Stress signal, mediated by a Hog1-like MAP kinase, controls sexual development in fission yeast

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Abstract We identified the *phh1*$^+$ gene that encodes a MAP kinase as the effector of Wis1 MAP kinase kinase in fission yeast, which is highly homologous with HOG1 of *S. cerevisiae*. Heterothalic *phh1* dsiruptant is phenotypically indistinguishable from *wis1* deletion mutant, both displaying the same extent of partial sterility and enhanced sensitivity to a variety of stress. In *phh1* disruptant, nitrogen starvation-induced expression of *ste11*$^+$, a key controller of sexual differentiation, is markedly diminished. Ectopic expression of *ste11*$^+$ effectively restores fertility, but not stress resistance, to the *phh1* disruptant. These data show that stress signal, mediated by a MAP kinase, is required for efficient start of sexual differentiation.

Key words: Signal transduction; MAP kinase; Stress; Sexual differentiation; Fission yeast

1. Introduction

The mitogen-activated protein kinase (MAP kinase; MAPK, also called ERK) cascades are major signal pathways for eliciting intracellular responses to extracellular conditions [1–3]. They have been classified into subgroups based on their structural features and the cellular responses they invoke [4,5]. The cellular responses in which they are involved include growth stimulation, the start of differentiation and stress responses, as recently be shown by studies with mammalian cells, Caenorhabiditis, *Drosophila* and yeast [6–11].

The *wis1*$^+$ gene of the fission yeast *Schizosaccharomyces pombe* was identified as a multicopy suppressor of the triple mutant *cdc25 wee1 win1* strain [12,13]. The *wis1*$^+$ gene product encodes a member of MAPK kinase (MAPKK) with the highest homology to Pbs2 of *S. cerevisiae*. Cells deleted for *wis1*$^+$ display complex phenotypes; elevated sensitivity to heat and high osmotic conditions, rapid cell death at confluence during culture, and partial sterility ([12]; T.K. Jr. and H.M., unpublished observation). These phenotypes have been attributed to a defect in cell cycle control, perhaps due to the inability of *wis1*$^-$ cells to integrate nutritional and some other extracellular signals into growth control [12,14,36].

S. pombe cells of opposite mating types conjugate, and undergo meiosis and sporulation in response to nitrogen starvation [15,16]. Conjugation is initiated by the action of Ste11, which is negatively regulated by cAMP [17,18,19] and other factors, which respond to nitrogen- and carbon-starvation [18]. Ste11 is a transcription factor with a HMG-box motif and activates the *cis*-element called TR box, which is present in genes required for conjugation and the initiation of meiosis [20].

In this study, we isolated from *S. pombe* a new member of the MAPK family designated Phh1, which possesses amazingly high homology with Hog1 of *S. cerevisiae*. This kinase mediates stress signals as the putative immediate downstream component of Wis1. We show that the Wis1-Phh1 cascade mediates the stress signal, which is required not only for self-protective cellular responses but also for the start of sexual development in *S. pombe*.

2. Experimental

2.1. Strains, media and genetic methods

Strains of *S. pombe* used in this study are listed in Table 1. Media were prepared as described previously [22,23,24]. Auxotrophic cells were grown in media with appropriate auxotrophic supplements. Standard genetic methods were performed as described previously [23,24].

