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Two-Component Mediated Peroxide Sensing and Signal Transduction in Fission Yeast

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Abstract

Two-component related proteins play a major role in regulating the oxidative stress response in the fission yeast, *Schizosaccharomyces pombe*. For example, the peroxide-sensing Mak2 and Mak3 histidine kinases regulate H_2O_2-induced activation of the Sty1 stress-activated protein kinase pathway, and the Skn7-related response regulator transcription factor, Prr1, is essential for activation of the core oxidative stress response genes. Here, we investigate the mechanism by which the *S. pombe* two-component system senses H_2O_2, and the potential role of two-component signaling in the regulation of Prr1. Significantly, we demonstrate that PAS and GAF domains present in the Mak2 histidine kinase are essential for redox-sensing and activation of Sty1. In addition, we find that Prr1 is required for the transcriptional response to a wide range of H_2O_2 concentrations and, furthermore, that two-component regulation of Prr1 is specifically required for the response of cells to high levels of H_2O_2. Significantly, this provides the first demonstration that the conserved two-component phosphorylation site on Skn7-related proteins influences resistance to oxidative stress and oxidative stress-induced gene expression. Collectively, these data provide new insights into the two-component mediated sensing and signaling mechanisms underlying the response of *S. pombe* to oxidative stress. *Antioxid. Redox Signal.* 15, 153–165.

Introduction

Reactive oxygen species (ROS) are produced as a by-product of aerobic metabolism, and in excess lead to oxidative stress by damaging DNA, proteins, and lipids (22). Consequently, all organisms have evolved defense mechanisms to protect against oxidative damage. However, ROS are also beneficial to organisms. For example, there is increasing evidence of the important role played by lower levels of ROS as signal transduction effector molecules (57), whereas higher levels of ROS are utilized to kill invading pathogens by the immune system (7). Hence, it is important that cells can distinguish the levels of ROS present allowing the appropriate responses to be initiated.

Studies in the model eukaryote *Schizosaccharomyces pombe* have provided significant insight into the cellular responses to ROS (26). Many of the signaling pathways that regulate transcriptional responses to H_2O_2 in *S. pombe* are conserved in higher eukaryotes and include the mammalian p38/JNK-related Sty1 (also known as Phh1 and Spc1) stress-activated protein kinase (SAPK) pathway (34, 48), which phosphorylates and regulates the bZip transcription factor Atf1 (49, 53), and the AP-1-like transcription factor Pap1 (55, 56), which is related to mammalian Jun. *S. pombe* also contains a multi-step two-component related system (1, 8, 38, 44), comprised of three histidine kinases, Mak1 (Phk3), Mak2 (Phk1), and Mak3 (Phk2), a single phosphorelay protein Mpr1 (Spy1), and the response regulators Mcs4 and Prr1. Mak2, Mak3, Mpr1, and Mcs4 constitute a phosphorelay system that transduces H_2O_2 signals to the Sty1 SAPK (8, 38), whereas the response regulator transcription factor Prr1 directly regulates oxidative stress response genes independently of Sty1 (39, 40). Significantly, studies on these oxidative stress signaling pathways have revealed that distinct pathways govern the transcriptional responses to peroxide stress depending on the level of H_2O_2. For example, the Sty1 SAPK is activated by H_2O_2 in a dose-dependent manner (8, 42). At low to intermediate levels (0.07–1 m*M*) of H_2O_2 stress the Mak2 and Mak3 histidine kinases play a major role in the relay of stress signals to Sty1 (34). However, at high levels (6 m*M*) of H_2O_2 a

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significant level of Sty1 activation occurs independently of Mak2 and Mak3 (42). In addition, the transcriptional response to low/intermediate levels of H_2O_2 stress is dependent upon Pap1, whereas the Sty1-regulated Atf1 is more important at higher levels of H_2O_2 (11, 42). Consistent with these distinct roles, increasing amounts of H_2O_2 trigger oxidation of Sty1, which is important for increasing levels of *atf1*$^+$ mRNA (15), and cause an increase in Sty1-dependent phosphorylation of Atf1 (42), which stabilizes Atf1 by preventing interaction with the ubiquitylation E3 ligase Fbh1 (30, 31). Whereas, oxidation of Pap1, which activates the protein, occurs more rapidly at low levels of H_2O_2 (42, 60). Furthermore, the molecular switch from Pap1- to Sty1/Atf1-regulated gene expression involves the H_2O_2 concentration-dependent oxidation of the 2-Cys peroxiredoxin Tpx1 (6, 59). Thus, the regulation of several different signal transduction pathways determines the pattern of oxidative stress-induced gene expression at specific levels of H_2O_2 in *S. pombe*.

Whilst the two-component related phosphorelay system is central to responses to H_2O_2 in *S. pombe*, there remain significant gaps in our knowledge regarding the sensing mechanisms and the precise role of phosphorelay signaling in fission yeast. This system is highly related to two-component signaling pathways prevalent in prokaryotes. However, similar to *Saccharomyces cerevisiae* (41), a more complex phosphorelay is present in *S. pombe* (44), which comprises of three hybrid histidine kinases: two highly related proteins Mak2 and Mak3, and a third kinase Mak1 (1, 8); the phosphorelay protein Mpr1, and two response regulators Mcs4 (38) and Prr1. Based on studies of the Sln1 phosphorelay system in *S. cerevisiae* (41), stimulation of the sensor kinase is predicted to result in autophosphorylation of a conserved histidine residue, which is subsequently transferred to an aspartic acid residue in the receiver domain of the histidine kinase. This phosphate is then transferred to a histidine residue in the phosphorelay protein which completes transfer to an aspartic acid residue in the receiver domain of the response regulator. Indeed, consistent with this, mutation of either the conserved aspartic acid phosphorylation site in the receiver domain of Mcs4 (Asp412) or of the conserved histidine phosphorylation site on Mpr1 (His221), or deletion of the *mak2*$^+$ or *mak3*$^+$ (but not *mak1*$^+$) histidine kinase-encoding genes, significantly impairs the relay of H_2O_2 signals to the Sty1 SAPK (8, 38, 42). Recently, it has been demonstrated that the glycolytic enzyme, GAPDH, interacts with the two MAPKKKs in the Sty1 pathway and the Mcs4 response regulator and, moreover, is involved in promoting phosphorelay in response to peroxide (37). However, despite the identification of this phosphorelay pathway the mechanism underlying H_2O_2 sensing and regulation of Mak2 and Mak3 activity has not been established.

The second response regulator in *S. pombe*, the Prr1 transcription factor, is also a major regulator of the oxidative stress response. Prr1 is a member of the Skn7-like two-component transcription factors, found in evolutionarily divergent fungi, and contains a receiver domain characteristic of response regulator proteins (39). Recent transcript-profiling studies have revealed H_2O_2-concentration dependent differences in the requirement for Prr1 (11). In particular, induction of the expression of core oxidative stress genes at low levels of H_2O_2 is largely independent of Prr1, whereas Prr1 is almost as critical as Pap1 in the response to intermediate levels of H_2O_2 (11). However, the role of Prr1 at higher levels of H_2O_2 where the Sty1/Atf1 pathway is more important has not been investigated. Significantly, although Skn7-like transcription factors appear to have a common role in the oxidative stress response in fungi, little is known about how they are regulated by oxidative stress. For example, whilst a recent study reported oxidative stress-induced serine/threonine phosphorylation of Skn7, the precise role of this stress-induced modification in the oxidative stress response remains unclear (23). In addition, previous studies have reported that two-component-mediated aspartic acid phosphorylation of *S. cerevisiae* Skn7 (36) and *S. pombe* Prr1 (40) is not important for resistance to peroxide stress.

In this article, we further characterize the role and regulation of two-component signal transduction in the response to oxidative stress in *S. pombe*. In particular, we identify domains of Mak2 important for H_2O_2 sensing and signaling. We also demonstrate that the two-component response regulator, Prr1, is essential for the transcriptional response to a range of H_2O_2 concentrations. Importantly, we present evidence that two-component signaling to Prr1 is specifically required for the response to high levels of H_2O_2. These data provide the first evidence that two-component signal transduction is important for the regulation of a fungal Skn7-family protein in response to oxidative stress and has significant implications for understanding the response of fungi to different levels of stress.

