Multiple modes of activation of the stress-responsive MAP kinase pathway in fission yeast

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The *Schizosaccharomyces pombe* *wis1*⁺ gene is essential for cell survival under stress conditions. The MAPKK homologue Wis1 is required for activation of the MAPK homologue Spc1, and integrity of the Wis1–Spc1 pathway is required for survival in extreme conditions of heat, osmolarity, oxidation or limited nutrition. We show here that Wis4, a protein kinase of a new MAPKKK class, phosphorylates Wis1 *in vitro* and activates it *in vivo*. Win1 is also required for full activation of Wis1, and Win1 rather than Wis4 mediates the osmotic stress signal. Surprisingly, the pathway can still be activated by heat or oxidative stress independently of the phosphorylation of two conserved Wis1 residues. Evidence is presented that the Pyp1 protein tyrosine phosphatase, which dephosphorylates Spc1, is central to this alternative activation mechanism.

Keywords: cell cycle control/fission yeast/MAP kinase signalling pathway/protein tyrosine phosphatase/stress response

Introduction

Eukaryotic cells typically respond to external stimuli by activation of signalling pathways, which eventually leads to changes in cellular activities such as gene expression and cell cycle control. In many cases, activation of a MAP kinase (MAPK) homologue is crucial (Ruis and Schuller, 1995; Kyriakis and Avruch, 1996). MAPKs are activated by phosphorylation at conserved threonine and tyrosine residues in the catalytic domain by a MAPK kinase (MAPKK), which is itself activated by phosphorylation at specific conserved serine/threonine residues (Payne *et al.*, 1991; Nakielnny *et al.*, 1992; Alessi *et al.*, 1994; Zheng and Guan, 1994). Several MAPK cascades can exist in a single cell and operate in parallel, each mediating a specific signal (Herskowitz, 1995). In some systems, activation of a single MAPK can be effected by different types of signal. Mammalian SAPK/JNK, for example, is activated by UV irradiation, heat, high osmolarity or inflammatory cytokines (Hibi *et al.*, 1993; Galcheva-Gargova *et al.*, 1994; Kyriakis *et al.*, 1994). One important question is how the various signals are integrated to activate a single response pathway.

In the fission yeast *Schizosaccharomyces pombe*,

integrity of the Wis1–Spc1 pathway is required for survival under several stress conditions, and the MAPKK homologue Wis1 is required for activation of the MAPK homologue Spc1 following osmotic, heat or oxidative stresses (Degols *et al.*, 1996; Kato *et al.*, 1996). Other genes relevant to the pathway are *pyp1*⁺ and *pyp2*⁺, whose products are protein tyrosine phosphatases that dephosphorylate the phosphotyrosine on Spc1 (Millar *et al.*, 1995; Shiozaki and Russell, 1995). *pyp2*⁺ is believed to serve for negative feedback regulation of the pathway (Millar *et al.*, 1995). The physiological function of *pyp1*⁺ is unknown. The functional resemblance of mammalian SAPK/JNK and p38 pathways and the fission yeast Wis1–Spc1 pathway (Millar *et al.*, 1995; Shiozaki and Russell, 1995, 1996; Degols *et al.*, 1996), in combination with the genetic amenability and physiology of fission yeast, make the organism an ideal model system for investigating stress response pathways.

In addition to involvement in stress responses, *wis1*⁺, *spc1*⁺ and *pyp1*⁺ have roles in cell cycle progression. Mutations in these genes as well as their overexpression result in changes in cell cycle regulation at the G₂–mitosis transition. *wis1*⁺ originally was isolated in a context of cell cycle control, and was shown to be a mitotic inducer (Warbrick and Fantes, 1991). *pyp1*⁺ and *pyp2*⁺ have been shown negatively to regulate entry into mitosis (Millar *et al.*, 1992). *wis1* and *spc1* (also known as *phh1*; Kato *et al.*, 1996) were re-isolated as *sty2* and *sty1*, respectively, in a screen for mutations that suppress the synthetic lethal phenotype caused by loss of the two tyrosine phosphatases (Millar *et al.*, 1995). Neither *wis1*⁺ nor *spc1*⁺ are essential genes (Warbrick and Fantes, 1991; Millar *et al.*, 1995; Shiozaki and Russell, 1995), but their combinations with *cdc25-22* are synthetically lethal (Shiozaki and Russell, 1995, 1996).

