Multistep Phosphorelay Proteins Transmit Oxidative Stress Signals to the Fission Yeast Stress-activated Protein Kinase

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In response to oxidative stress, eukaryotic cells induce transcription of genes required for detoxification of oxidants. Here we present evidence that oxidative stress stimuli are transmitted by a multistep phosphorelay system to the Spc1/Sty1 stress-activated protein kinase in the fission yeast *Schizosaccharomyces pombe*. The fission yeast $mpr1^+$ gene encodes a novel protein with a histidine-containing phosphotransfer domain homologous to the budding yeast Ypd1. Spc1 activation upon oxidative stress is severely impaired in the $\Delta mpr1$ mutant as well as in the $mpr1HQ$ strain, in which the putative phosphorylation site Mpr1-His221 is substituted with glutamine. In response to oxidative stress, Mpr1 binds to the Mcs4 response regulator that functions upstream of the Spc1 cascade, suggesting that Mcs4 is a cognate response regulator for Mpr1. Unexpectedly, when exposed to hydrogen peroxide, $\Delta mpr1$ cells can induce the catalase gene $ctt1^+$, one of the transcriptional targets of the Spc1 pathway, and survive oxidative stress in the absence of significant Spc1 activation. We have found that Pap1, a bZIP transcription factor homologous to human c-Jun, can mediate induction of $ctt1^+$ expression upon oxidative stress independently of the Spc1 stress-activated protein kinase. These studies show that oxidative stress stimuli are transmitted by multiple pathways to induce specific gene expression.

INTRODUCTION

Stress-activated protein kinases (SAPKs) form an evolutionarily conserved subfamily of the MAPKs and are responsive to diverse environmental stress stimuli rather than growth factors and other mitogenic stimuli (Waskiewicz and Cooper, 1995; Kyriakis and Avruch, 1996; Ip and Davis, 1998). The prototype of SAPKs, Hog1, was first identified in the budding yeast *Saccharomyces cerevisiae* (Brewster *et al.*, 1993). In response to high-osmolarity stress, Hog1 is activated by a MAPK kinase (MEK) homologue, Pbs2, which is in turn activated by three redundant MEK kinase (MEKK) homologues, Ssk2, Ssk22, and Ste11 (Maeda *et al.*, 1995; Posas and Saito, 1997). This Hog1 MAPK cascade is known to be regulated by a variation of the two-component system, multistep phosphorelay, which is composed of the Sln1, Ypd1, and Ssk1 proteins (Posas *et al.*, 1998). Under low-osmolarity conditions, the "sensor kinase" Sln1 autophosphorylates a histidine residue within its kinase domain, and this phosphoryl group is subsequently transferred to an aspartate residue in the "receiver domain" of Sln1 (Maeda *et al.*, 1994). Ypd1 acts as a phosphotransferase and transfers the phosphoryl group from Sln1 to an aspartate residue in the receiver domain of Ssk1, with a high-energy phosphohistidine

intermediate at Ypd1-His64 (Posas *et al.*, 1996). Phosphorylation inhibits the Ssk1 "response regulator" from activating the Ssk2 MEKK. Once cells are exposed to a high-osmolarity environment, the Sln1 kinase is inactivated, and binding of unphosphorylated Ssk1 to the N terminus of Ssk2 induces the activation of Ssk2 (Posas and Saito, 1998). Thus, the four-step His-Asp-His-Asp phosphorelay in the Sln1-Ypd1-Ssk1 pathway negatively regulates the Hog1 MAPK cascade. The two-component system is widespread among prokaryotic signaling pathways to sense and respond to a variety of environmental conditions, but it is less common among eukaryotic species (Loomis *et al.*, 1997). Thus, regulation of the Hog1 MAPK cascade by a two-component system is an intriguing connection between eukaryotic and prokaryotic signaling modules.

In contrast to Hog1, which is responsive only to osmolarity stress (Schüller *et al.*, 1994), SAPKs in mammals and the fission yeast *Schizosaccharomyces pombe* are activated by diverse forms of stress (reviewed by Banuett, 1998). In *S. pombe*, a SAPK called Spc1 (also known as Sty1 or Phh1) is activated by high osmolarity, oxidative stress, and heat shock, and $\Delta spc1$ mutant cells rapidly lose viability under these stress conditions (Millar *et al.*, 1995; Shiozaki and Russell, 1995; Kato *et al.*, 1996). Spc1 is activated through phosphorylation of Thr-171 and Tyr-173, which is carried out by a MEK homologue, Wis1. Wis1 is strongly activated in re-

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sponse to osmostress and oxidative stress, whereas heat shock induces relatively weak and transient Wis1 activation (Nguyen and Shiozaki, 1999). However, heat shock inhibits the Pyp1 and Pyp2 tyrosine phosphatases, negative regulators of Spc1, which results in strong activation of Spc1 (Nguyen and Shiozaki, 1999). Thus, there are at least two independent pathways that transmit different stress stimuli to Spc1: osmostress and oxidative stress are transmitted by Wis1 activation, and heat shock is mediated by inhibition of Pyp1/Pyp2.

It remains largely unknown how osmostress and oxidative stress are sensed and transmitted to the Wis1 MEK. Activation of Wis1 requires phosphorylation by a MEKK, and mutations of the MEKK phosphorylation sites in Wis1 abolish Wis1 activation in response to stress (Shiozaki *et al.*, 1998; Nguyen and Shiozaki, 1999), implying that stress signals are transmitted to Wis1 by MEKKs. Two MEKKs activating Wis1 have been identified, Wis4 (also known as Wik1 and Wak1) (Samejima *et al.*, 1997; Shieh *et al.*, 1997; Shiozaki *et al.*, 1997) and Win1 (Samejima *et al.*, 1998). Proceeding farther upstream, Mcs4, a homologue of the Ssk1 response regulator, is believed to regulate the Wis4 MEKK (Cottarel, 1997; Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). Stress-induced activation of Spc1 is partially impaired in strains lacking functional Mcs4, suggesting that Mcs4 positively regulates the Spc1 MAPK cascade. High sequence homology between Mcs4 and budding yeast Ssk1 implies that fission yeast also has a two-component signaling system upstream of the SAPK pathway.

The outcome of Spc1 activation in response to stress is induction of various stress-response genes, such as $gpd1^+$ and $ctt1^+$ (Degols *et al.*, 1996; Wilkinson *et al.*, 1996; Degols and Russell, 1997). $gpd1^+$ encodes glycerol-3-phosphate dehydrogenase (Pidoux *et al.*, 1990), a key enzyme in glycerol synthesis that is important to increase intracellular osmolarity in a high-osmolarity environment (Ohmiya *et al.*, 1995). Cytoplasmic catalase encoded by $ctt1^+$ decomposes hydrogen peroxide (H_2O_2) and protects cells from oxidative stress (Nakagawa *et al.*, 1995). Transcription of these stress-response genes is under the regulation of a bZIP transcription factor, Atf1 (also known as Gad7) (Takeda *et al.*, 1995; Kanoh *et al.*, 1996). Both $\Delta spc1$ and $\Delta atf1$ mutants are defective in stress-induced expression of the same set of genes and are sensitive to various stress treatments (Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996). Moreover, Atf1 is phosphorylated by Spc1 both in vivo and in vitro (Shiozaki and Russell, 1996). These results strongly suggest that Atf1 is a downstream target of Spc1. Atf1 is most homologous to ATF-2, a key substrate of the human SAPKs, p38 and c-Jun N-terminal kinases (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995; Raingeaud *et al.*, 1996), underscoring the high conservation between the *S. pombe* and human SAPK pathways. Recently, Toone *et al.* (1998) proposed that the Pap1 transcription factor, which has significant homology and similar DNA-binding specificity to mammalian c-Jun (Toda *et al.*, 1991, 1992), is a target transcription factor for the Spc1 MAPK under oxidative stress conditions (reviewed by Wilkinson and Millar, 1998). Pap1 is required for induction of $ctt1^+$ and other genes in response to oxidative stress. In addition, oxidative stress brings about nuclear accumulation of Pap1 in a $spc1^+$-dependent manner (Toone *et al.*, 1998). It should be noted, however, that Pap1 is not a substrate of the

Spc1 MAPK (Wilkinson *et al.*, 1996), and accumulation of Pap1 in the nucleus is much slower than rapid induction of gene expression after oxidative stress (Toone *et al.*, 1998). Thus, the physiological meaning of Pap1 regulation by Spc1 is not well understood.

