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Peroxide Sensors for the Fission Yeast Stress-activated Mitogen-activated Protein Kinase Pathway

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The *Schizosaccharomyces pombe* stress-activated Sty1p/Spc1p mitogen-activated protein (MAP) kinase regulates gene expression through the Atf1p and Pap1p transcription factors, homologs of human ATF2 and c-Jun, respectively. Mcs4p, a response regulator protein, acts upstream of Sty1p by binding the Wak1p/Wis4p MAP kinase kinase kinase. We show that phosphorylation of Mcs4p on a conserved aspartic acid residue is required for activation of Sty1p only in response to peroxide stress. Mcs4p acts in a conserved phospho-relay system initiated by two PAS/PAC domain-containing histidine kinases, Mak2p and Mak3p. In the absence of Mak2p or Mak3p, Sty1p fails to phosphorylate the Atf1p transcription factor or induce Atf1p-dependent gene expression. As a consequence, cells lacking Mak2p and Mak3p are sensitive to peroxide attack in the absence of Prr1p, a distinct response regulator protein that functions in association with Pap1p. The Mak1p histidine kinase, which also contains PAS/PAC repeats, does not regulate Sty1p or Atf1p but is partially required for Pap1p- and Prr1p-dependent transcription. We conclude that the transcriptional response to free radical attack is initiated by at least two distinct phospho-relay pathways in fission yeast.

INTRODUCTION

The mitogen-activated protein (MAP) kinase (MAPK) signaling pathways are critical for the response of cells to changes in their environment (Marshall, 1994; Herskowitz, 1995; Waskiewicz and Cooper, 1995; Treisman, 1996). They serve to transduce signals generated at the cell surface or in the cytoplasm to the nucleus, where changes in gene expression result. In mammalian cells, multiple distinct MAP kinases have been identified, including a large subset whose members are activated by a variety of environmental stress conditions, DNA-damaging agents, inflammatory cytokines, and certain vasoactive neuropeptides (Dérijard *et al.*, 1994; Freshney *et al.*, 1994; Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994; Kyriakis *et a*l., 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994; Sluss *et al.*, 1994). These stress-activated MAP kinases (SAPKs) fall into two distinct classes, termed the C-Jun N-terminal kinase (JNK) and p38 kinases, based on their sequences (Davies, 1994; Waskiewicz and Cooper, 1995). A number of transcription factors are phosphorylated in response to SAPK activation; for example, the c-Jun factor is regulated by JNK (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Kyriakis *et al.*, 1994) but not by p38, whereas ATF2 is phosphorylated and regulated by both JNK (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995) and p38 (Raingeaud *et al.*, 1995). Although a number of MAPK kinases (MAPKKs) and MAPKK kinases (MAPKKKs) that activate the SAPKs have been identified in mammalian cells, very little is known about how these are regulated by stress stimuli (reviewed in Ichijo, 1999; Tibbles and Woodgett, 1999). This is probably due to the multiplicity of SAPK pathways in mammalian cells and the difficulties of genetic analysis in these organisms.

Recently, a single member of the SAPK family, called Sty1p (also known as Spc1p or Phh1p), has been identified in the fission yeast *Schizosaccharomyces pombe* (Millar *et al.*, 1995; Shiozaki and Russell, 1995; Kato *et al.*, 1996). The Sty1p MAP kinase stimulates gene expression via the Atf1p and Pap1p transcription factors, homologs of human ATF2 and c-Jun, respectively, suggesting that the transcriptional targets of the *S. pombe* pathway are closely related to those in mammalian cells (Toda *et al.*, 1991; Takeda *et al.*, 1995; Wilkinson *et al.*, 1996; Shiozaki and Russell, 1996; Gaits *et al.*, 1998;

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Toone *et al.*, 1998; Wilkinson and Millar, 1998). Stress-induced nuclear translocation of activated Sty1p causes it to bind and phosphorylate Atf1p, although the precise mechanism by which this phosphorylation induces transcriptional activation is not known (Wilkinson *et al.*, 1996; Shiozaki and Russell, 1996; Gaits *et al.*, 1998). Sty1p also binds to Pap1p but in this case the mechanism of activation appears to be distinct. In contrast to Atf1p, the bulk of the Pap1p protein is cytoplasmic in unstressed cells and is translocated to the nucleus only in response to an oxidative stress (Toone *et al.*, 1998). This translocation requires Sty1p, although activation of Pap1p may not involve phosphorylation, because Pap1p is not a phosphorylation target of Sty1p in vitro. Instead, interaction of Pap1p with the nuclear export factor Crm1p is disturbed by oxidation of the cysteine residues in the C terminus of Pap1p. The role of Sty1p in this process is not understood (reviewed in Wilkinson and Millar, 1998). Nevertheless, many of the genes induced after Sty1p activation in *S. pombe* are similar to those induced by SAPK activation in mammals.

Sty1p is activated by a similar range of environmental stimuli to the mammalian SAPKs, including oxidative stress, UV light, DNA-damaging agents, osmotic stress, physical stresses, heat shock, and the protein synthesis inhibitor anisomycin (Millar *et al.*, 1995; Shiozaki and Russell, 1995; Degols *et al.*, 1996; Degols and Russell, 1997; Shieh *et al.*, 1997, 1998). Thus, one might postulate that the mechanisms controlling SAPK activation are similar in *S. pombe* and mammalian cells. However, we recently demonstrated that activation of Sty1p is regulated by Mcs4p, a protein that is homologous to the *Saccharomyces cerevisiae* Ssk1p response regulator protein, which is similar to a number of two-component systems that function in bacteria but have yet to be identified in mammals (Stock *et al.*, 1989; Parkinson, 1993). Ssk1p acts in a multistep phospho-relay system to control the activity of the Hog1p MAPK (Maeda *et al.*, 1994, 1995; Posas *et al.*, 1996; Shieh *et al.*, 1997; Posas and Saito, 1998). Although Hog1p is structurally related to the SAPK family, it is activated only by increases in external osmolarity (Brewster *et al.*, 1993; Schüller *et al.*, 1994). In hypotonic medium, a transmembrane histidine kinase, Sln1p, initiates a phospho-relay system in which a phosphate group is transferred from the Sln1p response-regulator domain to a histidine residue in Ypd1p and then to a conserved aspartate residue in Ssk1p (Posas *et al.*, 1996). In response to hypertonic stress, Sln1p is inactivated and Ssk1p is dephosphorylated. In this state, Ssk1p binds and activates one of two functionally overlapping MAPKKKs, Ssk2p and Ssk22p (Maeda *et al.*, 1994, 1995; Posas and Saito, 1998). Ssk2p and Ssk22p kinase activation leads to the sequential phosphorylation and activation of the Pbs2p MAPKK and Hog1p MAPK (Brewster *et al.*, 1993; Maeda *et al.*, 1994, 1995). Activation of Hog1p leads to the induction of a number of genes whose products protect the cells in hypertonic medium.

It has been shown previously that in *S. pombe*, Mcs4p acts upstream of the Wak1/Wis4p and Win1p kinases, homologs of the Ssk2p and Ssk22p MAPKKKs, which in turn transmit the stress signal to the Wis1p MAPKK, the direct activator of Sty1p (Warbrick and Fantes, 1991; Millar *et al.*, 1995; Shiozaki and Russell, 1995; Shieh *et al.*, 1997, 1998; Samejima *et al.*, 1998). Recently, an *S. pombe* homolog of Ypd1p, Mpr1p, was found to be required for the activation of Sty1p in response to an oxidative but not osmotic stress (Nguyen *et al.*, 2000). This suggests that the Sty1p MAPK pathway may be regulated by a phospho-relay system similar to the Sln1p-Ypd1p-Ssk1p pathway in *S. cerevisiae*. However, the sensors that initiate this signal in *S. pombe* have not been identified. Moreover, because deletion of *mcs4* abrogates the activation of Sty1p in response to multiple environmental stresses, it is not clear what role Mcs4p phosphorylation may play in transmitting these signals.

In this article, we describe the role of Mcs4p in stress-induced activation of Sty1p in more detail. We present evidence that *S. pombe* contains at least two phospho-relay signaling systems that are specifically involved in sensing peroxide stress and that one of these directly controls the activity of the Sty1p MAPK. We discuss the implications of these results both in relation to the ability of microbial pathogens to survive free radical attack and the mechanisms controlling SAPK activation in metazoans.

MATERIALS AND METHODS

Media and General Techniques

Media and genetic methods for studying *S. pombe* were as described by Moreno *et al.* (1991). Standard DNA methods were used (Sambrook *et al.*, 1989). Cell length measurements were made by using log-phase cells with a Nikon filar eyepiece drum micrometer at 1200× magnification. Transformations were regularly performed by the lithium acetate method (Moreno *et al.*, 1991) or by electroporation (Prentice, 1991) with a Bio-Rad gene pulser.

To isolate DNA *S. pombe* cells were cultured in YES medium to stationary phase. Chromosomal DNA isolated (Moreno *et al.*, 1991) from a 10-ml culture was dissolved in 25 ml of TE, and one-fifth was digested and subjected to electrophoresis and Southern blot hybridization. To isolate RNA, *S. pombe* cells were cultured in YES to exponential phase. Approximately 10 μg of total RNA was isolated (Moreno *et al.*, 1991) and resolved by agarose gel electrophoresis before transfer to nitrocellulose for hybridization as described previously (Wilkinson *et al.*, 1996). Probes for *pyp2*, *gpx1*, *ctt1*, and *cdc2* were prepared as described previously (Wilkinson *et al.*, 1996; Toone *et al.*, 1998).

Detection of Mcs4p–Wak1p Interaction and of Activated Sty1p

The catalytic domain of *wak1* was cloned by polymerase chain reaction (PCR) amplification from *S. pombe* genomic DNA. The 5′ oligonucleotide TAACT*AGATCT*ATGGCTTTCTGTTAACGCAT, incorporating a *Bgl*II site (italicized), hybridized to sequences 5′ to the catalytic domain, whereas the 3′ oligonucleotide TATTA*GCGGCCGC*GGTCAACACTATAGTTTATTGTG, incorporating a *Not*I site (italicized), hybridized to sequences surrounding the TGA termination codon. The fragment generated was digested with *Bgl*II and *Not*I and cloned into the *Bgl*II and *Not*I sites of pBSSK-ura4 (Millar, unpublished data) to form pBSSK-ura4-wak1. A tandem 9-myc epitope was amplified by PCR from plasmid pC3280 (a gift of K. Nasmyth, Research Institute of Molecular Pathology, Vienna, Austria) by using the 5′ oligonucleotide GAAAAAG*GCGGCCG*CATGGTTCAC and the 3′ oligonucleotide ATATATAT*GCGGCCGC*CTTATGTCGGCATATTCGAG, which incorporate *Not*I sites (italicized). The resulting fragment was cloned into pBSSK-ura4-wak1 that had been digested with *Not*I to form pBSSK-ura4-wak1(9myc). pBSSK-ura4-wak1(9myc) was linearized with *Nde*I before being transformed into CH429 cells. Stable integration of the tagged *wak1–9myc* gene at the genomic *wak1* locus was confirmed by Southern blot and PCR.

