Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast

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The stress-activated Wis1–Spc1 protein kinase cascade links mitotic control with environmental signals in Schizosaccharomyces pombe. Fission yeast spc1Δ mutants are delayed in G2 during normal growth and undergo G2 arrest when exposed to osmotic or oxidative stress. Here we report that Spc1 also has an important role in regulating sexual development in S. pombe. This discovery arose from the observation that Spc1 is activated in response to nitrogen limitation, a key signal that promotes conjugation in fission yeast. Mutant spc1Δ cells are defective at arresting in G1 during nitrogen starvation and exhibit a poor mating ability. These deficiencies correlate with a failure to induce transcription of ste11Δ, a gene that encodes a transcription factor responsible for expression of various meiotic genes. Two genes, atf1Δ and atf21Δ, were cloned as multicopy suppressors of the spc1Δ mating defect. Atf1 and Atf21 are bZIP transcription factors that are most closely related to human ATF-2/CRE-BP1. Spc1 is required for stress-induced phosphorylation of Atf1. Atf1 is required for induction of meiotic genes and stress-response genes, such as gpd1Δ and pyp2Δ, that are transcriptionally regulated by Spc1. atf1Δ and spc1Δ mutants are sensitive to osmotic stress and impaired for sexual development, showing that fission yeast uses a common pathway to respond to cytotoxic stress and nitrogen starvation. However, unlike spc1Δ mutants, atf1Δ cells have no mitotic cell-cycle defect, indicating that the stress response pathway bifurcates at Spc1 to regulate independently meiosis and mitosis.

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the threonine and tyrosine residues of the Thr-Gly-Tyr motif (Shiozaki and Russell 1995). Inactivation of Spc1 is carried out by tyrosine-specific phosphatases Pyp1 and Pyp2 (Millar et al. 1995; Shiozaki and Russell 1995; Degols et al. 1996), unlike other MAP kinase phosphatases that dephosphorylate both the threonine and tyrosine residues (Alessi et al. 1993; Sun et al. 1993; Doi et al. 1996), unlike other MAP kinase phosphatases that regulates Spc1 negatively, indicating the existence of negative feedback loop to attenuate Spc1 activity (Millar et al. 1995; Degols et al. 1996). Various forms of stress induce expression of these genes in a spc1+-dependent manner. Budding yeast Hog1p also regulates stress-inducible genes such as CTT1, GPDI, and HSP12, although Hog1p is reported to be specifically responsive to osmotic stress (Albetyn et al. 1994; Schüller et al. 1994; Varela et al. 1995).

In addition to cytotoxic stress, Spc1 is activated when cells are grown in a synthetic minimal medium (Shiozaki and Russell 1995), suggesting that the Spc1 pathway might also be involved in sensing the nutritional environment. In this paper we extend this observation by showing that Spc1 is activated in response to limitation of a specific nutrient, namely, nitrogen. This finding is important because nitrogen limitation induces a program of sexual development in fission yeast. This program involves secretion of mating pheromones and expression of mating pheromone receptors, followed by G1 arrest and conjugation between cells of opposite mating types (Egel 1989). Nitrogen starvation also stimulates the meiotic pathway, thus newly formed zygotyes normally proceed directly to meiosis, with the end product being four spores enclosed in an ascus. Our findings suggest that activation of the Wis1–Spc1 kinase cascade is important for sexual development, because spc1- mutants are severely defective at arresting in G1 and inducing mating genes upon nitrogen starvation. Two multicopy suppressors of the spc1- sterile phenotype have been isolated, atf1+ and atf21+ encode basic zipper (bZIP) transcription factors that are related most closely to human activating transcription factor 2 (ATF-2)/cAMP response element (CRE)–BP1 binding protein. atf1- and spc1- mutants share various phenotypes, including failure to respond to nitrogen starvation, osmoregulation, and a defect in expression of stress-response genes. Atf1 undergoes Spc1-dependent phosphorylation during stress. These results strongly suggest that Atf1 is a downstream target of Spc1. However, unlike spc1- cells, atf1- cells do not show a G2 delay phenotype, indicating that Spc1 phosphorylates another substrate to regulate mitosis. We propose that the Wis1–Spc1 signal transduction pathway links mitotic and meiotic controls with changes in the extracellular environment that affect cell physiology.

Results

Spc1 is activated in response to nitrogen starvation

To examine the activation state of Spc1 after nitrogen starvation, we used a haploid S. pombe strain in which the single chromosomal copy of spc1+ was tagged with a sequence encoding two copies of the HA epitope and six consecutive histidine residues (Shiozaki and Russell 1995). This allowed us to purify Spc1 using Ni2+-NTA beads and detect it using anti-HA antibodies. Cells growing exponentially in standard EMM2 minimal medium were collected and resuspended in EMM2 lacking nitrogen. Activation of Spc1 was assessed by monitoring the level of activating tyrosine phosphorylation using antiphosphotyrosine antibodies. Spc1 tyrosine phosphorylation increased within 0.5 hr of nitrogen depletion and peaked at ~1 hr, then decreased to the initial level by 2.5 hr (Fig. 1A). This result indicates that Spc1 undergoes transient activation upon nitrogen limitation.

Activation of Spc1 upon nitrogen starvation is required for G1 cell-cycle arrest and induction of ste11+ expression

In cultures of homothallic wild-type cells, nitrogen starvation initiates a program of sexual development in which cells arrest in G1, mate with partners of the opposite mating type, and proceed directly to meiosis, with the end product being four spores enclosed in an ascus. Our findings suggest that activation of the Wis1–Spc1 kinase cascade is important for sexual development, because spc1- mutants are severely defective at arresting in G1 and inducing mating genes upon nitrogen starvation. Two multicopy suppressors of the spc1- sterile phenotype have been isolated, atf1+ and atf21+ encode basic zipper (bZIP) transcription factors that are related most closely to human activating transcription factor 2 (ATF-2)/cAMP response element (CRE)–BP1 binding protein. atf1- and spc1- mutants share various phenotypes, including failure to respond to nitrogen starvation, osmoregulation, and a defect in expression of stress-response genes. Atf1 undergoes Spc1-dependent phosphorylation during stress. These results strongly suggest that Atf1 is a downstream target of Spc1. However, unlike spc1- cells, atf1- cells do not show a G2 delay phenotype, indicating that Spc1 phosphorylates another substrate to regulate mitosis. We propose that the Wis1–Spc1 signal transduction pathway links mitotic and meiotic controls with changes in the extracellular environment that affect cell physiology.

