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Activation and Regulation of the Spc1 Stress-Activated Protein Kinase in *Schizosaccharomyces pombe*

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Spc1, an osmotic-stress-stimulated mitogen-activated protein kinase (MAPK) homolog in the fission yeast *Schizosaccharomyces pombe*, is required for the induction of mitosis and survival in high-osmolarity conditions. Spc1, also known as Sty1, is activated by Wis1 MAPK kinase and inhibited by Pyp1 tyrosine phosphatase. Spc1 is most closely related to *Saccharomyces cerevisiae* Hog1 and mammalian p38 kinases. Whereas Hog1 is specifically responsive to osmotic stress, we report here that Spc1 is activated by multiple forms of stress, including high temperature and oxidative stress. In this regard Spc1 is more similar to mammalian p38. Activation of Spc1 is crucial for survival of various forms of stress. Spc1 regulates expression of genes encoding stress-related proteins such as glycerol-3-phosphate dehydrogenase (*gpd1*$^+$) and trehalose-6-phosphate synthase (*tps1*$^+$). Spc1 also promotes expression of *pyp2*$^+$, which encodes a tyrosine phosphatase postulated as a negative regulator of Spc1. This proposal is supported by the finding that Spc1 associates with Pyp2 in vivo and that the amount of Spc1 tyrosine phosphorylation is lower in a Pyp2-overproducing strain than in the wild type. Moreover, the level of stress-stimulated *gpd1*$^+$ expression is higher in Δ*pyp2* mutants than in the wild type. These findings demonstrate that Spc1 promotes expression of genes involved in stress survival and that Spc1-regulated transcription of one of these genes, *pyp2*$^+$, serves to attenuate Spc1 activity. This mechanism of regulation may be commonly employed to modulate MAPK signal transduction pathways in eukaryotic species.

Eukaryotic cells have highly effective mechanisms of adapting to environmental changes which cause physiological stress. In several cases it is clear that exposure to environmental insults, such as high temperature, high osmolarity, genotoxic agents, or hydroxyl radicals, leads to stimulation of kinases that phosphorylate transcription factors and thereby modulate gene expression (21). Spc1, a mitogen-activated protein kinase (MAPK) homolog in the fission yeast *Schizosaccharomyces pombe*, is an example of a stress-stimulated kinase (23, 36). Spc1, also known as Sty1 (23), is activated following exposure to high-osmolarity media or growth in suboptimal nutrient conditions. Like all MAPK homologs, Spc1 is activated by a MAPK kinase homolog, in this case Wis1 (40), which phosphorylates threonine 171 and tyrosine 173 of Spc1 (36). The activating tyrosine phosphorylation is convenient for analytical studies because it is easily monitored by immunoblotting with antiphosphotyrosine antibodies. The level of Spc1 tyrosine phosphorylation increases in response to osmotic stress, and this phosphorylation is dependent on Wis1 in vivo (23, 36).

The *spc1*$^+$ gene was first discovered in a genetic screen that identified mutations of five loci that rescued lethal phenotypes caused by loss of protein phosphatase 2C activity (37). Our interest in the stress response arose from the discovery that *spc1*$^-$ and *wis1*$^-$ (*spc2*$^-$) mutants grow to a large cell size before undergoing mitosis (23, 36, 40). This phenotype is greatly accentuated in cells grown in high-osmolarity media. In fact, the division of *spc1*$^-$ and *wis1*$^-$ mutants was arrested, and these mutants were highly elongated in high-osmolarity media. This observation suggests that the Wis1-Spc1 MAPK cascade is linked to the G_2/M cell cycle control mechanism. The core of G_2/M control consists of the Cdc2-Cdc13 M-phase-inducing kinase being inhibited by Wee1 tyrosine kinase and activated by Cdc25 tyrosine phosphatase (11, 22, 24, 28, 32, 33). Interaction of the osmosensing and cell control processes was underscored by the discovery of synthetic lethal interactions involving *spc1* and *cdc25* mutations (23, 36).

Discovery of the Wis1-Spc1 MAPK cascade led to an additional important new insight into the function and regulation of MAPK cascades. A protein tyrosine phosphatase, Pyp1, which was previously identified as a mitotic inhibitor (25, 27), was found to function by inactivating Spc1. Key observations supporting this conclusion included the following: Pyp1-overproducing cells underwent cell cycle arrest in high-osmolarity media, the level of Spc1 tyrosine phosphorylation was higher in Δ*pyp1* cells, Spc1 and Pyp1 coprecipitated from *S. pombe* lysates, and purified Pyp1 specifically dephosphorylated Tyr-173 of Spc1 in vitro (36). Moreover, Pyp1 overproduction abolished Spc1 tyrosine phosphorylation in vivo (23, 36). Pyp1 was unique among known MAPK phosphatases because it specifically dephosphorylated Tyr-173 of Spc1 (36), whereas previously identified MAPK phosphatases dephosphorylated both the threonine and tyrosine residues that were phosphorylated by MAPK kinases (10, 39).

A second fission yeast tyrosine phosphatase gene, *pyp2*$^+$, has been postulated to negatively regulate Spc1 (23, 36). There are several findings that are consistent with this proposal, including the demonstration that *spc1*$^-$ mutations rescue the lethality of Δ*pyp1* Δ*pyp2* double mutants (23, 36) and that Pyp2 overproduction results in a G_2/M cell cycle delay that is similar to that caused by Pyp1 overproduction (25, 27). This result indicates that Pyp1 and Pyp2 may share an essential function in dephosphorylating Spc1. Interestingly, expression of *pyp2*$^+$ mRNA is elevated by a Wis1-dependent mechanism in response to osmotic stress, suggesting that Spc1 might be involved in promoting expression of a gene whose protein product negatively

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regulates Spc1 (23). However, there is no data demonstrating an in vivo physical interaction between Pyp2 and Spc1, nor is it clear whether overproduction or inactivation of *pyp2*$^+$ affects Spc1 tyrosine phosphorylation in vivo (23, 36). Therefore, the suggestion that Pyp2 negatively regulates Spc1 is an unproved hypothesis.

A number of other important issues related to Spc1 function and regulation remain to be addressed. One concerns the specificity of stress signals that stimulate Spc1. Protein sequence comparisons have shown that Spc1 is most similar to Hog1 protein kinase of *Saccharomyces cerevisiae* (~80% identity), mammalian kinases variously known as p38, CSBP, and Mpk2 (~50 to 60% identity) and Jnk1 and stress-activated protein kinase (SAPK) (23, 36). Hog1 is reported to be stimulated only by osmotic stress (5, 35), whereas the mammalian homologs appear to be activated by various types of stress and inflammatory agents including high osmolarity, bacterial endotoxins, UV irradiation, heat stress, and cytokines, such as interleukin-1 and tumor necrosis factor (8, 12, 13, 18, 19, 31). Another issue concerns events that occur after stimulation of Spc1 kinase activity. Mammalian Jnk1 plays a central role in the response to extracellular stresses by phosphorylating and activating c-Jun and possibly ATF-2 transcription factors (7, 16). Budding yeast Hog1 kinase has also been indirectly implicated in the regulation of stress-related genes (2, 5, 35), but it remains to be established whether Spc1 has a role in regulating gene expression. This report focuses on three issues: the types of stress that lead to activation of Spc1, the processes that are regulated by Spc1, and whether Pyp2 directly regulates Spc1.