The *mcs4* open reading frame (ORF) was amplified by PCR from *S. pombe* genomic DNA. The 5′ oligonucleotide CCC*GGATCC*ATGCGCATTTGGTTTAAAAAAGTT incorporates a *Bam*HI site (italicized) and hybridized to sequences near the start codon, whereas the 3′ oligonucleotide CCC*GGATCC*TCATCGACCGCGAAAACGGCA incorporates a *Bam*HI site (italicized) and hybridized to sequences surrounding the TGA termination codon. The amplified fragment was digested with *Bam*HI and cloned into the *Bam*HI site of pDS472a (Forsburg and Sherman, 1997) to form pDS472a-mcs4, which expresses GST-Mcs4 from an attenuated version of the thiamine-repressible *nmt* promoter (*nmt41*) (Basi *et al.*, 1993). *mcs4::his7 wak1–9myc* cells (HM1830) transformed with either pDS472a or pDS472a-mcs4 were grown in minimal medium lacking leucine and thiamine for at least 24 h. Pelleted cells were lysed by using glass beads into lysis buffer (0.5% NP-40, 150 mM NaCl, 50 mM NaF, 10% glycerol, 2 mM Na-orthovanadate, 10 mM β-mercaptoethanol, 10 μg/ml aprotonin, 10 μg/ml benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 50 mM HEPES; pH 7.4) and proteins were partially purified by affinity precipitation on glutathione-agarose beads. Precipitated proteins were eluted in the presence of 10 mM reduced glutathione, resolved by SDS-PAGE, and blotted to nitrocellulose membranes. Membranes were probed with a monoclonal antibody to the *myc* epitope (9E10; Covance, Richmond, CA) according to the manufacturer's instructions. Phosphorylated and activated Sty1p was detected by Western blot exactly as described previously (Shieh *et al.*, 1997; Gaits *et al.*, 1998).

Construction of mcs4::his7 *and* mcs4(D412N) *Alleles*

mcs4 was disrupted with *his7* as follows. First, the *Eco*RI/*Sph*I fragment of pURB1-Mcs4-1 (Shieh *et al.*, 1997) was cloned into pT7T318u (Amersham-Pharmacia, Little Chalfont, Buckinghamshire, United Kingdom) to create pT7T318u-mcs4. Next, a 642-bp *Xba*I/*Kpn*I fragment of pT7T318u-mcs4 was replaced with an *Xba*I/*Kpn*I fragment from pEA2 (a gift from C. Hoffman, Boston College, MA) that carries *his7*. The *mcs4::his7* fragment was then excised from pT7T318u-mcs4::his7 by digestion with *Eco*RI and *Sph*I, gel purified, and transformed into CH429 cells. Haploid disruptants were isolated on minimal medium lacking histidine and stable integrants were confirmed by Southern blot.

The wild-type *mcs4* genomic locus was replaced with *mcs4(D412N)* as follows. A 1.56-kb fragment carrying the *mcs4(D412N*) allele was excised from pREP41HM-Mcs4(D412N) (Shieh *et al.*, 1997) by using *Nde*I and *Bam*HI and cotransformed with plasmid pIRT2(LEU2) (Basi *et al.*, 1993) into a *mcs4::ura4* haploid (Shieh *et al.*, 1997). Transformants were selected on minimal medium lacking leucine and then replica-plated onto complete minimal medium containing 5-fluoro-orotic acid to select for uracil auxotrophs. Genomic DNA from these strains was subjected to PCR amplification by using the oligonucleotides Mcs4-N1 and Mcs4-C1 (Shieh *et al.*, 1997), which generated a single 1.56-kb band in strains carrying the *mcs4(D412N)* allele. Successful gene replacement was confirmed by sequencing of PCR-amplified DNA.

Isolation and Sequencing of mak1

PCR amplification was performed on genomic *S. pombe* DNA by using the 5′ oligonucleotide CTI(A/G/C)TIGTIGA(G/A)GA(C/T)(A/G/C)A and the 3′ oligonucleotide GGCAT(C/T)TGI(A/C)I(A/G)TCCATIA, where I is inosine. The reaction was performed for 30 cycles at an annealing temperature of 40°C. The PCR products were separated by PAGE and a 160-bp fragment was purified and ligated directly into pCRII (Invitrogen, San Diego, CA) before being transformed into *Escherichia coli* DH5α. Plasmid inserts from nine resulting colonies were sequenced by using M13 and T7 primers. Six were found to contain the same sequence, which has homology to the conserved region of all response regulators. The160-bp fragment was then used to screen a pURB1 genomic library (Barbet *et al.*, 1992) and three overlapping clones were identified. Sequencing of the largest clone, pURB1-mak1, was performed by using custom-synthesized oligonucleotide primers, after constructing unidirectional deletions by using exonuclease III and S1 nuclease (Henikoff, 1984).

Sequence Alignments

GAF domain alignments were identified by using PSI-BLAST (Altschul *et al.*, 1997) and Mak3p as the query sequence. Reiterative searching identified Mak2p with an e-value of 2e-06 after round 1. Round 4 picked up the C.a. HK1 with an e-value of 1e-37. Round 5 picked up *E. coli* FhlA with an e-value of 5e-05. Round 7 picked up the ethylene receptor (Etr1) with an e-value of 2e-11. The PSI-BLAST converged before the PHYE sequences were picked up with e-values below the threshold. The program THREADER (Jones *et al.*, 1992) was run on the putative PAS/PAC domains Mak1a, Mak1b, Mak2, and Mak3. The top hits with high p were either the FixL or the ERG potassium channel PAS/PAC domains. The serine/threonine kinase sequences could also be picked up using PSI-BLAST with low e-values (<0.05) and columns of sequence identity can be seen in the alignment.

Deletion of mak1, mak2, mak3, *and* prr1 *Sequences*

To delete *mak1* sequences, the *Hin*dIII site in pBluescript II (Stratagene, La Jolla, CA) was first destroyed by digesting with *Hin*dIII, filling the 5′ overhangs with Klenow polymerase and religating to form pBSSK*. Next, pURB1-mak1 was digested with *Bam*HI and *Pst*I, and the resulting 4.0-kb fragment carrying 60% of the *mak1* ORF was ligated into pBSSK* that had been linearized with *Bam*HI and *Pst*I, thus forming pBSSK*-mak1. pBSSK*-mak1 was digested with *Hin*dIII to remove ~2.0 kb of the *mak1* ORF, including sequences critical for histidine kinase function, which were replaced with a 1.6-kb *Hin*dIII fragment bearing the *ura4* marker from pREP42 (Basi *et al.*, 1993). The resulting construct, pBSSK-mak1::ura4, was digested with *Bam*HI and *Pst*I and transformed into a *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-210/ade6-216 his7-366/his7-366 h−/h+* diploid strain (JM1429) (Table 1). Transformants were selected on minimal medium lacking uracil and stable heterozygous diploids were sporulated. Haploid colonies segregated 2:2 with respect to uracil prototrophy, indicating that *mak1* is a nonessential gene. Disruptants were confirmed by Southern blot. A similar strategy was used to disrupt *mak1* sequences with the *LEU2* marker.

To delete *mak2* sequences, a 8.7-kb *Pvu*II fragment carrying 98% of the *mak2* ORF was first excised from cosmid clone c27E2 (Ressourcenzentrum, Berlin, Germany) and ligated into the large *Pvu*II fragment of pBSSK to create pBSSK-mak2. pBSSK-mak2 was digested with *Hin*dIII to remove 4.6 kb (67%) of the *mak2* ORF, including the histidine kinase domain, which was replaced with a 2.2-kb *Hin*dIII fragment bearing the *LEU2* marker from pREP41 (Basi *et al.*, 1993) to create pBSSK-mak2::LEU2. The resulting construct, pBSSK-mak2::LEU2, was digested with *Pvu*II and the resulting 6.8-kb fragment was transformed into the diploid strain JM1429. Haploid *mak2* disruptants were isolated as described above for *mak1*. A similar strategy was used to disrupt *mak2* sequences with *ura4*.

To delete *mak3* sequences, the kanamycin-resistance gene was amplified from pFA6a-kanMX6 (Bahler *et al.*, 1998) by PCR with the 5′ oligonucleotide GTTCTTCCTTATGAAAATTGAAGTTCAATAACTTATAACATGGGAGTAAATGATAATTAACAATTGTTGCC*CGGATCCCCGGGTTAATTAA*, which is homologous both to sequences immediately upstream of the *mak3* ORF and to the 5′ region of the kanMX6 cassette (italics), and the 3′ oligonucleotide TGCCAAAATGTAACGAGCTCTTCTATGGTTTAGCATAGACATTCACGAGATTTTCCAAGAAAACAAAACA*GAATTCGAGCTCGTTTAAAC*, which is homologous both to sequences downstream of the *mak3* termination codon and to the 3′ region of the kanMX6 cassette (italics). The resulting fragment was transformed into CH429 cells

Table 1. Strains used in this study

Strain	Genotype	Source
CH429	*h*$^-$	C. Hoffman
HM1830	*mcs4::his7 wak1-9myc: ura4 h*$^-$	This study
VB1692	*mcs4 (D412N) h*$^-$	This study
JM2280	*mcs4 (D412N) sty1::ura4 h*$^-$	This study
JM1468	*mcs4::his7 h*$^+$	This study
KZ1535	*mak1::ura4 h*$^+$	This study
KZ1536	*mak1::LEU2 h*$^+$	This study
VB1530	*mak2::ura4 h*$^-$	This study
VB1834	*mak2::LEU2 h*$^-$	This study
VB1932	*mak3::kan*^{R} *h*$^-$	This study
VB1930	*mak2::LEU2 mak3::kan*^{R} *h*$^-$	This study
VB1971	*mak1::LEU2 mak2::ura4 mak3::kan*^{R} *h*$^+$	This study
VB1925	*mak1::ura4 mak2::LEU2 mak3::kan*^{R} *mcs4::his7 h*$^-$	This study
JM1521	*sty1(HA6His):ura4 h*$^+$	Shieh *et al.*, 1997
VB1700	*sty1(HA6His):ura4 mcs4 (D412N) h*$^-$	This study
VB1828	*sty1(HA6His):ura4 mak1::LEU2 h*$^+$	This study
VB1829	*sty1(HA6His):ura4 mak2::LEU2 h*$^-$	This study
VB1934	*sty1(HA6His):ura4 mak3::kanR h*$^-$	This study
KS1479	*atf1(HA6His):ura4 h*$^-$	Shiozaki and Russell, 1995
TS2029	*atf1(HA6His):ura4 mak1::LEU2 h*$^+$	This study
TS2030	*atf1(HA6His):ura4 mak2::LEU2 h*$^-$	This study
TS2031	*atf1(HA6His):ura4 mak3::kanR h*$^-$	This study
JM1516	*prr1::his7 h*$^-$	This study
JM1546	*prr1::his7 atf1::ura4 h*$^-$	This study
JQ51	*prr1::his7 mak2::LEU2 mak3::kanR h*$^-$	This study
JM1429	*h+/h−*	This study

All strains are *leu1-32 ura4-D18 ade6-M210* (or *ade6-M216*) *his7-366*.

and G418-resistant colonies were isolated as described (Bahler *et al.*, 1998). *mak3::kanR* haploid deletion strains were confirmed by PCR and Southern blot.

