The Mcs4 response regulator coordinately controls the stress-activated Wak1–Wis1–Sty1 MAP kinase pathway and fission yeast cell cycle

Jia-Ching Shieh, Marc G. Wilkinson, Vicky Buck, Brian A. Morgan,¹ Kozo Makino,² and Jonathan B.A. Millar³

Division of Yeast Genetics, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

The fission yeast Sty1 MAP kinase is required for cell cycle control, initiation of sexual differentiation, and protection against cellular stress. Like the mammalian JNK/SAPK and p38/CSBP1 MAP kinases, Sty1 is activated by a range of environmental insults including osmotic stress, hydrogen peroxide, menadione, heat shock, and the protein synthesis inhibitor anisomycin. We have identified an upstream regulator that mediates activation of the Sty1 MAP kinase by multiple environmental stresses as the product of the mitotic catastrophe suppressor, *mcs4*. *Mcs4* is structurally and functionally homologous to the budding yeast SSK1 response regulator, suggesting that the eukaryotic stress-activated MAP kinase pathway is controlled by a conserved two-component system. *Mcs4* acts upstream of Wak1, a homolog of the SSK2 and SSK22 MEK kinases, which transmits the stress signal to the Wis1 MEK. We show that the Wis1 MEK is controlled by an additional pathway that is independent of both *Mcs4* and the Wak1 MEK kinase. Furthermore, we demonstrate that *Mcs4* is required for the correct timing of mitotic initiation by mechanisms both dependent and independent on Sty1, indicating that *Mcs4* coordinately controls cell cycle progression with the cellular response to environmental stress.

[Key Words: Stress-activated MAP kinase; response regulator; two-component system; cell cycle; *Schizosaccharomyces pombe*]

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In recent years it has become apparent that the molecular machinery that governs the eukaryotic cell cycle has been evolutionarily conserved. In eukaryotic cells both the onset of DNA replication (S phase) and initiation of mitosis (M phase) are triggered by members of the cyclin-dependent kinase (Cdk) family in association with a regulatory cyclin subunit. In the fission yeast both major transitions are catalyzed by a single gene product, Cdc2 (Nurse and Bissett 1981). At the initiation of mitosis the Cdc2–Cdc13 (cyclin B) complex is activated by tyrosine dephosphorylation of the catalytic kinase subunit by the Cdc25 phosphatase (for review, see Millar and Russell 1992). The complex is maintained in an inactive state in interphase by phosphorylation of Cdc2 by two functionally overlapping tyrosine kinases, Wee1 and Mik1 (Rus-

sell and Nurse 1987; Lundgren et al. 1991). The Cdc2 kinase is additionally controlled in the G₁ phase of the cell cycle by direct binding of an inhibitor, Rum1 (Correa-Bordes and Nurse 1995). Extensive genetic analysis in *Schizosaccharomyces pombe* has revealed several other loci that show genetic interaction with the core elements described above and, by inference, also encode proteins that control the fission yeast cell cycle. For example in *cdc2-3w wee1-50* cells, which express a dominantly active Cdc2 kinase and a temperature-sensitive Wee1 kinase, hyperactivation of the Cdc2 kinase induces a premature, lethal mitotic initiation known as mitotic catastrophe (Russell and Nurse 1987). This phenotype is suppressed by mutation in one of six genes (*mcs1–mcs6*, for mitotic catastrophe suppression) (Molz et al. 1989). The *mcs2* and *mcs6* (also known as *crk1/mop1*) genes encode for a cyclin H-like molecule and its cyclin-dependent kinase, respectively, that interact to form fission yeast Cdc2-activating kinase (or CAK) (Buck et al. 1996; Damagnez et al. 1996). Despite the identification of these gene products our understanding of how the fission yeast cell cycle is controlled either by external growth regulatory cues, such as environmental stress, or inter-

Present addresses: ¹Department of Biochemistry and Genetics, The Medical School, The University of Newcastle-upon-Tyne, Newcastle-upon-Tyne NE2 4HH, UK; ²Department of Molecular Microbiology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka Suita, Osaka 565, Japan.

³Corresponding author.

E-MAIL j-millar@nimr.mrc.ac.uk; FAX (0181) 913 8536.

nal checkpoint mechanisms, such as DNA damage, remains rudimentary.

Recently we have uncovered a mitogen-activated protein (MAP) kinase pathway that controls mitotic initiation in fission yeast in response to environmental stress, the central elements of which are the Sty1 MAP kinase (also known as Spc1 and Phh1) and the Wis1 MAP kinase kinase (MEK) (Warbrick and Fantes 1991; Millar et al. 1995; Shiozaki and Russell 1995; Kato et al. 1996). Cells lacking either Sty1 or Wis1 are considerably elongated at cell division and display strong genetic interactions with known mitotic entry mutants, in particular the Cdc25 tyrosine phosphatase (Warbrick and Fantes 1991; Millar et al. 1992, 1995; Millar and Russell 1992; Shiozaki and Russell 1995). A direct downstream target of the Sty1 MAP kinase has been identified as Atf1, a homolog of the human ATF2 transcription factor, which is required for induction of genes required for meiosis and protection against environmental stress (Takeda et al. 1995; Shiozaki and Russell 1996; Wilkinson et al. 1996). Cells lacking Atf1, however, have no cell size at division defect, suggesting that an alternative Sty1 substrate controls mitotic initiation.

The Sty1 MAP kinase is most structurally similar to the budding yeast HOG1 MAP kinase, both of which are required for the cellular response to osmotic stress in their respective organisms (Brewster et al. 1993; Millar et al. 1995; Shiozaki and Russell 1995). The upstream activators of the budding yeast HOG1 MAP kinase have been identified genetically and biochemically. In particular, hyperosmolarity is thought to cause inactivation of a transmembrane histidine kinase, SLN1, that normally maintains the HOG1 MAP kinase inactive (Maeda et al. 1994, 1995). SLN1 autophosphorylation initiates a phospho-relay system in which a phosphate group is transferred from the SLN1 response regulator domain to a histidine residue in the YPD1 protein and then to a conserved aspartate residue in the SSK1 response regulator (Posas et al. 1996). This phospho-relay system is similar to a number of two-component systems that function in bacteria (Stock et al. 1989; Parkinson 1993). The unphosphorylated form of the SSK1 response regulator activates one of two functionally overlapping MEK kinases, SSK2 and SSK22, though the mechanism by which this is achieved is not known (Maeda et al. 1994, 1995). SSK2 and SSK22 kinase activation leads to the sequential phosphorylation and activation of the HOG4/PBS2 MEK and HOG1 MAP kinases (Brewster et al. 1993; Maeda et al. 1994, 1995). A second transmembrane osmosensor, SHO1, has been described in budding yeast, which interacts directly with a proline rich domain in the amino terminus of the PBS2/HOG4 MEK (Maeda et al. 1995). Importantly, although the budding yeast HOG1 MAP kinase is activated only by osmotic stress, the fission yeast Sty1 MAP kinase and the mammalian stress-activated SAPK/JNK and p38/CSBP1 MAP kinases are activated by a range of environmental insults, including osmotic stress, oxidative stress, ultraviolet (UV) light, DNA damaging agents, heat shock, and the protein synthesis inhibitor anisomycin (Brewster et al.

