The Win1 Mitotic Regulator Is a Component of the Fission Yeast Stress-activated Sty1 MAPK Pathway

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The fission yeast Sty1 mitogen-activated protein (MAP) kinase (MAPK) and its activator the Wis1 MAP kinase kinase (MAPKK) are required for cell cycle control, initiation of sexual differentiation, and protection against cellular stress. Like the mammalian JNK/SAPK and p38/CSBP1 MAPKs, Sty1 is activated by a range of environmental insults including osmotic stress, hydrogen peroxide, UV light, menadione, heat shock, and the protein synthesis inhibitor anisomycin. We have recently identified two upstream regulators of the Wis1 MAPKK, namely the Wak1 MAPKKK and the Mcs4 response regulator. Cells lacking Mcs4 or Wak1, however, are able to proliferate under stressful conditions and undergo sexual differentiation, suggesting that additional pathway(s) control the Wis1 MAPKK. We now show that this additional signal information is provided, at least in part, by the Win1 mitotic regulator. We show that Wak1 and Win1 coordinately control activation of Sty1 in response to multiple environmental stresses, but that Wak1 and Win1 perform distinct roles in the control of Sty1 under poor nutritional conditions. Our results suggest that the stress-activated Sty1 MAPK integrates information from multiple signaling pathways.

INTRODUCTION

One of the most common mechanisms by which eucaryotic cells sense and respond to changes in the extracellular environment is via activation of a mitogen-activated protein (MAP) kinase cascade. Signal transduction through MAPK cascades involves sequential phosphorylation and activation of three distinct kinases; the MAP kinase kinase kinase (or MAPKKK), the MAP kinase kinase (or MAPKK), and the MAP kinase (MAPK) itself. Although the precise mechanisms by which plasma membrane-associated receptors induce activation of the MAPKKK are still unclear, MAPKKK activation leads to MAPKK activation by direct phosphorylation. The MAPKK, in turn, activates the MAPK by dual phosphorylation on two closely spaced residues, a threonine and a tyrosine. The most widely studied of the MAPKs in mammalian cells is the ERK family of kinases, which are activated by a wide range of peptide growth factors and hormones. More recently, a new family of MAPKs has been identified in metazoan cells that are activated by a variety of stress conditions including osmotic stress, heat shock, oxidative stress, UV radiation, and the protein synthesis inhibitor anisomycin, as well as by inflammatory cytokines and certain vasoactive neuropeptides (Dérijard et al., 1994; Galcheva-Gargova et al., 1994; Han et al., 1994; Kyriakis et al., 1994; Lee et al., 1994; Rouse et al., 1994). Pharmacological, biochemical, and genetic evidence indicates multiple roles for these stress-activated MAPKs (SAPKs) in a wide variety of physiological and pathological conditions including development, control of cell proliferation, cell death, inflammation, and response to ischemic injury. As such, these enzymes are drawing considerable attention as potential targets for novel therapeutics. The mechanism(s) by which this class of MAPK is activated is, however, unknown.

We have identified a stress-activated MAPK pathway in the unicellular fission yeast, Schizosaccharomyces pombe, the central elements of which are the Sty1 MAPK (also known as Spc1 and Pfh1) and the Wis1 MAPKK (Warbrick and Fantes, 1991; Millar et al., 1995; Shiozaki and Russell, 1995; Kato et al., 1996). Impor-

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tantly, the fission yeast Sty1 MAPK, like its mammalian counterparts, is activated by a range of environmental stimuli including osmotic stress, oxidative stress, UV light, certain DNA-damaging agents, heat shock, and the protein synthesis inhibitor anisomycin (Millar et al., 1995; Shiozaki and Russell, 1995; Degols et al., 1996, Degols and Russell, 1997; Shieh et al., 1997). This suggests that an evolutionarily conserved sensor may regulate both the mammalian and fission yeast SAPKs. This possibility is consistent with the recent finding that a direct phosphorylation target of fission yeast Sty1 is the Atf1 transcription factor, a structural homologue of human ATF2 that binds and is activated by the SAPKαII/JNK2 MAPK (Gupta et al., 1995; Shiozaki and Russell, 1996; Wilkinson et al., 1996). The Sty1 MAPK controls multiple cellular events in fission yeast including the initiation of sexual differentiation, prolonged viability in stationary phase, and the cellular response to environmental stress. Cells deleted for Atf1 also display many of these phenotypes, suggesting Atf1 is a physiologically important target for Sty1 (Shiozaki and Russell, 1996; Wilkinson et al., 1996). Importantly, cells lacking wis1 or sty1 are highly elongated at cell division. Since the timing of mitotic initiation in fission yeast requires attainment of a critical cell size, these observations suggest a crucial role for the stress-activated Sty1 MAPK pathway in control of the cell cycle (Nurse, 1975; Warbrick and Fantes, 1991; Millar et al., 1995; Shiozaki and Russell, 1995). Since mitotic initiation is triggered by activation of the catalytic subunit of the Cdc13 (Cyclin B)/Cdc2 kinase, genes that when mutated alter cell size at division are, by inference, required for the correct timing of Cdc2 activation. Two such genes are the wee1 and cdc25 mitotic regulators, which code for a tyrosine kinase and phosphatase, respectively, that directly control the activity of the Cdc13/Cdc2 complex (Russell and Nurse, 1987; Millar and Russell, 1992). At present, the mechanism by which the Sty1 MAPK influences mitotic initiation is unknown, but it is likely to be independent of both the Wee1 tyrosine kinase and Cdc25 protein phosphatase, since wee1 and sty1 mutations can reverse the suppression of cdc25–22 temperature-sensitive mutants by wee1 inactivation (Warbrick and Fantes, 1991; Shiozaki and Russell, 1995).

