive to β-glucanase treatment. Together these results suggest that Mkh1 regulates cell morphology, cell wall integrity, salt resistance, cell cycle reentry from stationary-phase arrest, and filamentous growth in response to stress. These phenotypes are essentially identical to those exhibited by cells lacking Pmk1/Spm1, a recently identified mitogen-activated protein kinase. Our evidence suggests that Pmk1/Spm1 acts downstream from Mkh1 in a common pathway. Our results also suggest that Mkh1 and Pck2 act independently to maintain cell wall integrity, cell morphology, and salt resistance but act in opposition to regulate filamentous growth.

Mitogen-activated protein (MAP) kinase cascades constitute a fundamental regulatory mechanism that has been conserved among eucaryotic organisms, including yeasts and mammals. Such cascades consist of a trio of sequentially acting protein kinases: MEK kinases (MEKKs), which phosphorylate and activate MAP kinase kinases (MAPKKs or MEKs), which in turn phosphorylate and activate MAP kinases (MAPKs) (reviewed by Seger and Krebs [47]). These cascades are involved in mediating a variety of cellular responses, including cell growth, differentiation, and responses to various stress conditions. In *Saccharomyces cerevisiae*, several distinct MAPK pathways have been defined (reviewed by Herskowitz [14] and Levin and Errede [21]). The mating pheromone response pathway is perhaps the best-characterized MAPK pathway in this yeast. This response is transmitted by a MAPK cascade consisting of Ste11 (MEKK), Ste7 (MEK), and Kss1 and Fus3 (MAPKs) (4, 7, 12, 36, 50, 60). In addition to mediating the mating response, Ste11, Ste7 and Kss1 (but not Fus3) are required for pseudohyphal and invasive growth in response to nutritional starvation (41). Thus, a single MAPK cascade can specify two different developmental responses. A distinct pathway involving Hog1 (MAPK) regulates intracellular glycerol concentrations in response to hyperosmotic conditions (3). In contrast, the Bck1 (MEKK) pathway is activated in response to either hypotonic shock or heat (6, 15). Loss of Bck1 results in a temperature-sensitive cell lysis defect which is suppressed by osmotic stabilizing agents and has been attributed to a deficiency in cell wall construction (20, 22, 24, 39). Genetic and biochemical experiments indicate that Bck1 is activated by Pkc1, the *S. cerevisiae* protein kinase C (PKC) homolog (8, 24). Another *S. cerevisiae* pathway that appears to regulate sporulation involves Smk1 (MAPK) and Sps1, a STE20 homolog (10, 18).

The existence of several different MAPK pathways in *S. cerevisiae* and mammals suggests that other organisms may have similar MAPK pathways that mediate various cellular responses. We are interested in defining such pathways in the fission yeast *Schizosaccharomyces pombe*. Previous studies have identified components of three distinct MAPK cascades in fission yeast. The Ras1-activated pathway, which is required for conjugation and sporulation, includes Byr2 (MEKK), Byr1 (MEK), and Spk1 (MAPK) (34, 35, 37, 51, 54). This MAPK cascade is structurally and functionally conserved with the mating pheromone MAPK cascade of *S. cerevisiae* (37). A distinct MAPK pathway in fission yeast involving Wis1 (MEK) and Sty1/Spc1 (MAPK) regulates intracellular osmotic pressure and initiation of mitosis in response to osmotic and nutritional signals, and this pathway appears to be functionally related to the *S. cerevisiae* Hog1 pathway (32, 48, 49, 55). The MEKK that regulates Wis1 has not yet been reported. Very recently a novel MAPK, Pmk1/Spm1, that is most closely related to *S. cerevisiae* Mpk1, a component of the Bck1 cascade, was identified (53, 59). Pmk1/Spm1 is required for normal cell wall integrity, cell shape, cytokinesis, and response to hyperosmotic stress.

Here we report the identification of a gene, *mkh1*, encoding a MEKK homolog that is most closely related to Bck1. Mkh1 appears to be required for cellular responses identical to those mediated by Pmk1/Spm1, suggesting that these proteins function in a common MAPK pathway. We have performed genetic studies to further investigate the relationship of Mkh1 with Pmk1/Spm1. We also present evidence that this pathway is related to but distinct from that mediated by Pck1 and Pck2, the *S. pombe* PKC homologs.

* Corresponding author. Phone: (403) 220-3030. Fax: (403) 283-8727.

TABLE 1. *S. pombe* strains

Strain	Genotype	Source or reference
SP826	h^+/h^+ *leu1-32/leu1-32 ade6-210/ade6-216 ura4-D18/ura4-D18*	D. Beach, Cold Spring Harbor Laboratory
SP870	h^{90} *leu1-32 ade6-210 ura4-D18*	D. Beach
AS1	h^{90} *leu1-32 ade6-210 ura4-D18 mkh1::ura4*	This study
AS2	h^{90} *leu1-32 ade6-210 ura4-D18*	This study
AS7	h^{90} *leu1-32 ade6-210*	This study
TP47-2B	h^- *leu1-32 pck2-8*	52
TP169-1C	h^- *leu1-32 ura4-D18 pck2::LEU2*	52
AS13	h^- *leu1-32 ura4-D18 pck2::LEU2 mkh1::ura4*	This study
TZS69	h^- *leu1-32 ura4-D18 spm1::LEU2*	59
AS14	h^- *leu1-32 ade6-210 ura4-D18 spm1::LEU2 mkh1::ura4*	This study
ED904	h^+ *leu1-32 wis1::LEU2*	P. Fantes, University of Edinburgh
AS15	h^+ *leu1-32 ura4-D18 wis1::LEU2 mkh1::ura4*	This study

MATERIALS AND METHODS

Yeast strains and genetic analysis. The genotypes of *S. pombe* strains used in this study are listed in Table 1. Yeast culture, transformation, iodine staining, mating, tetrad analysis, and other genetic manipulations were performed as previously described (33).

DNA manipulation and analysis. Procedures used for DNA manipulation and analysis (purification, restriction site mapping, electrophoresis, transformation, etc.) have been previously described (45). The DNA sequences of both strands of sequenced clones were determined by a modified dideoxy chain-terminating method (46), using a Taq DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer).

PCR. The PCR method was used to amplify DNA fragments from an *S. pombe* cDNA library, using the degenerate oligonucleotides 5′TTGCGGCCGCATGG C[N]GT[N]AA[AG]CA[AG]GT and 5′TTGCGGCCGCTC[N]GG[N][GC][CA T]CATCCA[AG]AA as primers. Construction of the *S. pombe* cDNA library has been previously described (16, 28). Reaction mixtures (100 μl) containing 100 pmol of each primer and 1 μg of *S. pombe* cDNA library were incubated for 30 cycles (1 min at 94°C, 2 min at 50°C, and 1 min at 72°C) on a Perkin-Elmer Cetus 4800 DNA ThermoCycler as previously described (27). The 450-bp PCR product was purified and cloned into the *Not*I site of pBluescript II SK− (Stratagene).

Plasmids. pMKH1 contains a 7.3-kb *Spe*I fragment, which includes the entire coding region of *mkh1*, cloned in pBluescript II SK− (Stratagene), as described in Results. pUMKΔ1 was derived from pMKH1 by replacing the 1.5-kb *Bam*HI-*Sph*I fragment encoding the catalytic domain of Mkh1 with a 1.8-kb fragment containing *ura4*. pAALN is an *S. pombe* expression vector containing *LEU2*, the *ars1* sequence, and *adh1* promoter sequence flanking a polylinker site (56, 58). pLMK1 contains the coding sequence of *mkh1* cloned into the *Bam*HI-*Sac*I sites of pAALN. pREP1, pREP3, and pREP41X are *S. pombe* expression vectors containing the repressible *nmt1* promoter (2, 29). pR4L-GSPM1 contains the coding sequence for a glutathione *S*-transferase (GST)–Spm1 fusion in pREP-41X (59). pREP1-Wis1 and pREP1-GSTSpc1 contain the coding sequences for Wis1 and a GST-Spc1 fusion in pREP1, respectively (49). pR3L-PCK2 was constructed by inserting the coding sequence of Pck2 into the *Sal*I-*Bam*HI sites of pREP3. pCK11-2 and pPCK2-2B are multicopy plasmids containing the *pck1* and *pck2* genes, respectively (52).

Gene disruption. The *mkh1* gene was disrupted in the *S. pombe* haploid strain SP870 and the diploid strain SP826 by the gene replacement method (43, 44). SP826 was transformed with the 6.8-kb *Bss*HII fragment of pUMKΔ1, in which the catalytic domain of *mkh1* has been replaced with *ura4*, and Ura^+ transformants were selected on phorbol myristate acetate (PMA)-Leu medium. Six independent transformants were tested for stability of the Ura^+ phenotype, and they were analyzed by Southern blotting to confirm that they contained the proper disruption in one copy of the endogenous *mkh1* gene. h^{90}/h^{+n} revertants of these strains, which occur at a frequency of approximately 10^{-3}, were detected by the iodine vapor staining test. The haploid strains AS1 (Ura^+) and AS2 (Ura^-) were derived from spores of a single ascus from one such revertant. AS7 (Ura^+) was derived from AS2 by replacing the *ura4*-d18 allele with the wild-type *ura4* allele.