1993; Dérjard et al. 1994; Galcheva-Gargova et al. 1994; Han et al. 1994; Kyriakis et al. 1994; Lee et al. 1994; Rouse et al. 1994; Schüller et al. 1994; Millar et al. 1995; Shiozaki and Russell 1995; Degols et al. 1996). This suggests that the upstream regulators of the mammalian and fission yeast pathways may be structurally similar. However, the mechanism by which these environmental stresses are sensed and transduced to the fission yeast Sty1 and mammalian SAPK/JNK and p38/CSBP1 MAP kinases is, at present, a mystery.

In this paper we demonstrate that activation of the Sty1 MAP kinase by multiple stresses requires a response regulator encoded by the mitotic catastrophe suppressor gene *mcs4*. *Mcs4* is structurally and functionally similar to the budding yeast SSK1 gene product and acts upstream of Wak1, a homolog of the SSK2 and SSK22 MEK kinases, which transmits the stress signal to the Wis1 MEK. These results indicate a remarkable structural conservation in the upstream components of the budding yeast HOG1 and fission yeast Sty1 MAP kinase pathways, even though the two pathways are regulated by distinct extracellular stimuli. In addition we show that *Mcs4* is required for controlling the timing of mitotic initiation by a Sty1-independent pathway, suggesting that cell cycle progression and Sty1 MAP kinase activation are coordinately controlled by a two-component system in fission yeast.

Results

Mcs4 is a novel mitotic regulator required for Wis1 function

Hyperactivation of the Sty1 MAP kinase pathway causes fission yeast cells to cease proliferating and undergo a lethal cell lysis. This is observed when two functionally overlapping MAP kinase phosphatases, Pyp1 and Pyp2, are inactivated (Millar et al. 1995). A similar phenotype is observed when the Wis1 MEK is highly overexpressed from the thiamine-repressible *nmt1* promoter (Fig. 1A). We have identified two mutants that suppress this phenotype. These are *sty1-1*, which harbors a null allele of the Sty1 MAP kinase, and *mcs4-13* cells (Fig. 1A). The *mcs4-13* mutation was originally isolated by its ability to suppress the lethal hyperactivation of the Cdc2 kinase in a *cdc2-3w wee1-50* strain (Molz et al. 1989). Like cells lacking either *wis1* or *sty1*, *mcs4-13* mutant cells display a cell size at division defect in that they divide at 19.1 ± 1.1 μm relative to 14.2 ± 0.3 μm for wild type (Table 1). As is the case with $\Delta wis1$ and $\Delta sty1$ mutants, the effect of *mcs4-13* on cell size at division is dramatically exaggerated in *cdc25-22* cells that harbor a temperature-sensitive version of the Cdc25 protein phosphatase and are partially defective for mitotic initiation at 26°C. Whereas *Cdc25-22* cells divide at 21.9 ± 0.8 μm at 26°C, *mcs4-13 cdc25-22* double mutants are extremely long, dividing at 38.9 ± 10.9 μm (Table 1). These results confirm that *mcs4* has an important role in controlling mitotic initiation in fission yeast and further suggest that *mcs4* acts either as an upstream activator or downstream target of the Sty1 kinase.

Figure 1. Mcs4 is a mitotic inducer that interacts genetically with the Wis1 MEK. (A) Loss of *mcs4* suppresses overexpression of *wis1*. Wild-type (PR109), *sty1-1* (JM1144), or *mcs4-13* cells (JM1354) were transformed with pREP1-*wis1*. Transformants were selected on minimal medium lacking leucine containing 10 μ M thiamine and then streaked onto the same medium (+thiamine) or the same medium lacking thiamine (–) and growth of the cells monitored after 3 days at 30°C. (B) Mcs4 regulates the initiation of mitosis. Wild-type (WT) (PR109), *mcs4-13* (JM1354), *cdc25-22* (JM1351), or *mcs4-13 cdc25-22* (JM1303) cells were grown on YEPD at 25°C and then streaked onto YEPD plates at 25°C, 30°C, or 36°C. Growth of the strains after 3 days is shown.

To examine the mechanism by which the *mcs4-13* mutant suppresses the lethal hyperactivation of *wis1*, we first attempted to clone the *mcs4* gene. To do this we took advantage of the observation that *mcs4-13 cdc25-22* double-mutant cells undergo cell cycle arrest at 30°C, at which temperature *mcs4-13* and *cdc25-22* single mutants are able to proliferate normally (Fig. 1B). A genomic library was introduced into *mcs4-13 cdc25-22 ura4-D18* cells, and from a total of 50,000 transformants isolated at 25°C, 53 plasmid dependent colonies grew at 30°C. Of these suppressors, 45 represented already known genes, *cdc25* (34 clones), *nim1/cdr1* (five clones), and *wis1* (six

clones). The restriction maps of the remaining eight suppressors were found to be related and represented three distinct overlapping clones. The restriction map of the smallest clone, pURB1-Mcs4-1, is not similar to mitotic regulators identified previously (Fig. 2A).

To determine the relationship between the *mcs4-13* mutation and the pURB1-Mcs4-1 genomic clone, the open reading frame (ORF) of pURB1-Mcs4-1 was interrupted by the *ura4⁺* gene (Fig. 2A). The resulting plasmid pURB1-Mcs4::ura4 was unable to rescue a *mcs4-13 cdc25-22* mutant (data not shown). pURB1-Mcs4::ura4 was linearized and stably integrated into a *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M216/ade6-M210 his7-366/his7-366 h⁺/h[–]* diploid strain. Tetrad dissection of asci from heterozygous diploids gave rise to four viable spores on germination that showed a 2:2 segregation of uracil auxotrophs to uracil prototrophs (data not shown). All uracil prototrophs were found to be elongated to the same extent as *mcs4-13* cells and synthetically lethal in combination with the *cdc25-22* mutation at 30°C (data not shown). Random spore analysis of a cross between these uracil prototrophs and *mcs4-13* cells revealed that out of 3000 spores analyzed no wild-type recombinants could be isolated. We conclude that *mcs4-13* cells harbor a null mutation in a novel mitotic regulator in fission yeast.

Mcs4 is part of a conserved two-component system

Sequencing of the pURB1-Mcs4-1 clone revealed a single open reading frame of 1566 bp capable of encoding a polypeptide of 522 amino acids. The predicted protein sequence was analyzed against current databases and found to contain a response regulator domain in its carboxyl terminus, which is most commonly found in components of bacterial two-component systems, but more recently also have been found in eukaryotes such as *Dicystostelium discoideum*, *Saccharomyces cerevisiae*, and *Arabidopsis thaliana* (Chang et al. 1993; Ota and Varshavsky 1993; Schuster et al. 1996; Wang et al. 1996).