We have found that some of the upstream components of the fission yeast Sty1 pathway are structurally and functionally similar to those of the budding yeast HOG1 pathway, which is activated only by osmotic stress (Brewster et al., 1993; Schüller et al., 1994). These are the Mcs4 mitotic catastrophe suppressor and Wak1 MAPKKK (also known as Wik1). Mcs4 and Wak1 are structurally and functionally similar to the budding yeast SK1 response regulator and SKK2/SKK2 MAPKKKs from budding yeast, respectively (Maeda et al., 1994, 1995; Shieh et al., 1997; Shiozaki et al., 1997). SK1 acts as part of a “two-component phospho-relay system” that is initiated by inactivation of a transmembrane osmosensor, the SLN1 histidine kinase (Ota and Varshavsky, 1993; Posas et al., 1996). These results indicate that the fission yeast SAPK pathway is also controlled by a two-component system. It is not clear, however, whether the two-component system is responsible for activation of Sty1 by multiple stresses or whether additional pathways exist (Shieh et al., 1997). Importantly neither Wak1 nor Mcs4, however, are absolutely required for proliferation in stressful conditions, suggesting that additional elements do control the Wis1 MAPKK.

We have sought additional regulators of Sty1 with the goal of understanding how the pathway is activated by multiple environmental stresses. We focused initially on a recessive mutant, win1–1, that was found to be required for cell division in the simultaneous absence of both the Cdc25 phosphatase and Wee1 tyrosine kinase (Ogden and Fantes, 1986). Importantly, the cell cycle arrest phenotype of a wee1–50 cdc25–22 win1–1 strain is suppressed by overexpression of the Wis1 MAPKK (Warbrick and Fantes, 1991). In this paper we show that the Win1 mitotic regulator is a component of the Sty1 pathway and contributes to activation of Sty1 MAPK by multiple environmental stimuli.

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 (Garden City, NY) filar eyepiece drum micrometer at 1200× magnification. Transformations were regularly performed by the lithium acetate method (Moreno et al., 1991) or by electroporation (Prentice, 1991) using a Bio-Rad (Richmond, CA) Gene Pulser.

Assessment of Mating Efficiency

Homothallic (h+) cells were grown to log phase in liquid Edinburgh minimal media (EMM) and then incubated in the same medium lacking NH4Cl as a nitrogen source. Mating efficiency was determined microscopically by the appearance of cells undergoing conjugation or spore-containing asci.

Overexpression of Tagged Wak1 Protein

The catalytic domain of the wak1 gene was cloned by polymerase chain reaction (PCR) amplification from S. pombe genomic DNA. The oligonucleotide TAAC-TAGATCTATGCG-TTTCGTTAACG-CAT incorporated a BgII site (shown italicized) and hybridized to sequences 5′ to the catalytic domain, whereas the 3′ oligonucleotide TATAGCCCGCCGTCACACATATGATTTATGTG incorporated a NotI site (shown italicized) and hybridized to sequences surrounding the TGA termination codon. PCR amplification generated a fragment that was cleaved with BgII and NotI and cloned into the BgII and NotI sites of pREP41(6HisHA) downstream of an attenuated version of the nmt1 promoter (Basi et al., 1993) to form pREP41-wak1(6HisHA). Expression of this protein in S. pombe was confirmed by Western blot analysis.
Integration and Detection of Tagged Sty1 Protein
A C-terminal fragment of a 6-histidine and hemagglutinin (HA)-tagged sty1 gene was excised from pREP41-sty1(6HisHA) by digesting with NruI and BamHI (Millar et al., 1995) and cloned into the SnaI and BamHI sites of pBSKK-Ura to generate pBSKK-Ura-sty1(6HisHA). pBSKK-Ura4-sty1(6HisHA) was linearized with PacI, and the resulting fragment was transformed into wild-type S. pombe cells bearing the ure4-D18 auxotrophic marker. Stable integration of the tagged sty1 gene at the genomic sty1 locus was confirmed by Southern blot analysis and PCR.

