Spm1, a stress-activated MAP kinase that regulates morphogenesis in *S.pombe*

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A gene encoding a novel MAP kinase family member, Spm1, was isolated from the fission yeast *Schizosaccharomyces pombe*. Overproduction of Spm1 inhibits proliferation. Disruption of the *spm1*⁺ gene interferes with cell separation and morphogenesis. Under conditions of nutrient limitation, hypertonic stress or elevated temperature, *spm1*Δ cells grow as short branched filaments in which the cell walls and septa are thickened, suggesting defects in polarized growth and cell wall remodeling. At high osmolarity, *spm1*Δ cells fail to form colonies. The Spm1 protein is tyrosine phosphorylated and activated in response to osmotic and heat stress, consistent with a role for Spm1 in adaptation to these conditions. Two other *S.pombe* MAP kinases are known, Spk1, required for sexual differentiation and sporulation, and Spc1/Sty1/Phh1, which is activated in hypertonic conditions. However, the distinctive features of the *spm1*Δ mutant phenotype and direct biochemical assays suggest that Spm1 does not lie on other known MAP kinase pathways. Our results demonstrate the existence of a new MAP kinase pathway that regulates cell wall remodeling and cytokinesis in response to environmental stresses.

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Introduction

Protein kinase cascades regulate cytoplasmic and nuclear events in response to many types of external stimuli (Cano and Mahadevan, 1995; Herskowitz, 1995; Levin and Errede, 1995). MAP kinase (mitogen-activated protein kinase) cascades are ancient and conserved signaling cassettes found in unicellular and multicellular eukaryotes. Each MAP kinase cassette comprises a series of three or more protein kinases, each phosphorylating and thereby activating the next in line. The last kinase of the series (the MAP kinase or MAPK) is activated by dual phosphorylation on a specific threonine and a specific tyrosine residue. The two activating phosphorylation sites are separated by a single residue, and the Thr–Xaa–Tyr activation motif is a hallmark of MAP kinases. Both residues are phosphorylated by a single activating kinase, the MAPK kinase (MAPKK), which is activated in turn by phosphorylation on one or more serine or threonine residues by a MAPKK kinase (MAPKKK). There is some

cross-talk between pathways, but MAP kinase cassettes appear to be insulated from each other by the intrinsic specificity of the MAPKKs and MAPKKKs, and possibly by binding interactions that may organize the cassettes into multienzyme complexes (Cano and Mahadevan, 1995; Herskowitz, 1995; Levin and Errede, 1995).

The distinct biological functions of different MAP kinases have been demonstrated genetically in the budding yeast, *Saccharomyces cerevisiae*. The MAP kinase Smk1 is needed for sporulation, Hog1 for adaptation to hypertonic conditions, Mpk1/Slt2 for cell wall and morphological changes involved in polarized growth and the two MAP kinases Kss1 and Fus3 for transcriptional and cell cycle responses to mating pheromone. Parts of the signaling pathway that regulates Kss1 and Fus3 are also required for induction of pseudohyphal growth of diploids and invasive growth of haploids in response to nitrogen deprivation.

In contrast with the four or more MAP kinase cascades in budding yeast, until recently only a single MAP kinase cascade was known in the fission yeast, *Schizosaccharomyces pombe*. This cascade is required for mating and sporulation and includes the MAP kinase Spk1, the MAPKK Byr1, the MAPKKK Byr2 and the small GTPase, Ras1 (Nadin-Davis and Nasim, 1990a,b; Wang *et al.*, 1991; Gotoh *et al.*, 1993; Hughes *et al.*, 1993; Neiman *et al.*, 1993). Mutant haploid cells lacking any one of these gene products are unable to mate and mutant diploid cells do not sporulate. The Spk1 cascade regulates expression of genes such as *mat1-P_m* that are required for conjugation and for induction of meiosis in diploids (Nielsen *et al.*, 1992; Nielsen, 1993; Aono *et al.*, 1994). Surprisingly, the transcription of another pheromone-induced gene, *mam2*⁺, depends on Byr1 and Byr2 but not Spk1 (Xu *et al.*, 1994). This means that potentially there is another Byr1- and Byr2-dependent MAP kinase in *S.pombe* which is required for *mam2*⁺ expression and consequently for mating.

Recently, a second MAP kinase, Spc1/Sty1/Phh1 (called here Spc1), has been identified in *S.pombe* (Millar *et al.*, 1995; Shiozaki and Russell, 1995a; Kato *et al.*, 1996). The activator of Spc1 is the MAPKK, Wis1 (Warbrick and Fantes, 1991; Millar *et al.*, 1995; Shiozaki and Russell, 1995a). Spc1 and Wis1 are required for cells to adapt to hypertonic conditions, high temperature and oxidative stress, and to suppress the calcium sensitivity of phosphatase-deficient cells (Shiozaki and Russell, 1995b; Degols *et al.*, 1996). Spc1 is needed for the expression of specific stress-induced genes (Degols *et al.*, 1996). Hypertonic stress also induces a morphological change in *spc1*[−] or *wis1*[−] mutant but not wild-type cells. Mutant cells become elongated, due to prolonged growth in G₂ and delayed entry into M phase (Millar *et al.*, 1995; Shiozaki and

Russell, 1995a). This MAP kinase pathway may also be important for sensing nutrients. When starved for nitrogen, *wis1Δ* and *spc1Δ* cells fail to prepare for mating. They do not arrest in G₁, induce gene expression or mate efficiently (Kano *et al.*, 1996; Stettler *et al.*, 1996). Spc1 may normally induce gene expression through the transcription factors Atf1 and Pcr1 (Takeda *et al.*, 1995; Shiozaki and Russell, 1996; Stettler *et al.*, 1996; Watanabe and Yamamoto, 1996; Wilkinson *et al.*, 1996).

We have identified a third MAP kinase, Spm1 (*S.pombe* MAPK), in *S.pombe*. Like Spc1, Spm1 is required for survival in hypertonic conditions. *spm1Δ* cells undergo unusual morphological changes in media of increased tonicity, low glucose, or at elevated temperature. Sibling cells remain attached end-to-end through multiple divisions, and non-axial growth is observed. The septa are thicker than usual and fail to lyse. Perhaps as a consequence of these changes, cells lacking Spm1 mate inefficiently. Overproduction of Spm1 inhibits cell proliferation. Consistent with the influence of environment on *spm1Δ* cells, we show that Spm1 is phosphorylated on tyrosine and activated under hypertonic and heat shock conditions. Unlike Spc1, Spm1 is not activated by Wis1 and, unlike Spk1, Spm1 activation does not depend on Ras1. Thus Spm1 is a member of a novel MAP kinase cascade linking morphogenesis to environmental conditions.