Of the five *wis* genes that were isolated as multicopy suppressor genes of the triple mutant *win1-1 cdc25-22 weel-50* (Warbrick and Fantes, 1991, 1992; Weisman *et al.*, 1996), only *wis1*⁺ and *wis4*⁺ suppress the cell length phenotype of the *win1-1* mutation (Ogden and Fantes, 1986), suggesting a close relationship among the three genes (Warbrick and Fantes, 1992). We have characterized the *wis4*⁺ gene and the *win1-1* mutation, and show here that these genes encode components of the fission yeast stress-activated MAPK pathway. Wis4, a protein kinase of a new MAPKKK class, phosphorylates Wis1 *in vitro* and activates it *in vivo*. Win1 is also required for full activation of Wis1, and Win1 rather than Wis4 mediates the osmotic stress signal. Surprisingly, the pathway can still be activated by heat or oxidative stress in cells expressing only a mutant Wis1 protein lacking two conserved phosphorylation/activation residues, suggesting an independent mechanism for activation of the pathway. We present evidence that Pyp1, a tyrosine phosphatase

that dephosphorylates Spc1, is crucial to the activation of the pathway by heat and oxidative stresses.

Results

***wis4⁺* encodes a MAPKKK homologue of a new class**

The plasmid pWF7 is the minimum subclone of the original *wis4⁺* clone pWF1 (pN2 in Warbrick and Fantes, 1992) able to suppress the temperature-sensitive phenotype of the triple mutant *win1-1 cdc25-22 wee1-50* (Figure 1A). Subsequent DNA sequence analysis identified a single open reading frame (ORF) which encodes a polypeptide of 1399 amino acid residues, with a set of motifs conserved among protein kinases (Hanks *et al.*, 1988) located at the C-terminus (residues 1037–1304) (Figure 1A and B). Wis4 is most similar to the protein kinases encoded by two *Saccharomyces cerevisiae* genes, *SSK2* and *SSK22*, which function upstream of *PBS2* in the osmosensing signal transduction pathway (Maeda *et al.*, 1995). In addition to high sequence homology within the kinase domain (57%), the three proteins share >30% identity overall (Figure 1B), and appear to define a distinct subfamily. Wis4, Ssk2 and Ssk22 share substantial homology (up to 40%) within the kinase domain with other MAPKKKs. However, much of this similarity is due to the presence of residues shared among all protein kinases (so that, for example, the Wis4 group show 35% identity to MAPKKs), and nearly half of the residues specifically conserved among conventional MAPKKKs are not shared with those of any member of the Wis4 subfamily (Figure 1C). In addition, the characteristic sequences specific to the Wis4 subfamily are most evident in domains IV, VIa and X (Figure 1C). In these regions, conservation is weak across serine/threonine protein kinases in general, and the residues are not directly involved in the catalytic reaction (Hanks *et al.*, 1988).

Wis4* phosphorylates and activates *Wis1

wis1⁺ and *wis4⁺* were isolated in the same genetic screen (Warbrick and Fantes, 1992) and encode protein kinases homologous to members of the MAPKK and MAPKKK families respectively, suggesting that Wis4 would phosphorylate and activate Wis1. We therefore assayed Wis4 kinase activity towards Wis1 *in vitro* using a recombinant substrate, GST–Wis1(K349R). This substrate was phosphorylated by wild-type Wis4, but very poorly by Wis4(K1066R), a mutant protein in which the conserved lysine residue within the ATP-binding site (Hanks *et al.*, 1988) had been replaced with arginine (Figure 2A). Phosphoamino acid analysis of the phosphorylated GST–Wis1 showed that both serine and threonine were phosphorylated (data not shown).

Overexpression of the full-length *wis4⁺* gene did not result in any notable phenotype. Wis4, in common with other known MAPKKKs, has a long, non-catalytic domain at its amino-terminus; deletion of this domain in other MAPKKKs has been reported to result in constitutively active gene products (Cairns *et al.*, 1992; Lee and Levin, 1992; Stevenson *et al.*, 1992). When the Wis4 catalytic domain lacking the amino-terminal 958 residues (Wis4ΔN) was overexpressed in wild-type *S.pombe* cells, they became swollen (Figure 2B). No such effect was shown

by the kinase-inactive allele of Wis4ΔN(K1066R) (Figure 2B). This phenotype, similarly to that conferred by Wis1 overexpression (Shiozaki and Russell, 1995), was abolished in a *wis1Δ* background (Figure 2B), showing that the *wis4*-conferred phenotype is dependent on activity of the *wis1⁺* gene.

We next assayed Wis1 kinase activity *in vivo*, by determining the level of phosphotyrosine on the Spc1 protein (Figure 2C), because Wis1 is the sole kinase able to phosphorylate Tyr173 on Spc1 (Shiozaki and Russell, 1995). An increased level of phosphotyrosine was detected on Spc1 in *wis1⁺* cells overexpressing Wis4ΔN, but not in *wis1Δ* cells, indicating that the action of Wis4 is Wis1-dependent. Expression of the catalytically inactive mutant protein Wis4ΔN(K1066R) did not increase the Spc1 phosphotyrosine level. These data suggest that Wis4 activates Wis1 *in vivo*.