Materials and Methods

Growth media and yeast strains

The *S. pombe* and *Candida albicans* strains used in this study are listed in Table 1. *S. pombe* strains were grown in YE5S rich medium or EMM2 synthetic minimal medium (35) and *C. albicans* strains were grown in YPD rich medium (46). All strains were grown at 30°C.

Sensitivity tests

Ten-fold dilutions of mid-log-phase-growing cultures were spotted onto YE5S media containing the indicated concentrations of stress agent. Plates were incubated at 30°C for 2 or 3 days.

Gene tagging and strain construction

All the oligonucleotide primers used in this study are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertonline.com/ars). To tag Mak1 and Mak2 with 13myc epitopes at the C-terminus, the oligonucleotides mak1/2-13myc:kanF and mak1/2-13myc:kanR and the plasmid template pFA6a-13myc-kanMX6 (2) were used to generate a *mak1*- or a *mak2-13myc-kanMX6* cassette by PCR. These cassettes were introduced into CHP429 to create strains JM1915 and JM1918, respectively. The functionality of the tagged Mak2 construct was checked by crossing JM1918 with JM1521 expressing *sty1-6HisHA* to generate strain JP52. To tag Mak3, Mpr1, and Mcs4, with 3Pk epitopes at the C-terminus, the 3′ region of these genes was amplified using the oligonucleotide pairs Mak3PKF/Mak3PKR, MprPKF/MprPKR, and Mcs4PKF/Mcs4PKR, respectively. Subsequent PCR products were digested with *Pst*I and *Bam*HI and ligated with pRIP42Pk C (52) digested with the same enzymes. pRIP42Pk

Table 1. Strains Used in this Study

S. pombe	*Genotype*	*Source*
CHP429	h^-	Gift from C. Hoffman
CHP428	h^+	Gift from C. Hoffman
JM1915	h^- *mak1-13myc:kan*^{R}	This study
JM1918	h^- *mak2-13myc:kan*^{R}	This study
JP254	h^- *mak2gaf:ura4*$^+$*:pas-13myc:kan*^{R}	This study
JP277	h^- *mak2Δpas-13myc:kan*^{R}	This study
JP279	h^- *mak2Δgafpas-13myc:kan*^{R}	This study
JP331	h^- *mak2Δgaf-13myc:kan*^{R}	This study
JM1521	h^+ *sty1(HA6His):ura4*$^+$	(47)
JP52	h^+ *sty1(HA6His):ura4*$^+$ *mak2-13myc:kan*^{R}	This study
JM1829	h^+ *sty1(HA6His):ura4*$^+$ *mak2::LEU2*	This study
JP281	h^+ *sty1(HA6His):ura4*$^+$ *mak2Δpas-13myc:kan*^{R}	This study
JP283	h^- *sty1(HA6His):ura4*$^+$ *mak2Δgafpas-13myc:kan*^{R}	This study
JP333	h^- *sty1(HA6His):ura4*$^+$ *mak2Δgaf-13myc:kan*^{R}	This study
JP43	h^- *sty1(HA6His):ura4*$^+$ *mak2::LEU2 mak3::kan*^{R}	This study
JP162	h^+ *sty1(HA6His):ura4*$^+$ *nmt41(3HA)-mak2:kan*^{R} *nmt41(3HA)-mak3:kan*^{R}	This study
VB2091	h^+ *sty1(HA6His):ura4*$^+$ *nmt41(3HA)-mak2(ΔN):kan*^{R} *nmt41(3HA)-mak3(ΔN):kan*^{R}	This study
JP358	h^- *mak2Δgafpas+CHK1gaf −13myc:kan*^{R}	This study
JP359	h^- *mak2Δgaf+CHK1gaf-13myc:kan*^{R}	This study
JP361	h^- *mak2Δgafpas+CHK1gaf −13myc:kan*^{R} *sty1(HA6His):ura4*$^+$	This study
JP363	h^+ *mak2Δgaf+CHK1gaf-13myc:kan*^{R} *sty1(HA6His):ura4*$^+$	This study
JP329	h^- *mak3Pk:ura4*$^+$	This study
JP289	h^- *mpr1Pk:ura4*$^+$	This study
JP287	h^- *mcs4Pk:ura4*$^+$	This study
SW97	h^+ *prr1::ura4*$^+$	(19)
PM2	h^+ *prr1*^{D418N}	This study
PM3	h^+ *prr1*^{D418E}	This study
	all strains are *leu1-32 ura4-D18 ade6-M216 (or ade6-M210) his7-366*	
C. albicans		
CAI4	*Δura3::imm434/Δura3::imm434*	(17)
CHK21	*Δura3::imm434/Δura3::imm434 Δchk1::hisG/Δchk1::hisG*	(9)

C is an integrating vector that allows the construction and then expression of proteins from their own genomic locus that are epitope-tagged at the C-terminus with 3 x Pk epitopes. The resulting pRIP42Pk C-Mak3, pRIP42Pk C-Mpr1, and pRIP42Pk C-Mcs4 plasmids were linearized by digestion with either *Kpn*I, *Sal*I, or *Xho*I present in the respective open reading frames, and introduced into CHP429 at the wild-type loci to generate strains JP329, JP289, and JP287, respectively. Chromosomal insertion of these constructs was confirmed by PCR and DNA sequencing. In addition, the functionality of the tagged constructs was confirmed as all displayed wild-type levels of peroxide-induced Sty1 activation.

To delete the sequences encoding the putative Ser/Thr kinase domain in Mak2 and Mak3, the oligonucleotides truncNmak2/3F and truncNmak2/3R were used in combination with the plasmid template pFA6a-kanMX6-P41nmt-3HA (2) to generate nmt41-3HA-truncated N-terminal *mak2* and *mak3* cassettes by PCR. These cassettes were introduced into JM1521 or JM1827 (47), respectively, and correct integration confirmed by PCR. The strains were crossed and a strain containing both mutant *mak2* and *mak3* alleles and *sty1-6HisHA* (VB2091) was obtained by sporulation and tetrad dissection. The same strategy was used to obtain a control strain in which the wild-type alleles of *mak2* and *mak3* were under the control of the *nmt41* promoter (JP162).

To delete the coding sequences for one or both of the GAF and PAS domains in Mak2, initially the *ura4*$^+$ gene was inserted into the *mak2*$^+$ gene between the GAF and PAS domain-encoding sequences. This was achieved by generating a PCR-derived cassette, comprised of the *ura4*$^+$gene flanked by 100 bp corresponding to the 3′ region of the GAF domain-encoding sequence and 100 bp corresponding to the 5′ region of the PAS domain-encoding sequence, using the oligonucleotides Mak2UraF and Mak2UraR and the pREP42HA plasmid template (12). The cassette was introduced into JM1918 cells to create strain JP254. Deletion cassettes for the PAS domain, the GAF domain, or both the PAS and GAF domains were created by PCR using the oligonucleotide pairs PASdelF/HK-R, GAFdelF/HK-R, or GAFPASdelF/HK-R, respectively, and genomic DNA as template. These deletion cassettes were introduced together with plasmid pREP41 (*LEU2*) into JP254 cells. Leu$^+$ transformants were replica-plated onto EMM media containing 5-FOA to select for uracil auxotrophs (5). Successful strain construction of JP277 (mak2ΔPAS), JP331 (mak2ΔGAF) and JP279 (mak2Δ GAFPAS) was confirmed by PCR and DNA sequencing. Subsequent crosses with JM1521 generated JP281, JP333, and JP283, containing both mutant *mak2* and *sty1-6HisHA* alleles.

To replace the GAF domain, or both the GAF and PAS domains, within the Mak2 sequence with the *C. albicans* Chk1 GAF domain two different cassettes were created by PCR using the oligonucleotide pairs CaGAF1/CaGAF2 or CaGAF1/CaGAF3, respectively, and *C. albicans* genomic DNA as template. These cassettes were introduced together with

plasmid pREP41 (*LEU2*) into JP254 cells and correct strain construction of JP358 (mak2ΔGAFPAS+Chk1GAF) and JP359 (mak2ΔGAF+Chk1GAF) confirmed as before. Subsequent crosses with JM1521 generated JP361 and JP363 containing both mutant *mak2* and *sty1-6HisHA* alleles.