Results

Identification of novel MAP kinase gene in *S.pombe*

To identify potential MAP kinase genes in *S.pombe*, genomic DNA was subjected to PCR with primers based on sequences conserved between MAP kinases (see Materials and methods). Fragments of *spk1*⁺ (Toda *et al.*, 1991) and a novel gene, *spm1*⁺, were obtained. Using the *spm1*⁺ fragment as a probe, a full-length clone was isolated. The *spm1*⁺ gene contains a potential open reading frame interrupted by three potential introns of 100, 272 and 82 bp, respectively (Figure 1A). These potential introns begin and end with consensus *S.pombe* splicing signals (Russell, 1989), and are absent from *spm1*⁺ mRNA (PCR analysis of cDNA; data not shown).

The predicted Spm1 protein of 422 residues starts with an initiation codon in a consensus sequence preceded by an in-frame stop codon. The putative kinase domain (residues 28–322) shows strong similarity to MAP kinases, being most similar to *S.cerevisiae* Mpk1 (61% identity; Figure 1B and C). The residue between the two phosphate acceptors is glutamate, as in Mpk1, Spk1 and the pheromone (*S.cerevisiae*) and mitogen (vertebrate) responsive MAP kinases (Figure 1C). Spm1 has a long, hydrophilic, C-terminal non-catalytic region, containing many serine and threonine residues that may be phosphorylation sites. Hog1, Mpk1 and some vertebrate JNK family members similarly have long, hydrophilic, C-terminal tails, but the sequences are divergent. The predicted M_r of Spm1 is 48 261, but the protein has an electrophoretic mobility of ~54 kDa, perhaps because of abnormal SDS binding to the C-terminal tail.

Disruption of *spm1*⁺ affects morphology and colony formation in high osmolarity

To determine the function of Spm1, the *spm1*⁺ gene was disrupted by homologous recombination, using a cassette with residues 19–359 replaced by the *S.cerevisiae* LEU2 gene. The *spm1::LEU2* DNA was transformed into both *h*[−] and *h*⁺ *leu1*[−] *S.pombe* strains and leucine prototrophs were isolated. Gene disruptions were confirmed by PCR and by Southern blotting. The phenotypes described below were observed in independently derived *h*⁺ *spm1Δ* and *h*[−] *spm1Δ* strains, and segregated with leucine prototrophy at meiosis (Materials and methods).

spm1Δ mutant strains formed normal sized colonies on rich medium agar at 25, 30 or 37°C, but formed colonies of reduced size on synthetic minimal agar. Colony size was reduced under hypertonic conditions, and colony formation was completely inhibited on synthetic minimal agar containing 1.5 M KCl (Figure 2), 0.9 M NaCl (Figure 5) or 1.5 M sorbitol (data not shown). Sensitivity to hypertonic conditions was partly suppressed by rich media. Deletion of *spm1*⁺ also caused morphological aberrations when cells were grown on synthetic minimal agar, with the proportion of altered cells increasing at elevated temperature (37°C) or in hypertonic conditions. The aberrant *spm1Δ* cells grew as short trains joined end to end, resembling short pseudohyphae (Figure 3B). Each compartment in the short filament had a nucleus. The inner cells frequently were wider and shorter than average wild-type cells. The trains of cells occasionally were branched. The branching was probably due to growth and division of a cell within the train, since the ends of the inner cells are not available for the growth process (Sipiczki *et al.*, 1993). In contrast, wild-type cells appeared normal under hypertonic or 37°C growth conditions (Figure 3A). When *spm1Δ* cells were cultured in liquid minimal medium, short filaments were also observed, especially at high osmotic strength (Table I) or in glucose-starved but not nitrogen-starved cultures. Trains were less frequent in liquid rich media, or when cultures in synthetic media approached saturation. These observations suggest that Spm1 is needed for normal morphology and cytokinesis under conditions of nutrient deprivation, increased tonicity or temperature.

To investigate why *spm1Δ* cells fail to separate, we used transmission electron microscopy. The external walls and septa of *spm1Δ* cells are 50–100% thicker than normal (Figure 3D and E). Frequently, the septal material appeared to delaminate. We also frequently detected groups of cells joined via a continuous external cell wall (Figure 3C). Apparently, these cells had almost finished cytokinesis, but had not completely lysed the external wall, perhaps explaining the aggregates seen by light microscopy. Consistent with the increased thickness of *spm1Δ* cell walls and septa, *spm1Δ* cells stained more intensely than wild-type cells with calcofluor, a fluorescent compound that binds to *S.pombe* cell walls (Robinow and Hyams, 1989) (Figure 4A and B). The external walls of wild-type cells stain relatively dimly, with unstained stripes of division scars marking the sites of septa in prior divisions (Robinow and Hyams, 1989) (Figure 4A). The external cell walls

Fig. 1. Sequence analysis of the *spm1*⁺ gene and protein. (A) Sequence of the *spm1*⁺ gene and predicted protein sequence. Underlined bases in the nucleotide sequence indicate the upstream in-frame termination codon and the potential splicing signals in the three predicted introns. Colons in the protein sequence indicate the predicted splice junctions and the shaded areas indicate introns. The asterisks mark the phosphorylation sites, T186 and Y188. The introns were identified by searching for potential splice donor signals [GGTAAxGT (invariant bases underlined)], potentialariat signals (consensus TACTAACA) and potential splice acceptor signals (consensus TAG, 3–15 nucleotides from theariat signal) (Langford *et al.*, 1984; Mertins and Gallwitz, 1987; Russell, 1989). The sequences found in the predicted introns satisfy these consensus sequences almost perfectly. (B) Homology between the kinase domains of Spm1, other *S.pombe* MAP kinases, and *S.cerevisiae* Mpk1. Sequences were aligned using Pileup (Genetics Computer Group software package) (Devereux *et al.*, 1984). (C) Phylogram (prepared using GROWTREE algorithm of the GCG package) comparing sequences of protein kinase domains of representative MAP kinases from *S.pombe* (Sp), *S.cerevisiae* (Sc) and mammals (Mm). The sequence of *spm1*⁺ is available from databases under accession No. U65405.

and septa of *spm1*Δ cells stained much more brightly than wild-type cells, and division scars were not usually seen (Figure 4B). The walls and septa of *spm1*Δ cells may stain brightly with calcofluor because they are thicker than normal.

To confirm that the morphological and growth defects of *spm1*Δ cells are due to the mutation in *spm1*, a plasmid expressing Spm1, fused to an epitope tag, was introduced into *spm1*Δ cells. Cells expressing tagged Spm1, but not the tag alone, were able to grow on hypertonic agar (Figure 2), and the morphological defects were rescued (data not shown).

Partial sterility of *spm1*Δ cells

A requirement for Spm1 for mating and sporulation was evident when crossing heterothallic wild-type and *spm1*Δ

cells. Microscopic examination showed that the number of zygotes and ascii was greatly reduced by deletion of *spm1*⁺, suggesting decreased adhesion or fusion of conjugating cells. The efficiency of conjugation and sporulation was quantified by counting viable spores after killing vegetative cells with glusulase (Table II). Formation of spores by *spm1*Δ cells was strongly decreased after 3 days on low-nitrogen plates, but increased after longer times, suggesting that mating is retarded. Spore formation was partially rescued when one parent was *spm1*⁺ and the other *spm1*Δ (data not shown), consistent with the hypothesis that Spm1 contributes to events prior to cytoplasmic mixing, such as sexual differentiation, adhesion or fusion. The mating phenotype of *spm1*Δ cells is distinct from that of *spk1*Δ cells, which are completely sterile (Table II) (Toda *et al.*, 1991).