**Figure 1.** (A) Spc1 undergoes activating tyrosine phosphorylation upon nitrogen starvation. Strain KS1376, in which the genomic copy of spc1+ encodes an epitope-tagged form of Spc1 that has two copies of the HA epitope and six consecutive histidine residues at the carboxyl terminus, was grown to mid-log phase at 30°C in EMM2 medium and then cells were collected and resuspended into the same medium lacking a nitrogen source (NH4Cl). Spc1 was purified using Ni-NTA–agarose beads and analyzed by immunoblotting with anti-HA antibodies. (B) FACScan analysis of DNA content in wild-type (wt; PR109) and ∆spc1 (KS1366) strains after nitrogen starvation. Within 6 hr of nitrogen deprivation a majority of wild-type cells arrested in G1, with a 1C DNA content, whereas only very few of the ∆spc1 cells had a 1C DNA content. [C] ∆spc1 cells are defective in ste11+ expression. ste11+ mRNA in wild-type (PR109) and ∆spc1 (KS1366) strains was monitored at 2 hr intervals after nitrogen starvation by Northern blotting (upper). Ethidium bromide staining of ribosomal RNA shown below indicates nearly equal loading of RNA in each lane.
posite mating type, and then undergo meiosis. A key event in this program is the induction of ste11+ expression (Sugimoto et al. 1991). Ste11 is a transcription factor containing a high mobility group (HMG) motif that is required for transcription of mei2+, a gene encoding an essential factor for the initiation of meiosis (Watanabe and Yamamoto 1994). Interestingly, G1 arrest and induction of ste11+ expression are not dependent on the presence of cells of opposite mating types, thus events that occur purely in response to nitrogen limitation can be distinguished from those that are dependent on a response to mating pheromones. The role of Spcl in these processes was evaluated by examining the behavior of a heterothallic h− Δspcl strain as it was starved of nitrogen. FACScan analysis of DNA content revealed that Δspcl cells failed to arrest in G1 after nitrogen starvation, whereas a large population of G1 cells with a 1C DNA content was detected with wild-type cells (Fig. 1B). Expression of ste11+ mRNA was examined by Northern blotting. Under the experimental conditions used here, accumulation of ste11+ mRNA was observed 2 hr after withdrawal of nitrogen from the growth medium and returned to the initial level by 4 hr (Fig. 1C). On the other hand, in Δspcl cells the amount of ste11+ mRNA was very low before and during nitrogen starvation (Fig. 1C), indicating that Spcl is important for expression of ste11+. Defects in G1 arrest and ste11+ expression after nitrogen starvation were also observed with a Δwis1 strain (data not shown), suggesting that activation of the Wis1–Spcl pathway is important for cellular response to nitrogen limitation.

Isolation of spcl− multicopy suppressors

Our studies suggested that Spcl has a key early role in transmitting the nitrogen starvation signal that triggers the initiation of the sexual development. This proposal was supported by the observation that the efficiency of mating and sporulation was very low in a homothallic h° spc1−M13 strain (Fig. 2A). With the aim of identifying downstream elements of the Wis1–Spcl signal transduction pathway, we screened a S. pombe genomic DNA library and a cDNA expression library for plasmid suppressors of the spc1− mating defect. Strain KS1440 [h° leu1 ura4 spc−M13] was transformed with library DNA, and plasmids were recovered from sporulating colonies identified by iodine staining (see Materials and Methods). In addition to clones containing spc1+, plasmids named p2B from the cDNA library and p4U from the genomic library were found to rescue the sterility of the spc1− strain (Fig. 2A). In comparison to vector controls, the p2B transformants had an enhanced ability to undergo G1 arrest after nitrogen starvation (Fig. 2B) and grew much better in high-osmolarity medium (Fig. 2C). Plasmid p4U exhibited a similar ability to rescue the G1 arrest and osmosensitive defects of spc1−M13 cells (data not shown). Plasmid p2B contained a 1.2-kb insert and sequence analysis indicated that the insert cDNA lacked the 5' end (Fig. 3A). A full-length genomic clone was isolated from the genomic library by hybridization screening using the cDNA insert of p2B as a probe (p5A; Fig. 3A). Genomic clone p5A contained a 1698-bp open reading frame [ORF] encoding a protein of 566-amino acid residues, which was named atfl+ because of its homology to human ATF-2 (see below). Very recently atfl+ was discovered during the process of sequencing chromosome II of S. pombe (Takeda et al. 1995). Like p2B, a multi-copy plasmid pREP1−atfl+ (Fig. 3A), which expresses full-length atfl+ under the control of thiamine-repressible nmt1 promoter (Maundrell 1993), also complemented the mating defect and high-osmolarity sensitivity of spc1− mutants in the presence of thiamine [data not shown], indicating that leaky expression of atfl+ from the repressed nmt1 promoter was sufficient to rescue the spc1− phenotypes. Sequence analysis of the 3.9-kb insert of p4U identified two ORFs (Fig. 3B). A subclone of p4U, p4U-1, containing the pce1+ ORF encod-
Figure 3. Restriction maps of atf1+ and atf21+.
(A) Clone p2B isolated from S. pombe cDNA library lacks the 5' end of the atf1+ open reading frame and expresses the carboxy-terminal half of the protein under the nmt1 promoter (nmt1p). The atf1+ genomic clone p5A was isolated from a S. pombe genomic library by hybridization screening using a 1.2-kb insert from p2B. Full-length atf1+ expressed from the nmt1 promoter (pREPl-atf1+) also rescues spc1− mating and high osmolarity defects. The plasmid construct used for atf1+ disruption with the ura4+ marker is also shown. Restriction enzyme sites: (Ec) EcoRI, (Sc) SacI, (Sp) SphI, (Xb) XbaI. (B) Clone p4U isolated from S. pombe genomic library was found to contain two open reading frames atf21+ and pce1+. The pce1+ ORF was named atf21+. (A) Clone p4U-1 containing only pce1+ failed to rescue spc1−, whereas atf21+ expressed from the nmt1 promoter (pREPl-atf21+) complemented spc1−. Disruption of atf21+ was performed with the plasmid construct shown above. Restriction enzyme sites: (Hd) HindIII, (Na) NsiI, (RV) EcoRV.