Fluorescent staining. Cells were fixed in phosphate-buffered saline (PBS) containing 3.7% formaldehyde for 60 min at 37°C, washed twice in PBS, stained with calcofluor (1 μg/ml; Sigma), washed in PBS, stained with 4′,6-diamidino-2-phenylindole (DAPI; 10 μg/ml; Sigma) or Hoechst 33258 (20 ng/ml; Aldrich), and examined by fluorescence microscopy, using a UV filter (1).

Glucanase sensitivity and cell wall regeneration experiments. Glucanase sensitivities of various mutant strains were determined by using a previously described procedure (23). Cells were grown in PMA to an optical density at 600 nm (OD_{600}) of 1.0, washed in 10 mM Tris (pH 7.5)–1 mM EDTA–1 mM 2-mercaptoethanol, and incubated in the same buffer containing 100 μg of β-glucanase (Zymolyase-20T; ICN) per ml at 30°C with vigorous shaking. Cell lysis was monitored by measuring OD_{600}. In independent experiments, cell viability was correlated and found to be proportional to OD_{600}.

Cell wall regeneration was also determined by a previously described procedure (17). Strains were grown in PMA to an OD_{600} of 1.0. Cells were washed in 50 mM sodium citrate–100 mM sodium phosphate (pH 6.0) and incubated for 1 h at room temperature in the same buffer containing 1.2 M sorbitol and 5 mg of β-glucanase (Zymolyase-20T; ICN) per ml. Cells were washed in the same buffer without β-glucanase and divided into two equal aliquots. One aliquot was incubated in PMA (nonregeneration medium) and the other was incubated in PMA–1.2 M sorbitol (regeneration medium) for 15 h at 30°C with shaking. After incubation, cells were lysed by resuspension in H_2O, plated on PMA plates, and incubated for 3 to 5 days at 30°C.

Nucleotide sequence accession number. The nucleotide sequence of *mkh1* has been submitted to GenBank (accession no. U53872).

RESULTS

Cloning of the *S. pombe mkh1* gene. Yeast and mammalian MEKKs have significant homology in their catalytic domains. To identify MEKK homologs in *S. pombe*, we designed degenerate oligonucleotides encoding short peptides that are highly conserved and characteristic of MEKK catalytic domains. These oligonucleotides were used as primers to amplify DNA by PCR from a *S. pombe* cDNA library (see Materials and Methods). The 450-bp PCR product was purified and cloned, and the DNA sequences of seven independent clones were determined. As predicted, some of the clones contained inserts that were derived from *byr2*. However, one clone, pM3K4b, encodes a peptide that has strong homology to the catalytic domains of known MEKKs but is distinct from Byr2. Thus, we suspected that it was derived from a novel gene, which we named *mkh1*. Based on a restriction map of genomic DNA, we estimated that this gene is contained within a 7.3-kb *Spe*I DNA fragment. We constructed an *S. pombe* genomic DNA library in the λ ZAPII vector (Stratagene), and we screened this library by filter hybridization using a probe derived from the insert of pM3K4b. We isolated several independent clones containing 7.3-kb *Spe*I inserts that hybridized to our probe. The DNA sequences of the 7.3-kb inserts from two independently derived clones, pMKH1 and pMKH2, were determined and found to be identical (Fig. 1). The predicted protein encoded by this sequence is 1,116 amino acid residues in length. There is an in-frame stop codon located 18 bp from the 5′ end of the start codon, and there are no sequences in the coding region that fit the *S. pombe* consensus splicing sequences (31). Thus, the entire coding region of *mkh1* appears to be contained within a single exon.

Comparison of Mkh1 with known MEK kinases. Known yeast MEKKs have 35 to 55% identity among their C-terminal catalytic domains, but there is very little homology among their N-terminal regulatory domains. These proteins range in size from 659 (*S. pombe* Byr2) to 1,579 (*S. cerevisiae* Ssk2) amino acids in length. The putative catalytic domain of Mkh1 has strong homology with other yeast and mammalian MEKK cat-