Fig. 2. *spm1*Δ cells are sensitive to osmotic stress. *spm1*Δ (TZs70) cells, transformed with Rep4U-GST or Rep4U-GSpm1, and wild-type (GP20) cells were grown on EMM-2 agar without thiamine or uracil, to induce expression of GST or GST-Spm1. Plates contained 0 (left) or 1.5 M (right) KCl. Wild-type cells and *spm1*Δ cells expressing GST-Spm1 formed colonies on both plates, but *spm1*Δ cells expressing GST only did not form colonies in the presence of high salt. Plates were incubated at 30°C for 4 (control) or 6 days (KCl).

One of the initial steps in the sexual differentiation pathway of *S.pombe* is G_1 arrest in response to nitrogen starvation (Costello *et al.*, 1986). We used flow cytometry of propidium iodide-stained cells to investigate whether *spm1*Δ cells arrest in G_1 phase in low nitrogen media. *spm1*Δ and wild-type cells were indistinguishable by flow cytometry, arresting with 2n DNA content when starved for glucose and with 1n DNA content when starved for nitrogen (Costello *et al.*, 1986) (data not shown). These data are compatible with the hypothesis that the reduced and delayed mating of *spm1*Δ cells is a consequence of reduced adhesion or fusion due to their altered cell walls, but do not exclude the possibility of additional roles for Spm1 in meiosis or sporulation.

Interaction of Spm1 and Spk1 pathways

The morphological phenotype associated with the deletion of *spm1* is reminiscent of the pseudohyphal growth of diploid *S.cerevisiae*, which requires elements of the pheromone-responsive MAP kinase pathway. In *S.pombe*, the pheromone response pathway is dependent on the MAP kinase Spk1. Therefore, we tested whether deletion of *spk1* would affect the phenotype of *spm1*Δ cells.

The *spm1* gene was disrupted by homologous recombination in an *spk1*Δ strain. The double mutant partially integrated the phenotypes of the single mutants: absolute sterility characteristic of *spk1*Δ and some morphological abnormalities characteristic of *spm1*Δ. However, the morphological defects of *spm1*Δ cells were partially rescued, and the salt sensitivity of *spm1*Δ cells was completely corrected (Figure 5). We also noticed that both *spk1*Δ cells and *byr1*Δ cells (i.e. cells lacking an upstream activator of Spk1) formed larger colonies than wild-type cells on agar containing sorbitol or NaCl (Figure 5 and data not shown). This suggests that the Spk1 pathway may normally oppose the action of Spm1, and inhibits proliferation under hypertonic conditions.

Overproduction of Spm1 inhibits growth

To test the effect of overproduction of Spm1, we initially used the pART1 vector, containing the strong constitutive *adh1* promoter (Russell, 1989). Cells overproducing Spm1 formed much smaller colonies on selective plates than cells expressing the vector alone. Whether grown on agar or in liquid media, the majority of Spm1-overproducing cells had unusual and variable morphologies. Many of the cells were rounded but others were elongated and occasionally branched (data not shown).

The inhibitory effect of overproduction of Spm1 on cell growth was also observed when we expressed Spm1 under the strong thiamine-repressible *nmt1* promoter (Maundrell, 1993). Under derepressing conditions, colony size on agar and growth rate in liquid culture were strongly inhibited. We followed the induction of the protein with an antibody raised to the C-terminus of Spm1 (Figure 6). Under these conditions, the 54 kDa Spm1 protein was first detected after 11 h induction and expression increased with time. In five independent experiments (one of which is presented in Figure 6), growth rate was normal until ~11–12 h of induction, and then decreased. Cultures were diluted at 15 h and followed for an additional 20 h. Virtually no further growth of the induced culture was detected. Propidium iodide staining and flow cytometry did not reveal any difference in cell cycle distribution between cells overproducing Spm1 and controls.

Phosphorylation and activation of the Spm1 protein

Since Spm1 is required for colony formation in high osmolarity media, we tested whether the Spm1 protein is active under hypertonic conditions. Initially, we studied endogenous Spm1 using antiserum to a synthetic peptide based on the sequence of the 18 C-terminal residues of Spm1. The affinity-purified antiserum, when used to probe Western blots, detected a fusion protein made in *Escherichia coli* that encoded the C-terminal 98 residues of Spm1, as well as full-length Spm1 overproduced in *S.pombe* (Figure 6). Western blots allowed the detection of endogenous Spm1 in wild-type cells (Figure 7). The endogenous Spm1 migrates as a single 54 kDa band, whereas overexpressed Spm1 migrates as a major 54 kDa band and two minor bands of lower electrophoretic mobility, perhaps indicative of phosphorylation (Figure 7). As expected, the 54 kDa endogenous Spm1 was not detected in *spm1*[−] cells. Exposure of cells to 0.5 M NaCl for 10 min prior to analysis induced ~50% of Spm1 protein to migrate with decreased electrophoretic mobility (Figure 7). Many MAP kinases undergo a similar stimulus-induced mobility shift due to phosphorylation at the activating Thr–Xaa–Tyr phosphorylation sites. We also tested whether deletion of *spk1* would affect Spm1 expression or phosphorylation. No differences in expression or electrophoretic mobility relative to wild-type cells were detected (Figure 7).

We were unable to immunoprecipitate Spm1 from cells to assess its phosphorylation state or activity, so Spm1 was expressed as a GST fusion protein from the regulated *nmt1* promoter, adapted to give low levels of expression (Basi *et al.*, 1993). GST-Spm1 appeared to be functional, since it complemented the osmosensitivity of *spm1*[−] cells (Figure 2). GST-Spm1 was expressed in wild-type cells and purified using immobilized glutathione. Immuno-

Fig. 3. *spm1Δ* cells are defective for cytokinesis. Wild-type (GP970; **A** and **D**) and *spm1Δ* (TZs70; **B**, **C** and **E**) cells were grown on EMM-2 agar for 48 h and examined using phase contrast (**A** and **B**) or transmission electron microscopy (**C**–**E**). Open arrows indicate split layers of thickened septum in *spm1Δ* cells. Closed arrows indicate undigested portions of the cell wall joining a group of cells together.