The wild-type $prr1^{+}$ genomic locus was replaced with either the $prr1^{D418N}$ or $prr1^{D418E}$ mutant allele to create strains PM2 and PM3, respectively. Mutagenesis of $prr1^{+}$ to create $prr1^{D418N}$ or $prr1^{D418E}$ was performed by overlapping PCR using the oligonucleotides Prr1-HindF and Prr1-ClaR, and either DMUTE or DMUTN, and pBluescript-*prr1* as a template. The PCR products were digested with *Hin*dIII and *Cla*I and introduced into pBluescript-*prr1* to generate pBluescript-$prr1^{D418N}$ and pBluescript-$prr1^{D418E}$. A *Stu*I/*Pst*I fragment from these plasmids carrying either the $prr1^{D418N}$ or the $prr1^{D418E}$ allele was introduced, together with pREP81 (*LEU2*), into SW97 ($prr1::ura4^{+}$). Leu^{+} transformants were replica-plated onto EMM media containing 5-FOA to select for uracil auxotrophs (5). Successful gene replacement was confirmed by PCR and DNA sequencing.

Plasmids

The full-length *prr1* open reading frame was amplified by PCR from genomic DNA obtained from either CHP429, PM2 ($prr1^{D418N}$), or PM3 ($prr1^{D418E}$) using the oligonucleotide pair Prr1SalF/Prr1SalR. The PCR products were digested with *Sal*I and introduced into pREP41HM N (12) to create pREP41 HM-Prr1, pREP41HM-$Prr1^{DN}$, and pREP41HM-$Prr1^{DE}$, respectively.

SAPK phosphorylation assays

S. pombe strains expressing *sty1-6HisHA* from the normal chromosomal locus were grown to mid-log phase and incubated with 1 m*M* H_2O_2 for the indicated times. Detection of phosphorylated Sty1 was determined as described previously (47) by Western blotting with anti-phospho-p38 antibody (New England Biolabs, Ipswich, MA). Total levels of Sty1 were determined with an anti-HA antibody (Sigma, St. Louis, MO). *C. albicans* strains were grown to mid-log phase and incubated with either 2.5 or 5 m*M* H_2O_2 for the indicated times, and phosphorylated Hog1 was detected as described previously (51).

Fluorescence microscopy

Immunolocalization of tagged proteins was performed as described previously (42), using either an anti-myc 9E10 antibody (Sigma) or anti Pk-TAG antibody (Serotec, Kidlington, UK). DAPI and FITC fluorescence were captured by exciting cells with 365 nm and 450–490 nm wavelengths, respectively, using a Zeiss Axioscope microscope, with a 63x oil immersion objective and Axiovision imaging system.

RNA analysis

RNA was prepared and Northern blot analyses performed as described previously (4). Gene-specific probes were amplified by PCR from genomic DNA. Probed membranes were analyzed using a phosphorimager (Fuji Bas-1500) and the data quantified using Tina 2.0 software (Raytest, Sheffield, UK).

Supplementary methods

Methods specifically pertaining to the supplementary data can be found online at www.liebertonline.com/ars

Results

The PAS and GAF domains of Mak2 are required for H_2O_2-induced Sty1 activation

Although we previously reported that the Mak2 and Mak3 histidine kinases are involved in the sensing and relay of H_2O_2 stress signals to the Sty1 SAPK in *S. pombe* (8, 42), the peroxide-sensing domains were not elucidated. Sequence analysis of Mak2 and Mak3 revealed several structural motifs not present in the *S. cerevisiae* Sln1 osmo-sensing histidine kinase (7; Fig. 1). In addition to the histidine kinase and receiver domains, potential PAS and GAF domains are located adjacent to the histidine kinase domain. PAS domains and GAF domains, which are structurally similar but unrelated in amino acid sequence to PAS domains (25), are evolutionarily conserved protein motifs that are implicated in signal transduction (54). Notably, the *C. albicans* Chk1 histidine kinase has similar domain architecture to Mak2 and Mak3, with the exception that Chk1 lacks a detectable PAS domain adjacent to the histidine kinase domain (Fig. 1). In addition, a region with significant homology to Ser/Thr kinases is located towards the N-terminus of Mak2 and Mak3 (between amino acids 118 and 296 in Mak2 and 122 and 291 in Mak3) and Chk1. This homology is restricted to domains VIa-XI found in Ser/Thr-kinases which are largely responsible for binding peptide substrates and initiating phosphotransfer. Although domains I–V involved in ATP binding are missing in Mak2 and Mak3, a P-loop motif, which could potentially be involved in ATP/GTP binding, is present in both kinases near the N-terminus. To investigate the role of these Ser/Thr kinase domains in the oxidative stress signaling function of Mak2 and Mak3, a strain expressing truncated versions of Mak2 and Mak3 lacking the Ser/Thr kinase domain (*Pnmt-ΔNmak2/3*) was constructed (Fig. 2A). However, Sty1 phosphorylation was not impaired in cells expressing such truncated versions of Mak2 and Mak3 in response to H_2O_2, in contrast to cells lacking Mak2 and Mak3, indicating that the Ser/Thr kinase domain is not essential for the H_2O_2 signaling function of these histidine kinases (Fig. 2B).

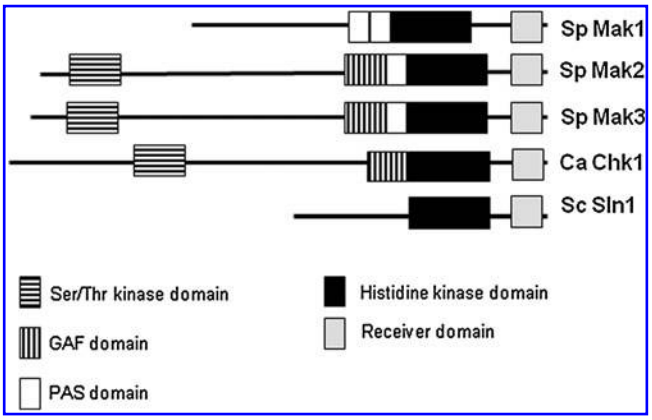

FIG. 1. The domain structures of Mak1, Mak2 and Mak3 (*S. pombe*), Sln1 (*S. cerevisiae*), and Chk1 (*C. albicans*) histidine kinases.

