Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast

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Simultaneous inactivation of *pyp1* and *pyp2* PTPases in fission yeast leads to aberrant cell morphology and growth arrest. Spontaneous recessive mutations that bypass the requirement for *pyp1* and *pyp2* and reside in two complementation groups were isolated, *sty1* and *sty2*. *sty1*[−] and *sty2*[−] mutant cells are substantially delayed in the timing of mitotic initiation. We have isolated the *sty1* gene, which encodes a MAP kinase that is closely related to a subfamily of MAP kinases regulated by osmotic stress including *Saccharomyces cerevisiae* HOG1 and human CSBP1. We find that *sty2* is allelic to the *wis1* MAP kinase kinase and that Δ *sty1* and Δ *wis1* cells are unable to grow in high osmolarity medium. Osmotic stress induces both tyrosine phosphorylation of Sty1 and a reduction in cell size at division. Pyp2 associates with and tyrosine dephosphorylates Sty1 in vitro. We find that *wis1*-dependent induction of *pyp2* mRNA is responsible for tyrosine dephosphorylation of Sty1 in vivo on prolonged exposure to osmotic stress. We conclude that Pyp1 and Pyp2 are tyrosine-specific MAP kinase phosphatases that inactivate an osmoregulated MAP kinase, Sty1, which acts downstream of the Wis1 MAP kinase kinase to control cell size at division in fission yeast.

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Growing fission yeast cells tend to divide at a constant size at division. The fission yeast cell cycle is controlled at two major points, in G₁ at entry into S phase (DNA replication) and at the initiation of mitosis. Genetic and physiological studies have revealed that the timing of both transitions requires attainment of a critical cell size and has led to the proposal that mass sensors govern these cell cycle transitions (Nurse 1975; Fantes and Nurse 1977). Under conditions of normal growth the mass sensor governing the initiation of DNA synthesis is cryptic, such that cell size at division is rate-limiting for cell cycle progression (Fantes and Nurse 1977). Because *Schizosaccharomyces pombe* cells grow by length extension, this is expressed as the attainment of a critical cell length. Cell size at division can be influenced either by the genotype of the cell or by the prevailing nutritional conditions. The molecular nature of the mass sensors governing these transitions is not known.

A considerable advance in our understanding of the molecular basis of mitotic initiation, nevertheless, has come from the analysis of mutants in fission yeast that uncouple size control from cell cycle progression. In particular the cell division cycle (*cdc*) mutants have been particularly valuable in identifying the central components of this control (Nurse et al. 1976; Thuriaux et al. 1978; Nasmyth and Nurse 1981). It is now recognized

that the timing of mitotic initiation is controlled by rapid tyrosine dephosphorylation and activation of the catalytic kinase subunit of the Cdc13 (cyclin B)/Cdc2 kinase complex by the Cdc25 phosphatase (for review, see Millar and Russell 1992). This complex is maintained in an inactivate state during interphase by the action of the *wee1* and *mik1* tyrosine kinases (Russell and Nurse 1987a; Lundgren et al. 1991). Structural and functional homologs of the *cdc2*, *cdc13*, *cdc25*, and *wee1* genes have been shown to exist in metazoa supporting the view that entry into mitosis is controlled by a universally conserved mechanism (Nurse 1990).

Extensive genetic analysis in *S. pombe* has revealed several other sets of genes that show genetic interaction with the core elements described above and, by inference, also encode gene products that control the timing of mitosis. These include *win1* and five multicopy suppressors of *win1*, *wis1*–*wis5* (Ogden and Fantes 1986; Warbrick and Fantes 1991, 1992), the *nim1*/*cdr1* and *cdr2* genes (Russell and Nurse 1987b; Young and Fantes 1987), and six mitotic catastrophe suppressors *mcs1*–*mcs6* (Molz et al. 1989). The Nim1 kinase acts to inhibit the Wee1 tyrosine kinase by direct phosphorylation (Coleman et al. 1993; Parker et al. 1993; Wu and Russell 1993). The *wis1* gene, which acts as a dose-dependent initiator of mitosis, encodes a protein that is homologous to a family of MAP kinase kinases (Ogden and Fantes 1986; Warbrick and Fantes 1991).

