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Skh1, the MEK component of the Mkh1 signaling pathway in *Schizosaccharomyces pombe*

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SUMMARY

We previously reported the identification of Mkh1, a MEK kinase in *Schizosaccharomyces pombe* that is required for cell wall integrity, and we presented genetic evidence that Pmk1/Spm1, a MAP kinase, functions downstream from Mkh1 in the same pathway. Here, we report the identification of Skh1, a MEK (MAP kinase kinase) in *S. pombe*. The sequence of Skh1 is nearly identical to that of the recently reported Pek1 sequence. We present biochemical and genetic evidence that Skh1 is the MEK component of the Mkh1-Spm1 MAP kinase cascade. Our yeast two-hybrid results indicate that Mkh1, Skh1, and Spm1 physically interact to form a ternary complex. Deletion of *mkh1*, *skh1* or *spm1* results in identical phenotypes, including sensitivity to β-glucanase treatment, growth inhibition on media containing KCl, and filamentous growth on medium containing caffeine. Double mutant strains exhibit phenotypes that are identical to the single mutant strains. Furthermore, expression of an activated HA-Skh1^{DD} protein suppressed these defects in *mkh1*Δ cells, and overexpression of Spm1 suppressed these defects in *skh1*Δ cells. We also show that HA-Spm1 is hyper-phosphorylated on tyrosine residues in cells co-expressing the activated HA-Skh1^{DD} protein. Furthermore, we found the phosphorylated/activated form of GFP-HA-Spm1 at detectable levels in wild-type cells, but not at appreciable levels in *mkh1*Δ or *skh1*Δ cells expressing this fusion protein. Together, our results indicate that Mkh1, Skh1 and Spm1 constitute a MAPK cascade in fission yeast.

Key words: Fission yeast, Mkh1, MAP kinase, Filamentous growth, Caffeine

INTRODUCTION

MAP kinase (MAPK) cascades constitute a fundamental signaling mechanism that has been conserved among eukaryotic organisms. Such cascades consist of a core MAPK module containing a trio of sequentially acting protein kinases: MEK kinases (MEKKs), which phosphorylate and activate MEKs (MAP kinase kinases), which in turn phosphorylate and activate MAPKs (reviewed by Seger and Krebs, 1995). In *Saccharomyces cerevisiae*, six MAPKs that mediate distinct biological responses to specific stimuli have been defined, including pathways that regulate the mating pheromone response, pseudohyphal development and invasive growth, intracellular osmotic pressure, cell wall biosynthesis, and sporulation (Herskowitz, 1995; Levin and Errede, 1995). However, in fission yeast, only three MAPK pathways have been identified so far. These include the Ras1-activated Byr2 pathway that regulates sexual development and meiosis (Neiman et al., 1993), and the Wis1 pathway that regulates the initiation of mitosis, as well as the appropriate response to osmotic, heat or oxidative stress (Warbrick and Fantes, 1991; Degols et al., 1996; Samejima et al., 1998).

We previously identified Mkh1, a MEKK component of a third MAPK pathway in fission yeast (Sengar et al., 1997). Mkh1 is most closely related to *S. cerevisiae* Bck1. Our genetic analysis indicated that Mkh1, like Bck1, is required for proper cell wall integrity since mutant strains lacking *mkh1* are hypersensitive to β-glucanase. However, *mkh1*Δ cells do not exhibit some phenotypes, such as a temperature sensitive cell lysis or decreased induced thermotolerance, seen in *bck1*Δ cells (Kamada et al., 1995). Also, *mkh1*Δ cells exhibit other phenotypes that are distinct from those seen in *bck1*Δ cells. Most strikingly, *mkh1*Δ cells exhibit filamentous, multi-septate growth when cells are grown at high temperature or in hyper-osmotic conditions, suggesting that Mkh1 is required for normal cellular responses to such stress conditions.

Two other labs independently identified Spm1/Pmk1, a MAP kinase in fission yeast that is most closely related to *S. cerevisiae* Mpk1, the MAPK component of the Bck1 cascade (Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). Deletion of *spm1* results in phenotypes that are essentially identical to those exhibited by *mkh1*Δ cells, suggesting that Mkh1 and Spm1 are components of the same MAPK pathway. Our genetic analysis supports this model. We found that overexpression of Spm1 suppressed the phenotypes resulting from deletion of *mkh1*, whereas overexpression of Mkh1

failed to suppress *spm1*Δ phenotypes; the phenotypes of a *mkh*1Δ*spm1*Δ double mutant strain were essentially identical to those of the single mutants (Sengar et al., 1997).

In this paper, we report the identification of Skh1, a MEK in fission yeast, and we present both biochemical and genetic evidence indicating that Skh1 is the MEK component of the Mkh1/Spm1 MAPK module.

MATERIALS AND METHODS

Yeast strains and genetic analysis

The *S. cerevisiae* strain L40 (*MAT**a** his3 trp1 leu2 ade2 LYS2::lexA-HIS3 URA3::lexA-lacZ*; Vojtek et al., 1993; Hollenberg et al., 1995) has been previously described. The genotypes of *S. pombe* strains used in this study are listed in Table 1. *S. pombe* culture, transformation, iodine staining, mating, tetrad analysis, and other genetic manipulations were performed as previously described (Moreno et al., 1990).

DNA manipulation and analysis

Procedures used for DNA manipulation and analysis (i.e. purification, restriction site mapping, electrophoresis, transformation, etc.) have been previously described (Sambrook et al., 1989). The DNA sequence of both strands of sequenced clones were determined by a modified dideoxy chain-termination method (Sanger et al., 1977), using the Taq DyeDeoxy^{TM} Terminator Cycle Sequencing Kit (Applied BioSystems). PCR was performed as previously described (Matviw et al., 1992).