Table I. Frequency of septa in *spm1Δ* cells

Concentration of NaCl	No. of septa per 100 cell units
0	15
0.4	35
0.5	44
0.75	97

Cells were grown from early log phase in EMM-2 with the indicated concentration of NaCl for 18 h, then the number of septa per 100 cell units was scored. A cell unit was defined as a single cell or short filament. For example, a filament containing four compartments was counted as a single cell unit with three septa. In wild-type cultures, the number of septa per 100 cell units was ~10–15 at all concentrations of NaCl. Approximately 500 cell units were counted for each condition.

blotting with either anti-GST or anti-Spm1 C-terminal antibodies showed one or more bands of full-length GST–Spm1 (~80 kDa) and C-terminally truncated forms of ~75 kDa (Figure 8A). The number and intensity of 80 kDa bands varied according to culture conditions, suggesting that post-translational modification of GST–Spm1 may

occur. Stimulation of cells with 0.5 M NaCl for 5 or 15 min induces tyrosine phosphorylation of the low-mobility form of GST–Spm1, as detected with antibodies to phosphotyrosine or to a phosphopeptide corresponding to the phosphorylation site in Erk1 (Figure 8C). The latter antibodies do not recognize most proteins that are phosphorylated at tyrosine, only phosphorylated MAP kinases (Materials and methods). Both the full-length and C-terminally processed forms of GST–Spm1 are tyrosine phosphorylated. In most experiments, tyrosine phosphorylation was reduced by 1 h (data not shown). These results suggest that GST–Spm1 is phosphorylated at tyrosine in the Thr–Glu–Tyr motif following NaCl treatment of cells.

To test whether tyrosine phosphorylation of GST–Spm1 correlated with increased *in vitro* kinase activity, we made use of myelin basic protein (MBP) as substrate (Figure 8B). Phosphorylation of MBP was increased when GST–Spm1 was isolated from salt-stimulated cells. However, phosphate incorporation was low, possibly indicating that MBP is a poor substrate for Spm1. Phosphorylation of proteins of ~80 kDa was also detected. This

Fig. 4. Calcofluor staining of cell walls and septa. Wild-type (GP970, **A**), *spm1*Δ (TZs70, **B**), *wis1*Δ (JM504, **C**) and *spm1*Δ*wis1*Δ (TZsw9, **D**) cells were grown on EMM-2 agar and examined after staining with calcofluor. The inset in (**D**) shows a negative image to illustrate the multiple septa found between cells in *spm1*Δ*wis1*Δ strains.

Table II. Spore formation by *spm1*Δ cells

Time of mating (days)	Relative spore formation (% wild-type)	
	<i>spm1</i> Δ× <i>spm1</i> Δ	<i>spk1</i> Δ× <i>spk1</i> Δ
3	0.3	<0.03
3	1.8	<0.02
3	3.0	<0.03
7	14.0	<0.001
10	31.0	<0.001

Spore production (glusulase-resistant spores per parental cell mated) was assayed for crosses of heterothallic *spm1*Δ and *spk1*Δ strains. Spore production for the mutants was normalized to spore production in a parallel, congenic wild-type cross. The results are from five independent experiments.

may represent autophosphorylation of GST-Spm1. These results indicate that Spm1 is tyrosine phosphorylated and activated under hypertonic conditions.

Because the morphological defects of *spm1*Δ cells are also more apparent at elevated temperature, we also tested whether GST-Spm1 is activated by heat stress. An induced culture was shifted from 30 to 48°C for various times.

GST-Spm1 was tyrosine phosphorylated after 5 or 30 min of heat stress (Figure 8D). This suggests that Spm1 may also be regulated by thermal stresses.

Spm1* function is not dependent on *Wis1

Since Spm1 and Spc1 are similarly activated by hypertonic and heat stress, and since the *spm1*Δ and *spc1*Δ phenotypes are exacerbated by the same environmental stimuli (Millar *et al.*, 1995; Shiozaki and Russell, 1995a), we suspected that both kinases may be activated by the same mechanism. Since Spc1 is activated by Wis1 (Millar *et al.*, 1995; Shiozaki and Russell, 1995a), we tested whether Wis1 activates Spm1.

First, we studied *wis1*Δ *spm1*Δ cells. If Wis1 is necessary to activate Spm1, then the phenotype of a *wis1*Δ *spm1*Δ mutant should be the same as that of a *wis1*Δ mutant. *h*⁺ *wis1*Δ and *h*⁻ *spm1*Δ haploids were crossed and tetrads dissected. Wild-type, *wis1*Δ (Ura⁺) and *spm1*Δ (Leu⁺) phenotypes segregated with the prototrophic markers. The *wis1*Δ*spm1*Δ (Ura⁺Leu⁺) progeny showed an additive phenotype when grown on agar (Figure 4D). Each cell was increased in length, similar to *wis1*Δ cells analyzed

Fig. 5. Rescue of osmosensitivity of *spm1Δ* cells by disruption of *spk1*. Wild-type (GP969), *spm1Δ* (TZs69), *spk1Δ* (TZk69) and *spm1Δspk1Δ* (TZsk9) cells were grown on EMM-2 agar containing 0 or 0.9 M NaCl. Note that the plating efficiency of *spm1Δ* cells on EMM-2 lacking NaCl is poor, but the colony size is similar to wild-type. In contrast, *spk1Δ* and *spm1Δspk1Δ* cells form larger colonies than wild-type on EMM-2 containing 0 or 0.9 M NaCl, suggesting that Spk1 inhibits growth on synthetic media or under hypertonic stress conditions.

Fig. 6. Induction of Spm1 overexpression inhibits cell proliferation. Wild-type (GP54) cells were transformed with pREP3X or pREP3X-*spm1*⁺. Two transformants were grown to early log phase in EMM-2 containing thiamine and lacking leucine. Cells were then transferred to media containing or lacking thiamine. At 11, 13 or 15 h after induction, culture density was measured (B) and protein extracts prepared. Spm1 protein was detected by Western blotting with antibody 551H raised to the C-terminus of Spm1 (A).

Fig. 7. Modification of endogenous Spm1 in response to increased osmotic strength. Wild-type (GP970), *spm1Δ* (TZs70) or *spk1Δ* (TZk70) cells were grown in YEL to early log phase, and exposed to 0.5 M NaCl for 30 min. Extracts were prepared and analyzed by Western blotting with 551H antibody to Spm1. Wild-type cells (GP54) containing pART1-*spm1*⁺ were used as a control for the migration of the Spm1 protein (left lane). Spm1 protein was not detected in *spm1Δ* cells.

in parallel (Figure 4C). In addition, the double mutant cells resembled *spm1Δ* cells (Figure 4B) in their failure to complete cytokinesis, intensity of calcofluor staining, slight increase in width and branching. The additive phenotypes indicate that Wis1 and Spm1 act independently. Curiously, the double mutant also showed a phenotype not seen in either parent. At some of the junctions between adjacent cells in a train, there were multiple septa, cutting off small compartments at the ends of the cells (Figure 4D inset). This phenotype resembles that of *cdc16-116* cells (Minet *et al.*, 1979), and suggests that the Wis1/Spk1 and Spm1 pathways may negatively regulate an early step in septation.

To test directly whether Wis1 is needed for Spm1 regulation, the phosphorylation of GST-Spm1 was assayed in *wis1Δ* cells. Expression of GST-Spm1 was induced in wild-type or *wis1Δ* cells, and phosphorylation in response to hypertonic stimulation analyzed (Figure 9A). In three independent transformants, GST-Spm1 was tyrosine phosphorylated, indicating that Wis1 is not needed for Spm1 phosphorylation in response to hypertonic stress.