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This latter observation is particularly intriguing because MAP kinase cascades exist in all eucaryotic cells and have been implicated in a wide range of cellular processes including mitogenic signal transduction, cellular differentiation, and response to environmental stress (for review, see Ammerer 1994; Davis 1994; Marshall 1994; Herskowitz 1995). At least four distinct MAP kinase pathways have been identified in the unicellular budding yeast *Saccharomyces cerevisiae*, which are required for mating, sporulation, control of cell polarity, and the cellular response to osmotic stress. Signal transduction through MAP kinase cascades involves sequential phosphorylation and activation of three distinct kinases: the MAP kinase kinase kinase (or MEKK), the MAP kinase kinase (or MEK), and the MAP kinase itself. Although there appear to be multiple mechanisms by which plasma membrane-associated receptors can induce activation of MEKK, it has now been demonstrated clearly that MEKK activation leads to MAP kinase kinase activation by direct phosphorylation. The MAP kinase kinase in turn activates the MAP kinase by dual phosphorylation on two closely spaced residues, a threonine and a tyrosine. Less is understood of the mechanisms that terminate signal transduction through MAP kinase cascades, although inactivation of MAP kinases has been ascribed to the action of dual specificity phosphatases that remove both of the phosphates from these residues (Alessi et al. 1993; Sun et al. 1993; Doi et al. 1994; Ward et al. 1994).

We and others recently have identified two additional components of fission yeast mitotic control, *pyp1* and *pyp2*, which encode tyrosine-specific phosphatases. Overexpression of either *pyp1* or *pyp2* causes a delay in mitotic initiation. Conversely, disruption of *pyp1* and, to a lesser extent, *pyp2* causes cells to divide at a reduced cell size. Simultaneous disruption of these genes is lethal, demonstrating that *pyp1* and *pyp2* share an essential overlapping function (Millar et al. 1992b; Otilie et al. 1991, 1992). We have constructed a conditional double mutant and undertaken a suppression analysis to investigate the role of *pyp1* and *pyp2* in the fission yeast cell cycle. We demonstrate that the *pyp1* and *pyp2* PTPases regulate a MAP kinase cascade that controls the timing of mitotic initiation in fission yeast. We have identified the central components of this pathway as the *wis1* MAP kinase kinase and a novel MAP kinase, encoded by the *sty1* gene. We show that the *sty1* MAP kinase pathway is regulated by changes in external osmolarity. These studies provide an important link between the cellular response to environmental stress and control of the cell cycle.

Results

Inactivation of Pyp1 and Pyp2 causes aberrant cell morphology and growth arrest

Inactivation of either the *pyp1* or *pyp2* tyrosine-specific phosphatases leads to a reduction in cell size at division in fission yeast. Simultaneous disruption of *pyp1* and

pyp2 gives rise to inviable spores that do not germinate (Millar et al. 1992b). To examine the role of *pyp1* and *pyp2* in the mitotic cell cycle in fission yeast, a conditional allele of *pyp1* under the control of the thiamine-repressible *nmt1* promoter was constructed (see Materials and methods). In the absence of thiamine *NMT-pyp1* cells divided at $14.3 \pm 0.4 \mu\text{m}$, a size similar to wild-type cells, whereas in the presence of thiamine, cells divided at $11.1 \pm 0.3 \mu\text{m}$, similar to $\Delta pyp1$ cells (Millar et al. 1992b). To examine the effects of a loss of *pyp1* expression in a $\Delta pyp2$ strain, *NMT-pyp1* $\Delta pyp2$ cells were grown in the absence of thiamine and then streaked onto minimal medium in the presence of $10 \mu\text{M}$ thiamine. In contrast to wild-type, *NMT-pyp1* or $\Delta pyp2$ cells, *NMT-pyp1* $\Delta pyp2$ cells were greatly retarded for growth on medium containing thiamine (Fig. 1A). Microscopic ex-

Figure 1. Suppression of *pyp1* and *pyp2* expression leads to abnormal cell morphology. (A) Cells were grown on minimal medium lacking thiamine and then streaked onto the same medium in the absence (left plate) or presence of $10 \mu\text{M}$ of thiamine (right plate) and allowed to grow for 3 days at 25°C . The genotypes of the cells were wild-type strain 972 (top left-hand segment), *pyp2::LEU2* (top right-hand segment), *NMT-pyp1(ura4)* (bottom left-hand segment), and *NMT-pyp1(ura4) pyp2::LEU2* (bottom right-hand segment). On prolonged incubation *NMT-pyp1* $\Delta pyp2$ cells eventually recovered, presumably because of metabolic depletion of thiamine in the medium. (B) The phenotype of *NMT-pyp1(ura4) pyp2::LEU2* cells was examined by phase-contrast microscopy after 3 days growth on minimal medium either in the absence (left) or presence of $10 \mu\text{M}$ of thiamine (right).