***Wis1* activation requires two conserved phosphorylation residues**

Wild-type cells strongly overexpressing the *wis1⁺* gene from the pREP1 *nmt1* promoter (Maundrell, 1990) are barely able to form colonies (Shiozaki and Russell, 1995). The toxicity of high levels of Wis1 is due to its kinase activity, because overexpression of a ‘kinase-dead’ Wis1 construct (in which the conserved lysine residue at the ATP-binding site is replaced with arginine) is not toxic (data not shown). Furthermore, loss of Spc1, the only known substrate of Wis1, abolishes lethality (Millar *et al.*, 1995; Shiozaki and Russell, 1995). MAPKKs contain two conserved sites within the kinase domain whose phosphorylation is necessary for full activity (Alessi *et al.*, 1994; Zheng and Guan, 1994). The corresponding residues in Wis1 are serine and threonine residues at positions 469 and 473 respectively. To investigate the possible role of these residues, we mutated both either to alanine (Wis1AA) or to glutamate (Wis1EE), to mimic unphosphorylated and phosphorylated states respectively. Both constructs were expressed in wild-type cells. Cells overexpressing Wis1AA were able to form colonies similar in size to control cells carrying only the plasmid vector (data not shown) while overexpression of the Wis1EE construct was toxic (Figure 3), suggesting that Wis1 toxicity requires phosphorylation of the S469 and T473 residues, mimicked by the substitution of these residues by glutamate. Consistent with these observations, Wis1AA shows greatly reduced kinase activity *in vivo* (data not shown), while Wis1EE is moderately active (Figure 5).

***Wis4* and *Win1* activate *Wis1* via two conserved phosphorylation sites**

We exploited the toxicity of high Wis1 activity to investigate the relationship between Wis4 and Wis1 further. In a *wis4Δ* strain (see below and Materials and methods) the toxicity of overexpression of the wild-type *wis1⁺* gene was alleviated (Figure 3), indicating that Wis4 acts within the same pathway as Wis1. In contrast, overexpression of Wis1EE in the *wis4Δ* strain was lethal (Figure 3). Thus Wis1 activation requires or is greatly enhanced by activation by Wis4, but this requirement is bypassed if the conserved phosphorylation sites on Wis1 are replaced by glutamate residues, which mimic phosphorylation. The simplest explanation for these observations is that Wis4

phosphorylates Wis1 on S469 and/or T473, which results in its activation. An alternative explanation—that Wis4 acts downstream of Wis1—seems very unlikely since loss of Spc1 (which is known to act downstream of Wis1) abolished the toxicity of overexpression of either wild-type Wis1 or Wis1EE (Figure 3).

wis4Δ cells are viable, and show modest elongation during growth on minimal medium (Figure 4), a phenotype reminiscent of that of *win1-1* cells. This, and the observation that modest overexpression of *wis4⁺* suppresses the *win1-1* phenotype (Warbrick and Fantes, 1992), led us to investigate interactions between *win1* and *wis1*. The *win1-1*

mutation, like *wis4Δ*, prevented the toxicity caused by overexpression of Wis1, but not of Wis1EE (Figure 3), suggesting that Win1 could activate Wis1 through its conserved phosphorylation sites. Win1 acts independently of Wis4 since the double mutant *wis4Δ win1-1* shows stronger phenotypes than either of the single mutants. Cell length of the double mutant is substantially greater than that of the respective single mutants (Figure 4), and the double mutant cannot form colonies on high osmolarity medium (Figure 4) or at 36°C (data not shown). Overall, the double *wis4Δ win1-1* mutant is very similar to a *wis1Δ* strain, suggesting that Wis4 and Win1 might act independently and additively to activate Wis1. In this context, we note that the predicted *win1*⁺ product contains a protein kinase domain with high similarity to that of Wis4 (I.S.Samejima and P.Fantes, unpublished data), making it likely that Win1 acts directly on Wis1.

As a further test, we constructed a strain in which the chromosomal copy of *wis1*⁺ is replaced by the *wis1EE* allele (henceforth referred to as *wis1-4^{EE}*). The Spc1 phosphotyrosine level of a *wis1-4^{EE}* strain was somewhat reduced compared with that of a *wis1*⁺ strain (Figure 5), perhaps because glutamate residues at positions 469 and

473 are less potent activators than phosphorylated serine and threonine residues. Consistent with the requirement for Wis4 and Win1 to effect full Wis1 activation, the Spc1 phosphotyrosine level of a *wis1*⁺ strain was reduced by either *wis4Δ* or *win1-1* mutations. The double mutant *wis4Δ win1-1* strain showed an even lower phosphotyrosine level (Figure 5). However, the *wis1-4^{EE} wis4Δ win1-1* strain showed a very similar phosphotyrosine level to the *wis1-4^{EE}* strain, indicating that Wis1EE bypasses the requirement for activation by Wis4 or Win1. This is consistent with observations that the mutant phenotypes due to the *win1-1 wis4Δ* combination were suppressed by the presence of the *wis1-4^{EE}* allele (data not shown). Furthermore, Wis1EE cannot be activated by Wis4, as shown by the observations that overexpression of *wis4ΔN* in a *wis1-4^{EE}* strain causes neither the increase in Spc1 phosphotyrosine nor the toxicity observed in a *wis1*⁺ strain (data not shown).