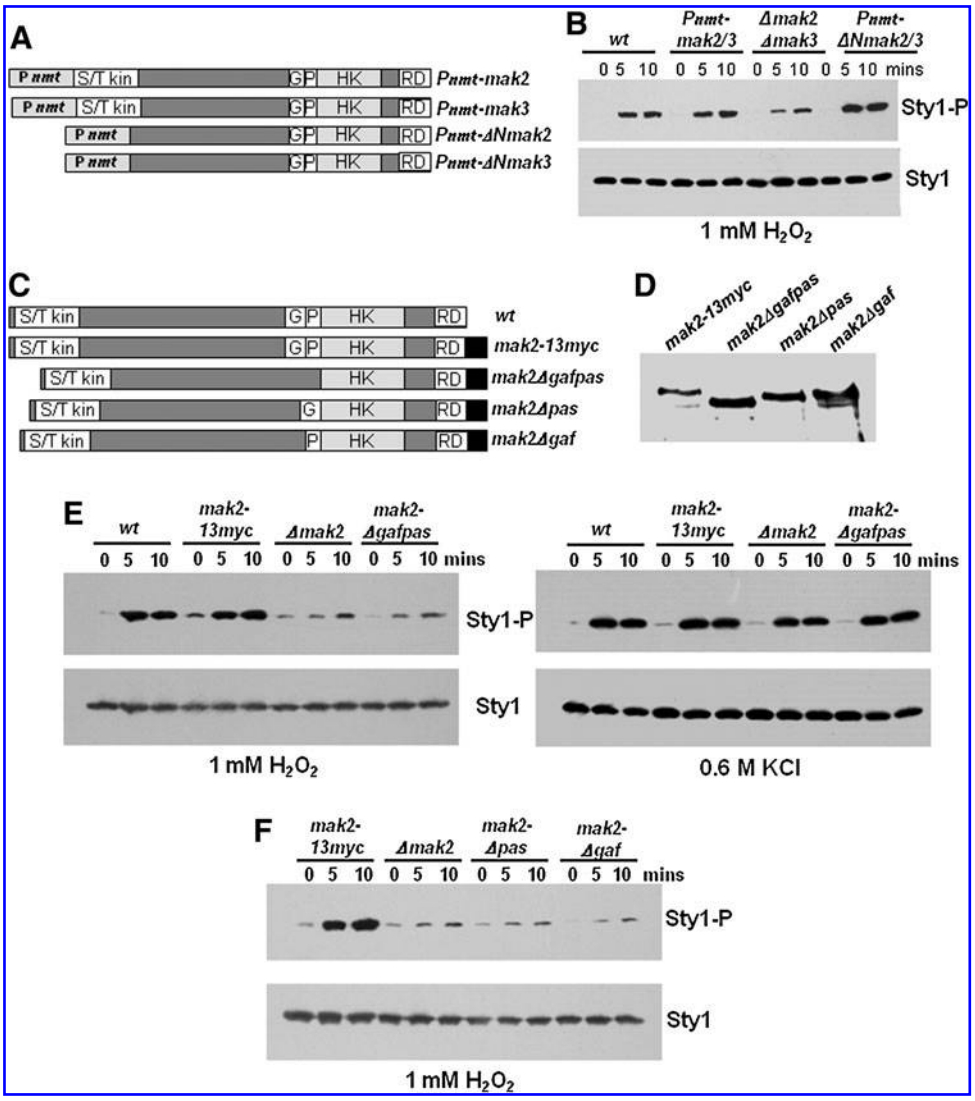

FIG. 2. The GAF and PAS domains of Mak2 are required for H_2O_2-induced Sty1 activation, whereas the putative Ser/Thr kinase domains of Mak2 and Mak3 are dispensable. (A) Schematic illustration of the Mak2 and Mak3 constructs used to investigate the role of the Ser/Thr kinase domain in H_2O_2 signaling: G, GAF domain; HK, histidine kinase domain; P, PAS domain; RD, receiver domain; S/T kin, serine/threonine kinase domain. Full length *mak2*$^+$ and *mak3*$^+$ (*Pnmt-mak2*, *Pnmt-mak3*) or constructs lacking the N-terminal Ser/Thr kinase (S/T kin) domain sequences (*Pnmt-ΔNmak2*, *Pnmt-ΔNmak3*) were expressed from the *nmt41*-promoter (*Pnmt*). **(B)** Western blot analysis of Ni^{2+}-NTA agarose-purified Sty1-His_6-HA from wild-type cells (JM1521, *wt*), Δ*mak2*Δ*mak3* cells (JP43), or from cells expressing either full-length Mak2 and Mak3 (JP162, *Pnmt-mak2/3*), or N-terminal truncations of Mak2 and Mak3 (VB2091; *Pnmt-ΔNmak2/3*) from the *nmt41* promoter. Samples were taken following treatment with 1 m*M* H_2O_2 for the indicated times. **(C)** Schematic illustration of the Mak2 constructs used to investigate the role of the PAS and GAF domains in H_2O_2 signaling: the same abbreviations were used as described in **(A).** The *mak2*$^+$ gene was tagged with a sequence encoding 13myc epitopes (*black box*), and mutant versions of *mak2*$^+$ with deletions of either or both of the PAS and GAF domains were created. **(D)** Western blot analysis of the expression levels of wild-type and mutant Mak2-13myc proteins. Equal amounts of extract isolated from cells expressing Mak2-13myc (JM1918), or Mak2-13myc with deletion of both the GAF and PAS domains (JP279 - *mak2Δgafpas*) or the single GAF (JP331 - *mak2Δgaf*) or PAS (JP277 - *mak2Δpas*) domain, were separated by SDS-PAGE and the levels of the various Mak2 constructs determined by Western blotting using an anti-myc antibody. **(E)** Western blot analysis of Ni^{2+}-NTA agarose-purified Sty1-His_6-HA isolated from either wild-type cells (*wt* - JM1521), Δ*mak2* cells (JM1829), or cells expressing *mak2-13myc* (JP52) or a derivative of *mak2-13myc* in which the PAS and GAF domains had been deleted (JP283; *mak2Δgafpas*). Samples were taken after treatment with either 1 m*M* H_2O_2 or 0.6 *M* KCl for the indicated times. **(F)** Western blot analysis of Ni^{2+}-NTA agarose-purified Sty1-His_6-HA isolated from either Δ*mak2* cells (JM1829), or cells expressing *mak2-13myc* (JP52) or *mak2-13myc* in which either the GAF (JP333; *mak2Δgaf)* or the PAS (JP281; *mak2Δpas*) domain had been deleted, treated with 1 m*M* H_2O_2 for the indicated times. Phosphorylated Sty1 (Sty1-P) was detected with an anti-phospho-p38 antibody and total levels of Sty1 protein (Sty1) were determined with an anti-HA antibody.

To investigate the potential role of the PAS and GAF domains in H_2O_2 signaling within the Mak2 histidine kinase, strains were constructed in which *mak2*$^+$ was replaced with mutant alleles expressing Mak2 derivatives lacking either the GAF (amino acids 1450–1602), PAS (amino acids 1627–1740), or both domains (Fig. 2C). These mutant versions of Mak2 were expressed at the normal chromosomal locus and tagged with sequences encoding 13myc epitopes. Importantly, deletion of the PAS and GAF domains did not decrease the stability of Mak2 (Fig. 2D). Furthermore, wild-type levels of H_2O_2-induced Sty1 phosphorylation were observed in cells expressing *mak2-13myc*, confirming the functionality of the epitope-tagged histidine kinase (Fig. 2E). However, similar to that observed in Δ*mak2* cells, deletion of either one, or both, of the PAS and GAF domains, significantly impaired H_2O_2-induced activation of Sty1 (Figs. 2E and 2F). In contrast, activation of Sty1 in response to osmotic stress was not affected in cells expressing Mak2 lacking the GAF and PAS domains

(Fig. 2E). These results are consistent with the specific role of the Mak2 histidine kinase in H_2O_2 sensing and signaling to Sty1 and indicate that both the PAS and GAF domains are essential for this function.

The GAF domain from C. albicans Chk1 can functionally replace the GAF domain of Mak2

Although the Chk1 histidine kinase in *C. albicans* is a close homologue of Mak2 and Mak3 in *S. pombe* (8, 9), deletion of *CHK1* does not prevent H_2O_2-induced activation of the *C. albicans* Hog1 SAPK (26; Fig. 3A). Interestingly, Chk1 contains a GAF domain adjacent to the histidine kinase domain but lacks the PAS domain found in Mak2 and Mak3 (Fig. 1). As the GAF domain of Mak2 is required for H_2O_2 signal transduction (Fig. 2F), we investigated whether the *C. albicans* GAF domain could function in the Mak2 kinase. To test this, two chimeras were constructed in which either the GAF domain or both the PAS and GAF domains of Mak2 were replaced with the GAF domain from Chk1 (Fig. 3B). As illustrated in Figure 3, the Chk1 GAF domain can functionally replace the GAF domain of Mak2, but not both the PAS and GAF domains, to regulate H_2O_2-induced activation of Sty1 (Fig. 3C). This result illustrates that, whilst the GAF domains from the *S. pombe* Mak2 and *C. albicans* Chk1 proteins are functionally conserved, both GAF and PAS domains are required for the H_2O_2 signaling function of Mak2.

Cellular localization of two-component related proteins in S. pombe

To further characterize two-component signal transduction in *S. pombe*, the cellular location of all the known two-component proteins (Mak1, Mak2, Mak3, Mpr1, Mcs4, and Prr1; Fig. 4) was determined by indirect immunofluorescence. To facilitate this analysis, either myc- or Pk epitope-tagged versions of these proteins were expressed from their native loci, or in the case of myc-tagged Prr1, expressed from the thiamine repressible *nmt41* promoter (see below). The phenotypes of cells expressing the epitope-tagged constructs were analyzed, and in each case were indistinguishable from cells expressing the untagged wild-type protein (our unpublished data). The three histidine kinases, Mak1, Mak2, and Mak3, were found to be cytoplasmic and visibly excluded from the nucleus (Fig. 4). Moreover, Mak2 and Mak3 appear in "dots" in the cytoplasm, suggesting concentration at specific places. The Mcs4 response regulator, which interacts with the cytoplasmic Wak1 MAPKKK and regulates activation of the Sty1 SAPK (8), showed a similar staining pattern to that of the histidine kinases (Fig. 4). Attempts to analyze the localization of the second potential response regulator, the Prr1 transcription factor, expressed from its normal locus failed. Hence, myc epitope-tagged Prr1 was expressed from the *nmt41* promoter (3, 12), and found to be predominantly nuclear (Fig. 4). In contrast to Mak1, Mak2, Mak3, Mcs4, and Prr1, the single phosphorelay protein Mpr1 was located in both the cytoplasmic and the nuclear compartments of the cell (Fig. 4), suggesting that Mpr1 may function to relay signals to the nuclear response regulator Prr1, in addition to Mcs4 (see below). Treatment of cells with H_2O_2 did not alter the cellular localization pattern of any of the two-component proteins (Supplementary Fig. S1). Consistent with this, the nuclear and cytoplasmic localization pattern of a mutant version of Mpr1, in which the phosphohistidine (H221) was mutated to glutamine, was the same as wild-type Mpr1 (Supplementary Fig. S2). Furthermore, the nuclear and cytoplasmic localization of Mpr1 was maintained in cells lacking the Mak1, Mak2, or Mak3 histidine kinases (Supplementary Fig. S2).