amination revealed that these cells arrested after three or four divisions as large, spherical, swollen cells, a proportion of which lysed (Fig. 1B). In the absence of thiamine, cells were indistinguishable from wild type (Fig. 1B). Growth arrest in the presence of thiamine was not suppressed by the presence of 1 M sorbitol, an osmotic stabilizing agent (data not shown). We conclude that the *pyp1* and *pyp2* genes, in addition to control of mitotic initiation, are required for cell shape control in fission yeast.

sty1 and sty2 mutants suppress a pyp1⁻ pyp2⁻ growth defect

We utilized the conditional arrest of *NMT-pyp1 Δpyp2* cells to isolate mutations that bypass the requirement for the *pyp1* and *pyp2* PTPases by pseudoreversion analysis. From a total of 10⁷ cells plated, 56 fast-growing colonies were identified, of which 17 grew in the absence of thiamine. Cells from all 17 mutant strains were highly elongated relative to wild-type (see below). When outcrossed to a wild type strain the elongated phenotype was found to be unlinked to either the *NMT-pyp1* or *Δpyp2* alleles. Additionally, cross-mating of these strains revealed that these 17 mutants fell into two complementation groups, *sty1* (11 alleles) and *sty2* (6 alleles) (for suppressor of tyrosine phosphatase). Because all mutants within each complementation group appeared to be elongated to the same extent, a single mutant from each complementation group, *sty1-1* and *sty2-1*, was chosen for further study. Further crosses revealed that both the *sty1-1* and *sty2-1* mutations segregated 2:2 with respect to the elongated phenotype, indicating that both were the consequence of a single nuclear lesion. To determine whether the *sty1-1* and *sty2-1* mutations could completely bypass the requirement for *pyp1* and *pyp2*, *sty1-1 pyp2::LEU2* or *sty2-1 pyp2::LEU2* cells were crossed to *pyp1::ura4* cells. Spores from both crosses gave rise to colonies of elongated cells that were able to grow in the absence of both uracil and leucine indicating that they had inherited both *Δpyp1* and *Δpyp2* alleles. Southern blot analysis was used to confirm the genotypes of the *sty1-1 Δpyp1 Δpyp2* and *sty2-1 Δpyp1 Δpyp2* triple mutants (data not shown). In a control cross, *pyp2::LEU2* was mated to *pyp1::ura4*. From >3000 spores analyzed, no colonies grew in the absence of both uracil and leucine, confirming previous observations that simultaneous loss of *pyp1* and *pyp2* function is lethal (Millar et al. 1992b; Otilie et al. 1992). These results suggest that both the *sty1-1* and *sty2-1* mutations were able to bypass the synthetic lethal combination of a *Δpyp1 Δpyp2* double mutant.

sty1 and sty2 mutants are delayed in mitotic initiation

The role of *sty1* and *sty2* in the mitotic cell cycle was examined. Growth rates of *sty1-1* or *sty2-1* cells were found to be identical to wild type with a doubling time of 2.5 hr in rich medium, or 3.5 hr in synthetic minimal medium at 30°C, indicating that neither mutant was de-

fective in cellular growth rate or division. However, when grown to mid-log phase, *sty1-1* and *sty2-1* mutant cells were found to be substantially longer at division than the wild-type control (Table 1), suggesting that they are delayed in the timing of mitotic initiation. These cells initiate mitosis at a size similar to *cdc25-22* cells, which express a partially defective *cdc25* phosphatase (Table 1). To investigate further the role of *sty1* and *sty2* in mitotic control, *sty1-1* cells were crossed to *cdc25-22* cells and random spore analysis performed. Approximately 25% of the spores from this cross germinated on YEPD plates at 26°C but arrested after one or two divisions as highly elongated cells, suggesting that the *sty1-1 cdc25-22* double mutants are synthetically lethal. To confirm this, asci from the same cross were subjected to tetrad dissection. The expected number of PD, TT, and NPD were observed, indicating that *cdc25* and *sty1* are unlinked genes. At least five NPD tetrads from the same cross had two spores that gave rise to wild-type colonies and two that underwent cell cycle arrest at 26°C (Table 1). The inviable cells from these NPDs were classified with the genotype *sty1-1 cdc25-22*. Identical results were found when crossing *sty2-1* to *cdc25-22* (Table 1). These data indicate that the products of the *sty1* and *sty2* genes are required for the control of cell size at division in fission yeast.