Win1 is responsible for osmostress signalling

Exposure of wild-type cells to 0.4 M KCl leads to Wis1 kinase activation which results in elevated phosphotyrosine on Spc1 (Millar *et al.*, 1995; Shiozaki and Russell, 1995).

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Fig. 1. (A) Subcloning of pWF1. A restriction map of pWF1 is shown, with the *wis4* ORF as a black box and its kinase domain in grey. pWF7 was the minimal clone suppressing the phenotype of the triple mutant *win1-1 cdc25-22 weel-50*, although it lacks the amino-terminal portion. The nucleotide sequence is available under the DDBJ/EMBL/GenBank accession number Y07750. For construction of the *wis4* deletion allele, the insert of pWF37 was integrated into the *S.pombe* chromosome by gene transplacement (see Materials and methods). (B) Amino acid sequence alignment of protein kinases Wis4, Ssk2 and Ssk22. Residues identical to *S.pombe* Wis4 are shown in white letters on black, and those conserved between *S.cerevisiae* Ssk2 and Ssk22, but not with Wis4, are shown in black letters on grey. (C) Amino acid sequence alignment of catalytic domains of MAPKKK types. The sequences of protein kinases that phosphorylate MAPKKs are aligned; Wis1 is also included, to distinguish the residues conserved among MAPKKs (black letters on grey) and those beyond MAPKKs (white letters on black). Some residues conserved among Wis4, Ssk2 and Ssk22 are indicated by white letters on grey. Roman numerals indicate subdomains defined by Hanks *et al.* (1988). Arabic numerals indicate the number of residues omitted here. DDBJ/EMBL/GenBank accession numbers for referred sequences are: *wis1*, X62631; *wis4*, Y07750; SSK22, P25390; SSK2, L41927; BCK1, X60227; STE11, X53431; BYR2, M74293; MKH1, U53872; MEKK1, U23470; RAF1, P04049; TAK1, D76446.

Fig. 2. Wis4 phosphorylates and activates Wis1. (A) Wis4 phosphorylates Wis1 *in vitro*. A catalytically inactive mutant, GST-Wis1(K349R), expressed in *Escherichia coli*, was purified and used as the substrate for the Wis4 kinase assay. GST-Wis1(K349R), which lacks autophosphorylation activity (lane 1), was incubated with wild-type (lane 2) or mutant (lane 3) Wis4 protein purified from *S. pombe* cell extracts. The mutant Wis4 has an arginine instead of the conserved lysine at position 1066 within the ATP-binding site. Lanes 4 and 5 show reactions run without substrate. (B) Phenotypes resulting from overexpression of Wis4ΔN are dependent on *wis1*⁺ function. The wild-type or mutant Wis4 catalytic domain was overexpressed from the pREP1 *nmf* promoter (Maundrell, 1990). Cells became swollen when the Wis4 catalytic domain (left, top row), but not the corresponding inactive domain (middle, top row), was overexpressed. The phenotypes were abolished in the *wis1*Δ background (bottom row). (C) Wis4 activates Wis1 *in vivo*. The level of phosphotyrosine on Spc1 protein was assayed in cell extracts from various strains (upper row). The wild-type strain (KS1376) was transformed with plasmids that express the wild-type (lanes 1 and 3) or mutant (lanes 2 and 4) Wis4 catalytic domain. When the *nmf* promoter was repressed by thiamine, both strains had a phosphotyrosine content (lanes 1 and 2) similar to that in cells carrying a vector plasmid (lane 7). Overexpression of wild-type Wis4 (lane 3), but not the mutant Wis4 (lane 4), resulted in increased phosphotyrosine on Spc1 protein. No phosphotyrosine signal was detected in a *wis1*Δ background (lanes 5 and 6). The lower row shows the amount of Spc1 protein detected by anti-HA antibody.

A wild-type strain, a strain expressing only the mutant Wis1AA protein and a *wis1*-4^{EE} strain were exposed to KCl and their Spc1 phosphotyrosine levels assayed (Figures 6 and 7). Before the shock, the strain expressing Wis1AA showed a very low level of phosphotyrosine, while the *wis1*-4^{EE} strain showed a moderate level. The Spc1 phosphotyrosine level increased substantially after exposure of a *wis1*⁺ strain to KCl, but in neither the Wis1AA nor the *wis1*-4^{EE} strain was any increase observed (Figures

6 and 7). These observations suggest strongly that phosphorylation of Wis1 at S469/T473 is required for the osmotic response.