2.2. Isolation, sequencing and disruption of phh1$^+$ gene

Degenerate oligonucleotides used for PCR amplification of conserved regions in MAP kinases are as follows: *primer MK1* 5′-CA(T/C)(C/A)GIGA(T/C)(T/C/A)TIAA(A/G)CCI(T/A/G)(C/G)IAA-3′ and *primer MK3* 5′-TCIGGIGCIC(T/G)(A/G)TA(C/A/G)(T/C)AIC(T/G)IGT-3′, in which I denotes inosine. PCR amplification was carried out with *S. pombe* genomic DNA prepared from L972 cells as a template, under the following conditions; 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min. Amplified DNA fragments were subcloned and sequenced. One clone containing a 135 bp insert (MAPK#28) had an open reading frame (ORF) with a deduced amino acid sequence highly homologous to a portion of HOG1 of *S. cerevisiae*. A *S. pombe* *Spe*I-genomic library [22] was screened for clones hybridizable with the 135 bp MAPK#28 DNA, and a 9 kb genomic clone containing the entire MAPK#28 gene (pAL*phh1*$^+$-1) was isolated. The clones, pAL*phh1*$^+$-2 and pAL*phh1*$^+$-3, which contain a 4 kb *Bam*HI-*Spe*I and 1.9 kb *Hin*dIII-*Spe*I fragment, respectively, were obtained by self-ligating the *Bam*HI or *Hin*dIII digested pAL*phh1*$^+$-1. *phh1* deletion strain was constructed as following. The 559 bp *Hha*I-*Eae*I fragment of pAL*phh1*$^+$-3 gene was replaced with a 1.8 kb *Hin*dIII fragment of the *S. pombe ura4*$^+$ gene. The *Hin*dIII-*Spe*I *phh1*::*ura4*$^+$ fragment was transfected into DK1. Stable Ura$^+$ cells were selected and successful gene disruption was confirmed by Southern hybridization. The resulting *Δphh1/phh1*$^+$ diploid strain Dphh1+/− was sporulated and haploid Ura$^+$ progenies were isolated showing that *phh1*$^+$ is not essential gene.

2.3. Determination of viability of S. pombe cells during culture and after osmotic or heat shock

Cell viability after entry into stationary phase was determined as follows: cells were grown in minimal medium (EMM) at 25°C to confluence and aliquots were sampled at the indicated time points. Cells were counted, appropriately diluted and plated onto EMM plates. Viability was calculated by dividing the number of colonies by the

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number of cells inoculated at each time point. Viability of cells exposed to osmotic or heat shock was determined as follows: cells were grown in EMM to 2–3 × 10^6 cells/ml at 25°C, reinoculated in fresh EMM at 0.8–1 × 10^6 cells/ml, and grown to 3–4 × 10^6 cells/ml. Aliquots were taken and incubated at 45°C for varying times. Cells were counted, diluted and plated onto EMM plates for incubation at 25°C. For osmotic shock, cells were similarly precultured, washed with EMM supplemented with NaCl at final concentration of 0.9M (EMM + 0.9 M NaCl), re-inoculated into EMM+0.9 M NaCl and further incubated at 25°C. After incubating for the times indicated, aliquots were taken, washed with EMM, the cell number was counted and plated on EMM plates at 25°C for 3–5 days. Viability was calculated as above.

2.4. Assay for mating efficiency

Exponentially growing cells in appropriate medium were washed with EMM and inoculated in EMM at a density of 0.8–1 × 10^6 cells/ml and grown to 4–5 × 10^6 cells/ml at 25°C. Cells were washed with, and reinoculated in, nitrogen-free minimal medium [EMM(−N)] at a density of 5–8 × 10^6 cells/ml and further incubated at 25°C. Aliquots were taken at the indicated time points, and after gentle sonication, the number of cells, zygotes and spores were counted under a microscope. The percentage of mating frequencies was calculated by dividing the number of zygotes and spores by the number of total cells (one zygote counted as two cells and one spore counted as a half cell).

2.5. Northern blot analysis

Cells were grown under the same conditions as for assaying the mating efficiency. Northern blot analysis was performed as described [21] with the 821bp *Pvu*II-*Fba*I fragment of the *ste11*$^+$ gene as a probe.