To delete *prr1* sequences, part of the *prr1* gene was amplified by PCR from cosmid SPAC8D9 (Ressourcenzentrum) by using the 5′ oligonucleotide Prr1.1, GTGTTGGATGTAA*GAATTC*CTTGAAAATTTGTTTGCTGG and the 3′ oligonucleotide Prr1.2, AAAGTAGGTAACTGCA*GAATTC*ATCGATATATTTGAAGTTATAGC (*Eco*RI sites in italics). The resulting 2.2-kb fragment was digested with *Eco*RI and ligated into pBR322 digested with the same enzyme to form pBR-prr1. pBR-prr1 was then digested with *Nsi*I and *Xba*I and a *Pst*I/*Xba*I fragment containing *his7* was isolated from pBSSK-his7(R) (Millar, unpublished data) and cloned into these sites to generate pBR:prr1::his7. This cloning removes codons 67 to 209 of the *prr1* ORF. pBR:prr1::his7 was digested with *Eco*RI and transformed into CH429 cells. Stable histidine prototrophs were selected and disruption of *prr1* confirmed by PCR.

RESULTS

Phosphorylation of Mcs4p Controls Sty1p Kinase Only in Response to Peroxide

Previous genetic data indicate that the Mcs4p cell cycle regulator acts upstream of two functionally redundant MAPKKKs, Wak1p/Wis4p and Win1p, to control the activity of the Sty1p MAP kinase (Shiozaki *et al.*, 1997; Shieh *et al.*, 1998; Samejima *et al.*, 1998). To examine whether Mcs4p interacts with either of these MAPKKKs, a GST-Mcs4p fusion protein was expressed in cells lacking endogenous Mcs4p but expressing Wak1p MAP fused to a 9-myc epitope tag. Proteins associating with GST-Mcs4p were identified after affinity precipitation from cell extracts. Wak1p associated with GST-Mcs4p but not glutathione *S*-transferase (GST) itself, even in unstressed cells (Figure 1A). Binding of Mcs4p to Wak1p was not altered when cells were challenged with various environmental stresses (our unpublished data). This result indicates that Mcs4p associates with Wak1p and is thus an integral component of the Sty1p MAPK complex. In this respect, Mcs4p acts in a similar manner to the Ssk1p response regulator in *S. cerevisiae*, which binds directly to the N termini of the Ssk2p and Ssk22p MAPKKKs.

In *S. cerevisiae*, inactivation of the Sln1p histidine kinase in response to changes in external osmolarity results in dephosphorylation of Ssk1p on a conserved aspartate residue (D554) in the response-regulator domain (Maeda *et al.*, 1994). The equivalent residue in Mcs4p is aspartate D412. In a previous article, we reported that deleting *mcs4* prevented activation of Sty1p in response to multiple environmental insults (Shieh *et al.*, 1997). Because the total absence of a protein could disrupt the integrity of multiprotein complexes, we reexamined the role of Mcs4p with respect to signal transmission via phosphorylation of D412 by constructing a strain in which *mcs4*$^+$ is replaced by a mutant allele bearing a nonphosphorylatable asparagine at residue 412. These *mcs4(D412N)* cells were viable but divided at a smaller size (11.4 $\pm$ 0.4 μm) relative to wild-type cells (14.3 $\pm$ 0.2 μm), although growth rate was unaffected (Figure 2B; our unpublished data). This phenotype is similar to that of cells lacking the Pyp1p MAP kinase phosphatase, suggesting that it is due to partial activation of Sty1p (Millar *et al.*, 1992). Consistent with this we observed that *mcs4(D412N) sty1::ura4* cells divided at 22.9 $\pm$ 1.8 μm, a size similar to that of *sty1::ura4* cells (23.1 $\pm$ 2.0 μm).

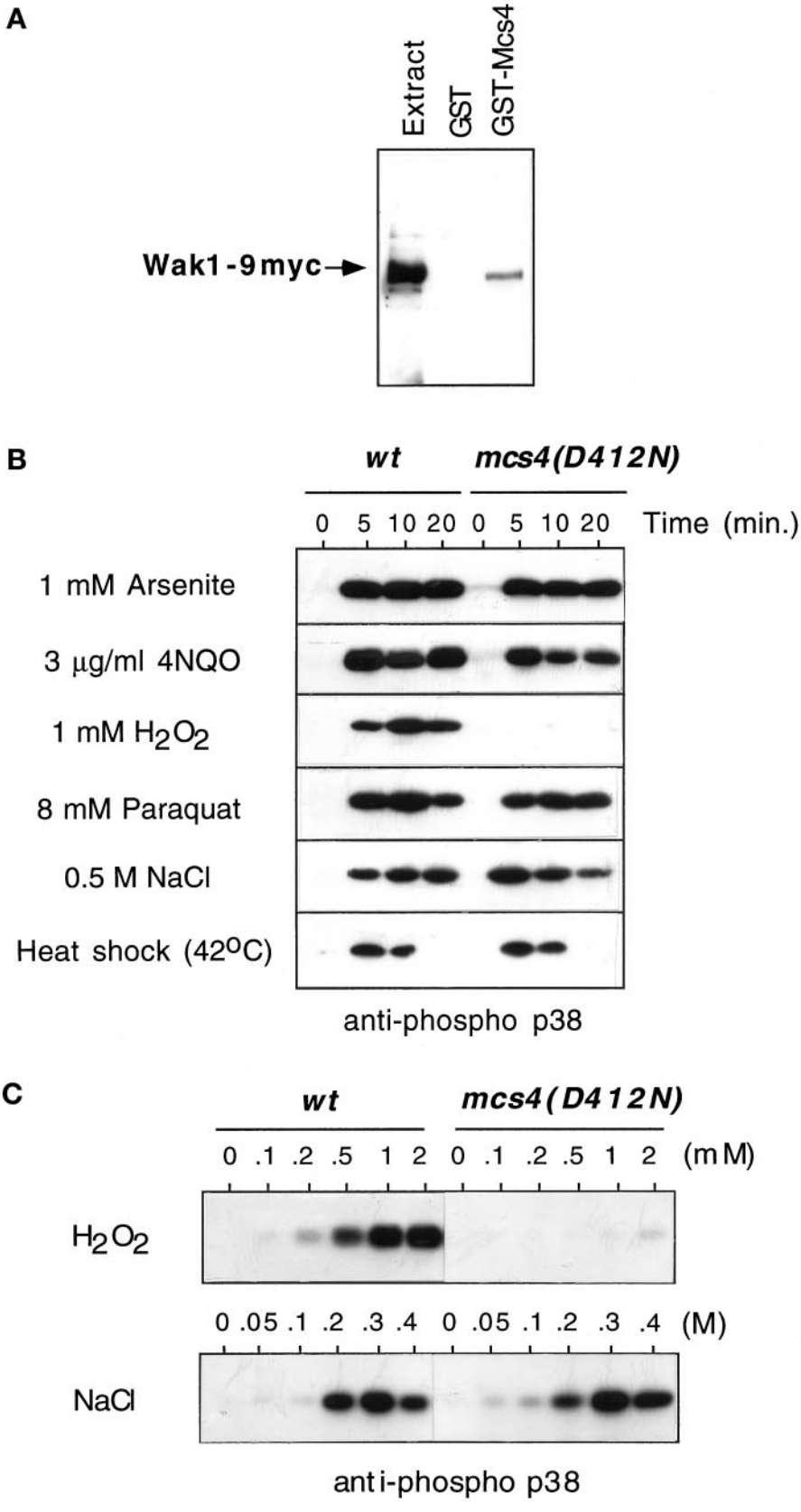

Figure 1. Mcs4p is required for activation of Sty1p MAPK specifically in response to peroxide stress. (A) Mcs4p interacts with the Wak1p MAPKKK. Cells expressing Wak1p-9myc and either GST or GST-Mcs4 were grown and extracts were prepared and processed as described in MATERIALS AND METHODS. Proteins in the extract or those purified on glutathione sepharose beads were analyzed by SDS-PAGE and Western blotting by using a monoclonal antibody to the myc epitope. (B) D412 of Mcs4p is required specifically for activation of the Sty1p MAPK in response to peroxide stress. Wild-type (JM1521) or *mcs4(D412N)* cells (VB1700) bearing an integrated, six-histidine and hemagglutinin-tagged version of Sty1p growing in YEPDA (2% bacto-peptone, 1% yeast extract, 2% glucose, 100 mg/l adenine) medium at 30°C were incubated for the times indicated in the same medium containing 1 mM sodium arsenite, 3 μg/ml 4-nitroquinoline oxide (4NQO), 1 mM hydrogen peroxide (H_2O_2), 8 mM paraquat or 0.5 M NaCl, or subjected to a mild heat shock at 42°C. Approximately 2×10^8 cells were harvested at each time point and lysed under nondetaturing conditions before the Sty1p protein was precipitated by using Ni^{2+}-nitriloacetic acid agarose. Precipitated proteins were separated by SDS-PAGE and Western blots were probed for the presence of phosphorylated Sty1p by using anti-phospho-p38 antibodies (see MATERIALS AND METHODS). (C) Dose response for activation of Sty1p in response to an oxidative or osmotic stress. Wild-type (JM1521) or *mcs4(D412N)* cells (VB1700) growing in YEPD at 30°C were incubated for 10 min in the same medium containing increasing doses of either hydrogen peroxide or NaCl. After this time, cells were harvested and phosphorylation of Sty1p was measured as described above.