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   1                ACTAGTGATTCAACATTTCTCTCCTTTCTTAATATTTTATTTACCTGCAAGAAATTGGGCTATTACTAACATCCCAAGTCTGTCTTTTGCAAATTAGTTCACTCGTTT
 109 CAGCCGAATCTTAATTTTTTTAATCATAAACTACTTAAGCAGCAATCTCCAATTCGTTTGACAAAAAAAAAGGTTTTTTATCTTGAATTCTTTTCAAAGTAACTAATACTGTTCTGGGTAT
                                                                                                                        ***
 229 ATGGCTGCCGATATCGGATCGCAGTCATCAGGCTCTTTGGAAGAACGGTTTGAACAGTCTCTTCATCTTCAGAATGTCGATAAGCAAGATTGGTCACTTAACAGTGTACTTCAGTTTTTA
   1  M  A  A  D  I  G  S  Q  S  S  G  S  L  E  E  R  F  E  Q  S  L  H  L  Q  N  V  D  K  Q  D  W  S  L  N  S  V  L  Q  F  L
 349 AAACTATACAAATTTAACAAAGAATGGGAAGACGTTTTTATTAAAAGTCGAATCGAAATGGATTTATTTATCAATTTGGCCGATCAATCAAAAGCCGAGGAATTCGCCTTTAAAAATAAA
  41  K  L  Y  K  F  N  K  E  W  E  D  V  F  I  K  S  R  I  E  M  D  L  F  I  N  L  A  D  Q  S  K  A  E  E  F  A  F  K  N  K
 469 TTGAGCAAGGAGTCTGCCATCCAATTGAGTAGCTGTATTCGCAAAACACTTTTAGCACCTTCTTCGACTCGCGTACCTAGCAAAAACTCGTCTTACGAAACATTAACTTACAGCGCCAAA
  81  L  S  K  E  S  A  I  Q  L  S  S  C  I  R  K  T  L  L  A  P  S  S  T  R  V  P  S  K  N  S  S  Y  E  T  L  T  Y  S  A  K
 589 GATAGTTCGGATGACGTTTTTACAGAAACTAACTCTGGTTTCCGCTCTTCAAATCAAAATTCGTCCCTCAAAAGCTTTCAGAGTGTTCCTGATAGCAATGTGAACGTGTTTGGTGGCTTC
 121  D  S  S  D  D  V  F  T  E  T  N  S  G  F  R  S  S  N  Q  N  S  S  L  K  S  F  Q  S  V  P  D  S  N  V  N  V  F  G  G  F
 709 GGTGGATCAGTAGTCGACAACAATGAGCTATTGAGCACAGGAAAAAACTCCCATCAAACAACCTCTTTAAATTTGGAAGGCTCTCCTATAAACTTACACGCTTACAAAGGAACTGTCACT
 161  G  G  S  V  V  D  N  N  E  L  L  S  T  G  K  N  S  H  Q  T  T  S  L  N  L  E  G  S  P  I  N  L  H  A  Y  K  G  T  V  T
 829 TCAATAATTAACGATGACAGCAGAAACATTAATAAAAAAACATTGTCGAAACAACCTGTATCCGAACATAAAGAAAAACAAACTAGCTTTCTCCGTCGTTTCCGGGTACCTGGGTTTTCT
 201  S  I  I  N  D  D  S  R  N  I  N  K  K  T  L  S  K  Q  P  V  S  E  H  K  E  K  Q  T  S  F  L  R  R  F  R  V  P  G  F  S
 949 CGTGACAAGGATAAAACTAAAGATTGCCCTTCTTCAAATTCGAACCCATTCCATTTAGCTTCTTCAAATGTGAAAACATTAGACGCGTCTTTGGATCAAGGTGAGTGGGTACCTCGTATT
 241  R  D  K  D  K  T  K  D  C  P  S  S  N  S  N  P  F  H  L  A  S  S  N  V  K  T  L  D  A  S  L  D  Q  G  E  W  V  P  R  I
1069 CATCGTTTAGAAAGTCAAATTGGTTTAATATCCAAAAAGAAGTCATTTGTTCTTGCTACTATGGATGATATGAAATTCACAGTAGTGGATATTACCAACGTCCAAAATGCTACTCAGCTA
 281  H  R  L  E  S  Q  I  G  L  I  S  K  K  K  S  F  V  L  A  T  M  D  D  M  K  F  T  V  V  D  I  T  N  V  Q  N  A  T  Q  L
1189 CGTAAGCTAATAGCTAAGAGTATGTATTTAGACATTTCAATTGACCAGTTTGATTTGTTTCTCACGGAAGTCGGCGGGGCTCAATACATAGAAATATTAGATGATAGAAAGCTTGATATT
 321  R  K  L  I  A  K  S  M  Y  L  D  I  S  I  D  Q  F  D  L  F  L  T  E  V  G  G  A  Q  Y  I  E  I  L  D  D  R  K  L  D  I
1309 GCAAGGCTTTATTCTGATGAATTTGGAACTATTAAATTTTTCGTAAAGCCATCACAAAATGAAGAATCGGGTATGGATAGTGATACTTATTTATCTTTTGGCACAAAATCAAGTTCAACT
 361  A  R  L  Y  S  D  E  F  G  T  I  K  F  F  V  K  P  S  Q  N  E  E  S  G  M  D  S  D  T  Y  L  S  F  G  T  K  S  S  S  T
1429 TATAAAGCTGATGATGACTCAATATATCATCGCAAGGAAGATTTTAAAAAGCAACCAAGTTACCCTGTGCTTACTTCGGATTTTGAAATTACTGATGCAGGACCTAATTTATCATTATCA
 401  Y  K  A  D  D  D  S  I  Y  H  R  K  E  D  F  K  K  Q  P  S  Y  P  V  L  T  S  D  F  E  I  T  D  A  G  P  N  L  S  L  S
1549 GGGCATCAACCTGATAATAAATACTACAAAGGTTTTAGTTCGGCACCGAATTTGGCAGTTGTTCCAGAATTACCATCTCGACGTTTTCGAGGGTTTGAAAAAATCCGTGGTGCTAAAGGA
 441  G  H  Q  P  D  N  K  Y  Y  K  G  F  S  S  A  P  N  L  A  V  V  P  E  L  P  S  R  R  F  R  G  F  E  K  I  R  G  A  K  G
1669 GAAATGGCTACAAAAATTTTGGATGCCACTGAAGCCCAAAGTGAAAAAAACAAATTTACCGTTTGTAGACCTCACAAGAAGGTCACATTGAAAATGCCACTTAATTCCGGCTCTTCCGCT
 481  E  M  A  T  K  I  L  D  A  T  E  A  Q  S  E  K  N  K  F  T  V  C  R  P  H  K  K  V  T  L  K  M  P  L  N  S  G  S  S  A
1789 CCCCAAAGTCCTTCATCTAATACTTCTGCTTCTGTTTTAACTAGAAATTTTGTGGCACATAGAGATCCTCCACCCCCACCCACAGAGACATCTAGTTTACGTCGAAAAAATACATTGACT
 521  P  Q  S  P  S  S  N  T  S  A  S  V  L  T  R  N  F  V  A  H  R  D  P  P  P  P  P  T  E  T  S  S  L  R  R  K  N  T  L  T
1909 CGTAGACCAAGTATTCGTCACGCTCGGTCCTCTCCTTACATTGATACCGGACATAACGAAGCTAGCAAATTTTCACATACGTCTTTTGACCCCAAAGCATCTAGTAAATCTTCTAATTCA
 561  R  R  P  S  I  R  H  A  R  S  S  P  Y  I  D  T  G  H  N  E  A  S  K  F  S  H  T  S  F  D  P  K  A  S  S  K  S  S  N  S
2029 TTAAAGGAAAGTGTGGAAGCTTTATCAGAAATACCTTTTGAAGATGCGCCTGCACTAGACGAATCGGATCTTTCTGGGGATCCCTTTTGGGCTATACAGCCCAAACAATCTTCCTCCCAA
 601  L  K  E  S  V  E  A  L  S  E  I  P  F  E  D  A  P  A  L  D  E  S  D  L  S  G  D  P  F  W  A  I  Q  P  K  Q  S  S  S  Q
2149 GTACCTAAAGAAAATCATCACAACATTCAATCCAAACTTTCCATTAACACAGAGGCTGCTACGGATTTGAAAGCAAATGAACTATCTTCGCCTAAAACTCCTGAATACTGTAGAGGTGAT
 641  V  P  K  E  N  H  H  N  I  Q  S  K  L  S  I  N  T  E  A  A  T  D  L  K  A  N  E  L  S  S  P  K  T  P  E  Y  C  R  G  D
2269 GACAGATCCATTAGTTTATCACCGTTATCTTATCGTTTAAGAAAGTCCAAACATATTCGTGAATCCCCACCGTCTTCAAAGGTTATCAATTCTGGTAACTGGGAAGTTCGTCCATCTGCT
 681  D  R  S  I  S  L  S  P  L  S  Y  R  L  R  K  S  K  H  I  R  E  S  P  P  S  S  K  V  I  N  S  G  N  W  E  V  R  P  S  A
2389 GATGATCTTTATGAGGATGTTGATCGATTTTTTCCCCGTTATGATTTGGATAAAGTACTTGTAGTGGACCAAAGCCGCATGGTTTCTTCCCCTTCAAAGGTATCGATACGTCCGAAAATG
 721  D  D  L  Y  E  D  V  D  R  F  F  P  R  Y  D  L  D  K  V  L  V  V  D  Q  S  R  M  V  S  S  P  S  K  V  S  I  R  P  K  M
2509 AAGTCTGTACGATTATTAGCCCGCGAAGCATCTGAAGCTCGGAAAGAAATACGACATAACGCGAGACGCAATAAATCTGGAAATCTTCTACGTCGATCAAGTACGAAACTTTGGGGCTCT
 761  K  S  V  R  L  L  A  R  E  A  S  E  A  R  K  E  I  R  H  N  A  R  R  N  K  S  G  N  L  L  R  R  S  S  T  K  L  W  G  S
2629 AGGATTGTAGAACTAAAACCAGATACTACTATAACTTCTGGATCAGTTGTTTCACAAAATGCCACGTTCAAATGGATGAAAGGAGAATTGATTGGAAATGGTACTTATGGTAAGGTATTT
 801  R  I  V  E  L  K  P  D  T  T  I  T  S  G  S  V  V  S  Q  N  A  T  F  K  W  M  K  G  E  L  I  G  N  G  T  Y  G  K  V  F
2749 TTGGCTATGAACATTAATACGGGTGAATTGATTGCAGTAAAGCAAGTTGAAATACCACAAACTATTAATGGCCGTCATGACCAATTACGCAAAGATATCGTGGATTCCATTAATGCAGAA
 841  L  A  M  N  I  N  T  G  E  L  I  A  V  K  Q  V  E  I  P  Q  T  I  N  G  R  H  D  Q  L  R  K  D  I  V  D  S  I  N  A  E
2869 ATTTCTATGATTGCCGATTTGGATCACTTAAATATAGTGCAATATCTGGGTTTCGAAAAGACGGAAACGGATATAAGTATATTCCTGGAATATGTTTCAGGTGGTTCAATTGGTCGATGT
 881  I  S  M  I  A  D  L  D  H  L  N  I  V  Q  Y  L  G  F  E  K  T  E  T  D  I  S  I  F  L  E  Y  V  S  G  G  S  I  G  R  C
2989 TTGCGGAATTATGGTCCTTTCGAAGAGCAACTGGTCCGTTTTGTATCACGCCAGGTGCTCTACGGGTTGTCTTACTTACATTCTAAAGGTATTATACATCGAGATTTAAAGGCTGACAAT
 921  L  R  N  Y  G  P  F  E  E  Q  L  V  R  F  V  S  R  Q  V  L  Y  G  L  S  Y  L  H  S  K  G  I  I  H  R  D  L  K  A  D  N
3109 TTGCTCATTGATTTTGATGGAGTTTGCAAAATTTCAGACTTTGGAATATCTAAGCATAGTGATAATGTGTATGACAATGACGCAAACCTGTCCATGCAAGGATCCATCTTTTGGATGGCA
 961  L  L  I  D  F  D  G  V  C  K  I  S  D  F  G  I  S  K  H  S  D  N  V  Y  D  N  D  A  N  L  S  M  Q  G  S  I  F  W  M  A
3229 CCTGAAGTAATTCATAATGATCATCAAGGATATAGTGCTAAGGTCGACGTCTGGTCCTTGGGATGTGTAGTGTTGGAAATGTTAGCTGGTCGTAGACCGTGGTCTACAGATGAGGCTATC
1001  P  E  V  I  H  N  D  H  Q  G  Y  S  A  K  V  D  V  W  S  L  G  C  V  V  L  E  M  L  A  G  R  R  P  W  S  T  D  E  A  I
3349 CAAGCTATGTTCAAGTTAGGTACCGAGAAAAAGGCGCCTCCTATTCCTAGTGAATTGGTGTCTCAGGTATCACCCGAAGCGATTCAATTTTTGAATGCATGCTTTACTGTGAATGCTGAT
1041  Q  A  M  F  K  L  G  T  E  K  K  A  P  P  I  P  S  E  L  V  S  Q  V  S  P  E  A  I  Q  F  L  N  A  C  F  T  V  N  A  D
3469 GTAAGGCCAACCGCAGAGGAATTATTAAATCACCCGTTTATGAAATGTGACGAAGAATTCAACTTTAAGGACACGAATCTTTACGACATGCTTTGTAAAAGAAAGAGCTAAATTGGTCGC
1081  V  R  P  T  A  E  E  L  L  N  H  P  F  M  K  C  D  E  E  F  N  F  K  D  T  N  L  Y  D  M  L  C  K  R  K  S ***
3589 AATTATTATTTATTACTTAGCGTTTCCTCGCAGGGGCATGCATTCTCAATTTCTTCTTATAGCTATTTATTTAATATTGAAATT
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FIG. 1. Nucleotide and deduced protein sequences of *S. pombe mkh1*. This gene encodes a 1,116-amino-acid protein. Numbers of nucleotides (beginning from the *Spe*I site) and amino acid residues (beginning from the first encoded Met) are indicated on the left. The positions of in-frame stop codons flanking the coding sequence are indicated by asterisks.

alytic domains, and it contains regions that are conserved among Ser/Thr kinases, including motifs that are specifically conserved among MEKKs (Fig. 2). Of known proteins, *S. cerevisiae* Bck1 has the highest sequence homology with Mkh1. The putative catalytic domains of these proteins are 54% identical, but there is no significant homology between the N-terminal domains. Thus, Mkh1 appears to be a member of the MEKK family.

