Evidence for a novel MAPKKK-independent pathway controlling the stress activated Sty1/Spc1 MAP kinase in fission yeast

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SUMMARY

The fission yeast Sty1/Spc1 MAP kinase, like the mammalian JNK/SAPK and p38/CSBP1 kinases, is activated by a range of environmental insults including osmotic stress, hydrogen peroxide, heat shock, UV light and the protein synthesis inhibitor anisomycin. Sty1 is activated by a single MAPKKK, Wis1. We demonstrate that the conserved MAPKKK phosphorylation sites Ser 469 and Thr 473 in the catalytic domain of Wis1 are normally essential for Sty1 activation. However, when mildly overexpressed, a mutant Wis1 kinase lacking these conserved phosphorylation sites is able to support stress inducible gene expression and activation of the Sty1 MAP kinase in response to an oxidative or osmotic stress or to a mild heat shock. We show that phosphorylation and activation of Sty1 under these conditions is not due to inactivation of the Pyp1 MAP kinase phosphatase. These results reveal a novel MAPKKK-independent pathway by which the Wis1 MAPKK can activate the Sty1 MAPK in response to stress in fission yeast.

Key words: Stress activated MAP kinase, Cell cycle, Schizosaccharomyces pombe, Sexual differentiation

INTRODUCTION

It is currently thought that activation of MAP kinase cascades is initiated by the catalytic activation of MAPKKK(s) which activate a MAPKK via phosphorylation on two conserved serine and threonine residues in its catalytic domain. The activated MAPKK in turn activates a MAPK by phosphorylation on two conserved residues, threonine and tyrosine. This view has come primarily from biochemical analysis of the Raf1-MEK-ERK pathway in mammalian cells and genetic analysis in yeast (reviewed by Marshall, 1994; Herskowitz, 1995). The residues in MKK1 that are phosphorylated by the Raf kinase have been identified as Ser 218 and Ser 222, and analogous residues are phosphorylated on the STE7 MAPKK in budding yeast by the STE11 MAPKKK (Alessi et al., 1994; Neiman and Herskowitz, 1994; Zheng and Guan, 1994). Mutation of these residues to non-phosphorylatable alanine inhibits the catalytic activity both in vivo and in vitro. This has led to the belief that phosphorylation on these residues is the primary mechanism of MAPKK activation and, by inference, MAPK activation.

Recently, a family of MAP kinases has been identified in metazoan cells whose members are activated by a variety of environmental stress conditions, DNA damaging agents, 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 MAP kinases (SAPKs) in a wide variety of physiological and pathological conditions including development, control of cell proliferation, cell death, inflammation and response to ischaemic injury. The precise mechanism(s) by which this class of MAP kinases are activated, however, remains a mystery. We and others have identified a stress activated MAP kinase pathway in the unicellular fission yeast, Schizosaccharomyces pombe, the central elements of which are the Sty1 MAP kinase (also known as Spc1 and Phh1) and the Wis1 MAPKK (Warbrick and Fantes, 1991; Millar et al., 1995; Shiozaki and Russell, 1995; Kato et al., 1996). The Sty1 MAP kinase is required for multiple cellular events in fission yeast including protection of the cell in response to multiple environmental stresses, initiation of sexual differentiation and the timing of mitotic initiation. Importantly, the fission yeast Sty1 MAP kinase, like its mammalian counterparts, is activated by a similar range of environmental insults including osmotic stress, oxidative stress, ultraviolet (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). We have undertaken a detailed analysis of the mechanism by which this cascade is activated in the hope that it will shed light onto the mechanism by which SAPK pathways are activated in other organisms.

The Sty1 MAPK is activated by a single MAPKK, Wis1 (Warbrick and Fantes, 1991; Millar et al., 1995; Shiozaki and...
Russell, 1995). We and others have found that two upstream regulators of the Wis1 MAPKK are the Wak1 (also known as Wik1/Wis4) and the Win1 MAPKKs (Shieh et al., 1997, 1998; Shiozaki et al., 1997; Samejima et al., 1997). Wak1 is highly homologous to the budding yeast SSK2 and SSK2 MAPKKs which directly phosphorylate the PBS2 MAPKK on residues Ser 514 and Thr 518, leading in turn to phosphorylation and activation of the HOG1 MAPK. Phosphorylation of PBS2 on these sites is also controlled by the STE11 MAPKK in budding yeast (Posas and Saito, 1997). PBS2 is unable to function in strains lacking the Ssk2, Ssk2 and Ste11 MAPKKs even when it is overexpressed, indicating that phosphorylation on these residues is absolutely required for PBS2 activity (Maeda et al., 1995; Posas et al., 1996; Posas and Saito, 1997). In contrast we have previously found that overexpression of the Wis1 MAPKK can bypass the requirement for the Wak1 and Win1 MAPKKs, suggesting that Wis1 may be controlled by an alternative pathway (Shieh et al., 1997). In this paper we have identified the phosphorylation sites in Wis1 that are catalysed by the Wak1 MAPKK and determined their role in signal transmission to the Sty1 MAP kinase. These results reveal a novel stress activated MAPKK-independent pathway controlling the Sty1 MAP kinase that can be detected only when the Wis1 MAPKK is overexpressed. The implications of these results for signalling to the mammalian SAPKs is discussed.