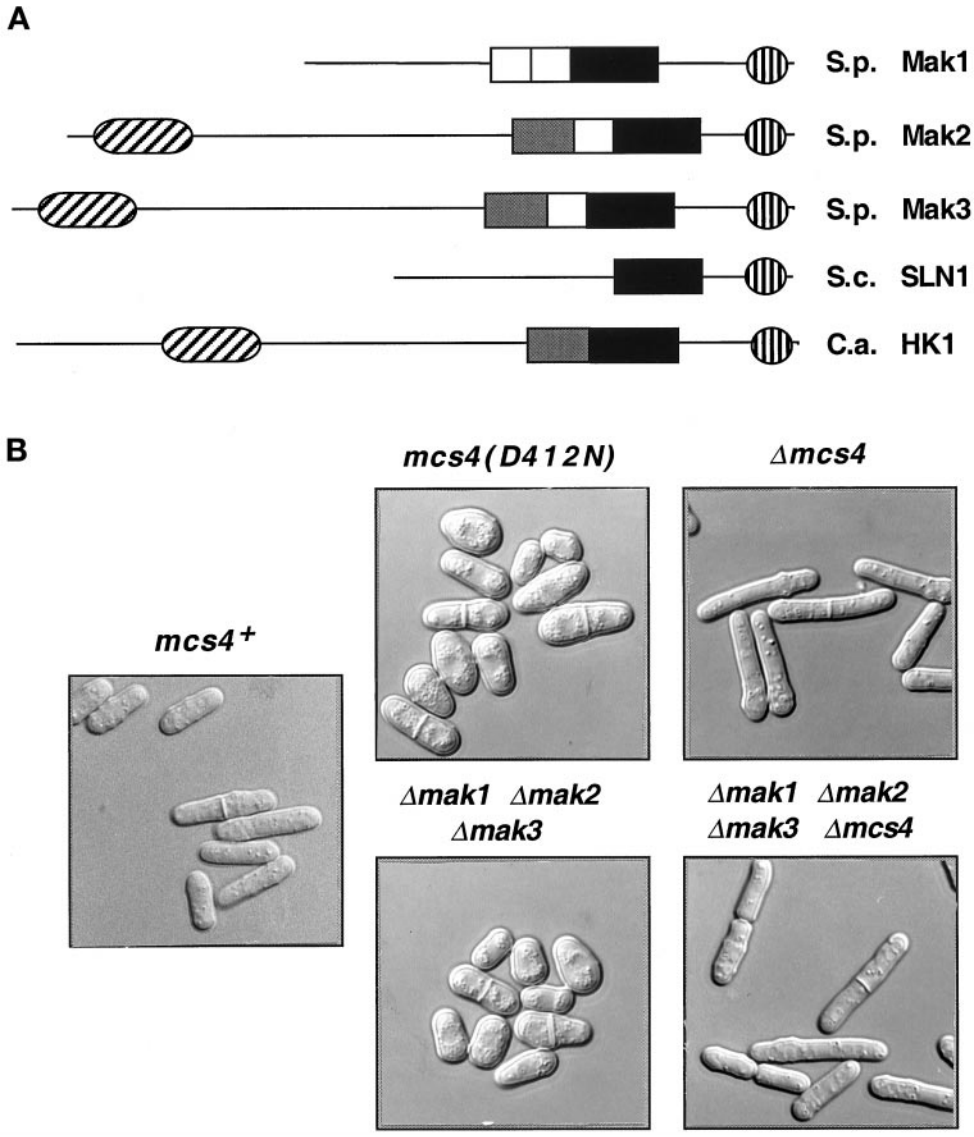

Figure 2. Identification of three nonessential histidine kinases in fission yeast. (A) Diagrammatic representation of the domain architecture of *S. pombe* (S.p.) Mak1p, Mak2p, and Mak3p compared with the *S. cerevisiae* (S.c.) Sln1p and *C. albicans* (C.a.) HK1 histidine kinases. The positions of the following domains are shown in these molecules: putative serine/threonine kinase domain (diagonally hatched bar), GAF domain (gray bar), PAS/PAC motifs (open bar), histidine kinase (black bar), and response-regulator domain (vertically hatched bar). The Mak1p, Mak2p, and Mak3p sequences appear in the SWISSPROT and EMBL databases under the accession numbers AL157734, Z98978, and AL031543, respectively. (B) *S. pombe* histidine kinases control cell size at cell division via Mcs4p. Wild-type (*mcs4*$^+$) (JM 1059), *mcs4(D412N)* (VB1692), Δ*mcs4* (JM1468), Δ*mak1* Δ*mak2* Δ*mak3* (VB1971), or Δ*mak1* Δ*mak2* Δ*mak3* Δ*mcs4* (VB1925) cells were grown to log phase in minimal medium and photographed by using Normarski optics.

Wild-type or *mcs4(D412N)* strains bearing a six-histidine and hemagglutinin-tagged *sty1* allele were subjected to various stresses, including 1 mM sodium arsenite, 3 μg/ml 4-nitroquinoline oxide, 1 mM hydrogen peroxide, 8 mM paraquat, 0.5 M NaCl, or subjected to a mild heat shock at 42°C. Phosphorylation of Sty1p was monitored by Western blot with an antibody that recognizes only the phosphorylated and, by inference, activated form of Sty1p (see MATERIALS AND METHODS). With the sole exception of hydrogen peroxide, which failed to activate Sty1p in a *mcs4(D412N)* background, phosphorylation of Sty1p was observed after all other stresses in both the wild-type and *mcs4(D412N)* cells (Figure 1B). To confirm this result, wild-type or *mcs4(D412N)* cells were subjected to increasing doses of either NaCl or hydrogen peroxide and the phosphorylation of Sty1p was monitored after 10 min of incubation. With NaCl, no difference was observed between wild-type and *mcs4(D412N)* cells in the activation of Sty1p, indicating that phosphorylation of Mcs4p D412 is not required for activation of Sty1p by hypertonic stress (Figure 1C). In contrast, activation of Sty1p by hydrogen peroxide was almost completely abolished in *mcs4(D412N)* cells irrespective of the dose (Figure 1C), suggesting that phosphorylation of Mcs4p D412 is required only for the activation of Sty1p MAP kinase to peroxide stress. This contrasts sharply with the results obtained with *mcs4*-deletion cells, suggesting either that Mcs4p is required for the structural integrity of the Sty1p MAP kinase complex or that modifications other than D412 phosphorylation of Mcs4p are required for signal transmission in response to other environmental stimuli.

Identification of a Family of Histidine Kinases in Fission Yeast

We next sought the putative peroxide receptor(s) that might control Mcs4p phosphorylation, postulating that they might

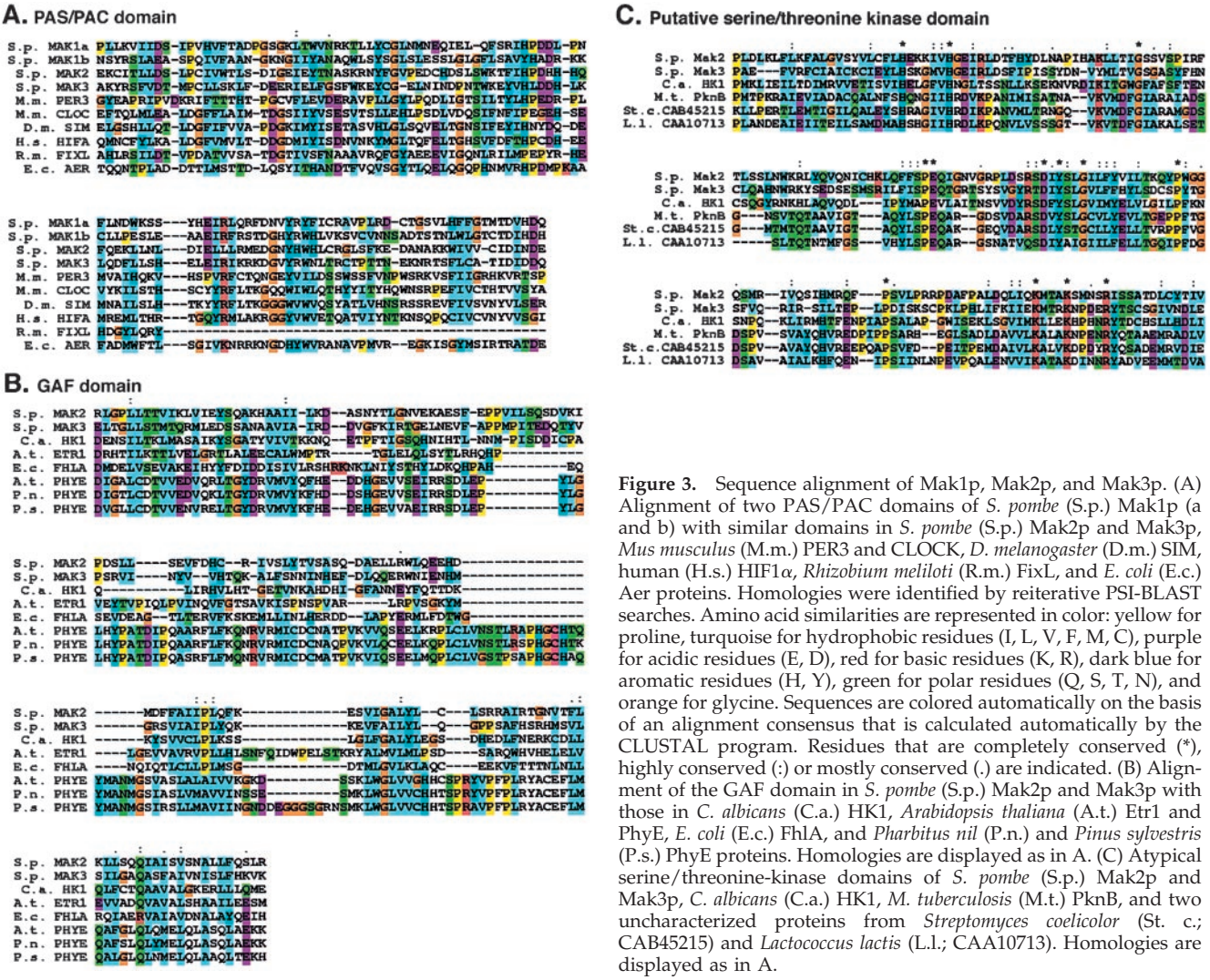

Figure 3. Sequence alignment of Mak1p, Mak2p, and Mak3p. (A) Alignment of two PAS/PAC domains of *S. pombe* (S.p.) Mak1p (a and b) with similar domains in *S. pombe* (S.p.) Mak2p and Mak3p, *Mus musculus* (M.m.) PER3 and CLOCK, *D. melanogaster* (D.m.) SIM, human (H.s.) HIF1α, *Rhizobium meliloti* (R.m.) FixL, and *E. coli* (E.c.) Aer proteins. Homologies were identified by reiterative PSI-BLAST searches. Amino acid similarities are represented in color: yellow for proline, turquoise for hydrophobic residues (I, L, V, F, M, C), purple for acidic residues (E, D), red for basic residues (K, R), dark blue for aromatic residues (H, Y), green for polar residues (Q, S, T, N), and orange for glycine. Sequences are colored automatically on the basis of an alignment consensus that is calculated automatically by the CLUSTAL program. Residues that are completely conserved (*), highly conserved (:) or mostly conserved (.) are indicated. (B) Alignment of the GAF domain in *S. pombe* (S.p.) Mak2p and Mak3p with those in *C. albicans* (C.a.) HK1, *Arabidopsis thaliana* (A.t.) Etr1 and PhyE, *E. coli* (E.c.) FhlA, and *Pharbitus nil* (P.n.) and *Pinus sylvestris* (P.s.) PhyE proteins. Homologies are displayed as in A. (C) Atypical serine/threonine-kinase domains of *S. pombe* (S.p.) Mak2p and Mak3p, *C. albicans* (C.a.) HK1, *M. tuberculosis* (M.t.) PknB, and two uncharacterized proteins from *Streptomyces coelicolor* (St. c.; CAB45215) and *Lactococcus lactis* (L.l.; CAA10713). Homologies are displayed as in A.