3. Results

3.1. phh1 kinase is the downstream effector of Wis1 kinase

To understand the molecular nature and the biological role of *wis1*$^+$, we attempted to identify components of the Wis1 signal cascade, particularly its downstream component. For this purpose, we employed degenerated oligonucleotide-derived PCR and isolated pAL*phh1*$^+$-3 which can rescue the *cdc25–22* mutant at 33.5°C (see section 2.2 and below). pAL*phh1*$^+$-3 contained an ORF encoding a protein homologous to known

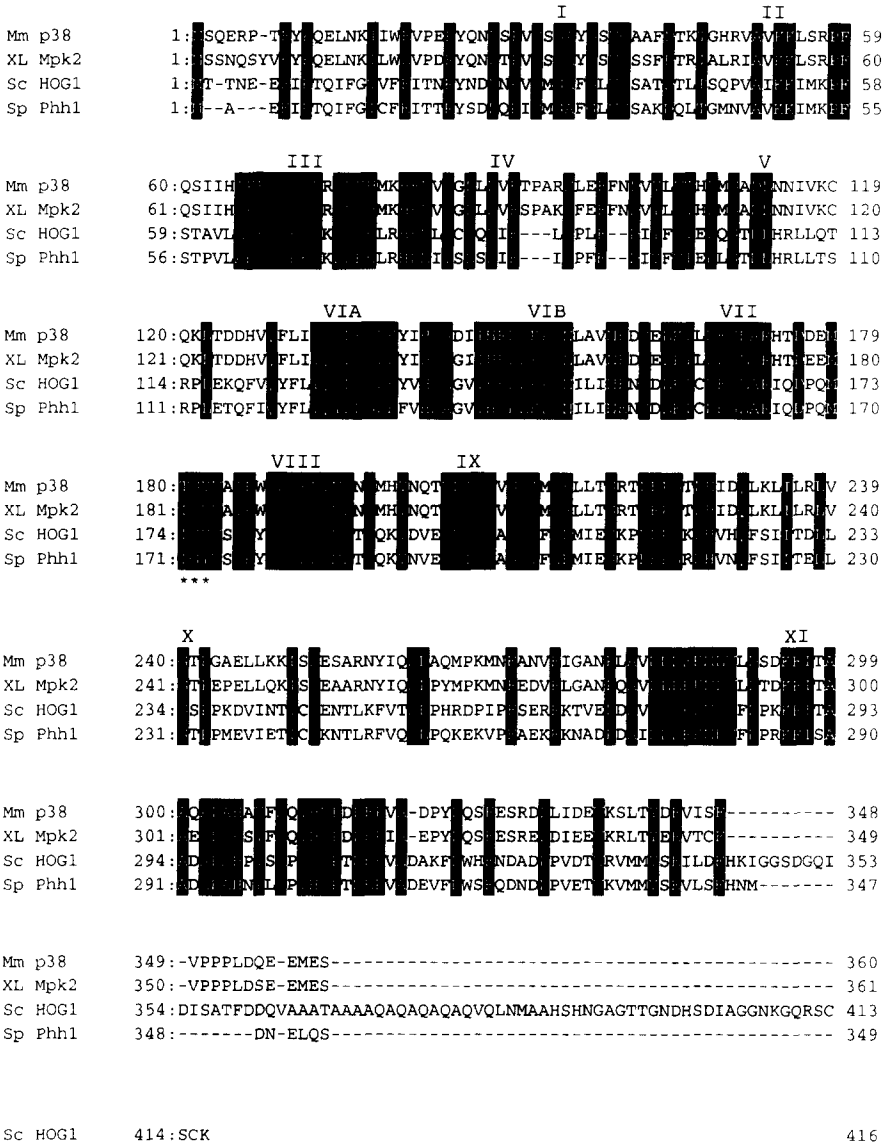

Fig. 1. Comparison of the amino acid sequences of Phh1p, mouse p38, *Xenopus* RK/Mpk2 and *Saccharomyces cerevisiae* Hog1p. Amino acids identical among all four proteins are shown in white against black. Gaps in the sequences that were introduced to optimize the alignment are illustrated with dashes. The tripeptide dual phosphorylation motif, TGY, in this subgroup of MAP kinase is indicated by asterisks. Protein kinase subdomains are indicated with Roman numerals.