Table 1. Loss of *mcs4* or *wak1* delays the timing of mitosis

	Temp. (°C)	Cell size at division (μ m)
Wild type	26	14.2 $\pm$ 0.3
<i>mcs4-13</i>	26	19.1 $\pm$ 1.1
<i>wak1::ura4</i>	26	16.5 $\pm$ 1.3
<i>wis1::ura4</i>	26	23.1 $\pm$ 2.1
<i>cdc25-22</i>	26	21.9 $\pm$ 0.8
<i>mcs4-13 cdc25-22</i>	26	38.9 $\pm$ 7.9
<i>wak1::ura4 cdc25-22</i>	26	28.5 $\pm$ 4.5
<i>wis1::ura4 cdc25-22</i>	26	Cdc ^a

Cell size measurements of septated cells grown in minimal medium. The temperature at which the measurements were made is indicated. Measurements were the mean of at least 30 individual determinations ($\pm$ S.D.).

^aCell division cycle arrest.

Figure 2. Mcs4 is a response regulator. (A) Restriction map of the insert of pURB1-Mcs4-1. The position of the ORF is shown. The arrow indicates the direction, with the arrowhead at the 3' end of the gene. One-kilobase intervals are marked under the map. A map of the pURB1-Mcs4::ura4 construction is also shown. (B) Mcs4 is homologous to response regulator proteins. Alignment of the complete Mcs4 amino acid sequence to that of budding yeast SSK1 protein is shown. Boxes indicate identities between the two proteins. An asterisk marks the location of the phosphate acceptor residue in SSK1 and Mcs4 response regulator domains. The nucleotide sequence of the *mcs4* gene will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession no. Y11927.

Mcs4 is most closely related to the SSK1 protein from the budding yeast *S. cerevisiae*, which is involved in a phosphorelay system that regulates the HOG1 MAP kinase cascade in response to external osmolarity (Maeda et al. 1994). Mcs4 is 55% identical to SSK1 in the carboxy-terminal response regulator domain, as compared with an average of 18% identity to a variety of bacterial response regulators, but shares little homology outside of this region (Fig. 2B). Budding yeast contains two non-transmembrane proteins that contain response regulator domains, namely, SSK1 and SKN7 [also known as BRY1/POS9]. The SKN7 protein plays an important role in cell wall biosynthesis, G₁ cyclin expression, and induction of gene expression in response to oxidative stress (Brown et al. 1993; Morgan et al. 1995; Krems et al. 1996). To assess whether Mcs4 may be functionally related to these gene products, SSK1 and SKN7 were cloned behind the thiamine-repressible *nmt1* promoter and expressed in *mcs4-13 cdc25-22* cells. As the results in Figure 3A demon-

strate, SSK1 but not SKN7 was able to rescue the cell cycle arrest resulting from loss of *mcs4*. Together these results indicate that Mcs4 is both structurally and functionally related to the SSK1 gene product of budding yeast.

Inactivation of the SLN1 histidine kinase results in dephosphorylation of SSK1 on a conserved aspartate residue (D554) in the response regulator domain, and promotes activation of the HOG1 MAP kinase pathway (Maeda et al. 1994). The equivalent residue in Mcs4 is aspartate D412. To assess whether phosphorylation of Mcs4 in the putative response regulator domain is required for its mitotic function, aspartate D412 was mutated to either asparagine (D412N) or glutamic acid (D412E), which are not substrates for phosphorylation. Full-length forms of either wild-type *mcs4* or the site-directed mutants were expressed behind a crippled version of the *nmt1* promoter and introduced into *mcs4-13 cdc25-22* cells. Both *mcs4*(D412N) and *mcs4*(D412E) mutants were able to rescue the cell cycle arrest of this strain as efficiently as wild-type *mcs4* at 30°C, indicating that phosphorylation of Mcs4 at aspartate D412 is not required for its cell cycle function. We assume that glutamic acid does not mimic a constitutively phosphorylated aspartic acid residue in the response regulator domain.

Mcs4 is required for stress-induced gene expression and activation of the Sty1 MAP kinase

The Sty1 MAP kinase, like the human JNK/SAPK and p38 enzymes, is activated by a variety of environmental insults including osmotic stress, heat shock, and oxidative stress (Millar et al. 1995; Degols et al. 1996). We show here that Sty1 can also be activated by the protein synthesis inhibitor anisomycin. Activation of the Wis1-Sty1-Atf1 pathway by these stresses causes induction of a number of genes, including the *pyp2* MAP kinase phosphatase, which acts in a feedback loop to attenuate Sty1 activity (Millar et al. 1995; Degols et al. 1996; Wilkinson et al. 1996). To examine whether *mcs4* is required for activation of the Sty1 MAP kinase pathway, wild-type or $\Delta mcs4$ cells were incubated in the presence of 0.6 M KCl, 1 mM H₂O₂, or 10 μ g/ml of anisomycin or given a mild heat shock for various times and then the level of *pyp2* expression examined by Northern blot analysis. As the results in Figure 4A show, induction of *pyp2* was reduced dramatically in $\Delta mcs4$ cells in response to every stress employed, although some expression of the *pyp2* mRNA was observed after the peak expression of *pyp2* observed in wild type cells. Notably, induction of *pyp2* mRNA by these stresses is absolutely dependent on the Sty1 MAP kinase as no induction is observed in $\Delta sty1$ cells (Fig. 4A). Northern blots were reprobed with the *cdc2*, *act1*, *wis1*, or *sty1* genes, the corresponding mRNAs of which were not altered by loss of *mcs4* or in response to stress (data not shown). To confirm this result, we took advantage of previous observations that the catalase (*ctt1*) and glyceraldehyde-3-phosphate dehydrogenase (*gpd1*) genes are also under control of the Wis1-Sty1-Atf1 pathway

Figure 3. Mcs4 is functionally related to budding yeast SSK1. (A) SSK1 rescues loss of Mcs4. *mcs4-13 cdc25-22* cells (JM1303) were transformed with a control plasmid pREP42 (pControl), pREP42-SSK1 (pSSK1), or pREP42-SKN7 (pSKN7) expressing either the SSK1 or SKN7 ORFs, respectively, behind the thiamine-repressible *mtl1* promoter. Transformants were grown and streaked on minimal medium lacking thiamine and uracil and growth of the cells monitored after 3 days at temperatures shown. (B) Phosphorylation of residue D412 is not required for Mcs4 function. *mcs4-13 cdc25-22* cells (JM1303) were transformed with a control plasmid pREP42, pREP42-*mcs4*, pREP42-*mcs4*(D412N), or pREP42-*mcs4*(D412E) expressing either the wild-type or mutant versions of the *mcs4* ORF, respectively, behind the thiamine-repressible *mtl1* promoter. Transformants were grown on minimal medium lacking thiamine and uracil and growth of the cells monitored after 3 days at temperatures shown.

(Wilkinson et al. 1996). Wild-type or $\Delta mcs4$ cells were stimulated with either 0.6 M KCl or 1 mM H_2O_2 and the induction of the *ctt1* and *gpd1* genes analyzed by Northern blot. Stress-induced expression of both the *ctt1* and *gpd1* mRNAs also was reduced dramatically in $\Delta mcs4$ cells relative to wild type. These results indicate that the Mcs4 response regulator is required for induction of gene expression by multiple environmental insults.