be structurally related to Sln1p, which contains both histidine-kinase and response-regulator domains. Thus, degenerate primers were designed to regions corresponding to the amino acid sequences L(I/V/L)VEDN and (L/F)MD(I/L/V)QMP and used in low-temperature PCR amplification of *S. pombe* genomic DNA. Six of nine individual PCR clones contained the same nucleotide sequence, which translated to a polypeptide with homology to response regulators. One of these was used to isolate a full-length clone from a genomic library. By this means, an uninterrupted 4.8-kb ORF was identified that gives rise to a predicted protein of 1615 amino acids. The protein was found to contain, in addition to a response-regulator domain, a region most closely related to members of the histidine-kinase family (Figure 2A). We termed this gene *mak1*, for Mcs4-associated kinase. By screening the *S. pombe* genome sequence for similar sequences, we identified two additional histidine-kinase–like ORFs. These intronless genes translate to predicted proteins of 2310 and 2344 amino acids, which we designated Mak2p and Mak3p, respectively (Figure 2A). Comparison of these proteins revealed a high degree of homology to each other throughout, including both histidine-kinase and response-regulator domains in the C termini. The Mak1p, Mak2p, and Mak3p histidine kinases contain several additional domains in their N termini that are absent in Sln1p. Adjacent to the histidine-kinase domain are two (in the case of Mak1p) or one (in the case of Mak2p and Mak3p) copy of a PAS/PAC domain (Crews and Fan, 1999), which is found throughout the evolutionary spectrum in proteins associated with light reception and regulation (plant phytochromes), oxygen or redox (*E. coli* Aer, ArcB, and FixL), oxygen-regulated transcription (human HIF1α), and regulation of circadian rhythms (such as mouse PER and CLOCK and *Drosophila* SIM) (Figures 2A and 3A). Adjacent to this region in Mak2p and Mak3p, but not Mak1p, is a GAF domain, which has also been identified in phytochromes (such as *Arabidopsis* PhyE), redox-regulated transcription factors (*E. coli* FhlA), and plant ethylene receptors (e.g., *Arabidopsis* Etr1) (Aravind and Ponting, 1997) (Figures 2A and 3B). Finally, at the extreme N termini of Mak2p and Mak3p, we identified a

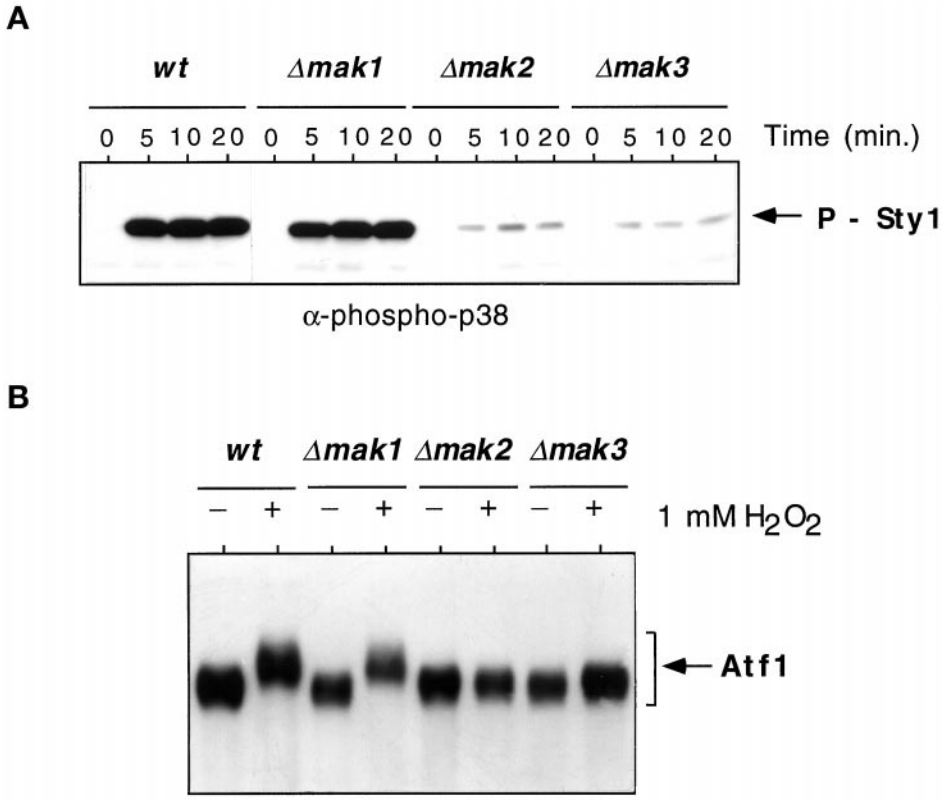

Figure 4. Mak2p and Mak3p are required for Sty1p activation in response to peroxide stress. (A) Mak2p and Mak3p are required for Sty1p phosphorylation. Log-phase cultures of wild-type (JM1521), Δ*mak1* (VB1828), Δ*mak2* (VB1829), or Δ*mak3* (VB1934) cells bearing a hemagglutinin- and six-histidine–tagged version of Sty1p growing in YEPD medium at 30°C were incubated in the same medium containing 1 mM hydrogen peroxide for the times indicated. Approximately 2×10^8 cells were harvested at each time point and phosphorylation of Sty1p was measured as described in MATERIALS AND METHODS. (B) Mak2p and Mak3p are required for Sty1p-dependent Atf1p phosphorylation. Log-phase cultures of wild-type (KS1479), Δ*mak1* (TS2029), Δ*mak2* (TS2030), or Δ*mak3* (TS2031) cells bearing a six-histidine– and hemagglutinin-tagged version of Atf1p growing in YEPD medium at 30°C were incubated in the same medium containing 1 mM hydrogen peroxide for 5 min. Atf1p was precipitated from each sample using Ni^{2+}-nitriloacetic acid agarose and analyzed by probing Western blots with the 12CA5 anti-hemagglutinin antibody.

domain that has considerable homology to a family of atypical serine/threonine kinases from prokaryotes, in particular *Mycobacterium tuberculosis* PknB (Figures 2A and 3C). Although this domain is absent in Mak1p and Sln1p, a similar domain is found in the *Candida albicans* HK1 histidine kinase, which has a similar overall domain architecture to *S. pombe* Mak2p and Mak3p (Figures 2A and 3C).

To investigate the cellular functions of *mak1*, *mak2*, and *mak3*, internal sequences were replaced with auxotrophic markers. Disruption of *mak1* with *ura4* gave rise to viable haploids that divided at a marginally (~10%) smaller size than wild-type (our unpublished data). *mak2::LEU2* and *mak3::kanR* haploid strains were also viable but had no discernible growth or cell-size defect. We also made doubly and triply deleted strains. The triple-deletion cells were viable and divided at 11.2 ± 0.5 μm (as opposed to 14.3 ± 0.2 μm for wild-type cells) and were phenotypically indistinguishable from *mcs4(D412N)* cells (Figure 2B). All double deletion strains divided at an intermediate size (our unpublished data). To test whether the Mak histidine kinases may function through Mcs4p, we constructed a *mak1::ura4 mak2::LEU2 mak3::kanR mcs4::his7* quadruple mutant. These cells divided at 19.3 ± 1.2 μm, a size similar to that of the *mcs4::his7* single mutant (19.1 ± 1.4 μm) (Figure 2B). This result suggests that the Mak1p, Mak2p, and Mak3p histidine kinases act upstream of Mcs4p.

Mak2p and Mak3p Regulate Sty1p Activity in Response to Peroxide

To determine whether any of the three histidine kinases is involved in signaling to Sty1p, wild-type cells or cells individually disrupted for *mak1*, *mak2*, or *mak3* were incubated in the presence of 1 mM hydrogen peroxide and phosphorylation of Sty1p was monitored after various lengths of time. Whereas a rapid increase in Sty1p phosphorylation was observed in both wild-type and Δ*mak1* cells, the response to peroxide was almost completely absent in Δ*mak2* or Δ*mak3* cells (Figure 4A). The Sty1p MAP kinase is cytoplasmic in unstressed cells and translocates to the nucleus, where it associates with and phosphorylates the Atf1p transcription factor, only in response to stress (Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996; Gaits *et al.*, 1998). Phosphorylation of Atf1p can be monitored by a mobility shift on SDS-PAGE (Shiozaki and Russell, 1996). Cells expressing a six-histidine and hemagglutinin-tagged *atf1* allele (KS1479) or derivatives independently deleted for *mak1* (TS2029), *mak2* (TS2030), or *mak3* (TS2031) were incubated in the presence of 1 mM hydrogen peroxide for 5 min and phosphorylation of Atf1p was monitored by SDS-PAGE. Although a rapid hyperphosphorylation of Atf1p was observed in both wild-type and Δ*mak1* cells, no change in Atf1p mobility was observed in Δ*mak2* or Δ*mak3* cells (Figure 4B). These results imply that Sty1p is not fully phosphorylated, activated, or translocated to the nucleus in response to peroxide stress in the absence of Mak2p or Mak3p. Given the similarity to the Sln1p-Ypd1p-Ssk1p pathway in *S. cerevisiae*, we infer that Mak2p and Mak3p are responsible for the phosphorylation of Mcs4p (presumably via Mpr1p) and consequently the activity of the Sty1p MAP kinase.