MATERIALS AND METHODS

Yeast strains, media, and general methods. *S. pombe* PR109 (*h*$^-$ *leu1-32 ura4-D18*), KS1147 (*h*$^+$ *leu1-32 ura4-D18 spc1-M13*), KS1366 (*h*$^-$ *leu1-32 ura4-D18 spc1*::*ura4*$^+$), JM418 (*h*$^+$ *leu1-32 ura4-D18 pyp2*::*ura4*$^+$), JM544 (*h*$^-$ *leu1-32 ura4-D18 wis1*::*ura4*$^+$), and KS1376 [*h*$^-$ *leu1-32 ura4-D18 spc1-Ha6H*(*ura4*$^+$)] have been described elsewhere (36). GD1498 [*h*$^+$ *leu1-32 ura4-D18 pyp2*::*ura4*$^+$ *spc1-Ha6H*(*ura4*$^+$)] was constructed during the course of these experiments. YES and synthetic EMM2 media were used for growth media. Fission yeast experimental methods and media have been described elsewhere (3, 26).

RNA isolation and hybridization. Northern (RNA) hybridization analyses were performed as described previously (34). A 2.3-kb *Bam*HI fragment from the pRep3-pyp2 plasmid was used to probe for *pyp2*$^+$ mRNA. A 1-kb *Eco*RV fragment from pJK148 was used for the control *leu1*$^+$ probe. A 750-bp fragment of the *tps1*$^+$ gene was amplified by PCR with the 5′ oligonucleotide TCACT CA CGG GTGAT GCTTC TAACT C and the 3′ oligonucleotide TCGAC ACCCA CAATC ACCTT AACGC C. A 1,139-bp fragment containing the *gpd1*$^+$ gene (29) was amplified by PCR with the 5′ oligonucleotide GGAAT TCCAT AT GTC TGGAT ATGGT CAACA AGGT and the 3′ oligonucleotide TAGTT TAGCG GCCGC CCTCG GTTTC AGTAC CGCCC TCG.

Detection of Spc1. Strain KS1376 contains a chromosomal copy of *spc1*$^+$-*Ha6H* (36). This gene encodes Spc1 protein having a C-terminal tag containing two copies of the influenza virus hemagglutinin (HA) epitope followed by six consecutive histidine residues. Spc1 protein was purified by Ni^{2+}-nitrilotriacetic acid (NTA)-agarose chromatography, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted to a nitrocellulose membrane, and then detected with either an anti-HA (12CA5) or antiphosphotyrosine (4G10 [Upstate Biotechnology]) antibody. Immunoreactive bands were detected with horseradish peroxidase-conjugated second antibodies and the ECL Western blotting detection system (Amersham).

***pyp2*$^+$, *spc1*$^+$, and *wis1*$^+$ expression plasmids.** Plasmids pREP3-pyp2, pREP1-GST-spc1, and pREP1-wis1 have been described elsewhere (36). The *pyp2-C630S* mutation was constructed as follows. The 5′ end of *pyp2* (codons 1 to 629) was amplified by PCR with the 5′ oligonucleotide 1 (GCGCG GCGGC ATATG CTCCA TCTTC TGTCT AAAGA CG [*Nde*I site underlined]) and the 3′ oligonucleotide 2 (GTGAA CGAAC ATTGG TCCAT C) in the presence of Vent polymerase (New England Biolabs). The 3′ end of *pyp2* (codons 630 to 711) was PCR amplified with the mutagenic 5′ oligonucleotide 3 (TCTTC AGCAG GCG TAG GACGC) and the 3′ oligonucleotide 4 (GGCGC GCGCG CGGCC GC AAG TCATC AAGGG CTTGG AAGCC TGG [*Not*I site underlined]). After phosphorylation with T4 polynucleotide kinase, amplified fragments were ligated and the entire *pyp2* open reading frame was amplified from the ligation mixture by using oligonucleotides 1 and 4. The PCR fragment was then cleaved by *Nde*I and *Not*I and cloned in the pREPKZ vector to form plasmid pREP-KZ-pyp2-C630S. This plasmid directs expression of *GST-pyp2-C630S* from the thiamine (vitamin B_1)-repressible *nmt1* promoter. KS1376 or KS1366 cells transformed with pREP-KZ-pyp2-C630S or a pREPGST control plasmid were grown for 16 h in the absence of thiamine. Cell extracts were prepared with extraction buffer, which consisted of 50 mM Tris HCl (pH 8.3), 200 mM NaCl, 50 mM NaF, 5 mM EDTA (pH 8), 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 5 μg (each) of leupeptin, pepstatin, and aprotinin per ml. The cell extracts were mixed with glutathione (GSH)-Sepharose beads and incubated for 1 h at 4°C. The complexes were pelleted and washed three times with extraction buffer before being resolved by SDS-PAGE.

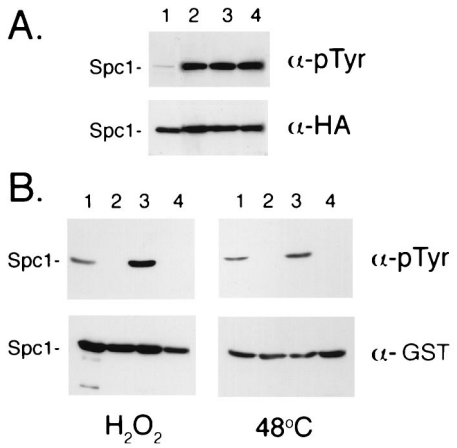

FIG. 1. Wis1-dependent tyrosine phosphorylation of Spc1 by high-temperature and oxidative-stress signals. (A) Cells expressing epitope-tagged Spc1 at wild-type levels (Spc1-Ha6H; KS1376 strain) were grown to log phase in YES medium (lane 1) and then exposed to osmotic stress (15 min of exposure to YES plus 0.6 M KCl [lane 2]), oxidative stress (15 min of growth in YES plus 0.3 mM H_2O_2 [lane 3]), or heat stress (10 min of growth at 48°C in YES [lane 4]). Spc1 was isolated by Ni^{2+}-NTA-agarose affinity precipitation and then analyzed by immunoblotting with an antiphosphotyrosine (α-pTyr) or anti-HA (α-HA) antibody. Activating tyrosine phosphorylation of Spc1 was dramatically increased in response to all three forms of stress. (B) Wis1 is required for induction of Spc1 tyrosine phosphorylation in response to oxidative and heat stress. Wild-type (PR109) and Δ*wis1* (JM544) strains were transformed with the pREP1-GST-spc1 plasmid, grown to mid-log phase in EMM2 medium, and then incubated for 15 min in YES plus 0.3 mM H_2O_2 or for 10 min at 48°C. GST-Spc1 was isolated with GSH-Sepharose and then analyzed by immunoblotting with an antiphosphotyrosine (α-pTyr) or anti-GST (α-GST) antibody. Lane 1, wild-type cells before stress; lane 2, Δ*wis1* cells before stress; lane 3, wild-type cells after stress; lane 4, Δ*wis1* cells after stress.