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 micrometric eyepiece at ×1200 magnification. Transformations were regularly performed by lithium acetate method (Moreno et al., 1991) or by electroporation (Prentice, 1991) using a Bio-Rad Gene Pulser™.

Assessment of mating efficiency

Homothallic (h⁹⁰) cells were grown to log phase for two days in liquid EMM and then transferred for various lengths of time to the same medium lacking a nitrogen source. Mating efficiency was determined microscopically by scoring the percentage of cells undergoing conjugation or spore-containing asci.

Site specific mutagenesis of wis1

Serine 469, serine 471 and threonine 473 were mutated to alanine (A) by mutagenic PCR amplification of the gene in two parts using the oligonucleotides CCCCCTCGAGCATAATGGTCATGATCTTCTCTCCAAAT- AAATCAACCC (Wis1-1) incorporating XhoI, NdeI, XbaI and BglII sites (shown italicized) that hybridizes to sequences surrounding the ATG initiation codon in combination with the 3’ mutagenic oligonucleotide CCAATGGTGCTGGCCATATGACCAAGCCAGGACATGTAAGAACG (Wis1-AAA) and the 5’ mutagenic oligonucleotide CTCGCGTTGCTGATGTTGCGTATAGCGGACAGCAGAG (Wis1-AAG) in combination with the 3’ oligonucleotide CAGTGTATGGTGCTGGCCATATGACCAAGCCAGGACATGTAAGAACG- GTCTTGTTTGGTCTCAGG (Wis1-2) incorporating a PsiI and NotI site (shown italicized) which hybridizes to sequences surrounding the TGA termination codon. The mutated bases are underlined. Products from each reaction were then combined and a second round of amplification performed using the oligonucleotides Wis1-N1 and Wis1-2. The resulting 1.82 kb product was cleaved with NdeI and NotI and cloned in to the NdeI and NotI sites of pREP81(6HisHA) to form pREP81-Wis1(6HisHA), respectively. A similar strategy was used to mutate only residue Ser469 and Thr473 using the 5’ mutagenic oligonucleotide CTTGGTGGCTGATGTTGCGTATAGCGGACAGCAGAG (Wis1-AA5) and the 3’ mutagenic oligonucleotide TCCAGTGTTATGGGCAATTAGGCGGACAGCAGAG (Wis1-AAA) and the product cloned into pREP81(6HisHA) to form pREP81-Wis1(KR5)(6HisHA). All mutants were confirmed by DNA sequencing.

Transplacement of the wild-type wis1* locus with a wis1 (AAA) or wis1(AA) mutant allele

pREP81-Wis1(6HisHA) was cleaved with BamHI to release a 1.6 kb fragment containing most of the mutated wis1 gene that was ligated into the BamHI site of pBSSK-Ura4 to create pBSSK-Ura4-Wis1(6HisHA). This plasmid was linearised with MscI and transformed into the wild-type strain MJ1059 bearing the ura4-D18 mutation. Stable integrants were selected on minimal plates lacking uracil and were shown by Southern blot hybridisation and PCR to have a single integrated plasmid at the wis1 locus. The wis1(6HisHA):ura4 strain expressed a full length and haemaglutinin tagged version of the Wis1 protein as analysed by SDS-PAGE and western blot analysis. Transplacement of the wild-type wis1* locus was performed by selection on 5-fluoroorotic acid (Boeke et al., 1987) to select for uracil auxotrophs. Correct transplacement of the wild-type locus was verified by Southern blot, PCR and the loss of a tagged Wis1 protein by western blot analysis. This strain has the genotype wis1(6HisHA). A similar strategy was used to create a strain producing Wis1 with Ser 469 and Thr 473 only mutated to alanine residues. This strain has the genotype wis1(AAA).

Construction and production of GST-Wis1 fusion proteins in bacteria

The full length wis1 gene was excised from pREP81-wis1(6HisHA) by digestion with NdeI and NotI and ligated into the NdeI and NotI sites of pGEX-KGN. Escherichia coli DH5 was transformed with pGEX-KGN-wis1 and protein was isolated from overexpressing cells exactly as previously described (Millar et al., 1992). GST-Wis1(KR) and GST-Wis1(AAA) were made by a similar strategy. A second round of mutagenic PCR was performed on a wis1(6HisHA) gene using the mutagenic oligonucleotides Wis1-KR5 and Wis1-KR3 described above and the product cloned into pGEX-KGN to create pGEX-KGN-wis1(KR)(6HisHA) and this protein was purified from overexpressing bacteria as described above. Synthesis of full length proteins was confirmed by SDS-PAGE and Coomassie blue staining.

Detection of tagged Sty1 protein

The Sty1 protein was partially purified from cells bearing an integrated tagged version of sty1 (6HisHA):ura4 using Ni²⁺-NTA agarose exactly as previously described (Millar et al., 1995). Precipitated proteins were resolved by SDS-PAGE and transferred electrotherophoretically to nitrocellulose membranes. Membranes were probed with either a monoclonal antibody to the haemaglutinin epitope (12CA5) or with a monoclonal antibody to phosphotyrosine (4G10, UBI). Detection was performed using a peroxidase-conjugated anti-mouse IgG (Amersham, UK) and chemiluminescence visualisation (ECL, Amersham) according to the manufacturer’s instructions.

