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# Mcs4 Mitotic Catastrophe Suppressor Regulates the Fission Yeast Cell Cycle through the Wik1-Wis1-Spc1 Kinase Cascade

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Spc1 in *Schizosaccharomyces pombe* is a member of the stress-activated protein kinase family, an evolutionary conserved subfamily of mitogen-activated protein kinases (MAPKs). Spc1 is activated by a MAPK kinase homologue, Wis1, and negatively regulated by Pyp1 and Pyp2 tyrosine phosphatases. Mutations in the $spc1^+$ and $wis1^+$ genes cause a $G_2$ cell cycle delay that is exacerbated during stress. Herein, we describe two upstream regulators of the Wis1-Spc1 cascade. $wik1^+$ (Wis1 kinase) was identified from its homology to budding yeast *SSK2*, which encodes a MAPKK kinase that regulates the *HOG1* osmosensing pathway. Δ*wik1* cells are impaired in stress-induced activation of Spc1 and show a $G_2$ cell cycle delay and osmosensitive growth. Moreover, overproduction of a constitutively active form of Wik1 induces hyperactivation of Spc1 in a $wis1^+$-dependent manner, suggesting that Wik1 regulates Spc1 through activation of Wis1. A mutation of $mcs4^+$ (mitotic catastrophe suppressor) was originally isolated as a suppressor of the mitotic catastrophe phenotype of a *cdc2–3w wee1–50* double mutant. We have found that $mcs4^-$ cells are defective at activation of Spc1 in response to various forms of stress. Epistasis analysis has placed Mcs4 upstream of Wik1 in the Spc1 activation cascade. These results indicate that Mcs4 is part of a sensor system for multiple environmental signals that modulates the timing of entry into mitosis by regulating the Wik1-Wis1-Spc1 kinase cascade. Inactivation of the sensor system delays the onset of mitosis and rescues lethal premature mitosis in *cdc2–3w wee1–50* cells.

## INTRODUCTION

Recent progress in the study of eukaryotic cell cycle regulation has succeeded in attributing the initiation of mitosis to a biochemical process, activation of Cdc2-cyclin B protein kinase complex. During interphase, Cdc2-cyclin B activity is maintained at a low level due to inhibitory phosphorylation carried out by Wee1 and related protein kinases. Activation of Cdc2-cyclin B at the $G_2$-M boundary is performed by Cdc25 phosphatase, which removes inhibitory phosphates from Cdc2 (Dunphy, 1994). Genetic analysis in the fission yeast *Schizosaccharomyces pombe* has played a leading role in the identification of Cdc2, Cdc25, Wee1, and other key regulators of mitosis, in large part because growth and division of *S. pombe* cells are tightly coupled and, therefore, defects in cell cycle regulation are often easily visualized as changes in cell size. $G_2$ cell cycle arrest caused by loss of functional Cdc2 or Cdc25 produces a highly elongated cell morphology, whereas cells carrying $wee1^-$ or dominant *cdc2-w* mutations divide at a shorter cell length than wild-type cells, producing the wee phenotype. Combinations of *cdc2–3w* and temperature-sensitive *wee1–50* mutation or overproduction of Cdc25 in *wee1–50* mutant cells bring about a more extreme version of the wee phenotype known as mitotic catastrophe (Russell and Nurse, 1986, 1987b). The lethality observed in cells undergoing mitotic catastrophe is believed to result from the uncoupling of the onset of mitosis from the completion of DNA replication.

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Several genetic screens have been performed to identify additional regulators of the central cell cycle machinery in fission yeast. A number of candidate cell cycle regulatory genes have been found, although the biochemical mechanisms by which most of them function remain largely unknown. Two notable exceptions are Nim1 and Mik1 protein kinases. Nim1 kinase, also known as Cdr1 (Russell and Nurse, 1987a; Feilotter *et al.*, 1991), promotes mitosis by performing inhibitory phosphorylation of Wee1 (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). Mik1 was first identified as a multicopy suppressor of *cdc2–3w wee1–50* mitotic catastrophe, subsequent studies showed that Mik1 is a functional homologue of Wee1 (Lundgren *et al.*, 1991; Lee, M.S. *et al.*, 1994). Mutants lacking Mik1 activity have no phenotype, but simultaneous mutational inactivation of Wee1 and Mik1 causes mitotic catastrophe, showing that Wee1 and Mik1 share an essential function in preventing lethal premature activation of Cdc2-cyclin B kinase. Six *mcs* genes were identified as extragenic suppressor mutations of the mitotic catastrophe phenotype in a *cdc2–3w wee1–50* double mutant (Booher and Beach, 1987; Molz *et al.*, 1989). Only $mcs2^+$ and $mcs6^+$ genes have been cloned so far: $mcs2^+$ encodes a protein related to cyclins (Molz and Beach, 1993), and $mcs6^+$, also known as $crk1^+$ and $mop1^+$, encodes a protein kinase that forms a functional complex with Mcs2 (Buck *et al.*, 1995; Damagnez *et al.*, 1995). The $mcs4^+$ gene, defined by the single recessive allele *mcs4–13*, is of particular interest, because *mcs4–13* cells exhibit a nutrition-sensitive cell cycle delay in $G_2$. Growth of *mcs4–13* cells in synthetic minimal medium leads to a significant cell elongation that is not observed with *mcs4–13* cells grown in rich yeast extract medium, implying a role for $mcs4^+$ gene product in mediating a nutritional signal to the cell cycle machinery (Molz *et al.*, 1989).