Detection of Tagged Sty1 Protein
The Sty1 protein was partially purified from cells bearing an integrated tagged version of sty1 (see above) using Ni++-nitrilo-triacetic acid (NTA) agarose exactly as previously described (Millar et al., 1995). Precipitated proteins were resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were probed with either a monoclonal antibody to the HA epitope (12CA5) or with a monoclonal antibody to phosphotyrosine (4G10, Upstate Biotechnology, New York, NY). Detection was performed using a peroxidase-conjugated anti-mouse IgG (Amersham, Buckinghamshire, U.K.) and enhanced chemiluminescence visualization (ECL, Amersham) according to the manufacturer's instructions.

Assay of Endogenous Sty1 Kinase Activity
Endogenous Sty1 kinase was precipitated from cell extracts and activity was assayed using a glutathione-S-transferase (GST)-Atf1 fusion protein prebound to glutathione beads as previously described (Shieh et al., 1997). Protein concentration in cell extracts was measured by the Lowry assay and adjusted before 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, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 10 mM EGTA, 10 mM β-mercaptoethanol, 0.2 μCi/ml γ³²P-ATP for 20 min at 30°C. Reactions were terminated by the addition of SDS-sample buffer, and the products were separated by SDS-PAGE. Phosphorylation of Atf1 was determined by autoradiography.

Analysis of DNA Content by Flow Cytometry
Samples containing ~10⁷ cells were fixed with 70% ethanol, treated successively with RNase and pepsin, and stained with 50 μg/ml propidium iodide essentially as previously described (Corliss and White, 1981). DNA content was then analyzed with a Becton Dickinson (Oxford, U.K.) FACSscan and CELL Quest software.

RNA Isolation and Hybridization
To isolate RNA, S. pombe cells were cultivated in YEPD to exponential phase. Approximately 10 μg of total RNA were isolated and resolved by agarose gel electrophoresis before transfer to nitrocellulose for hybridization as previously described (Aves et al., 1985). Probes for ppy2 and ct1 were as previously described (Millar et al., 1995; Takeda et al., 1995).

RESULTS
Evidence for an Additional Pathway Controlling the Wis1 MAPKK
In poor nutritional conditions fission yeast cells enter a quiescent state either in the G₁ or G₂ phases of the cell cycle. In defined media, arrest in the G₁ phase of the cell cycle can be promoted by depletion of an exogenous nitrogen source. Under these conditions cells lacking either the Wis1, Sty1, or Atf1 proteins fail to arrest in G₁ and arrest preferentially in G₂ (Takeda et al., 1995; Kato et al., 1996; Shiozaki and Russell, 1996). We have assessed the role of two upstream regulators of the Wis1-Sty1-Atf1 cascade, the Wak1 MAPKK and Mcs4 response regulator, in this process. Analysis of DNA content by fluorescence-activated cell sorter indicates that cells deleted for either wak1 or mcs4 arrest normally with a 1 N content of DNA after either 12 or 24 h incubation in nitrogen-free medium, indicating a G₁ arrest (Figure 1A). In contrast, approximately only 5% of ∆wis1 or ∆sty1 cells arrest in G₁ under the same conditions as previously observed (our unpublished data; Shiozaki and Russell, 1996). In homothallic (h90) strains, entry into the G₁ phase is accompanied by sexual conjugation and differentiation. Since the Wis1 MAPKK is required for proper arrest in G₁, cells lacking either the Wis1, Sty1, or Atf1, pointing to the existence of an alternative pathway controlling the Wis1 MAPKK that is independent of either Wak1 or Mcs4 proteins. Mutants in the Sty1 pathway lose viability in stationary phase, which is likely to contribute to the mating deficiency (see below).