Plasmids

The yeast two-hybrid expression vector pBTM116 contains the DNA-binding LexA coding sequence under the control of the *ADH1* promoter, the 2μ origin of replication, and the *TRP1* gene (Bartel et al., 1993). Plasmids that express LexA fusion proteins were generated by inserting DNA fragments into the polylinker of pBTM116 located 3′ to the LexA coding sequence. The yeast two-hybrid expression vector pVP16 contains the coding sequence of a nuclear localized VP16 acidic activation domain under the control of the *ADH1* promoter, the 2μ origin of replication, and the *LEU2* gene (Vojtek et al., 1993; Hollenberg et al., 1995). Plasmids that express VP16 fusion proteins were generated by inserting DNA fragments in the polylinker of pVP16.

pAD-HA-Skh1 was generated by cloning the open reading frame of Skh1 downstream of the HA epitope of pAD4H (Hubberstey et al., 1996). pADHis-HA-Skh1 was generated by cloning the 1.7 kb *HIS3* sequence into the *Bst*EII site of the *LEU2* gene of pAD-HA-Skh1, thus disrupting *LEU2* expression. pSKH1, contains a 3.07 kb *Bam*HI/*Hin*dIII genomic DNA fragment, which includes the entire coding region and flanking sequences of *skh1*, cloned in pBluescript II SK− (Stratagene). pSKHΔ was derived from pSKH1 by replacing the 1.0 kb *Sex*AI-*Nde*I fragment, containing the coding region, with a 1.8 kb fragment containing *ura4*. pSPM1, contains a 3.7 kb DNA fragment amplified from genomic DNA, which includes the entire coding region and flanking sequences (5′ 0.8 kb and 3′ 1.2 kb) of *spm1*, cloned in pBluescript II SK−. pSPMΔ was derived from pSPM1 by replacing the 1.65 kb *Sal*I-*Bcl*I fragment with a 1.8 kb fragment containing *ura4*.

pREP3X, pREP41X, and pREP42X are *S. pombe* expression vectors containing the repressible *nmt1* promoter (Maundrell, 1993; Basi et al., 1993). pREP3X-HA contains the DNA sequence (5′CT-CGAGATGTATCCTTATGACGTGCCTGACTATGCCAGCCTGGG-AGGACCGTCGACAACTAGTAGCGGCCGCAGGATCC) encoding the HA epitope and *Sal*I, *Spe*I, and *Not*I restriction sites cloned into the *Xho*I/*Bam*HI sites of pREP3X. pREP41X-HA and pREP42X-HA were made in a similar fashion. pREP41X-GFP-HA was constructed by cloning the coding sequence of enhanced green fluorescent protein (Clontech) into the *Xho*I site of pREP41X-HA such that an eGFP-HA fusion protein is expressed.

pREP41X-HA-Skh1 was generated by cloning the open reading frame of Skh1 downstream of the HA epitope of pREP41X-HA. pREP41X-HA-Skh1^{DD} contains the coding sequence for Skh1^{DD} cloned into pREP41X-HA. Skh1^{DD} contains two mutations that change amino acid residues 234 and 238 each to Asp. This was accomplished by PCR using the mutagenic primer: 5′GAATTG-GTTAACGATCTCGCTGGCGATTTCACTGGA, and a 3′ primer 5′TTGCGGCCG CTAATCAGACCAGACTTG. The 0.4 kb fragment generated was cut with *Hin*cII and *Not*I and cloned in pBluescript II SK− for sequencing. After confirming the generation of only the desired mutations, the wild-type *Hin*cII-*Not*I fragment of pREP41X-HA-Skh1 was replaced by the mutant *Hin*cII-*Not*I fragment to generate pREP41X-HA-Skh^{DD}. The full length HA-Skh1^{DD} fragment was shuttled to pREP42X to generate pREP42X-HA-Skh1^{DD}.

pREP3X-HA-Spm1 was generated by cloning the open reading frame of Spm1 downstream of the HA epitope of pREP3X-HA. pREP41X-HA-Spm1 and pREP41X-GFP-HA-Spm1 were generated in a similar manner.

Yeast two hybrid screen

A *Schizosaccharomyces pombe* cDNA library cloned in the vector pGAD-GH (Clonetech) was obtained from G. Hannon and D. Beach, Cold Spring Harbor Laboratory. The *S. cerevisiae* strain L40 containing pLexA-Mkh1 was transformed with *S. pombe* cDNA library using a high-efficiency transformation method (Schiestl and Gietz, 1989; Hill et al., 1991; Vojtek et al., 1993). These transformants were grown in synthetic medium (YC-Trp-Ura-Leu-Lys) for 16 hours at 30°C to obtain efficient expression of the *HIS3* reporter gene. The transformants were then plated on synthetic plates (Yc-Trp-His-Ura-Leu-Lys) to select for cells that express His3. The plates were incubated for 3-5 days, and colonies were picked and tested for β-galactosidase activity by a filter assay (Breeden and Nasmyth, 1985). Approximately 500 His$^+$ transformants were obtained from 5×10^6 primary transformants. A subset of these were further analyzed by segregation analysis to yield 41 colonies that were dependent on the pLexA-fusion plasmid for their His$^+$LacZ$^+$ phenotype. The pVP16-fusion plasmids were isolated from plasmid-dependent colonies, re-transformed into L40 expressing either LexA-Mkh1 or LexA-lamin, and the assays for His3 expression and β-galactosidase activity were repeated to verify the plasmid-dependent phenotype.