Figure 4. (A) Comparison of predicted amino acid sequences of Atf1, human ATF-2/CRE-BP1 (Hs ATF-2) and Atf21. Residues conserved among all three proteins are boxed and shaded residues are conserved between two of the proteins. MAPK phosphorylation sites in ATF-2 (Thr-69 and Thr-71) (Gupta et al. 1995; Livingstone et al. 1995; van Dam et al. 1995) are indicated by filled circles. Residues important for formation of the “leucine zipper” structure are marked by asterisks. GenBank accession nos.: S. pombe atf1+, U38237, atf21+, U51566; human ATF-2/CRE-BP1, X15875. Alignments were performed using GeneWorks software (IntelliGenetics). (B) Schematic representation of the structures of human (Hs) ATF-2 and S. pombe transcription factors carrying CREB/ATF-like bZIP domain, Atf1, Atf21, and Pcr1. Human ATF-2 carries a zinc finger motif in its amino-terminal domain.

**atf1+ and atf21+ encode transcription factors related to human ATF-2**

The atf1+ and atf21+ genes encode 65-kDa and 41-kDa proteins, respectively. The deduced primary sequences of Atf1 and Atf21 contain a carboxy-terminal bZIP structure that consists of a DNA-binding domain composed of basic residues followed by a leucine zipper structure (Fig. 4A). A data-base search revealed that Atf1 and Atf21 are most homologous to the human ATF-2/CRE-BP1 transcription factor (Hai et al. 1989; Maekawa et al. 1989). S. pombe Atf1 and Atf21 show 50% and 55% identity to ATF-2 in the bZIP domains, respectively. Weak similarity is also seen in the amino-terminal domains of human ATF-2 and Atf1, whereas, unlike ATF-2, neither of Atf1 nor Atf21 have a zinc finger motif (Fig. 4B).

**atf1+ is required for the cellular response to nitrogen starvation and sexual development**

Gene disruption experiments were performed to reveal the cellular functions of Atf1 and Atf21 (see Fig. 3A, B). FACScan analyses of cells revealed that Δatf1 cells were highly defective in undergoing G1 arrest upon nitrogen starvation (Fig. 5A). On the other hand, Δatf21 cells had
no defect in undergoing G₁ arrest. Homothallic Δatfl strains had a low mating frequency, whereas ΔatflΔΔspc1 cells appeared to be normal in mating and sporulation (data not shown). Δatfl and ΔΔspc1 mutants were also defective in meiosis, 31% of diploid cells homozygous for Δatfl and 20% of ΔΔspc1 diploids performed meiosis in sporulation medium, compared to a value of 78% with wild-type diploid cells. In addition, the Δatfl and ΔΔspc1 mutations suppressed meiosis induced by the temperature-sensitive patl1-114 mutation. patl1⁺, also known as ran1⁺, encodes a protein kinase [McLeod and Beach 1986] that regulates negatively the initiation of meiosis [Iino and Yamamoto 1985; Nurse 1985]. Growth of patl1-114 mutants at the restrictive temperature results in the initiation of meiosis, regardless of cell ploidy or the presence of nitrogen in the growth medium. It was found that Δatfl patl1-114 and ΔΔspc1 patl1-114 double mutants formed colonies of mitotically dividing cells at 32°C, whereas patl1-114 cells did not form colonies be-

cause they underwent lethal haploid meiosis (data not shown). The Δatfl conjugation defects are consistent with those recently described by Takeda and colleagues [1995]. Our findings suggest that upon nitrogen starvation Spc1 and Atfl function in the same pathway to promote mating and meiosis by bringing about G₁ cell cycle arrest and inducing ste11⁺ transcription.

Δatfl⁻ cells are defective in the response to osmostress

The findings described above, indicating that Atfl and Spc1 have important roles in mediating the cellular response to nitrogen starvation, prompted an examination of whether Atfl might also have a role in regulating the cellular response to osmotic stress. We found that Δatfl cells grew very poorly on medium supplemented with 1 M KCl [Fig. 5B], although the growth defect of Δatfl cells was less severe than that of ΔΔspc1 cells. Importantly, the osmosensitive growth caused by ΔΔspc1 was not enhanced in ΔΔspc1 Δatfl double mutants (data not shown), which implies that Atfl and Spc1 function in a linear pathway. The ΔatflΔΔspc1 mutation did not affect the high osmolality sensitivity of wild-type and Δatfl cells [Fig. 5B], indicating that Atfl does not have a major role in osmoregulation.

These findings suggested that Atfl may be involved in promoting transcription of stress response genes that are regulated by the Wis1–Spc1 signal transduction pathway, such as gpd1⁺, tsp1⁺, and pyp2⁺ [Miller et al. 1995; Degols et al. 1996]. Indeed, osmostress-induced expression of gpd1⁺, which encodes glycerol-3-phosphate dehydrogenase [Pidoux et al. 1990], was abolished in Δatfl cells (Fig. 5C). Expression of pyp2⁺ was similarly defective in Δatfl cells [Fig. 5C], indicating that Atfl is a part of the negative feedback loop in which Spc1-stimulated induction of pyp2⁺ expression attenuates Spc1 activity [Miller et al. 1995; Degols et al. 1996].

Atfl regulation by Spc1

Our genetic findings indicated that Atfl probably functions downstream of Spc1 in the regulation of sexual differentiation and stress response, therefore, we examined the biochemical relationship between Atfl and Spc1 kinase. To facilitate analysis of Atfl protein, the chromosomal atfl⁺ gene was tagged with a sequence encoding the hemagglutinin antigen [HA] epitope and six consecutive histidine residues [see Materials and Methods]. The resultant strain was indistinguishable from wild type, indicating that Atfl protein with the carboxy-terminal tag was fully functional [data not shown]. Atfl protein purified from unstressed wild-type cells migrated as a somewhat diffuse band with an apparent molecular mass of ~85 kD in SDS-PAGE [Fig. 6A, lane 1]. When cells were stressed in a medium supplemented with 0.6 M KCl for 10 min, Atfl migrated with a reduced mobility of ~90 kD (Fig. 6A, lane 2). In contrast, Atfl protein purified from a ΔΔspc1 strain migrated as a less diffuse ~80 kD protein species both before and during osmospres-
mobility shifts were caused by phosphorylation, Atf1 protein isolated from osmostressed wild-type cells was incubated with calf intestine alkaline phosphatase (CIP).