D418 of Prr1 is important for the response to peroxide in S. pombe

Although the Prr1 response regulator is important for the oxidative stress response in *S. pombe*, a previous study sug-

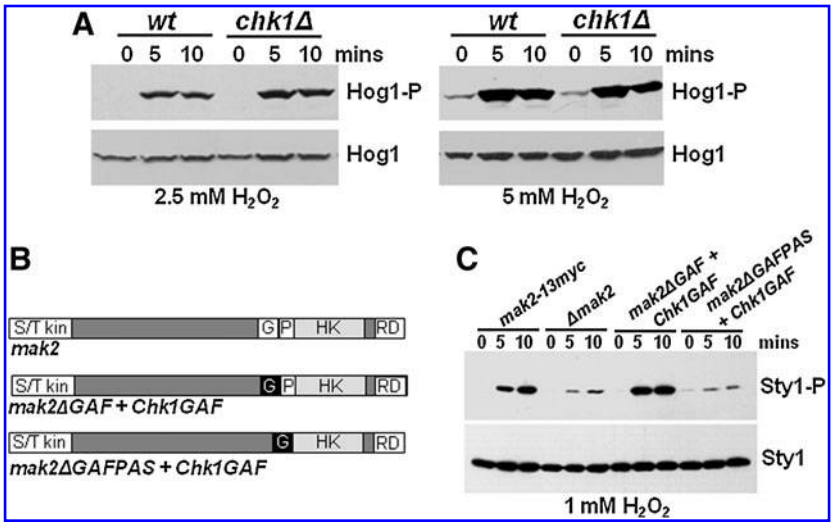

FIG. 3. The *C. albicans* Chk1 GAF domain can functionally replace the GAF domain of Mak2. (A) Western blot analysis of H_2O_2-induced phosphorylation of the *C. albicans* Hog1 SAPK in wild-type cells (CAI4 – *wt*) and in cells lacking *CHK1* (CHK21 – *chk1*Δ). Samples were isolated following treatment with 2.5 or 5 m*M* H_2O_2 for the indicated times. **(B)** Schematic illustration of the Mak2 constructs (domain abbreviations are as described in Fig. 2 with the exception that the Chk1 GAF domain is designated as a *black box*). Chimeras in which the *C. albicans* Chk1 GAF domain replaced the Mak2 GAF domain (JP359; *mak2*Δ*gaf*+*Chk1GAF*) or the Mak2 GAF and PAS domains (JP358; *mak2*Δ*gafpas*+*Chk1 GAF*) were created. **(C)** Western blot analysis of Ni^{2+}-NTA agarose-purified Sty1-His_6-HA isolated from JM1829 (Δ*mak2*), JP52 (*mak2-13myc*), JP363 (*mak2*Δ*gaf*+*Chk1GAF*; Chk1 GAF domain replaces the Mak2 GAF domain) and JP361 (*mak2*Δ*gafpas*+*Chk1GAF*; Chk1 GAF domain replaces the Mak2 GAF and PAS domains) cells treated with 1 m*M* H_2O_2 for the indicated times. Phosphorylated Hog1 (Hog1-P) and phosphorylated Sty1 (Sty1-P) were detected with an anti-phospho-p38 antibody and total levels of Sty1 (Sty1) or Hog1 (Hog1) protein were determined with either an anti-HA or an anti-Hog1 antibody, respectively.

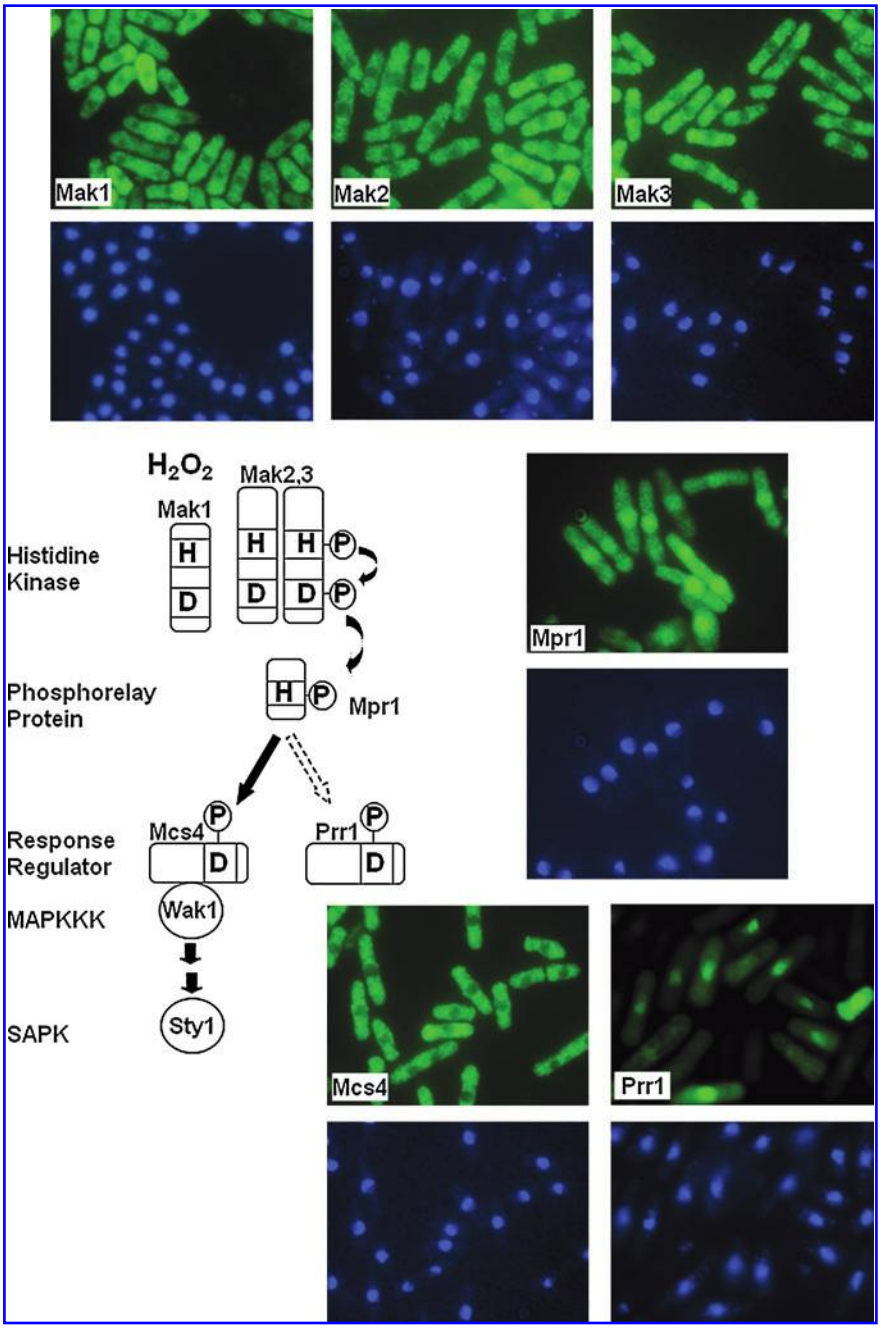

FIG. 4. Histidine kinases and response regulators, but not the phosphorelay protein, are located in distinct cellular compartments in *S. pombe*. Cellular localization of *S. pombe* two-component proteins. The localization of all the two-component proteins identified in *S. pombe* was examined by immunofluorescence microscopy in cells expressing epitope-tagged versions of the proteins. Strains used were JM1915 (*mak1-13myc*), JM1918 (*mak2-13myc*), JP329 (*mak3-Pk*), JP289 (*mpr1-Pk*), and JP287 (*mcs4-Pk*). Nuclei were observed by DAPI staining of the cells (*bottom panels*). Prr1 could not be detected in cells expressing epitope-tagged Prr1 from the normal chromosomal locus, hence wild-type cells containing pREP41HM-Prr1 were examined. *Inset* is a schematic illustration of two-component signal transduction in *S. pombe*. Based on studies in *S. cerevisiae* (41) phosphate is predicted to be relayed from the histidine kinases to the Mcs4 response regulator via the Mpr1 phosphorelay protein. However, the regulation of the Prr1 response regulator by two-component signaling is unclear (*hatched arrow*).