Identification of *skh1* genomic clones

A 0.8 kb *Eco*RI fragment derived from a plasmid isolated in the two-hybrid screen was used as a probe to screen an *S. pombe* genomic library by filter hybridization. Positive clones were purified and analyzed by restriction mapping. Subsequently, a 3073 bp *Bam*HI-*Hin*dIII fragment containing the entire *Skh1* coding region and flanking sequences was subcloned into pBluescript II (Stratagene) and the DNA sequence was determined.

Nucleotide accession number

The DNA sequence of *skh1* has been deposited in GenBank (accession no. AF157632).

Gene disruption

The *skh1* gene was disrupted in the *S. pombe* diploid strain SP826 by the gene replacement method (Rothstein, 1983; Russel and Nurse, 1986). SP826 was transformed with the 2.4 kb *Hin*cII-*Hin*dIII fragment of pSKH1Δ, in which the *skh1* coding region has been replaced with *ura4*, and Ura+ transformants were selected on PMA + Leu media. Ten independent transformants were tested for stability of the Ura+ phenotype, and they were analyzed by Southern blots to confirm that they contained the proper disruption in one copy of the endogenous *skh1* genes. h^{90}/h^{+n} revertants of these strains, which

Table 1. *S. pombe* strains

Strain	Genotype	Source
SP826	*h⁺/h⁺ leu1-32/leu1-32 ura4-d18/ura4-d18 ade6-M210/ade6-M216*	Dr David Beach
RL127	*h⁺ leu1-32 ade6-M210*	This study
RL143	*h⁺ leu1-32 ura4-d18 ade6-M210*	This study
AS1-11b	*h⁺ leu1-32 ura4-d18 ade6-M210 mkh1::ura4*	Sengar et al., 1997
RL142	*h⁺ leu1-32 ura4-d18 ade6-M210 skh1::ura4*	This study
RL167	*h⁺ leu1-32 ura4-d18 ade6-M210 spm1::ura4*	This study
RL233	*h⁺ leu1-32 ura4-d18 ade6-M210 mkh1::ura4 skh1::ura4*	This study
RL313	*h⁺ leu1-32 ura4-d18 ade6-M210 skh1::ura4 spm1::ura4*	This study

occur at a frequency of approximately 10^{-3}, were detected by the iodine vapor staining test. The haploid strains RL142 (Ura$^+$) and RL143 (Ura$^-$) were derived from spores of a single ascus from one such revertant.

The *spm1* gene was disrupted in the haploid strain RL143 by transformation with the 3.2 *Bam*HI-*Aat*II fragment of pSPMΔ. Stable Ura+ transformants were analyzed by Southern blots to confirm that they contained the proper disruption of the endogenous *spm1* gene.

β-Glucanase sensitivity and cell wall regeneration experiments

β-Glucanase sensitivity of various mutant strains was determined using a previously described procedure (Levin and Bishop, 1990). Briefly, cells were grown in PMA without thiamine to an OD_{600} = 1.0, washed in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, and incubated in the same buffer containing 100 μg/ml β-glucanase (Zymolyase-20T, ICN) 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}.

Activation and detection of HA-Spm1

For heat shock activation of Spm1, 250 ml cultures were grown to an OD_{600} = 1 at which point 50 ml were aliquoted to pre-warmed 250 ml flasks and incubated at 50°C for either 0, 5 or 15 minutes. Cells were collected by centrifugation, resuspended in Breaking Buffer (200 mM NaCl, 200 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.2 mM PMSF, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM benzamidine HCl) and lysed with glass beads using the Mini-Beadbeater-8 (Biospec Products). Beads and cellular debris were removed by centrifugation and protein concentrations were determined. 30 μg of protein from total cell lysates was separated by SDS-PAGE, electrophoretically transferred to nitrocellulose and immunoblotted with either 12CA5 (anti-HA epitope), 4G10 (anti-phosphotyrosine, kind gift of S. Robbins), or anti-activated MAPK (affinity-purified rabbit antiserum to a phosphopeptide: DHTGFLpTEpYVATRWC from NEB). Primary antibodies were detected with goat anti-mouse HRP conjugate (Pierce) (12CA5, 4G10) or goat anti-rabbit HRP conjugate (Pierce) (anti-activated MAPK) and visualized with enhanced chemiluminescence (ECL) (Amersham) as per the manufacturer's recommendations.

For immunoprecipitation of samples in Fig. 6B, 10 mg protein lysate was diluted to 1.5 ml with Breaking Buffer and precleared with 50 μl of a 50% Protein A Sepharose slurry. Subsequently, 7 μl of 12CA5 ascites fluid was added, samples were incubated overnight at 4°C, immune complexes were collected with 50 μl of Protein A Sepharose slurry, and the beads were washed 4× for 10 minutes each with 0.8 ml Breaking Buffer at 4°C. One third of immunoprecipitates were analyzed by western blots, as described above.

RESULTS

Identification of Skh1, and comparison with known MAPK kinases

In order to identify components of the Mkh1 signaling pathway we performed a yeast two-hybrid screen for proteins that interact with a LexA-Mkh1 fusion protein. A *S. pombe* cDNA library, encoding GAD fusion proteins, was screened using LexA-Mkh1 as a bait (Materials and Methods). We identified and analyzed forty one clones that exhibited plasmid-dependent transactivation of reporter genes when co-expressed with LexA-Mkh1, but failed to transactivate reporter genes when co-expressed with LexA-lamin. Restriction mapping and

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Skh1      1 MSKKPVLNLDTSNGFSEEYISHPERNDNQGIVEITDLVFSSESKLTQRKESRDSKTFVPSFLEELDDDHLHELVTNGGILYMNS