It seemed likely that Wis4 would be involved in activating Wis1 in response to osmotic stress, since it is required for basal Wis1 activity and acts through the S469/T473 phosphorylation sites which are essential for osmosignalling, and because the homologous MAPKKKs Ssk2 and Ssk22 are involved in osmosignalling in *S. cerevisiae* (Maeda *et al.*, 1995). We therefore examined the response of *wis4*Δ cells to osmotic shock, by monitoring the phosphotyrosine level on Spc1. Surprisingly, *wis4*Δ cells showed an increase in the level of Spc1 tyrosine phosphorylation after KCl treatment similar to that of wild-type cells (Figure 6). Furthermore, the kinetics of the increase in the phosphotyrosine level in *wis4*Δ paralleled those in wild-type cells, with a maximal level attained after 15 min, which persisted until 40 min, after which it started to fall (data not shown). These results indicate that Wis4 is not essential for osmosignalling. This suggested that Win1 might be involved, and indeed the increase in Spc1 phosphotyrosine level after KCl treatment of a *win1*-1 strain was greatly reduced (Figure 6). The Spc1 phosphotyrosine level remained low in the *win1*-1 mutant for 60 min (data not shown), during which the phosphotyrosine level rises and falls in wild-type (Millar *et al.*, 1995; Degols *et al.*, 1996) and *wis4*Δ strains (our data). This suggests strongly that the major route for osmosignalling upstream of Wis1 is through Win1 rather than Wis4.

Cells defective in osmotic stress signalling, such as *wis1*Δ or *spc1*Δ mutants, show a substantial increase in cell length during growth on KCl medium. *win1*-1 cells showed a similar but less pronounced effect (Figure 4), consistent with Win1 having a major role in osmosignalling. In contrast, *wis4*Δ cells were not elongated noticeably during growth on KCl medium, indicating that the osmosignalling capacity remains, presumably mediated by Win1. Double mutant *wis4*Δ *win1*-1 cells were highly elongated during growth on KCl medium, similarly to *wis1*Δ cells, and the double mutant showed a level of Spc1 tyrosine phosphorylation lower than that of *win1*-1 (Figure 5). The enhancement by *wis4*Δ of the *win1*-1 phenotype may reflect loss of basal Wis1 phosphorylation mediated by Wis4.

A novel mode of activation of the Wis1-Spc1 pathway

The Wis1-Spc1 pathway is activated by conditions other than osmotic stress, such as heat shock and oxidative stress (Degols *et al.*, 1996). We were interested to find out whether Wis4 or Win1 were required for activation of the pathway by these stresses. After exposure to heat or oxidative shocks, the Spc1 phosphotyrosine level increased in *win1*-1, *wis4*Δ and *win1*-1 *wis4*Δ double mutant strains to almost the same extent as a wild-type control (Figure 7A). This surprising result suggested that neither Wis4 nor Win1 was required for activation of the pathway following these treatments. One possible interpretation is that activation of Wis1 by phosphorylation independently of both Wis4 and Win1 might be involved. However, a strain expressing only the mutant Wis1AA protein showed an essentially normal increase in Spc1

Fig. 3. Wis4 and Win1 are activators of Wis1. The wild-type (*wis1*⁺) or an activated allele of *wis1* (*wis1EE*) was overexpressed from the pREP1 *nmt* promoter in wild-type, *wis4*Δ, *win1-1* or *spc1*Δ cells. Expression of *wis1* is repressed on medium containing thiamine (bottom row).

Fig. 4. Phenotypes of *wis4*Δ, *win1-1* and *wis4*Δ *win1-1*. The cell morphology of wild-type and mutant cells on minimal medium (EMM) without (upper row) or with (lower row) 1.2 M KCl is shown.

phosphotyrosine level after heat or oxidative stress (Figure 7A), showing that phosphorylation at the S469 and T473 residues of Wis1 is not required for increased tyrosine phosphorylation of Spc1 under these conditions. A *wis1-4^{EE}* strain showed a similar response to heat stress, and responded, although less strongly, to oxidative stress (Figure 7A).

What other mechanisms might account for the activation of Spc1 by heat and oxidative stresses? It is unlikely that any kinase other than Wis1 might phosphorylate Spc1, since *wis1*Δ strains show no detectable Spc1 phosphotyrosine (Millar *et al.*, 1995; Shiozaki and Russell, 1995). However, there may be ways of activating Wis1 other than by phosphorylation at S469 and T473: for instance by phosphorylation elsewhere on the molecule or by interaction with other proteins. Alternatively, the increase in Spc1 phosphotyrosine level following heat or oxidative stresses might result from loss of function of either of the tyrosine phosphatases Pyp1 or Pyp2, which dephosphorylate Spc1 (Millar *et al.*, 1995; Shiozaki and Russell, 1995).