Mkh1 is required for growth in high salt concentrations and proper cell cycle reentry. To investigate the function of Mkh1, we constructed and examined the phenotypes of *S. pombe* strains in which *mkh1* is disrupted. The coding sequence of the catalytic domain of *mkh1* was replaced with *ura4* in a diploid strain (see Materials and Methods). Tetrad analysis was performed on several independently derived diploid transformants containing the *mkh1* disruption. In most cases, asci

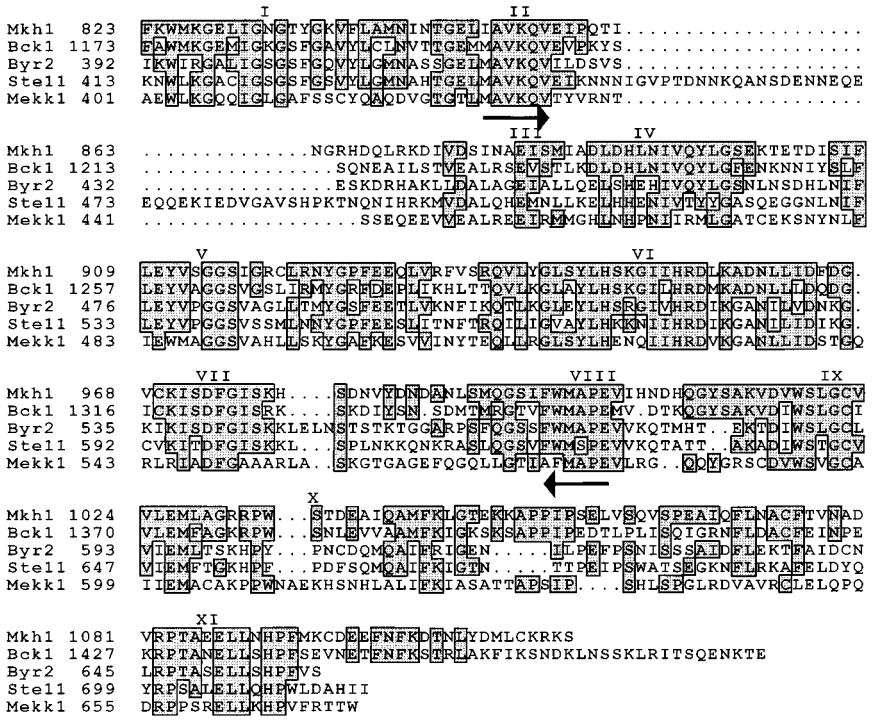

FIG. 2. Comparison of the catalytic domains of *S. pombe* Mkh1, *S. cerevisiae* Bck1 (20), *S. pombe* Byr2 (54), *S. cerevisiae* Ste11 (40), and mammalian Mekk1 (19). Shaded boxes enclose regions with sequence identity to Mkh1. Roman numerals I to XI indicate the positions of conserved kinase subdomains (13). Arrows indicate the positions of peptides that were used to design the degenerate oligonucleotides used to identify *mkh1*. The putative catalytic domain of Mkh1 (residues 823 to 1116) is 54% identical to that of Bck1, 42% identical to that of Byr2, 41% identical to that of Ste11, and 33% identical to that of Mekk1.

contained four viable spores, and two of the spores were Ura$^+$, indicating that they contained the disrupted *mkh1* allele. Thus, *mkh1* is not required for germination or cell growth. We also found that *mkh1*Δ cells are capable of mating but at a slightly lower efficiency than normal cells (data not shown).

The *mkh1*Δ and *mkh1*$^+$ haploid strains grew at similar rates in standard growth conditions, as measured by both cell number and OD_{600} (Table 2). We also found that overexpression of Mkh1 had no significant affect on growth rate (Table 2). Thus, Mkh1 does not appear to be involved in regulating cell growth or division during logarithmic growth in standard conditions. However, we found that *mkh1*Δ cells were unable to grow in the presence of very high KCl concentrations, while growth of *mkh1*$^+$ cells was only partially inhibited (Fig. 3A). We similarly found that *mkh1*Δ cell growth was inhibited by high NaCl concentrations (data not shown). These results indicate that Mkh1 provides an essential function when cells are confronted with extreme hyperosmotic stress.

TABLE 2. Growth rates

Strain[a]	Mean doubling time (h)[b] ± SD	
	PMA-Leu	YEA
SP870(pAALN)	4.45 ± 0.07	
SP870(pLMK1)	4.38 ± 0.08	
AS1 (*mkh1*Δ *ura4*$^+$)		2.04 ± 0.04
AS2 (*mkh1*+ *ura4*Δ)		2.32 ± 0.04
AS7 (*mkh1*+ *ura4*$^+$)		2.11 ± 0.04

[a] pAALN is a control plasmid. pLMK1 encodes Mkh1 under the control of the *adh1* promoter.

[b] Determined by a least-squares algorithm for strains grown in the indicated media at 30°C.

We also found that *mkh1*Δ cells required a significantly longer period of time to reenter the cell cycle upon reinoculation into fresh medium following prolonged stationary-phase arrest compared to their sister *mkh1*$^+$ cells (Fig. 4). This phenotype was not a function of cell viability but rather represented a change in the response to nutrient conditions. Thus, Mkh1 either is important for establishing stationary-phase physiology or is involved in nutrient sensing and cell cycle reentry.

***mkh1*Δ mutants exhibit defects in cell shape and cytokinesis.** Deletion of *mkh1* also resulted in a subtle but distinct effect on cell shape. Most *mkh1*Δ cells were more rounded than *mkh1*$^+$ cells, which exhibited a normal elongated rod-like shape (Fig. 5). We also found that cells overexpressing Mkh1 exhibited a greater (~8%) average length than control cells. Thus, Mkh1 is required for normal cell morphogenesis, and it may actively promote polarized cell shape. These phenotypes were apparent only during late-logarithmic-phase growth or in stationary-phase cultures, suggesting that Mkh1 is required for the normal response to nutrient depletion.

A more dramatic phenotype was observed when *mkh1*Δ cells were grown on synthetic (PMA) medium at an elevated temperature (35°C). Under such conditions, a significant fraction (~10%) of *mkh1*Δ cells formed filaments containing multiple septa and nuclei, suggesting that completion of cytokinesis was inhibited (Fig. 5). This multiseptate phenotype was observed much less frequently (<1%) in *mkh1*Δ cells grown at 23 or 30°C. Also, this phenotype was only seen after *mkh1*Δ cells reached stationary phase in liquid cultures, or after at least 3 days of growth on agar plates, suggesting that it is induced by a combination of both high temperature and nutrient depletion. The multiseptate phenotype was much more dramatic when *mkh1*Δ cells were grown on medium containing 1.0 M sorbitol or 0.6 M KCl (Fig. 6). Under such conditions, a large

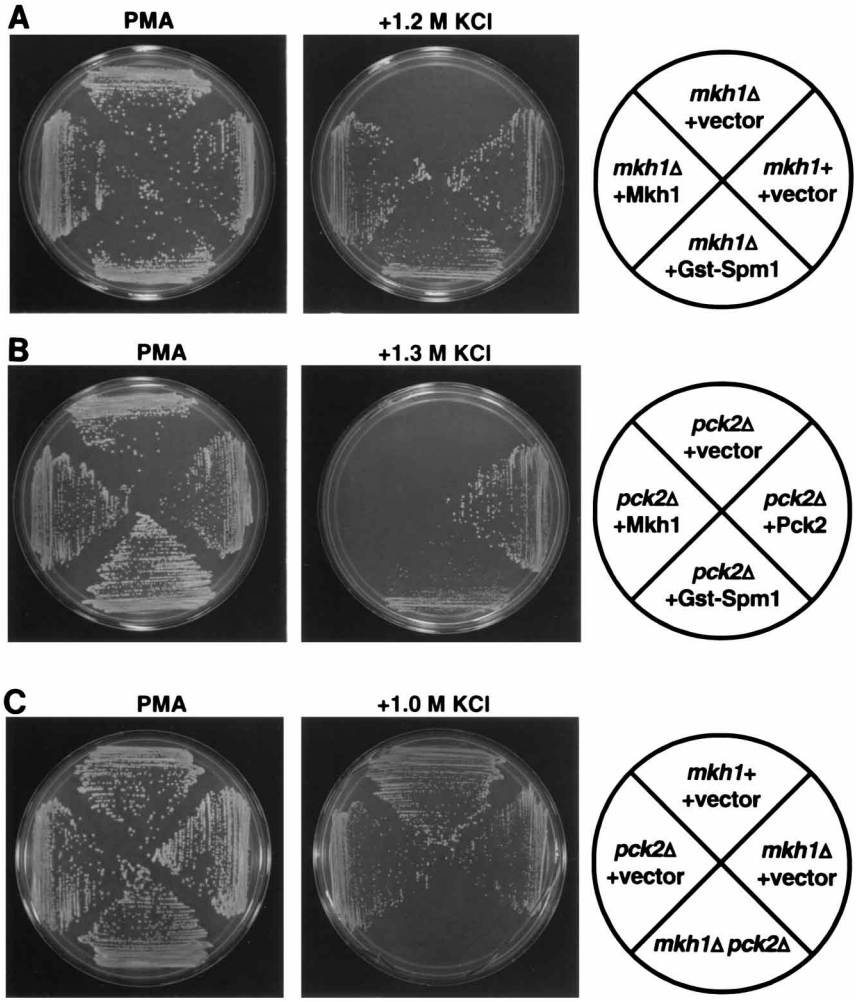

FIG. 3. Growth inhibition of *mkh1*Δ and *pck2*Δ mutants by KCl. AS1 (*mkh1*Δ), AS7 (*mkh1*$^+$), TP47-2B (*pck2*Δ), and AS13 (*pck2*Δ *mkh1*Δ) strains overexpressing the indicated proteins were grown on PMA plates for 3 days, or on PMA containing the indicated concentrations of KCl for 6 days, at 35°C. Plasmids used to overexpress various proteins were pAALN (vector), pLMK1 (Mkh1), pPCK2-2B (Pck2), and pR4L-GSPM1 (GST-Spm1).