DNA and RNA isolation and hybridisation

S. pombe cells were cultured in YEPD medium (0.5% yeast extract, 3% glucose, 50 mg/l adenine) to stationary phase. Chromosomal DNA isolated from a 10 ml culture was dissolved in 25 ml of TE, of which
one fifth was digested and subjected to electrophoresis and Southern blot hybridisation. To isolate RNA, S. pombe cells were cultured in YEPD to exponentially growing phase. Approximately 10 μg of total RNA was isolated and resolved by agarose gel electrophoresis before transfer to nitrocellulose for hybridisation as previously described (Aves et al., 1985). Probes for pyp2, cdc2 and gpd1 were as previously described (Wilkinson et al., 1996).

RESULTS

Wak1 MAPKKK phosphorylates and activates Wis1 MAPKK on Ser 469 and Thr 473

Previous studies have identified two sites within subdomain VII of MAPKKs as the phosphorylation targets of MAPKKKs. Sequence comparison of this region of the Wis1 MAPKK with several unrelated MAPKKs from various species suggested that the analogous sites in Wis1 are Ser 469 and threonine 473 (Fig. 1a). Wis1 contains an additional serine at residue 471 which is not conserved in other MAPKKs but which we reasoned may also be a potential target for phosphorylation. We have simultaneously mutated both Ser 469 and Thr 473, or Ser 469, Ser 471 and Thr 473 to non-phosphorylatable alanine residues and compared the ability of the resulting proteins to act as substrates for the Wak1 MAPKKK both in vitro and in vivo. To eliminate Wis1 autophosphorylation while assessing the in vitro phosphorylation of the various mutants, the catalytic activity of the Wis1 protein itself was abolished by site-directed mutagenesis of the catalytic lysine residue (K349) to arginine. Constructs were made that express fusion proteins of either the kinase-inactive full length wild-type Wis1 (Wis1), catalytically inactive double phosphorylation site mutant (Wis1(AA)) or catalytically inactive triple phosphorylation site mutant (Wis1(AAA)) linked to glutathione-S-transferase. Following expression in bacteria, the purified fusion proteins were used as substrates for Wak1 kinase in vitro. Active Wak1 was immunoprecipitated from fission yeast lysates expressing an HA tagged N-terminally truncated wak1 gene. As the results in Fig. 1b show, Wak1 is able to effectively phosphorylate Wis1 and this phosphorylation is abolished when only Ser 469 and Thr 473 are mutated to non-phosphorylatable alanine. These results suggest that Ser469 and Thr 473 are the targets for the Wak1 MAPKKK in vitro.

Massive overexpression of the catalytic domain of Wak1 MAPKKK is toxic to wild-type fission yeast cells, an effect which can be bypassed by the inactivation of Wis1 (Shiozaki and Russell, 1996; Shieh et al., 1997). To determine whether Wak1 controls activation of Wis1 via phosphorylation of Ser 469 and Thr 473 in vivo, integrative transplacement was first used to replace the wild-type wis1+ locus with a wis1 allele mutated either at Ser 469 and Thr 473 (wis1(AA)) or at Ser 471.

Fig. 1. Wak1 MAPKKK phosphorylates Ser 469 and Thr 473 in the Wis1 MAPKK. (A) Alignment of amino acids 459 to 484 of Schizosaccharomyces pombe (S.p.) Wis1 with homologous regions in subdomain VII of Saccharomyces cerevisiae (S.c.) Pbs2, S.p. Byr1, Homo sapiens (H.s.) MKK3 and MKK4, Mus musculus (M.m.) MKK6 and MKK7 and S.c. Ste7 proteins. Residues S469 and T473 encoded by wild-type wis1+ are shown mutated to alanine (A) encoded by the wis1(AA) allele or S469, S471 and T473 encoded by the wis1( AAA) allele. (B) Wak1 phosphorylates Wis1 on serine 469 and threonine 473 in vitro. Full length GST-Wis1(KR) fusion proteins containing either no mutation (Wis1) or with S469 and T473 mutated to alanine (Wis1 (AA)) or with S469, S471 and T473 mutated to alanine (Wis1 (AAA)) were produced in bacteria and purified on glutathione-agarose. 1 μg of each fusion protein was incubated in kinase buffer in the presence of [γ-32P]ATP and anti-HA (anti-haemaglutinin monoclonal antibody 12CA5) immunoprecipitates from extracts of log phase S. pombe expressing either untagged (Control) or an HA-tagged Wak1 kinase (+Wak1). Labelled proteins were separated by SDS-PAGE and visualised by autoradiography. Arrows indicate the position of the phosphorylated GST-Wis1 and HA-Wak1 proteins. (C) Wak1 phosphorylates Wis1 on serine 469 and threonine 473 in vivo. Wild type (WT) (PR109), wis1(AA) (JM 1730) or wis1(AAA) (JM1684) cells were transformed with a plasmid pREP1-wak1ΔN that expresses, from the thiamine repressible nmt1 promoter, a truncated version of the Wak1 MAPKKK lacking the regulatory N-terminal domain. Transformants were grown on minimal medium lacking leucine and growth of the cells monitored after 3 days at 33°C either in the presence (left hand plate) or absence of thiamine (right hand plate).
The Sty1/Spc1 MAPK and Wis1 MAPKK are required for several events in *S. pombe* including stress resistance, sexual conjugation, sexual differentiation and the initiation of mitosis. Phosphorylation of Wis1 MAPKK on MAPKKK sites is normally required for Sty1/Spc1 activity. This study has used the *wis1* (AAA) allele to prevent any possible phosphorylation at this residue, although this precaution was probably unnecessary. Our results suggest that the *wis1* (AAA) allele, when expressed under normal conditions, cannot not support signal transmission to the Sty1 MAP kinase. To directly this *wis1* (AAA) cells were challenged with various environmental stresses and the tyrosine phosphorylation state of Sty1 determined. As the results in Fig. 2b show, no detectable tyrosine phosphorylation of Sty1 was observed after challenging *wis1* (AAA) cells to a temperature shock, even after a prolonged period of incubation (Fig. 2b). Similarly, no induction of Sty1 tyrosine phosphorylation was observed in the same cells after either osmotic or oxidative stress, or in response to the protein synthesis inhibitor anisomycin, all of which activate Sty1 (data not shown). Together these results indicate that, in direct contrast to previous findings (Samejima et al., 1997), phosphorylation of Wis1 on MAPKKK sites is normally required for Sty1/Spc1 activation.