Stress sensitivity assays. For heat shock experiments, cells were grown in YES medium to mid-log phase at 30°C and then transferred to 48°C. Samples were taken at 10-min intervals, diluted in ice-cold YES medium, and plated onto YES agar. The plates were incubated for 5 days at 30°C before counting the number of colonies. Mid-log-phase cells grown in YES medium at 30°C were also used to check the sensitivity to hydrogen peroxide (H_2O_2). Aliquots of the culture were incubated for 1 h in the presence of increasing amounts of H_2O_2 (up to 8 mM). Cells were then washed, diluted, and plated on YES agar. The number of colonies was counted after 5 days of incubation at 30°C. These experiments were repeated three times.

RESULTS

Spc1 MAPK is activated by high-temperature and oxidative-stress signals. One aim of our study was to determine whether activation of Spc1 kinase pathway was specific to osmotic stress and nutrient limitation. This study utilized a strain that has a single copy of *spc1*$^+$-*Ha6H* expressed from the *spc1*$^+$ promoter (36). The *spc1*$^+$-*Ha6H* gene encodes Spc1 protein having a C-terminal tag containing two copies of the HA epitope and six consecutive histidine residues, allowing Spc1 purification with Ni^{2+}-NTA and detection with an anti-HA antibody. As previously reported (23, 36), exposure of cells to high-osmolarity conditions, in this case YES medium containing 0.6 M KCl, led to the rapid increase of activating tyrosine phosphorylation of Spc1 (Fig. 1A, lane 2). Oxidative stress, accomplished by exposure of cells to 0.3 mM hydrogen peroxide (H_2O_2) for 15

min, likewise caused a large increase in the amount of Spc1 tyrosine phosphorylation (Fig. 1A, lane 3). Heat stress, performed by incubation of cells at 48°C for 10 min, also caused a similar large increase in Spc1 tyrosine phosphorylation (Fig. 1A, lane 4). These findings demonstrate that Spc1 is activated by several different types of environmental stress.

Wis1 MAPK kinase transmits high-temperature and oxidative-stress signals. Earlier studies showed that Wis1 was essential for the osmotic-stress-induced tyrosine phosphorylation of Spc1 (23, 36). Mammalian stress-stimulated MAPKs appear to be activated by multiple species of MAPK kinases that may be preferentially responsive to different stimuli (8, 19). For this reason it was important to determine if Wis1 was also required for transmission of the high-temperature and oxidative-stress signals. These studies utilized *wis1*$^+$ and Δ*wis1* strains that were transformed with a plasmid expressing functional GST-Spc1 fusion protein. The GST moiety was used to purify that fusion protein with GSH-Sepharose. These studies showed that Wis1 was required for the tyrosine phosphorylation of Spc1 that is induced by high temperature and oxidative stress (Fig. 1B). These findings show that the Wis1 MAPK kinase transmits a variety of stress-stimulated signals to Spc1 MAPK.

Spc1 is crucial for survival of high temperature and oxidative stress. Fission yeast *spc1*$^-$ and *wis1*$^-$ mutants are unable to form colonies in high-osmolarity media, showing that activation of the Wis1-Spc1 kinase pathway is critical for survival of osmotic stress (23, 36). We investigated whether Spc1 was also important for survival of heat and oxidative stress. Survival of heat stress was analyzed by incubating *spc1*$^+$ and *spc1-M13* cells at 48°C for 0 to 50 min. The mortality of *spc1-M13* cells during heat stress was dramatically higher than that of the wild type (Fig. 2A). Less than 1% of the *spc1-M13* cells were viable after 30 min of incubation at 48°C, whereas >80% of the wild-type cells survived this degree of heat stress. Survival of oxidative stress was evaluated by incubating cells for 1 h in medium containing 0.5 to 8 mM hydrogen peroxide. Approximately 86% of the *spc1*$^+$ cells survived exposure to 1 mM H_2O_2, whereas only 2% of the *spc1-M13* cells survived the same treatment (Fig. 2B). Higher concentrations of hydrogen peroxide caused almost 100% mortality of *spc1-M13* cells, whereas 60 to 80% of the wild-type cells survived this treatment. Other studies showed that *wis1*$^-$ cells were also extremely sensitive to heat stress and oxidative stress (data not shown). These observations show that activation of the Wis1-Spc1 pathway is of critical importance for survival of many forms of environmental stress.

Cell cycle defect of *spc1*$^-$ mutants exposed to oxidative stress. Previous studies showed that ~50% of *spc1*$^-$ mutant cells undergo cell cycle arrest when exposed to high-osmolarity medium, while the remaining cells appear to undergo rapid lysis (36). We investigated whether exposure of *spc1*$^-$ cells to oxidative stress also caused a cell cycle defect. As shown in Fig. 3, *spc1-M13* cells became elongated when incubated in media containing 0.3 mM hydrogen peroxide for 20 h, whereas moderate oxidative stress had no effect on the cell division size of wild-type cells. These observations indicate that Spc1 is crucially important for the induction of mitosis in several different types of stress.

Spc1 regulates stress-induced transcription of *gpd1*$^+$ and *tps1*$^+$. It seemed plausible that the Spc1 pathway regulates the transcription of stress response genes. The gene *gpd1*$^+$, which encodes glycerol-3-phosphate dehydrogenase, a key enzyme required for glycerol synthesis, was considered likely to have a key role in the survival of osmotic stress (2). As predicted, exposure to 0.6 M KCl led to a large increase in the amount of *gpd1*$^+$ mRNA (Fig. 4A). The kinetics of induction was compatible with the kinetics of activating tyrosine phosphorylation of Spc1 MAPK (data not shown). Stress-induced transcription of *gpd1*$^+$ was totally deficient in the *spc1-M13* strain (Fig. 4A).