gested that two-component-mediated phosphorylation of Prr1 is not important for function (39). However, the nuclear localization of both Prr1 and the Mpr1 phosphorelay protein (Fig. 4) prompted us to further investigate the potential role of two-component-mediated regulation of Prr1. To facilitate this study, the conserved aspartic acid phosphorylation site (D418) in the receiver domain of Prr1 (39) was substituted with either asparagine (Prr1D418N), which is predicted to prevent phosphorylation (13, 21, 33), or glutamic acid (Prr1D418E), which is predicted to mimic constitutive phosphorylation (27–29). Both mutations were incorporated into the $prr1^{+}$ gene at the normal genomic locus. We examined the sensitivity of wild-type (CHP428), Δ*prr1* (SW97), $prr1^{D418N}$ (PM2), and $prr1^{D418E}$ (PM3) cells to H_2O_2 and the heavy metal cadmium (Fig. 5). In agreement with previous studies, the Δ*prr1* mutant was found to be more sensitive than the wild-type control strain to both of these agents (39). However, whilst mutation of D418 to asparagine had no detectable affect on the function of Prr1 in response to cadmium, this mutation of Prr1 did affect the response of cells to H_2O_2 (Fig. 5). For example, although $prr1^{D418N}$ cells were not as sensitive as Δ*prr1* cells to H_2O_2, they are more sensitive to higher levels of H_2O_2 than wild-type cells (Fig. 5). However, mutation of D418 of Prr1 to glutamic acid resulted in cells that displayed similar levels of resistance to H_2O_2 as wild-type cells (Fig. 5). Importantly, such results were replicated in response to the alkyl hydroperoxide, *t*-BOOH, another oxidative stress generator (Fig. 5). Hence, these data suggested that D418 of Prr1

has a role in the cellular response to peroxides such as H_2O_2 and *t*-BOOH but not to cadmium toxicity. Significantly, mutation of D418 of Prr1 does not appear to significantly affect the stability of this transcription factor or the phosphorylation of other sites (Supplementary Fig. S3).

Prr1 is required for the induction of gene expression at high levels of H_2O_2

Recent transcript profiling experiments revealed that gene induction in response to low levels of H_2O_2 (0.07 m*M*) was largely Pap1-dependent and Prr1-independent, whereas at intermediate levels of H_2O_2 (0.5 m*M*) both Prr1 and Pap1 were critical for the induction of the core oxidative stress response genes (11). This significantly extends earlier work which demonstrated that Prr1 regulates *ctt1*$^+$ and *trr1*$^+$ expression in response to 0.2 m*M* H_2O_2 (39). However, neither study examined the role of Prr1 in activating gene expression in response to high levels (6 m*M*) of H_2O_2. Hence, the induction of *ctt1*$^+$ and *trr1*$^+$ expression at intermediate (0.25 m*M*) and high (6 m*M*) levels of H_2O_2 was examined. Both *ctt1*$^+$ and *trr1*$^+$ expression were induced in wild-type cells when exposed to both intermediate and high levels of H_2O_2, and this was largely dependent on Prr1 (Fig. 6). These results are consistent with the transcript profiling data at intermediate levels of H_2O_2 (11) but also extend these data by demonstrating that Prr1 is important for the regulation of gene expression at high levels of H_2O_2.

D418 of Prr1 plays a role in gene induction specifically at high levels of H_2O_2

Due to the specific effect of mutation of D418 of Prr1 on the resistance of cells to high but not intermediate levels of peroxide, we examined the potential role of D418 of Prr1 in stress-induced gene expression at intermediate and high levels of H_2O_2. Strikingly, consistent with the sensitivity data, the *prr1*^{D418N} and *prr1*^{D418E} mutations have opposing effects on *ctt1*$^+$and *trr1*$^+$ expression at high (6 m*M*) levels of H_2O_2 (Fig. 6). In particular, at high levels of H_2O_2 the fold induction of *ctt1*$^+$and *trr1*$^+$ gene expression was increased to a greater extent in *prr1*^{D418E} cells compared with *prr1*$^+$ control cells while reduced induction of *ctt1*$^+$and *trr1*$^+$ gene expression was observed in *prr1*^{D418N} cells compared with *prr1*$^+$control cells (Fig. 6). In contrast, very similar induction of *ctt1*$^+$and *trr1*$^+$ gene expression occurred in *prr1*$^+$ and *prr1*^{D418} mutant cells at intermediate (0.25 m*M*) levels of H_2O_2 (Fig. 6). This analysis suggests that the relative sensitivity of *prr1*^{D418N} mutant cells at high levels of peroxide is associated with the effects of this specific mutation on *ctt1*$^+$ and *trr1*$^+$expression. The effects of mutation of D418 of Prr1 on gene expression at high levels of H_2O_2 are specific for certain genes as the expression of the Sty1/Atf1-regulated *gpx1*$^+$ gene, encoding glutathione peroxidase, is induced to similar levels in *prr1*$^+$, Δ*prr1*, *prr1*^{D418E}, and *prr1*^{D418N} cells (Fig. 6). Significantly, whilst homologues of Prr1 such as Skn7 in *S. cerevisiae* and *C. albicans* also have roles in the oxidative stress response (36, 50), this is the first demonstration that mutation of the conserved phosphorylation site influences resistance to oxidative stress and oxidative stress-induced gene expression.

Discussion

We have investigated two-component signaling proteins in the regulation of the response of *S. pombe* cells to peroxide stress. Our data have identified the potential peroxide sensing domains in the Mak2 histidine kinase that are essential for the activation of the Sty1 SAPK pathway by H_2O_2. Furthermore, we find that the Prr1 response regulator is required for H_2O_2-induced gene expression at both intermediate and high levels of H_2O_2 and, significantly, that the conserved aspartic acid residue (D418) in the receiver domain plays a role in the response to high levels of peroxide stress. Taken together, these data suggest that two-component signal transduction plays a

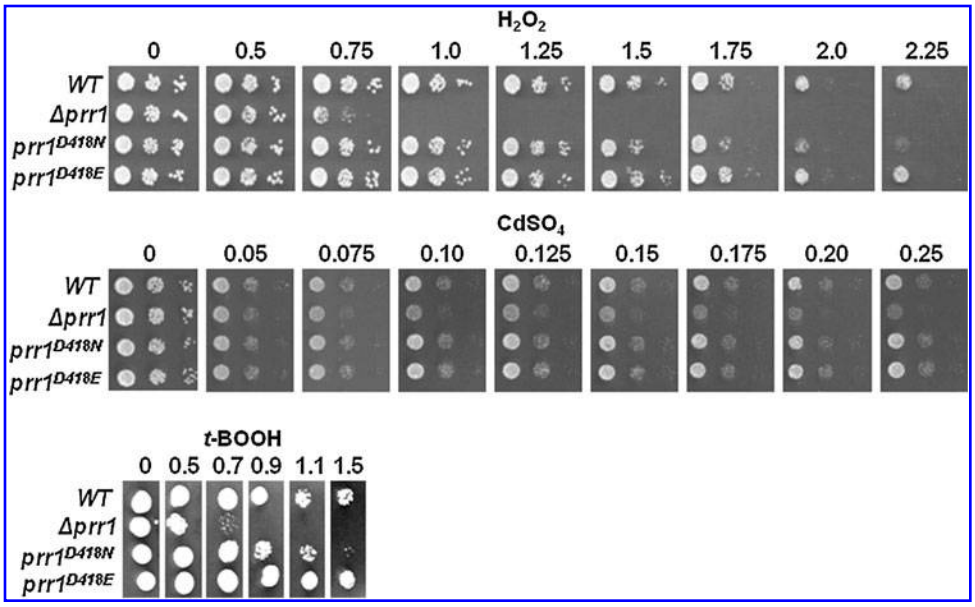

FIG. 5. D418 of Prr1 is important for the cellular response to peroxides but not cadmium. *prr1*$^+$ (*WT*-CHP428) and mutant *prr1* cells, containing either a disruption of the *prr1*$^+$ gene (Δ*prr1*-SW97) or a substitution of the conserved aspartic acid residue (D418) in the receiver domain with either an asparagine residue (*prr1*^{D418N}-PM2) or a glutamic acid residue (*prr1*^{D418E}-PM3), were grown to mid-log phase and 10-fold dilutions of $\sim 10^3$ cells spotted onto plates containing the indicated concentrations (m*M*) of H_2O_2 and $CdSO_4$. In addition, 10^3 cells were spotted onto plates containing the indicated concentrations (m*M*) of *t*-BOOH. The H_2O_2 and *t*-BOOH plates were incubated at 30°C for 2 days and the $CdSO_4$ plates for 3 days.