**Phosphorylation of Wis1 MAPKK on MAPKKK sites is normally required for Sty1/Spc1 activity**

The Sty1/Spc1 MAPK and Wis1 MAPKK are required for several events in *S. pombe* including stress resistance, sexual conjugation, sexual differentiation and the initiation of mitosis. To assess the role of MAPKKK phosphorylation of Wis1, we have analysed the ability of the *wis1* (AA) and *wis1* (AAA) mutants to support signal transmission to the Sty1 MAP kinase. Firstly, wild-type cells, *wis1* (AA) and *wis1* (AAA) mutants were grown either on rich medium at 30°C, or in the same medium containing 1.5 M sorbitol at 30°C or on rich medium with no additions at 37°C, conditions under which *Δwis1* cells are unable to proliferate. As the results in Fig. 2a demonstrate, cells expressing only a Wis1 protein lacking MAPKKK phosphorylation sites were unable to grow at high temperature or under hyperosmolar conditions, indicating that activation of Wis1 by one or more MAPKKKs is required for multiple stress resistance in fission yeast. Secondly we also find that *wis1* (AA) and *wis1* (AAA) mutants are as defective in sexual conjugation as cells completely deleted for Wis1 suggesting that the meiotic function of Wis1 also requires phosphorylation by MAPKKKs (Table 2). Lastly we observe that cells expressing non-phosphorylatable *wis1* (AA) or *wis1* (AAA) alleles undergo cell division at a similar size to cells lacking Wis1, suggesting that phosphorylation by MAPKKKs is normally essential for Wis1 function (Table 2). No difference in the phenotypes of the *wis1* (AA) or *wis1* (AAA) mutants could be observed in any of these assays, suggesting that phosphorylation of Ser 471 is not important for Wis1 function in vivo. In many subsequent experiments we have used the *wis1* (AAA) allele to prevent any possible phosphorylation at this residue, although this precaution was probably unnecessary. Our results suggest that the *wis1* (AAA) allele, when expressed under normal conditions, cannot not support signal transmission to the Sty1 MAP kinase.

**Overexpression of a MAPKKK phosphorylation site mutant of Wis1 can rescue loss of Wis1 function**

One possible trivial explanation of our previous observations is that mutation of the MAPKKK sites in Wis1 to alanine residues cause the protein to be degraded in vivo. To assess this the Wis1 protein or the phosphorylation site derivatives were epitope tagged at the C terminus with 6 histidine and a haemaglutinin tag and integrated at the Wis1 locus (see Table 2). Measurements were the mean of 50 individual determinations (± s.d.)

### Table 1. Strains used in this study

<table>
<thead>
<tr>
<th><em>S. pombe</em> strains</th>
<th>Genotype</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR 109</td>
<td><em>h</em></td>
<td>P. Russell</td>
</tr>
<tr>
<td>JM 1730</td>
<td><em>wis1</em>(AA) <em>ade6-M210 his7-366 h</em></td>
<td>This study</td>
</tr>
<tr>
<td>JM 1684</td>
<td><em>wis1</em>(AAA) <em>ade6-M216 his7-366 h</em></td>
<td>This study</td>
</tr>
<tr>
<td>JM 504</td>
<td><em>wis1::ura4 h</em></td>
<td>Millar et al., 1995</td>
</tr>
<tr>
<td>JM 1664</td>
<td><em>wis1::ura4 ppy1::ura4 his1-102</em></td>
<td>This study</td>
</tr>
<tr>
<td>JM 1521</td>
<td><em>sty1</em>(6HisHA)::ura4 <em>ade6-M216 his7-366 h</em></td>
<td>This study</td>
</tr>
<tr>
<td>JM 1574</td>
<td><em>sty1</em>(6HisHA)::ura4 *ppy1::ura4 <em>ade6-M216 his7-366 h</em></td>
<td>This study</td>
</tr>
<tr>
<td>JM 1685</td>
<td><em>sty1</em>(6HisHA)::ura4 *wis1(6HisHA)::ura4 <em>ade6-M216 his7-366 h</em></td>
<td>This study</td>
</tr>
<tr>
<td>JM 1690</td>
<td><em>sty1</em>(6HisHA)::ura4 <em>wis1::ura4 ppy1::ura4 h</em></td>
<td>This study</td>
</tr>
<tr>
<td>JY 878</td>
<td><em>ade6-M216 h</em></td>
<td>D. Hughes</td>
</tr>
<tr>
<td>JM 1731</td>
<td><em>ade6-M216 his7-366 wis1(AA) h</em></td>
<td>This study</td>
</tr>
<tr>
<td>JM 1732</td>
<td><em>ade6-M216 his7-366 wis1(1AA) h</em></td>
<td>This study</td>
</tr>
<tr>
<td>JM 1260</td>
<td><em>ade6-M216 wis1::ura4 h</em></td>
<td>This study</td>
</tr>
</tbody>
</table>