fraction (~30%) of cells exhibited filamentous, multiseptate growth, and cells appeared to be swollen. Also, the morphologies of *mkh1*$^+$ and *mkh1*Δ colonies grown on media containing 0.6 M KCl were strikingly different. While *mkh1*$^+$ colonies exhibited a normal round shape, colonies of *mkh1*Δ cells exhibited an irregular shape with filamentous protrusions (Fig. 6). Filamentous growth of *mkh1*Δ cells in hyperosmotic media was observed at low to high temperatures (23 to 35°C), although it was enhanced at 35°C. In contrast, *mkh1*$^+$ cells very rarely (<0.1%) exhibited a multiseptate phenotype in response to high temperature or hyperosmotic growth conditions after several days (Fig. 6 and 7). These observations suggest that Mkh1 is required for normal cellular responses that inhibit filamentous growth under certain stress conditions.

A multiseptate phenotype similar to that observed in *mkh1*Δ cells has been previously observed in *S. pombe* grown in medium containing cyclosporin A (30 μg/ml) at 23°C but not at higher temperatures (57). Cyclosporin A has been shown to specifically inhibit calcineurin in different organisms, including yeast. Also, *S. pombe* lacking Ppb1, a calcineurin-related protein phosphatase, exhibits a multiseptate phenotype (57). To determine if Mkh1 and the cyclosporin A target act in a common pathway, we examined the effect of cyclosporin A treat-

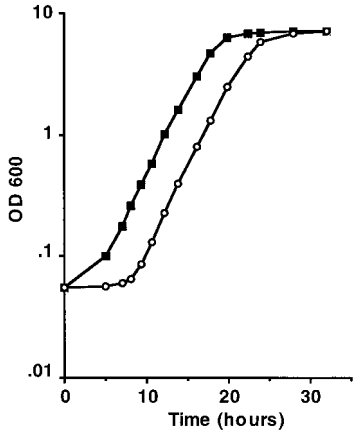

FIG. 4. Reentry into the cell cycle. AS1 (*mkh1*Δ) (○) and AS7 (*mkh1*$^+$) (■) cells were grown to mid-log phase and reinoculated into fresh liquid YEA medium at an OD_{600} of 0.05. After the cells reached stationary phase, they were incubated for an additional 4 days. Cell viability was determined at intervals during this period by plating approximately 1,000 cells/plate in triplicate and counting the number of colonies after 5 days of growth at 30°C. Both strains were found to remain >90% viable during this period. After 4 days, stationary-phase cultures were used to inoculate fresh YEA at an OD_{600} of 0.05, and growth curves were determined.

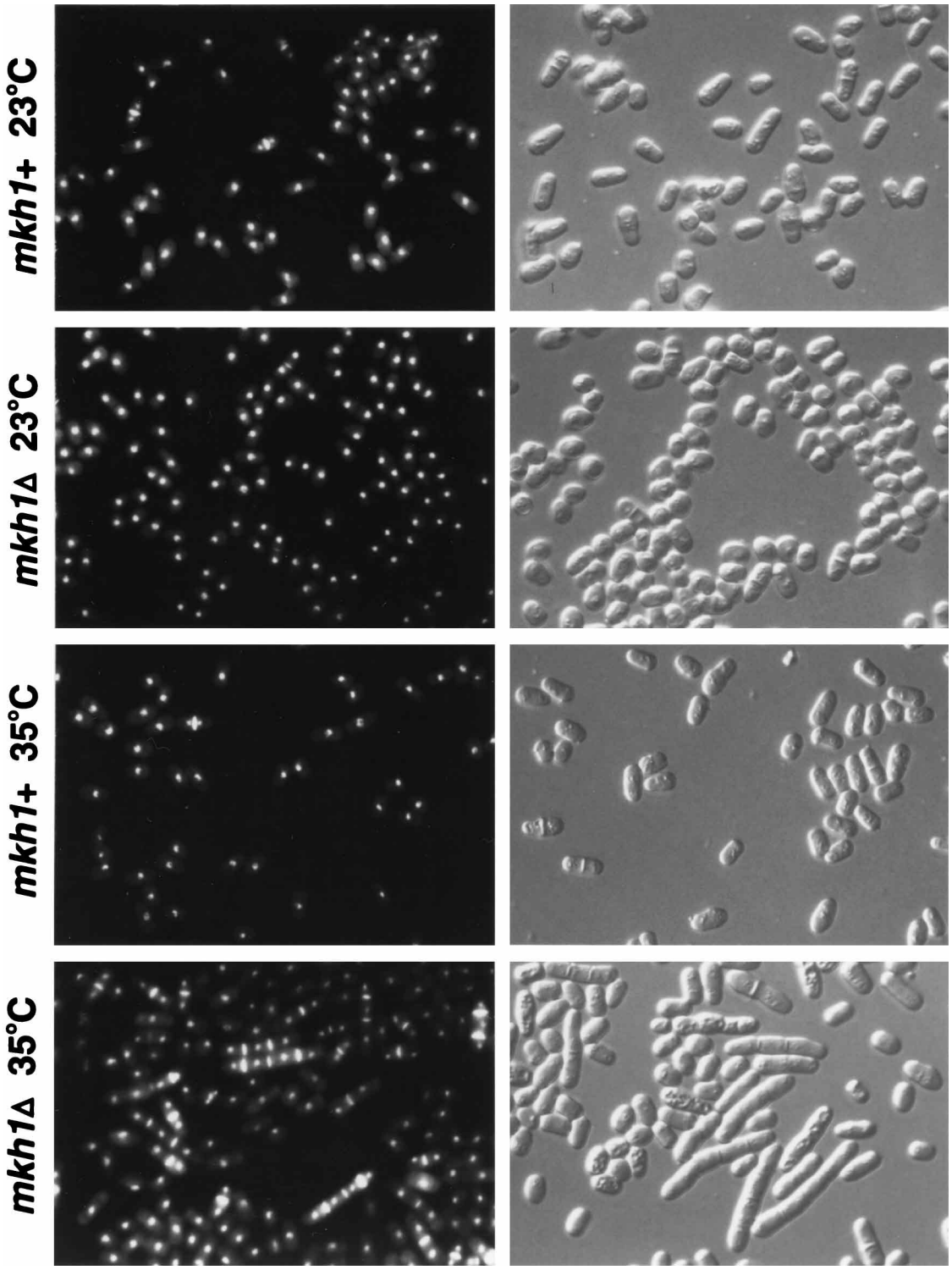

FIG. 5. Morphology of *mkh1*Δ cells. AS1 (*mkh1*Δ) and AS7 (*mkh1*$^+$) cells were grown for 96 h on YEA agar plates at 23 or 35°C. The left panels show cells that were fixed with formaldehyde and stained with DAPI and calcofluor. Identical results were observed when cells were stained with Hoechst 33258. The right panels show unstained cells viewed by differential interference contrast microscopy.

ment on *mkh1*Δ cells. We found that cyclosporin A (30 μg/ml; Sandoz) had no apparent effect on *mkh1*$^+$ cells grown at 35°C, as previously observed, but enhanced the multiseptate phenotype of *mkh1*Δ cells: a greater fraction (~20%) of cells exhibited filamentous growth, and this phenotype was induced at an earlier time (<24 h). This observation suggests that Mkh1 and the cyclosporin A target act synergistically to regulate filamentous growth.