Recently, a group of mitotic regulator genes in *S. pombe* were shown to encode elements of a stress and nutrient-sensing signal transduction pathway. These include $wis1^+$ (Warbrick and Fantes, 1991, 1992), $pyp1^+$ (Ottilie *et al.*, 1991), $pyp2^+$ (Millar *et al.*, 1992; Ottilie *et al.*, 1992), and $spc1^+/sty1^+/phh1^+$ (Millar *et al.*, 1995; Shiozaki and Russell, 1995a; Kato *et al.*, 1996). The studies leading to the discovery of $wis1^+$ were rooted in the observation that $cdc25^-$ mutations, in particular the temperature-sensitive *cdc25–22* allele, are suppressed by $wee1^-$ mutations (Fantes, 1979; Russell and Nurse, 1986). A mutation, *win1–1*, was found to reverse the suppression of *cdc25–22* by $wee1^-$ mutations (Ogden and Fantes, 1986). Interestingly, the *win1–1* effect was specific for cells grown in synthetic minimal medium, *cdc25–22* $wee1^-$ *win1–1* cells incubated at the restrictive temperature underwent cell cycle arrest in minimal medium but were viable in rich medium. The $wis1^+$ gene was one of five multicopy suppressors of the *cdc25–22* $wee1^-$ *win1–1* cell cycle defect (Warbrick and Fantes, 1991). $wis1^+$ encodes a protein kinase most similar to *Saccharomyces cerevisiae* Pbs2p, a mitogen-activated protein kinase kinase (MAPKK) homologue that functions in the osmosensing pathway (Boguslawski, 1992; Brewster *et al.*, 1993). Wis1 phosphorylates and activates a mitogen-activated protein kinase (MAPK) encoded by $spc1^+$, a gene also known as $sty1^+$ and $phh1^+$, which belongs to a family of evolutionary conserved stress-activated protein kinases including *S. cerevisiae* Hog1 (Brewster *et al.*, 1993), murine p38 (Han *et al.*, 1994), human CSBP1 (Lee, J. *et al.*, 1994), and *Xenopus* Mpk2 (Rouse *et al.*, 1994). The Wis1-Spc1 MAPK cascade is activated by high osmolarity, oxidative stress, heat shock, and nutritional limitation by nitrogen starvation or growth in synthetic minimal medium (Millar *et al.*, 1995; Shiozaki and Russell, 1995a; Degols *et al.*, 1996; Shiozaki and Russell, 1996). Loss of functional $wis1^+$ or $spc1^+$ leads to a $G_2$ cell cycle delay, which is accentuated under stress conditions and in minimal medium (Warbrick and Fantes, 1991; Millar *et al.*, 1995; Shiozaki and Russell, 1995a). On the other hand, $pyp1^-$ and $pyp2^-$ mutants undergo advanced initiation of mitosis (Millar *et al.*, 1992; Ottilie *et al.*, 1992) because of high Spc1 activity (Shiozaki and Russell, 1995a; Degols *et al.*, 1996). It has been proposed that the Wis1-Spc1 pathway links the control regulating the onset of mitosis with changes in the extracellular environment that affect cell physiology.

The upstream components regulating the Wis1-Spc1 pathway in response to environmental signals have not been identified. In *S. cerevisiae*, the Pbs2p-Hog1p pathway is activated by Ssk2p/Ssk22p MAPKK kinases (MAPKKKs), which are under the regulation of a two component osmosensor composed of Sln1p histidine kinase, Ypd1p, and a response regulator, Ssk1p (Maeda *et al.*, 1994; Posas *et al.*, 1996). Pbs2p is also regulated by Sho1p, which contains a SH3 domain that is believed to directly interact with the N-terminal region of Pbs2p (Maeda *et al.*, 1995). However, unlike the fission yeast Spc1 pathway, the Hog1 pathway in budding yeast is reported to be unresponsive to forms of stress other than high osmolarity (Schüller *et al.*, 1994) and is not implicated in cell cycle regulation.

With the goal of understanding the mechanism that links environmental signals and cell cycle control, we have sought to identify genes encoding proteins functioning upstream of Wis1 and downstream of Spc1. One of these screens led to the identification of $atf1^+$, encoding a transcription factor similar to mammalian ATF-2, as a key substrate of Spc1 kinase (Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996). Herein we describe two genes encoding upstream elements of the signal transduction pathway. $wik1^+$ encodes a MAPKKK homologue closely related to *S. cerevisiae* Ssk2p/Ssk22p. Wik1 plays a crucial role in the activation of

Spc1. Genetic and biochemical studies have revealed that one of the mitotic catastrophe suppressor genes, *mcs4*$^+$, encodes a protein acting upstream of Wik1. Our data strongly suggest that *mcs4*$^+$ gene product is a component of the sensor system monitoring multiple environmental signals and regulates the $G_2$-M transition through the Wik1-Wis1-Spc1 kinase cascade.

## MATERIALS AND METHODS

### *S. pombe Strains and General Techniques*

All strains used in this study (Table 1) are derivatives of *972h*$^-$ and *975h*$^+$ (Mitchison, 1970). Yeast extract medium YES and synthetic minimal medium EMM2 were used for *S. pombe* cells (Moreno *et al.*, 1991; Alfa *et al.*, 1993). Basic yeast techniques and procedures for manipulation of nucleic acids have been described (Sambrook *et al.*, 1989; Alfa *et al.*, 1993). Cell length measurements were performed with early logarithmic phase cultures in YES and EMM2. Twenty septated cells were measured using a 15× eyepiece with a micrometer and a 100× objective lens.

### *wik1*$^+$ *Gene Disruption*

The DNA sequence of *wik1*$^+$ was compiled from residues 1985 to 6189 of cosmid c9G1, sequenced as part of the *S. pombe* chromosome I sequencing project at the Sanger Centre (Cambridge, United Kingdom), with corrections by our DNA sequence determination of the *wik1*$^+$ gene. The DNA sequence of the *wik1*$^+$ gene encoding amino acid residues 935-1401 was amplified by polymerase chain reaction (PCR) with a pair of primers WK1 (5′-GGAAGA TCTTATGGCTTT-TCTGTTAACGCATTTCG-3′, *Bgl*II restriction site is underlined) and WK2 (5′-GGAAGATCTTAGTCAACACTATAGTTTATTGTGTC-3′, *Bgl*II restriction site is underlined) and wild-type *S. pombe* genomic DNA as template. Amplified DNA fragment was cloned into pCRII (Invitrogen, San Diego, CA), and a 1.8-kb fragment of the *ura4*$^+$ marker gene (Grimm *et al.*, 1988) was inserted into the *Nde*I restriction site of the *wik1*$^+$ fragment (see Figure 2). The resultant plasmid was digested by *Bgl*II to release the *wik1::ura4*$^+$ fragment and then used to transform wild-type (PR109) cells. Stable Ura$^+$ transformants were selected and disruption of *wik1*$^+$ was confirmed by PCR and Southern blot hybridization with genomic DNA isolated from the transformants. Backcross experiments showed cosegregation of the uracil prototrophy and osmosensitive growth phenotype.

### *Overexpression of Wik1ΔN Protein and Detection of Spc1 Tyrosine Phosphorylation*

The PCR-amplified 1.4-kb fragment of *wik1*$^+$ described above was cloned into the *Bam*HI site of pREP1 vector, which expresses inserted genes from the thiamine-repressible *nmt1* promoter (Maundrell, 1990). The resultant plasmid pREP1-*wik1*ΔN and empty pREP1 vector were used to transform wild-type (KS1376), Δ*wis1* (GD1682), and *mcs4–13* (KS1559) strains, which carry a chromosomal copy of *spc1*$^+$ tagged with a sequence encoding two copies of hemagglutinin (HA) epitope and six consecutive histidine residues, otherwise known as the HA6H tag (Shiozaki and Russell, 1995a). Transformants were grown in EMM2 supplemented with 1 μM thiamine and then incubated in EMM2 lacking thiamine for 16 h to induce expression from the *nmt1* promoter. Spc1 protein was purified using Ni-NTA-agarose (QIAGEN, Chatsworth, CA) under denaturing condition as described previously (Shiozaki and Russell, 1995), which was followed by immunoblotting with anti-HA (12CA5) and anti-phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY) monoclonal antibodies.