To assess whether the effect of *mcs4* on stress-induced gene expression is at the level of transcription or via controlling the activity of the Sty1 MAP kinase, we developed a coupled affinity-kinase assay to measure endogenous Sty1 activity. This assay is based on our previous observation that a glutathione *S*-transferase (GST)–Atf1 fusion protein specifically associates with and is a substrate for the Sty1 kinase (Wilkinson et al. 1996). Sty1 was precipitated from extracts of either wild-type or $\Delta mcs4$ cells that had been challenged with either 0.6 M KCl or 1 mM H_2O_2 or given a mild heat shock and assayed for activity against the GST–Atf1 protein as substrate. As the results in Figure 4D show, activation of Sty1 was dramatically lowered in cells lacking *mcs4* in response to all three stresses, although some activation was again observed at later time points (Fig. 4D). Under the conditions employed in these experiments, no detectable activity could be precipitated from cells lacking the Wis1 MEK (data not shown). Similar results were found when cells were challenged with either the superoxide generator menadione or the protein synthesis inhibitor anisomycin (data not shown). It should be noted that the effect of anisomycin is not dependent on protein

synthesis inhibition, because cycloheximide at concentrations that inhibit 98% of protein synthesis does not induce *pyp2* expression or activate the Sty1 MAP kinase (Wilkinson et al. 1996). Together these results indicate that the Mcs4 protein is required for Sty1 MAP kinase activation in response to multiple independent environmental stresses. These results are in striking contrast to budding yeast SSK1, which is required for activation of HOG1 only in response to osmotic stress. Furthermore, because Sty1 is activated by the Wis1 MEK, a prediction from this is that Wis1 activity is lower in the absence of Mcs4. This would explain why lethal overexpression of *wis1* is suppressed in *mcs4-13* cells (Fig. 1).

Mcs4 acts upstream of Wak1, a homolog of the SSK2 and SSK22 MEK kinases

The budding yeast SSK1 gene product is thought to regulate the HOG1 MAP kinase pathway by controlling the activity of the SSK2 and SSK22 MEK kinases (Maeda et al. 1994). We reasoned that Mcs4 may control the activity of a structural homolog of SSK2 and SSK22 in fission yeast. In a search of the *S. pombe* sequence databases we identified an ORF on chromosome I with high sequence homology to SSK2 and SSK22. The restriction map of this ORF is presented in Figure 5A. We have termed this kinase Wak1, for *Wis1*-activating *kinase*. Wak1 encodes a predicted 1307-amino-acid protein that is 50% identical to SSK2 and 49% identical to SSK22 in the carboxy-terminal catalytic kinase domain as compared with an average of 26% identity with other kinases. Importantly

Figure 4. Mcs4 is required for stress-induced gene expression and Sty1 activation. (A) Expression of the Pyp2 MAP kinase phosphatase is attenuated in cells lacking *mcs4*. Log-phase cultures growing in YEPD at 30°C of wild-type (wt) (PR109), *sty1::ura4* ($\Delta sty1$) (JM 1160), or *mcs4::ura4* ($\Delta mcs4$) (JM 1354) cells were incubated in the same medium containing 0.6 M KCl, 1 mM H₂O₂, or shifted to 42°C for the times indicated. Total RNA was extracted and equal quantities separated by electrophoresis and probed using DNA specific to the *pyp2* gene. Represented blots were probed with the same probe and for the same length of time. Reprobing with both actin- and *cdc2*-specific probes verified equal loading of RNA. (B) Induction of catalase (*ctt1*) is attenuated in cells lacking *mcs4*. An experiment similar to that described in A was performed in which wild-type (wt) (PR109), *sty1::ura4* ($\Delta sty1$) (JM 1160), or *mcs4::ura4* ($\Delta mcs4$) (JM 1354) cells were incubated in medium containing 1 mM H₂O₂ for the times indicated. In this experiment total RNA was extracted and probed using DNA specific to the *ctt1* gene. (C) Induction of *gpd1* is attenuated in cells lacking *mcs4*. An experiment similar to that described in A was performed in which wild-type (wt) (PR109), *sty1::ura4* ($\Delta sty1$) (JM 1160), or *mcs4::ura4* ($\Delta mcs4$) (JM 1354) cells were incubated in medium containing 0.6 M KCl for the times indicated. Total RNA was extracted and probed using DNA specific to the *gpd1* gene. (D) Activation of Sty1 in cells lacking *mcs4*. Log-phase cultures of either wild-type (wt) (PR109) or *mcs4::ura4* ($\Delta mcs4$) (JM 1354) cells growing in YEPD at 30°C were incubated in the same medium containing 0.6 M KCl, 1 mM H₂O₂ or shifted to 42°C for the times indicated. Cell extracts were prepared and Sty1 precipitated with GST-Atf1 prebound to glutathione beads. Precipitates were washed extensively and incubated in the presence of [γ -³²P]ATP for 20 min at 30°C. Phosphorylation of Atf1 was visualized after SDS-PAGE and autoradiography.

Wak1 shares extensive structural homology in the amino-terminal noncatalytic half of the protein to both SSK2 and SSK22 kinases (Fig. 5B). To assess whether *wak1* is required for Sty1 MAP kinase pathway function, a chromosomal deletion of the *wak1* locus was constructed by replacement of at least 50% of the *wak1* ORF with the *ura4*⁺ marker (Fig. 5A). Tetrad dissection of asci from heterozygous diploids gave rise to four viable spores on germination that showed a 2:2 segregation of uracil auxotrophs to uracil prototrophs, indicating that *wak1* is not an essential gene. Importantly, $\Delta wak1$ cells were found to be delayed in the timing of mitotic initiation dividing at 16.5 ± 1.3 μ m relative to 14.2 ± 0.3 μ m for wild type. As is the case with *mcs4*, *wis1*, and *sty1*, the effect of loss of *wak1* activity on cell size at division is greatly enhanced in combination with the *cdc25-22* mu-

tant (Table 1).