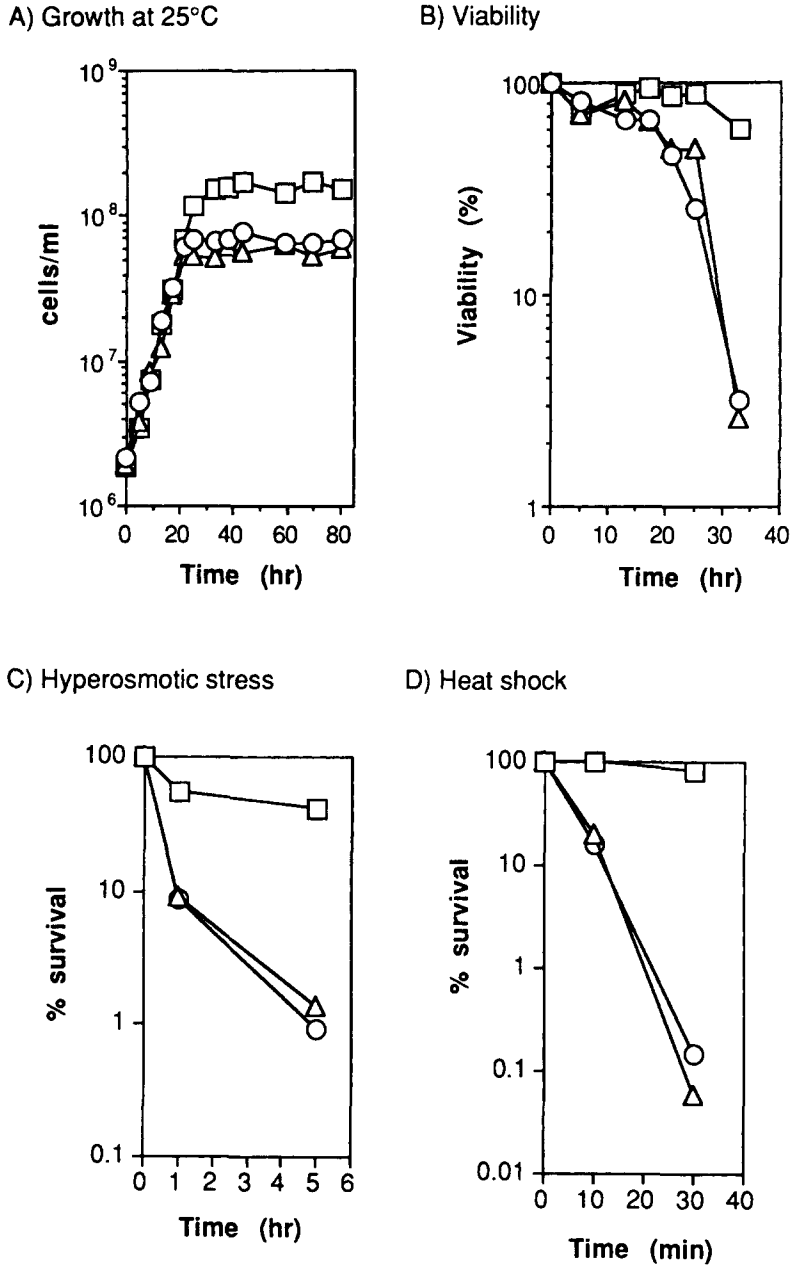

Fig. 2. Viability of wild type, *Δwis1* and *Δphh1* strains after entry into stationary phase. (A) Growth of TK003 (h^- wild type; open squares), TK102 (h^-*Δwis1*; open triangles) and TK107 (h^-*Δphh1*; open circles) strains in EMM medium at 25°C. (B) Viability of wild type and mutant strains. (C) Hypersensitivity of TK003 (h^- wild type; open squares), TK102 (h^- *Δwis1*; open triangles) and TK107 (h^-*Δphh1*; open circles) strains to hyperosmotic stress. (D) Hypersensitivity of TK003 (h^- wild type; open squares), TK102 (h^-*Δwis1*; open triangles) and TK107 (h^-*Δphh1*; open circles) strains to heat shock.