Mak1p, Mak2p, and Mak3p Control the Transcriptional Response to Peroxide

In *S. pombe,* a number of genes, including those encoding the tyrosine-specific MAP kinase phosphatase (*pyp2*) and glutathione peroxidase (*gpx1*), are induced in response to peroxide in a Sty1p- and Atf1p-dependent manner (Millar *et al.*, 1995; Degols *et al.*, 1996; Wilkinson *et al.*, 1996; Toone *et al.*, 1998; Yamada *et al.*, 1999). Induction of other genes such as those for thioredoxin reductase (*trr1*) and catalase (*ctt1*) are also dependent on Sty1p but require one of two distinct transcription factors, Pap1p and Prr1p (Toone *et al.*, 1998; Ohmiya *et al.*, 1999). To determine whether peroxide-induced gene expression is affected by loss of one or more of the Mak histidine kinases, wild-type cells or those deleted for *mak1*, *mak2*, or *mak3* were incubated in the presence of 1 mM hydrogen peroxide and the levels of various transcripts were assessed by Northern blot analysis. Induction of both *pyp2* and *gpx1* was severely reduced in Δ*mak2* and Δ*mak3* cells but not in Δ*mak1* cells (Figure 5). Conversely, induction of *ctt1* was significantly reduced in Δ*mak1* cells but unaffected in Δ*mak2* and Δ*mak3* cells (Figure 5). These results indicate that Mak2p and Mak3p are required for Atf1p-dependent, but not Pap1p/Prr1p-dependent, gene expression in response to peroxide stress. This is presumably because Sty1p is not activated and therefore unable to phosphorylate and activate Atf1p. On the other hand, Mak1p appears to be required for effective transcription through a Pap1p- or Prr1p-dependent pathway. Deletion of *mak1*, *mak2*, or *mak3* had no effect on the activation of Sty1p or induction of gene expression in response to all other stresses tested (our unpublished data). We conclude that at least two distinct phospho-relay systems control the SAPK-dependent transcriptional response to peroxide stress in *S. pombe*.

Two Phospho-relay Systems Are Required for Protection against Free Radical Attack

Previous studies have shown that the Atf1p and Pap1p transcription factors control distinct but complementary roles in the protection of *S. pombe* cells from free-radical attack. Cells lacking both Atf1p and Pap1p are more sensitive to peroxide attack than cells lacking either protein alone (Nguyen *et al.*, 2000). In *S. cerevisiae*, Yap1p (the Pap1p homolog) plays a functionally overlapping role with the response-regulator protein Skn7p in the transcriptional response to peroxide stress (Morgan *et al.*, 1997). Because Prr1p, a homolog of *S. cerevisiae* Skn7p, is also required for peroxide-induced catalase expression in *S. pombe*, Prr1p and Pap1p may share a similar relationship (Ohmiya *et al.*, 1999). To determine the role of various two-component signaling molecules in the protection of *S. pombe* from peroxide attack, wild-type cells or cells lacking Atf1p or Prr1p were tested for their ability to survive exposure to an acute dose of hydrogen peroxide either in the presence or absence of the Mak2p and Mak3p histidine kinases. Whereas cells lacking Atf1p alone showed little sensitivity to peroxide (our unpublished data), cells lacking Prr1p were more sensitive than wild-type but not as sensitive as cells lacking both Atf1p and Prr1p (Figure 6). These results suggest that Atf1p and Prr1p control distinct aspects of the response to peroxide in *S. pombe*. Because Mak2p and Mak3p appear to control Atf1p-dependent gene expression, we next tested the sensitivity to peroxide of cells lacking Mak2p or Mak3p. We found that cells lacking both Mak2p and Mak3p were no more sensitive to peroxide than wild-type when Prr1p was present but displayed increased sensitivity when Prr1p was absent (Figure 6). These results suggest that at least two distinct phospho-relay pathways contribute to the protection of *S. pombe* cells from peroxide attack. The role of Mak1p in this process will be dealt with in detail in a subsequent study.

DISCUSSION

As reported previously, we and others have identified Mcs4p as an upstream regulator of the *S. pombe* Sty1p MAPK pathway (Shiozaki *et al*., 1997; Shieh *et al*., 1997). Here we show that Mcs4p associates with Wak1p, one of two MAPKKKs that control phosphorylation of Sty1p. Thus, Mcs4p acts in a manner similar to that of the *S cerevisiae* Ssk1p response-regulator protein, which binds the Ssk2p and Ssk22p MAPKKKs to regulate the Hog1p MAP kinase (Posas and Saito, 1998). The activity of Ssk1p is controlled by phosphorylation on aspartate 554, an event that is initiated by a transmembrane histidine kinase, Sln1p. Phosphotransfer between Sln1p and Ssk1p requires phosphorylation of an intermediate protein, Ypd1p, on histidine 64. In this article, we report that phosphorylation of the equivalent residue in Mcs4p (D412) is required for activation of the Sty1p MAP kinase, but only in response to hydrogen peroxide and not to a battery of other environmental insults, including osmotic stress. Surprisingly, paraquat, which induces superoxide radicals and, as a consequence, the production of hydrogen peroxide, still activates Sty1p in *mcs4(D412N)* cells. We conclude that either the superoxide radical is sensed by a distinct mechanism or, at the concentration used, paraquat activates stress signals other than the overproduction of peroxide. Similarly, heavy metal toxicity, also regarded as an oxidative stress, appears to require a distinct pathway to activate Sty1p that does not involve phosphorylation of Mcs4p. Together, these results suggest that Mcs4p acts in a two-component system that specifically senses peroxide stress. Indeed, Nguyen et al. (2000) have recently shown that an *S. pombe* homolog of *S. cerevisiae* Ypd1p, Mpr1p, is also required for activation of Sty1p by peroxide and that this is mediated through a conserved histidine residue. In this article, we present evidence that deletion of either the Mak2p or Mak3p histidine kinase, but not Mak1p, prevents phosphorylation of Sty1p in response to hydrogen peroxide. As a consequence, Sty1p is not activated as judged by the absence of Atf1p phosphorylation and failure of Atf1-dependent gene expression. These results strongly suggest that Sty1p is controlled by a phospho-relay system that is similar to the Sln1p-Ypd1p-Ssk1p pathway in *S. cerevisiae*, but which is initiated by two histidine kinases, Mak2p and Mak3p (see model in Figure 7).

An important difference between the *S. pombe* and *S. cerevisiae* histidine kinases is that Mak2p and Mak3p specifically sense oxidative stress, whereas Sln1p senses changes in external osmolarity. The domain architecture of the Mak2p and Mak3p proteins provides some clue as to why this may be. In particular, both proteins contain a single repeat of a PAS/PAC domain, which has been identified in a variety of prokaryotic sensors of oxygen or redox, including other members of the histidine-kinase superfamily such as *E. coli* Aer and ArcB and *Rhizobium meliloti* FixL, but not Sln1p (Crews and Fan, 1999). Importantly, the PAS/PAC domain of FixL binds heme as a prosthetic group. Changes in the

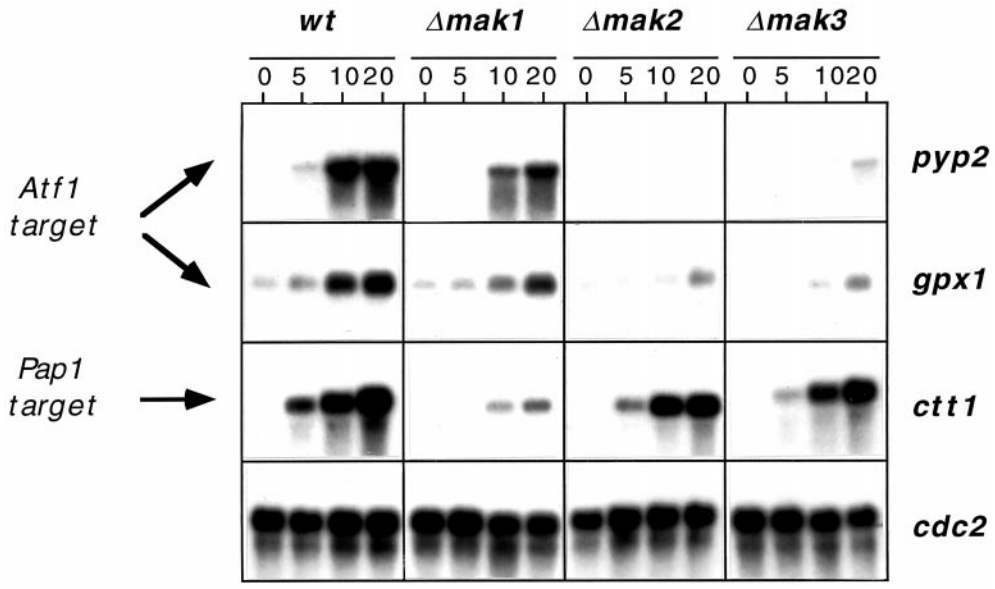

Figure 5. Mak2p and Mak3p control Atf1p-dependent gene transcription in response to peroxide stress. Log-phase cultures of wild-type (CH429), Δ*mak1* (KZ1535), Δ*mak2* (VB1530), or Δ*mak3* (VB1932) cells growing in YES at 30°C were incubated in the presence of 1 mM hydrogen peroxide for the times indicated. Total RNA was extracted and equal quantities were separated by electrophoresis. Northern blots were then probed sequentially by using DNAs specific to the *pyp2*, *gpx1*, *ctt1*, and *cdc2* genes.

availability of oxygen to the heme moiety induce a conformational change in the PAS/PAC domain that causes an alteration in the activity of the adjoining histidine-kinase domain (Gong *et al.*, 1998; Pellequer *et al.*, 1999). It is possible that a similar mechanism may regulate the activity of Mak2p and Mak3p. The PAS/PAC domain may also explain why both of these histidine kinases are required for signal transmission to Sty1p, because this domain has been shown to mediate protein–protein interaction in other species (Huang *et al.*, 1993, 1995). Although Mak2p and Mak3p appear to be exclusively cytoplasmic in *S. pombe*, we have not been able to demonstrate an interaction between them (Quinn, Buck, and Millar, unpublished data). Thus, we cannot yet rule out the possibility that Mak2p and Mak3p initiate independent signals that are both required for activation of Wak1p.

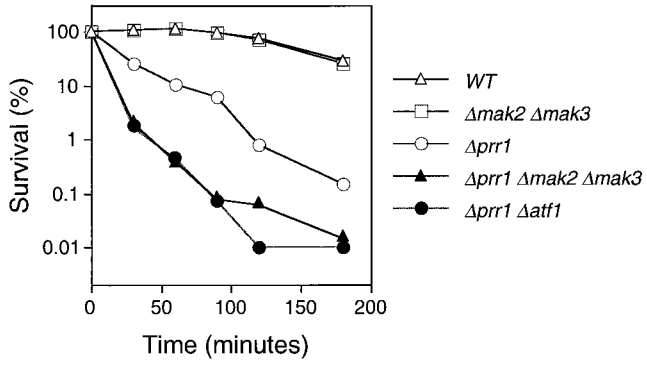

Figure 6. Mak2p and Mak3p protect cells against peroxide attack. Exponentially growing cultures of wild-type (CHP429), Δ*prr1* (JM1516), Δ*prr1* Δ*atf1* (JM1546), Δ*mak2* Δ*mak3* (JM1930), and Δ*mak2* Δ*mak3* Δ*prr1* (JM2232) cells growing in YES medium at 30°C were incubated in the same medium containing 50 mM hydrogen peroxide. Cells were removed at the times indicated, washed, and plated on YES agar plates to determine the number of viable cells. The data shown represent the mean values from three independent experiments.