Spm1 activation is not dependent on Pkc1 or Ras1

Spm1 phosphorylation was also studied in a *pkc1Δ* strain. Pkc1, also known as *pck2*, is one of two protein kinase C (PKC) homologs in *S.pombe* (Mazzei *et al.*, 1993; Toda *et al.*, 1993). In *S.cerevisiae*, PKC regulates the Spm1 homolog, Mpk1 (Lee *et al.*, 1993; Zarzov *et al.*, 1996). When GST-Spm1 was expressed in *pkc1Δ* cells, it was constitutively tyrosine phosphorylated (Figure 9B). Therefore, it seems that Pkc1 antagonizes rather than assists Spm1 activation. We conclude that Spm1 activation involves a novel MAP kinase pathway.

The small GTPase Ras1 controls pheromone signaling via the Spk1 MAP kinase (Neiman *et al.*, 1993; Xu *et al.*, 1994). Ras1 also regulates morphogenesis by another mechanism (Wang *et al.*, 1991). To test whether Spm1 activation depends on Ras1, GST-Spm1 was expressed in a *ras1Δ* strain (Materials and methods), and its phosphorylation in response to hypertonic stress was assayed (Figure 9C). GST-Spm1 was tyrosine phosphorylated in response to high salt in the absence of Ras1. Therefore, Ras1 is not needed for Spm1 activation by this stimulus. In addition, overexpression of Spm1 did not change the morphology of *ras1Δ* cells.

Fig. 8. Tyrosine phosphorylation and activation of Spm1 is induced under hypertonic conditions. Wild-type (GP970) cells were transformed with pR4L-GST or pR4L-GSpm1, and grown in EMM-2 containing thiamine and lacking leucine. Cells were then transferred to media containing or lacking thiamine for 18 h. Cultures were treated with 0 or 0.5 M NaCl before preparing extracts. Proteins were purified using glutathione-Sepharose and analyzed by Western blotting or protein kinase assays. (A) Induction of GST-Spm1 expression, detected with antiserum 551H to the C-terminus of Spm1 or anti-GST antibodies. Full-length GST-Spm1 (80 kDa) was detected with both antibodies. Smaller proteins (75 kDa) detected with anti-GST but not anti-Spm1 antibodies are presumably truncated at the C-terminus. (B) Effect of hypertonicity on protein kinase activity of Spm1. GST and GST-Spm1 proteins bound to glutathione-Sepharose were incubated with [γ - 32 P]ATP and myelin basic protein (MBP). To control for possible contamination of MBP with protein kinases, one reaction contained MBP alone (left lane). Phosphorylated products were analyzed by electrophoresis and autoradiography (upper panel). Phosphorylation of MBP was quantified using a Phosphorimager (Molecular Dynamics) and is represented in arbitrary units (histogram). (C) Phosphorylation of GST-Spm1 under hypertonic conditions. Western blots were probed with antibodies to GST, phosphotyrosine or phosphorylated MAP kinase. (D) Phosphorylation of GST-Spm1 in cells heat shocked at 48°C for 5 or 10 min, detected with antibody to phosphorylated MAP kinase.

Fig. 9. Phosphorylation of GST-Spm1 expressed in mutant cells. Matched cultures of wild-type (GP970, Sp12), *wis1* Δ (JM504), *pkc1* Δ (GE111) and *ras1* Δ (Sp525) cells, expressing GST-Spm1 from pR4U-GSpm1 or pR4L-GSpm1, were stimulated with 0.5 M NaCl for 15 min (**A** and **B**) or various times (**C**) before purification of GST-Spm1 on glutathione-agarose and Western blotting. Expression of GST-Spm1 was low in *ras1* Δ cells, for unknown reasons.

Discussion

Spm1 has features of two *S.cerevisiae* MAP kinases

The new MAP kinase, Spm1, appears to be a sequence homolog of *S.cerevisiae* Mpk1, sharing 61% sequence identity in the catalytic domain, and possessing a long hydrophilic C-terminal region that may provide binding sites for other proteins, localization signals or sites for post-translational modification. Functionally, Spm1 and Mpk1 also have some similarities: both MAP kinases appear to regulate cell wall remodeling, perhaps by controlling the polarized secretion of enzymes that make and break bonds between cell wall subunits (Mazzoni *et al.*, 1993). Both *mpk1* Δ *S.cerevisiae* and *spm1* Δ *S.pombe* are partially sterile but mate slowly. However, in other respects, the phenotypes are quite different. *mpk1* Δ *S.cerevisiae* have weak cell walls and lyse at points of growth at elevated temperature (Lee *et al.*, 1993), whereas *spm1* Δ *S.pombe* have thick walls and septa and do not lyse at temperatures tested. *mpk1* Δ *S.cerevisiae* are stabilized by hypertonic conditions, whereas *spm1* Δ *S.pombe* are sensitive to hypertonic conditions. To what extent these differences indicate evolutionary divergence in the kinases and to what extent they are due to differences in the ways the two yeasts grow is not known.

Perhaps the most striking difference between Spm1 and Mpk1 is that the former is activated by hypertonic stress and the latter by hypotonic stress (Davenport *et al.*, 1995). In this regard, Spm1 resembles the *S.cerevisiae* kinase Hog1 (Ballard *et al.*, 1991; Brewster *et al.*, 1993). Like

Hog1 in *S.cerevisiae* (Brewster *et al.*, 1993), Spm1 is required for *S.pombe* to form colonies in high osmolarity media. Activation of Hog1 induces the expression of various gene products including enzymes that synthesize glycerol and thus raise the intracellular tonicity to offset the increased extracellular tonicity (Brewster *et al.*, 1993; Albertyn *et al.*, 1994; Schuller *et al.*, 1994; Varela *et al.*, 1995). Hog1 is also required to organize the cytoskeleton in osmotically stressed cells (Brewster and Gustin, 1994). Spm1 could perform similar functions in *S.pombe*, regulating gene expression or the cytoskeleton in response to osmotic stress.

Function of Spm1 in cytokinesis and cell wall metabolism

Under conditions of hypertonic, nutrient or temperature stress, the majority of rapidly growing *spm1* Δ cells fail to separate for one to three generations after division, forming short filaments or trains that are two to eight cells long. The cells in a train remain attached across the entire division plate. Even when cytokinesis occurs, *spm1* Δ cells sometimes remain physically attached by a continuous layer of outer cell wall, as seen by electron microscopy, and appear as aggregates by light microscopy. However, when *spm1* Δ cultures reach stationary phase, trains become infrequent, suggesting either that cell separation occurs eventually or that Spm1-independent mechanisms for cell separation are induced in slowly growing cells.