All strains are also *leu1-32 ure4-D18* unless stated otherwise.

### Table 2. Phenotypes of Wis1 phosphorylation site mutants

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Mating efficiency (%)</th>
<th>Cell size at division (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>wt</em></td>
<td>42.3 ± 0.3</td>
<td>14.2 ± 0.3</td>
</tr>
<tr>
<td><em>wis1</em>(AA)</td>
<td>2.7 ± 0.3</td>
<td>25.9 ± 0.5</td>
</tr>
<tr>
<td><em>wis1</em>(AAA)</td>
<td>2.7 ± 0.3</td>
<td>26.8 ± 0.5</td>
</tr>
<tr>
<td><em>Δwis1</em></td>
<td>5.8 ± 0.3</td>
<td>23.9 ± 0.3</td>
</tr>
</tbody>
</table>

Mating efficiency was determined microscopically by scoring the percentage of cells undergoing conjugation or spore-containing ascii. Measurements were the mean of 300 individual cells. Cell size measurements of septated cells grown in either liquid synthetic minimal medium (EMM). The temperature at which the measurements were made is indicated. Measurements were the mean of 50 individual determinations (± s.d.)
Materials and Methods). The Wis1 proteins were then precipitated under denaturing conditions using Ni^{2+}-NTA affinity chromatography and the amount of Wis1 protein analysed using a haemagglutinin antibody (α-HA). As the results in Fig. 3a show the Wis1 phosphorylation site mutants were expressed at a comparative level to wild-type protein (Fig. 3a). An alternative explanation to explain the lack of signal transduction in the absence of MAPKKK-mediated phosphorylation is that the mutated proteins may misfold into a conformationally inactive form. To test this, a GST-Wis1(AAA) fusion protein was overexpressed in bacteria and the catalytic activity of the purified protein assessed. Importantly, both full length GST-Wis1 and GST-Wis1(AAA) proteins autophosphorylate in vitro, whereas a GST-Wis1(KR) protein is completely inactive (Fig. 3b). Furthermore the purified GST-Wis1 and GST-Wis1(AAA) proteins were able to phosphorylate Sty1 that had been immunoprecipitated from fission yeast extracts whereas a GST-Wis1(KR) protein was not. These results indicate that although Ser 469 and Thr 473 are essential for signalling in vivo, they are not required for catalytic activity in vitro.

Since the Wis1 protein retains catalytic activity, we next determined whether a Wis1 protein lacking MAPKKK phosphorylation sites could rescue loss of Wis1 function if overexpressed from a partially crippled version of the strong thiamine repressible nmt1 promoter in fission yeast (Basi et al., 1993). Western blot analysis reveals that the Wis1 proteins is expressed at least five times a higher level from the nmt81 promoter than from its endogenous chromosomal promoter (Fig. 3a). Surprisingly we find that overexpression of the mutant wis1(AAA) gene is able to restore the ability of Δwis1 cells to grow at high temperature whereas a similarly overexpressed catalytic inactive wis1(KR) mutant is not (Fig. 3b).

### Fig. 2. MAPKKK-dependent phosphorylation of Wis1 is required for Wis1 function in vivo. (A) Phosphorylation of Wis1 on Ser 469 and Thr 471 is essential for stress resistance. Wild type (WT) (PR109), wis1(AA) (JM 1730) or wis1(AAA) (JM1684) cells were grown on YEPE and then streaked on the same medium (top left plate) or on YEPD at 37°C (bottom left plate) for three days. (B) Phosphorylation of Wis1 on Ser 469 and Thr 471 is essential for activation of the Sty1 MAP kinase. Log phase cultures of wild-type (WT) (JM1520) or wis1(AAA) (JM1684) cells bearing an integrated 6His and haemagglutinin epitope tagged version of Sty1 were incubated at 42°C for the times indicated. Approximately 2×10^{9} cells were harvested at each time point, lysed and the Sty1 protein precipitated using Ni^{2+}-NTA agarose. Precipitates were probed by western blot for the presence of phosphotyrosine (α-pTyr) or the haemagglutinin epitope tag (α-HA).