Win1 Mitotic Regulator Interacts Genetically with Components of the Sty1 Pathway
Cells deleted for either the Sty1 MAPK or Wis1 MAPKK are delayed in the timing of mitotic initiation. Since ∆mcs4 and ∆wak1 cells are shorter at division than ∆wis1 or ∆sty1 cells, we presumed that other genes controlling Wis1 may also control the timing of mitotic initiation. For this reason we focused initially on the win1 mitotic regulator in an attempt to identify other components of the Sty1 pathway (Ogden and Fantes, 1986). To examine genetic interactions of a win1–1 mutant with components of the Sty1 pathway, two approaches were taken: first, win1–1 cdc25–22 cells were transformed with plasmids expressing either the wis1, wak1, or the mcs4 genes. At 33°C win1–1 cdc25–22 cells undergo cell cycle arrest, whereas win1–1 and cdc25–22 single mutants are able to proliferate normally (our unpublished data). Overexpression of either wis1 or wak1 could suppress the cell cycle arrest of a win1–1 cdc25–22 strain at 33°C, indicating that ectopically increasing the activity of the Sty1 MAPK can bypass the mitotic delay of a win1–1 mutant. (Figure 2A). We have subsequently noticed that the restriction map of wak1 is identical to a previously identified
multicopy suppressor of win1–1, namely wis4 (Warbrick and Fantles, 1992). In contrast, overexpression of mcs4 was unable to suppress the win1–1 cdc25–22 arrest, the reasons for which are discussed below.

In a second genetic test to assess the role of win1, wild-type, win1–1 mutants, or cells deleted for either wak1 or sty1 were transformed with a vector expressing wis1 from the strong thiamine-repressible nmt1 promoter (Maundrell, 1991). Hyperactivation of the Sty1 MAPK by massive overexpression of the Wis1 MAPKK is toxic to wild-type cells but not, for instance, to mcs4–13 cells, which are defective in Wis1 activation (Shieh et al., 1997). As the results in Figure 2B show, massive overexpression of wis1 is also not toxic in either win1–1 cells or in cells lacking either the Wak1 MAPKKK or Sty1 MAPK. These results confirm that win1 has an important role in controlling mitotic initiation in fission yeast and further suggest that win1 acts either as an activator or downstream target of the Sty1 pathway.

**Win1 Controls Stress-induced Gene Expression and Activation of the Sty1 MAPK**

Activation of the Wis1-Sty1-Atf1 pathway by a variety of environmental insults including osmotic stress, heat shock, oxidative stress, and anisomycin causes induction of a number of genes including the pyp2 MAPK phosphatase, which acts in a feedback loop to attenuate Sty1 activity (Millar et al., 1992, 1995; Degols et al., 1996; Wilkinson et al., 1996). To
Win1 is required for stress-induced gene expression. Expression of the Pyp2 MAPK phosphatase is attenuated in cells lacking win1. Top panel, log phase cultures growing in YEPD at 30°C of either wild-type (WT), (PR109), win1–1 (win1–1) (JM1413), wak1::ura4 (Δwak1) (JM 1436), or wak1::ura4 win1–1 (Δwak1 win1–1) (JM1504) cells were incubated in the same medium containing 0.6 M KCl, 1 mM H2O2, 10 μg/ml anisomycin or shifted to 42°C for the times indicated. Total RNA was extracted, and equal quantities were separated by electrophoresis and then probed using DNA specific to the pyp2 gene. Middle panel, induction of catalase (ctt1) is attenuated in cells lacking win1–1. Log phase cultures of wild type (WT) (PR109), win1–1 (win1–1) (JM1413), wak1::ura4 (Δwak1) (JM 1436), or wak1::ura4 win1–1 (Δwak1 win1–1) (JM1504) cells growing in YEPD were incubated in the same medium containing either 10 μg/ml anisomycin or 1 mM H2O2 for the times indicated. In this experiment total RNA was extracted as described previously and probed using DNA specific to the ctt1 gene. Bottom panel, blots were reprobed with a cdc2-specific probe to verify equal loading of RNA.

To assess whether the effect of win1–1 on stress-induced gene expression is at the level of transcription or via controlling the activity of the Sty1 MAPK, we measured both phosphotyrosine content and activity of Sty1. Strains bearing a single integrated C-terminally epitope-tagged sty1 gene in either wild-type or a win1–1 background were constructed. The Sty1 protein was affinity precipitated from log phase cultures of these strains after challenge with 0.6 M KCl. The phosphorylation state of the Sty1 protein is dramatically reduced in win1–1 cells relative to wild type. Duplicate samples probed using a monoclonal antibody to phosphotyrosine. Figure 4A demonstrates that the increase in phosphotyrosine on the Sty1 protein is dramatically reduced in win1–1 cells relative to wild type. Duplicate samples probed using a monoclonal antibody to the epitope (HA) tag showed that the level of protein did not change through the course of the experiment (Figure 4 A). Similar results were obtained when cells were stimulated with other stresses (our unpublished data). To confirm this result the activity of endogenous untagged Sty1 protein was determined by a coupled affinity precipitation-kinase assay using a GST-Atf1 fusion protein as a substrate, as previously described (Wilkinson et al., 1996). Wild-type and win1–1 cells were heat shocked at 42°C for various times, and the Sty1 protein was precipitated and its kinase activity determined. As the results in Figure 4B show, stimulation of Sty1 by heat shock was also dramatically reduced in win1–1 cells, although some residual induction was evident. Similar results were found when cells were challenged with either an osmotic stress or the protein synthesis inhibitor anisomycin (our unpublished data). Together these results indicate that Win1 is required for Sty1 MAPK activation in response to multiple independent environmental stresses.