Cell separation requires dissolving both the primary septum and the cylinder of cell wall that surrounds it

(Robinow and Hyams, 1989). The primary septum, which is rich in β -glucans (Horisberger and Rouvet-Vauthey, 1985), is the first part of the septum to be laid down, late in anaphase. The position of the primary septum is determined by an actin ring that forms around the middle of the dividing nucleus (Marks and Hyamns, 1985). An annulus of primary septum is then laid down on the inner surface of the old wall, around the actin ring. The septum grows centripetally until the annulus closes completely and compartmentalizes the two daughter cells (Johnson *et al.*, 1973). Each daughter then contributes cell wall material to her side of the primary septum, building up a secondary septum rich in α -galactomannan (Horisberger and Rouvet-Vauthey, 1985). The three-layered septum ordinarily is completed during the short G1 phase of the cell cycle, and then the primary septum and surrounding cylinder of old wall are dissolved, liberating the two daughters, at about the time of S phase entry.

spm1 Δ mutant cells show obvious differences in cell wall and septum structure that could contribute to reduced cytokinesis. Electron microscopy shows dramatically thickened septa and cell walls. There are more layers in the mutant than wild-type septum, and there are cavities between layers, suggesting delamination. A thickened septum can result from activation of β -glucan synthase (Arellano *et al.*, 1996), and may contribute to the decreased separation of *spm1* Δ cells after division. Alternatively, or in addition, lysing enzymes may not be synthesized or targeted properly in *spm1* Δ mutant cells. Presumably the enzymes that dissolve the primary septum are either packaged in latent form at the time of primary septum assembly or are transported to the primary septum following completion of the three-layered structure. The decreased separation of *spm1* Δ daughter cells may be a consequence of the increased deposition of septum and wall material, or failure to synthesize or target lysing enzymes.

An additional feature of *spm1* Δ cells is that the trains are branched, suggesting that growth is not always axial, as in wild-type cells. Defects in axial growth are also suggested by the shorter, wider shape of *spm1* Δ cells. Possibly all the morphological phenotypes of *spm1* Δ mutant cells are caused by a primary defect in vectorial transport of cell wall materials and enzymes. It will be interesting to examine the cytoskeleton of *spm1* Δ mutant cells.

Two other mutants that give rise to branched trains resembling *spm1* Δ cells have been described (Sipiczki *et al.*, 1993; Yoshida *et al.*, 1994). The *sep1-1* mutant forms branched trains and is not defective in β -glucanase (Sipiczki *et al.*, 1993). The mutant is not osmosensitive and the molecular identity of the Sep1 protein is unknown. Cells mutant for *ppb1*, a homolog of calcineurin (protein phosphatase 2B), also form short filaments (Yoshida *et al.*, 1994). Since the *ppb1* Δ mutant of *S.pombe* is cold sensitive (Yoshida *et al.*, 1994), whereas the phenotype of the *spm1* Δ mutant is exaggerated at high temperature, it seems likely that Ppb1 and Spm1 are on different pathways. In *S.cerevisiae*, Mpk1 and calcineurin are functionally redundant for calcium resistance and vanadate sensitivity, suggesting that they operate on different but converging pathways (Nakamura *et al.*, 1996).

Interactions with other signaling pathways

The distinctive phenotype of *spm1* Δ cells suggests that the functions of Spm1 are different from those of Spk1, which is required for pheromone signaling (Gotoh *et al.*, 1993). We also found that Ras1, which is also required for pheromone signaling and is supposed to be important for Spk1 activation by pheromone (Neiman *et al.*, 1993; Albertyn *et al.*, 1994), is not needed for Spm1 activation by hypertonic stress, and GST-Spm1 underwent indistinguishable post-translational modification in *spk1* Δ and wild-type cells. Curiously, however, the growth of *spm1* Δ cells in hypertonic media was rescued by an additional mutation in *spk1*. Deletion of the genes for either Spk1 or its activator Byr1 increased colony size on hypertonic media relative to wild-type cells. This suggests that Spk1 may be active during vegetative growth in hypertonic media, and that Spk1 activity inhibits cell proliferation and increases the rate of cell separation following septation. A function for Spk1 in vegetative growth was not described before, but Spk1 expression is increased in vegetative cells by staurosporine (Toda *et al.*, 1991), suggesting that it may be induced in stressed cells. The epistasis could be explained if Spm1 negatively regulates Spk1 expression or activation, and Spk1 inhibits growth under stress conditions. Alternatively, Spk1 and Spm1 pathways could converge downstream. For example, Spk1 could induce expression of a growth inhibitor that is inactivated by Spm1.

The similarities between Spm1 and *S.cerevisiae* Mpk1, and the proposed regulation of Mpk1 by PKC (Lee *et al.*, 1993; Zarzov *et al.*, 1996), raise the possibility that Spm1 may be activated by a PKC-dependent pathway. However, several arguments suggest that this is unlikely. Two PKC genes, *pck1* and *pck2* (also called *pkc1*), have been studied in *S.pombe* (Mazzei *et al.*, 1993; Toda *et al.*, 1993). Disruption of both genes is lethal, and defective cytokinesis was not noted in either single mutant (Toda *et al.*, 1993). *pkc1* Δ cells are not osmosensitive (data not shown). Overexpression of *pck1* causes short filaments reminiscent of *spm1* Δ (Mazzei *et al.*, 1993; Toda *et al.*, 1993). Moreover, *S.cerevisiae* PKC is activated by the Rho1 GTPase (Nonaka *et al.*, 1995), and overexpression of activated Rho1 in *S.pombe* causes cell wall thickening and inhibits cytokinesis (Arellano *et al.*, 1996). Therefore, the phenotypes of PKC mutants and Rho1 overexpression are not consistent with a model where PKC opposes Spm1. Spm1 is constitutively tyrosine phosphorylated in *pkc1* Δ cells (Figure 9), suggesting that Pkc1 may negatively regulate Spm1 activation. Therefore, PKC is not on a simple linear biochemical pathway with Spm1, and may actually inhibit Spm1 activation or function.

Mechanism of stress activation of Spm1

The protein kinase activity of Spm1 is stimulated in response to hypertonic or heat stress. Spc1 is also activated by hypertonic and heat stress, raising the possibility that Spm1 and Spc1 are regulated by the same pathway. However, two lines of evidence suggest that the Spc1 MAPKK, Wis1, does not activate Spm1. First, the phenotype of a *wis1* Δ *spm1* Δ double mutant is additive. The cells are elongated, like *wis1* Δ cells, and form branching mycelia like *spm1* Δ cells. Second, Spm1 is tyrosine phosphorylated normally in *wis1* Δ cells. Thus we propose

a second, Spm1-specific MAPKK that is stimulated under hypertonic conditions, although our data do not exclude the possibility of a common MAPKKK.