### Fig. 3. Wis1 is active in the absence of MAPKKK-dependent phosphorylation. (A) Expression of Wis1 phosphorylation site mutants. Log phase cultures of chromosomally tagged wis1(6HisHA):ura4, wis1(AA)(6HisHA):ura4, wis1(AAA)(6HisHA):ura4 or Δwis1 cells expressing wis1 from the thiamine repressible nmt81 promoter (nmt81-wis1(6HisHA)) were grown in liquid minimal medium. Approximately 1×10^{7} cells were collected, lysed in denaturing lysis buffer (6 M GuHCl, 1% NP40) and the Wis1 precipitated using Ni-NTA agarose beads. Precipitates were separated by SDS-PAGE and probed by western blot for the presence of haemagglutinin epitope tag (α-HA). (B) Activity of Wis1 in vitro. HA-tagged Sty1 was immunoprecipitated from log phase extracts of wild-type cells expressing pREP41-sty1(6HisHA) grown in EMM lacking leucine using an anti-haemagglutinin (HA) monoclonal antibody (12CA5). Immunoprecipitates were incubated in kinase buffer in the presence of [γ-^{32}P]ATP and either 1 μg GST, 1 μg GST-Wis1, 1 μg GST-Wis1(6HisHA) containing the K349R mutation or 1 μg GST-Wis1(6HisHA) in which S469, S471 and T473 are mutated to alanine. Labelled proteins were separated by SDS-PAGE and visualised by autoradiography. Arrows indicate the position of the GST-Wis1 and HA-Sty1 proteins. The GST-Wis1 and GST-Wis1(6HisHA) proteins autophosphorylate even in the absence of Sty1 immunoprecipitates.
In addition, overexpression of the \( \text{wis1(AAA)} \) allele partially corrected the cell size at division defect of a \( D\text{\textit{wis1}} \) strain whereas the catalytically inactive \( \text{wis1(KR)} \) allele could not (Fig. 4b). Similarly, expression of \( \text{wis1(AAA)} \) partially rescued the ability of \( D\text{\textit{wis1}} \) cells to undergo sexual conjugation (Fig. 4c). We reason that the \( \text{wis1(AAA)} \) allele is not as active as wild-type \( \text{wis1} \) since, unlike \( \text{wis1} \), it is not toxic to wild-type cells when expressed from the full strength \( \text{nmt1} \) promoter (data not shown). These results indicate that a Wis1 protein lacking MAPKKK sites retains catalytic activity in vitro and is able to partially rescue loss of Wis1 function only if overexpressed in vivo.

Sty1 is activated by stress in the absence of MAPKKKs when Wis1 is overexpressed

We have previously demonstrated Wis1 can bypass the requirement for the Wak1 and Win1 MAPKKs when overexpressed (Shieh et al., 1997). We next wished to determine whether overexpression of a Wis1 protein lacking MAPKK phosphorylation sites is able to support stress inducible signalling to Sty1. In particular, we and others have shown that Sty1 is activated by the same range of environmental stresses as the mammalian SAPK/JNK and p38/CSBP1 MAP kinases, such as an osmotic stress, heat shock or oxidative stress. Exposure to these environmental stimuli causes induction of a number of genes in fission yeast, including \( \text{pyp2} \), \( \text{ctt1} \) and \( \text{gpd1} \) in a manner dependent on both Sty1 and Wis1 (Millar et al., 1995; Degols et al., 1996; Wilkinson et al., 1996; Shieh et al., 1997). Cells lacking endogenous \( \text{wis1} \) but ectopically expressing either wild-type \( \text{wis1} \), a \( \text{wis1(AAA)} \) allele lacking MAPKKK phosphorylation... 

Fig. 4. Overexpression of Wis1 restores Wis1 function in the absence of MAPKKK phosphorylation. (A) Thermosensitivity of cells lacking Wis1 is rescued by overexpression of a Wis1 phosphorylation site mutant. \( \text{\Delta wis1} \) (JM 504) cells were transformed with either the control plasmid pREP81 (pCont.), or pREP81 derivatives expressing wild-type \( \text{wis1} \) (pWis1), \( \text{wis1} \) mutated at Ser469, Ser471 and Ser 473 (pWis1(AAA)) or a catalytically inactive \( \text{wis1} \) (pWis1(KR)) from the thiamine repressible \( \text{nmf81} \) promoter. Transformants were streaked on minimal medium lacking thiamine and leucine and colony formation monitored after 3 days incubation at 37°C. (B) Overexpression of a Wis1 phosphorylation site mutant partially rescues the cell size at division defect of \( \text{\Delta wis1} \) cells. \( \text{\Delta wis1} \) (JM 504) cells were transformed with either a control plasmid (open bar), or plasmids expressing wild-type \( \text{wis1} \) (grey bar), a \( \text{wis1} \) mutated at Ser469, Ser471 and Ser 473 (black bar) or a catalytically inactive \( \text{wis1} \) (striped bar) from the thiamine repressible \( \text{nmf81} \) promoter. Transformants were grown to log phase in minimal medium lacking thiamine and leucine and cell size at division measured. (C) Overexpression of a Wis1 phosphorylation site mutant partially rescues the mating defect of \( \text{\Delta wis1} \) cells. \( \text{\Delta wis1} \) \( ^{\text{h90}} \) (JM 1260) cells were transformed with either a control plasmid (open bar), or plasmids expressing wild-type \( \text{wis1} \) (grey bar), a \( \text{wis1} \) mutated at Ser469, Ser471 and Ser 473 (black bar) or a catalytically inactive \( \text{wis1} \) (striped bar) from the thiamine repressible \( \text{nmf81} \) promoter. Transformants were grown to log phase in liquid EMM lacking leucine and thiamine and then transferred to the same medium lacking \( \text{NH}_{4}\text{Cl} \) for 24 hours at 30°C. Mating efficiency was assessed microscopically.
sites or a catalytically inactive allele of *wis1* (*wis1(KR)*) were challenged with various environmental stresses and induction of various genes assessed by northern blot. Surprisingly, *pyp2* and *gpd1* were induced by a mild heat shock or challenge with an oxidative stress (1 mM H₂O₂) in cells overexpressing the *wis1(6HisHA)* allele (Fig. 5). No induction was observed in Δ*wis1* cells bearing either an empty plasmid or a plasmid expressing the catalytically inactive *wis1(KR) allele*. It should be noted that the strength of induction was lower and the kinetics of induction slower in Δ*wis1* cells bearing *wis1(6HisHA)* compared to the wild-type allele. Similar results were obtained when blots were probed with *ctt1*, which is also under control of the Sty1 MAP kinase pathway (data not shown). We find that *gpd1* is also induced in cells overexpressing a *wis1* allele lacking MAPKKK phosphorylation sites by osmotic stress, although this induction was quite weak (Fig. 5). To determine whether stress induced gene expression under these conditions is through increased Sty1 activity, the tyrosine phosphorylation state of Sty1 was assessed after affinity precipitation from cell extracts. Strikingly, we observe that Sty1 underwent a time dependent increase in tyrosine phosphorylation in Δ*wis1* cells overexpressing the *wis1(6HisHA) mutant* in response to a mild heat shock or to an oxidative stress, as previously observed (Samejima et al., 1997) (Fig. 6a). These results indicate that overexpressed *Wis1* can support stress inducible gene expression in the absence of MAPKKKs.