In this report, we have characterized the fission yeast Mpr1, which is highly homologous to the Ypd1 response regulator phosphotransferase in budding yeast. $\Delta mpr1$ cells are defective in Spc1 activation by oxidative stress but not other forms of stress. In addition, the conserved phosphorylation site, His-221, is essential for Mpr1 function, and Mpr1 binds to the Mcs4 response regulator in response to oxidative stress. These results strongly suggest that Mpr1 and Mcs4 are part of a multistep phosphorelay that transmits oxidative stress signals to the Spc1 MAPK cascade. Unexpectedly, $\Delta mpr1$ cells are not sensitive to oxidative stress or defective in induced expression of $ctt1^+$ after oxidative stress. Our data strongly suggest that the Pap1 transcription factor can mediate oxidative stress–induced $ctt1^+$ expression independently of Spc1 activation. We propose that multiple independent pathways mediate oxidative stress signals to $ctt1^+$ expression, which may be important to sensing and responding to different forms of oxidative stress.

MATERIALS AND METHODS

Yeast Strains and General Techniques

S. pombe strains used in this study are listed in Table 1. They are derivatives of *972 h⁻* and *975 h⁺* (Mitchison, 1970). Growth media as well as basic genetic and biochemical techniques for *S. pombe* have been described (Moreno *et al.*, 1991; Alfa *et al.*, 1993). *S. pombe* cells were grown in yeast extract medium YES and synthetic minimal medium EMM2.

Gene Disruption of mpr1⁺

Using *S. pombe* genomic DNA as a template, we amplified a 1.4-kilobase (kb) DNA fragment that contains the entire $mpr1^+$ ORF by PCR with a pair of primers, EcNd-mpr1 (5′-CC GGA ATT CAT ATG AGT GTA TAT CGT GAT AAC ATG-3′; *Eco*RI restriction site is underlined) and mpr1-3Kp (5′-CCG GGG TAC CAT GCC ACG ACC GTA AGA ACG-3′; *Kpn*I restriction site is underlined). After digesting with *Eco*RI and *Kpn*I, this fragment was cloned into pBluescript (Stratagene, La Jolla, CA). The resultant plasmid (pBS-$mpr1^+$) was digested with *Hin*dIII and *Xho*I, to which a 1.9-kb *Xba*I–*Sma*I fragment of $his7^+$ (Apolinario *et al.*, 1993) was ligated. The $mpr1::his7^+$ construct was released by restriction digestion with *Eco*RI and *Kpn*I and used to transform a diploid strain constructed by mating CHP429 and KS1575. Stable His⁺ transformants were selected, and disruption of one of the $mpr1^+$ loci in the diploid was confirmed by Southern hybridization. Sporulation and tetrad dissection generated viable haploid segregants of $mpr1::his7^+$ $spc1::ura4^+$ and $mpr1::his7^+$ $spc1^+$, which indicated that $mpr1^+$ is not essential for cellular viability.

Construction of the mpr1:HA6H and mpr1HQ:HA6H Alleles

The $mpr1HQ$ mutant gene, which has the His-221→Gln substitution, was created by the overlap extension method with the use of PCR (Higuchi *et al.*, 1988; Ho *et al.*, 1989). Two separate amplification reactions were performed with pBS-$mpr1^+$ as the template with the use of a first pair of primers, EcNd-mpr1 and MPR3HQ (5′-CC TTT

Table 1. *S. pombe* strains used in this study

Strain	Genotype	Source or reference
PR109	*h⁻*	Laboratory stock
CHP429	*h⁻ his7-366 ade6-M216*	Laboratory stock
JM544	*h⁻ wis1::ura4⁺*	Laboratory stock
TP108-3c	*h⁻ pap1::ura4⁺*	Toda *et al.* (1991)
KS1366	*h⁻ spc1::ura4⁺*	Laboratory stock
KS1376	*h⁻ spc1:HA6H(ura4⁺)*	Shiozaki and Russell (1995)
KS1497	*h⁻ atf1::ura4⁺*	Shiozaki and Russell (1996)
KS1533	*h⁻ spc1::ura4⁺ atf1::ura4⁺*	Shiozaki and Russell (1996)
KS1575	*h⁺ his7-366 ade6-M210 spc1::ura4⁺*	Shiozaki and Russell (1996)
KS2081	*h⁻ wis1DD:12myc(ura4⁺)*	Shiozaki *et al.* (1998)
KS2088	*h⁻ spc1:HA6H(ura4⁺) wis1DD:12myc(ura4⁺)*	Shiozaki *et al.* (1998)
KS2096	*h⁻ spc1:HA6H(ura4⁺) wis1:12myc(ura4⁺)*	Shiozaki *et al.* (1998)
CA220	*h⁻ mcs4::ura4⁺ spc1:HA6H(ura4⁺)*	This study
CA279	*h⁻ his7-366 mpr1::his7⁺ spc1:HA6H(ura4⁺)*	This study
CA288	*h⁺ his7-366 mpr1::his7⁺*	This study
CA290	*h⁻ mcs4:12myc(ura4⁺) spc1:HA6H(ura4⁺)*	This study
CA334	*h⁻ atf1::ura4⁺ pap1::ura4⁺*	This study
CA337	*h⁻ his7-366 mcs4:12myc(ura4⁺) mpr1::his7⁺*	This study
CA356	*h⁻ pap1::ura4⁺ wis1DD:12myc(ura4⁺)*	This study
CA385	*h⁻ mpr1:HA6H(ura4⁺) spc1:HA6H(ura4⁺)*	This study
CA403	*h⁻ mpr1HQ:HA6H(ura4⁺) spc1:HA6H(ura4⁺)*	This study
CA420	*h⁻ his7-366 mcs4::ura4⁺ mpr1::his7⁺ spc1:HA6H(ura4⁺)*	This study
CA423	*h⁺ his7-366 mcs4::ura4⁺ prr1::his7⁺ spc1:HA6H(ura4⁺)*	This study
CA425	*h⁻ his7-366 prr1::his7⁺ spc1:HA6H(ura4⁺)*	This study

All strains are *leu1-32 ura4-D18*.

AAG GAA **TTG** CCC CAA CG-3′; the codon corresponding to the substitution from His to Gln is in boldface), and a second pair of primers, MPR5HQ (5′-CG TTG GGG **CAA** TTC CTT AAA GG-3′) and mpr1-3Kp. These PCR products were purified by agarose gel electrophoresis, mixed at a 1:1 ratio, and then subjected to another PCR with the use of the primers PS288-MPR (5′-T GCA CTG CAG GCT GCT AAT GAG ACG GCT GGC GC-3′; *Pst*I restriction site is underlined) and MPR-NT2 (5′-GT TTA GCG GCC GCC TGT AGA AGA ATT TTT TTC ATA AAA GTC AAG-3′; *Not*I restriction site is underlined). The same pair of primers was also used to amplify the wild-type *mpr1⁺* sequence with the use of the pBS-*mpr1⁺* plasmid as a template. The 0.6-kb *Pst*I–*Not*I fragments of these PCR products were cloned in pUra4-HA6H to add a C-terminal tag of two copies of hemagglutinin (HA) epitope and six consecutive histidine residues (Shiozaki and Russell, 1997). The resultant pUra4-mpr1-HA6H and pUra4-mpr1HQ-HA6H plasmids were used to transform wild-type strain PR109 after being linearized at the *Bcl*I site in the *mpr1* ORF. Stable Ura⁺ transformants were selected, and integration of the plasmids at the *mpr1⁺* locus was confirmed by Southern hybridization. Furthermore, the *mpr1* sequence was amplified by PCR with the use of the genomic DNA from the transformants, and the introduced mutation was confirmed by DNA sequencing. In these strains, the *mpr1:HA6H* and *mpr1HQ:HA6H* constructs are expressed from the chromosomal *mpr1⁺* promoter.

Purification and Detection of the Spc1, Mpr1, and Mpr1HQ Proteins with the HA6H Tag

The HA6H sequence encoding two copies of the HA epitope and six consecutive histidine residues (Shiozaki and Russell, 1995, 1997) was used to tag the chromosomal *spc1⁺*, *mpr1⁺*, and *mpr1HQ* genes. Cells were grown to midlog phase in liquid YES medium at 30°C and subjected to stress treatments with 0.6 M KCl, 0.3 mM H₂O₂, or heat shock at 48°C, as described previously (Shiozaki *et al.*, 1997). Cells were harvested by rapid filtration (Shiozaki and Russell, 1997),

and HA6H-tagged proteins were purified on Ni-nitrilotriacetic acid (NTA)–agarose beads under denaturing conditions, followed by immunoblotting with anti-HA (12CA5; Boehringer Mannheim, Indianapolis, IN) and anti-phospho-p38 MAPK (New England Biolabs, Beverly, MA) antibodies (Shiozaki and Russell, 1997). HRP-conjugated secondary antibodies (Promega, Madison, WI) and ECL Plus Reagent (Amersham Pharmacia, Arlington Heights, IL) were used for detection and quantification with the Storm System (Molecular Dynamics, Sunnyvale, CA).