**Table 1.** *S. pombe* strains used

| Strain | Genotype | Source or reference |
|---|---|---|
| PR100 | *h*$^+$ *leu1 ura4* | Laboratory stock |
| PR109 | *h*$^-$ *leu1 ura4* | Laboratory stock |
| JM544 | *h*$^-$ *leu1 ura4 wis1::ura4*$^+$ | Laboratory stock |
| SP849 | *h*$^-$ *mcs4-13* | Molz *et al.*, 1989 |
| KS1376 | *h*$^-$ *leu1 ura4 spc1HA6H(ura4*$^+$*)* | Shiozaki and Russell, 1995a |
| KS1559 | *h*$^-$ *leu1 ura4 mcs4-13 spc1HA6H(ura4*$^+$*)* | This study |
| KS1598 | *h*$^-$ *leu1* | Laboratory stock |
| KS1664 | *h*$^-$ *leu1 ura4 wik1::ura4*$^+$ | This study |
| KS1667 | *h*$^-$ *leu1 ura4 spc1HA6H(ura4*$^+$*) wik1::ura4*$^+$ | This study |
| KS1669 | *h*$^-$ *leu1 ura4 mcs4-13 wik1::ura4*$^+$ | This study |
| KS1675 | *h*$^-$ *leu1 mcs4-13* | This study |
| GD1682 | *h*$^-$ *leu1 ura4 spc1HA6H(ura4*$^+$*)wis1::ura4*$^+$ | G. Degols |

All alleles of *leu1* and *ura4* are *leu1-32* and *ura4-D18*, respectively.

### *Stress Treatment of S. pombe Cells*

Wild-type (KS1376), Δ*wik1* (KS1667), and *mcs4–13* (KS1559) strains were grown to early logarithmic phase in YES medium at 30°C. For high-osmolarity stress, a one-third volume of prewarmed YES containing 2.4 M KCl was added to the culture, producing a final KCl concentration of 0.6 M. For oxidative stress, 7.5 μl of 30% (wt/wt) hydrogen peroxide solution (Sigma, St. Louis, MO) was added to a 100-ml culture. For heat stress, cultures at 30°C were shifted to a 48°C water bath and incubated for 10 min with shaking. For carbon starvation, logarithmic phase cultures ($OD_{600}$ = ~0.5) of KS1376 and KS1559 in EMM2 at 30°C were harvested and washed three times with EMM2 medium without glucose (EMM2-C) on a filtration apparatus. Cells were reinoculated into prewarmed EMM2-C at a cell concentration of $OD_{600}$ = 0.5. In all experiments, cells were harvested by filtration and immediately frozen in liquid nitrogen.

## RESULTS

### *Identification of wik1*$^+$ *as a Homologue of Budding Yeast SSK2*

To identify a MAPKKK homologue activating Wis1 MAPKK, we performed a computer search for *S. cerevisiae SSK2* homologues in the *S. pombe* genome sequences submitted to the European Molecular Biology Laboratory database by the Sanger Center. One of the open reading frames in cosmid c9G1 (Gentles, Hunt, Churcher, and Barrell, unpublished data), derived from chromosome I, was found to encode a protein highly homologous to *SSK2* (Figure 1). This putative MAPKKK gene, named *wik1*$^+$ (Wis1 kinase), has a 4203-bp uninterrupted open reading frame encoding a protein of 1401 amino acids with a calculated molec-

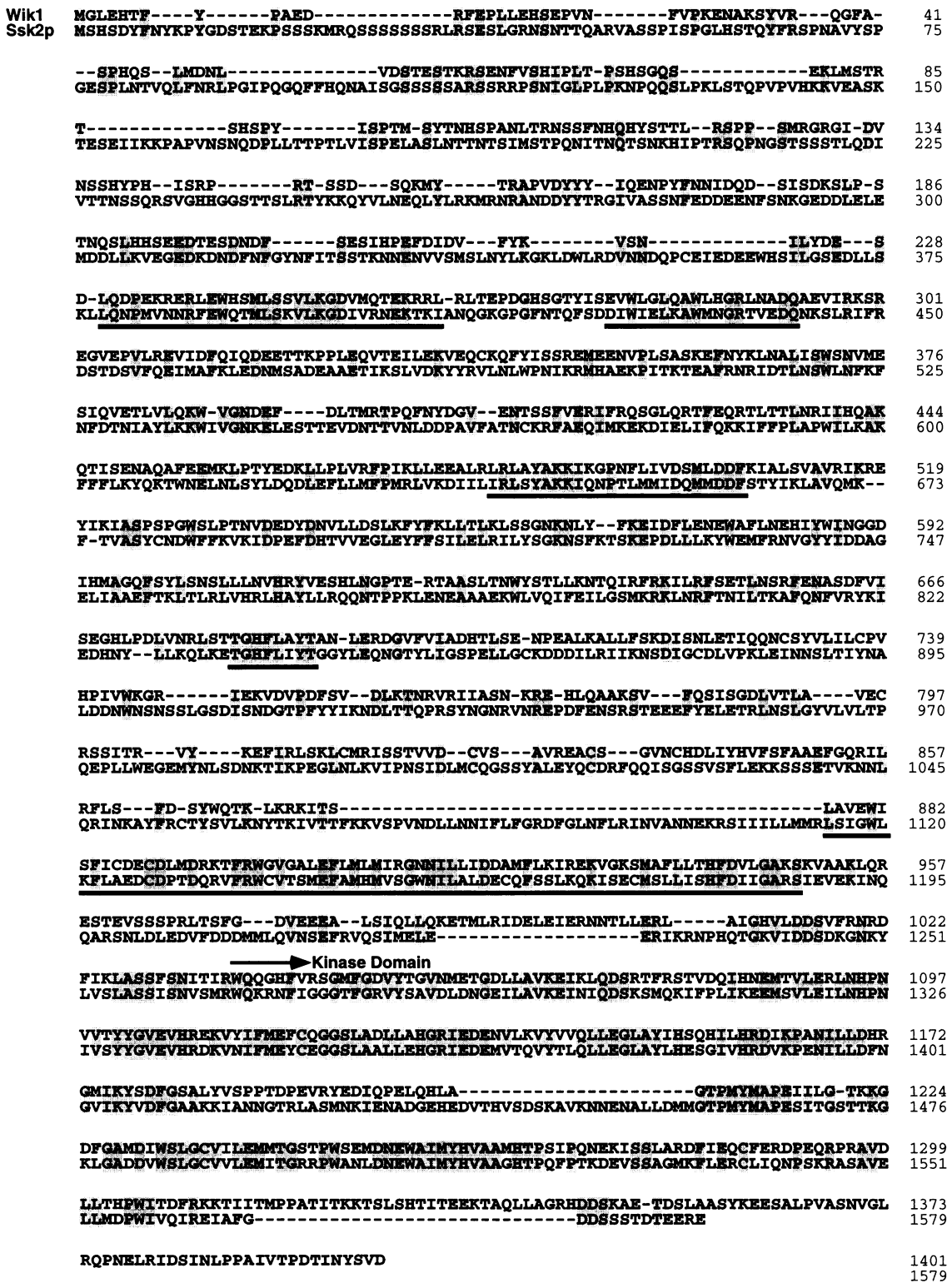


**Figure 1.** Predicted amino acid sequence of *S. pombe* Wik1 and its budding yeast homologue Ssk2p. The alignment was performed by the GeneWorks program (IntelliGenetics, Mountain View, CA) and identical residues are shaded. Both proteins have a kinase domain in the C-terminal region (starting at the position indicated by the arrow). Regions of high sequence similarity in the N-terminal noncatalytic domain are underlined. The GenBank accession number of the *wik1*$^+$ DNA sequence is U81521.