**MAPKKK-independent activation of Sty1 does not involve Pyp1 MAPK phosphatase**

The tyrosine phosphorylation state of Sty1 is a balance between MAPKK and MAPK phosphatase function. Two MAPK phosphatases for Sty1 are encoded by the *pyp1* and *pyp2* genes (Millar et al., 1995; Shiozaki and Russell, 1995). We have examined whether increased tyrosine phosphorylation of Sty1 in the absence of MAPKKKs could be due to stress-induced inactivation of Pyp1, as has been recently suggested (Samejima et al., 1997). Δ*wis1 Δpyp1* cells overexpressing the *wis1(6HisHA) allele* were challenged with either 1 mM H₂O₂ or heat shocked at 42°C and the tyrosine phosphorylation state of Sty1 was assessed after affinity precipitation from cell extracts. We observe that in these cells, Sty1 still undergoes a marked increase in tyrosine phosphorylation in response to either an oxidative stress or when subjected to a mild heat shock (Fig. 6b). The induction of *pyp2* mRNA expression is similarly unaffected in Δ*wis1 Δpyp1* cells expressing the *wis1(6HisHA) allele* when challenged with the same stresses that cause tyrosine phosphorylation of Sty1 (data not shown). These results indicate that MAPKKK-independent activation of Sty1 can be stimulated by multiple environmental stresses not just heat shock and oxidative stress and does not involve inactivation of the Pyp1 MAP kinase phosphatase, as previously suggested (Samejima et al., 1997).

**DISCUSSION**

The fission yeast Sty1 MAP kinase is activated by many of the same environmental stresses that activate the mammalian JNK/SAPK and p38/CSBP1 MAP kinases. This has led us to investigate the mechanism by which Sty1 is activated in the hope of understanding how the SAPKs are activated in mammalian cells and the molecular basis of stress sensing. Sty1 is activated by a single MAPKK, Wis1, which is in turn controlled by two functionally overlapping MAPKKKs, Wak1 and Win1 (Millar et al., 1995; Shiozaki and Russell, 1995; Samejima et al., 1997; Shieh et al., 1997, 1998; Shiozaki et al., 1997). In this paper we have defined the residues in the fission yeast *Wis1 MAPKKK* that are phosphorylated by the Wak1 MAPKKK in vitro and in vivo as serine 469 and threonine 473. These residues lie in a conserved region of the catalytic domain and are found in all other MAPKKKs. We have also examined the role of Ser 471 in *Wis1*, but conclude that this is not phosphorylated by Wak1 in vivo or in vitro and does not contribute to signal transmission through the pathway. Substitution of serine 469 and threonine 473 in the genome with non-phosphorylatable alanine residues effectively abolishes all stress-inducible signalling to the Sty1 MAP kinase, indicating that MAPKKKs are essential for activation of Sty1. This is in direct contrast to the observations previously reported (Samejima et al., 1997). Our conclusion is supported by the observations that cells expressing a mutant *Wis1* protein lacking these phosphorylation sites from the natural promoter have identical defects when undergoing cell division, initiating sexual differentiation or resisting the toxic effects of environmental stress as cells completely deleted for *wis1*. These results are also in agreement with our previous findings that simultaneous inactivation of functionally overlapping MAPKKKs, Wak1/Wik1/Wis4 and Win1, also prevents *Sty1* activation (Shieh et al., 1998).