To investigate whether activation of Wis1 or inactivation of Pyp1 or Pyp2 was involved in the activation of Spc1 by heat and oxidative stresses, we examined the responses of strains lacking each of the phosphatases. The *pyp2*Δ strain showed responses very similar to the wild-type control (Figure 7B). In contrast, the *pyp1*Δ strain showed a high basal level of Spc1 phosphotyrosine, which was not increased by any stress (Figure 7B). However, since

Fig. 5. Spc1 phosphotyrosine levels in wild-type and mutant strains. Phosphotyrosine and (HA-tagged) Spc1 proteins were detected by antibodies 4G10 (upper panel) and 12CA5 (lower panel) respectively. Lane 1, wild-type; lane 2, *wis4*Δ; lane 3, *win1-1*; lane 4, *win1-1 wis4*Δ; lane 5, *wis1-4* (*wis1EE*); lane 6, *wis1-4 win1-1 wis4*Δ.

Pyp1 acts directly on the phosphotyrosine of Spc1 (Shiozaki and Russell, 1995) and is the major cellular source of this activity (Millar *et al.*, 1992), a high phosphotyrosine level in the *pyp1*Δ strain might simply reflect saturation of the system, so that even in the absence of stress the phosphorylation activity of Wis1 would overcome the dephosphorylation activity of Pyp2 (and perhaps other phosphatases). We therefore constructed strains in which the activity of Wis1 was reduced in different ways, and tested their responses in a *pyp1*Δ background.

First, we examined a *pyp1*Δ strain in which the sole Wis1 activity in the cell derived from a chromosomal copy of the *wis1*⁺ coding region, expressed from the weak pREP81 promoter. When grown in the presence of thiamine (which represses transcription from this promoter), this strain showed a low level of Spc1 phosphotyro-

Fig. 6. Spc1 phosphotyrosine levels in wild-type and mutant strains in response to 0.6 M KCl. Each strain was treated with 0.6 M KCl for 15 min before harvest. Phosphotyrosine and (HA-tagged) Spc1 proteins were detected by antibodies 4G10 (upper panel) and 12CA5 (lower panel) respectively.

sine (Figure 7B). Osmotic stress led to a substantial increase in phosphotyrosine level, showing that we could, in this *pyp1Δ* strain, detect the effect of activating Wis1. However, heat shock induced no increase in the Spc1 phosphotyrosine level, suggesting that the absence of Pyp1 had blocked the normal response.

Second, we tested a *pyp1Δ* strain in which the only source of Wis1 activity was the WisAA protein. None of the stresses tested elicited any increase in the Spc1 phosphotyrosine level (Figure 7B). This contrasts with the strong responses of the *pyp1+* WisAA strain to heat and oxidative stresses (Figure 7A). The most likely explanation for the complete absence of response in the *pyp1Δ* Wis1AA strain is that osmotic stress is ineffective because of the absence of phosphorylation/activation sites on Wis1, while the effect of heat shock is blocked by the absence of Pyp1.

Neither phosphorylation of Wis1 on S469 and T473 (Figure 7A) nor Pyp1 (Figure 7B) is required for a response to oxidative stress, but loss of both (in *pyp1Δ* Wis1AA) abolishes the response (Figure 7B). This suggests that both mechanisms might contribute to the oxidative stress response.

Discussion

While the basic mechanisms by which eukaryotic cells respond to environmental stresses have become much better understood in the last few years, little is known of how diverse stress signals are integrated (Kyriakis and Avruch, 1996). This study describes the identification of different modes of activation of the stress-responsive Wis1–Spc1 signalling pathway, which depends on the type of imposed stress. We describe the identification of Wis4 as a MAPKKK homologue that phosphorylates Wis1 *in vitro*. While Wis4 is required for basal Wis1 activity *in vivo*, it is not required for normal response to osmotic stress, which does however require function of the *win1+* gene product. The responses of the pathway to heat or oxidative stress occur independently of both Wis4 and Win1, and of the conserved activating phosphorylation sites of Wis1. We present evidence that the tyrosine phosphatase Pyp1, which is known to dephosphorylate Spc1, is crucial for activation of the pathway by heat or oxidative stresses.

The regulation of MAPKs has until now been considered primarily in terms of activation by MAPKKs, which specifically phosphorylate threonine and tyrosine residues on their cognate MAPK and thereby increase its kinase activity (Kyriakis and Avruch, 1996). MAPKKs are themselves subject to regulatory activation. The best known mode of activation of MAPKKs is by phosphorylation of

Fig. 7. Spc1 phosphotyrosine levels in wild-type and mutant strains in response to different types of stress. First column, cells growing without imposed stress; second column, osmotic stress (0.5 M KCl); third column, oxidative shock (1 mM H₂O₂); fourth column, heat shock (48°C) (see Materials and methods). Phosphotyrosine and (HA-tagged) Spc1 proteins were detected by antibodies 4G10 (upper panel for each strain) and 12CA5 (lower panel) respectively. *wis1-81* is an allele of the *wis1+* gene, with the wild-type ORF being transcribed from the pREP81 *nmt* promoter instead of the native promoter. Under the conditions used, the promoter is repressed.