To assess whether *wak1* is required for stress-induced gene expression or Sty1 activation, wild-type or $\Delta wak1$ cells were grown to log phase and then given a temperature shock. As the results in Figure 6A demonstrate, induction of *pyp2* and activation of Sty1 were dramatically lowered in $\Delta wak1$ cells, although some residual induction was observed. Similar results were obtained when the cells were challenged with an osmotic or oxidative stress or anisomycin (data not shown). These results strongly suggest that Wak1 is required for activation of the Sty1 MAP kinase pathway by directly phosphorylating Wis1. To assess the relationship between Wak1 and Mcs4, stress-induced gene expression and activation of the Sty1 MAP kinase were assessed in double $\Delta wak1 \Delta mcs4$ mutants. As the results in Figure 6 demonstrate,

Figure 6. *Wak1* is required for activation of the *Sty1* MAP kinase cascade. (A) *Wak1* is required for induction of the *Pyp2* MAP kinase phosphatase. Log-phase cultures growing in YEPD at 30°C of wild-type (wt) (PR 109), *wak1::ura4* ($\Delta wak1$) (JM1436), or *wak1::ura4 mcs4::ura4* ($\Delta wak1 \Delta mcs4$) (JM 1483) cells were shifted to 42°C for the times indicated. Total RNA was extracted and probed using DNA specific to the *pyp2* gene. Blots were reprobed with both actin- and *cdc2*-specific probes for normalization of RNA levels. (B) *Wak1* is required for activation of *Sty1*. Log-phase cultures of cells as in A were shifted to 42°C for the times indicated. Cell extracts were prepared and the *Sty1* MAP kinase isolated by incubation with GST-Atf1 and affinity precipitation on glutathione-agarose beads. Precipitates were washed extensively and incubated in the presence of [γ -³²P]ATP for 20 min at 30°C. Products were visualized after separation on a 10% SDS-PAGE gel and autoradiography.

high temperature or under hyperosmolar conditions as observed previously (Millar et al. 1995), $\Delta wak1$ and $\Delta mcs4$ cells were still able to form colonies, though not as effectively as wild type (Fig. 8A). These results indicate the existence of a second pathway for activating the *Wis1* MEK by environmental stress that is independent of both the *Wak1* and *Mcs4* upstream regulators. We note that cells deleted for either *mcs4* or *wak1* are also not as severely delayed in the timing of mitotic initiation as $\Delta wis1$ or $\Delta sty1$ cells (Table 1; Millar et al. 1995), suggesting that this alternative pathway also contributes to cell cycle control by the *Sty1* MAP kinase pathway.

Mcs4 controls *Cdc2* activation by a *Sty1*-independent pathway

The previous results suggest that *Mcs4* is an important regulator of *Sty1* activity. Because the *mcs4-13* mutant was originally identified based on its ability to suppress the premature and lethal mitotic catastrophe caused by hyperactivation of *Cdc2* kinase, we wished to determine whether this phenotype is a result of a reduction in *Sty1* MAP kinase activity (Molz et al. 1989). To assess this the *mcs4*, *sty1*, and *wis1* genes were independently disrupted in *cdc2-3w wee1-50* cells, and the viability of the cells determined at the permissive temperature of 25°C and restrictive temperature of 32°C. At these temperatures the growth rates of $\Delta sty1$, $\Delta wis1$, or $\Delta mcs4$ cells are not detectably different from wild type (Warbrick and

Fantes 1991; Millar et al. 1995; Shiozaki and Russell 1995; J.B.A. Millar, unpubl.). As the results in Figure 9 demonstrate, loss of either *wis1* or *sty1* does not prevent *cdc2-3w wee1-50* cells from undergoing a lethal mitotic catastrophe at 32°C, whereas loss of *mcs4* does, in agreement with previous observations (Molz et al. 1989). These results indicate that *Mcs4* controls the *G*₂/M transition in fission yeast both by regulating *Sty1* MAP kinase activity and by influencing a second *Sty1*-independent pathway required for the correct timing of *Cdc2* kinase activation.

Discussion

The fission yeast *Sty1* MAP kinase is activated by a wide range of environmental insults, including osmotic stress, oxidative stress, DNA damaging agents, heat shock, and the protein synthesis inhibitor anisomycin. This is similar to the mammalian JNK/SAPK and p38/CSBP1 MAP kinases, but directly contrasts with the budding yeast HOG1 MAP kinase, which is activated only by osmotic stress (Schüller et al. 1994). We have identified two upstream regulators of the *Sty1* MAP kinase cascade as the *Mcs4* response regulator and the *Wak1* MEK kinase. *Mcs4* is both structurally and functionally related to the SSK1 gene product of budding yeast, which is a component of a phospho-relay system that regulates the HOG1 MAP kinase cascade in response to osmotic stress (Mada et al. 1994, 1995; Posas et al. 1996). Activation of HOG1 is caused by inactivation of SLN1 and subsequent dephosphorylation of SSK1 on aspartate 544. In a similar manner we have shown that phosphorylation of *Mcs4* on the equivalent aspartate residue is not necessary for *Mcs4* activity. In budding yeast SSK1 is thought to act directly upstream of the MEK kinases SSK2 and SSK22. We have identified a homolog of the SSK2 and SSK22 MEK kinases in fission yeast, *Wak1*, that is required for *Sty1* activation in response to multiple stresses. Although the precise relationship between *Mcs4* and *Wak1* is at present unclear, we show that $\Delta wak1$ cells are epistatic to changes in *mcs4* expression, suggesting that *Wak1* transmits the stress signal from *Mcs4* to the *Wis1* MEK and *Sty1* MAP kinase (Fig. 10). These results indicate that a response regulator protein acts as part of a multistress sensor in fission yeast to control the activity of the *Sty1* MAP kinase cascade.

In contrast to SSK1, *Mcs4* is required for activation of the *Sty1* MAP kinase in response to multiple stresses including osmotic stress, heat shock, oxidative stress, the superoxide generator menadione, and the protein synthesis inhibitor anisomycin. One might predict from this that the putative histidine kinase that controls *Mcs4* in fission yeast must be regulated by multiple stresses and is not simply an osmosensor. An alternative explanation for this difference is that multiple histidine kinases for *Mcs4* exist in fission yeast, each of which is responsible for sensing a distinct cellular stress. Intriguingly, using a PCR-based approach, recently we have isolated two transmembrane histidine kinases in fission yeast, called Mak1 and Mak2, suggesting this latter pos-

Figure 7. Mcs4 acts upstream of the Wak1 MEK kinase. (A) *Wak1* is epistatic to *mcs4*. $\Delta wak1::ura4 cdc25-22$ cells (JM1469) were transformed with a control plasmid pREP41 (pControl), pREP41-*wis1* (pWis1), or pREP41-*mcs4* (pMcs4) behind the thiamine-repressible *nmt1* promoter. Transformants were grown and streaked on minimal medium lacking thiamine and leucine and cultured for 3 days at temperatures shown. (B) Lethal overexpression of *mcs4* is suppressed by loss of *wak1*. Wild-type (WT) (PR109), *sty1::ura4* ($\Delta sty1$) (JM 1160), *wis1::ura4* ($\Delta wis1$) (JM 544), or *wak1::ura4* ($\Delta wak1$) (JM1436) cells were transformed with pREP1-*mcs4*. Transformants were selected on minimal medium lacking leucine containing 10 μ M thiamine and then streaked onto the same medium (+ thiamine) or the same medium lacking thiamine (-thiamine) and growth of the cells monitored after 5 days at 30°C.

sibility may be the case (K. Makino and J. Millar, unpubl.). In this light we note that the time course of Sty1 activation by hyperosmolarity or anisomycin is considerably slower than that observed with heat shock or oxidative stress. This could be a result of either differential utilization of histidine kinases or slower generation of a common second messenger. Currently we are trying to distinguish between these possibilities. It is important to point out that we have not provided formal proof that Mcs4 becomes dephosphorylated on aspartate 412 in response to multiple stresses, and as such the mechanism by which cellular stress is sensed in fission yeast is still unclear.