In addition to their distinct functions in hypertonic conditions, *wis1* and *spm1* have an interesting genetic interaction. *wis1Δspm1Δ* cells have a novel phenotype, the presence of multiple septa between sister cells. This resembles the phenotype of *cdc16-116* cells, where multiple closely spaced septa separate daughter cells (Minet *et al.*, 1979; Fankhauser *et al.*, 1993). Cdc16 has been proposed to act as a checkpoint in the septation process (Chang and Nurse, 1993; Fankhauser and Simanis, 1994). In this model, Cdc16 is activated by septum completion, and then inhibits septum initiation. In *cdc16-116* cells, new septa are initiated prior to septum completion. Our data suggest that Spm1 and Spc1 may play a redundant role in regulating septum initiation in *S.pombe*.

In summary, we have identified a new MAPK that is involved in the response of *S.pombe* to nutrient limitation, increased osmotic strength and increased temperature. The phenotype of *spm1Δ* cells suggests that the normal role of Spm1 is to regulate cell wall remodeling and polarized growth under adverse growth conditions. The growth of *spm1Δ* cells as short branched filaments when stressed resembles that of wild-type cells under certain conditions of low temperature and low dissolved oxygen (Johnson and McDonald, 1983), and may be a consequence of uncoupling of nuclear and septation cycles. Filamentous growth may be advantageous for wild yeast, allowing invasion into the substrate in times of stress (Liu *et al.*, 1993; Roberts and Fink, 1994). The requirement for Spm1 for survival on hypertonic agar, and the inhibition of growth by Spm1 overproduction, should permit genetic analysis to identify other proteins in the Spm1 signaling pathway.

Materials and methods

Cloning of the *spm1* gene

Oligonucleotides for amplification of MAP kinase relatives were designed based on the conserved peptide sequences VA[VI]KKI (CGCGAATT-CGTNGCNAT[ACT]AA[AG]AA[AG]AT; primer 1a; square brackets enclose alternative residues found at a specific location), YF[TLI]YQI (CGCGAATTCTA[CT]TT[CT][CT]TNTA[CG]CAAAT; primer 2a), [ML]TEYVA (CGCGAATTCGCNAC[AG]TA[CT]TCNGTCAT; primer 2b), and [TCV]GCILA (CGCGAATTCGCNAR[AGT]AT[AG]CANCC-[AG]CA; primer 1b). These peptides are found in the order 1a, 2a, 2b, 1b in the MAP kinases Spk1, Erk1, Erk2, Kss1 and Fus3.

PCR was performed with primers 1a and 1b and *S.pombe* DNA as template for 30 cycles (94°C 1 min, 52°C 1.5 min, 72°C 1 min). The reaction was analyzed by electrophoresis, and ~807 and 1161 bp products were detected. Each product was purified and PCR repeated with primers 2a and 2b. In both cases an ~190 bp product was detected, cloned and sequenced. The product of the 807 bp band was derived from the *spk1*⁺ gene, and the product of the 1161 bp band was derived from a novel gene. The PCR product was labeled and used to probe a library of *Sau3A* partial digestion products of *S.pombe* DNA in pFL20 (Losson and Lacroute, 1983). Ten clones appeared to contain the same 6.2 kb insert based on restriction mapping. A 3.2 kb *SpeI* fragment was subcloned into BluescriptSK+ (clone 3.2) and a region of 1917 bp containing the full-length *spm1*⁺ gene was sequenced on both strands, making use of specific primers and subcloned fragments. The accession number for the Spm1 sequence is U65405.

Strains, media and genetic techniques

The *S.pombe* strains used in this study are listed in Table III. Rich YEL media (3% glucose, 0.5% yeast extract), minimal EMM-2 (Alfa *et al.*, 1993) and sporulating SPA (1% glucose, 0.1% KH₂PO₄ and vitamins)

Table III. Fission yeast strains used in this study

Strain	Genotype	Source
GP20	<i>h</i> ⁺ <i>leu1-32</i>	G.Smith
GP54	<i>h</i> ⁹⁰ <i>leu1-32 ade6-M375</i>	G.Smith
GP970	<i>h</i> ⁺ <i>leu1-32 ura4-D18</i>	P.Szankasi
GP969	<i>h</i> ⁻ <i>leu1-32 ura4-D18</i>	P.Szankasi
TZs70	<i>h</i> ⁺ <i>leu1-32 ura4-D18 spm1::LEU2</i>	T ^a of GP970, this study
TZs69	<i>h</i> ⁻ <i>leu1-32 ura4-D18 spm1::LEU2</i>	T of GP969, this study
TZk70	<i>h</i> ⁺ <i>leu1-32 ura4-D18 spk1::ura4</i> ⁺	T of GP970, this study
TZk69	<i>h</i> ⁻ <i>leu1-32 ura4-D18 spk1::ura4</i> ⁺	T of GP969, this study
TZsk9	<i>h</i> ⁻ <i>leu1-32 ura4-D18 spm1::LEU2 spk1::ura4</i> ⁺	T of TZk69, this study
JM504	<i>h</i> ⁺ <i>leu1-32 ura4-D18 wis1::ura4</i> ⁺	P.Russell ^b
Sp12	<i>h</i> ⁺ <i>leu1-32 ura4-D18 ade6-704</i>	K.Maundrell ^c
GE111	<i>h</i> ⁺ <i>leu1-32 ura4-D18 ade6-704 pkc1::ura4</i> ⁺	K.Maundrell ^c
TZsw-9	<i>leu1-32 ura4-D18 spm1::LEU2 wis1::ura4</i> ⁺	TZs69 X JM504, this study
SpBU	<i>h</i> ⁹⁰ <i>leu1-32 ura4-D18 ade6.210 byr1::ura4</i> ⁺	M.Wigler ^d
Sp525	<i>h</i> ⁹⁰ <i>leu1-32 ura4-D18 ade6.216 ras1::LEU2</i>	M.Wigler ^e

^aT, transformant.

^bShiozaki and Russell, 1995b.

^cMazzei *et al.*, 1993.

^dWang *et al.*, 1991.

^eNadin-Davis *et al.*, 1986.

(Gutz and Heslot, 1974) were employed. EMM-2 with 0.5% glucose was used for glucose starvation. Yeast were transformed using lithium acetate (Alfa *et al.*, 1993).

Gene disruptions

Codons 19–359 of the *spm1*⁺ gene in clone 3.2 were replaced with the *S.cerevisiae* *LEU2* gene (Rothstein, 1983). The body of a plasmid containing the 3.2 kb *spm1*⁺ region was amplified by PCR using primers including *Bgl*II sites, and ligated to the *Bam*HI–*Bgl*II-digested *LEU2*⁺ gene. We also disrupted the *spk1*⁺ gene. The *spk1*⁺ gene was cloned from the pFL20 library by PCR using primers from the published DNA sequence (Toda *et al.*, 1991). The entire Spk1 coding region was replaced with the *S.pombe* *ura4*⁺ gene. Because MAP kinases are typically not essential for survival, the *spm1*⁺ and *spk1*⁺ genes were disrupted in haploid strains. Strains GP969 and GP970 (Table III) were transformed with gel-purified inserts from the above plasmids, and plated on selective media. Transformants were recovered with high frequency and identified by PCR and Southern blotting. To exclude the possibility that *Leu*⁺ transformants contained unlinked mutations that modify the *spm1Δ* phenotype, *h*⁺ *spm1Δ* and *h*⁻ *spm1Δ* cells were mated to GP969 and GP970 and tetrads dissected. In 19 meioses, leucine prototrophy segregated 2:2 and all leucine prototrophs showed the characteristic morphological defects of *spm1Δ* cells.