After phosphatase treatment, Atf1 protein migrated as a less diffuse protein species with faster mobility (Fig. 6A, lane 6), whereas no change was seen if phosphatase inhibitors were included in the incubation (Fig. 6A, lane 7).

As shown in Figure 6A, the amount of Atf1 protein recovered from a wild-type strain was two- to threefold higher than in a Δspc1 strain. Therefore, we examined whether atf1^+ mRNA expression was regulated by Spc1. Northern blotting analysis demonstrated that in unstressed conditions the level of atf1^+ mRNA was reduced ~50% in spc1^−M13 cells as compared with wild type (Fig. 6B, time 0). A transient increase in the amount of atf1^+ mRNA was observed when wild-type cells were stressed by 0.6 M KCl (Fig. 6B), whereas no increase in the amount of atf1^+ mRNA was observed in spc1−M13 mutant cells. This finding indicates that expression of atf1^+ mRNA is also regulated by Spc1, possibly through the regulation of Atf1 itself.

There is considerable evidence from studies of mammalian cells indicating that the stress-activated Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) phosphorylate ATF-2 directly (Gupta et al. 1995; Livingstone et al. 1995; van Dam et al. 1995). This conclusion is supported by the detection of stable in vitro binding between JNK1 and ATF-2. Interestingly, this binding is mediated by a region of ATF-2 that is separate from the sites of phosphorylation. We carried out experiments to evaluate whether the Spc1-dependent phosphorylation of Atf1, as established by the data shown in Figure 6A, might involve direct phosphorylation of Atf1 by Spc1 kinase. As a first step in this analysis, we asked whether Atf1 coprecipitated with a protein kinase. These studies were facilitated by expression of glutathione S-transferase (GST)-Atf1 fusion protein in S. pombe, which rescued both spc1^− and atf1^− osmosensitive phenotypes (data not shown). GST–Atf1 was purified by glutathione (GSH)–Sepharose affinity precipitation and then incubated with Mg^2+ and [γ-^32P]ATP (Fig. 7A). GST–Atf1 became highly phosphorylated in this assay, indicating that GST–Atf1 coprecipitated with a protein kinase that phosphorylates GST–Atf1 efficiently. Importantly, this phosphorylation was abolished when GST–Atf1 was expressed and recovered from Δspc1 cells (Fig. 7A).

These findings indicate that Spc1 or another kinase whose activity is dependent on Spc1 copurifies with and phosphorylates GST–Atf1. To distinguish between these possibilities, we first asked whether Spc1 was present in the GST–Atf1 samples prepared by GSH–Sepharose affinity precipitation. GST–Atf1 and unfused GST were expressed in a strain in which endogenous Spc1 was tagged with the HA epitope. GST–Atf1 and GST were detected in the GST–Atf1 sample but not with GST alone (Fig. 7B). Control experiments, in which GST–Atf1 was coexpressed with HA epitope-tagged forms of Wee1 kinase, Nim1 kinase, and Cig2 B-type cyclin, confirmed that the detection of HA-tagged Spc1 in GST–Atf1 sample was not attributable to a nonspecific interaction between the HA epitope tag and GST–Atf1 (data not shown).

These results supported the conclusion that Spc1 may be directly responsible for the phosphorylation of GST–Atf1 in the GSH–Sepharose precipitates. This possibility was explored further by comparing the two-dimensional tryptic phosphopeptide map of GST–Atf1 phosphorylated by its coprecipitating kinase with the map generated by direct phosphorylation of GST–Atf1 with purified Spc1 kinase. The GST–Atf1 isolated from Δspc1 cells was treated with irreversible protein kinase inhibitor p-flurosulfonyl-benzoyl 5'-adenosine (FSBA) (Zoller and Taylor 1979) to inactivate coprecipitating kinases, and then incubated with Mg^2+ and [γ-^32P]ATP together

Figure 6. (A) Spc1-dependent phosphorylation of Atf1 in vivo. Wild-type [KS1479, lanes 1,2,5-7] and Δspc1 [KS1572, lanes 3,4] strains carrying a chromosomal copy of epitope tagged atf1^+ were grown in YES medium at 30°C, and aliquots were harvested before [lanes 1,3] and after osmotic stress in YES + 0.6 M KCl for 10 min [lanes 2,4-7]. Atf1 protein tagged with HA epitopes and six His residues was purified on Ni-NTA beads and analyzed by SDS-PAGE followed by immunoblotting with anti-HA antibodies. For lanes 5-7, samples were incubated with buffer alone [lane 5], alkaline phosphatase (CIP, lane 6), or alkaline phosphatase and phosphatase inhibitors [CIP + inh, lane 7] before electrophoresis. (B) atf1^+ mRNA accumulates with osmotic stress in a spc1^−-dependent manner. Wild-type [PR109, solid bar] and spc1−M13 [KS1147, open bar] strains were treated with 0.6 M KCl as in Fig. 5C, and atf1^+ mRNA was examined by Northern blotting. Data were quantified and calibrated using a control blot with leu1^+ probe as a standard. Even before osmotic stress (at time 0), the level of atf1^+ mRNA detected in spc1^− cells was lower than wild type.
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Figure 7. (A) Spcl-dependent phosphorylation of Atfl by its coprecipitating protein kinase. GST–Atfl proteins were purified from spcl– (wt, KS1376) and Δspcl [KS1366] strains that had been grown in EMM + 0.6 M KCl for 10 min. GST–Atfl proteins were incubated in the presence of Mg2+ and [γ-32P]ATP and subjected to SDS-PAGE. Coomassie blue (CB) staining of purified GST–Atfl shows that approximately equal levels of GST–Atfl were recovered from the two strains. An autoradiogram of the gel shows that phosphorylation affects the electrophoretic mobility of GST–Atfl. Note that the phosphorylated GST–Atfl protein was detected in the GST–Atfl sample, and not detected in the GST control sample. (B) Physical interaction of Atfl and Spcl. GST and GST–Atfl fusion proteins expressed in KS1376 strains were precipitated using GSH-Sepharose beads. After extensive washes, proteins bound to the beads were analyzed by immunoblotting with anti-GST (upper) and anti-HA (lower) antibodies. SpclHA protein was detected in the GST–Atfl sample and not detected in the GST control sample. (C) Tryptic phosphopeptide maps of GST–Atfl proteins phosphorylated by a coprecipitating “Atfl kinase” (top), purified GST–Spcl [middle], and a mixture of the same amount of both samples [bottom]. 32P-labeled GST–Atfl proteins were subjected to trypsin digestion and spotted to origins shown by circles. Peptides were developed by electrophoresis at pH 1.9 (left, anode; right, cathode) in the first dimension, which is followed by ascending chromatography in the second dimension. Because Spcl does not phosphorylate GST in vitro [data not shown], all of the phosphopeptides in the middle panel are derived from Atfl. Note that the map of the top panel is more expanded than the others because of faster ascension of the chromatography buffer.