                    I           II                III         IV                    V
Skh1     85 LGEGVSGSVRKCRIRGTQMIFAMKTVLAAP-NTALQKQLLRELKINRSCTSPYIVKYYGACYNNAECQLNIAMEYCGAGSLDAIYKRVRSQGGRTGE
Mkk1    227 LGEGAGGSVSKCKLKNGSKIFALKVINTLNTDPEYQKQIFRELQFNRSFQSEYIVRYYGMFTDDENSSIYIAMEYMGGRSLDAIYKNLLERGGRISE
Byr1     72 LGEGNGGAVSLVKHRN---IFMARKTVYVGSDSKLQKQILRELGVLHHCRSPYIVGFYGAFQYKNN--ISLCMEYMDCGSLDAILR----EGGPIPL
Mek5b   173 LGHGNGGTVYKAYHVPSGKILAVKVILLDI-TLELQKQIMSELEILYKCDSSYIIGFYGAFFVENR--ISICTEFMDGGSLDVYRK--------MPE

                          VI                VII                   VIII          IX
Skh1     RPLGKIAFGVLSGLSYLHDR-KIIHRDIKPSNILLTSKGQVKLCDFGVSGELVNSLAGTFTGTSYYMAPERISGGSYTISSDIWSLGLTLMEVALNRFPF
Mkk1     KVLGKIAEAVLRGLSYLHEK-KVIHRDIKPQNILLNENGQVKLCDFGVSGEAVNSLATTFTGTSFYMAPERIQGQPYSVTSDVWSLGLTILEVANGKFPC
Byr1     DILGKIINSMVKGLIYLYNVLHIIHRDLKPSNVVVNSRGEIKLCDFGVSGELVNSVAQTFVGTSTYMSPERIRGGKYTVKSDIWSLGISIIELATQELPW
Mek5b    HVLGRIAVAVVKGLTYLWSL-KILHRDVKPSNMLVNTRGQVKLCDFGVSTQLVNSIAKTYVGTNAYMAPERISGEQYGIHSDVWSLGISFMELALGRFPY