How is the peroxide signal actually transduced to the Wak1p MAPKKK? It is possible that the peroxide signal activates the histidine-kinase domains of Mak2p and/or Mak3p. In this manner, the response-regulator domains of Mak2p, Mak3p, or both would become phosphorylated and the phosphate group would, in turn, be transferred to Mpr1p. This conclusion is supported by the observation that peroxide induces Mpr1p to associate with Mcs4p and mutation of histidine 221 in Mpr1p abolishes this association (Nguyen *et al.*, 2000). Conversely, it is also formally possible that the peroxide signal inhibits the activity of Mak2p and/or Mak3p, leading to dephosphorylation of Mpr1p and Mcs4p, and that the dephosphorylated form of Mcs4p activates Wak1p. Notably, *mcs4(D412N)* cells show a higher basal level of Sty1p phosphorylation than *mcs4*$^+$ cells, suggesting that the dephosphorylated form of Mcs4p is active. Analysis of the in vivo phosphorylation status of Mak2p, Mak3p, Mpr1p, and Mcs4p will be necessary to distinguish between these possibilities.

In contrast to Mak2p and Mak3p, Mak1p does not control Sty1p activity or Atf1p-dependent gene expression. Instead, we find that Mak1p is partially required for the induction of catalase in response to peroxide stress. It is intriguing in this respect that Mak1p also contains two PAS/PAC repeats, suggesting that Mak1p may also function as a peroxide sensor. Induction of catalase requires both the Pap1p transcription factor and Prr1p, a response-regulator protein that is homologous to the *S. cerevisiae* Skn7p transcription factor (Ohmiya *et al.*, 1999). Skn7p shares an overlapping function with the Yap1p transcription factor in the response to oxidative stress in *S. cerevisiae* (Brown *et al.*, 1993; Morgan *et al.*, 1995; Krems *et al.*, 1996; Morgan *et al.*, 1997). It is possible that Mak1p regulates the activity of Prr1p via phosphorylation of an independent pool of Mpr1p, but the details of such a pathway are unclear because deletion of *mpr1* causes superinduction of catalase expression (Nguyen *et al.*, 2000). Nevertheless, these results suggest that the transcriptional response to free radical attack is regulated by at least two distinct phospho-relay pathways in fission yeast.

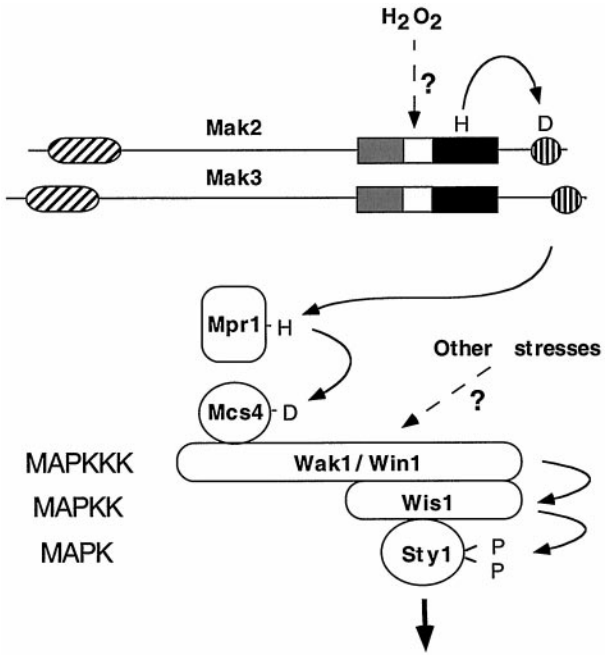

Figure 7. Regulation of the Sty1p MAP kinase cascade by a two-component peroxide receptor. We propose that peroxide stress regulates the activity of a Mak2p/Mak3p histidine kinase heterodimer to control Sty1p activity. According to this model, activation of the Mak2p/Mak3p kinase via alterations in the PAS/PAC domain leads to sequential phosphorylation of histidine 221 in Mpr1p and aspartate 412 in Mcs4p. Mcs4p interacts directly with the N terminus of the Wak1p MAPKKK both before and after stress stimulation. Phosphorylation of Mcs4p alters the activity of Wak1p (and probably also the Win1p MAPKKK), leading to sequential phosphorylation of the Wis1p MAPKK and Sty1p MAPK. Other stresses control the activity of Wak1p and Win1p by an as-yet-unidentified mechanism(s).

In addition to the PAS/PAC motif, Mak2p and Mak3p contain a GAF domain, which has been identified in a diverse array of phototransducing proteins, including cGMP-specific and -stimulated phosphodiesterases, *Anabena* adenylate cyclase, and the *E. coli* FhlA transcriptional regulator (Aravind and Ponting, 1997). One function of the GAF domain that has been well characterized is that of cGMP binding (Charbonneau *et al.*, 1990). Intriguingly, the chromophore attachment site in plant and cyanobacterial phytochromes occurs on a conserved cysteine residue within their single GAF domain (Figure 3B; Yeh and Lagarias, 1998). Although the overall domain architecture of Mak2p and Mak3p is similar to that of the phytochrome family, their GAF domains lack this residue. Thus, the function of this domain in Mak2 and Mak3 is at present unknown. Intriguingly, Mak2p and Mak3p are most closely related to a histidine kinase in the human pathogen *C. albicans*, C.a. HK1 (Calera *et al.*, 1998). In addition to histidine-kinase, response-regulator and GAF domains, all three proteins possess an N-terminal domain that is strikingly similar to a series of atypical serine/threonine kinase in prokaryotes, in particular *M. tuberculosis* PknB. To our knowledge, these are the only known proteins to have two separate kinase domains of apparently distinct specificity. Our initial analysis suggests that these N-terminal domains in Mak2p and Mak3p are not required for signal transmission to Sty1p (Quinn, Buck, and Millar, unpublished data). Nevertheless, it is tempting to speculate that Mak2p, Mak3p, and C.a. HK1 are derived from a fusion of distinct ORFs in an ancestral prokaryotic operon that functioned in a common signaling pathway to sense and respond to free radical attack. Although it is not clear whether C.a. HK1, which lacks a PAS/PAC motif, acts as a peroxide sensor, homozygous null diploids for HK1 are nonvirulent in a murine model (Calera *et al.*, 1998, 1999). Furthermore, a homolog of Ssk1p and Mcs4p is also required for virulence in this system, suggesting that C.a. HK1 may function in a phospho-relay system similar to that described here (Calera and Calderone, 1999; Calera *et al.*, 2000).

The absence of structural homologs of Mak2p and Mak3p in *S. cerevisiae* could explain why the Hog1p MAP kinase is activated only in response to osmotic stress and not to hydrogen peroxide. Indeed, *S. cerevisiae* has only one histidine kinase (Sln1p), whereas at least three have been identified in *C. albicans* and *S. pombe*. In addition, the *C. albicans* and *S. pombe* histidine kinases are not identical to each other. Although an osmosensing Sln1p homolog exists in *C. albicans* (C.a. Sln1), it is unlikely that Sty1p is controlled by a Sln1p-like molecule in *S. pombe*, because activation of Sty1p in response to osmotic stress does not require phosphorylation of Mcs4p. Conversely, no homologs of Mak1p or C.a. Nik1 have been identified in *C. albicans* and *S. pombe*, respectively. It seems possible, therefore, that all fungi are derived from a common eukaryotic ancestor that possessed multiple two-component regulatory systems that have been selectively lost by evolutionary pressure.

To date, no structural homologs of the phospho-relay proteins have been identified in mammals. Thus, control of SAPK pathways by two-component systems is likely to be unique to lower eukaryotes. Importantly, we have shown that the phospho-relay system is not required for activation of the *S. pombe* Sty1p MAPK in response to environmental stresses that activate the mammalian SAPKs, including heat shock, the protein-synthesis inhibitor anisomycin, agents that cause oxidative damage such heavy metals (e.g., arsenic or cadmium), superoxide anion generators (e.g., paraquat), or UV light mimetics (e.g., 4-nitroquinoline oxide). We tentatively propose that each environmental stress is sensed by a specific cellular receptor(s) that triggers activation of Sty1p, possibly by regulating the activity of the Wak1p/Wis4p and/or Win1p MAPKKKs (Figure 7). We will need to identify other upstream activators of the Sty1p pathway to determine whether these are structurally or functionally conserved in mammals.

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REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zheng, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST:

a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389–3402.

Aravind, L., and Ponting, C.P. (1997). The GAF domain: an evolutionary link between diverse phototransducing proteins. Trends Biochem. Sci. *22*, 458–459.

Bahler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A., Steever, A.B., Wach, A., Philippsen, P., and Pringle, J.R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast *14*, 943–951.

Barbet, N., Muriel, W.J., and Carr, A.M. (1992). Versatile shuttle vectors and genomic libraries for use with *Schizosaccharomyces pombe*. Gene *114*, 59–66.

Basi, G., Schmid, E., and Maundrell, K. (1993). TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcriptional efficiency but not the transcription start point or thiamine repressibility. Gene *123*, 131–136.

Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E., and Gustin, M.C. (1993). An osmosensing signal transduction pathway in yeast. Science *259*, 1760–1763.

Brown, J.L., North, S., and Bussey, H. (1993). *SKN7*, a yeast multicopy suppressor of a mutation affecting cell wall β-glucan assembly, encodes a product with domains homologous to prokaryotic two-component regulators and to heat shock transcription factors. J. Bacteriol. *175*, 6908–6915.

Calera, J.A., and Calderone, R.A. (1999). Identification of a putative response regulator two-component phospho-relay gene (CaSSK1) from *Candida albicans*. Yeast *15*, 1243–1254.

Calera, J.A., Choi, G.H., and Calderone, R.A. (1998). Identification of a putative histidine kinase two-component phospho-relay gene (CaHK1) in *Candida albicans*. Yeast *14*, 665–674.

Calera, J.A., Zhao, X.J., De Bernardis, F., Sheridan, M., and Calderone, R. (1999). Avirulence of *Candida albicans* CaHK1 mutants in a murine model of hematogenously disseminated candidiasis. Infect. Immunol. *67*, 4280–4284.

Calera, J.A., Zhao, X.J., and Calderone, R. (2000). Defective hyphal development and avirulence caused by a deletion of the *SSK1* response regulator gene in *Candida albicans*. Infect. Immunol. *68*, 518–525.

Charbonneau, H., Prusti, R.K., LeTrong, H., Sonnenburg, W.K., Mullaney, P.J., Walsh, K.A., and Beavo, J.A. (1990). Identification of a noncatalytic cGMP-binding domain conserved in both the cGMP-stimulated and photoreceptor cyclic nucleotide phosphodiesterases. Proc. Natl. Acad. Sci. USA *87*, 288–292.

Crews, S.T., and Fan, C.M. (1999). Remembrance of things PAS: regulation of development by bHLH-PAS proteins. Curr. Opin. Genet. *9*, 580–587.

Degols, G., Shiozaki, K., and Russell, P. (1996). Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. Mol. Cell. Biol. *16*, 2870–2877.