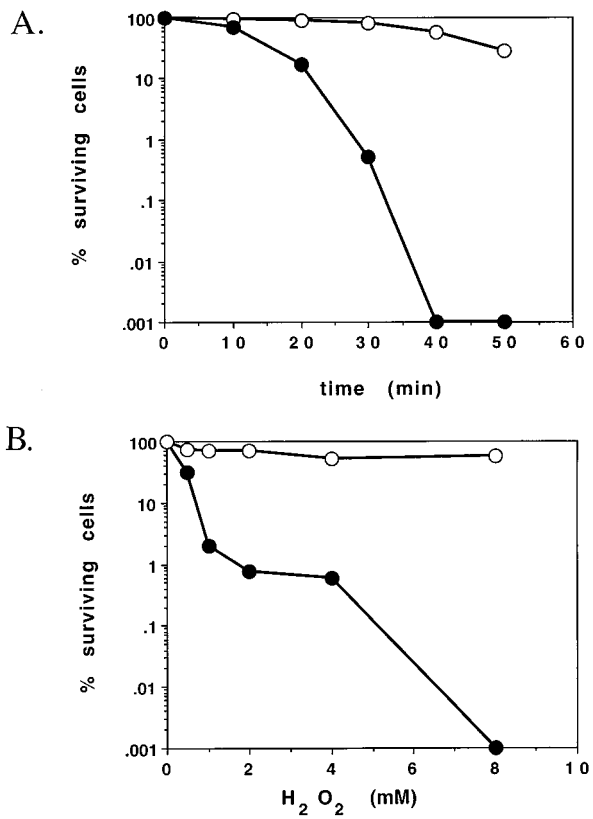

FIG. 2. *spc1-M13* cells do not survive high temperature and oxidative stress well. (A) Wild-type (open circles) or *spc1-M13* mutant (closed circles) cells were grown to mid-log phase in YES medium at 30°C, and then the temperature was raised to 48°C. Cell viability was measured at regular intervals by plating dilutions of the culture on YES agar and incubating at 30°C for 5 days. (B) Wild-type (open circles) or *spc1-M13* mutant (closed circles) cells were grown to mid-log phase in YES at 30°C and then incubated for 1 h with increasing concentrations of H_2O_2 in YES. Cells were then washed, diluted, and assayed for their colony-forming ability.

The gene *tps1*$^+$, which encodes trehalose-6-phosphate synthase, an enzyme that is required for trehalose synthesis, has an important role in the heat shock response (4, 9). As shown in Fig. 4B, the amount of *tps1*$^+$ mRNA was dramatically increased in response to incubation at 42°C. Studies carried out with a *spc1-M13* strain revealed that the heat shock-induced transcription of *tps1*$^+$ was largely dependent on Spc1 activity (Fig. 4B). Interestingly, induction of *tps1*$^+$ expression was not completely abolished in *spc1-M13* cells, suggesting that there may be also a Spc1-independent mechanism of inducing *tps1*$^+$ expression in response to heat stress.

We also explored whether multiple forms of stress caused induction of *gpd1*$^+$ and *tps1*$^+$ expression. We found that *gpd1*$^+$ expression was elevated in response to oxidative stress and heat stress (Fig. 4C) and that *tps1*$^+$ expression was induced following exposure to osmotic stress (Fig. 4D). These findings demonstrate that Spc1 has a key role in promoting the stress-induced transcription of at least two genes involved in the survival of several different types of cytotoxic stress.

Expression of *pyp2*$^+$ is induced by several forms of stress. Earlier studies indicated that expression of *pyp2*$^+$ mRNA, which encodes a tyrosine phosphatase implicated in the regulation of Spc1 (25, 27), is elevated in response to heat shock

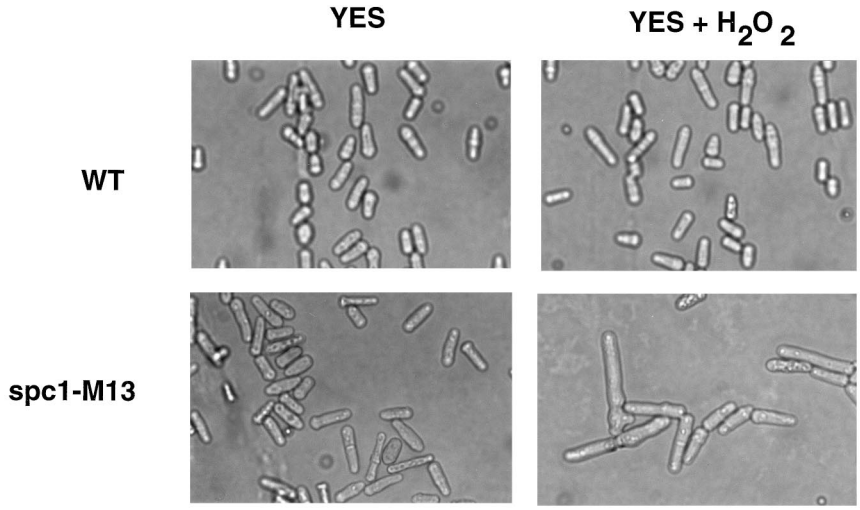

FIG. 3. *spc1-M13* cells exhibit a cell cycle defect when exposed to oxidative stress. Wild-type (WT) (PR109) or *spc1-M13* (KS1147) cells were grown on YES agar plates or on the same medium supplemented with 0.3 mM H_2O_2 for 20 h at 30°C. Wild-type cells formed colonies that were streaked immediately before the photograph was taken, whereas *spc1-M13* cells exhibited defective cell division and were not streaked before the photograph was taken.

(27a). This prompted us to investigate whether *pyp2*$^+$ expression is induced by various types of stress in a Spc1-dependent manner. These investigations revealed that the amount of *pyp2*$^+$ mRNA was highly elevated in response to exposure to high-salt media (0.6 M KCl), oxidative stress (0.3 mM hydrogen peroxide), and high temperature (48°C) (Fig. 5A). Induction of *pyp2*$^+$ expression was completely deficient in *spc1-M13* cells (Fig. 5B). The finding that *pyp2*$^+$ mRNA expression is elevated in response to osmotic stress has also recently been reported in an independent study (23). The kinetics of *pyp2*$^+$ mRNA expression was monitored in more detail in cells exposed to 0.6 M KCl (Fig. 6A). Significant amounts of *pyp2*$^+$ mRNA appeared within 10 min of exposure to osmotic stress and were almost entirely eliminated within 30 min, so the response was extremely transient. The pattern of *pyp2*$^+$ mRNA accumulation and disappearance lags slightly behind Spc1 tyrosine phosphorylation (see below), a finding consistent with the supposition that *pyp2*$^+$ expression is regulated by Spc1 MAPK. As predicted, induction of *pyp2*$^+$ mRNA expression in response to osmotic stress was abolished in a *spc1-M13* strain (Fig. 6A).

Wis1 overproduction stimulates *pyp2*$^+$ expression in the absence of stress. We investigated whether *pyp2*$^+$ expression could be induced by activation of the Wis1-Spc1 pathway in the absence of environmental stress. Wild-type and *spc1-M13* cells transformed with a plasmid containing *nmt1*:*wis1*$^+$ were grown in media that induced *wis1*$^+$ expression from the *nmt1* promoter. Wis1 overproduction caused a large increase in the amount of *pyp2*$^+$ mRNA in the wild-type background (Fig. 6B, lane 1). In contrast, *pyp2*$^+$ mRNA was not detected in the *spc1-M13* cells that overproduced Wis1 (Fig. 6B, lane 2). These findings strongly suggest that activation of the Spc1 pathway is both necessary and sufficient to cause induction of *pyp2*$^+$ expression.