ular weight of 161 kDa. The protein kinase domain of Wik1 (residues 1037 to the C terminus) shares high similarity with members of the MAPKKK family, including budding yeast Ssk2p (43% identity), Ssk22p (40%; Maeda *et al.*, 1995), and Bck1p (31%; Lee and Levin, 1992) and fission yeast Byr2 (28%; Wang *et al.*, 1991). On the other hand, the N-terminal noncatalytic domain of Wik1 shows significant similarity only to Ssk2p and Ssk22p, which is limited to relatively short stretches (Figure 1, underlined sequences).

### *Stress-induced Activation of Spc1 Is Impaired in Δwik1 Cells*

A *wik1*$^+$ gene disruption experiment was performed to determine whether Wik1 is an activator of the Wis1-Spc1 pathway (Figure 2A). A *wik1::ura4*$^+$ construct was used to transform a wild-type haploid strain and stable Ura$^+$ transformants were subjected to genomic Southern blot analysis to confirm the *wik1* gene disruption (Δ*wik1*). Backcrosses to a wild-type strain produced four viable segregants of 2$^+$:2$^-$ uracil auxotrophy, confirming that *wik1*$^+$ is not essential for growth. However, Δ*wik1* was found to cosegregate with following phenotypes. Δ*wik1* cells are largely defective in activation of Spc1 when exposed to high-osmolarity stress. We used wild-type and Δ*wik1* strains carrying a chromosomal copy of the *spc1*$^+$ gene tagged with the HA6H sequence encoding two copies of HA epitope and six consecutive histidine residues. These strains use the *spc1*$^+$ promoter to express a wild-type level of Spc1 protein, which can be purified by Ni-NTA-agarose chromatography and detected with anti-HA epitope antibodies (Shiozaki and Russell, 1995a). Spc1 was purified from both strains before and after osmostress by 0.6 M KCl, and activation of Spc1 was assessed by immunoblotting with anti-phosphotyrosine monoclonal antibodies (Figure 2B). In wild-type cells, Spc1 was highly activated within 10 min after exposure to high osmolarity, while only a very low level of tyrosine phosphorylation was detected with Spc1 from Δ*wik1* cells before and after the stress. Consistent with this observation, Δ*wik1* cells grew slowly in high-osmolarity medium (Figure 3). Wild-type, Δ*wik1*, and a strain lacking a functional *wis1*$^+$ MAPKK gene (Δ*wis1*) were grown on agar plates of YES medium and YES containing 1 M KCl at 30°C. In the absence of osmostress, all three strains formed colonies of similar size. On the other hand, on the KCl medium, the Δ*wik1* colonies were significantly smaller than those of wild-type, and Δ*wis1* cells could not form colonies on the same medium as reported previously (Millar *et al.*, 1995; Shiozaki and Russell, 1995). These results strongly suggest that Wik1 is a positive regulator of the Wis1-Spc1 pathway, presumably by directly phosphorylating and activating Wis1 MAPKK.

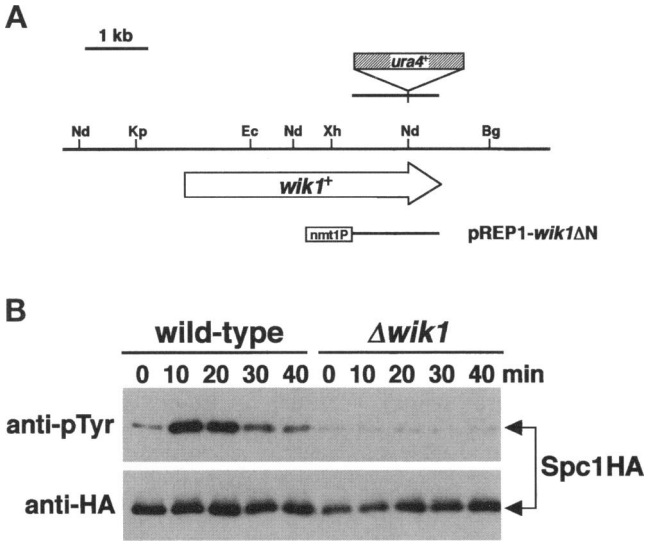


**Figure 2.** (A) Restriction map of an 8-kb region around the *wik1*$^+$ locus and the plasmid construct used for the *wik1*$^+$ gene disruption. A 1.4-kb fragment encoding the protein kinase domain of Wik1 was amplified by PCR. The *Nde*I site was used to insert the *ura4*$^+$ gene fragment. The same 1.4-kb *wik1* fragment was also used to construct a pREP1-*wik1*ΔN plasmid for overexpression of the constitutively active Wik1ΔN from the *nmt1* promoter (nmt1P). Restriction enzyme sites: Bg, *Bgl*II; Ec, *Eco*RI; Kp, *Kpn*I; Nd, *Nde*I; Xh, *Xho*I. (B) *wik1*$^-$ cells are defective in the activating tyrosine phosphorylation of Spc1 upon osmostress. Wild-type (KS1376) and Δ*wik1* (KS1667) mutant strains carrying a chromosomal *spc1*$^+$ gene tagged with the HA6H sequence encoding two copies of the HA epitope and six consecutive histidine residues were grown in YES medium at 30°C. KCl was added to a final concentration of 0.6 M at time 0, and aliquots of cells were harvested every 10 min. Spc1 protein was purified by Ni-NTA-chromatography and analyzed by immunoblotting with anti-phosphotyrosine (pTyr) and anti-HA antibodies, as indicated.