Figure 3. Win1 is required for stress-induced gene expression. Expression of the Pyp2 MAPK phosphatase is attenuated in cells lacking win1. Top panel, log phase cultures growing in YEPD at 30°C of either wild-type (WT), (PR109), win1–1 (win1–1) (JM1413), wak1::ura4 (Δwak1) (JM 1436), or wak1::ura4 win1–1 (Δwak1 win1–1) (JM1504) cells were incubated in the same medium containing 0.6 M KCl, 1 mM H2O2, 10 μg/ml anisomycin or shifted to 42°C for the times indicated. Total RNA was extracted, and equal quantities were separated by electrophoresis and then probed using DNA specific to the pyp2 gene. Middle panel, induction of catalase (ctt1) is attenuated in cells lacking win1–1. Log phase cultures of wild type (WT) (PR109), win1–1 (win1–1) (JM1413), wak1::ura4 (Δwak1) (JM 1436), or wak1::ura4 win1–1 (Δwak1 win1–1) (JM1504) cells growing in YEPD were incubated in the same medium containing either 10 μg/ml anisomycin or 1 mM H2O2 for the times indicated. In this experiment total RNA was extracted as described previously and probed using DNA specific to the ctt1 gene. Bottom panel, blots were reprobed with a cdc2-specific probe to verify equal loading of RNA.

examine whether win1 is required for activation of the Sty1 MAPK pathway, wild-type or win1–1 cells were incubated in the presence of either 0.6 M KCl, 1 mM H2O2, 10 μg/ml anisomycin or given a mild heat shock for various times and then the level of pyp2 expression was examined by Northern blot analysis. As the results in Figure 3, top, show, induction of pyp2 was dramatically reduced in win1–1 cells in response to osmotic or heat shock or to anisomycin, although significant delayed expression of the pyp2 mRNA was observed after stimula-
Win1 and the Wak1 MAPKKK Cooperative to Control Activation of the Sty1 MAPK

Both Wak1 and Win1 are required for the correct timing of mitotic initiation (Ogden and Fantes, 1986; Shieh et al., 1997; Shiozaki et al., 1997). To assess the relationship of Win1 to the Wak1 MAPKK, double Δwak1 win1–1 mutant cells were constructed and cell size at division was analyzed. We observe that double mutant Δwak1 win1–1 cells divide at 21.4 ± 1.8 μm, larger than either Δwak1 cells (16.5 ± 0.5 μm) or win1–1 single mutants (17.1 ± 1.1 μm), indicating that the effect of Wak1 and Win1 on the timing of mitotic initiation is additive. To examine the relationship of wak1 and win1 in controlling Sty1 MAPK function, stress-induced expression of the pyp2 and ctt1 genes was determined in double Δwak1 win1–1 single mutants. Expression of both pyp2 and ctt1 was virtually abolished in single wak1 and win1–1 mutants in response to osmotic stress, heat shock, or anisomycin (Figure 3). However, we found that considerable residual expression of pyp2 and to a lesser extent ctt1 was observed in both single mutants in response to hydrogen peroxide (Figure 3). Importantly this residual expression was also lost in double Δwak1 win1–1 mutants, suggesting that win1 contributes to acute activation of Sty1 in the presence or absence of wak1 (Figure 3).

We have previously shown that Wak1 is not required for long-term survival either at high temperature or in high osmolarity medium (Shieh et al., 1997; Shiozaki et al., 1997). To assess the role of win1 in the long-term response of the cell to environmental stress, wild-type cells, Δwak1 and win1–1 single mutants, or Δwak1 win1–1 double mutants were grown either on rich medium at 30°C, on the same medium containing 1.5 M sorbitol, or on the same medium at 37°C. As previously observed neither Δwis1 nor Δsty1 cells were able to grow at high temperature or under hyperosmolar conditions whereas Δwak1 cells were unaffected (Millar et al., 1995; Figure 5). In contrast, win1–1 cells grew poorly at high temperature or on high osmolarity medium, and this effect was exacerbated in Δwak1 win1–1 double mutants (Figure 5).
Together, these results indicate that Wak1 and Win1 act in concert to control stress-induced activity of the Sty1 MAPK in response to multiple environmental stimuli.