Pyp2 overexpression decreases Spc1 tyrosine phosphorylation. It is well established that Pyp1 directly regulates Spc1 in vivo, but the role of Pyp2 in regulating Spc1 is less certain (23, 36). In particular, it is not known whether Pyp2 overexpression causes a decrease of Spc1 tyrosine phosphorylation, nor is it known whether Spc1 and Pyp2 interact directly in vivo. With the goal of establishing whether a Spc1-Pyp2 negative-feedback loop actually exists in fission yeast, we first inquired whether

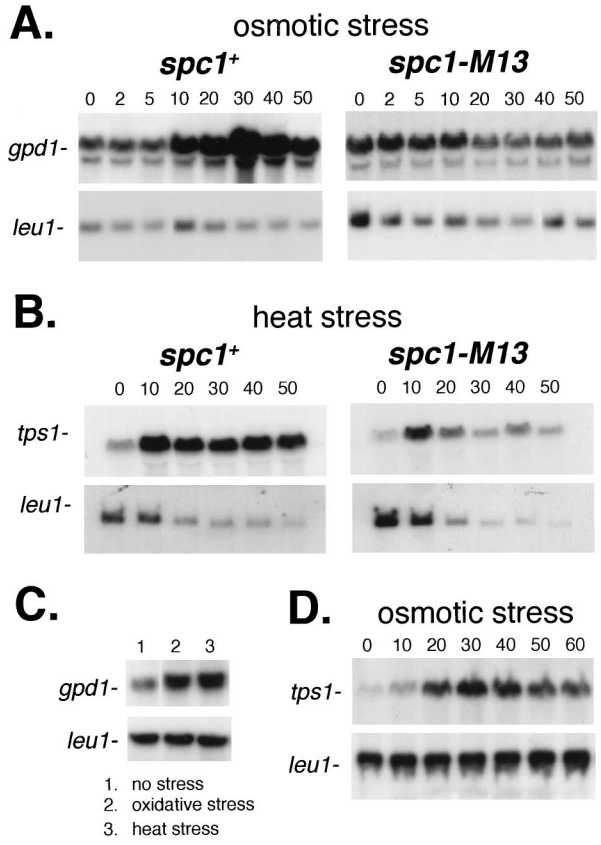

FIG. 4. Spc1 is required for the stress-induced transcription of *gpd1*$^+$ and *tps1*$^+$. (A) Northern hybridization analysis of *gpd1*$^+$ and *leu1*$^+$ mRNAs from total RNA extracted from wild-type cells or *spc1-M13* mutant cells following exposure to YES medium plus 0.6 M KCl for the indicated periods of time (in minutes). (B) Northern hybridization analysis of *tps1*$^+$ and *leu1*$^+$ mRNAs from total RNA extracted from wild-type cells or *spc1-M13* mutant cells following exposure to YES at 42°C. (C) Northern hybridization analysis of *gpd1*$^+$ and *leu1*$^+$ mRNAs in wild-type cells grown in YES at 30°C (lane 1) or incubated in YES plus 0.3 H_2O_2 at 30°C for 20 min (lane 2) or incubated in YES at 42°C for 10 min (lane 3). (D) Northern hybridization analysis of *tps1*$^+$ and *leu1*$^+$ mRNA in wild-type cells following exposure to YES plus 0.6 M KCl for the indicated periods of time (in minutes). Experiments studying *tps1*$^+$ expression in cells exposed to oxidative stress have not yet been carried out.

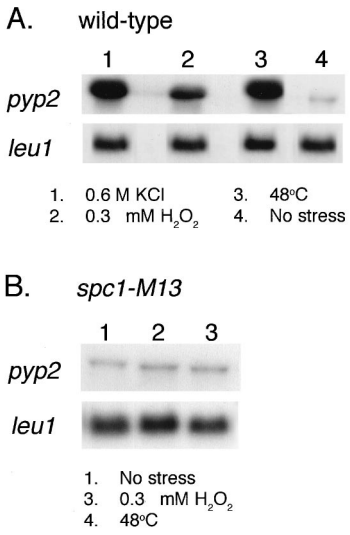

FIG. 5. Induction of the *pyp2*$^+$ mRNA in stressed cells. (A) Log-phase cultures of wild-type cells were incubated for 20 min in YES medium plus 0.6 M KCl (lane 1) on YES plus 0.3 mM H_2O_2 (lane 2) or were incubated for 10 min at 48°C in YES (lane 3) or not treated (lane 4). Total RNA was extracted and subjected to Northern hybridization analysis with *pyp2*$^+$ and *leu1*$^+$ probes. (B) A similar analysis was carried out with *spc1-M13* cells, showing that induction of pyp2 expression in response to oxidative stress (lane 2) and heat stress (lane 3) is dependent on Spc1 activity.

Pyp2 overexpression led to a decrease of Spc1 tyrosine phosphorylation. Cells transformed with a plasmid carrying *nmt1*:*pyp2*$^+$ were grown in medium that induced the *nmt1* promoter and then harvested immediately before and after a 10-min exposure to 0.6 M KCl. Spc1-Ha6H protein was purified and analyzed by immunoblotting with anti-HA and anti-pTyr antibodies (Fig. 7). This analysis revealed that Pyp2 overproduction greatly reduced the amount of Spc1 tyrosine phosphorylation both in unstressed and stressed conditions, indicating that Pyp2 is able to regulate Spc1 tyrosine phosphorylation in vivo.

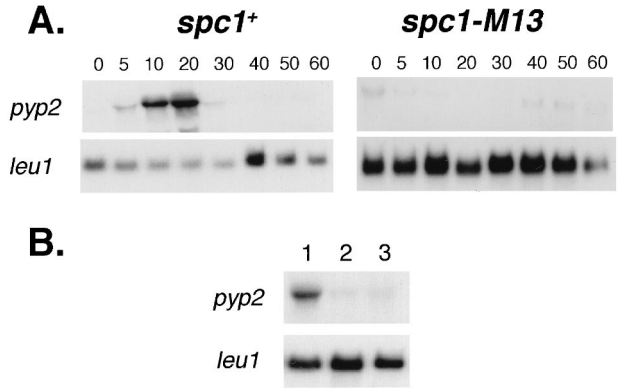

FIG. 6. Spc1 is required for stress-stimulated induction of *pyp2*$^+$ expression. (A) Total RNAs extracted from wild-type and *spc1-M13* cells at the indicated times (in minutes) after osmotic stress (growth in YES medium plus 0.6 M KCl) were subjected to Northern hybridization analysis with *pyp2*$^+$ and *leu1*$^+$ probes. (B) Induction of *pyp2*$^+$ mRNA in cells overexpressing Wis1 kinase. Wild-type (lane 1) and *spc1-M13* (lane 2) cells transformed with a pREP1-wis1 plasmid were grown for 17 h at 30°C in EMM2 medium in the absence of thiamine to induce *wis1*$^+$ overexpression. Total RNA was extracted and probed with *pyp2* and the *leu1* control. Wild-type cells carrying no plasmid were included as a control (lane 3).