The Sty1 MAP kinase and mammalian SAPK/JNK and p38 MAP kinase are stimulated by a remarkably similar range of environmental stimuli. This data would imply that a two-component system may act upstream of the SAPK/JNK and p38/CSBP1 pathways in mammalian cells. This possibility is strengthened by the recent finding that a direct downstream target of Sty1 is the Atf1 transcription factor, a structural homolog of human ATF2, which binds and is activated by the SAPKaII/JNK2 MAP kinase (Gupta et al. 1995; Shiozaki and Russell 1996; Wilkinson et al. 1996). However, no mammalian homologs of the Mcs4 response regulator or Mak1 and Mak2 histidine kinases have yet been identified. Several upstream regulators of the mammalian the SAPK/JNK and p38/CSBP1 MAP kinases have been cloned, including MEKs that are responsible for either p38 activation (MKK3 and MKK6) or JNK/SAPK activa-

Figure 8. *Wis1* is activated by a *Wak1* and *Mcs4*-independent pathway. (A) Cells lacking *Wak1* or *Mcs4* are only partially stress sensitive. Wild-type (WT) (PR109), $\Delta sty1$ (JM 1160), $\Delta wis1$ (JM 544), $\Delta wak1$ (JM 1436), or $\Delta mcs4$ (JM 1354) cells were grown on YEPD and then streaked on the same medium at 30°C and 37°C or on the same medium containing 1.2 M sorbitol. Growth after 3 days is shown.

Figure 9. *Mcs4* controls mitotic initiation by a Sty1-independent mechanism. *Cdc2-3w wee1-50* (*wt*), *cdc2-3w wee1-50 mcs4::ura4* ($\Delta mcs4$), *cdc2-3w wee1-50 wis1::ura4* ($\Delta wis1$), or *cdc2-3w wee1-50 sty1::ura4* ($\Delta sty1$) cells were grown on YEPD at 25°C and then streaked on the same medium either at 25°C or 32°C and growth of colonies examined after 6 days.

tion (MKK4) (Sanchez et al. 1994; Yan et al. 1994; Dériard et al. 1995; Lin et al. 1995; Raingeaud et al. 1996). MKK4 is activated by overexpression of a number of MEK kinases, including MEKK1, TAK1, MUK, SPRK/MLK3, and Tpl2/Cot1, perhaps in a tissue-specific manner (Yan et al. 1994; Yamaguchi et al. 1995; Hirai et al. 1996; Rana et al. 1996; Salmeron et al. 1996). In addition, a number of upstream regulators of MEKK1 have been proposed, also based on transient transfection studies in mammalian cells, including the STE20-related protein kinases PAK-1, PAK-3, germinal center kinase (GCK), and HPK1 (Bagrodia et al. 1995; Pombo et al. 1995; Hu et al. 1996; Zhang et al. 1996). Some of the PAKs bind and are activated by members of the Rho family of GTPases, Rac1 and Cdc42, constitutively activated versions of which can also stimulate the JNK/SAPK and p38 pathways (Bagrodia et al. 1995; Coso et al. 1995; Minden et al. 1995). These latter results are somewhat curious because no role for the STE20-like kinases or Cdc42 proteins has yet been found in signal transmission to the budding yeast HOG1 or fission yeast Sty1 MAP kinases. It should be noted, however, that the mammalian stress-activated MAP kinases are also stimulated by receptor occupancy at the plasma membrane by the proinflammatory cytokines [tumor necrosis factor (TNF α) and IL-1], bacterial lipopolysaccharide (LPS), certain G-protein coupled receptors, and the lymphocyte costimulatory receptors CD28 and CD40 (Fresney et al. 1994; Han et al. 1994; Sakata et al. 1994; Sluss et al. 1994; Su et al. 1994; Raingeaud et al. 1995). It is possible that distinct upstream regulators may regulate activation of the mammalian SAPK/JNK and p38/CSBP1 MAP kinases in response to different stimuli.

Importantly, the HOG1 MAP kinase is activated by hyperosmolarity in cells lacking either the SSK1 or the SSK2 and SSK22 MEK kinases (Maeda et al. 1995). We provide evidence in this paper that alternative stress-inducible pathways also exist for activating the Wis1 MEK in fission yeast that are independent of the *Mcs4* response regulator and *Wak1* MEK kinase (Fig. 10). First, stress-induced expression of the *pyp2*, *ctt1*, and *gpd1* genes is observed in $\Delta mcs4$ and $\Delta wak1$ cells, albeit to a greatly reduced degree, whereas all induction is abolished in either $\Delta wis1$ or $\Delta sty1$ cells. Second, we consistently observe inducible activation of Sty1 MAP kinase

in $\Delta mcs4$ and $\Delta wak1$ cells, whereas all activation is abolished in the absence of Wis1. This is consistent with the observation that both the Wis1 and Sty1 kinases are ab-

Figure 10. A model for control of the fission yeast stress-activated Sty1 MAP kinase cascade and cell cycle by the *Mcs4* response regulator. We propose that multiple stresses are sensed by an as-yet-defined histidine kinase. This leads to dephosphorylation of *Mcs4* on a conserved aspartate residue D412, which relieves inhibition of the *Wak1* MEK kinase, perhaps by direct binding to *Wak1*. Activation of *Wak1* leads to phosphorylation of *Wis1*, which in turn leads to phosphorylation and activation of the *Sty1* MAP kinase. We propose that *Mcs4* influences cell cycle progression by pathways both dependent and independent on *Sty1*.

Table 2. *Strains used in this study*

Strain no.	Genotype	Reference/source
PR 109	<i>leu1-32 ura4-D18 h⁻</i>	P. Russell
JM 1351	<i>leu1-32 ura4-D18 ade6-M210 his7-366 cdc25-22 h⁺</i>	this study
JM 1144	<i>leu1-32 ura4-D18 sty1-1 h⁻</i>	Millar et al. (1995)
JM 1160	<i>leu1-32 ura4-D18 ade6-216 sty1::ura4 h⁻</i>	Millar et al. (1995)
JM 544	<i>leu1-32 ura4-D18 wis1::ura4 h⁻</i>	Millar et al. (1995)
SP 890	<i>mcs4-13 h⁻</i>	Molz and Beach (1989)
JM 1303	<i>ura4-D18 mcs4-13 cdc25-22 h⁻</i>	this study
JM 1347	<i>leu1-32 ura4-D18 ade6-M210 his7-366 mcs4-13 h⁺</i>	this study
JM 1354	<i>leu1-32 ura4-D18 ade6-M210 his7-366 mcs4::ura4 h⁺</i>	this study
JM 1468	<i>leu1-32 ura4-D18 ade6-M210 his7-366 mcs4::his7 h⁺</i>	this study
JM 1436	<i>leu1-32 ura4-D18 ade6-M210 his7-366 wak1::ura4 h⁺</i>	this study
JM 1469	<i>leu1-32 ura4-D18 ade6-M210 his7-366 wak1::ura4 cdc25-22 h⁺</i>	this study
JM 1483	<i>leu1-32 ura4-D18 ade6-M210 his7-366 wak1::ura4 mcs4::his7 h⁺</i>	this study
PR 12	<i>leu1-32 ura4-D18 cdc2-3w wee1-50 h⁻</i>	P. Russell
JM 1367	<i>leu1-32 ura4-D18 ade6-M210 sty1::ura4 cdc2-3w wee1-50 h⁺</i>	this study
JM 553	<i>leu1-32 ura4-D18 wis1::ura4 cdc2-3w wee1-50 h⁻</i>	this study
JM 1480	<i>leu1-32 ura4-D18 ade6-M210 mcs4::ura4 cdc2-3w wee1-50 h⁺</i>	this study