MAPKs. A particularly high homology was noticed with HOG1 of *S. cerevisiae*; 68.8% identity in amino acids. Hence it was designated as *phh1*$^+$ (*p*ombe *h*omologue of *H*OG1). Recently, mammalian homologues of HOG1 were also isolated [25–28]. Amino acid sequence alignment of these proteins is shown in Fig. 1. This group of MAPKs possesses Thr-Gly-Tyr for the tripeptide dual phosphorylation motif (asterisks in Fig. 1) that is required for activation [5]. The fact that Phh1 fell into the Thr-Gly-Tyr class of MAPKs suggested that it might be a putative downstream effector of Wis1 because Wis1 is most highly related to PBS2 of *S. cerevisiae*, the upstream activator of HOG1.

To examine whether Phh1 is the downstream effector of Wis1 or not, we tested the ability of the *phh1*$^+$ gene to rescue the *cdc25-22* mutant at 33.5°C. It indeed suppressed the mutant to the same extent as *wis1*$^+$ does (data not shown). For further analysis, cells deleted for *phh1*$^+$ (*Δphh1*) were constructed by one-step gene replacement (see section 2.2). *Δphh1* cells divided at increased cell length, just like the *wis1* disruptant [12]. *Δphh1* cells also showed a drastic reduction in viability upon entry into stationary phase. Wild type, *Δwis1* and *Δphh1* cells were grown in minimal medium to confluence, and their cell number and viability were monitored (Fig. 2A and B). They all grew at almost the same rate in a logarithmic phase. However, once