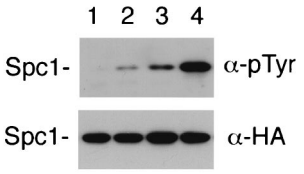

FIG. 7. Spc1 tyrosine phosphorylation is decreased in cells that overexpress *pyp2*$^+$. Wild-type cells expressing Spc1-Ha6H (KS1376) transformed with a pREP3-pyp2 plasmid (lanes 1 and 3) or cells carrying no plasmid (lanes 2 and 4) were grown for 16 h at 30°C in EMM2 medium in the absence of thiamine to induce the overexpression of *pyp2* from the thiamine-repressible *nmt1* promoter. Cells were harvested immediately before (lanes 1 and 2) or after 10 min of exposure to YES plus 0.6 M KCl (lanes 3 and 4). The Spc1 protein was precipitated with Ni^{2+}-NTA-agarose and analyzed by immunoblotting with an antiphosphotyrosine (α-pTyr) or anti-HA (α-HA) antibody.

A limitation of the aforementioned Pyp2 overproduction experiment was that it did not exclude the possibility that Pyp2 also dephosphorylates another tyrosine-phosphorylated protein, a protein which could be the key physiological substrate of Pyp2 in vivo. Therefore, a second experiment was carried out to evaluate whether Spc1 is the only tyrosine-phosphorylated protein that associates with Pyp2 in vivo. A plasmid containing *nmt1*:*GST-pyp2-C630S* was constructed and transformed into *spc1*$^+$*-Ha6H* and Δ*spc1* backgrounds. The GST-Pyp2-C630S fusion protein was purified by GSH-Sepharose affinity precipitation. The rationale of this experiment was that the Pyp2-C630S protein, in which the active-site cysteine has been substituted with serine, should have a stable interaction with its substrate, as was observed for the equivalent mutant form of Pyp1 (36). Expression of GST-Pyp2-C630S from the *nmt1* promoter caused cells to become highly elongated, consistent with an earlier report of the effects of Pyp2-C630S overexpression (14). Immunoblotting revealed that a single tyrosine-phosphorylated protein coprecipitated with GST-Pyp2-C630S (Fig. 8, middle gel, lanes 3 to 6). No other proteins were detected even

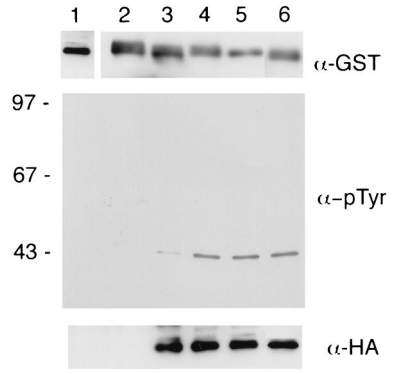

FIG. 8. Spc1 is the in vivo substrate of Pyp2. Wild-type cells expressing Spc1-Ha6H (KS1376) transformed with a pREP-GST control plasmid (lane 1) or a pREP-KZ-pyp2-C630S plasmid (lanes 3 to 6) or Δ*spc1* cells (KS1366) transformed with pREP-KZ-pyp2-C630S (lane 2), were grown for 17 h at 30°C in EMM2 medium in the absence of thiamine to induce the expression of the GST and GST-Pyp2-C630S proteins. Cells were then incubated for 15 min in YES medium (lane 3) or YES with 0.6 M KCl (lane 4) or with 0.3 mM H_2O_2 (lane 5) or incubated for 10 min at 48°C (lane 6). GST and GST-Pyp2-C630S proteins were precipitated with GSH-Sepharose beads under native conditions, washed extensively, subjected to SDS-PAGE, and immunoblotted with an anti-GST (α-GST), antiphosphotyrosine (α-pTyr), or anti-HA (α-HA) antibody. Note that lane 1 in the top gel, in which GST is detected, was taken from the ~30-kDa region of the gel, whereas lanes 2 to 5, in which GST-Pyp2-C630S is detected, were taken from the ~110-kDa region of the gel. A ~43-kDa tyrosine-phosphorylated protein specifically coprecipitates with GST-Pyp2-C630S. It is absent in Δ*spc1* cells and comigrates with Spc1-Ha6H.

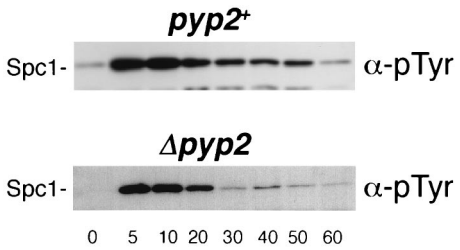

FIG. 9. Spc1 tyrosine phosphorylation patterns are very similar in wild-type and Δ*pyp2* strains during osmotic stress. Wild-type cells (KS1376) and Δ*pyp2* cells (GD1498) containing epitope-tagged Spc1 protein were incubated for the indicated times (in minutes) in the presence of 0.6 M KCl. The Spc1 protein was precipitated with Ni_2^+-NTA agarose and probed by Western blotting for the presence of phosphotyrosine (α-pTyr). Immunoblot analysis with an anti-HA antibody confirmed that the Spc1 levels were approximately constant throughout (data not shown).

after a ~10-fold-longer exposure of the immunoblot. This protein migrated at ~43 kDa, which is the predicted size of Spc1-Ha6H. The protein was not detected in a control GST precipitate, nor was it detected in the GST-Pyp2-C630S complex prepared from Δ*spc1* cells (Fig. 8, lanes 1 and 2). Moreover, the 43-kDa anti-pTyr signal was greatly enhanced in cells exposed to 0.6 M KCl, 0.3 mM H_2O_2, or 48°C heat stress (Fig. 8, lanes 4 to 6). Indeed, probing of a duplicate immunoblot with anti-HA antibody revealed that the 45-kDa protein comigrated with Spc1-Ha6H (Fig. 8, bottom gel). These data provide strong evidence that Spc1 is the major and perhaps the sole physiological substrate of Pyp2 in vivo.