two conserved residues located between subdomains VII and VIII (Alessi *et al.*, 1994; Zheng and Guan, 1994). We show here that the conserved residues of the MAPKK homologue Wis1, Ser469 and Thr473, are important in the regulation of Wis1 activity *in vivo*. Replacement of these residues with non-phosphorylatable alanine residues causes a reduction in the basal level of phosphotyrosine on the MAPK substrate of Wis1, Spc1. A similar effect is seen in strains lacking Wis4 and/or Win1, activators of Wis1. Ser469 and Thr473 are also crucial for Spc1 phosphorylation and activation following osmotic stress, since cells in which the Ser469 and Thr473 residues of Wis1 have been mutated to alanine or glutamate do not respond (Figures 6 and 7A).

Strikingly, activation of Spc1 in response to other types of stress is not mediated through the phosphorylation of Wis1 on Ser469 and Thr473. Strains lacking both Wis4 and Win1 still activate Spc1 following heat and oxidative stresses, as do Wis1AA and Wis1EE strains. Clearly some other mechanism must be involved, two likely candidates being activation of Wis1 by some mechanism other than phosphorylation on Ser469 and Thr473, or activation of

Spc1 by a mechanism that does not involve regulation of Wis1 activity. We present evidence in favour of the latter hypothesis. Pyp1, the major tyrosine phosphatase responsible for dephosphorylation of Tyr173 of Spc1, is crucial to activation of the pathway by heat stress. Cells lacking *pyp1*⁺ have a high basal level of phosphotyrosine on Spc1, and this does not increase after osmotic, heat or oxidative stress. This is not merely due to saturation phosphorylation of Spc1 in the absence of Pyp1: in a *pyp1*Δ strain with reduced Wis1 activity, an increase in Spc1 phosphorylation response was observed after osmotic stress, but no response was observed after heat shock. This suggests strongly that heat shock activates the pathway by functional inactivation of Pyp1. The mechanism by which Pyp1 is inactivated remains to be determined. We note that although inactivation of Pyp1 is sufficient to explain the response to heat stress, we cannot exclude the formal possibility that activation of Wis1 by a mechanism not involving phosphorylation on Ser469 and Thr473 might additionally be involved, though in our opinion there is no evidence to support this.

During the preparation of this manuscript, two other groups reported the identification of the *mcs4* gene, whose product activates Wis1 (Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). They show that the action of Mcs4 on Wis1 requires the function of genes they named *wak1*⁺ and *wik1*⁺, which are both identical to *wis4*⁺. While both these groups demonstrated that Wis4/Wak1/Wik1 acts upstream of Wis1, their analysis did not extend to investigating the mechanism of this activation, nor to other possible modes of activation. In this study, we show that residues Ser469 and Thr473 of Wis1 are essential for its activation by Wis4. Taken together, these observations imply that the activation of Wis1 by Mcs4 is mediated through Ser469 and Thr473 and, indeed, the response of *mcs4* cells to stresses is very similar to that of cells expressing non-phosphorylatable Wis1AA (Figure 7A).

Shiozaki *et al.* (1997) propose that several types of stress are mediated through Mcs4 and Wik1 (=Wis4), because the levels of phosphotyrosine on Spc1 after heat or oxidative stress are lower in *mcs4* strains than in a wild type. Shieh *et al.* (1997) present similar observations. Both reports show that *mcs4* cells do indeed show a substantial increase in Spc1 phosphotyrosine from the basal level following these stresses, although the final level attained is less than in wild-type cells. These observations, taken together with our findings, led us to a different interpretation. Our experiments show that the major response mode following heat or oxidative stress is through functional inactivation of Pyp1, which changes the dynamic balance between phosphorylation and dephosphorylation of Tyr173 on Spc1, resulting in a net increase in the phosphotyrosine level. This explanation accounts for the persistence of a response in Wis1AA cells, where no activation of Wis1 by phosphorylation is possible. It also accounts for the responses of *wis4*Δ and *wil1-1* strains to heat and oxidative stresses, where basal Wis1 activity is reduced due to underphosphorylation on Ser469 and Thr473. This is also likely to be true for *mcs4* cells, since Mcs4 acts through Wis1, as shown by the observation that loss of Mcs4 function attenuates the toxicity of Wis1 overexpression (Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). In *mcs4* strains, loss of Pyp1 activity after heat stress

leads to an increase in the tyrosine phosphorylation level of Spc1, but the reduced Wis1 activity prevents it from attaining the wild-type level, explaining why the response is similar to that of Wis1AA cells (Figure 7A). We therefore believe that the roles of Mcs4, and its effector Wis4, should be reassessed, particularly with respect to their proposed roles in heat and oxidative stress signalling.