H₂O₂ Sensitivity Assay and RNA Analysis

S. pombe cells were grown in YES medium to midlog phase at 30°C. Aliquots of the culture were incubated for 1 h in the presence of 0, 1, 2, 4, and 8 mM H₂O₂. Cells were washed, diluted, and plated on YES agar (Degols *et al.*, 1996). Percent viability was determined after 3 d of incubation at 30°C. Survival curves in Figures 6 and 8 represent the mean values of three independent experiments. Northern hybridization analyses of *ctt1⁺* and *leu1* have been described by Degols and Russell (1997).

Detection of Mpr1-Mcs4 Association In Vivo

The pREP1-KZ-mpr1 and pREP1-KZ-mpr1HQ plasmids were constructed by cloning of the wild-type *mpr1⁺* and mutant *mpr1HQ* genes in pREP1-KZ vector (Shiozaki and Russell, 1997); they express GST-Mpr1 or GST-Mpr1HQ fusion proteins, respectively, under the regulation of the thiamine-repressible *nmt1* promoter (Maundrell, 1990). The pREP1-KZ-mpr1 plasmid can complement the Δ*mpr1* defect in Spc1 activation upon oxidative stress, indicating that the GST tag does not disturb the Mpr1 function. CA337 (Δ*mpr1 mcs4:myc*) cells were transformed with the plasmids pREP1-KZ-mpr1 and pREP1-KZ-mpr1HQ, and transformants were grown at 30°C for 23 h in EMM2 medium supplemented with 0.03 μM thiamine to induce expression of the

GST fusion proteins at a low level. After stress treatments, cells were harvested by rapid filtration and disrupted in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl, 1 mM 2-mercaptoethanol, 10% glycerol, 0.1 mM Na_3VO_4). The protein concentration of each lysate was normalized with the use of a Bio-Rad (Richmond, CA) protein assay before immunoblot analysis of Mcs4 (see Figure 4A, bottom panel) and incubation with 10 μl (bed volume) of glutathione (GSH)-Sepharose beads (Amersham Pharmacia). After extensive washes with lysis buffer containing 0.7% Triton X-100, proteins bound to the beads were analyzed by immunoblotting with anti-GST and anti-myc (BabCO, Richmond, CA) antibodies (Shiozaki and Russell, 1997).

RESULTS

Mpr1 Is Homologous to Ypd1, a Response Regulator Phosphotransferase in Budding Yeast

Previous studies identified Mcs4 as a regulator of the Spc1 MAPK cascade. Mcs4 is most homologous to the Ssk1 response regulator in budding yeast (Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). To examine whether the Spc1 cascade is regulated by a multistep phosphorelay similar to the budding yeast Sln1-Ypd1-Ssk1 pathway, we performed a computer search for Ypd1 homologues in the *S. pombe* genome sequences submitted to GenBank by the Sanger Center. An 888-base pair uninterrupted ORF (SPBC725.02) in chromosome II was found to encode a protein of 295 amino acid residues with a significant homology to Ypd1 (Figure 1A). This putative 32.5-kDa protein is significantly larger than Ypd1, at 165 amino acid residues; however, its C-terminal region of 130 residues is 43% identical to the Ypd1 sequence. Notably, residues 62–71 of Ypd1, containing the histidine phosphorylation site, are completely conserved in this fission yeast gene, which was therefore named *mpr1+* (multistep phosphorelay). On the other hand, no apparent homology to any known proteins was found in the N-terminal 160 residues of Mpr1.

Mpr1 Is Important for Oxidative Stress Signaling to SAPK

To study the cellular function of Mpr1, gene disruption of *mpr1+* was performed. In *S. cerevisiae*, mutational inactivation of *YPD1* brings about lethal hyperactivation of the Hog1 MAPK, which can be suppressed by the *hog1Δ* mutation (Posas *et al.*, 1996). Therefore, a *spc1::ura4+/spc1+* heterozygous diploid strain, which has one of the *spc1* loci disrupted with the *ura4+* gene, was transformed with the *mpr1::his7+* plasmid construct (Figure 1B). Integration of this plasmid to one of the *mpr1+* loci replaced the Mpr1 sequence homologous to Ypd1 with the *his7+* marker gene, which was confirmed by Southern hybridization. The resultant diploid *mpr1::his7+/mpr1+ spc1::ura4+/spc1+* strain was sporulated and subjected to tetrad analysis. All of the dissected tetrads gave rise to four viable spores with 2:2 segregation of His+ and His− phenotypes regardless of the uracil auxotrophy, indicating that *mpr1+* is not essential for viability in both *spc1+* and *spc1−* backgrounds. A haploid strain with the disrupted *mpr1+* gene (Δ*mpr1*), however, showed ~12% longer doubling time than that of wild-type cells under the standard growth condition (in YES medium at 30°C). Δ*mpr1* cells also grew and formed colonies on YES agar plates
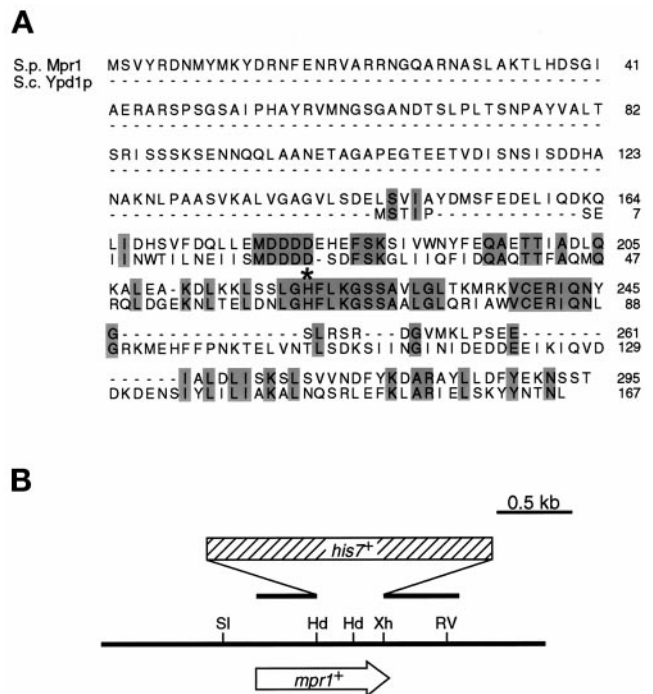

Figure 1. Mpr1 is a fission yeast homologue of the budding yeast Ypd1 protein, a response regulator phosphotransferase. (A) Alignment of the amino acid sequence of Mpr1 with that of Ypd1. Identical residues are shown by shaded boxes. An asterisk marks the phosphorylation site His-64 of Ypd1, which is also conserved in Mpr1. The nucleotide sequence of *mpr1+* is available in the GenBank database under accession number AL034352 (ORF, SPBC725.02). (B) Restriction map of the *mpr1+* locus and the *mpr1::his7+* construct for gene disruption. The *Hin*dIII–*Xho*I fragment encoding residues 136–281 was replaced with the *his7+* marker gene (Apolinario *et al.*, 1993) to disrupt the *mpr1+* gene. Restriction enzyme sites: Hd, *Hin*dIII; RV, *Eco*RV; Sl, *Sal*I; Xh, *Xho*I.

containing 1 M KCl (our unpublished results), suggesting that *mpr1+* is not essential for cellular survival of high-osmolarity conditions.

We next examined whether, like Ypd1, Mpr1 is involved in stress signaling to the SAPK cascade in fission yeast. Activation of Spc1 in response to stress treatments was compared between wild-type and Δ*mpr1* cells with the use of strains in which chromosomal *spc1+* is tagged with the HA6H sequence encoding the HA epitope and six consecutive histidine residues (Shiozaki and Russell, 1995). Anti-phospho-p38 antibodies were used in immunoblotting to detect the active form of Spc1 phosphorylated on both Thr-171 and Tyr-173 (Shiozaki and Russell, 1997; Nguyen and Shiozaki, 1999). Under normal growth conditions, the level of active Spc1 in Δ*mpr1* cells is 60–70% higher than that in wild-type cells (Figure 2, lanes 0), whereas no apparent difference was observed in the amount of the Spc1 protein detected by anti-HA antibodies. High-osmolarity stress induced by 0.6 M KCl (Figure 2A) and oxidative stress induced by 0.3 mM H_2O_2 (Figure 2B) increased active Spc1 four- to fivefold in wild-type cells. On the other hand, oxidative stress–induced activation of Spc1 in Δ*mpr1* cells was only 60% of that in wild-type cells (Figure
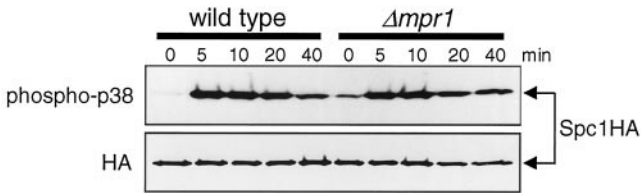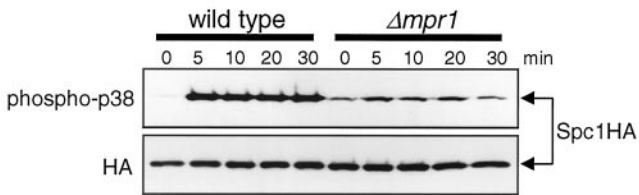
A Osmostress

B Oxidative stress

Figure 2. Mpr1 is important for activation of Spc1 in response to oxidative stress. Wild-type (KS1376) and Δ*mpr1* (CA279) strains carrying the *spc1:HA6H* allele were grown to midlog phase at 30°C in YES medium and treated with either high-osmolarity stress induced by 0.6 M KCl (A) or oxidative stress induced by 0.3 mM H$_2$O$_2$ (B). Aliquots of cells were harvested at the indicated times, and Spc1 was purified by Ni-NTA chromatography, followed by immunoblotting with anti-phospho-p38 and anti-HA antibodies. Δ*mpr1* mutant cells showed a significant defect in Spc1 activation upon oxidative stress, whereas osmostress induced strong activation of Spc1 in Δ*mpr1* mutants and wild-type cells.