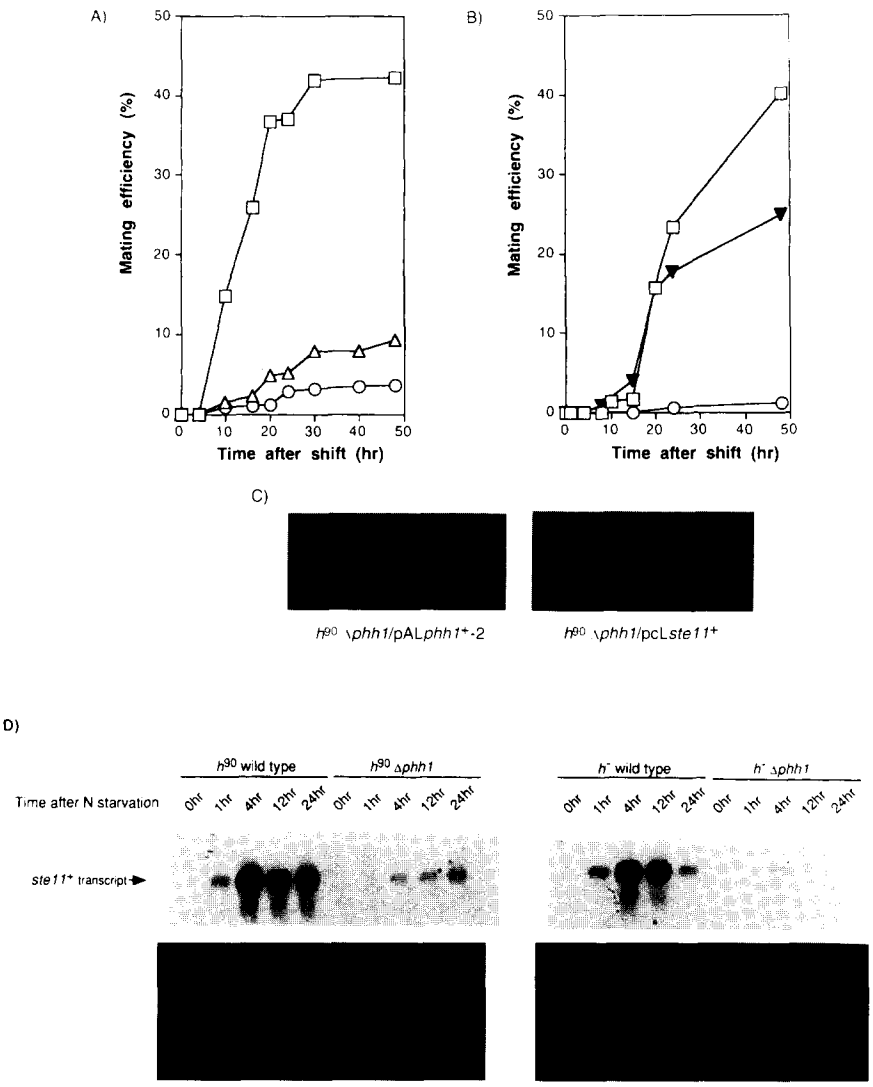

Fig. 3. (A) Mating efficiency of TK001 (h^{90}wild type; open squares), TK100 ($h^{90}\Delta wis1$; open triangles) and TK105 ($h^{90}\Delta phh1$; open circles) mutant strains. (B) Mating efficiencies of *Δphh1* cells transformed with pALSK (open circles), pAL*phh1*$^+$−2 (open squares), or pcL*ste11*$^+$ (closed inverse triangles). (C) Morphological characteristics of pAL*phh1*$^+$−2 or pcL*ste11*$^+$ transformed *Δphh1* cells undergoing sexual development. (D) Northern blot analysis of *ste11*$^+$ transcript in wild type and *Δphh1* cells (upper). 10 μg of total RNAs prepared from wild type and *Δphh1* strains of both homothallic and heterothallic genetic backgrounds were subjected to northern blotting analysis. Photograph of ethidium stained agarose gels are shown below.

they reached the end of logarithmic phase, both *Δwis1* and *Δphh1* cells started to lose their viability to the same extent and in the same time course.

Pbs2 and Hog1 are key components of a osmosensing signaling cascade in *S. cerevisiae* [29–31], and therefore, inactivation of either gene leads to cell death in high osmolarity medium [31,32]. In addition, *wis1*$^+$ is allelic to *spc2*$^+$, a suppressor of cell death in high salt medium of a protein phosphatase 2C mutant [14]. We therefore examined the response of the *Δphh1* strain to hyperosmotic stress. Wild type, *Δwis1* and *Δphh1* cells rapidly growing in minimal medium were exposed to hyperosmotic shock (0.9 M NaCl) and their viability was determined. As shown in Fig. 2C, both *Δwis1* and *Δphh1* cells were hypersensitive to osmotic shock to the same extent.

Unlike a *pbs2* strain of *S. cerevisiae*, the *Δwis1* cells are highly sensitive to various kinds of stress ([33,34]; T.K. Jr. unpublished observation). Recently, a mammalian homologue of Hog1 was identified as a kinase activated by lipopolysaccharides, cytokines and a variety of stress ([25–28]; for review see [4,5,35]). This led us to investigate whether *Δphh1* strain is sensitive to stress other than hyperosmotic stress. Wild type, *Δwis1* and *Δphh1* cells rapidly growing in minimal medium were exposed to heat shock at 45°C for various times. As shown in Fig. 2D, both *Δwis1* and *Δphh1* strains were hypersensitive to heat shock. Furthermore, both *Δwis1* and *Δphh1* cells were sensitive to UV, γ-ray, hydroxyurea and bleomycin (data not shown). In addition, *Δwis1 Δphh1* double disuptant cells were phenotypically indistinguishable from *Δphh1* cells, and consistent with the general property of MAPK that MAPK is inactive without activation by MAPKK, overexpression of *phh1*$^+$ was unable to suppress the stress sensitivity of *Δwis1* cells (data not shown). These results, together with the protein structure, led us to tentatively conclude that Phh1 is the downstream effector of Wis1 and, moreover, that the Wis1 and Phh1 signal cascade is deeply