Microscopy

Cells were stained with calcofluor and propidium iodide as described (Alfa *et al.*, 1993) and observed with a Zeiss fluorescence microscope (63× objective). For electron microscopy, yeast were pelleted, washed and fixed in half-strength Karnovsky's fixative for at least 2 h. Samples were rinsed in cacodylate buffer, post-fixed in 1% collidine-buffered osmium tetroxide, stained with 2% uranyl acetate, dehydrated, embedded in Pelco low viscosity embedding media overnight and cured at 70°C overnight. Sections (7–900 μm) were photographed at ×6000 and ×25 000 magnification.

Mating assays

Cultures of *h*⁺ and *h*⁻ strains were grown to OD₅₉₅ 0.5 in YEL, counted, collected, mixed, washed twice with sterile water and spotted on SPA plates. At 3, 7 and 10 days later, the cell walls were treated with glucanase at 30°C overnight, washed, and viable spores assayed by plating serial dilutions on YEA.

Hypertonic induction and sensitivity

Exponentially growing cultures were exposed to 0.5 M NaCl for various times, cooled on ice, collected by centrifugation and lysed for protein

analysis. Colony formation was assayed on EMM-2 agar plates containing 1.5 M KCl, 0.9 M NaCl or 1.5 M sorbitol.

Antibodies and immunological methods

A synthetic peptide corresponding to the 19 C-terminal residues of Spm1 was synthesized and conjugated to keyhole limpet hemocyanin using glutaraldehyde. Rabbits were immunized according to standard procedures and the antiserum (551H) was purified over Affigel 10 (BioRad) conjugated to the peptide immunogen. Rabbit antiserum 1608H was raised against bacterially expressed and purified GST-Spm1 fusion protein containing the 98 C-terminal amino acids of Spm1. Anti-GST rabbit antiserum 38.4 was a gift of A.Waskiewicz. Antibodies to phosphotyrosine (mouse monoclonal 4G10) and phospho-MAP kinase (affinity-purified rabbit antiserum to a phosphopeptide: DHTGFLTEpY-VATRWC) were from Upstate Biotechnology and New England Biolabs, respectively.

Overproduction of Spm1

The *spm1* open reading frame was cloned by PCR behind the *adh1* promoter in pART1 (McLeod *et al.*, 1987) using *Pst*I and *Sma*I sites, for constitutive expression. The same PCR fragment was also cloned into pCRII (Invitrogen), and a *Bam*HI-*Sma*I fragment subcloned behind the regulated *nmt1* promoter in pREP3X (LEU2⁺) (Maundrell, 1993). Lower, regulated expression was achieved using pREP-42X, which contains a mutated *nmt1* promoter and a *ura4*⁺ selectable marker (Basi *et al.*, 1993; Forsburg, 1993; Maundrell, 1993). The coding region for GST was amplified using PCR from pGEX-3X and a 5' primer that places the initiator methionine in a good context for initiation in eukaryotes. GST was inserted into the *Bam*HI site of pREP-42X as a *Bgl*2-*Bam*HI fragment, to give pR4U-GST. The *spm1* open reading frame was then inserted as a *Bam*HI fragment, to give pR4U-GSpm1. A parallel construct was made in pREP-41X, which contains a *LEU2*⁺ selectable marker (Basi *et al.*, 1993; Forsburg, 1993), by replacing the *Xho*I-*Sma*I region with the *Xho*I-*Sma*I fragment from pR4U-GSpm1, to give pR4L-GSpm1. Digestion of this plasmid with *Bam*HI and recircularization gave the control pR4L-GST.

To induce the production of GST or GST-Spm1, transformants were grown to early exponential phase in EMM-2 containing thiamine, washed three times in the same medium lacking thiamine, and resuspended in EMM-2 lacking thiamine for 18 h of growth. Cells from exponentially growing cultures were broken by vortexing with glass beads in NP-40 buffer [1% NP-40; 10 mM HEPES pH7.4; 2 mM EDTA; 50 mM NaF; 0.2 mM Na₃VO₄; 0.1% 2-mercaptoethanol; 1 mM phenylmethylsulfonyl fluoride (PMSF); 1% aprotinin] and centrifuged at 14 000 g for 20 min. Cleared extract from a 30 ml culture (OD 0.25) was incubated with glutathione-Sepharose (Pharmacia; 10 µl of bed volume) for 30 min, washed twice with cold NP-40 buffer, twice with NP-40 + 1 M NaCl, and eluted with 2× loading buffer (5 mM EDTA; 4% SDS; 5.6 M 2-mercaptoethanol; 20% glycerol; 0.2 M Tris, pH 6.8; 0.02% bromophenol blue). Proteins were resolved on 15% SDS-PAGE and transferred to Immobilon membrane.

Kinase assays

Protein extracts were prepared from stimulated or non-stimulated cultures and purified with glutathione-Sepharose as described above with three additional washes with ice-cold PAN buffer (0.1 M NaCl; 10 mM PIPES pH 7.0; 40 µg/ml aprotinin) and then with kinase buffer (20 mM HEPES pH 7.5; 2 mM dithiothreitol; 10 mM MgCl₂; 1 mM PMSF; 2 mM Na₃VO₄). Kinase assays were performed in kinase buffer containing 0.5 mg/ml MBP and 5 µCi at 30°C for 30 min. For each reaction, 10 µl of bed volume of GSH was used to purify the GST-tagged proteins from ~30 ml of exponentially growing cultures (OD₅₉₅ 0.25).

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Note added in proof

The *spm1⁺* gene has been identified and characterized independently by T.Toda *et al.*, and named *pmk1⁺* [Toda,T., Dhut,S., Superti-Furga,G., Gotoh,Y., Nishida,E., Sugiura,R. and Kuno,T. (1996) The fission yeast *pmk1⁺* gene encodes a novel mitogen-activated protein kinase homolog

which regulates cell integrity and functions coordinately with the protein kinase C pathway. *Mol. Cell. Biol.*, **16**, 6752–6764]. Some aspects of the phenotypes of *pmk1Δ* and *spm1Δ* cells differ. It is possible that the *ura4⁻* mutation in our strains exacerbates the penetrance of the morphological phenotype and the severity of NaCl- and sorbitol-sensitivity of *spm1Δ* cells. However, the stimulation of tyrosine phosphorylation of GST–Spm1 by NaCl suggests that Spm1 function is indeed regulated by NaCl. Background effects may also affect interpretation of the phenotypes of *spm1Δspk1Δ* and *spm1Δwis1Δ* cells, since these cells are also *ura4⁺*. We are very grateful to T.Toda for alerting us to this possibility and for communicating unpublished results.