Spc1 is the protein kinase that coprecipitates with and phosphorylates GST–Atfl.

Atfl is not involved in G2–M cell cycle control

Our findings indicate that Atfl is a downstream target of Spcl that regulates G1 arrest, sexual development, and the osmotic stress response. However, unlike spcl– mutants that show a G2 cell-cycle delay, Δatfl cells divided at a wild-type cell length in both rich YES and minimal EMM2 media [data not shown]. This observation was confirmed by the following findings. The cell lengths at which Δatfl cdc25-22 double mutants and cdc25-22 single mutants divided were nearly identical at 25°C, a permissive temperature of cdc25-22 mutation, which is in contrast to cdc arrest phenotype exhibited by spcl– M13 cdc25-22 double mutant grown at 25°C [Fig. 8A]. Furthermore, the cdc phenotype of a spcl– M13 cdc25-22 wee1-50 triple mutant at 35°C [Shiozaki and Russell 1995] was not rescued by overexpression of atfl+ [data not shown]. These observations strongly suggest that the mitotic induction activity of Spcl is transmitted by an Atfl-independent pathway.

Bifurcation of the Spcl pathway was also implied by comparing the effects of Wis1 overproduction in Δspcl and Δatfl cells. Previous studies showed that Δspcl cells with GST–Spcl purified from S. pombe. This reaction led to extensive phosphorylation of GST–Atfl, and the two-dimensional tryptic phosphopeptide pattern of GST–Atfl phosphorylated by purified Spcl kinase was very similar to the pattern generated by analysis of GST–Atfl that had been phosphorylated by coprecipitating “Atfl kinase” [Fig. 7C]. A mixing experiment confirmed that the four major phosphopeptides [labeled 1–4 in Fig. 7C] in the two samples migrated with identical properties. These data provide strong evidence indicating that

Figure 8. (A) The Δatfl mutation does not cause a G2 cell-cycle delay. A cdc25-22 mutant strain (GL84) was crossed with spc1– M13 [KS1310] or Δatfl [KS1497] strains and segregants of cdc25-22, spc1– M13 cdc25-22, and Δatfl cdc25-22 were grown at 25°C. cdc25-22 and Δatfl cdc25-22 double mutant cells grew well at this temperature with a moderate elongated cell morphology, whereas spc1– M13 cdc25-22 cells showed a cdc arrest phenotype. Bar, 10 μm. (B) Phenotype of wild-type (wt; PR109), Δspcl [KS1366], and Δatfl [KS1497] strains upon Wis1 overexpression. Strains transformed with either pREPl vector or pREPl–wisl+ were streaked on an EMM z plate without thiamine to induce the expression of Wis1 from the nmt1 promoter. The plate was photographed after 3 days at 30°C. Wis1 overproduction severely impaired growth in wild type, had a somewhat less severe effect in Δatfl cells, and had very little effect on Δspcl cells.
are almost completely refractive to the lethal cell lysis phenotype caused by Wis1 overproduction (Shiozaki and Russell 1995). In contrast, Wis1 overproduction severely decreased the size of Δatf1 colonies, although Δatf1 colonies were larger than those of wild-type cells overproducing Wis1 [Fig. 8B]. Microscopic examination revealed that Wis1 overproduction severely deformed cell shape in Δatf1 cells but did not cause extensive lysis. The Δatf21 mutation had no affect on the toxicity of Wis1 overproduction [data not shown]. This result shows that the toxic effect of Spcl hyperactivation is not solely dependent on Atf1 or Atf21, nor presumably on genes, such as gpd1 + that are regulated by Atf1.

Discussion

Our results indicate that the Wis1–Spcl pathway regulates at least two different downstream targets [Fig. 9]. One branch proceeds by Atf1 to cause G1 arrest and induce ste11 + expression in response to nitrogen limitation, and to induce expression of genes such as gpd1 + and pyp2 + in response to cytotoxic stress. The second branch regulates the onset of mitosis. Thus, the Wis1–Spcl pathway bifurcates to regulate both meiosis and mitosis in response to changes in the extracellular environment.

Identification of a S. pombe ATF-2 homolog as a downstream element of the stress-activated Wis1–Spcl kinase cascade

Two structurally related genes, atf1 + and atf21 +, encoding bZIP transcription factors, have been identified as multicopy suppressors of the sterility and osmosensitivity of spc1 – cells. Atf1 and Atf21 are most homologous to human ATF-2/CRE-BP1 (Hai et al. 1989; Maekawa et al. 1989). Evidence presented in this paper strongly indicates that Atf1 is a downstream component of the Wis1–Spcl pathway. First, Δatf1 and Δspc1 mutants exhibit very similar stress and sexual development deficiencies. Both mutants show osmosensitive growth and fail to induce the expression of gpd1 + and pyp2 +. Δatf1 and Δspc1 cells do not arrest in G1 and show severe defects in sexual differentiation after nitrogen deprivation. Second, Δspc1 mutant phenotypes are not enhanced in Δspc1 Δatf1 double mutants. Third, overexpression of atf1 + complements the spc1 – defects. Fourth, Atf1 is phosphorylated upon stress in a spc1 +–dependent manner. Fifth, GST–Atf1 coprecipitates with a kinase that phosphorylates Atf1; this kinase activity is abolished in a spc1 – mutant. Sixth, the tryptic phosphopeptide map derived from GST–Atf1 phosphorylated by its coprecipitating kinase closely matches the pattern derived from GST–Atf1 phosphorylated by purified GST–Spcl. Finally, Spcl was detected in GST–Atf1 samples purified by GSH–Sepharose precipitation.