solutely required for growth under conditions of environmental stress, whereas Mcs4 and Wak1 cells are not. The nature of this alternative pathway is not known but may involve a distinct MEK kinase from Wak1 or a homolog of the SH3 domain containing protein, SHO1, which is thought to act as an alternative osmosensor in budding yeast by binding to the amino terminus of the PBS2/HOG4 MEK (Maeda et al. 1995). Notably, whereas the Wis1-Sty1-Atf1 pathway is also required for viability in stationary phase, sexual conjugation, and meiotic progression (Warbrick and Fantes 1991; Takeda et al. 1995; Kato et al. 1996; Wilkinson et al. 1996), cells lacking either the *mcs4* or *wak1* genes only have a limited or undetectable defect in these processes (J.-C. Shieh and J.B.A. Millar, unpubl.). Because $\Delta mcs4$ and $\Delta wak1$ cells are shorter at division than $\Delta wis1$ or $\Delta sty1$ cells, we presume this alternative pathway also controls the timing of mitotic initiation.

The Sty1 MAP kinase was initially identified based on its effects on cell size at division (Millar et al. 1995; Shiozaki and Russell 1995). Because the timing of mitotic initiation requires attainment of a critical cell size before Cdc2 is activated, these results imply that Sty1 directly or indirectly influences the activity of the Cdc2 kinase. At present the mechanism underlying this control is unknown, but is likely to be independent of both the Wee1 tyrosine kinase and Cdc25 protein phosphatase, because *wis1* and *sty1* mutations can reverse the suppression of *cdc25-22* temperature-sensitive mutants by *wee1* inactivation (Warbrick and Fantes 1991; J.B.A. Millar, unpubl.). Notably the *mcs4* mutant, which was isolated by its ability to suppress the lethal hyperactivation of the central mitotic regulator Cdc2, also displays this phenotype (Molz et al. 1989; Warbrick and Fantes 1992). These results suggest that Mcs4 controls the timing of mitotic initiation by effecting Sty1 activity. Nevertheless loss of either *wis1* or *sty1* is not sufficient to suppress mitotic catastrophe caused by hyperactivation

of Cdc2, demonstrating that Mcs4 controls the timing of Cdc2 kinase activation by an additional Sty1-independent pathway (Fig. 10). Intriguingly the budding yeast MCM1 transcription factor, which is required for the periodic expression of a number of genes at the G₂/M transition, recently has been shown to be regulated by the SLN1 histidine kinase in a HOG1-independent manner (Yu et al. 1995). Whether a similar factor is under the control of Mcs4 in fission yeast is currently under investigation. In conclusion, these data illustrate an important role for a conserved two-component system in the coordination of cell cycle progression with the cellular response to environmental stress.

Materials and methods

Media and general techniques

Media and genetic methods for studying fission yeast have been reviewed recently (Moreno et al. 1991). General DNA methods were performed using standard techniques (Sambrook et al. 1989). Cell-length measurements were made using log-phase cells with a Nikon filar eyepiece drum micrometer at 1200 $\times$ magnification. Transformations were regularly performed by lithium acetate method (Moreno et al. 1991) or by electroporation (Prentice 1991) using a Bio-Rad Gene Pulser. Strains used in this study are listed in Table 2.

Overexpression of wis1

The *wis1* ORF was excised from pREP42-*wis1* (HA-6His) (Millar et al. 1995) by digestion with *NdeI* and *NotI* and ligated into the *NdeI* and *NotI* sites of pREP1 (HA-6His) (Maundrell 1993; J.B.A. Millar, unpubl.) bearing the LEU2 selectable marker to form pREP1-*wis1* (HA-6His). This plasmid was used to transform strains bearing the *leu1-32* mutation and leucine prototrophs selected. The phenotype of overexpression was determined after at least 48 hr growth in the absence of thiamine.

Isolation of the mcs4 genomic clone

A genomic library, pURB1 (Barbet et al. 1992), was introduced into a *mcs4-D18 cdc25-22 ura4-D18 h⁻* strain by electropora-

tion and plated on medium lacking uracil. A total of 55,000 transformants were screened. Transformants were replica plated twice to 30°C and 53 complementing colonies identified. DNA from these colonies were isolated by transformation into *Escherichia coli*. Plasmids encoding *cdc25*, *nim1*, or *wis1* were identified by Southern blot analysis. The eight remaining plasmids were related by restriction map. pURB1-Mcs4-1 contained a 3.25-kb insert (isolated five times); pURB1-Mcs4-2 (isolated twice) contained a 3.1-kb insert; and pURB1-Mcs4-3 (isolated once) contained a 5.6-kb insert. pMcs4-1 was used for further analysis.

Localization and sequencing of the *mcs4* gene

Simultaneous localization and sequencing was performed by transposon mutagenesis of the pURB1-Mcs4-1 genomic clone using TnHIS3 as described previously (Sedgewick and Morgan 1994). Complementing activity was tested by the transformation of transposed DNA into a *mcs4-13 cdc25-22 ura4-D18* strain, which was assayed for growth at 30°C. Dideoxy sequencing was performed using a T7 sequencing kit from Pharmacia using primers to the 5' and 3' ends of the transposon that were described previously.

Disruption of *Mcs4*

pCRII-Ura4 (J.B.A. Millar, unpubl.) was digested with *Xho*I and *Bam*HI and the resulting 1.6-kb Ura4⁺ fragment ligated into pURB1-Mcs4-1 that had been digested previously with *Bgl*III and *Xho*I to form pURB1-Mcs4::ura4(BX). The fragment removed contains >50% of the *Mcs4* ORF. Plasmid pURB1-Mcs4::ura4(BX) was linearized with *Sst*I and transformed into a *leu1-32/leu1-32 ade6-210/ade6-216 ura4-D18/ura4-D18 h⁻/h⁺* strain. Stable integrants were selected on medium lacking uracil and genomic DNA subjected to Southern blot hybridization using an *mcs4*-specific probe. Colonies bearing a disruption of *mcs4* were plated onto minimal medium containing adenine to induce sporulation. The resulting asci were used for tetrad dissection and DNA from the resulting haploid uracil prototrophs isolated to confirm disruption of *mcs4* by genomic Southern blot analysis.