Degols, G., and Russell, P. (1997). Discrete roles of the Spc1 kinase and the Atf1 transcription factor in the UV response of *Schizosaccharomyces pombe*. Mol. Cell. Biol. *17*, 3356–3363.

Dérijard, B., Hibi, M., Wu, I-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R.J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell *76*, 1025–1037.

Forsburg, S.L., and Sherman, D.A. (1997). General purpose tagging vectors for fission yeast. Gene *191*, 191–195.

Freshney, N.W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., and Saklatvala, J. (1994). Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. Cell *78*, 1039–1049.

Gaits, F., Degols, G., Shiozaki, K., and Russell, P. (1998). Phosphorylation and association with the transcription factor Atf1 regulate localization of Spc1 stress-activated kinase in fission yeast. Genes Dev. *12*, 1464–1473.

Galcheva-Gargova, Z., Dérijard, B., Wu, I-H., and Davis, R.J. (1994). An osmosensing signal transduction pathway in mammalian cells. Science *265*, 806–808.

Gong, W., Hao, B., Mansy, S.S., Gonzalez, G., Gilles-Gonzalez, M.A., and Chan, M.K. (1998). Structure of a biological oxygen sensor: a new mechanism for heme-driven signal transduction. Proc. Natl. Acad. Sci. USA *95*, 15177–15182.

Gupta, S., Campbell, D., Derijard, B., and Davies, R.J. (1995). Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science *267*, 389–393.

Han, J., Lee, J-D., Bibbs, L., and Ulevitch, R.J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science *265*, 808–811.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene *28*, 351–359.

Herskowitz, I. (1995). MAP kinase pathways in yeast: for mating and more. Cell *80*, 187–197.

Hibi, M., Anning, L., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. *7*, 2135–2148.

Huang, Z.J., Edery, I., and Rosbash, M. (1993). PAS is a dimerization domain common to *Drosophila* period and several transcription factors. Nature *364*, 259–262.

Huang, Z.J., Curtin, K.D., and Rosbash, M. (1995). PER protein interactions and temperature compensation of a circadian clock in *Drosophila*. Science *267*, 1169–1172.

Ichijo, H. (1999). From receptors to stress-activated MAP kinases. Oncogene *18*, 6087–6093.

Jones, D.T., Taylor, W.R., and Thornton, J.M. (1992). A new approach to protein fold recognition. Nature *358*, 86–89.

Kato, T., Okazaki, K., Murakami, H., Stettler, S., Fantes, P., and Okayama, H. (1996). Stress signal, mediated by a Hog1-like MAP kinase, controls sexual development in fission yeast. FEBS Lett. *378*, 207–212.

Krems, B., Charizannis, C., and Entian, K-D. (1996). The response regulator-like protein Pos9/Skn7 of *Saccharomyces cerevisiae* is involved in oxidative stress resistance. Curr. Genet. *29*, 327–334.

Kyriakis, J.M., Banerjee, P., Nikolakaki, E., Dai-T., Rubie, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J.R. (1994). The stress-activated protein kinase family of c-Jun kinases. Nature *369*, 156–160.

Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthal., M.J., Heys, J.R., Landvatter, S.W., Strickler, J.E., McLaughlin, M.M., Siemens, I.V., Fisher, S.M., Livi, G.P., White, J.R., Adams, J.L., and Young, P.R. (1994). A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature *372*, 739–746.

Livingstone, C., Patel, G., and Jones, N. (1995). ATF-2 contains a phosphorylation-dependent transcriptional activation domain. EMBO J. *14*, 1785–1797.

Maeda, T., Takekawa, M., and Saito, H. (1995). Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science *269*, 554–558.

Maeda, T., Wurgler-Murphy, S.M., and Saito, H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature *369*, 242–245.

Marshall, C.J. (1994). MAP kinase kinase kinase, MAP kinase kinase, and MAP kinase. Curr. Opin. Genet. Dev. *4*, 82–89.

Maundrell, K. (1993). Thiamine repressible expression vectors pREP and pRIP for fission yeast. Gene *123*, 127–130.

Millar, J.B.A., Buck, V., and Wilkinson, M.G. (1995). Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. Genes Dev. *9*, 2117–2130.

Millar, J.B.A., Russell, P., Dixon, J.E., and Guan, K-L. (1992). Negative regulation of mitosis by two functionally overlapping PTPases in fission yeast. EMBO J. *11*, 4943–4952.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. *194*, 795–823.

Morgan, B.A., Banks, G.R., Toone, W.M., Raitt, D., Kuge, S., and Johnston, L.H. (1997). The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast *Saccharomyces cerevisiae*. EMBO J. *16*, 1035–1044.

Morgan, B.A., Bouquin, N., Merrill, G.F., and Johnston, L.H. (1995). A yeast transcription factor bypassing the requirement for SBF and DSC1/MBF in *S. cerevisiae* has homology to bacterial signal transduction proteins. EMBO J. *14*, 5676–5689.

Nguyen, A.N., Lee, A., Place, W., and Shiozaki, K. (2000). Multistep phosphorelay proteins transmit oxidative stress signals to the fission yeast stress-activated protein kinase. Mol. Biol. Cell. *11*, 1169–1181.

Ohmiya, R., Kato, C., Yamada, H., Aiba, H., and Mizuno, T. (1999). A fission yeast gene ($prr1^+$) that encodes a response regulator implicated in oxidative stress response. J. Biochem. *125*, 1061–1066.

Parkinson, J.S. (1993). Signal transduction systems in bacteria. Cell *73*, 857–871.

Pellequer, J.L., Brudler, R., and Getzoff, E.D. (1999). Biological sensors: more than one way to sense oxygen. Curr. Biol. *9*, 416–418.

Posas, F., and Saito, H. (1998). Activation of the yeast Ssk2 MAP kinase kinase kinase by the Ssk1 two-component response regulator. EMBO J. *17*, 1385–1394.

Posas, F., Wurgler-Murphy, S.M., Maeda, T., Witten, E.A., Thai, T.C., and Saito, H. (1996). Yeast HOG1 MAP kinase cascade is regulated by a multi-step phospho-relay mechanism in the SLN1-YPD1-SSK1 "two component" osmosensor. Cell *86*, 865–875.

Prentice, H.L. (1991). High efficiency transformation of *Schizosaccharomyces pombe* by electroporation. Nucleic Acids Res. *20*, 621.

Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J. Ulevitch, R.J., and Davies, R.J. (1995). Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem. *270*, 7420–7426.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A. (1994). A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell *78*, 1027–1037.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Samejima, I., Mackie, S., Warbrick, E., Weisman, R., and Fantes, P.A. (1998). The fission yeast mitotic regulator $win1^+$ encodes a MAP kinase kinase kinase that phosphorylates and activates Wis1 MAP kinase kinase in response to high osmolarity. Mol. Biol. Cell. *9*, 2325–2335.

Schüller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C., and Ruis, H. (1994). The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae CTT1* gene. EMBO J. *13*, 4382–4389.

Shieh, J-C., Wilkinson, M.G., Buck, V., Morgan, B., Makino, K., and Millar, J.B.A. (1997). The Mcs4 response regulator co-ordinately controls the stress activated Wak1-Wis1-StyI MAP kinase pathway and fission yeast cell cycle. Genes Dev. *11*, 1008–1022.

Shieh, J.-C., Wilkinson, M.G., and Millar, J.B.A. (1998). The Win1 mitotic regulator is a component of the stress activated StyI MAP kinase pathway in fission yeast. Mol. Biol. Cell. *9*, 311–322.

Shiozaki, K., and Russell, P. (1995). Cell cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature *378*, 739–743.

Shiozaki, K., and Russell, P. (1996). Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. Genes Dev. *10*, 2276–2288.

Shiozaki, K., Shiozaki, M., and Russell, P. (1997). Mcs4 mitotic catastrophe suppressor regulates the fission yeast cell cycle through the Wik1-Wis1-Spc1 kinase cascade. Mol. Biol. Cell *8*, 409–419.

Sluss, H.K., Barrett, T., Derijard, B., and Davis, R.J. (1994). Signal transduction by tumor necrosis factor mediated by JNK protein kinases. Mol. Cell. Biol. *14*, 5037–5047.

Stock, J.B., Ninfa, A.J., and Stock, A.M. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev *53*, 450–490.

Takeda, T., Toda, T., Kominami, K., Kohnosu, A., Yanagida, M., and Jones, N. (1995). *Schizosaccharomyces pombe* $atf1^+$ encodes a transcription factor required for sexual development and entry into stationary phase. EMBO J. *14*, 6193–6208.

Tibbles, L.A., and Woodgett, J.R. (1999). The stress-activated protein kinase pathways. Cell Mol. Life Sci. *55*, 1230–1254.

Toda, T., Shimanuki, M., and Yanagida, M. (1991). Fission yeast genes that confer resistance to staurosporine encode an AP1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and *S. cerevisiae* FUS3 and KSS1 kinases. Genes Dev. *5*, 60–73.

Toone, W.M., Kuge, S., Samuels, M., Morgan, B.A., Toda, T., and Jones, N. (1998). Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress activated MAP kinase StyI. Genes Dev. *12*, 1453–1463.

Treisman, R. (1996). Regulation of transcription by MAP kinase cascades. Curr. Opin. Cell Biol. *8*, 205–215.

van Dam, H., Wilhelm, D., Herr, I., Steffen, A., Herrlich, P., and Angel, P. (1995). ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-*jun* induction in response to genotoxic agents. EMBO J. *14*, 1798–1811.

Warbrick, E., and Fantes, P. (1991). The Wis1 protein kinase is a dose-dependent regulator of mitosis in *Schizosaccharomyces pombe*. EMBO J. *10*, 4291–4299.

Waskiewicz, A.J., and Cooper, J.A. (1995). Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. Curr. Opin. Cell Biol. *7*, 798–805.

Wilkinson, M.G., Samuels, M., Takeda, T., Toda, T., Toone, M.W., Shieh, J.-C., Millar, J.B.A., and Jones, N.C. (1996). The Atf1 transcription factor is a target for the StyI stress activated MAP kinase pathway in fission yeast. Genes Dev. *10*, 2289–2301.

Wilkinson, M.G., and Millar, J.B.A. (1998). SAPKs and transcription factors do the nucleocytoplasmic tango. Genes Dev. *12*, 1391–1397.

Yamada, K., Nakagawa, C.W., and Mutoh, N. (1999). *Schizosaccharomyces pombe* homologue of glutathione peroxidase, which does not contain selenocysteine, is induced by several stresses and works as an antioxidant. Yeast *15*, 1125–1132.

Yeh, K.C., and Lagarias, J.C. (1998). Eukaryotic phytochromes: light-regulated serine/threonine protein kinases with histidine kinase ancestry. Proc. Natl. Acad. Sci. USA *95*, 13976–13981.