Human ATF-2 is phosphorylated on Thr-69 and Thr-71 by several kinases belonging to the extended MAP kinase family (Abdel-Hafiz et al. 1992; Gupta et al. 1995; Livingstone et al. 1995; Raingeaud et al. 1995, 1996; van Dam et al. 1995). Phosphorylation by MAP kinase increases in vitro DNA-binding activity of ATF-2, and alanine substitution of Thr-69 and Thr-71 abolishes the transcriptional activity of Gal4–ATF-2 fusion protein in vivo. ATF-2 activity is stimulated by UV and genotoxic agents, and bacterially produced GST–ATF-2 fusion protein binds to stress-activated kinases JNK/SAP kinase and p38. The identification of Atf1 as a downstream element of the Spcl signal transduction system further extends a similarity between yeast and mammalian stress-sensing MAP kinase pathways. Although MAP kinase cascades are conserved signal transduction modules, this is the first example that the structural similarity between the mammalian and yeast pathways has been detected to the level of downstream transcription factors. Because stress response is a basic and essential mechanism for cells to survive environmental changes, a high degree of conservation in the cellular mechanism to sense stress is perhaps not surprising.

Genes regulated by Atf1

Atf1 regulates the transcription of the stress response
gene gpd1\textsuperscript{+}, the pyp2\textsuperscript{+} tyrosine phosphatase gene, and the ste11\textsuperscript{+} gene that regulates the commitment to meiosis. It has also been reported that the glucose-repressible gene fbp1\textsuperscript{+}, which encodes fructose-1,6-bisphosphatase [Vassarotti and Friesen 1985], is also regulated by osis. It has also been reported that the glucose-repressible gene the response to glucose starvation {G. Degols and P. Russell, studies with ATF-2-deficient mice, which have severe chondrodysplasia and neurological abnormalities, have indicated that ATF-2 is required for stress-induced expression of the E-selectin gene but may not be required for expression of other genes whose promoters bind ATF-2 in vitro, including interferon \(\beta\) and c-jun [Reimold et al. 1996]. Like pyp2\textsuperscript{+}, transcription of genes encoding dual specificity phosphatases that are proposed to dephosphorylate MAP kinases are induced by various forms of stress [Keyse and Emslie 1992; Ishibashi et al. 1994]. More recent studies with ATF-2-deficient mice, which have severe chondrodysplasia and neurological abnormalities, have indicated that ATF-2 is required for stress-induced expression of the E-selectin gene but may not be required for expression of other genes whose promoters bind ATF-2 in vitro, including interferon \(\beta\) and c-jun [Reimold et al. 1996]. Like pyp2\textsuperscript{+}, transcription of genes encoding dual specificity phosphatases that are proposed to dephosphorylate MAP kinases are induced by various forms of stress [Keyse and Emslie 1992; Ishibashi et al. 1994, Liu et al. 1995]. It will be important to determine whether expression of these stress-induced MAP kinase phosphatase genes are regulated by ATF-2.

Induction of the Ste11 transcription factor is one of the early events in the process of sexual differentiation. Ste11 expression leads to transcription of a number of genes required for conjugation and meiosis, including the mating type genes and mei2\textsuperscript{+} [Sugimoto et al. 1991]. Expression of ste11\textsuperscript{+} is regulated negatively by the cAMP-dependent protein kinase [PKA] pathway [Sugimoto et al. 1991]. Nitrogen deprivation from the growth medium lowers the intracellular concentration of cAMP [Mochizuki and Yamamoto 1992], which leads to derepression of ste11\textsuperscript{+}. We have shown that nitrogen starvation also induces activation of Spc1 kinase and Spc1 is required for ste11\textsuperscript{+} transcription. Spores are extremely resistant to all forms of stress, thus it may be significant that S. pombe uses a single MAP kinase pathway to regulate induction of stress response genes and to promote initiation of sexual development. Counteracting activities of stress-activated MAP kinase homologs and PKA in transcriptional regulation has also been reported in budding yeast [Schüller et al. 1994], although it is not known which transcription factors are involved.

The Wis1–Spcl–Atf1 pathway is also essential to establish a G\textsubscript{2}–like quiescent state with nutritional deprivation. Cells lacking any component of the pathway quickly lose viability after they enter stationary phase [Warbrick and Fantes 1991; Takeda et al. 1995]. This spc1\textsuperscript{−} defect is complemented by overexpression of atf1\textsuperscript{+} [K. Shiozaki and P. Russell, unpubl.]. An important goal of future studies will be to identify the Atf1-regulated genes that are required for the asexual response to nutritional starvation.

Other downstream targets of Spc1
Unlike Δspc1, the Δatf1 mutation does not cause a G\textsubscript{2} delay or synthetic lethality in a cdc25-22 background, implying that a distinct branch of the pathway mediates the mitotic induction activity of Spc1. Little is known about this branch of the signal transduction pathway. It is possible that a protein kinase cascade extends downstream of Spc1, as p38 kinase, a mammalian homolog of Spc1, has been implicated as an activator of MAPK-associated protein kinase-2 [Freshney et al. 1994; Rouse et al. 1994]. The cdc arrest phenotype of a spc1–M13 cdc25-22 wee1-50 triple mutant at 35°C suggests that Spc1 can function independently of Wee1 and Cdc25 to regulate mitosis [Shiozaki and Russell 1995]. Mutational inactivation of Mkl1, a second kinase that inhibits Cdc2 [Lundgren et al. 1991], had no effect in a spc1–M13 strain, indicating that Mkl1 is not regulated by Spc1. The substate of Spc1 in this pathway could be another transcription factor, although Atf21 does not appear to be involved, as Δatf21 cells show no cell-cycle defect (data not shown). One might predict that such a transcription factor would be a high-copy suppressor of the spc1\textsuperscript{−} cdc25-22 synthetic lethal interaction, therefore experiments to identify such a gene are currently under way.