                          X                XI
Skh1     PPEGSPP--PMPIELLSYIINMPPPLLPQ-EPGIKWSKSFQHFLCVCLDKDKTRRPGPQKMLTHPWVKAFERIHVD-MEEFLRQVWSD          363
Mkk1     SSEKMAAN-IAPFELLMWILTFTPELKDEPESNIIWSPSFKSFIDYCLKKDSRERPSPQMINHPWIKGQMKKNVN-MEKFVRKCWKD          508
Byr1     SFSNIDDS-IGILDLLHCIVQEEPPRLPS-----SFPEDLRLFVDACLHKDPTLRASPQQLCAMPYFQQALMINVD-LASWASNFRSS          340
Mek5b    PQIQKNQGSLMPLQLLQCIVDEDSPVLPVG----EFSEPFVHFITQCMRKQPKERPAPEELMGHPFIVQFNDGNAAVVSMWVCRALEERRSQQGPP 448
```

Fig. 1. Comparison of MAPK kinases. *S. pombe* Skh1, *S. cerevisiae* Mkk1 (Irie et al., 1993), *S. pombe* Byr1 (Nadin-Davis and Nasim, 1988), and *H. sapiens* Mek5b (Zhou et al., 1995) were aligned by the Clustal-W program (Thompson et al., 1994). Regions of protein sequence identity with Skh1 are shaded. Roman numerals I to XI indicate the positions of conserved kinase subdomains (Hanks et al., 1988).

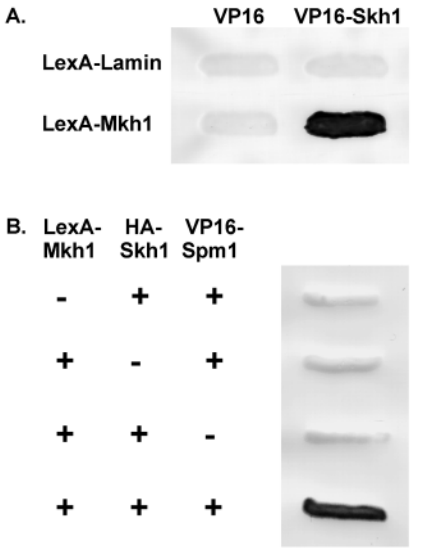

Fig. 2. Mkh1, Skh1, and Spm1 interact to form a complex. (A) VP16-Skh1 was tested for its ability to interact with LexA-Lamin or LexA-Mkh1 by the yeast two-hybrid test (Materials and Methods). Each patch represents an independent transformant of the yeast-two hybrid tester strain L40 expressing the indicated proteins. Interaction between VP16-Skh1 and LexA-fusion proteins was assayed by their ability to induce expression of β-galactosidase. (B) Simultaneous co-expression of LexA-Mkh1, HA-Skh1, and VP16-Spm1 result in transactivation of the β-galactosidase reporter gene. All three fusion proteins were required for transactivation, indicating that they form a ternary complex in vivo.

DNA sequence analysis revealed that these clones belonged to three classes. One class, comprised of seven clones, contained insert sequences derived from a gene that we have named *skh1*.

As a prelude to biochemical and genetic studies, the genomic locus of *skh1* was cloned and characterized (Materials and Methods). *skh1* encodes a 363 amino acid protein with significant homology to known yeast and metazoan MEKs, and it contains motifs that are specifically conserved among MEKs (Fig. 1). Thus, Skh1 appears to be a member of the MEK family of kinases. Skh1 is most closely related (46% identical) to Mkk1 and Mkk2, the MEK components of the *S. cerevisiae* Bck1 pathway. Byr1 is the most similar *S. pombe* kinase to Skh1; and MEK5b is the most similar human MEK. Unlike the dual MEKs of the Bck1 pathway, southern blot hybridization of the Skh1 locus did not suggest the existence of a closely related homologue (data not shown).

Skh1 forms a complex with Mkh1 and Spm1

To investigate whether Mkh1, Skh1, and Spm1 constitute a MAPK module, we first sought to demonstrate a physical interaction between these kinases. First, we demonstrated that VP16-Skh1 (full length) interacts with LexA-Mkh1 by the yeast two-hybrid test (Fig. 2A). Unfortunately, it was not possible to use a simple yeast two-hybrid test to determine if Skh1 interacts with Spm1 since both LexA-Skh1 and LexA-Spm1 transactivated the reporter genes on their own (unpublished results). Instead, we demonstrated that while co-expression of LexA-Mkh1 and VP16-Spm1 failed to cause transactivation of the reporter β-galactosidase gene, simultaneous co-expression with HA-Skh1 does induce expression of β-galactosidase (Fig. 2B). This suggests that Mkh1, Skh1, and Spm1 form a stable ternary complex.

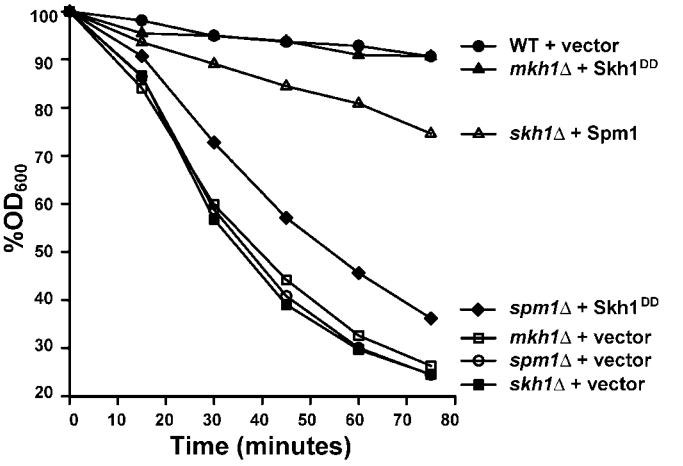

Fig. 3. Cell wall integrity. *mkh1*Δ, *skh1*Δ, and *spm1*Δ cells are hypersensitive to β-glucanase treatment. Sensitivity of *mkh1*Δ cells can be rescued by expression of HA-Skh1^{DD}, an activated allele of Skh1; and sensitivity of *skh1*Δ cells can be rescued by expression of HA-Spm1. Cell lysis was measured at different times during treatment of cell cultures with β-glucanase by determining the OD_{600} (Materials and Methods). The strains examined were RL127 (WT), AS1-11b (*mkh1*Δ), RL142 (*skh1*Δ), and RL167 (*spm1*Δ) containing either the vector pREP3X-HA, pREP41X-HA-Skh1^{DD} (HA-Skh1^{DD}) or pREP3X-HA-Spm1 (HA-Spm1).

Skh1 functions upstream of Spm1, and downstream of Mkh1

To further investigate the function of Skh1 we constructed and examined the phenotypes of *S. pombe* strains in which *skh1* is disrupted. The coding sequence of *skh1* was replaced with *ura4* in a diploid strain (Materials and Methods). Tetrad analysis was performed on several independently derived diploid transformants containing the *skh1* disruption. In most cases asci contained four viable spores, and two of the spores were

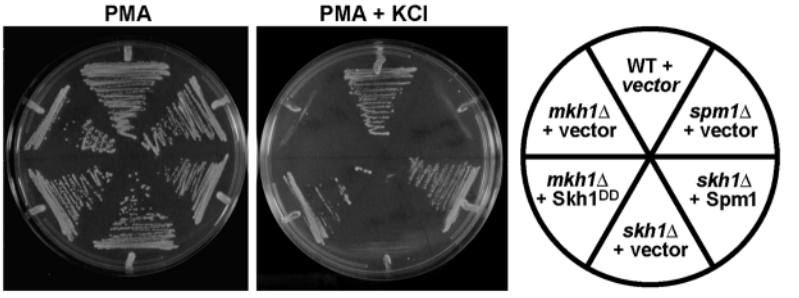

Fig. 4. Growth inhibition by KCl. Growth of *mkh1*Δ, *skh1*Δ, and *spm1*Δ cells is inhibited on media containing KCl. The growth defect of *mkh1*Δ cells can be rescued by expression of HA-Skh1^{DD}; and sensitivity of *skh1*Δ cells can be rescued by expression of HA-Spm1. RL127 (WT), AS1-11b (*mkh1*Δ), RL142 (*skh1*Δ), and RL167 (*spm1*Δ) containing either the vector pREP3X-HA, pREP41X-HA-Skh1^{DD} (HA-Skh1^{DD}) or pREP3X-HA-Spm1 (HA-Spm1) were grown on PMA plates containing 1.2 M KCl for 5 days at 35°C.