Spc1 tyrosine phosphorylation and *gpd1*$^+$ transcription in Δ*pyp2* cells. The final phase of these investigations dealt with the in vivo physiological importance of Pyp2. This was initially approached by comparing Spc1 tyrosine phosphorylation in wild-type and Δ*pyp2* cells following exposure to 0.6 M KCl. Remarkably, these experiments did not detect any reproducible difference between wild-type and Δ*pyp2* cells in the amount or duration of Spc1 tyrosine phosphorylation following exposure to osmotic stress (Fig. 9). There was also no apparent difference in the wild-type and Δ*pyp2* cells in the amount or duration of Spc1 tyrosine phosphorylation in cells exposed to 0.9 M KCl or 48°C heat shock (6). These results prompted us to examine whether the expression of Spc1-regulated genes was affected by the Δ*pyp2* mutation. Experiments were performed to measure the amount of *gpd1*$^+$ mRNA in cells exposed to 0.6 M KCl osmotic stress. This analysis revealed that at the 30-, 40-, and 50-min time points, the abundance of *gpd1*$^+$ mRNA was 1.5- to 2.5-fold higher in Δ*pyp2* cells than in the wild-type cells (Fig. 10). These findings were replicated in two independent experiments (Fig. 10). These results indicate that Pyp2 may have a greater effect on Spc1 activity than on Spc1 tyrosine phosphorylation itself. Mechanisms which may explain this phenomenon are described below.

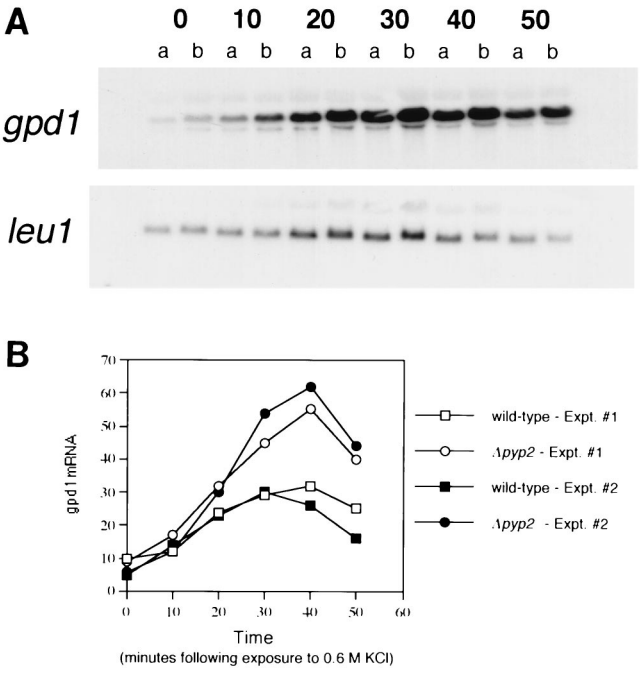

FIG. 10. Disruption of *pyp2*$^+$ results in a 1.5- to 2.5-fold increase of *gpd1*$^+$ expression during osmotic stress. (A) Total RNAs extracted from wild-type cells (lanes a) and Δ*pyp2* cells (lanes b) at the indicated times (in minutes) after high-salt exposure (YES plus 0.6 M KCl) was probed with *gpd1* and *leu1* probes. (B) The *gpd1* signals were measured with a PhosphorImager (Molecular Dynamics), normalized to the corresponding *leu1* signal, and plotted. Experiment 1 refers to the data shown in panel A, Experiment 2 was a different experiment. Nearly identical results were obtained in the two experiments.

DISCUSSION

The major findings of this report are schematized in Fig. 11. The first important discovery is that Spc1 MAPK is activated in response to diverse forms of stress, including nutrient limitation, osmotic stress, heat stress, and oxidative stress. In this respect the Spc1 pathway more closely resembles the stress-activated kinases of the Jnk1 and p38 families in mammalian cells than the *S. cerevisiae* Hog1 kinase that has been reported to be responsive only to osmotic stress (30, 35). A second important finding is that transmission of all of these signals requires Wis1, a MAPK kinase homolog. Our experiments have also shown that Spc1 is critically important for survival of oxidative stress, heat stress, and osmotic stress. Not only is Spc1 essential, but we can also state that activation of Spc1 is crucially important, since *spc1*$^-$ and *wis1*$^-$ cells exhibit similar mortality rates following exposure to these types of stress.

Another finding to emerge from this study is that Spc1 is required for the stress-induced transcription of two genes that are believed to be important for stress survival. The *gpd1*$^+$ gene, which encodes glycerol-3-phosphate dehydrogenase, a key enzyme required for glycerol synthesis, is induced after

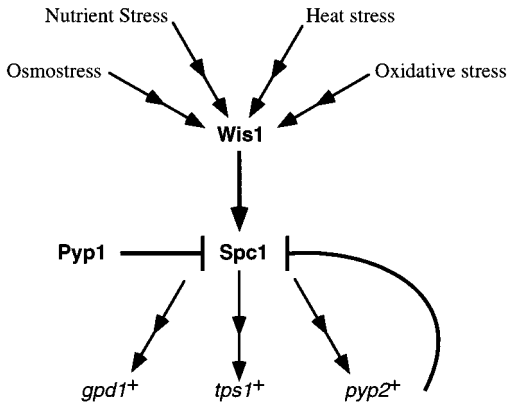

FIG. 11. Wis1-Spc1 pathway. Spc1 is activated by various forms of stress in a Wis1-dependent mechanism. Activation of Spc1 leads to the transcriptional induction of a number of genes believed to be important for the stress response, such as *gpd1*$^+$ and *tps1*$^+$. Spc1 also promotes the transcription of *pyp2*$^+$, which feeds back to negatively regulate Spc1. Expression of *pyp1*$^+$ is unaffected by Spc1.

osmotic stress in a Spc1-dependent manner. This finding is in agreement with the recent demonstration that elevation of *gpd1* expression in response to osmotic shock is defective in a Δ*wis1* mutant (1). In an analogous manner, osmotic-stress-stimulated expression of *S. cerevisiae GPD1*, which also encodes glycerol-3-phosphate dehydrogenase, is dependent on Hog1 kinase (2). We have also observed that expression of *tps1*$^+$, encoding trehalose-6-phosphate synthase, is regulated through the Spc1 MAPK pathway in response to heat shock. The downstream targets of Hog1 are not known, but if Spc1 pathway is analogous to mammalian stress-activated Jnk1 kinase, then we can predict that Spc1 will activate a transcription factor related to c-Jun. Indeed, recent studies have identified a fission yeast ATF-2 homolog that is directly regulated by Spc1 (38). We have found that induction of *gpd1*$^+$ and *tps1*$^+$ expression is not specific to the form of stress applied to the cells. For example, the level of *gpd1*$^+$ expression is higher in response to osmotic, oxidative, and heat stress. These findings are consistent with the model of a simple linear pathway of transmitting the transcriptional induction signal.