### *Δwik1 Mutant Shows a Cell Cycle Delay in $G_2$ Phase*

Mutations in the *wis1*$^+$ and *spc1*$^+$ genes cause a $G_2$ cell cycle delay that leads to an increase in the cell length at division (Warbrick and Fantes, 1991; Millar *et al.*, 1995; Shiozaki and Russell, 1995). As shown in Table 2, Δ*wis1* cells were ~20% longer than wild-type cells in the yeast extract medium YES and this cell elongation phenotype was further exacerbated in the synthetic minimal medium EMM2. Δ*wik1* cells are ~8% and ~25% longer at division than wild-type in YES and EMM2 media, respectively (Table 2). DNA flow cytometry and microscopic examination revealed that almost all asynchronously growing Δ*wik1* cells had a 2C DNA content (our unpublished results). In Δ*wik1* cells, there was no increase in the fraction of the cells with mitotic figures. These observations strongly indicate that the cell elongation phenotype of Δ*wik1* is attributed to a $G_2$ cell cycle delay. However, it is noteworthy that cell size measurements indicated that

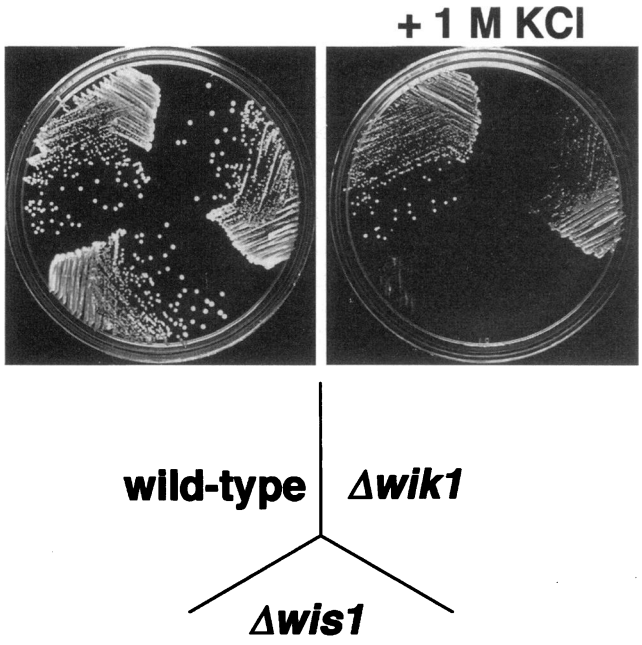


**Figure 3.** Osmosensitive growth of Δ*wik1* cells. Wild-type (KS1598), Δ*wis1* (JM544), and Δ*wik1* (KS1664) cells were streaked on agar plates of YES (left panel) and YES supplemented with 1 M KCl (right panel). Colonies of Δ*wik1* cells were significantly smaller than those of wild-type in the presence of KCl, although the osmosensitivity of Δ*wik1* cells was not as severe as for Δ*wis1* cells.

the $G_2$ delay of Δ*wik1* cells was not as severe as that of Δ*wis1* cells (Table 2).

### *Mitotic Catastrophe Suppressor Mutation* mcs4–13 *Impairs Spc1 Activation*

Mutations of six genes, *mcs1*$^+$ through *mcs6*$^+$, were originally identified as extragenic suppressors of the lethal mitotic catastrophe phenotype of a *cdc2–3w wee1–50* double mutant (Booher and Beach, 1987; Molz *et al.*, 1989). *mcs4*$^+$ has unique characteristics among *mcs* genes: *mcs4–13* mutant cells have a cell elongation phenotype that is exacerbated in synthetic minimal medium, and a *mcs4–13 cdc25–22* double mutant undergoes cell cycle arrest at 25°C, a temperature that is normally permissive for *cdc25–22*. These phenotypes are quite similar to those caused by *spc1*$^-$ and *wis1*$^-$ mutations (Millar *et al.*, 1995; Shiozaki and Russell, 1995a). Genetic crosses established that *wis1*$^+$ and *mcs4*$^+$ are different genes (Warbrick and Fantes, 1992). In addition, a multicopy plasmid carrying *wis1*$^+$ gene suppresses the *mcs4–13* defect (Warbrick and Fantes, 1992), suggesting that *mcs4*$^+$ and *spc1*$^+$ are unlikely to be the same gene, a fact established by genetic crosses (our unpublished results). These observations led us to hypothesize that Mcs4 functions in the same pathway as Wis1 and Spc1, possibly upstream of Wis1. Therefore, we examined whether the *mcs4–13* mutation affects Spc1 activation. Wild-type and *mcs4–13* strains were exposed to osmostress by incubation in medium containing 0.6 M KCl, and activation of Spc1 was monitored by immunoblotting with anti-phosphotyrosine antibodies (Figure 4A). In comparison to wild-type cells, the level of Spc1 tyrosine phosphorylation in *mcs4–13* cells was very low in the absence of stress and was only slightly increased after exposure to high-osmolarity medium. The defect in activating Spc1 was consistent with the slow growth of *mcs4–13* cells in high-osmolarity medium (Figure 4B). These data indicate that Mcs4 plays a crucial role in the activation of Spc1 under normal and high-osmolarity growth conditions.

**Table 2.** Initiation of mitosis is delayed in *wik1*$^-$ and *mcs4*$^-$ mutants

| Strain | Genotype | Length at cell division (μm) YES | EMM2 |
|---|---|---|---|
| KS1598 | Wild-type | 14.1 ± 0.3[a] | 13.7 ± 0.6 |
| JM544 | Δ*wis1* | 17.8 ± 1.1 | 21.0 ± 1.0 |
| KS1664 | Δ*wik1* | 15.2 ± 0.4 | 17.1 ± 0.8 |
| KS1675 | *mcs4-13* | 16.1 ± 0.6 | 18.4 ± 1.0 |
| KS1669 | *mcs4-13* Δ*wik1* | 16.4 ± 1.0 | 18.1 ± 0.7 |

[a]Data are the mean ± SD.

### *Mcs4 Functions Upstream of Wik1*

Having established that Mcs4 is involved in the activation of Spc1 kinase, we next carried out experiments to determine the relationship between Mcs4 and Wik1. In view of the similar phenotypes caused by *mcs4*$^-$ and *wik1*$^-$ mutations, we first explored whether the two genes were allelic, but genetic crosses established that *mcs4*$^+$ and *wik1*$^+$ were different. A *mcs4–13* Δ*wik1* double mutant strain was isolated and its phenotype was examined in comparison to a *mcs4–13* single mutant strain. As shown in Figure 4B, the osmosensitive growth phenotype of *mcs4–13* Δ*wik1* cells was no more severe than *mcs4–13* single mutant cells, indicating that the phenotypes caused by these two mutations are not additive. This conclusion was also confirmed by the observation that the $G_2$ cell cycle delay of the *mcs4–13* strain was not exacerbated by the Δ*wik1* mutation (Table 2). *mcs4–13* and *mcs4–13* Δ*wik1* double mutant cells divided at a similar cell length both in YES and synthetic EMM2 media. These results suggest that Mcs4 and Wik1 function in a linear pathway.