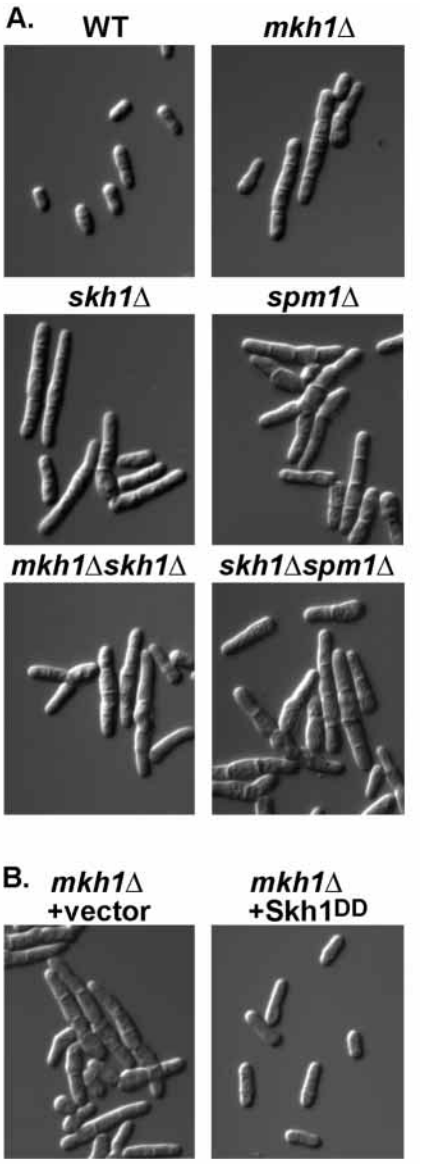

Fig. 5. Caffeine induced filamentous growth. (A) AS1-11b (*mkh1*Δ), RL142 (*skh1*Δ), and RL167 (*spm1*Δ) single mutant cells or RL233 (*mkh1*Δ/*skh1*Δ) and RL313 (*skh1*Δ/*spm1*Δ) double mutant cells were grown on either YEA or YEA with 8 mM caffeine for two days. (B) Caffeine induced filamentous growth of *mkh1*Δ cells is suppressed by expression of HA-Skh1^{DD}. AS1-11b (*mkh1*Δ) containing either vector pREP41X-HA or pREP41X-HA-Skh1^{DD} were grown on PMA with 5 mM caffeine for two days. Cells were examined by differential interference contrast microscopy.

Ura$^+$, indicating that they contained the disrupted *skh1* allele. Thus, *skh1* is not required for germination or cell growth. We also found that *skh1*Δ cells, like *mkh1*Δ and *spm1*Δ cells, have a significantly reduced mating efficiency than normal cells (data not shown).

We found that *skh1*Δ cells, like *mkh1*Δ and *spm1*Δ cells, are also hypersensitive to β-glucanase treatment, suggesting that they have a similar cell wall defect (Fig. 3). We previously reported that overexpression of GST-Spm1 could rescue the β-glucanase sensitivity of *mkh1*Δ cells (Sengar et al., 1997). Similarly, we have found that overexpression of HA-Spm1 rescues the β-glucanase hypersensitivity of *skh1*Δ cells, suggesting that Spm1 may function downstream from Skh1 (Fig. 3). Interestingly, overexpression of HA-Skh1 did not rescue β-glucanase hypersensitivity of *mkh1*Δ cells (data not shown). To determine if an activated form of Skh1 is capable of rescuing, we generated an activated allele of Skh1. MEKs are activated by phosphorylation of two Ser/Thr residues located between the kinase subdomains VII and VIII (Zheng and Guan, 1994; Johnson et al., 1996); and previous studies have shown that mutation of these residues to the charged amino acid Asp results in constitutive activity (Pagès et al., 1994; Shiozaki et al., 1998). Therefore, we generated a Skh1 mutant (HA-Skh1^{DD}) in which the conserved phosphorylation sites at positions 234 and 238 were both replaced with Asp (Materials and Methods). While HA-Skh1 did not rescue β-glucanase hypersensitivity of *mkh1*Δ cells, expression of the constitutively active mutant HA-Skh1^{DD} was able to rescue (Fig. 3). HA-Skh1^{DD} was not able to rescue the β-glucanase sensitivity of *spm1Δ* cells. These results are consistent with a model where Skh1 is activated by Mkh1, and Skh1 in turn activates Spm1.

Similar to *mkh1*Δ cells, both *skh1*Δ and *spm1*Δ cells fail to form colonies on synthetic medium containing 1.2 M KCl (Fig. 4). We previously reported that overexpression of GST-Spm1 complemented this phenotype in *mkh1*Δ cells. In corroboration with the β-glucanase rescue results, we have found that overexpression of HA-Spm1 also suppressed the growth sensitivity of *skh1*Δ cells to KCl, suggesting that Spm1 functions downstream from Skh1 (Fig. 4). Also, expression of HA-Skh1^{DD} suppressed the growth sensitivity of *mkh1*Δ cells, suggesting that Skh1 functions downstream from Mkh1 (Fig. 4).

*mkh1*Δ, *skh1*Δ, and *spm1*Δ cells display a striking morphology when grown in the presence of caffeine

S. cerevisiae cells deficient in Bck1 pathway signaling exhibit growth sensitivity in the presence of caffeine (Watanabe et al., 1997). Since the Bck1 pathway appears to be the orthologous pathway to the *S. pombe* Mkh1 pathway, we wished to determine if *mkh1*Δ cells are also sensitive to caffeine. Interestingly, we observed that *mkh1*Δ cells grown on medium containing 8 mM caffeine display a filamentous phenotype, similar to that we previously observed when these cells are grown under stress and nutrient limiting conditions (Fig. 5A) (Sengar et al., 1997). Under these growth conditions, *mkh1*Δ cells become greatly elongated; they fail to complete cytokinesis, as indicated by the presence of multiple septa; and they are often branched. We found that *mkh1*Δ, *skh1*Δ, and *spm1*Δ cells exhibit identical morphologies when grown in the presence of caffeine (Fig. 5A). Furthermore, *mkh1*Δ*skh1*Δ and *skh1*Δ*spm1*Δ double mutant strains exhibit morphologies identical to the single mutant strains, suggesting that the phenotypes are neither additive nor synergistic. Expression of HA-Skh1^{DD} suppressed the caffeine induced filamentous growth phenotype of *mkh1*Δ cells (Fig. 5B). These observations complement our epistasis results and suggest that Mkh1, Skh1 and Spm1 function in the same signaling pathway.

Skh1 regulates the activation of Spm1 in vivo

MAPKs are activated by phosphorylation at a pair of conserved

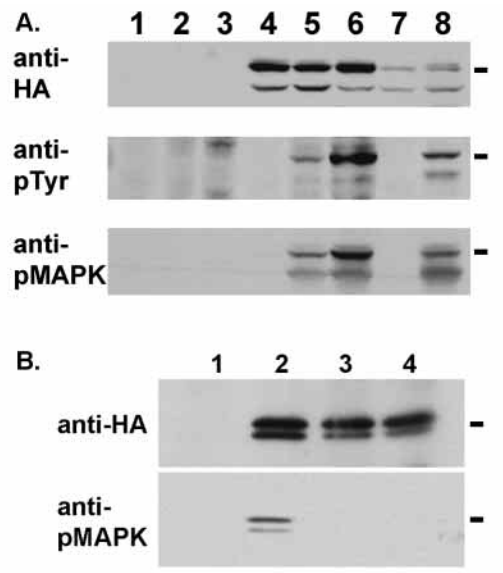