In this respect our results are consistent with other reports that phosphorylation of analogous residues in MAPKKs is
required for signal transmission to MAPKs. Activation of a number of mammalian MAPKKs by multiple environmental stresses has been observed in vivo, including MKK3, MKK4(SEK1), MKK6 and MKK7. These enzymes are catalytically stimulated by phosphorylation on the same conserved residues that are catalysed by the Raf MAPKKK in MKK1 (Yan et al., 1994; Raingeaud et al., 1996; Deacon and Blank, 1997). Mutant MKK3, MKK4 and MKK6 proteins bearing non-phosphorylatable residues at these sites are functionally inactive in vitro and behave as dominant negative mutants when overexpressed in vivo. In these cases it is not possible to know whether mutation of these residues merely generates a functionally inactive protein by preventing the formation of a correct three-dimensional structure. In contrast, we show that mutation of these phosphorylation sites in the Wis1 protein does not abolish the ability of a bacterially produced Wis1 to autophosphorylate or to phosphorylate Sty1 in vitro. Indeed, overexpression of a Wis1 protein lacking MAPKKK phosphorylation sites functionally complements the inability of *Δwis1* cells to proliferate in conditions of environmental stress, to undergo sexual conjugation and to maintain cell size control at cell division. Strikingly, Sty1 activity can be induced by osmotic stress, oxidative stress and heat shock in cells overexpressing only a Wis1 protein lacking MAPKKK phosphorylation sites, as judged by tyrosine phosphorylation of Sty1 and induction of several genes including *pyp2, ctt1* and *gpd1* whose expression is dependent on Sty1. These results suggest that Sty1 activity can be induced by a novel MAPKKK-independent mechanism in fission yeast, but only when the Wis1 MAPKK is overexpressed.

So how can Sty1 activity be induced by the Wis1 MAPKK without activation via a MAPKKK? Tyrosine phosphorylation and activation of Sty1 is, like other MAP kinases, controlled by specific MAP kinase phosphatases which for Sty1 have been shown to be encoded by the *pyp1* and *pyp2* genes (Millar et al., 1995; Shiozaki and Russell, 1995). One could imagine that inactivation of one or other of the Pyp1 or Pyp2 PTases could account for activation of Sty1 in the absence of MAPKKKs, as previously suggested (Samejima et al., 1997). However in direct contrast to this notion, we find that deletion of *pyp1* does not affect stress inducible Sty1 activation in cells overexpressing only the *wis1*(AAA) allele. We cannot, however, rule out the possibility that an as-yet unidentified PTase contributes to Sty1 activation. Importantly, we have recently found that the non-catalytic N-terminal domain of the Wis1 MAPKK is phosphorylated by a novel stress-inducible kinase that acts independently of the Wak1 and Win1 MAPKKKs in fission yeast (H. Martin, J.-C. Shieh and J. Millar, unpublished observations). This alternative pathway may account for the activation of Sty1 under these conditions. Since MAPKKK-independent signalling can only be observed when Wis1 is overexpressed, this pathway is clearly not sufficient to activate Wis1 by itself and suggests that the stoichiometry of the components of the Sty1 pathway maybe critically important. Indeed it is not at present known whether N-terminal phosphorylation of Wis1 alters its catalytic activity or ability to interact with Sty1. The identity of the kinase responsible for this phosphorylation is the subject of intense investigation.

We have previously established that the fission yeast Sty1 MAP kinase pathway differs from the budding yeast Hog1 MAP kinase pathway in that it is activated by a range of environmental insults including osmotic stress, hydrogen peroxide, heat shock and anisomycin, whereas Hog1 appears to be activated solely by osmotic stress. Like Sty1, Hog1 is activated by a single MAPKK, Pbs2. In contrast to Wis1, the budding yeast Pbs2 MAPKK is phosphorylated only on residues Ser514 and Ser518 by the Ssk2, Ssk22 and Ste11 MAPKKKs (Posas and Saito, 1997). Mutation of these residues completely abolishes signalling to Hog1, even when Pbs2 is overexpressed (Maeda et al., 1995; Posas and Saito, 1997). Thus the modes of Hog1 and Sty1 regulation in the two yeasts differ in two important aspects. Importantly the MAPKK-independent activation of Sty1 by the Wis1 MAPKK is stimulated by many of the same stresses that activate the SAPK/JNK and p38/CSBP1 enzymes in mammalian cells, suggesting that this alternative pathway may also be conserved. Indeed one is lead to consider whether this may be the primary mechanism by which SAPK are activated in animal cells. One could envisage a scenario that stress dependent complex formation of the components of the SAPK pathways could lead to activation of stress activated MAP kinases without necessarily activation of a MAPKKK. Intriguingly, although a number of MAPKKKs have been identified that activate the JNK and/or p38/CSBP1 MAP kinases when overexpressed in mammalian cells, including MEKK1, TAK1, MUK, SPRK/MLK3, TPL2/COT1 and ASK1, none of these enzymes appear to be catalytically stimulated by environmental stress (Yan et al., 1994; Yamaguchi et al., 1995; Hirai et al., 1996; Rana et al., 1996; Salmeron et al., 1996; Ichijo et al., 1997). Further experimentation will be required to ascertain the role of this alternative pathway in activation of the Sty1 MAP kinase in fission yeast and to determine whether a similar mechanism operates in metazoan.

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MAPKKK-independent activation of the Sty1 MAPK