The *mcs4*⁺ gene encodes a protein highly homologous to response elements of bacterial-type two-component systems, which acts through Wis4 (Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). In *S.cerevisiae*, Ssk2 and Ssk22 (homologues of Wis4 which activate Pbs2, a Wis1 homologue) are activated by a two-component signal transduction system following osmotic stress (Maeda *et al.*, 1994, 1995). The architectural homology between the two pathways does not, however, reflect identity of function. The *S.cerevisiae* HOG pathway is only responsive to hyperosmotic conditions (Schüller *et al.*, 1994), while Spc1 is activated by several different types of stress. Osmotic stress appears to be transduced in *S.pombe* by a similar mechanism to that in *S.cerevisiae*. We show here that, in striking contrast, other stresses can activate the MAPK homologue Spc1 independently of upstream kinases, by a molecular mechanism that involves the Spc1 phosphatase Pyp1. This provides a means by which different signals can be integrated to evoke a common downstream response. In mammalian cells, investigations into stress signalling have focused on the roles of protein kinases acting upstream of p38 and SAPK/JNK, which can be activated by different extracellular conditions. The mechanisms by which the various input signals are integrated have not been well characterized, and little attention has been paid to the possibility that different stresses might act in fundamentally different ways. In the light of our observations, it will be interesting to see whether multiple modes of activation operate in mammalian stress-responsive pathways, and whether specific phosphatases are involved as regulatory components.

Materials and methods

Handling of *S.pombe*

Standard techniques and media used in this study are described elsewhere (Moreno *et al.*, 1991). Cells of *S.pombe* were grown at 30°C unless otherwise specified. The *S.pombe* strains used for phosphotyrosine assays are derivatives of KS1376 (Shiozaki and Russell, 1995) which have a genomic copy of *spc1*⁺ tagged with 6×His and an epitope, haemagglutinin (HA).

Disruption of the *wis4* gene

The insert of the plasmid pWF37 was used to disrupt the *wis4*⁺ gene by homologous recombination. A 2186 bp *HindIII*–*BamHI* fragment that contains codons from 453 to 1182 of *wis4*⁺ was replaced by the *S.pombe ura4*⁺ gene (Figure 1A). Stable uracil prototrophic diploids were sporulated and dissected for tetrad analysis. All the four spores are viable, and uracil auxotrophy segregated 2:2. Disruption of the *wis4* gene in *ura*⁺ progeny was confirmed by PCR.

In vitro Wis4 kinase assay

Wis4 tagged with 6×His at the C-terminus was expressed from the pREP1 promoter without thiamine for 14 h. For cell breakage, 200 ml of 1.0×10⁷ cells/ml were disrupted in buffer [50 mM Tris–HCl pH 8.0, 0.15 M NaCl, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)], incubated with Ni-NTA beads (Qiagen), then eluted by 2×50 µl buffer (50 mM Tris–HCl pH 8.0, 0.15 M NaCl, 250 mM imidazole, 20% glycerol), followed by dialysis in buffer (50 mM Tris–HCl pH 7.5, 0.15 M NaCl, 20% glycerol). GST–Wis1 fusion protein

was expressed in *Escherichia coli* BL21(DE3) and purified by use of glutathione–Sepharose beads (Pharmacia). Purification of GST–Wis1 was essentially as described by Alessi *et al.* (1994). Kinase reactions were carried out in buffer (50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 6.7 µM ATP and 10 µCi of [γ -³²P]ATP) at 30°C for 30 min.

Stress conditions

Previously described procedures (Degols *et al.*, 1996) were followed. Osmotic, oxidative and heat stress were given by exposure of exponentially growing cells to 0.5 M or 0.6 M KCl, 1 mM hydrogen peroxide or shift to 48°C for 15 min. *wis1AA* is a *wis1Δ* strain transformed with the pREP41–*wis1AA* plasmid; the full genotype is *h⁺ leu1-32 ura4-D18 spc1* his1-102 wis1::his1⁺* [pREP41–*wis1AA*]. [*spc1** is the HA- and His6-tagged allele of *spc1* described by Shiozaki and Russell (1995).] This strain was incubated in EMM without thiamine for 14 h before stress treatment; other strains were cultured in YE medium.

Detection of phosphotyrosine on Spc1

A previously described method (Shiozaki and Russell, 1995) was followed. Briefly, cells were disrupted by glass beads under denaturing conditions (6 M guanidinium HCl, 0.1 M Na phosphate, 50 mM Tris–HCl pH 8.0). Spc1 proteins tagged with the HA epitope and 6×His were purified by using Ni–NTA–agarose beads. Immunoblotting was carried out with anti-HA (12CA5, Boehringer) and anti-phosphotyrosine (4G10, Upstate Biotechnology) antibodies; enhanced chemiluminescence (ECL; Amersham) was performed to visualize the bound antibodies.

In vitro mutagenesis of *wis1⁺* and *wis4⁺* genes

Mutations were introduced by site-directed mutagenesis following the method described by Kunkel (1985).

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