Materials and methods

Yeast strains and general techniques
Schizosaccharomyces pombe strains used in this study are listed in Table 1. They are derivatives of 972h\textsuperscript{−} and 975h\textsuperscript{−} [Mitchison 1970]. Growth media and basic genetic and biochemical techniques for fission yeast have been described [Alfa et al. 1993]. Yeast extract medium YES and synthetic minimal medium EMM\textsubscript{2} were used in growing S. pombe cells.

Purification and detection of Spc1HA\textsubscript{6}H protein
In strain KS1376 the genomic copy of spc1\textsuperscript{+} is tagged with a sequence encoding two copies of the HA epitope and six consecutive histidine [Shiozaki and Russell 1995]. KS1376 was grown on EMM\textsubscript{2} medium at 30°C to midlog phase. Cells were harvested and washed three times on a filtration apparatus with the same medium but lacking the nitrogen source NH\textsubscript{4}Cl [EMM\textsubscript{2}-N], then reincubated in EMM\textsubscript{2}-N at 30°C. Aliquots of cells were harvested by filtration and Spc1HA\textsubscript{6}H protein was purified on Ni\textsuperscript{2+}-NTA–agarose beads [Qiagen Inc.] as described [Shiozaki and Russell 1995]. Immunoblotting was performed using anti-HA [12CA5] and anti-phosphotyrosine (4G10, Upstate Biotechnology) monoclonal antibodies.

Isolation and disruption of atf1\textsuperscript{+} and atf21\textsuperscript{+} genes
Strain KS1440 was transformed with a S. pombe genomic library in pUR19 [Barbet et al. 1992] and a S. pombe cDNA library constructed using the pREP3 vector in which expression of insert CDNA is regulated by the nmt1 promotor [Maundrell 1993]. Transformants were plated onto EMM\textsubscript{2} [3% glucose] agar plates, and then replica-plated onto 1% glucose EMM\textsubscript{2} plates. Cells transformed with the cDNA library were plated on EMM\textsubscript{2} supplemented with 1 μM thiamine. After incubation at 26°C for 4 days, sporulating colonies were identified by staining with iodine vapor. Three of ~70,000 cDNA library transformants and two of ~50,000 genomic library transformants showed plasmid-dependent sporulation. Recovered plasmids were analyzed by PCR and DNA hybridization. Plasmids p2B from the cDNA
Table 1.  S. pombe strains used in this study (all strains are leu1-32 ura4-D18)

| Strains | Genotype | Source or reference |
|---------|----------|---------------------|
| PR109   | h-       | Lab stock           |
| PR636   | h<sup>90</sup> | Lab stock           |
| CHP428  | h<sup>+</sup> his7-366 ade6-M210 | C.S. Hoffman (Boston College, Chestnut Hill, MA) |
| CHP429  | h<sup>+</sup> his7-366 ade6-M216 | C.S. Hoffman |
| GL84    | h<sup>+</sup> cdc25-22 | Lab stock |
| LW117   | h<sup>+</sup> mil1HA6H(ura4<sup>+</sup>) | L. Wu and P. Russell, unpubl. |
| FY114   | h<sup>+</sup> pat1-114 | S.L. Forsburg (Salk Institute, La Jolla, CA) |
| JM544   | h<sup>-</sup> wis1::ura4<sup>+</sup> | Lab stock |
| KS1172  | h<sup>-</sup> pat1-114 spc1-M13 | Shiozaki and Russell 1995 |
| KS1310  | h<sup>-</sup> spc1-M13 | Shiozaki and Russell 1995 |
| KS1366  | h<sup>-</sup> spc1::ura4<sup>+</sup> | Shiozaki and Russell 1995 |
| KS1374  | h<sup>-</sup> spc1-M13 cdc25-22 wee1-50 | Shiozaki and Russell 1995 |
| KS1376  | h<sup>-</sup> spc1HA6H(ura4<sup>+</sup>) | Shiozaki and Russell 1995 |
| KS1440  | h<sup>90</sup>spc1-M13 | This study |
| OM1442  | h<sup>-</sup> HA-cig2<sup>+</sup> | Mondesert et al. 1996 |
| KS1479  | h<sup>-</sup> atf1HA6H(ura4<sup>+</sup>) | This study |
| KS1497  | h<sup>-</sup> atf1::ura4<sup>+</sup> | This study |
| KS1501  | h<sup>90</sup> atf1::ura4<sup>+</sup> | This study |
| KS1523  | h<sup>-</sup> atf1::ura4<sup>+</sup> cdc25-22 | This study |
| KS1533  | h<sup>-</sup> atf1::ura4<sup>+</sup> spc1::ura4<sup>+</sup> | This study |
| KS1542  | h<sup>-</sup> atf1::ura4<sup>+</sup> pat1-114 | This study |
| KS1557  | h<sup>-</sup> atf21::ura4<sup>+</sup> | This study |
| KS1562  | h<sup>-</sup> his7-366 ade6-M210 atf1::ura4<sup>+</sup> | This study |
| KS1565  | h<sup>-</sup> atf1::ura4<sup>+</sup> atf21::ura4<sup>+</sup> | This study |
| KS1570  | h<sup>-</sup> his7-366 ade6-M216 atf1::ura4<sup>+</sup> | This study |
| KS1572  | h<sup>-</sup> spc1::ura4<sup>+</sup> atf1HA6H (ura4<sup>+</sup>) | This study |
| KS1575  | h<sup>-</sup> his7-366 ade6-M210 spc1::ura4<sup>+</sup> | This study |
| KS1578  | h<sup>-</sup> his7-366 ade6-M216 spc1::ura4<sup>+</sup> | This study |
| KS1579  | h<sup>90</sup> atf21::ura4<sup>+</sup> | This study |
| LW1585  | h<sup>-</sup> wee1HA | L. Wu and P. Russell, unpubl. |

library and p4U from the genomic library, which did not hybridize to spc1<sup>+</sup> probe, were subjected to further analysis. Genomic clone of atf1<sup>+</sup> (p5A) was isolated from the genomic library in pUR19 by hybridization screening using a 1.2-kb insert of p2B as a probe. For the atf1<sup>+</sup> and atf21<sup>+</sup> gene disruption, a diploid strain constructed from CHP428 and CHP429 was transformed with the construct shown in Figure 3 carrying the ura4<sup>+</sup> marker gene (Grimm et al. 1988). Stable Ura<sup>+</sup> transformants were selected and disruption of one of atf1<sup>+</sup> or atf21<sup>+</sup> loci in the diploids was confirmed by Southern hybridization. After sporulation, haploid Ura<sup>+</sup> segregants were back-crossed with a wild-type strain [PR109] and used for further experiments.