To decipher the order of Mcs4 and Wik1 in the signal transduction pathway, we constructed a plasmid pREP1-*wik1*ΔN (Figure 2A) that expresses the C-terminal 466 residues of Wik1 protein (Wik1ΔN)

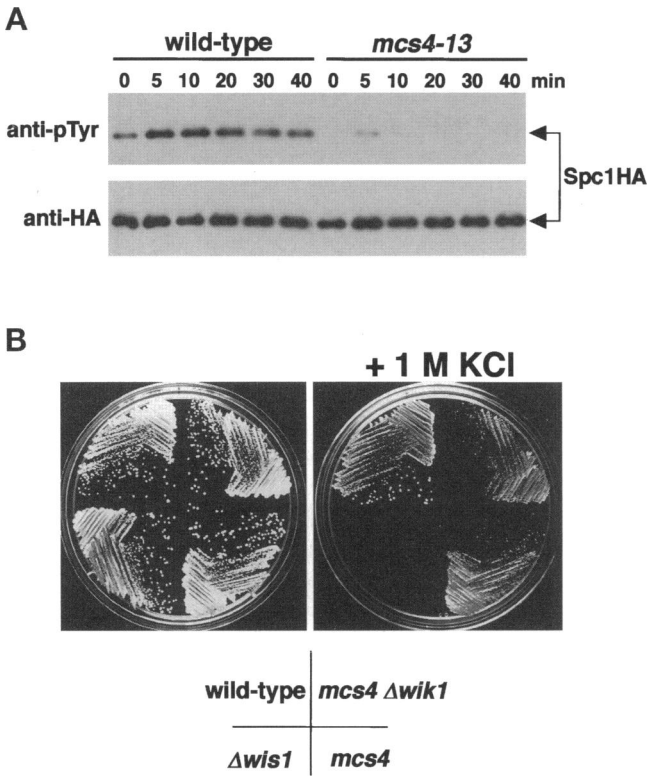


**Figure 4.** (A) Activation of Spc1 is defective in *mcs4–13* mutant cells. Wild-type (KS1376) and *mcs4–13* (KS1559) strains carrying a chromosomal *spc1*$^+$ gene tagged with the HA6H sequence were grown in YES medium at 30°C, and 0.6 M KCl was added to the cultures at time 0. Spc1HA6H protein was purified on Ni-NTA-agarose beads from cells harvested at each time point and analyzed by immunoblotting with anti-phosphotyrosine (pTyr) and anti-HA antibodies as indicated. (B) Osmosensitivity of *mcs4* and *mcs4* Δ*wik1* double mutant cells. Wild-type (KS1598), Δ*wis1* (JM544), *mcs4–13* (KS1675), and *mcs4–13* Δ*wik1* (KS1669) strains were streaked on agar plates of YES medium (left panel) and YES supplemented with 1 M KCl (right panel). In the presence of KCl, *mcs4–13* cells formed only small colonies in comparison to wild type. No visible Δ*wis1* colonies were observed. Colonies of *mcs4–13* Δ*wik1* double mutant cells had a size similar to those of *mcs4–13* cells.

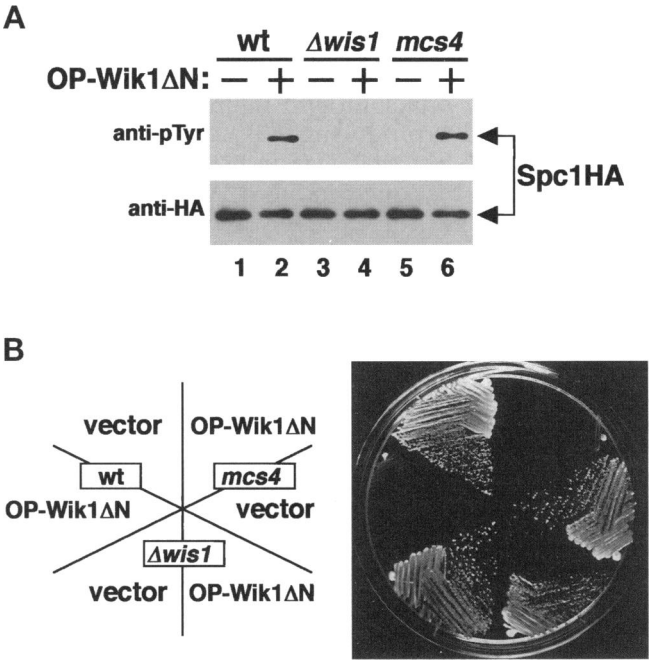


**Figure 5.** (A) Activation of Spc1 by overexpressing an N-terminal truncated form of Wik1. Wild-type (KS1376), Δ*wis1* (GD1682), and *mcs4–13* (KS1559) strains in which the chromosomal copy of *spc1*$^+$ expressed HA6H-tagged Spc1 protein were transformed with either pREP1 vector (lanes 1, 3, and 5) or the pREP1-*wik1*ΔN plasmid (lanes 2, 4, and 6), which uses the the thiamine-repressible *nmt1* promoter to express the C-terminal kinase domain of Wik1 protein truncated from the N-terminal 934 residues of the noncatalytic domain (Wik1ΔN). Spc1 protein was purified by Ni-NTA-chromatography from cells harvested after 16 h of thiamine depletion to induce expression from the *nmt1* promoter. A high level of Spc1 tyrosine phosphorylation was observed in wild-type and *mcs4–13* cells expressing Wik1ΔN but not in Δ*wis1* cells. (B) Effect of Wik1ΔN overexpression in wild-type (KS1376), Δ*wis1* (GD1682), and *mcs4–13* (KS1559) strains. Strains used in A were streaked on an EMM2 plate without thiamine to induce expression of Wik1ΔN from the *nmt1* promoter. The plate was photographed after 3 d at 30°C. Overproduction of Wik1ΔN was lethal in wild-type and *mcs4–13* cells, whereas Δ*wis1* cells that expressed Wik1ΔN readily formed colonies.

under the regulation of thiamine-repressible *nmt1* promoter (Maundrell, 1990). It is known that truncation of the N-terminal noncatalytic domain of MAPKKKs typically causes constitutive activation of kinase activity (Cairns *et al.*, 1992; Howe *et al.*, 1992; Maeda *et al.*, 1995). Wild-type, Δ*wis1*, and *mcs4–13* strains expressing HA6H-tagged Spc1 were transformed with either pREP1 or pREP1-*wik1*ΔN. Expression from the *nmt1* promoter was induced by depleting thiamine from the growth medium for 16 h. Immunoblotting with anti-phosphotyrosine antibodies showed that expression of Wik1ΔN activated Spc1 in wild-type cells in the absence of stress stimuli (Figure 5A, lane 2). Spc1 in Δ*wis1* cells was not responsive to Wik1ΔN expression (Figure 5A, lane 4), indicating that activation of Spc1 by Wik1ΔN is dependent on Wis1. This result confirmed that Wik1 functions upstream of Wis1. As was the case in wild-type cells, a high level of Spc1 activation was observed in *mcs4–13* cells upon Wik1ΔN overproduction (Figure 5A, lane 6).