Overexpression of the fission yeast *mcs4* and budding yeast SSK1 and SKN7 genes

The entire *mcs4* ORF was amplified from pURB1-Mcs4-1 by PCR by use of the 5' oligonucleotide CATAAGGATCCACATATGCGCATTGTTGTTAAAAAG (Mcs4-N1) containing a *Bam*HI and *Nde*I site (italicized) and the 3' oligonucleotide CAATAGGATCCGAATTCTGCAGTCATCGACCGCGAAAACGGCA (Mcs4-C1) containing *Bam*HI, *Eco*RI, and *Pst*I sites (italicized). The resulting 1.58-kb product was digested with *Nde*I and *Bam*HI and cloned into the *Nde*I and *Bam*HI sites of pREP41 bearing an attenuated version of the *nmt1* promoter (Basi et al. 1993) to form pREP41-Mcs4. The same fragment was cloned into the *Nde*I and *Bam*HI sites of pREP1 to form pREP1-Mcs4.

The entire SSK1 gene was amplified from *S. cerevisiae* cDNA using the 5' oligonucleotide CTGCTGTAAATCATATGCGAATCGATTGTTGGG (SSK1-1) and the 3' oligonucleotide ACATTCTATCATATGGTCCTCTACACGGTACAC (SSK1-2), both containing a *Nde*I site (italicized), digested with *Nde*I and cloned into the *Nde*I site of pREP41 to form pREP41-SSK1. In a similar manner the entire SKN7 response regulator protein was amplified by use of the 5' oligonucleotide ACTGAAGGGGATCCAATATTGAACGAAAAG (SKN7-1) and the 3' oligo-

nucleotide GCTTACTTTTGGGATCCACTATGAGC (SKN7-2), both containing a *Bam*HI site (italicized), digested with *Bam*HI and cloned into the *Bam*HI site of pREP41 to form pREP41-SKN7.

Mutation of putative phospho-acceptor residue in *Mcs4*

Aspartic acid (D) 412 in the *Mcs4* protein was mutated to asparagine (N) by mutagenic PCR amplification of the gene in two parts by use of the oligonucleotides Mcs4-N1 (see above) in combination with the 3' mutagenic oligonucleotide CGTGGGAAGTTGGATGTTTATTAGAAATCAAGTG (Mcs4-DN3) and the 5' mutagenic oligonucleotide CACTTGATTCTAATGAACATCCAACTTCCCACG (Mcs4-DN5) in combination with the 3' oligonucleotide Mcs4-C1 (see above). The mutated base is underlined. Products from each reaction were then combined and a second round of amplification performed by use of the oligonucleotides Mcs4-N1 and Mcs4-C1. The resulting 1.57-kb product was cloned to pREP41 as described above to form pREP41-Mcs4(D412N). A similar strategy was used to mutate residue 412 to glutamic acid (E) by use of the mutagenic oligonucleotides CGTGGGAAGTTGGATCTCCATTAGAAATCAAGTG (Mcs4-DE3) and CACTTGATTCTAATGGAGATCCAACCTTCCCACG (Mcs4-DE5) to form pREP41-Mcs4(D412E). Both mutations were confirmed by DNA sequencing.

Isolation and disruption of *Wak1*

A 4.2-kb clone of *wak1* was isolated from the fission yeast cosmid ICRFc60G109 by use of the 5' oligonucleotide ATAGAGCGGCCGCAGATCTTATGGGGCTAGAGCATACT bearing *Bgl*III and *Not*I sites (shown italicized) that hybridized to sequences in the promoter region of *wak1* and the 3' oligonucleotide CATAGGTACCAGATCTTTAGTCAACACTATAGTTTATTGTG bearing *Bgl*III and *Kpn*I sites (shown italicized) that hybridized to sequence around the termination codon. The fragment was digested with *Not*I and *Kpn*I and cloned into the *Not*I and *Kpn*I sites of pBSSK to form pBSSK-Wak1. pBSSK-Wak1 was digested with *Hind*III to release 1.57 kb of internal Wak1 ORF sequences and the *ura4⁺* gene ligated into these sites after digestion of pREP42 with *Hind*III to form pBSSK-Wak1::ura4. pBSSK-Wak1::ura4 was linearized with *Bgl*III and transformed into a *leu1-32/leu1-32 ade6-210/ade6-216 ura4-D18/ura4-D18 h⁻/h⁺* strain. Stable integrants were selected on medium lacking uracil and genomic DNA subjected to Southern blot hybridization by use of a *wak1*-specific probe. Colonies bearing a disrupted allele of *wak1* were plated onto minimal medium containing adenine to induce sporulation. The resulting asci were used for tetrad dissection and the resulting haploid uracil prototrophs were confirmed to bear a disrupted copy of *wak1* by genomic Southern blot analysis. A *wak1::LEU2* strain was constructed by a similar strategy.

Assay of endogenous Sty1 kinase activity

Endogenous Sty1 kinase was precipitated from cell extracts and assayed for kinase activity using a GST-Atf1 fusion protein bearing a fragment of Atf1-containing residues (81-194) that contains both the binding domain for Sty1 and three consensus Sty1 phosphorylation sites (Wilkinson et al. 1996; M. Samuels and N. Jones, unpubl.). Cells were lysed into lysis buffer (0.5% NP-40, 0.5% Na-deoxycholate, 50 mM NaF, 10% glycerol, 2 mM Na-orthovanadate, 10 mM β -mercaptoethanol, 10 μ g/ml of aprotinin, 10 μ g/ml of benzamidin, 2 mM PMSF, 10 μ g/ml of pepstatin A, 10 μ g/ml of leupeptin, 50 mM Tris-HCl at pH 7.4) and the Sty1 protein isolated by affinity precipitation on GST-Atf1

protein prebound to glutathione beads. Protein concentration in cell extracts were measured by Lowry and adjusted prior to precipitation. Precipitated proteins were washed three times with lysis buffer containing 0.5 M NaCl, washed once with kinase assay buffer without ATP, and then incubated in kinase assay buffer containing 50 mM HEPES at pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 10 mM EGTA, 10 mM β -mercaptoethanol, 0.2 μ Ci/ml of [γ -³²P]ATP for 20 min at 30°C. Reactions were terminated by the addition of SDS-sample buffer and the products separated by SDS-PAGE. Phosphorylation of Atf1 was determined by autoradiography.

DNA and RNA isolation and hybridization

S. pombe cells were cultured in YEA medium (0.5% yeast extract, 3% glucose, 50 mg/liter of adenine) to stationary phase. Chromosomal DNA isolated from a 10-ml culture was dissolved in 25 μ l of 10 mM Tris, 1 mM EDTA (TE), of which one-fifth was digested and subjected to electrophoresis and Southern blot hybridization. To isolate RNA, *S. pombe* cells were cultured in YEPD to exponentially growing phase. Ten micrograms of total RNA was isolated and resolved by agarose gel electrophoresis and transferred to nitrocellulose for hybridization. Probes for *pyp2*, *gpd1*, and *ctt1* were as described previously (Wilkinson et al. 1996). Equal loading of gels was verified by reprobing with *act1*, *cdc2*, *wis1*, or *sty1* cDNAs.

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