Fig. 6. Skh1 phosphorylates Spm1. (A) Cell extracts were examined by western blot analysis using 12CA5 antibody (anti-HA) (top panel) as a control for expression; 4G10 antibody (anti-phosphotyrosine) (middle panel); and anti-phospho-MAPK antibody (bottom panel) (Materials and Methods). The positions of HA-Spm1 are indicated by the dash on the right of each blot. RL127 (WT) cells containing vector (lanes 1 to 3), or expressing HA-Spm1 (lanes 4 to 6) were heat shocked at 50°C for 0, 5, or 15 minutes. Untreated RL143 (WT) cells expressing HA-Spm1 (lane 7) or co-expressing HA-Spm1 and HA-Skh1^{DD} (lane 8) were also examined. Plasmids used to express various proteins were pREP42X-HA (vector), pREP41X-HA-Spm1 (HA-Spm1), and pREP42X-HA-Skh1^{DD} (HA-Skh1^{DD}). (B) Extracts from (1) RL127 (WT) cells containing a control plasmid, or (2) RL127 (WT), (3) RL142 (*skh1*Δ), or (4) AS1-11b (*mkh1*Δ) cells expressing GFP-HA-Spm1 were immunoprecipitated with anti-HA (12CA5) antibody and western blots were performed using either anti-HA (top) or anti-phospho-MAPK (bottom) (Materials and Methods). The positions of GFP-HA-Spm1 are indicated on the right of each blot. Plasmids used were pREP41X-GFP-HA (control) and pREP41X-GFP-HA-Spm1.

Thr and Tyr residues (Marshall, 1994; Cobb and Goldsmith, 1995). It was previously reported that a GST-Spm1 fusion is phosphorylated and activated in response to heat-shock treatment (Zaitsevskaya-Carter and Cooper, 1997). We found a similar result using HA-epitope tagged Spm1 (Fig. 6A). In western blots of cell extracts expressing HA-Spm1, we observed two major HA-Spm1 bands (48 kDa and 52 kDa) which become hyper-phosphorylated in response to heat-shock. Multiple phosphorylated and non-phosphorylated forms of endogenous Spm1 have been previously reported in other studies (Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). We currently do not understand the nature of these bands: they may represent distinct isoforms of Spm1, but the lower band may be a C-terminal degradation product.

To demonstrate that Skh1 regulates Spm1 activity, we examined the phosphorylation of HA-Spm1 in strains expressing the activated HA-Skh1^{DD} mutant. Anti-phosphotyrosine, and anti-activated MAPK antibodies detected much higher levels of phosphorylated HA-Spm1 from cells coexpressing HA-Skh1^{DD} than from control cells (Fig. 6A). The anti-activated-MAPK antibody specifically recognizes

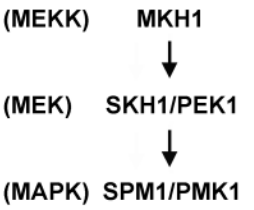

Fig. 7. Model of Mkh1 kinase cascade.

MAPKs that are phosphorylated at the Thr and Tyr residues required for activation (Marshall, 1994; Cobb and Goldsmith, 1995), and it has been previously shown to recognize activated Spm1 (Zaitsevskaya-Carter and Cooper, 1997). Also, the HA-Spm1 phosphorylation pattern induced by HA-Skh1^{DD} is identical to that observed in cells exposed to heat-shock, which has been previously shown to activate Spm1 (Zaitsevskaya-Carter and Cooper, 1997). Thus, our results strongly suggest that activated Skh1 is capable of phosphorylating and activating Spm1 in vivo.

To explore whether activation of Spm1 is dependent on Mkh1 and Skh1, we examined the phosphorylation of a GFP-HA-Spm1 fusion protein in *mkh1*Δ and *skh1*Δ cells (Fig. 6B). We found detectable levels of GFP-HA-Spm1 in an active phosphorylated state in wild-type cells, as determined by immunoprecipitation with anti-HA antibody followed by western blots using anti-phospho-MAPK antibody. This observation is consistent with previous reports (Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1996). However, we did not detect appreciable levels of the activated form of GFP-HA-Spm1 in *skh1*Δ or *mkh1*Δ cells. Thus, both Skh1 and Mkh1 are required for appropriate phosphorylation and activation of Spm1 in vivo.

DISCUSSION

Skh1 shares very strong homology with the catalytic domains of known MEKs. MEKs are dual-specificity kinases that phosphorylate Thr and Tyr residues in the conserved TXY motif of MAPKs, but they contain catalytic subdomains that are characteristic of Ser/Thr kinases (Hanks et al., 1988). However, MEKs also contain specific conserved motifs that are not found in other kinases, and thus they represent a distinct structural class of kinases. The strong homology between Skh1 and known MEKs, and our biochemical evidence that Skh1 can phosphorylate a putative MAPK, indicate that Skh1 is a member of the MEK family of dual-specificity kinases.

We previously reported genetic evidence that Spm1 (MAPK) functions downstream from Mkh1 (MEKK) in the same pathway: deletion of either or both *mkh1* or *spm1* result in the same phenotypes, and overexpression of Spm1 complements deletion of *mkh1* (Sengar et al., 1997). In this report we provide biochemical and genetic evidence that Skh1 is the MEK component of the Mkh1/Spm1 MAPK cascade. During the preparation of this manuscript, Sugiura et al. (1999) reported the identification of Pek1. The reported partial sequence of Pek1 appears to be nearly identical to Skh1. Similar to our observations, they found that Spm1 is hyper-phosphorylated on tyrosine residues in cells expressing an activated Pek1/Skh1 protein. Also, we similarly found that