Hyperactivation of the Spc1 kinase by overproduction of Wis1 MAPKK causes a lethal cell lysis phenotype (Shiozaki and Russell, 1995a). We found that Wik1ΔN is also toxic to wild-type cells: wild-type cells transformed with pREP1-*wik1*ΔN plasmid could not form colonies on an EMM2 agar plate lacking thiamine, whereas a control strain carrying the pREP1 vector grew normally (Figure 5B). Microscopic examination revealed that Wik1ΔN overproducer cells had a deformed swollen morphology often accompanied by cell lysis, which closely resembled the phenotype

caused by Wis1 overproduction (Shiozaki and Russell, 1995a). As expected from the result in Figure 5A, Δ*wis1* cells were refractive to the toxicity of Wik1ΔN, exhibiting only minor growth inhibition (Figure 5B). *mcs4–13* cells overproducing Wik1ΔN exhibited a severe growth defect that was indistinguishable from wild-type cells that overproduced Wik1ΔN. These results indicate that constitutively active Wik1ΔN promotes activation of Spc1 in the absence of Mcs4 function. Collectively, these results strongly suggest that Msc4 functions upstream of Wik1 to positively regulate the Spc1 kinase cascade.

### *Mcs4 Transmits Various Stress Signals to the Spc1 Pathway*

The responsiveness of the Spc1 pathway to very different forms of stress raises a question as to whether a single stress sensor regulates the Spc1 pathway. Alternatively, it is possible that multiple upstream sensors funnel various stress signals to Spc1. After the identification of Mcs4 as a positive regulator of the Wik1-Wis1-Spc1 kinase cascade, we then examined whether Mcs4 is involved in sensing different forms of stress.

Activation of Spc1 was monitored in wild-type and *mcs4–13* cells undergoing high-osmolarity stress, oxidative stress, or heat stress. Both strains were grown in YES medium at 30°C, and then either 0.6 M KCl (Figure 6A, lanes 2) or 0.3 mM hydrogen peroxide (lanes 3) was added or the incubation temperature was shifted to 48°C (lanes 4). As reported previously (Degols *et al.*, 1996), high osmolarity, oxidative stress, and heat shock induced strong tyrosine phosphorylation of Spc1 in wild-type cells. In contrast, each of these three forms of stress induced only a weak Spc1 tyrosine phosphorylation signal in *mcs4–13* cells (Figure 6A).

We have recently begun to explore the range of signals that lead to activation of Spc1. The data in Figure 6B show that Spc1 becomes activated in response to carbon source starvation. We therefore examined whether Mcs4 was important for sensing carbon source depletion. Wild-type and *mcs4–13* cells grown to logarithmic phase in EMM2 medium were washed and reinoculated in EMM2 medium without glucose. Spc1 was activated within 10 min in wild-type cells but not in *mcs4–13* cells (Figure 6B). These results suggest that Mcs4 is very important for the transmission of many types of stress signals to the Wik1-Wis1-Spc1 pathway.

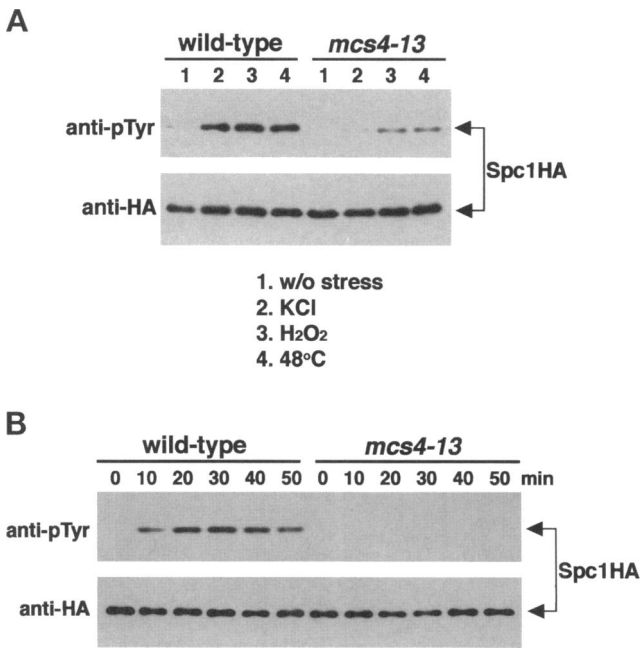


**Figure 6.** (A) Mcs4 function is important for activation of Spc1 in response to various forms of stress. Wild-type (KS1376) and *mcs4–13* (KS1559) cells expressing Spc1 tagged with HA6H were grown in YES medium at 30°C (lanes 1) and then exposed to 0.6 M KCl for 15 min (lanes 2), 0.3 mM $H_2O_2$ for 15 min (lanes 3), or 48°C for 10 min (lanes 4) in YES. Spc1 protein was purified on Ni-NTA-beads and subjected to immunoblotting with anti-phosphotyrosine (pTyr) and anti-HA epitope antibodies as indicated. In comparison to the wild-type strain, only a low level of Spc1 tyrosine phosphorylation was observed in *mcs4–13* cells under the stress conditions tested. (B) Activation of Spc1 in wild-type (KS1376) and *mcs4–13* (KS1559) cells after carbon starvation. Cells were grown in EMM2 medium at 30°C and then shifted to EMM2 lacking glucose at time 0. Aliquots of the cells were harvested every 10 min during the incubation at 30°C, and the tyrosine phosphorylation of Spc1 was examined as described above.

## DISCUSSION

In this study we have identified two genes, *wik1*$^+$ and *mcs4*$^+$, that encode positive regulators of the Wis1-Spc1 kinase cascade (Figure 7). *wik1*$^+$ encodes a MAPKKK homologue closely related to *S. cerevisiae* Ssk2p, which implies that Wik1 directly activates Wis1 MAPKK by phosphorylation. Data presented herein strongly support this hypothesis. First, Δ*wik1* cells fail to activate Spc1 upon osmostress. Second, disruption of *wik1*$^+$ causes phenotypes that are very similar to those exhibited by Δ*wis1* and Δ*spc1* strains. Δ*wik1* cells exhibit an osmosensitive growth and a $G_2$ cell cycle delay that is exacerbated by growth in synthetic minimal medium. Third, Wik1 protein lacking the N-terminal noncatalytic domain, Wik1ΔN, can activate Spc1 in the absence of stress stimuli, and this activation of Spc1 by Wik1ΔN is dependent on Wis1. Interestingly, we observed that the Δ*wik1* phenotypes were not as severe as those of Δ*wis1* strains. A low level of Spc1 tyrosine phosphorylation was detected in Δ*wik1* cells, whereas Δ*wis1* cells are totally defective in phosphorylating Spc1 (Millar *et al.*, 1995; Shiozaki and Russell, 1995a). This difference may account for the fact that Δ*wik1* cells are somewhat less osmosensitive than Δ*wis1* cells. Three possibilities may explain the differ-