Our studies have also shown that *pyp2*$^+$ transcription is induced in response to stress by a Spc1-dependent mechanism. This finding concurs with the recent report that the level of *pyp2*$^+$ transcription is higher following osmotic stress by a Wis1-dependent mechanism (23). Since previous studies indicated that Pyp2 might negatively regulate Spc1, these findings suggested that Spc1-dependent induction of *pyp2*$^+$ expression might serve as a negative-feedback mechanism to dampen the Spc1 signal transduction pathway. However, a number of crucial tests of this model were not carried out in previous studies (23, 36), so it became important to carry out a rigorous evaluation of the hypothesis that Pyp2 regulates Spc1 in vivo. Part of the proof of this hypothesis was the demonstration that Pyp2 overproduction reduces the in vivo amount of Spc1 tyrosine phosphorylation. This parallels results obtained with cells that overproduce Pyp1 (23, 36). As mentioned above, one limitation of this experiment was that it did not exclude the possibility that another tyrosine-phosphorylated protein is the primary substrate of Pyp2 in vivo, nor did it provide a direct connection between Pyp2 and Spc1. These deficiencies were addressed by showing that Spc1 is the only tyrosine-phosphorylated protein that is associated with Pyp2 at a detectable level in vivo, suggesting that Spc1 is the major and perhaps sole physiological substrate of Pyp2. An important feature of this experiment was that it utilized an active-site mutant form of Pyp2, because a previous study had shown that the equivalent mutation of Pyp1 greatly stabilizes the Pyp1-Spc1 interaction (36). These findings establish that Spc1 is the critical target of Pyp2 tyrosine phosphatase.

Although these findings demonstrate that Pyp2 negatively regulates Spc1 in vivo, conclusions regarding the significance of this regulation are tempered by the observation that Spc1 tyrosine phosphorylation is not changed by a Δ*pyp2* mutation. In fact, the pattern and time course of stress-induced Spc1 tyrosine phosphorylation and dephosphorylation are not noticeably different in the wild type and Δ*pyp2* mutants. These observations differ from those of a recent report (23), which indicated that the Δ*pyp2* mutation had two major effects on Spc1 tyrosine phosphorylation: one was to greatly dampen the osmotic stress-induced increase of Spc1 tyrosine phosphorylation, and the second was to increase the amount of Spc1 tyrosine phosphorylation during prolonged exposure to osmotic stress. As shown in Fig. 9, our experiments have shown that in Δ*pyp2* cells there is a large increase in the amount of Spc1 tyrosine phosphorylation in response to osmotic stress and heat stress. The dampening of the osmotic-stress-induced increase of Spc1 tyrosine phosphorylation observed in a previous study (23) cannot be easily resolved with the proposal that Pyp2 dephosphorylates Spc1 in a negative-feedback mechanism, in fact the opposite result is predicted by the model. The *pyp2*$^+$ gene is only very transiently expressed after exposure to stress, therefore the Δ*pyp2* mutation would be predicted to cause an exaggerated increase in the amount of Spc1 tyrosine phosphorylation following exposure to stress. The discordant findings may be partially explained by one or a combination of experimental differences. Our experiments utilized strains that expressed wild-type levels of Spc1, whereas in the previous study *spc1*$^+$ was highly overexpressed from a *nmt1*:*spc1*$^+$ construct on a multicopy plasmid (23). It is possible that Spc1 overexpression causes changes in the pattern of Spc1 tyrosine phosphorylation in ways that do not occur in cells expressing wild-type levels of Spc1. The second difference concerns the growth medium. We have previously shown that growth on a synthetic minimal medium, EMM2, causes a moderate increase of Spc1 tyrosine phosphorylation, well above that observed with cells grown in YES, a complex medium consisting of yeast extract and glucose. This difference accounts for the observation that the *spc1*$^-$ cell elongation phenotype is enhanced when cells are grown in EMM2 medium as opposed to YES medium (36). These observations indicate that growth in EMM2 can be considered to be moderately stressful to *S. pombe* cells. Most of our experiments, including those involving Δ*pyp2* strains, were carried out with YES medium, whereas the previous studies used cells grown in EMM2 (e.g., 23). It is likely that *spc1*$^+$ overexpression combined with growth in moderately stressful medium obscured the increase in Spc1 tyrosine phosphorylation that occurs when Δ*pyp2* cells experience osmotic stress. We have focused our investigations on the role of Pyp2 during acute stress, mainly because these are the circumstances in which one observes dramatic induction of *pyp2*$^+$ expression.

Our investigations demonstrated that *gpd1*$^+$ transcription is increased approximately twofold in a Δ*pyp2* mutant, showing that the loss of Pyp2 enhances one measure of Spc1 activity. It is curious that we detected an increase in *gpd1*$^+$ expression without observing a corresponding change in Spc1 tyrosine phosphorylation. This may be explained by downstream amplification of the Spc1 signal, but there may be other explanations. It is possible that Pyp2 preferentially regulates a subset of Spc1 molecules that are most directly involved in promoting stress-stimulated transcription. Perhaps Pyp2 has some affinity for Spc1 substrates, allowing Pyp2 to concentrate its activity on Spc1 molecules as they are in the act of transmitting the downstream signal. A second possibility is that Pyp2 inhibition of Spc1 is a two-step process that first involves binding of Pyp2 to Spc1, which is itself inhibitory, followed by dephosphorylation of Spc1. If the dephosphorylation reaction is relatively slow, then Pyp2 could inhibit Spc1 without dramatically affecting the amount of Spc1 tyrosine phosphorylation. Evaluation of these models will require a more detailed understanding of the enzymatic and in vivo functional properties of Pyp2.

The Spc1-Pyp2 negative-feedback loop has interesting parallels with the proposed Fus3-Msg5 negative-feedback loop in *S. cerevisiae* (10). Fus3 is a MAPK homolog that is activated in response to mating factors and plays a role in promoting the transcriptional activation of mating-specific genes and cell cycle arrest in G_1. The *MSG5* gene was identified as a high-copy-number suppressor of the cell cycle arrest phenotype of strain that could be induced to inappropriately activate Fus3. *MSG5* encodes a dual-specificity phosphatase that is able to dephosphorylate Fus3 in vitro. It is noteworthy that the amount of *MSG5* mRNA increases in response to mating factors, suggest-

ing that perhaps Fus3 and Msg5 participate in a negative-feedback loop. However, it has not been reported whether Msg5 directly regulates Fus3 in vivo, nor is it known whether Fus3 activating phosphorylation is decreased in Msg5-overproducing strains or if *MSG5* expression is dependent on Fus3 (10). These questions will need to be addressed in order to establish the existence of a Fus3-Msg5 negative-feedback loop in budding yeast.

In mammalian cells, several protein phosphatases that inactivate stress-activated MAP kinases and turn off intracellular signaling pathways have been identified. Like Pyp2, the expression of CL100, 3CH134, MKP-1, PAC-1, and B23 phosphatases is induced following exposure to mitogens or environmental stress (15, 17, 20, 39, 41). It has been proposed that induction of these phosphatases may lead to the deactivation of mitogen- or stress-activated protein kinases, thereby restoring the pathway to a sensitive state. However, it has yet to be established whether transcriptional induction of the mammalian stress-regulated phosphatases is dependent on the kinases that they negatively regulate. The Spc1-dependent induction of Pyp2 provides a clear example of negative-feedback control of a stress-stimulated signal transduction pathway.

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