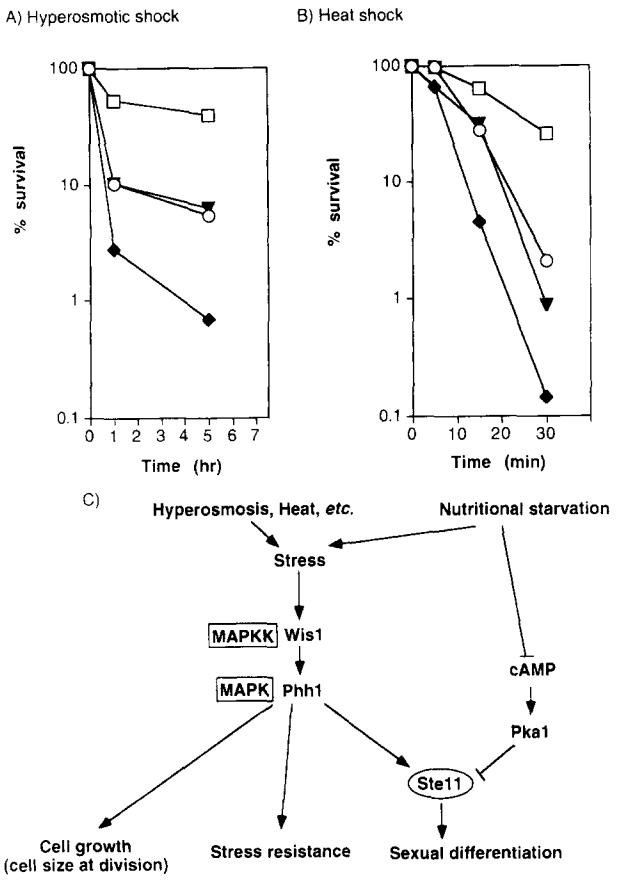

Fig. 4. Hypersensitivity of pALSK- (open circles), pAL*phh1*$^+$-2- (open squares), pAL*ste11*$^+$-(closed triangles) or pcL*ste11*$^+$-(closed diamonds) transformed *Δphh1* cells to osmotic stress and heat shock. (A) Viability after exposure to hyperosmotic stress. (B) Viability after exposure to heat shock. (C) The Wis1-Phh1 cascade mediates stress signal and controls both stress resistance and sexual development. Sexual development is controlled via induction of *ste11*$^+$ transcript. Phh1 is antagonistic to Pka1. The *Δphh1 Δpka1* double disruptant showed intermediate phenotype concerning fertility (data not shown).

involved in protective cellular responses to a wide variety of stress including hyperosmosis, heat shock, genotoxic agents and nutritional deprivation.

3.2. Δphh1 *strain is partially sterile and defective in nitrogen starvation-responsive* ste11$^+$ *induction*

In addition to a defect in stress response, *Δphh1* strain exhibits partial sterility. Upon nitrogen starvation, up to 45% of h^{90}wild type cells were able to conjugate whereas only 7–9% of h^{90}*Δwis1* and 3–4% of h^{90}*Δphh1* cells were so (Fig. 3A). Lower conjugational frequencies were constantly observed for h^{90}*Δphh1* cells. We attributed this at least partly to the slight ability of the mating pheromone pathway, perhaps Byr1 the MAPKK in the pathway, to activate Phh1. *Δwis1* cells with the h^{90} homothalic background were significantly more resistant to stress, particularly in the presence of *phh1*$^+$ overexpression, but deletion of *byr1*$^+$rendered *Δwis1* cells more susceptible to stress (data not shown).

The *ste11*$^+$ gene encodes a transcription factor with the HMG-box motif that plays a central role in the initiation of sexual differentiation [20]. To understand molecular mechanism for the sterility of *Δphh1* strain, we examined induction of the *ste11*$^+$ transcript in *Δphh1* cells upon nitrogen starvation. As shown in Fig. 3D, induction of *ste11*$^+$ was severely reduced in *Δphh1* cells with both homothalic and heterothalic genetic backgrounds. This result indicates that the sterile phenotype of *Δphh1* may be caused by a defect in *ste11*$^+$ induction.