2B), although no apparent difference was detected between the two strains in the level of Spc1 activation after osmostress (Figure 2A). We also observed that heat shock–induced activation of Spc1 in the Δ*mpr1* strain was comparable to that in the wild-type strain (our unpublished results). Thus, Spc1 activation in response to oxidative stress, but not other forms of stress, is defective in the Δ*mpr1* mutant. These results implicate Mpr1 in oxidative stress signaling to the Spc1 SAPK cascade, which contrasts with the function of Ypd1 in osmostress signaling of budding yeast (Posas *et al.*, 1996).

The Putative Histidine Phosphorylation Site of Mpr1 Is Crucial for Oxidative Stress Signaling

Sequence conservation of the histidine phosphorylation site between Mpr1 and Ypd1 (Figure 1A) prompted us to test whether phosphorylation of His-221 in Mpr1 is required for oxidative stress signaling. Site-directed mutagenesis was used to construct the *mpr1HQ* mutant gene, in which His-221 was substituted with an unphosphorylatable residue, glutamine. The chromosomal *mpr1*+ locus was replaced with the *mpr1HQ* gene, which was tagged with the HA6H sequence for purification by Ni-NTA chromatography and detection by anti-HA antibodies. Phenotypes of the control strain expressing wild-type Mpr1 tagged with the HA6H
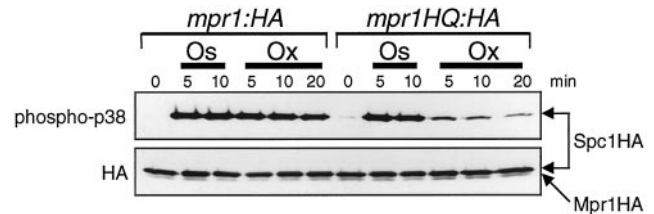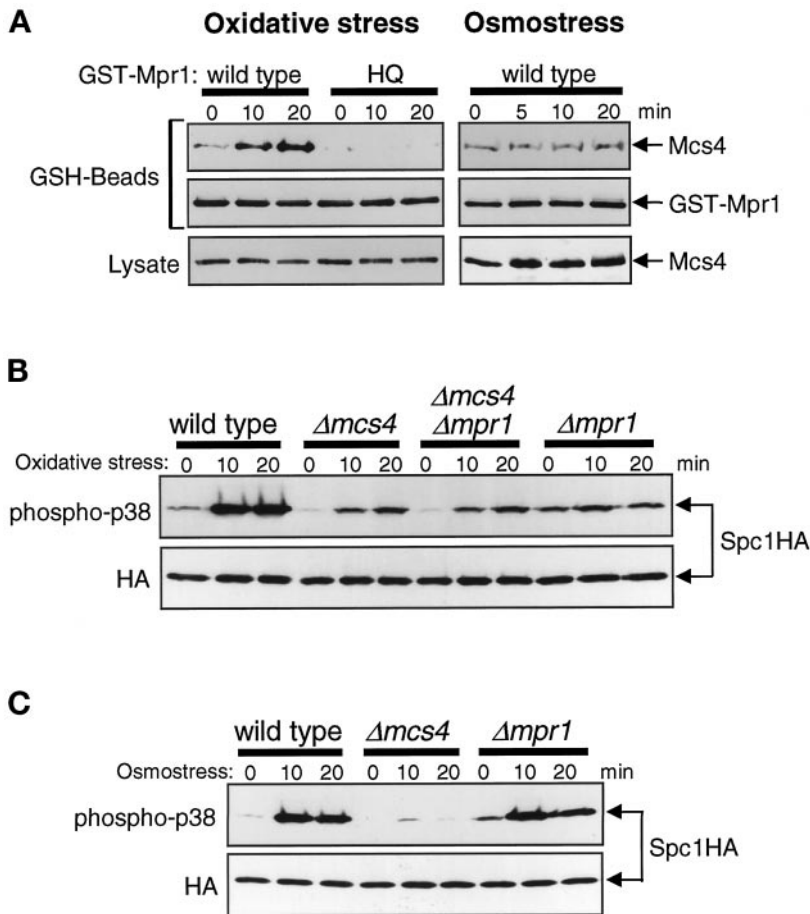

Figure 3. The putative histidine phosphorylation site, His-221, is required for Mpr1 function in oxidative stress signaling to Spc1. The *mpr1HQ* mutant gene with the His-221→Gln substitution was created by site-directed mutagenesis and fused to the HA6H tag, which was used to replace the *mpr1*+ locus of an *spc1:HA6H* strain. The resultant strain (CA403) and the control strain, which has the wild-type *mpr1*+ locus tagged with HA6H (CA385), were grown to midlog phase at 30°C in YES medium and then treated with osmostress induced by 0.6 M KCl (Os) and oxidative stress induced by 0.3 mM H$_2$O$_2$ (Ox) for the indicated times. Spc1 as well as wild-type and mutant Mpr1 were purified on Ni-NTA beads and analyzed by immunoblotting with anti-phospho-p38 and anti-HA antibodies. The Mpr1 and Mpr1HQ proteins were detected as weak bands below the Spc1 bands in anti-HA immunoblotting. Stress-induced Spc1 activation in the *mpr1:HA* strain was indistinguishable from that in strains expressing untagged Mpr1, indicating that the HA6H tag does not disturb the Mpr1 function.

sequence were indistinguishable from those of cells expressing untagged Mpr1; strong activation of Spc1 was observed in response to osmostress and oxidative stress (Figure 3, *mpr1:HA*). In contrast, oxidative stress–induced Spc1 activation was significantly reduced in the *mpr1HQ* mutant strain, whereas high osmolarity activated Spc1 in *mpr1HQ* cells to a level comparable to that in the control strain (Figure 3). Thus, like Δ*mpr1* cells, the *mpr1HQ* mutant is defective in oxidative stress–induced activation of Spc1, although anti-HA immunoblotting detected both the Mpr1 and Mpr1HQ proteins at similar levels (Figure 3, lower panel). These results indicate that the putative phosphorylation site, His-221, is required for the Mpr1 function in oxidative stress signaling to Spc1.

Mpr1 Interacts with the Mcs4 Response Regulator

The results described above suggest that, like Ypd1, Mpr1 may mediate stress signals by transferring a phosphoryl group from its histidine residue to a response regulator. Because a response regulator homologue, Mcs4, regulates the Spc1 MAPK cascade (Cottarel, 1997; Shieh *et al.*, 1997; Shiozaki *et al.*, 1997), it is likely that Mpr1 functions as a phosphotransferase for Mcs4. To explore this possibility, physical interaction between Mpr1 and Mcs4 in vivo was examined. We constructed a strain in which chromosomal *mcs4*+ was tagged with the sequence encoding the myc epitope for detection. Phenotypes of this *mcs4:myc* strain were indistinguishable from those of strains expressing untagged Mcs4 (our unpublished results), indicating that the Mcs4 function was not disturbed by the myc epitope. The *mcs4:myc* strain was transformed with pREP1-KZ-mpr1 and pREP1-KZ-mpr1HQ, which express wild-type Mpr1 and the His-221→Gln mutant (Mpr1HQ), respectively, as fusion with GST. Expression of GST-Mpr1 and GST-Mpr1HQ was