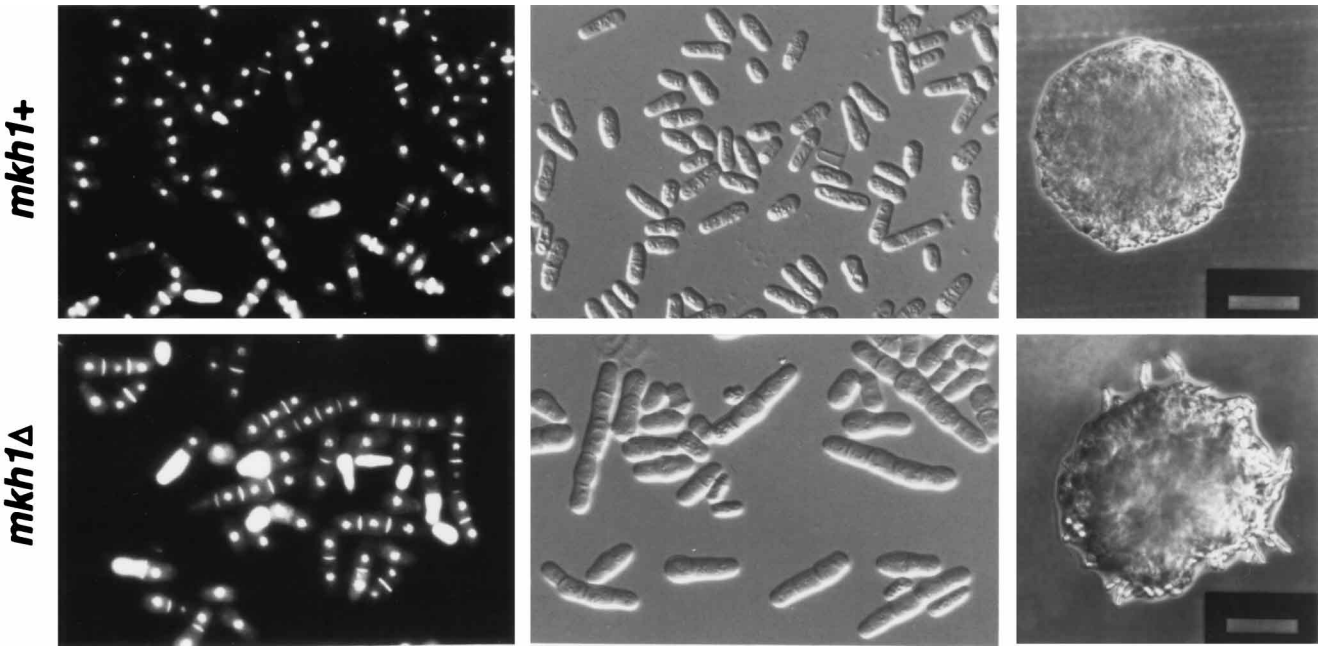

FIG. 6. *mkh1*Δ cells exhibit a multiseptate phenotype in hyperosomotic growth conditions. AS1 (*mkh1*Δ) and AS2 (*mkh1*$^+$) cells were grown for 48 h on YEA agar plates containing 0.6 M KCl at 30°C. Left panels show cells that were fixed with formaldehyde and stained with DAPI and calcofluor; middle panels show unstained cells viewed by differential interference contrast microscopy; right panels show colony morphology.

Mkh1 is required to maintain cell wall integrity. The relatively strong conservation between Mkh1 and Bck1 suggested that these proteins may be functionally related. However, we found that deletion of these genes resulted in very distinct phenotypes. Deletion of *mkh1* does not result in a temperature-sensitive cell lysis defect, like that exhibited by *bck1*Δ cells. Furthermore, while *bck1*Δ cells are stabilized by hypertonic conditions, *mkh1*Δ cell growth is sensitive to hypertonic conditions. We also found that deletion of *mkh1* has no apparent effect on induced thermotolerance (data not shown), whereas *bck1*Δ cells exhibit a reduced induced thermotolerance (15). Also, we found that expression of Mkh1 failed to complement the temperature-sensitive cell lysis defect associated with *bck1*Δ (data not shown). Thus, the functions of these proteins do not appear to be well conserved. Nevertheless, we further examined whether Mkh1, like Bck1, is required for normal cell wall integrity and whether its function is similarly regulated by PKC homologs. Two PKC-related genes, *pck1* and *pck2*/*pkc1*, have been identified in *S. pombe* (30, 52). Deletion of both *pck1* or *pck2* is lethal, while deletion of either gene results in hypersensitivity to staurosporine. *pck2*Δ cells also exhibit a rounded or pear-shaped morphology, somewhat similar to that exhibited by *mkh1*Δ cells. Also, *pck2*Δ cells are hypersensitive to β-glucanase treatment, and they fail to regenerate their cell walls after such treatment, indicating that they are defective in cell wall biosynthesis (17, 48).

To determine if Mkh1 is required for normal cell wall integrity, we examined the sensitivity of *mkh1*Δ cells to β-glucanase treatment. We found that logarithmically growing *mkh1*Δ cells are hypersensitive to β-glucanase but slightly less sensitive than *pck2*Δ cells (Fig. 7A). However, in late log phase, the sensitivity of *mkh1*Δ cells was more similar to that of wild-type cells, while *pck2*Δ cells remained hypersensitive (data not shown). We have also observed significant lysis and decreased viability of *mkh1*Δ cells upon β-glucanase treatment (data not shown). Thus, Mkh1 is required to maintain normal cell wall integrity

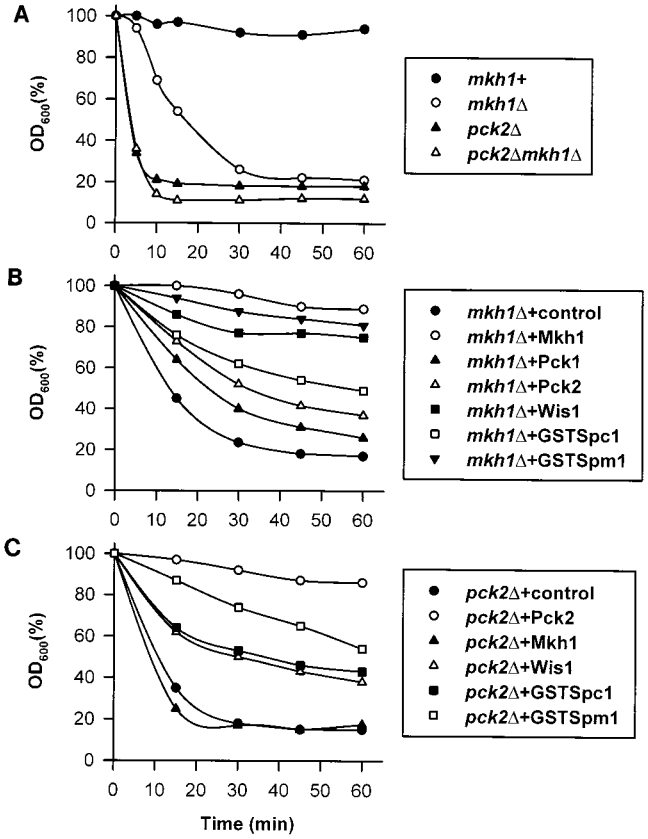

FIG. 7. Sensitivity of *mkh1*Δ and *pck2*Δ cells to β-glucanase. Cell lysis was measured at different times during treatment with β-glucanase by determining the OD_{600} (see Materials and Methods). The strains examined were AS1 (*mkh1*Δ), AS7 (*mkh1*$^+$), TP47-2B (*pck2*Δ), and AS13 (*pck2*Δ *mkh1*Δ) containing either vector (pAALN), pLMK1 (Mkh1), pPCK11-2 (Pck1), pPCK2-2B (Pck2), pREP-WIS1 (Wis1), pREP1-GSTSPC1 (GST-Spc1), or pR4L-GSPM1 (GST-Spm1).

TABLE 3. Cell wall regeneration

Strain	Plasmid	Regeneration[a]
AS7 (*mkh1*+)	pAALN (vector)	++
AS1 (*mkh1*Δ)	pAALN (vector)	++
TP47-2B (*pck2*Δ)	pAALN (vector)	−
TP47-2B (*pck2*Δ)	pPCK2-2B (Pck2)	++
TP47-2B (*pck2*Δ)	pLMK1 (Mkh1)	−
TP47-2B (*pck2*Δ)	pR4L-GSpm1 (GST-Spm1)	++
AS13 (*pck2*Δ *mkh1*Δ)		−
TZS69 (*spm1*Δ)	pAAUN (vector)	++
AS14 (*spm1*Δ *mkh1*Δ)		++

[a] Cell wall regeneration after treatment with β-glucanase was determined as described in Materials and Methods. The percentages of cells that recovered were estimated as indicated: −, <0.1%; ++, >90%.

upon β-glucanase treatment. This may reflect a defect in cell wall construction, a defect in osmoregulation, or both. The underlying molecular basis for this phenotype will require further investigation. Whether this defect is similar to that in *pck2*Δ cells is unclear. However, we found that unlike *pck2*Δ cells, *mkh1*Δ cells were not defective in cell wall regeneration after β-glucanase treatment (Table 3). Thus, the *mkh1*Δ defect is distinguishable from that associated with *pck2*Δ. We also found that overexpression of Mkh1 in *pck2*Δ cells failed to complement the β-glucanase sensitivity or cell wall regeneration defect (Fig. 7C and Table 3). Interestingly, however, we found that overexpression of Pck1 or Pck2 partially complemented the β-glucanase sensitivity of *mkh1*Δ cells (Fig. 7B), indicating that they can act independent of Mkh1 to partially compensate for this defect. However, such complementation could be due to a variety of undetermined cellular parameters and may not necessarily reflect related functions. To further examine the relationship of Pck2 and Mkh1, we constructed *pck2*Δ *mkh1*Δ double-mutant strains. These cells exhibited a slightly enhanced sensitivity to β-glucanase treatment compared to the single mutants (Fig. 7A). These cells also exhibited a defect in cell wall regeneration (Table 3). Together, these observations suggest that Pck1,2 and Mkh1 act independently to maintain cell wall integrity.

Mkh1 and Pck2 act cooperatively to maintain cell shape and KCl resistance. We found that growth of *pck2*Δ cells was hypersensitive to KCl (Fig. 3B) but not as sensitive as growth of *mkh1*Δ cells, since a slightly higher concentration of KCl was required to completely inhibit *pck2*Δ cell growth (data not shown). Overexpression of Mkh1 in *pck2*Δ cells failed to complement this defect (Fig. 3B). Similarly, overexpression of Pck1 or Pck2 failed to complement KCl sensitivity of *mkh1*Δ cells (data not shown). Furthermore, the *pck2*Δ *mkh1*Δ double mutant was more sensitive to KCl than either single-mutant strain (Fig. 3C). These observations suggest that Pck2 and Mkh1 act independently to regulate KCl resistance.