ence between the Δ*wik1* and Δ*wis1* phenotypes. One possibility is that Wis1 MAPKK may have a low basal activity in the absence of activating phosphorylation by Wik1. A second possibility is that there may be a second MAPKKK homologue capable of activating Wis1. In the homologous pathway of *S. cerevisiae*, two MAPKKKs, Ssk2p and Ssk22p, activate Pbs2p MAPKK (Maeda *et al.*, 1995). The fact that the *mcs4–13* mutation causes a cell elongation phenotype that is more severe than that of Δ*wik1* cells (Table 2) is most consistent with there being a second MAPKKK homologue regulated by Mcs4. It is very unlikely that *mcs4*$^+$ encodes a second MAPKKK that activates Wis1, because mutations in a second MAPKKK would be expected to cause only minor phenotypes relative to those caused by Δ*wik1* mutations. The third possibility is that there is another upstream activator of Wis1 that is independent of Wik1 MAPKKK. Budding yeast has two transmembrane osmosensors: the Sln1p histidine kinase and Sho1p carrying a cytoplasmic SH3 domain (Ota and Varshavsky, 1993; Maeda *et al.*, 1994, 1995). The SH3 domain of Sho1p is believed to directly interact with a proline-rich sequence in the noncatalytic region of Pbs2p MAPKK and thereby positively regulate the Pbs2p activity. *S. pombe* Wis1 also has a putative SH3-binding motif at residues 77–85, hinting at the possibility that a Sho1p-like protein in fission yeast might activate Wis1. However, it should be noted that the Δ*wik1* mutation has profound effects on Spc1 tyrosine phosphorylation and growth in high-osmolarity medium, whereas in *S. cerevisiae* the function of Ssk2p/Ssk22p is cryptic in the presence of Sho1p (Maeda *et al.*, 1995). Likewise, *sho1* mutations cause no obvious phenotype in strains that have a wild-type copy of *SSK2* or *SSK22*.

The mitotic catastrophe suppressor gene *mcs4*$^+$ was originally identified as a potential regulator of mitosis (Booher and Beach, 1987; Molz *et al.*, 1989). Loss of Mcs4 activity rescued the lethal premature mitosis of *cdc2–3w wee1–50* cells, suggesting that Mcs4 has a positive role in promoting the onset of mitosis. Our studies have revealed that Mcs4 has an important role in activating the Wik1-Wis1-Spc1 kinase cascade. This conclusion is supported by a series of findings. First, activation of Spc1 in response to various forms of stress is highly impaired in a *mcs4* mutant. Second, *mcs4* mutant cells show a growth defect in high-osmolarity medium and a nutrition-sensitive cell cycle delay. Third, the *mcs4* phenotypes are not accentuated in a Δ*wik1* genetic background, suggesting that Mcs4 and Wik1 function in a linear pathway. Finally, constitutively active Wik1ΔN does not require functional Mcs4 to promote activation of Spc1, which strongly suggests that Wik1 functions downstream of Mcs4 in the Spc1 activation pathway.

In budding yeast, a two-component sensor system that consists of Sln1p, Ypd1, and Ssk1p regulates the Ssk2p/Ssk22p MAPKKKs in the osmosensing pathway (Maeda *et al.*, 1994; Posas *et al.*, 1996). The current model is that Ssk1p response regulator protein activates Ssk2p/Ssk22p MAPKKKs, presumably by direct interaction, and Sln1p histidine kinase phosphorylates Ypd1p, which in turn negatively regulates Ssk1p by transferring a phosphate into the receiver domain of Ssk1p. If a homologous two-component system regulates Wik1 MAPKKK in *S. pombe*, then Mcs4, a positive regulator of Wik1, is expected to encode a response regulator protein rather than a histidine kinase. The *mcs4*$^+$ gene was recently cloned and found to encode a protein most homologous to Ssk1p (Cottarel, personal communication). We propose that Mcs4 is a component of the stress sensor system that regulates the Wik1-Wis1-Spc1 MAPK cascade in response to various environmental stimuli.

Spc1 phosphorylates and activates the Atf1 transcription factor (Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996). Atf1 is required for expression of many stress-responsive genes and also appears to be impor-

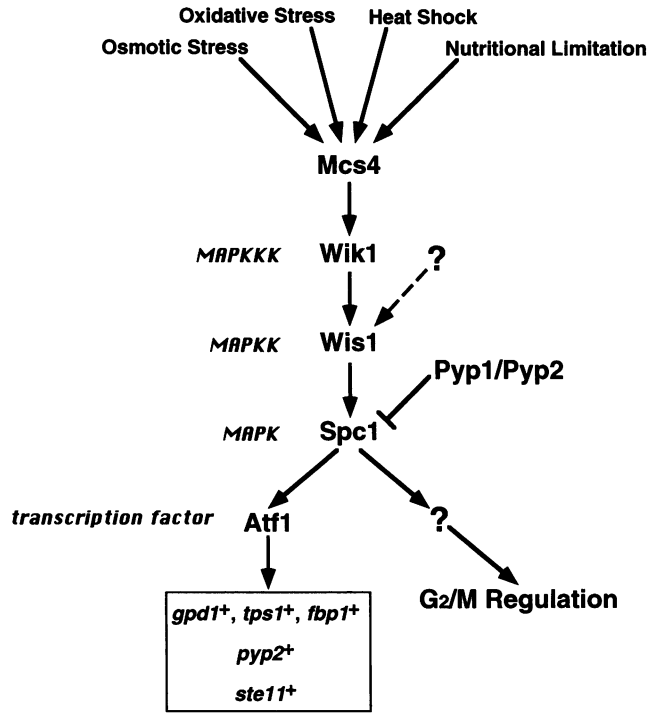


**Figure 7.** Model of the Spc1 signal transduction pathway. Various forms of cytotoxic stress and nutritional limitation activate the pathway through Mcs4. Mcs4 activates Wik1 directly or indirectly and Wik1, in turn, activates Wis1, presumably by direct phosphorylation. There might be another pathway to activate Wis1 since a low level of Spc1 activation has been observed in the absence of Wik1 or Mcs4. Spc1 is activated by Wis1 through phosphorylation of Thr-171 and Tyr-173 and is inactivated by Pyp1 and Pyp2 tyrosine-specific phosphatases. Spc1 promotes the $G_2$-M transition through an unidentified target and also activates Atf1 transcription factor to induce expression of stress-response genes and *ste11*$^+$.

tant for expression of *ste11*$^+$, which encodes a transcription factor that regulates commitment to meiosis (Takeda *et al.*, 1995; Kato *et al.*, 1996; Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996). However, unlike mutations in *spc1*$^+$ or upstream genes, Δ*atf1* cells do not show a $G_2$ cell cycle delay, suggesting that a distinct substrate of Spc1 promotes the $G_2$-M transition. The putative mitotic regulator that is regulated by Spc1 appears to function independently of Wee1 and Cdc25, since *spc1*$^-$ and *mcs4*$^-$ mutations cause a cell cycle arrest phenotype in *cdc25–22 wee1–50* mutants at 35°C (Molz *et al.*, 1989; Shiozaki and Russell, 1995). Experiments are underway to identify the substrate of Spc1 that regulates mitosis to understand how a stress-sensing signal transduction pathway is used to coordinate the mitotic control with changes in the environment that affect cell physiology.

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