A

B

C

Figure 4. Mpr1 functions upstream of the Mcs4 response regulator. (A) Oxidative stress induces physical association between Mpr1 and Mcs4. Strain CA337 has chromosomal $mcs4^+$ tagged with the sequence encoding the myc epitope. This strain was transformed with pREP1-KZ-mpr1 and pREP1-KZ-mpr1HQ plasmids, which express GST fusion proteins of wild-type and His-221→Gln mutant Mpr1, respectively, under the regulation of the thiamine-repressible *nmt1* promoter. The transformants were grown in EMM2 medium with 0.03 μM thiamine to induce expression of the GST fusion proteins at a low level and treated with either oxidative stress induced by 0.3 mM H_2O_2 (left panels) or high-osmolarity stress induced by 0.6 M KCl (right panels) for the indicated times. Cell lysates were absorbed to GSH-Sepharose beads, and after extensive washes, proteins bound to the beads (GSH-Beads) were analyzed by immunoblotting with anti-myc and anti-GST antibodies. The amount of Mcs4 detected in the crude cell lysates (Lysate) did not change significantly after the stress treatments. (B) Wild-type (KS1376), $\Delta mcs4$ (CA220), $\Delta mpr1$ (CA279), and $\Delta mcs4$ $\Delta mpr1$ (CA420) strains carrying the *spc1:HA6H* allele were grown to mid-log phase at 30°C in YES medium and treated with oxidative stress induced by 0.3 mM H_2O_2. Aliquots of cells were harvested at the indicated times, and Spc1 was purified by Ni-NTA chromatography, followed by immunoblotting with anti-phospho-p38 and anti-HA antibodies. The pattern of Spc1 activation in the $\Delta mcs4$ $\Delta mpr1$ double mutant is identical to that in the $\Delta mcs4$ mutant before and after oxidative stress. (C) Wild-type (KS1376), $\Delta mcs4$ (CA220), and $\Delta mpr1$ (CA279) strains were treated with high-osmolarity stress induced by 0.6 M KCl, and Spc1 activation was examined as described for B.

induced at a relatively low level from the thiamine-repressible *nmt1* promoter of the pREP1 vector (Maundrell, 1990) in the growth medium containing 0.03 μM thiamine. Isolation of the GST-Mpr1 protein on GSH-Sepharose beads resulted in copurification of Mcs4, whereas little Mcs4 was recovered with the mutant GST-Mpr1HQ protein (Figure 4A). Moreover, the amount of Mcs4 coprecipitated with GST-Mpr1 increased significantly during the experiment after cells were exposed to oxidative stress induced by H_2O_2. In contrast, osmostress did not affect the interaction between GST-Mpr1 and Mcs4 (Figure 4A, right panels), indicating that the induced interaction between Mpr1 and Mcs4 is specific to oxidative stress.

We next examined the phenotype of the $\Delta mcs4$ $\Delta mpr1$ double mutant in Spc1 activation upon oxidative stress. Compared with wild-type cells, exposure to H_2O_2 brought about a reduced level of Spc1 activation in $\Delta mcs4$ cells (Figure 4B), indicating that Mcs4 positively regulates Spc1 activation (Shieh *et al.*, 1997; Shiozaki *et al.*, 1997). In $\Delta mpr1$ cells, the level of active Spc1 before stress was somewhat higher than in wild-type cells, but only slight activation of Spc1 was induced by oxidative stress. We found that the pattern of Spc1 activation before and after oxidative stress in the $\Delta mcs4$ $\Delta mpr1$ double mutant was indistinguishable from that in the $\Delta mcs4$ strain (Figure 4B); therefore, $\Delta mcs4$ appears to be epistatic to $mpr1^+$.

This observation is consistent with the idea that Mpr1 transmits oxidative stress signals to the Spc1 cascade through the Mcs4 response regulator and that Mpr1 cannot affect Spc1 activity in the absence of Mcs4. However, $\Delta mpr1$ and $\Delta mcs4$ cells showed significantly different phenotypes under osmostress conditions (Figure 4C); only weak activation of Spc1 was observed in $\Delta mcs4$ cells exposed to high osmolarity, whereas Spc1 activation in $\Delta mpr1$ cells was comparable to that in wild-type cells under the same conditions.

In the $\Delta mcs4$ and $\Delta mcs4$ $\Delta mpr1$ strains, weak activation of Spc1 was induced in response to oxidative stress (Figure 4B), indicating that oxidative stress stimuli can be transmitted to Spc1 independently of Mpr1 and Mcs4. Recently, the $prr1^+$ gene encoding a novel response regulator was reported, which is important for cellular resistance against oxidative stress in *S. pombe* (Ohmiya *et al.*, 1999). To test whether the Prr1 response regulator is involved in oxidative stress signaling to Spc1, H_2O_2-induced activation of Spc1 in *prr1* null mutant ($\Delta prr1$) cells was examined. The $\Delta prr1$ mutation showed no apparent defect in Spc1 activation before or after oxidative stress in either the $mcs4^+$ (Figure 5, top) or the $\Delta mcs4$ (Figure 5, bottom) background, suggesting that Prr1 is not important for oxidative stress signaling to Spc1.
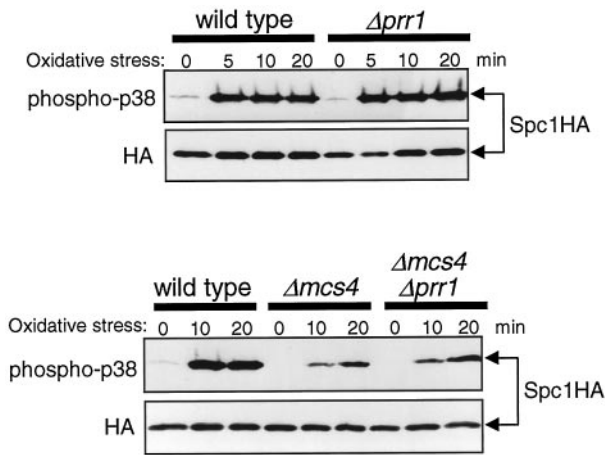
Figure 5. The Prr1 response regulator is not important for Spc1 activation after oxidative stress. Wild-type (KS1376), Δ*prr1* (CA425), Δ*mcs4* (CA220), and Δ*mcs4* Δ*prr1* (CA423) strains carrying the *spc1: HA6H* allele were grown to midlog phase at 30°C in YES medium and treated with oxidative stress induced by 0.3 mM H₂O₂. Aliquots of cells were harvested at the indicated times, and Spc1 was purified by Ni-NTA chromatography, followed by immunoblotting with anti-phospho-p38 and anti-HA antibodies.

Mpr1 Is Not Essential for Cellular Resistance against Oxidative Stress

Because *spc1* mutants are supersensitive to H₂O₂ (Degols *et al.*, 1996; Toone *et al.*, 1998), we also examined the H₂O₂ sensitivity of the Δ*mpr1* mutant, which is defective in Spc1 activation upon oxidative stress. Wild-type, Δ*spc1*, and Δ*mpr1* cells were incubated for 1 h with 1 and 2 mM H₂O₂ in the growth medium, and the survival of each strain was evaluated in terms of their colony-forming ability after plating. As shown in Figure 6A, Δ*mpr1* cells showed H₂O₂ resistance similar to that of wild-type cells, whereas significantly higher loss of viability was observed with Δ*spc1* cells under the same conditions.

In response to oxidative stress, Spc1 activation brings about induced expression of the *ctt1*⁺ gene encoding cytosolic catalase (Wilkinson *et al.*, 1996; Degols and Russell, 1997), which decomposes H₂O₂ to protect cells from oxidative damage (Nakagawa *et al.*, 1995). Northern blot experiments demonstrated that, in Δ*mpr1* cells, *ctt1*⁺ expression was higher than in wild-type cells in the absence of stress, and *ctt1*⁺ was further induced upon exposure to H₂O₂ (Figure 6B). On the other hand, *ctt1*⁺ mRNA was hardly detected in Δ*spc1* cells before or after oxidative stress. Although Mpr1 plays an important role in the activation of Spc1 in response to oxidative stress, these data indicate that Mpr1 is not essential for the induction of *ctt1*⁺ expression and other cellular responses to protect cells from oxidative stress.

Oxidative Stress Can Induce ctt1⁺ Expression Independently of Spc1 Activation

The results described above indicate that in Δ*mpr1* cells, expression of *ctt1*⁺ is strongly induced in the absence of a significant increase in cellular Spc1 activity. However, Δ*spc1* mutants are totally defective in *ctt1*⁺ expression upon oxi-

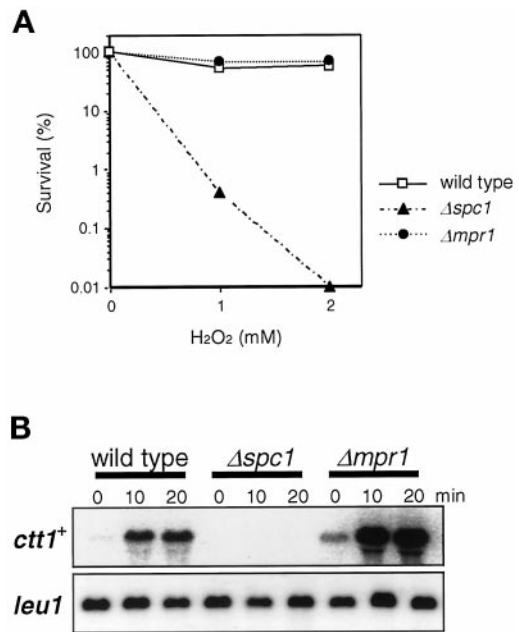**Figure 6.** Δ*mpr1* cells are not sensitive to oxidative stress. (A) Wild-type (PR109), Δ*spc1* (KS1366), and Δ*mpr1* (CA288) cells were grown to midlog phase in YES medium at 30°C, and H₂O₂ was added at concentrations of 0, 1, and 2 mM. After 1 h of incubation at 30°C, cells were washed, diluted, and plated on YES agar medium. The survival of each strain was evaluated in terms of its colony-forming ability after 3 d of incubation at 30°C. Results from an average of three independent experiments are shown. (B) Oxidative stress–induced expression of the catalase gene, *ctt1*⁺, in Δ*mpr1* cells. The strains used in A were treated with 0.3 mM H₂O₂ in YES medium, and aliquots of cells were harvested at the indicated times for Northern hybridization analysis of the *ctt1*⁺ mRNA. The *leu1*⁺ probe served as a control. *ctt1*⁺ expression was strongly induced in Δ*mpr1* cells but was not detectable in Δ*spc1* cells.