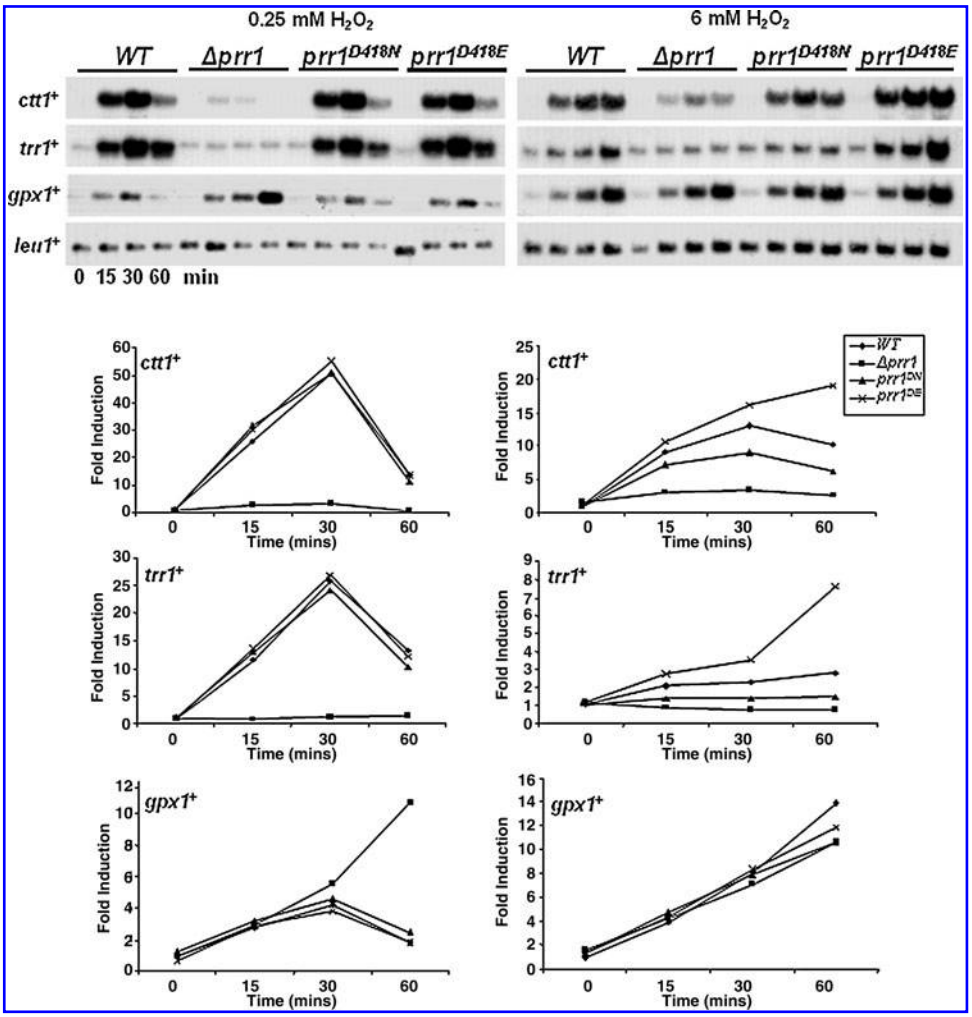

FIG. 6. Prr1 is important for gene expression at intermediate and high levels of H_2O_2 but D418 is only important at high levels. Northern blot analysis of RNA isolated from mid-log-phase growing cultures of *prr1*$^+$ (*WT*-CHP428), Δ*prr1* (SW97), *prr1*^{D418N} (PM2), and *prr1*^{D418E} (PM3) cells treated with 0.25 m*M* or 6 m*M* H_2O_2 for 0, 15, 30, and 60 min. Transcripts were detected using gene-specific probes. Fold induction of the indicated genes is calculated relative to time 0 of the *prr1*$^+$ (*WT*-CHP428) strain using the *leu1*$^+$ transcript as a loading control.

central role in the regulation of two distinct pathways in response to peroxide in *S. pombe*.

Our previous work demonstrated that the Mak2 and Mak3 histidine kinases are important for the regulation of the Sty1 SAPK in response to H_2O_2 (8). Although many histidine kinases are transmembrane proteins to allow sensing of external stimuli, we demonstrate that all three histidine kinases in *S. pombe*, Mak1, Mak2, and Mak3, are cytoplasmic (Fig. 4) which is consistent with the apparent absence of transmembrane spanning domains (8). In contrast, expression of the *S. cerevisiae* transmembrane Sln1 histidine kinase in *S. pombe* results in membrane staining (Supplementary Fig. S1). H_2O_2 is readily diffusible and thus the cytoplasmic localization of these histidine kinases may have evolved to allow the sensing of both external and internal signals. Our analysis also revealed that the PAS and GAF domains located near the histidine kinase domain of Mak2, but not a putative Ser/Thr kinase domain located towards the N-terminus, are essential for the relay of H_2O_2 signals to the Sty1 pathway. PAS domains are evolutionarily conserved domains that sense a diverse range of stimuli including redox potential, oxygen, light and voltage (for reviews, see Refs. 18, 20, 54). These domains have a characteristic three-dimensional fold which in some cases forms a binding site for co-factors, including FAD, heme, or other chromophores. GAF domains are similar to PAS domains in structure, but not primary sequence (24). Although many GAF domains bind cyclic nucleotides, recent studies have identified redox-sensing GAF domains which bind other co-factors. For example, a GAF domain in the hypoxia/NO-inducible *Mycobacterium tuberculosis* DosS histidine kinase binds heme (45), whereas the GAF domain in the *E. coli* NO sensor NorR has a mononuclear non-heme iron center that senses NO by formation of a mono-nitrosyl iron complex (14). However, we can not detect heme binding to the Mak2 PAS domain (our unpublished data). Nonetheless, PAS domains have also been implicated in protein–protein interactions (for examples, see 16, 61). Hence, we hypothesized that the PAS domains may function to mediate protein–protein interactions between Mak2 and Mak3, as both of these histidine kinases are required to relay H_2O_2 stress signals to Sty1. However, whilst Mak2 and Mak3 do form complexes consistent with heterodimerization, these interactions also occur in the absence of the PAS domains (Supplementary Fig. S4). Hence, the precise role of the PAS and GAF domains of Mak2 in H_2O_2 signaling is not yet known. Nonetheless, we do know that both the PAS and GAF domains are important for Mak2 function in response to H_2O_2. Consistent with this finding, the *C. albicans* Chk1 GAF domain can replace the Mak2 GAF domain, but not both the PAS and GAF domains, to restore activation of Sty1 by H_2O_2, and fungal histidine