phosphorylation of Spm1 requires Pek1/Skh1, but we further demonstrated that such phosphorylation is also dependent on Mkh1. Our genetic analyses also complement and extend their studies suggesting that *skh1* is epistatic to *mkh1*, while *spm1* is epistatic to *mkh1* and *skh1*. Similar to our findings, Sugiura et al. (1999) observed that expression of wild-type Pek1/Skh1 failed to rescue *mkh1*Δ phenotypes, whereas activated Pek1^{DD} did rescue. They further demonstrated that overexpression of Pek1/Skh1 or an unactivatable allele of Pek1/Skh1 repressed Spm1 signaling; possibly, by physically interacting with phosphorylated Spm1 and inhibiting its activity. Our results confirm their observations suggesting that Pek1/Skh1 is a novel MEK that acts upstream of Spm1 and downstream of Mkh1.

In addition, we provide evidence that these three kinases can physically interact to form a complex, as indicated by our yeast two-hybrid data. Our results indicate that while Mkh1 and Spm1 do not directly interact, they will form a ternary complex with Skh1 in a heterologous yeast. Some MEKs, such as *S. cerevisiae* Pbs2, have been shown to function as scaffolding proteins that facilitate the formation of specific MAPK modules (Posas and Saito, 1997). Although Skh1 does not contain a long N-terminal domain, like Pbs2 and other scaffolding MEKs, it may provide the specific interactions necessary for the assembly of a MAPK module in fission yeast. Perhaps overexpression of Pek1/Skh1 disrupts formation of the ternary kinase complex and contributes to the observed inhibition of Spm1 activity (Suguira et al., 1999).

Our genetic analyses support a model where Mkh1, Skh1, and Spm1 are components of the same MAPK pathway. Deletion of *skh1* results in phenotypes that are identical to those of *mkh1*Δ or *spm1*Δ mutants. This result suggests that another MEK with a redundant function does not exist. Furthermore, double mutants do not exhibit more exaggerated or distinct phenotypes. Our complementation analysis indicate that expression of the activated HA-Skh1^{DD} kinase can complement deletion of *mkh1*, but does not complement deletion of *spm1*; while expression of HA-Spm1 can complement deletion of either *mkh1* or *skh1*. These results are consistent with a model where Skh1 functions downstream from Mkh1, and Spm1 functions downstream from Skh1 in the same pathway (Fig. 7).

Deletion of *mkh1*, *skh1*, or *spm1* result in several phenotypes, including sensitivity to β-glucanase treatment, growth inhibition on medium containing KCl, and filamentous growth under stress conditions. The molecular defect(s) responsible for these phenotypes remain unclear. However, the similarity of the β-glucanase sensitive phenotype with that of *bck1*Δ cells suggests that the Mkh1/Skh1/Spm1 pathway may regulate genes involved in cell wall biosynthesis.

In our previous report we found that *mkh1*Δ cells exhibit filamentous growth in response to high temperature, KCl, and nutrient limitation. In this paper we show that mutant cells exhibit a similar morphological change when they are grown on media containing caffeine. The diverse cytological effects caused by caffeine in mammals and microorganisms have lead to the proposal that there are multiple cellular targets for this drug (Nehlig and Debry, 1994; Kumada et al., 1996). One reported effect of caffeine is the amplification of cAMP signaling by inhibiting cAMP phosphodiesterase activity in yeasts (Sutherland and Rall, 1958; Beach et al., 1985). However, we found that *mkh1*Δ cells have a normal morphology when grown in medium containing 10 mM cAMP (data not shown) suggesting that caffeine induces the aberrant morphology independently of cAMP signaling. Recently, several *S. pombe* genes involved in caffeine-resistance have been identified (Kumada et al., 1996; Benko et al., 1997, 1998). Further studies may shed light on possible relationships between Mkh1 signaling and these *caf* genes.

Mkh1 is most closely related to *S. cerevisiae* Bck1, and Spm1 is most closely related to the MAP kinase component of the Bck1 pathway. Not surprisingly, the most closely related proteins to Skh1 are the two MEK components of the Bck1 pathway. In addition to this structural conservation, the Mkh1 and Bck1 signaling pathways may share some functional similarities: mutations in both pathways result in defects in cell wall integrity. Also, heterologous expression of *S. cerevisiae* Mpk1 can partially suppress the cell wall integrity defect of *spm1*Δ cells (Toda et al., 1996). Thus the Mkh1 pathway seems to be orthologous to the *S. cerevisiae* Bck1 pathway. Whether regulatory or effector components of these MAPK pathways are conserved remains to be determined.

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