dative stress (Degols and Russell, 1997) (Figure 6B), suggesting that Spc1 activity is essential for induction of *ctt1*⁺ expression. One hypothesis to explain both of these observations is that *ctt1*⁺ expression has an absolute requirement for a certain level of Spc1 activity, but oxidative stress stimuli can trigger induction of *ctt1*⁺ in the absence of any increase in Spc1 activity. To test this hypothesis, we examined *ctt1*⁺ expression in the *wis1DD* mutant strain, which expresses a mutant form of the Wis1 MEK that is constitutively active because of aspartic acid substitutions at the MEKK phosphorylation sites (Shiozaki *et al.*, 1998; Nguyen and Shiozaki, 1999). In contrast to wild-type cells, the activation level of Spc1 in *wis1DD* cells was not affected by osmostress and oxidative stress (Figure 7A). As shown in Figure 7B, *ctt1*⁺ expression was strongly induced by oxidative stress in *wis1DD* cells, which suggests that induction of *ctt1*⁺ upon oxidative stress does not require an increase in Spc1 activity. In contrast, *ctt1*⁺ induction by high-osmolarity stress appeared to be mediated by Spc1 activation (Figure 7C). The level of *ctt1*⁺ mRNA was constant both before and after osmostress in *wis1DD* cells, whereas strong induction of *ctt1*⁺ was observed in wild-type cells. As in the Δ*spc1* cells, *ctt1*⁺ expression was not detectable in the Δ*wis1* cells.
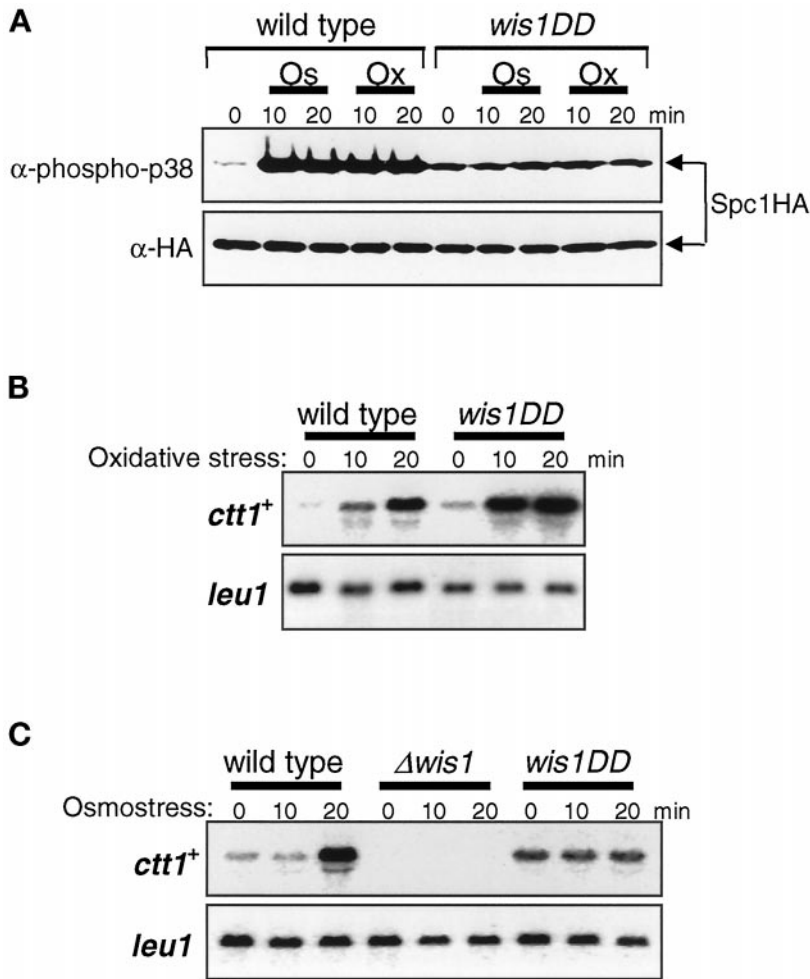
Figure 7. Oxidative stress but not osmostress can induce *ctt1*[+] expression independently of Spc1 activation. (A) Wild-type (KS2096) and *wis1DD* (KS2088) strains carrying the *spc1:HA6H* allele were grown to midlog phase at 30°C in YES medium and treated with either high-osmolarity stress induced by 0.6 M KCl (Os) or oxidative stress induced by 0.3 mM H_2O_2 (Ox). Aliquots of cells were harvested at the indicated times, and Spc1 was purified by Ni-NTA chromatography, followed by immunoblotting with anti-phospho-p38 and anti-HA antibodies. The level of active Spc1 does not change in *wis1DD* cells even after osmostress and oxidative stress. (B and C) Wild-type (PR109), *wis1DD* (KS2081), and Δ*wis1* (JM544) cells were grown to midlog phase in YES medium and treated with oxidative stress induced by 0.3 mM H_2O_2 (B) and high-osmolarity stress induced by 0.6 M KCl (C). Aliquots of cells were harvested at the indicated times for Northern hybridization analysis with the *ctt1*[+] and *leu1*[+] probes. In *wis1DD* cells, *ctt1*[+] expression was increased significantly in response to oxidative stress but not osmostress.

Thus, Spc1 activity is necessary for *ctt1*[+] expression, but oxidative stress can induce *ctt1*[+] expression independently of stress-induced activation of Spc1. In contrast, induction of *ctt1*[+] upon osmostress is dependent on an increase in Spc1 activity.

Both the Atf1 and Pap1 Transcription Factors Regulate ctt1[+] Expression in Response to Oxidative Stress

Two transcription factors, Atf1 and Pap1, are implicated in expression of the *ctt1*[+] gene (Wilkinson *et al.*, 1996; Degols and Russell, 1997; Toone *et al.*, 1998). Toone *et al.* (1998) recently proposed that *ctt1*[+] expression in response to high osmolarity is induced by the Spc1-Atf1 pathway, but oxidative stress–induced expression of *ctt1*[+] is wholly dependent on the Spc1-Pap1 pathway. In contrast to this model, we found that Atf1 is also important in induced expression of *ctt1*[+] after oxidative stress. Expression of *ctt1*[+] in Δ*atf1*, Δ*pap1*, and Δ*atf1* Δ*pap1* mutants was examined by Northern hybridization (Figure 8A). Compared with wild-type cells, expression of *ctt1*[+] upon H_2O_2 treatments was dramatically reduced in both Δ*atf1* and Δ*pap1* strains, and *ctt1*[+] mRNA was undetectable in Δ*atf1* Δ*pap1* double mutant cells. These data clearly indicate that both Atf1 and Pap1 are required for the full expression of *ctt1*[+] upon oxidative stress. In addition, the defective phenotypes of Δ*atf1* and Δ*pap1* in *ctt1*[+] expression appear to be additive, suggesting that Atf1 and Pap1 can independently regulate transcription of *ctt1*[+].