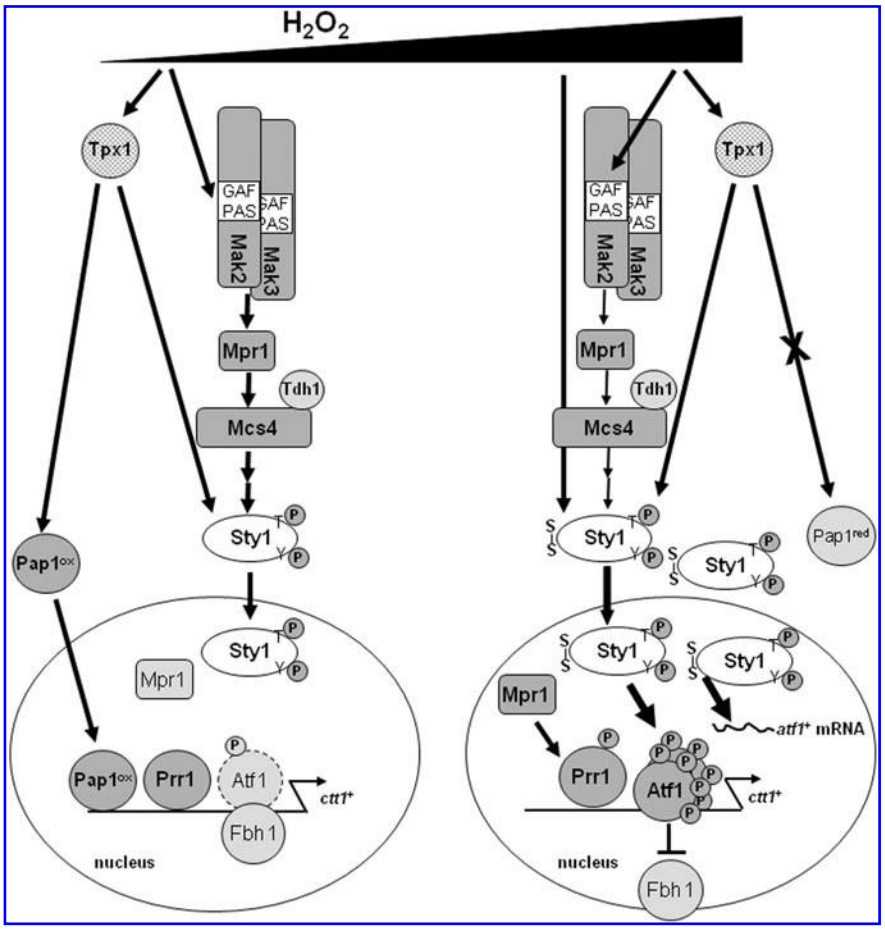

FIG. 7. Diagram summarizing the H_2O_2 concentration-dependent regulation of oxidative stress responses in *S. pombe*. Two-component signal transduction regulates the cellular response to different levels of H_2O_2 through two distinct pathways in *S. pombe*. Two-component signal transduction is more important in the regulation of the Sty1 SAPK at low and intermediate levels of H_2O_2 stress (42). Recently, the glycolytic enzyme GAPDH, encoded by *tdh1*$^+$, has been shown to be involved in promoting phosphorelay via Mcs4 in response to peroxide (37). In addition to the role of two-component signal transduction at lower levels of H_2O_2, the present study illustrated that two-component signaling is important for the regulation of Prr1 in response to higher levels of H_2O_2 stress. Prr1 also regulates gene expression at intermediate levels of peroxide stress, although under such conditions two-component regulation of Prr1 is dispensable for function. This contrasts to the previously defined H_2O_2 concentration-dependent roles and regulation of the Pap1 and Atf1 transcription factors. Pap1 regulates the transcriptional response to low levels of H_2O_2 as the oxidation (Pap1^{ox}) and nuclear accumulation of Pap1 depends upon the thioredoxin peroxidase activity of Tpx1, which is inhibited at high concentrations of H_2O_2 (6, 59). However, Tpx1 is still able to activate the Sty1 SAPK, at these higher levels of H_2O_2 that inhibit Pap1 (6, 58, 59), which subsequently phosphorylates the Atf1 transcription factor at multiple sites (31). Atf1 is a target for the ubiquitin–proteasome system and its degradation is dependent upon an SCF E3 ligase containing the F box protein Fbh1 (30). However, stress-induced phosphorylation of Atf1 stabilizes this transcription factor (31) by preventing its interaction with Fbh1 (30). Furthermore, in a distinct mechanism, oxidation of Sty1 by formation of a disulfide bond between Cys-153 and Cys-158 is important for maintaining *atf1*$^+$ mRNA stability at higher concentrations of H_2O_2 (15). Thus, the responses of *S. pombe* cells to peroxide are regulated by a diverse range of H_2O_2 dose-dependent regulatory mechanisms.

kinases which lack PAS and GAF domains (Fig. 1) are not peroxide sensors.

In this study, we also show that the Prr1 transcription factor regulates gene expression in response to intermediate (0.25 m*M*) and high (6 m*M*) levels of H_2O_2. These roles for Prr1 contrasts with those reported for Pap1 and Atf1 which are required for transcriptional responses to either low/intermediate or high levels of H_2O_2 stress, respectively (42). Furthermore, we demonstrate that two-component regulation influences the activity of the Prr1 transcription factor at high but not intermediate levels of H_2O_2. Prr1 contains a typical receiver domain found in response regulator proteins, including the predicted conserved phosphorylatable aspartic acid residue (D418) (39). However, previous work demonstrated that expression of a non-phosphorylatable (D418N) mutant version of Prr1 complements the sensitivity of Δ*prr1* cells to low levels of *t*-BOOH, suggesting that regulation of Prr1 is independent of two-component phosphorylation (40). In this study, however, we demonstrate that mutation of D418 of Prr1 to the non-phosphorylatable asparagine residue impacts on cell survival and gene expression specifically in response to high levels of peroxide stress. Although Prr1 is also required for tolerance to cadmium, mutating D418 had no detectable effect on the sensitivity of cells to this heavy metal, which is consistent with observations that the two-component system in *S. pombe* solely responds to peroxide (8). These data suggest that two-component regulation of Prr1 confers an additional layer of regulation, the effects of which are only manifested at high concentrations of peroxide stress. Currently, the molecular mechanisms underlying the role(s) of phosphorylation of Prr1 in the regulation of gene expression at different levels of H_2O_2 are unclear and are under investigation. Interestingly, mutation of the equivalent aspartic acid residue within the receiver domain of Skn7 in *S. cerevisiae* does not affect the function of Skn7 in the oxidative stress response (36). Instead, phospho-Skn7 is required for the expression of the mannosyltransferase encoding gene *OCH1* and other genes involved in cell wall integrity (32). Such results are consistent with the *S. cerevisiae* two-component signal transduction system monitoring changes in cell wall turgor pressures (43).

Our attempts to reconstruct the *S. pombe* two-component phosphorelay system *in vitro* have been unsuccessful. In our

hands, the recombinant histidine kinases are inactive, thus preventing investigation of phosphorelay to Mpr1 and the response regulators Mcs4 and Prr1. Furthermore, genetic approaches to directly relate Mpr1 to the activity of Prr1 or to identify which histidine kinase(s) may regulate Prr1 are complicated by the fact that these two-component proteins also regulate the Sty1 SAPK which is also implicated in the regulation of the Prr1-regulated genes tested (8, 10, 42). Nevertheless, taken together, our mutation analysis of the Mcs4 (8) and Prr1 (this study) response regulators suggest that two-component signal transduction regulates H_2O_2-induced gene expression through both the Mcs4-regulated Sty1/Atf1 pathway and the Prr1 transcription factor (Fig. 7). In addition, the results presented here, together with our previous work (42), demonstrate that two-component signal transduction also plays important roles in determining gene expression at different levels of H_2O_2, via the regulation of these two distinct pathways (Fig. 7). Specifically, two-component signaling is more important for Sty1 activation at low to intermediate levels of H_2O_2, whereas two-component regulation of Prr1 is more important at high levels of H_2O_2. This adds to our current knowledge of H_2O_2 concentration-dependent effects on the regulation of the Pap1 and Atf1 transcription factors (summarized in Fig. 7). Thus, *S. pombe* cells utilize a panoply of transcription factors and regulatory pathways to tailor distinct responses to different levels of H_2O_2, providing insights into the mechanisms used by cells to respond to different levels of an environmental stimulus.

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Authors Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

5-FOA = 5-fluoro-orotic acid
AP-1 = activator protein 1
ctt1$^+$ = *S. pombe* gene encoding catalase
DAPI = 4′,6-diamidino-2-phenylindole
FITC = fluorescein isothiocyanate
GAF = cGMP regulated cyclic nucleotide phosphodiesterase, Adenylyl cyclase, FhlA
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
MAPKKK = mitogen activated protein kinase kinase kinase
NO = nitric oxide
PAS = Per, Arnt, Sim
PCR = polymerase chain reaction
ROS = reactive oxygen species
SAPK = stress-activated protein kinase
t-BOOH = *tert*-butyl hydroperoxide
trr1$^+$ = *S. pombe* gene encoding thioredoxin reductase

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