As previously mentioned, *pck2*Δ cells exhibit a round shape that is similar to but distinct from that of *mkh1*Δ cells. The *pck2*Δ *mkh1*Δ double mutant also exhibited a morphology similar to that of *pck2*Δ cells (Fig. 8). Also, we found that overexpression of Mkh1 in *pck2*Δ cells restored a more normal, elongated cell shape in standard growth conditions (Fig. 8), indicating that Mkh1 can act independent of Pck2 to maintain normal cell morphology.

We confirmed a previous observation (52) that expression of very high levels of Pck2, from the repressible *nmt1* promoter, leads to cell growth arrest under normal growth conditions (Fig. 9A). However, we found that similar high-level expression of Pck2 in *mkh1*Δ cells did not lead to growth arrest (Fig. 9A), indicating that Mkh1 is required for growth inhibition by Pck2.

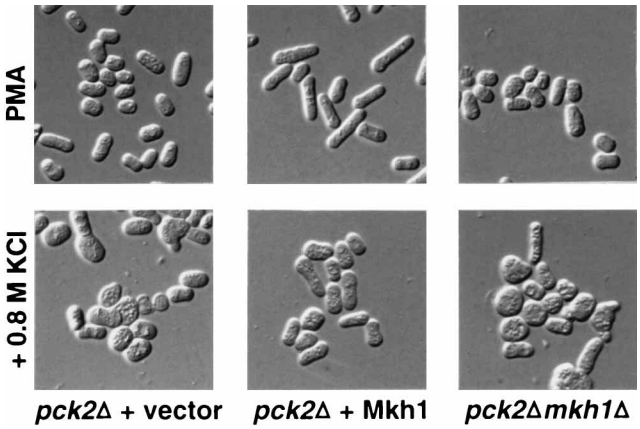

FIG. 8. Morphology of *pck2*Δ mutants. TP169-1C(pAAUN) (*pck2*Δ + vector), TP169-1C(pLMK1) (*pck2*Δ + Mkh1), and AS13 (*pck2*Δ *mkh1*Δ) cells were grown on PMA or PMA–0.8 M KCl at 30°C for 3 days and examined by differential interference contrast microscopy.

Mkh1 and Pck2 act in opposition to regulate filamentous growth. Many of the foregoing observations suggest that Mkh1 and Pck2 regulate similar cellular responses. In striking contrast, it has been previously shown that high-level expression of Pck2 results in filamentous, multiseptate growth very similar to that exhibited by *mkh1*Δ cells (30, 52), suggesting that Mkh1 and Pck2 act antagonistically. We confirmed this observation, and we found that high-level expression of Pck2 in either *mkh1*$^+$ or *mkh1*Δ cells resulted in a large fraction of multiseptated cells in standard growth conditions (Fig. 9B). Thus, the induction of filamentous growth by Pck2 does not require Mkh1, and the growth inhibition associated with high levels of Pck2 is not due to the induction of filamentous growth. We also found that the *pck2*Δ *mkh1*Δ double mutant does not exhibit a multiseptate phenotype when grown at a high temperature or on hyperosmotic media (Fig. 8). Together, these observations suggest that Pck2 and Mkh1 act in opposition to regulate filamentous multiseptate growth and that Pck2 is both necessary (in the absence of Mkh1) and sufficient (when it is highly expressed) to induce this phenotype.

Is Wis1 an effector of Mkh1? We have performed various genetic analyses to examine the relationship of Mkh1 with known MAPK pathways in *S. pombe*. Since the Wis1-Sty1/Spc1 pathway appears to be osmoregulated, we first investigated whether Mkh1 acts in a common pathway. We found that overexpression of Wis1 or a GST-Spc1 fusion in *mkh1*Δ cells failed to complement the multiseptate phenotype or sensitivity to KCl (data not shown). However, Wis1 or, to some extent, GST-Spc1 restored resistance to β-glucanase (Fig. 7B). Expression of Wis1 or GST-Spc1 in *pck2*Δ cells also partially restored resistance to β-glucanase (Fig. 7C). These results were somewhat surprising since we also found that *wis1*Δ cells were not hypersensitive to β-glucanase (data not shown). To further investigate the relationship between Mkh1 and Wis1, we constructed *mkh1*Δ *wis1*Δ double mutants, and we found that they exhibit a striking morphology that appears to reflect a combination of the *wis1*Δ and *mkh1*Δ phenotypes: cells appear highly elongated, even more than *wis1*Δ cells, and some are branched with multiple septa (Fig. 10). Thus, our evidence indicates that Wis1 can compensate for at least one defect associated with *mkh1*Δ but has additional functions unrelated to Mkh1.

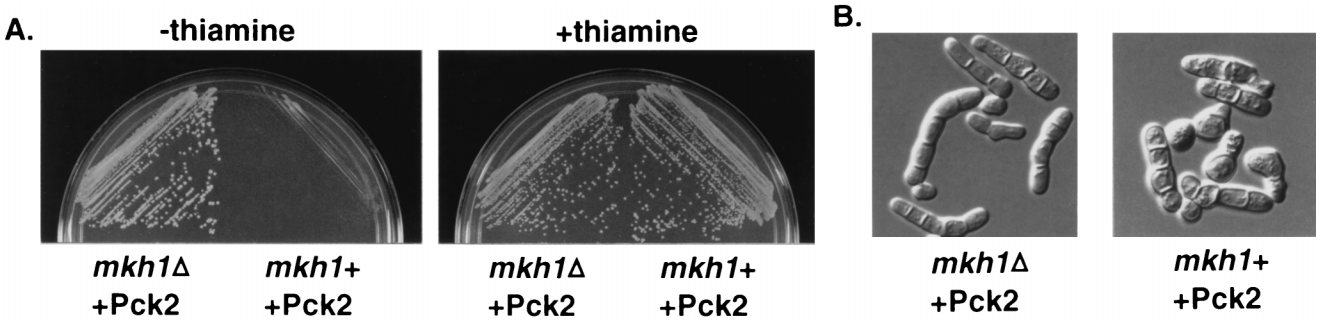

FIG. 9. Overexpression of Pck2. Strains AS1(pR3LPck2) (*mkh1*Δ + Pck2) and AS7(pR3LPck2) (*mkh1*$^+$+ Pck2) express very high levels of Pck2 when grown in the absence of thiamine. (A) Strains streaked on PMA plates with or without thiamine and grown at 30°C for 3 days. (B) Morphology of cells grown on PMA at 30°C for 3 days and examined by differential interference contrast microscopy.

Mkh1 and Pmk1/Spm1 mediate similar cellular responses. As mentioned earlier, an *S. pombe* gene, *pmk1/spm1*, encoding a novel MAPK was recently identified (53, 59). Deletion of *pmk1/spm1* results in phenotypes (round cell shape, filamentous growth, KCl sensitivity, and β-glucanase sensitivity) that are essentially identical to those of *mkh1*Δ cells except that a larger fraction of *spm1*Δ cells exhibit filamentous growth on synthetic medium (PMA) at 30°C. Thus, Pmk1/Spm1 appears to be a very strong candidate for a downstream MAPK component of the Mkh1 pathway. To investigate this possibility, we first examined whether overexpression of a GST-Spm1 fusion protein would complement the *mkh1*Δ phenotypes. We found that expression of GST-Spm1 in *mkh1*Δ cells suppressed the sensitivities to KCl (Fig. 3A) and β-glucanase (Fig. 7B) but only partially suppressed the filamentous growth phenotype (data not shown). However, this latter failure could be due to the inability of the GST-Spm1 fusion to be fully activated in the absence of Mkh1. In contrast, overexpression of Mkh1 in *spm1*Δ cells failed to complement any of the known mutant phenotypes, including round cell shape, filamentous growth, KCl sensitivity, and β-glucanase sensitivity (data not shown). We also constructed a *mkh1*Δ *spm1*Δ double mutant strain, and we found that the phenotypes of this strain were essentially identical to the single mutants (Fig. 10 and other data not shown). These observations are consistent with a model where Pmk1/Spm1 acts downstream from Mkh1 (Fig. 11).

We also found that expression of GST-Spm1 in *pck2*Δ cells partially restored normal cell morphology (data not shown), resistance to KCl (Fig. 3B), resistance to β-glucanase treatment (Fig. 7C), and cell wall regeneration (Table 3). This latter observation was surprising since *spm1*Δ cells are not defective in cell wall regeneration (Table 3). Thus, Pmk1/Spm1 can partially compensate for most of the *pck2*Δ phenotypes, but deletion of either gene results in distinct phenotypes. These results suggest that Pmk1/Spm1 and Pck2 function in related but distinct pathways (Fig. 11).