Consistent with these observations, we found that Δ*atf1* mutant cells are more sensitive to H_2O_2 than wild-type cells (Figure 8B), although it was previously reported that Δ*atf1* cells did not show increased sensitivity to oxidative stress (Toone *et al.*, 1998). Only a slight difference was observed between wild-type cells and Δ*atf1* cells when treated with 1 mM H_2O_2; however, only 1% of Δ*atf1* cells survived exposure to 2 mM H_2O_2, compared with 60% survival of the same treatment with the wild-type strain. In addition, the Δ*atf1* Δ*pap1* double mutant showed more severe sensitivity to H_2O_2 than the Δ*atf1* single mutant (Figure 8B), indicating that both Atf1 and Pap1 are involved in the cellular response to oxidative stress. With a higher concentration of H_2O_2, the Δ*pap1* single mutant also showed increased sensitivity; when exposed to 4 mM H_2O_2, the viability of Δ*pap1* and Δ*atf1* cells decreased to 1 and 0.01% of that of wild-type cells, respectively.
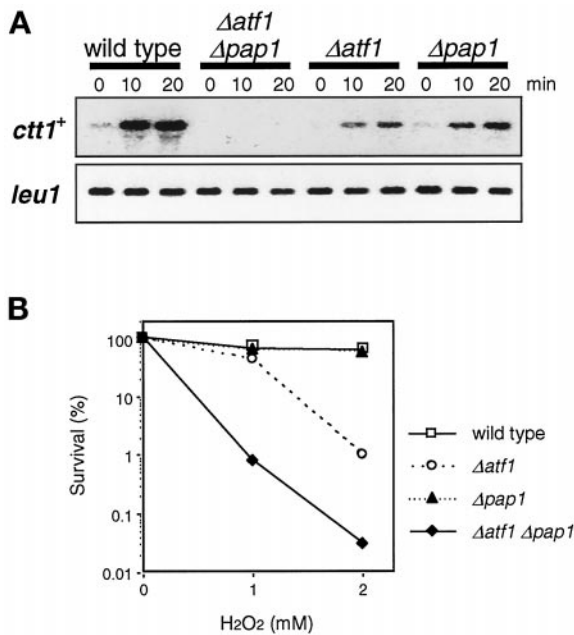
Figure 8. Two transcription factors, Atf1 and Pap1, regulate expression of $ctt1^+$ in response to oxidative stress. (A) Wild-type (PR109), $\Delta atf1$ (KS1497), $\Delta pap1$ (TP108-3c), and $\Delta atf1$ $\Delta pap1$ (CA334) strains were grown to midlog phase in YES medium at 30°C and treated with 0.3 mM H_2O_2. Aliquots of cells were harvested at the indicated times for Northern hybridization analysis with the $ctt1^+$ and $leu1^+$ probes. Compared with wild-type cells, $ctt1^+$ expression after oxidative stress was significantly impaired in $\Delta atf1$ and $\Delta pap1$ mutants and was not detectable in $\Delta atf1$ $\Delta pap1$ double mutants. (B) The strains used in A were grown to midlog phase in YES medium at 30°C, and H_2O_2 was added at concentrations of 0, 1, and 2 mM. After 1 h of incubation at 30°C, cells were washed, diluted, and plated on YES agar medium. The survival of each strain was evaluated in terms of its colony-forming ability after 3 d of incubation at 30°C. Results from an average of three independent experiments are shown. The $\Delta atf1$ mutant showed higher sensitivity to H_2O_2 than the wild-type cells, and this sensitivity was further accentuated in the $\Delta pap1$ background.

Spc1-independent Induction of $ctt1^+$ upon Oxidative Stress Is Mediated by Pap1

Having established that both Atf1 and Pap1 are involved in the expression of $ctt1^+$ upon oxidative stress, we next examined which factor, Atf1 or Pap1, is responsible for oxidative stress–induced $ctt1^+$ expression in the presence of constitutive Spc1 activity (Figure 7). Even in the absence of stress, activation of Spc1 by Wis1 overexpression induces the expression of various stress-response genes that are regulated by the Spc1 pathway (Degols et al., 1996). We found that $ctt1^+$ expression was also induced by Wis1 overexpression in wild-type and $\Delta pap1$ cells but not in $\Delta atf1$ cells (our unpublished results), which indicates that activated Spc1 induces $ctt1^+$ expression through Atf1 rather than Pap1. Therefore, we tested whether Pap1 is involved in the Spc1-independent induction of $ctt1^+$ in wis1DD cells upon oxidative stress. As shown in Figure 9A, the $\Delta pap1$ mutation largely impaired the induction of $ctt1^+$ after oxidative stress in wis1DD cells, and the level of $ctt1^+$ mRNA in the wis1DD
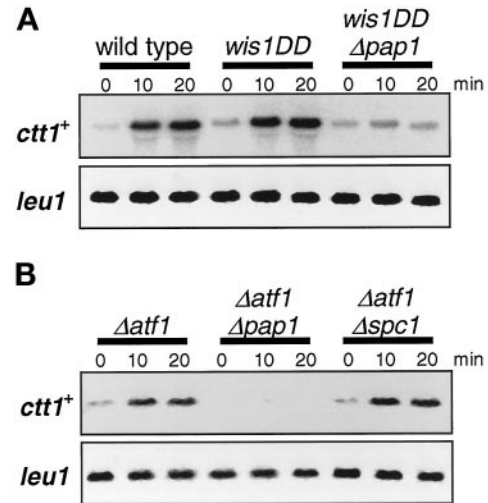

Figure 9. Pap1 can regulate $ctt1^+$ expression upon oxidative stress independently of the Spc1 pathway. (A) Wild-type (PR109), wis1DD (KS2081), and wis1DD $\Delta pap1$ (CA356) strains were grown to midlog phase in YES medium at 30°C and treated with 0.3 mM H_2O_2. Aliquots of cells were harvested at the indicated times for Northern hybridization analysis with the $ctt1^+$ and $leu1^+$ probes. In wis1DD cells, which have constitutive Spc1 activity, $ctt1^+$ induction upon oxidative stress was dependent on Pap1. (B) Northern analysis similar to that described for A was performed with $\Delta atf1$ (KS1497), $\Delta atf1$ $\Delta pap1$ (CA334), and $\Delta atf1$ $\Delta spc1$ (KS1533). In the $\Delta atf1$ background, the induction of $ctt1^+$ upon oxidative stress was dependent on Pap1 but not Spc1.

$\Delta pap1$ strain showed little change before and after H_2O_2 treatments. Thus, Pap1 has an essential role in inducing $ctt1^+$ expression upon oxidative stress when cells have constitutive Spc1 activity. In other words, in the $\Delta pap1$ background, induction of $ctt1^+$ expression is mediated by oxidative stress–induced activation of Spc1, presumably through Atf1.

A simple model to explain the data described above is that two independent inputs regulate $ctt1^+$ expression in response to oxidative stress. One input is the Spc1-Atf1 pathway in which stress-activated Spc1 induces $ctt1^+$ transcription through Atf1. The other input is mediated by the Pap1 transcription factor that can induce $ctt1^+$ expression upon oxidative stress independently of Spc1 activation. A prediction from this model is that in the $\Delta atf1$ mutant, induction of $ctt1^+$ by oxidative stress is dependent on Pap1 but not Spc1. As expected, the experiments shown in Figure 9B demonstrated that after oxidative stress, the pattern of $ctt1^+$ expression in $\Delta atf1$ $\Delta spc1$ double mutant cells was indistinguishable from that in $\Delta atf1$ cells, whereas $ctt1^+$ mRNA was not detectable in $\Delta atf1$ $\Delta pap1$ cells. Collectively, these results strongly suggest that Pap1 can regulate $ctt1^+$ transcription in response to oxidative stress independently of the Spc1 MAPK.

DISCUSSION

Reactive oxygen species (ROS) generated as by-products of aerobic metabolism or by environmental chemicals cause

damage to DNA, proteins, and cellular structures. Yeast has been used as a model system to study cellular oxidative stress responses in eukaryotes, and a set of genes required for detoxification of ROS has been identified (Moradas-Ferreira *et al.*, 1996). However, little is known about the mechanism whereby eukaryotic cells detect ROS and modulate expression of the antioxidant defense genes. The data presented in this paper strongly suggest that, in fission yeast, proteins homologous to members of two-component systems are involved in oxidative stress signaling to a MAPK cascade that regulates transcription of stress-response genes.

Mpr1 is most homologous to the budding yeast Ypd1 protein, which transfers a phosphoryl group from the receiver domain of Sln1 to the Ssk1 response regulator in osmostress signaling to the Hog1 MAPK cascade. Phosphorylation of Ypd1-His64 is an intermediate in the Sln1-Ypd1-Ssk1 phosphorelay, and substitution of His-64 with glutamine abolishes the Ypd1 function (Posas *et al.*, 1996). We found that the sequence encompassing His-64 of Ypd1 is completely conserved in Mpr1 and that the mutant Mpr1 protein with glutamine substitution of the putative phosphorylation site, His-221, is not functional. Furthermore, physical interaction between Mpr1 and the Mcs4 response regulator was observed. These data suggest that, like Ypd1, Mpr1 also functions as a response regulator phosphotransferase.