**Win1 Is Crucial for Controlling Sty1 MAPK Activity in Stationary Phase**

As previously demonstrated, cells lacking either the Wis1 MAPKK or the Sty1 MAPK fail to enter G1 when starved of a nitrogen source whereas cells lacking the Wak1 MAPKKK are able to do so (Warbrick and Fantes, 1991; Takeda *et al.*, 1995; Kanoh *et al.*, 1996). In parallel cultures to those shown in Figure 1A, win1−1 cells were grown to log phase in minimal medium, and the ability to arrest in G1 was determined by fluorescence-activated cell sorter analysis after either 12 or 24 h incubation in nitrogen-free medium. As the results in Figure 6A demonstrate, less than 50% of the cells had entered G1 after 24 h, suggesting that Wak1 and Win1 perform distinct functions in stationary phase (Figure 6C). To directly compare the relative roles of Wak1 and Win1 in stationary phase, the ability of mutant strains to initiate sexual conjugation and differentiation was assessed. Homothallic strains of wild-type cells, Δwak1 cells, win1−1 mutants, or Δwak1 win1−1 double mutants were grown to stationary phase in minimal medium lacking a nitrogen source, and the number of cells that had undergone sexual conjugation and meiosis were assessed after the times indicated. As the results in Figure 6B illustrate, win1−1 cells were profoundly defective in initiating sexual differentiation, but this was not exacerbated by simultaneous inactivation of wak1. In fission yeast, sexual conjugation is triggered in poor nutritional conditions that promote exit from the cell cycle and entry into a quiescent G0 state. Importantly, cells lacking the Wis1 MAPKK, the Sty1 MAPK, or the Atf1 transcription factor fail to maintain viability in stationary phase, a phenotype that is also displayed by the win1−1 mutant (Ogden and Fantes, 1986; Warbrick and Fantes, 1991; Takeda *et al.*, 1995; Kanoh *et al.*, 1996). To directly compare the roles of Wak1 and Win1 in this process, wild-type cells, win1−1 mutants, or cells deleted for either the Wak1 MAPKKK or Wis1 MAPKK were grown in rich medium and cell viability assessed as the culture reached saturation and stationary phase. Counting of total cell number revealed that all cultures ceased dividing after continuous incubation for approximately 24 h (our unpublished data). As the results in Figure 6C demonstrate, after this time Δwis1 and win1−1 cells underwent a rapid loss of viability, whereas Δwak1 cells were mostly unaffected. It is likely that mating efficiency reflects both the ability to enter G1 phase of the cell cycle and to maintain viability in stationary phase. Regardless of this, these results indicate that Wak1 and Win1 perform distinct roles in controlling the Sty1 MAPK in poor nutritional conditions.

**Wis1 and Sty1 Are Active at a Low Level in Δwak1 win1−1 Double Mutants**

To assess whether Win1 is the only regulator of Sty1 in the absence of Wak1, the phenotypes of Δwak1 win1−1
cells were compared with those of Δsty1 and Δwis1 cells. First, we note that Δwak1 win1–1 cells divide at a smaller size than Δwis1 cells, suggesting that Sty1 is not inactive in Δwak1 win1–1 cells (Table 1). This is supported by the observation that Δwak1 win1–1 cells can be propagated at 26°C in rich medium whereas Δwis1 cells cannot (Table 1; Millar et al., 1995; Shiozaki and Russell, 1995). Second, Δwak1 win1–1 h90 cells were able to undergo sexual conjugation more effectively than Δwis1 h90 or Δsty1 h90 cells (Figure 6B). These observations predict that increasing the expression of wis1 in double mutant Δwak1 win1–1 cells should reverse the phenotype resulting from simultaneous loss of wak1 and win1 function. Homothallic and heterothallic wild-type, Δwis1, or Δwak1 win1–1 cells were transformed with a vector expressing either wis1 or a truncated version of wak1 from the thiamine-repressible nmt1 promoter, and the ability of these cells to undergo sexual conjugation or grow at high temperature was assessed (Basi et al., 1993). Increasing the expression of wis1 completely suppresses the mating deficiency and thermosensitivity of Δwak1 win1–1 cells (Figure 7A and B). These effects are dependent on the catalytic activity of Wis1 since a catalytically inactive mutant in which the active site lysine has been mutated to an arginine is unable to complement these strains (our unpublished data). Overexpression of wak1 was also able to completely suppress the inability of win1–1 Δwak1 to mate or proliferate at high temperature, indicating that when overexpressed, wak1 can fully substitute for loss of win1 (Figure 7A and B). These data indicate that the Sty1 MAPK retains significant activity in the absence of both Wak1 and Win1, suggesting that either win1–1 is not a null allele or that additional elements control the Sty1 MAPK.