**Northern blotting**

RNA isolation and detection of gpd1<sup>+</sup> and pyp2<sup>+</sup> mRNAs by hybridization have been described [Degols et al. 1996]. The stel1<sup>+</sup> coding sequence was amplified by PCR using S. pombe genomic DNA as a template with a pair of primers XH-ST11 (5'-CCGCTGGACTTCTATTAACCGCGACCA-3') and ST11-NT (5'-TGCGGGCCTAAATGACATGATTGCC-3'). This PCR product was cloned into pCRII and used as a probe. The atf1<sup>+</sup> cDNA clone p2B was used as a probe to detect atf1<sup>+</sup> mRNA. Quantification of the results was performed using PhosphorImager [Molecular Dynamics].

**Meiotic assay of diploid strains**

Diploid strains were constructed by interrupted mating of wild-type CHP428 and CHP429, the isogenic atf1 derivatives KS1562 and KS1570, and the isogenic spc1 derivatives KS1575 and KS1578. Diploids were maintained by selection for adenine prototrophy generated by complementation between ade6-M210 and ade6-M216 alleles. Meiosis was induced by incubating diploid cells on sporulation medium malt extract for 2 days at 30°C. Cells were stained by 4',6-diamidino-2-phenylindole (DAPI) and observed under the fluorescence microscope. Frequency of meiosis/sporulation was calculated according to a previously described equation [Kunitomo et al. 1995].

**Expression of Atf1HA6H protein**

The -0.6-kb sequence encoding the carboxyl terminus of Atf1 [residues 363–566] was amplified by PCR with 5' primers NDE-363 (5'-GGAATTCCATATGCCATCTGTTTACCGCGATAC-3') and Ndel restriction site is underlined) and 2B-NOT (5'-TAGTTTAGCGGCCGCCTACCTAAATTGATTTTTGACC-3', NotI restriction site is underlined). After digesting with Ndel and NotI, this fragment was cloned into pRP42–HA6H, a derivative of pREP1–HA6H (Wu and Russell 1993) to add a carboxy-terminal tag of two copies of HA epitope and six consecutive histidine residues. The resultant plasmid was digested by Psfl and Ndel and religated to eliminate the nmtI promoter sequence, then used for transformation of wild-type S. pombe strain [PR109] after being linearized at the EcORI site in atf1<sup>+</sup>. Stable Ura<sup>+</sup> transformants were selected and integration of the plasmid at atf1<sup>+</sup> locus was confirmed by Southern hybridization. The Atf1HA6H construct is expressed from atf1<sup>+</sup> promoter. Atf1HA6H was purified and detected by the procedures used for Spc1HA6H [see above]. Phosphatase treatment of the
Atf1HA protein was performed with CIP [Boehringer Mannheim] following a published procedure (Lanker et al. 1996).

Expression of GST–Atf1 fusion protein

The atf1 + ORF was amplified by PCR with primers NDE-S1 [5’-GGATCCCATATGGTCCCCGCTCTCAGGTTAATCCCTCCAC-3’, Ndel restriction site is underlined] and A1-NOT [5’-TAATTCACAGCGCCGCTCTAGTACCTAACCAAATTT-3’, NotI restriction site is underlined and cloned into the Ndel–NotI sites of pREP1-KZ, which expresses GST fusion constructs from the nmt1 promoter. The resultant plasmid pREP1-KZ–atf1 + and a control plasmid pLl205 [Leatherwood et al. 1996], which expresses unfused GST from the nmt1 promoter, were used to transform strain KS1376, which expresses Spc1HA6H [Shiozaki and Russell 1995]. Plasmids pREP1-KZ–atf1 + and pLl205 were also transformed into strain LW1585, which expresses Wecl kinase tagged with three copies of the HA epitope; strain LW117, which expresses Nim1 kinase tagged with two copies of HA and six histidine residues; and strain OM1442, which expresses Gig2 B-type cyclin with a tag of three copies of HA (Mondesert et al. 1996). Expression from the nmt1 promoter was induced by thiamine depletion. GST and GST–Atf1 proteins were precipitated using glutathione [GSH]–Sephrose following the procedures described previously [Shiozaki and Russell 1995]. Proteins bound to the Sepharose beads were analyzed by immunoblotting with anti-GST and anti-HA antibodies.

In vitro phosphorylation of GST–Atf1

GST–Atf1 protein from strains KS1376 [spc1HA6H/ura4 +] and KS1366 [Δspc1] incubated in a medium containing 0.6 M KCl for 10 min was recovered on GSH–Sephrose beads as described above and washed three times in KA buffer [25 mM Tris-HCl (pH 7.2), 10 mM MgCl2, 0.1 mM EDTA, 0.1 mM Na3VO4, 1 mM DTT]. The phosphorylation reaction by the coprecipitated protein kinase was performed in the same buffer containing 50 μM [γ-32P]ATP for 10 min at 25°C. For in vitro phosphorylation of Atf1 by purified Spc1 kinase, GST–Atf1 was purified by GSH–Sephrose precipitation from strain KS1366 and subjected to three successive treatments by 1 mM FSBA at 30°C for 15 min in KA buffer without dithiothreitol (DTT). The Sepharose beads were then washed three times with KA buffer to eliminate the FSBA. GST–Spc1 protein expressed in wild-type PR109 strain (Shiozaki and Russell 1995) was purified on GSH–Sephrose beads and added to FSBA-treated GST–Atf1. The mixture was incubated at 30°C for 20 min in KA buffer supplemented with 5 mM glutathione and 50 μM [γ-32P]ATP. Phosphorylation of GST–Atf1 was analyzed by SDS-PAGE and the 32P-labeled GST–Atf1 bands were excised after autodigestion. Tryptic peptide mapping was performed as described [Boyle et al. 1991] using pH 1.9 buffer for the first dimension electrophoresis and phosphorochromatography buffer for the second dimension ascending chromatography.

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Note added in proof

The atf1 + gene was also recently independently identified as gad7 + by Kanoh et al. [1996].

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