Considering the highly conserved architecture of the Mpr1-Mcs4 pathway and the budding yeast Ypd1-Ssk1 pathway, it is surprising that Mpr1 and Mcs4 are involved in transmitting oxidative stress rather than osmostress signals to a SAPK cascade. Under low-osmolarity conditions, phosphotransfer from Ypd1 to the Ssk1 response regulator inhibits Ssk1 from activating the Ssk2 MEKK in budding yeast, and the *ypd1* null mutation causes hyperactivation of Hog1 (Posas *et al.*, 1996). Δ*mpr1* cells have a higher basal level of Spc1 activity, which is suppressed in the Δ*mcs4* Δ*mpr1* double mutant, implying that, like Ypd1, Mpr1 also suppresses the Mcs4 response regulator from activating the Spc1 MAPK cascade under normal growth conditions. However, when cells are exposed to oxidative stress, the Mpr1 function is required for induction of Spc1 activation; Spc1 activation in response to oxidative stress is significantly impaired in *mpr1* mutants. Thus, under oxidative stress conditions, Mpr1 may positively regulate Mcs4 to activate the downstream MEKKs. Importantly, significant activation of Spc1 was observed upon oxidative stress even in the Δ*mcs4* and Δ*mcs4* Δ*mpr1* mutants, indicating that oxidative stress stimuli can be transmitted to the Spc1 cascade independently of the Mpr1-Mcs4 pathway (Figure 10). Therefore, it is also possible that the Mpr1 function may be required for coordinated action of the two pathways transmitting oxidative stress signals to the MEKKs. In *mpr1* mutants, deregulated Mcs4 might hamper the other pathway in activation of the MEKKs.

It is very likely that Mpr1-Mcs4 forms part of a multistep phosphorelay similar to the Sln1-Ypd1-Ssk1 in budding yeast. The *S. pombe* genome-sequencing project has identified two ORFs (SPAC27E2.09 and SPCC74.06) encoding "sensor kinases" similar to Sln1. Both of these proteins have C-terminal receiver domains as well as histidine kinase domains and, therefore, can form His-Asp-His-Asp phosphorelays with Mpr1 and Mcs4. Interestingly, the sensor kinase
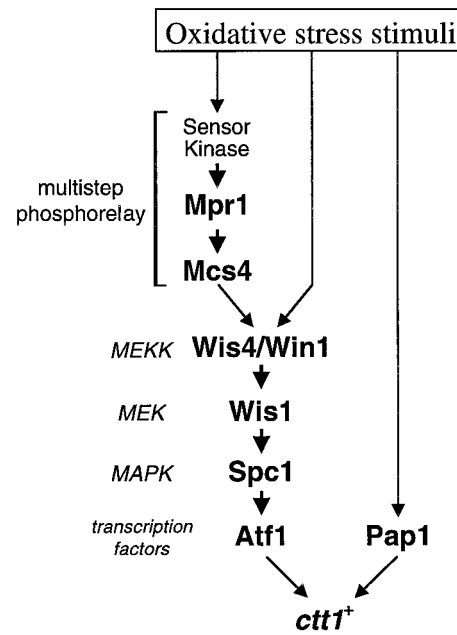

Figure 10. Oxidative stress signaling pathways that lead to transcription of the *ctt1*$^+$ gene. A multistep phosphorelay composed of an unknown sensor kinase, Mpr1, and the Mcs4 response regulator transmits oxidative stress signals to the Spc1 MAPK cascade. Oxidative stress induces partial activation of Spc1 even in the Δ*mcs4* Δ*mpr1* strain, suggesting that oxidative stress can be transmitted to the Spc1 cascade by additional pathways. Activated Spc1 induces transcription of *ctt1*$^+$ and other stress-response genes through the Atf1 transcription factor. The Pap1 transcription factor can also mediate *ctt1*$^+$ expression in response to oxidative stress independently of the Spc1 pathway.

encoded by SPCC74.06 is a transmembrane protein with a PAS domain that is common among the sensor proteins in bacterial two-component systems responsive to redox or related stimuli (reviewed by Zhulin *et al.*, 1997). Therefore, SPCC74.06 may be a good candidate as an oxidative stress sensor.

Interestingly, oxidative stress induces association between Mpr1 and the Mcs4 response regulator. We are not aware of any known example of stimuli-induced complex formation between phosphotransferase and response regulator proteins in two-component systems. The His-221→Gln mutation in Mpr1 abolishes interaction with Mcs4, implying that phosphorylation of Mpr1 modulates interaction with Mcs4. It is conceivable that signaling to the Spc1 MAPK cascade may involve not only phosphotransfer from Mpr1 to Mcs4 but also complex formation between these proteins, which is currently under investigation.

When Δ*mpr1* cells are exposed to oxidative stress, only a minor increase of Spc1 activity is detectable; however, the catalase gene *ctt1*$^+$ is strongly induced and Δ*mpr1* cells show H$_2$O$_2$ resistance comparable to that of wild-type cells. This is an unexpected result, because *ctt1*$^+$ expression is known to be under the regulation of the Spc1 pathway; *ctt1*$^+$ expression is undetectable in Δ*spc1* and Δ*wis1* cells, and these mutants are hypersensitive to oxidative stress (Degols *et al.*, 1996; Wilkinson *et al.*, 1996; Degols and Russell, 1997). We

found that oxidative stress, but not osmostress, can induce *ctt1⁺* expression in *wis1DD* cells, which have constitutive Spc1 activity, suggesting that *ctt1⁺* expression requires a certain level of Spc1 activity, but oxidative stress can induce *ctt1⁺* independently of an increase in Spc1 activity.

Spc1 regulates a number of stress-response genes through the Atf1 transcription factor (Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996), and induction of *ctt1⁺* upon osmostress (Wilkinson *et al.*, 1996) and UV irradiation (Degols and Russell, 1997) is impaired in Δ*atf1* mutants. Recently, Toone *et al.* (1998) reported that the Δ*pap1* mutant, but not the Δ*atf1* mutant, is defective in H_2O_2-induced expression of *ctt1⁺* and is sensitive to oxidative stress, phenotypes similar to those of Δ*spc1* mutants. These observations led to the model that Spc1 regulates Pap1 under oxidative stress but regulates Atf1 in response to other forms of stress (Toone *et al.*, 1998; reviewed by Wilkinson and Millar, 1998). However, this model raises perplexing questions. How does activated Spc1 choose either Atf1 or Pap1, depending on the stress that activates Spc1? Why is Spc1 unable to regulate Atf1 only when activated by oxidative stress? The data presented in this paper draw a rather different picture. First, oxidative stress–induced expression of *ctt1⁺* is partially impaired in both Δ*atf1* and Δ*pap1*, and *ctt1⁺* mRNA is undetectable in the Δ*atf1* Δ*pap1* double mutant, indicating that both Atf1 and Pap1 are involved in *ctt1⁺* expression upon oxidative stress. Consistently, Δ*atf1* cells lose viability rapidly in liquid medium with H_2O_2, although the H_2O_2 sensitivity of Δ*atf1* cells was not apparent when grown on agar medium containing H_2O_2 in the previous study (Toone *et al.*, 1998). Second, induced *ctt1⁺* expression upon oxidative stress is dependent on Pap1 in the *wis1DD* strain, which has constitutive Spc1 activity, suggesting that Pap1 can mediate *ctt1⁺* expression independently of stress-induced activation of Spc1. Third, in Δ*atf1* cells, *ctt1⁺* induction in response to oxidative stress is dependent on Pap1 but not Spc1. It was previously reported that in Δ*spc1* cells, unphosphorylated Atf1 represses *ctt1⁺* expression both before and after stress (Degols and Russell, 1997). However, in the absence of Atf1, induction of *ctt1⁺* by oxidative stress is detectable and appears to be mediated by Pap1 independently of Spc1. A simple model to explain these data is that *ctt1⁺* expression upon oxidative stress is regulated by two independent pathways, the Spc1-Atf1 pathway and Pap1 (Figure 10). Regardless of the form of stress, activated Spc1 phosphorylates Atf1 to induce *ctt1⁺* expression. Under oxidative stress conditions, Pap1 can also induce *ctt1⁺* expression independently of Spc1. Pap1 has a NES sequence sensitive to oxidative stress; the Pap1 NES contains cysteine residues essential for its function, and the thiol group of these residues might serve as a redox sensor (Kudo *et al.*, 1999). However, our data do not exclude the possibility that Pap1 is also subjected to auxiliary regulation by oxidative stress–activated Spc1. It should be noted that *ctt1⁺* is inducible by oxidative stress in the Δ*atf1* Δ*spc1* double mutant but not in the Δ*spc1* single mutant, suggesting that Pap1 cannot induce *ctt1⁺* under transcriptional repression by unphosphorylated Atf1.

In summary, the data presented here suggest that fission yeast has at least three pathways sensing oxidative stress stimuli to induce the catalase gene *ctt1⁺* (Figure 10). In addition to the phosphorelay system with Mpr1-Mcs4, an Mcs4-independent pathway transmits oxidative stress signals to the Spc1 MAPK cascade, which regulates *ctt1⁺* through Atf1. Oxidative stress can also induce *ctt1⁺* through the Pap1 transcription factor independently of the Spc1 pathway. Studies in budding yeast also suggest that cellular defense against oxidative stress is complex and may involve multiple signaling pathways (Moradas-Ferreira *et al.*, 1996). It is possible that different sensory mechanisms with partly overlapping specificities contribute to the detection of various ROS and prooxidants to minimize damage to the cell.

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