**DISCUSSION**

The stress-activated Sty1 MAPK pathway of fission yeast displays some remarkably similar features to the mammalian SAPK pathways. Most significantly, the Sty1 MAPK is activated by a similar range of environ-

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**Table 1.** Win1 regulates cell size at division independently of Wak1

<table>
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<th>Genotype</th>
<th>T°C</th>
<th>Cell size at division (μm)</th>
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<tr>
<td>wt</td>
<td>(30°C)</td>
<td>14.2 ± 0.3 μm</td>
</tr>
<tr>
<td>win1-1</td>
<td>(30°C)</td>
<td>17.1 ± 1.1 μm</td>
</tr>
<tr>
<td>wak1::ura4</td>
<td>(30°C)</td>
<td>16.5 ± 0.5 μm</td>
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<td>21.4 ± 1.3 μm</td>
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<td>23.1 ± 2.1 μm</td>
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<td>(26°C)</td>
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**Table 2.** Strains used in this study

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We have recently identified two upstream regulators of the Sty1 MAP kinase pathway as the Mcs4 response regulator and Wak1 MAPKK. These results suggest that the architecture of the fission yeast Sty1 MAPK pathway is similar to the HOG1 MAPK pathway in the related budding yeast, and that both pathways are controlled by a conserved two-component system (Shieh et al., 1997). Notably, however, cells lacking either Mcs4 or Wak1 are able to proliferate under stressful conditions and have limited detectable defects in either entering G1 phase or initiating sexual conjugation. Together these data indicate the existence of alternative signaling pathways controlling the Wis1 MAPK. Since cells lacking mcs4 or wak1 are not as severely delayed in the timing of mitotic initiation as are Δwis1 or Δsty1 cells, we presumed that this alternative pathway also controls the timing of mitotic initiation. For this reason we focused on a recessive mutant, win1–1, which is delayed in the timing of mitotic initiation and displays genetic interactions with the Wis1 MAPK (Ogden and Fant, 1986; Warbrick and Fant, 1991). The following lines of evidence suggest that Win1 and the Wak1 MAPKK cooperatively control the activity of the stress-activated Sty1 MAPK in response to multiple environmental stimuli. First, stress-mediated induction of several genes whose expression is regulated by Sty1, including pyp2, ctt1, and gpd1, is severely diminished in win1–1 mutant cells. Second, activation of the Sty1 MAPK by multiple environmental stresses is also dramatically attenuated in win1–1 cells, as assessed by phosphotyrosine content and ability to phosphorylate a GST-Atf1 fusion protein. To determine the relationship of Win1 to the Wak1 MAPKK, win1–1 Δwak1 double mutants were constructed. These cells divided at a larger size than either wak1 or win1–1 single mutants alone, suggesting that Wak1 and Win1 act independently (Table 1). This conclusion is supported by the observation that induction of pyp2 mRNA in double Δwak1 win1–1 mutants is considerably lower than either win1–1 or Δwak1 single mutants alone, and that Δwak1 win1–1 double mutants proliferate very poorly either at high temperature or in high osmolarity, whereas win1–1 and Δwak1 single mutants are able to form colonies under these conditions. These data, together with genetic evidence that ectopically increasing the activity of Sty1 can bypass loss of win1 function, incontrovertibly establish Win1 as a component of the Sty1 pathway. One possible explanation for these data is that win1 may encode a structural component that tethers components of the Sty1 pathway together in a manner analogous to the role that the STE5 gene product plays in the budding yeast-mating pheromone pathway (Choi et al., 1994). We believe a more likely explanation is that since Wis1 is the only MAPKK needed for Sty1 activation, win1 encodes a second MAPKK for Wis1 MAPK. This is not un-