DISCUSSION

Mkh1 is required for normal cellular responses to stress conditions. Our observations indicate that deletion of *mkh1* results in a defect in the normal response to stress, including

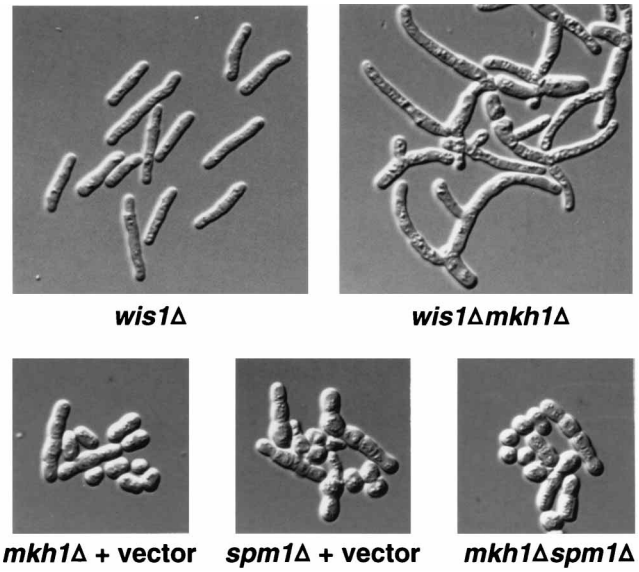

FIG. 10. Morphology of double mutants. ED904 (*wis1*Δ), AS15 (*wis1*Δ*mkh1*Δ), AS1(pAALN) (*mkh1*Δ + vector), TZS69(pAAUN) (*spm1*Δ + vector), and AS14 (*mkh1*Δ *spm1*Δ) strains were grown on PMA at 30°C for 3 days and examined by differential interference contrast microscopy.

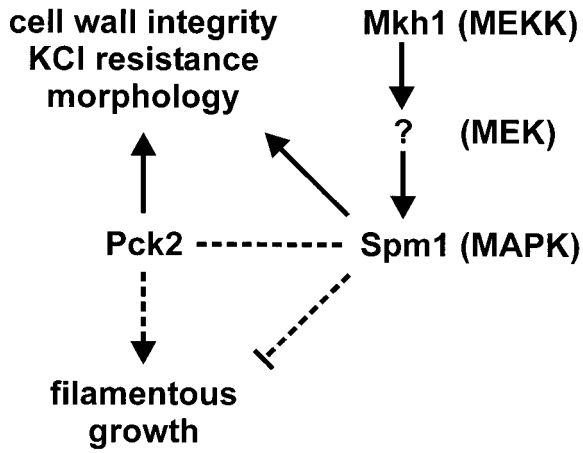

FIG. 11. Model for the possible relationships of Mkh1, Spm1, and Pck2. These proteins are required for cell wall integrity, cell morphology, and resistance to high KCl concentrations. Our evidence is consistent with a model where Spm1 acts downstream from Mkh1 in a novel MAPK cascade. Pck2 appears to act in an independent pathway to regulate cell wall integrity, cell morphology, and KCl resistance. In contrast, Pkc2 induces filamentous growth, while deletion of *mkh1* or *spm1* results in a similar phenotype. Our evidence is consistent with models where Pck2 and Spm1 act antagonistically either by independent mechanisms or in a common pathway to regulate filamentous growth.

nutrient limitation, high temperature, and hyperosmotic conditions. This defect results in an abnormal morphology, delayed reentry from stationary phase into the cell cycle, hypersensitivity to β-glucanase, hypersensitivity to high salt concentrations, and filamentous growth in response to stress. These observations suggest that Mkh1 may function in a MAPK pathway that responds to various stress conditions. However, biochemical studies will be required to determine if Mkh1 and/or other components of this pathway are activated in response to such conditions.

Mkh1 and filamentous growth. The filamentous multiseptate phenotype of cells lacking Mkh1 indicates that these cells are defective in cytokinesis. However, the heterogeneity of these cells indicates that either cytokinesis is only delayed or only a fraction of cells undergo a physiological change that results in multiseptate growth. Genetic studies in yeast have helped to define several steps and mechanisms involved in cytokinesis (9). In *S. pombe*, an actin-based contractile ring forms in the middle of the cell during early mitosis before anaphase. At the end of mitosis, the ring contracts as the septum forms at the site marked by the actin ring. Normally, the septum is then digested away, leading to the separation of the two daughter cells. However, under certain growth conditions, *S. pombe*, like other dimorphic yeasts, exhibits pseudohyphal growth in which the daughter cells remain attached, resulting in multiseptate filaments similar to those we observed in *mkh1*Δ cells (42). Several genes involved in actin ring and septum formation in fission yeast have been defined (5, 26, 38). However, very little is known about the mechanisms that regulate completion of cytokinesis after septum formation. The pseudohyphal filaments exhibited by *mkh1*Δ cells could result from a defect in cell wall construction, in septum structure, or in the regulation of enzymes that normally digest the septum away prior to cell separation.

Recent studies have shown that pseudohyphal and invasive growth of *S. cerevisiae* requires components of the mating pheromone-responsive MAPK cascade (25, 41). However, there is little information in the literature about pseudohyphal development in *S. pombe*. The filamentous growth of *mkh1*Δ cells suggests that Mkh1 may regulate dimorphic development in this yeast.

Relationship of Mkh1 to other *S. pombe* MAPK pathways. Our observations indicate that Mkh1 is important for a variety of cellular responses to stress and nutrient limitation. By analogy with known MAPK pathways, there are most likely two other kinases that act downstream from Mkh1. The *S. pombe* Wis1-Spc1/Sty1 pathway is also responsive to hypertonic stress, raising the possibility that Wis1 is activated by Mkh1. However, defects in the Wis1 pathway result in phenotypes that are very different from those exhibited by *mkh1*Δ cells. Wis1 was first identified as a mitotic inducer (55), and subsequent evidence suggests that it is required for the regulation of mitosis and intracellular osmotic pressure in response to extracellular osmotic signals (32, 48, 49). Deletion of *wis1* results in G_2 delay and concomitant cell elongation, whereas overexpression of Wis1 causes mitotic initiation to occur at a reduced cell size. Defects in this pathway also result in growth inhibition at high temperature or hyperosmotic conditions (0.8 M KCl). In contrast, overexpression of Mkh1 results in an elongated cell shape, while *mkh1*Δ cells exhibit a round cell shape in standard growth conditions and filamentous growth in hyperosmotic media. *mkh1*Δ cell growth is not temperature sensitive but is inhibited by very high salt levels (>1.2 M KCl). In view of the apparent differences in these phenotypes, it seems unlikely that Mkh1 is an activator of the Wis1-Spc1/Sty1 pathway. Our observation that deletion of *mkh1* and *wis1* results in a synergistic effect further suggests that these proteins act in different pathways. However, our observation that overexpression of Wis1 or GST-Spc1 in *mkh1*Δ or *pck2*Δ cells confers resistance to β-glucanase suggests that these pathways may have related, as well as distinct, functional roles.

As mentioned earlier, the phenotypes resulting from deletion of *pmk1/spm1* are strikingly similar to those of *mkh1*Δ cells. The *mkh1*Δ *spm1*Δ double mutant exhibits phenotypes identical to those of the single mutants. Also, our results indicate that expression of GST-Spm1 complements the *mkh1*Δ phenotypes. In addition, we found that the morphology of the *mkh1*Δ *wis1*Δ double mutant is essentially identical to that exhibited by *spm1*Δ *wis1*Δ cells (59). Together these observations are most consistent with a model where Pmk1/Spm1 acts downstream from Mkh1 in a novel MAPK cascade (Fig. 11); however, we have not completely ruled out the possibility that they act in parallel pathways.

Relationship of Pck2 with Mkh1 and Pmk1/Spm1. Many of our observations suggest that Pck1,2 and Mkh1 act to regulate similar cellular responses. However, the relationship between these proteins appears to be complex (Fig. 11). On the one hand, the similar morphologies, defects in cell wall integrity, and sensitivities to KCl exhibited by *pck2*Δ and *mkh1*Δ mutants suggest that they have related roles. On the other hand, the apparent opposite effects of these proteins on filamentous growth suggest that they function antagonistically. Together, our observations are most consistent with a model where Pck2 and Mkh1 act independent of each other to regulate common cell responses; in some cases they act cooperatively, and in others they act antagonistically.

Our observation that expression of GST-Spm1 partially complements many of the *pck2*Δ mutant phenotypes is also consistent with models where Pmk1/Spm1 acts either downstream or independently of Pck2. However, a previous report that *pck2*Δ and *pmk1*Δ mutations have synergistic effects on cell wall integrity favors a model where these proteins act independently (53). The relationship of these proteins in regulating filamentous growth is less clear. It is possible that they act independently or that one of these proteins negatively regulates the other. For instance, it is possible that Pck2 induces filamentous growth by inhibiting Pmk1/Spm1. Alternatively, deletion of *spm1* could result in activation of Pck2, which could then induce filamentous growth. Clarification of the relationship between these proteins and their functions will require further studies.

Conservation of Mkh1 and Bck1 pathways. In *S. cerevisiae*, the Bck1 MAPK pathway is required for normal cellular responses to various stress conditions. While both *mkh1*Δ and *bck1*Δ cells are defective in cell wall integrity, the phenotypes resulting from deletion of these genes are different. As mentioned earlier, loss of Bck1 results in a temperature-sensitive cell lysis defect. Whether the molecular basis for this defect is similar to that of *mkh1*Δ cells is not clear. Interestingly, growth defects exhibited by Bck1 pathway mutants are more severe in the absence of calcineurin (11). Our observation that expression of Mkh1 fails to complement *bck1*Δ indicates that the functional properties of these related MEKKs have not been highly conserved. However, a previous report indicates that expression of Mpk1, the MAPK downstream from Bck1, can complement deletion of *pmk1* (53). Thus, the specificity for targets of these related MAPK cascades may have been conserved during evolution.

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