3.3. ste11$^+$ *expression restores fertility but not stress resistance to* Δphh1 *strain*

To investigate whether the partial sterility of *Δphh1* was indeed caused by a defect in *ste11*$^+$ induction, the *ste11*$^+$ gene was expressed from a constitutive promoter in *Δphh1* cells, and its effect on the fertility and stress resistance of this strain was examined. As shown in Fig. 3B, the conjugational ability of *Δphh1* was restored by expressing the *ste11*$^+$ gene from the SV40 promoter, to a similar extent to that obtained with the *phh1*$^+$ gene. In this experiment, the control *Δphh1* cells expressing an empty vector conjugated at <2%. The slightly lowered mating frequency of *ste11*$^+$-expressing *Δphh1* cells after 30 h was perhaps due to their stress lability during meiosis (see below). This restoration was dependent on the level of *ste11*$^+$ expression. When *ste11*$^+$ with its own promoter was introduced, the restoration of mating efficiency was lower than this (data not shown). However, the post-conjugational processes were still defective in the *Δphh1* strain expressing *ste11*$^+$ and consequently, spore formation was significantly reduced (data not shown) perhaps due to stress lability. *ste11*$^+$-overexpressing *Δphh1* cells did not look as healthy as the *Δphh1* cells transformed with the *phh1*$^+$ gene (Fig. 3C).

Unlike fertility, stress resistance was not restored by expression of *ste11*$^+$. Enforced expression of *ste11*$^+$ failed to restore hyperosmotic and heat shock resistance to *Δphh1* cells and rather made the cells more sensitive (Fig. 4A and B).

4. Discussion

In this study we isolated a new member of the MAPK family from *S. pombe* and showed that the MAPK signaling cascade involving this kinase controls protective cellular responses to various kinds of stress and the start of sexual differentiation. In addition, this signaling cascade seems to be linked to the cell cycle control machinery as already suggested ([12]; T.K. Jr. and H.M., unpublished results).

Phh1 has the highest similarity in structure and function to Hog1 of budding yeast. But, unlike Hog1, Phh1 mediates sig-

Table 1
Strains used in this study

	Genotype	Source or reference
DK1	*h*$^+$/*h*$^-$ *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4D-18/ura4D-18*	21
Dphh1 +/–	*phh1*$^+$/*phh1::ura4*$^+$*h*$^+$/*h*$^-$ *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4D-18/ura4D-18*	this study
TK001	h^{90} *leu1-32*	our stock
TK003	*h*$^-$ *leu1-32*	our stock
ED1009	*h*$^-$ *wis1::his1*$^+$ *his1-102 leu1-32 ura4-D18*	S. Stetler and P.A. Fantes
TK100	h^{90} *wis1::his1*$^+$ *his1-102 leu1-32*	ED1009
TK102	*h*$^-$ *wis1::his1*$^+$ *his1-102 leu1-32*	ED1009
TK105	h^{90} *phh1::ura4*$^+$ *leu1-32 ura4D-18*	this study
TK107	*h*$^-$ *phh1::ura4*$^+$ *leu1-32 ura4D-18*	this study

nals not only from hyperosmotic stress but also from various other kinds of stress, such as heat, genotoxic drugs and nutritional starvation. More surprisingly, these stress signals, mediated by Phh1, are required for proper sexual development. Cells deleted for the *phh1*$^+$ gene are semi-sterile with markedly reduced induction of *ste11*$^+$ during nitrogen starvation. Enforced expression of *ste11*$^+$ in *Δphh1* cells restored fertility, but not stress resistance, to the cells. Thus, in fission yeast, stress signals positively control cellular differentiation. Unlike budding yeast, fission yeast starts sexual development when starved for nutrients. Therefore, this organism might have evolved a system that integrates stress signals into the control of differentiation. This appears to be highly analogous with the situation in mammalian cells. Lipopolysaccharides and lymphokines activate a Hog/Phh1-like pathway and induce growth and differentiation [26–28].

All the evidence presented in this study indicates that Wis1 is the upstream activator of Phh1 kinase. While this manuscript is in preparation, Millar et al. reported isolation of the same gene and showed that Wis1 phosphorylates and activates Phh1 in vitro [36]. Finally, a model for the Wis1-Phh1 pathway and its regulation of stress response, cell growth and sexual development, which is constructed on the data we presented, is illustrated in Fig. 4C. Efficient induction of *ste11*$^+$ requires at least a stress signal mediated by the Wis1-Phh1 pathway and a decrease in positive growth signal mediated by the cAMP-Pka1 pathway, as consequences of nitrogen starvation.

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