Figure 7. Evidence for a Wak1- and Win1- independent pathway controlling Wis1. (A) Wis1 suppresses the mating deficiency of a Δwak1 win1–1 strain. The homothallic strain Wak1:ura4 win1–1 leu1–32 k10 (Δwak1 win1–1) (JM 1509) was transformed either with a control plasmid pREP41 (Cont.) or with pREP41-wis1 (pWis1) or pREP41-wak1 (pWak1) (as above). Transformants were grown to log phase at 30°C in liquid EMM lacking leucine and transferred to the same medium lacking NH4Cl for 48 h, and mating efficiency was assessed microscopically. (B) Wis1 suppresses the temperature sensitivity of a Δwak1 win1–1 strain. Wak1::ura4 win1–1 leu1–32 (Δwak1 win1–1) (JM 1504) cells were transformed with a control plasmid pREP41 (Cont.) or either pREP41-wis1 (pWis1) or pREP41-wak1 (pWak1) in which the wis1 and wak1 genes were expressed from the thiamine-repressible mnt1 promotor. Transformants were streaked on minimal medium lacking thiamine and leucine, and colony formation was monitored after 3 d incubation at either 30°C (left hand plate) or 37°C (right hand plate).
reasonable since \textit{wak1} when overexpressed can fully substitute for loss of \textit{win1}. Indeed, three functionally overlapping MAPKKKs have been found to regulate the single PBS2 MAPKK in budding yeast in response to osmotic stress (Maeda \textit{et al.}, 1995; Posas and Saito, 1997). Moreover, our finding that \textit{win1–1} cells are epistatic to overexpression of \textit{mcs4} is consistent with the hypothesis that the \textit{mcs4}-response regulator acts upstream of both \textit{wak1} and \textit{win1}. We also tentatively suggest that the \textit{wis1} MAPKK may be controlled by an additional \textit{wak1}- and \textit{win1}-independent pathway.

![Diagram](image)

\textbf{Figure 8.} A model for the role of \textit{win1} in controlling the fission yeast stress-activated \textit{sty1} MAPK. We propose that \textit{win1} controls the activity of \textit{wis1} MAPKK in parallel with the \textit{wak1} MAPKKK and that the \textit{mcs4} response regulator acts upstream of both \textit{wak1} and \textit{win1}. We also tentatively suggest that the \textit{wis1} MAPKK may be controlled by an additional \textit{wak1}- and \textit{win1}-independent pathway.

in this process. Second, \textit{win1–1} cells are partially sterile whereas \textit{Δwak1} are able to mate with almost wild-type efficiency. One possibility is that distinct regulators control \textit{wak1} and \textit{win1} in response to either environmental stress or nutrient deprivation. It is important to point out, however, that there is no formal proof that either \textit{wak1} activity or \textit{win1} function are stimulated by environmental stimuli, so that the mechanism by which the stress signal is transduced to the \textit{sty1} MAPK is still unknown. The development of reagents to study the \textit{wak1} and \textit{win1} proteins in vivo will help resolve some of these issues.

A number of MAPKKKs that stimulate the JNK/SAPK and p38/CSBP1 MAPKs have been identified by transient transfection studies in mammalian cells, including MEKK1, TAK1, MUK, SPRK/MLK3, TPL2/COT1, and ASK1. It is curious that none of these enzymes have been shown to be catalytically stimulated by environmental stress (Yan \textit{et al.}, 1994; Yamaguchi \textit{et al.}, 1995; Hirai \textit{et al.}, 1996; Rana \textit{et al.}, 1996; Salmeron \textit{et al.}, 1996; Ichijo \textit{et al.}, 1997). It is possible that additional as-yet-undiscovered MAPKKKs control the SAPKs or that these pathways are triggered without necessarily activating a MAPKKK. In this regard it is intriguing that \textit{sty1} activity is not abolished in \textit{win1–1 Δwak1} mutants. Specifically, \textit{win1–1 Δwak1} double mutants are not as severely delayed in mitotic initiation as \textit{Δwis1} or \textit{Δsty1} cells but are more effective in initiating sexual conjugation. In addition, overexpression of the \textit{wis1} MAPKK can rescue the phenotypes of \textit{win1–1 Δwak1} double mutant cells suggesting that either additional pathway(s) regulate the \textit{sty1} MAPK or that the \textit{win1–1} mutant is not a complete loss-of-function allele. Further experimentation will be needed to establish which of these possibilities is true.

In conclusion, we have identified a new component of the stress-activated \textit{sty1} MAPK pathway as the product of the mitotic regulator, \textit{win1}. Although we have yet to decipher precisely how \textit{sty1} is activated, the similarity of the stimuli that activate both the fission yeast and mammalian SAPKs suggest that this information will dramatically improve our understanding of how the mammalian SAPK pathways are regulated. The amenability of fission yeast to genetic, biochemical, and immunocytochemical analysis indicates that this goal is attainable.

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