sty1 and sty2 mediate mitotic control by the pyp1 and pyp2 PTPases

We have shown previously that overexpression of *pyp1* or *pyp2* causes a delay in the timing of mitosis, whereas disruption of either gene has the opposite effect (Otilie et al. 1991, 1992; Millar et al. 1992b). Two lines of evidence suggest that *sty1* and *sty2* mediate control of cell size at division by the *pyp1* and *pyp2* PTPases. First, *sty1-1 Δpyp1 Δpyp2* and *sty2-1 Δpyp1 Δpyp2* cells initiate cell division at the same size as *sty1-1* and *sty2-1* single mutants alone (Table 1). Second, no additional delay in mitotic initiation was observed when *sty1-1* and

Table 1. Loss of *sty1* delays the timing of mitosis

	Temperature (°C)	Cell size at division (μm)
Wild type	30	14.2 ± 0.3
<i>cdc25-22</i>	26	21.9 ± 0.8
<i>sty1-1</i>	30	23.2 ± 1.6
<i>sty1-1 pyp1::ura4 pyp2::LEU2</i>	30	22.9 ± 2.1
<i>sty1-1 cdc25-22</i>	26	cdc
<i>sty2-1</i>	30	23.4 ± 1.3
<i>sty2-1 pyp1::ura4 pyp2::LEU2</i>	30	24.2 ± 1.3
<i>sty2-1 cdc25-22</i>	26	cdc

Cell size measurements of septated cells grown in either liquid synthetic minimal medium, (EMM). (cdc) Cell division cycle phenotype. The temperature at which the measurements were made is indicated. Measurements were the mean of 30 individual determinations (±S.D.).

sty2-1 cells were transformed with a plasmid constitutively expressing either *pyp1* or *pyp2* from the strong *adh* promoter as compared with empty vector alone (data not shown). From this we conclude that the *sty1-1* and *sty2-1* cells are completely epistatic to control of mitotic initiation by the *pyp1* and *pyp2* PTPases.

sty2 is allelic to the *wis1* MAP kinase kinase

We noted that mutants in the *wis1* gene, which encodes a putative MAP kinase kinase homolog, are delayed in mitotic initiation to a similar extent to the *sty1-1* and *sty2-1* mutants (Warbrick and Fantes 1991). The relationship of *wis1* to *sty1* and *sty2* was examined. During initial characterization we observed that the growth of single *sty1-1* and *sty2-1* mutant cells was defective at high temperatures. Neither *sty1-1* nor *sty2-1* mutant cells were able to grow at 36.5°C, whereas wild-type cells are unaffected (Fig. 2A). Importantly, *sty2-1* but not *sty1-1* cells ectopically expressing the *wis1* gene from the thiamine-repressible *nmt1* promoter were able to proliferate at the restrictive temperature of 36.5°C (Fig. 2B). We find that $\Delta wis1$ cells are also defective for cell division at 36.5°C (Table 2; data not shown). To determine the relationship between *sty2* and *wis1*, *sty2-1* cells were crossed to $\Delta wis1$ cells. Analysis of >5000 spores from this cross revealed no wild-type recombinants, strongly suggesting that *sty2* and *wis1* are allelic. Supporting this argument a $\Delta wis1 \Delta pyp1 \Delta pyp2$ triple mutant strain was found to be viable (data not shown). Because $\Delta wis1$ cells divide at an identical cell size to the *sty2-1* allele, we conclude that *sty2-1* harbors a null mutation in the *wis1* MAP kinase kinase.

Sty1 encodes a MAP kinase homolog

We used the observation that *sty1-1* cells are unable to proliferate at high temperature to isolate the *sty1* gene. A genomic library was introduced into *sty1-1* cells, and from a total of 50,000 transformants isolated at 30°C, 6 were able to divide at 36.5°C. Plasmids isolated from these six suppressors contain three different inserts that are related by restriction analysis and were subsequently shown to encode the same gene. pSTY1-4 containing the smallest insert is able to fully suppress the temperature sensitivity of a *sty1-1* but not a *sty2-1* mutant strain (Fig. 2C).

The restriction map of pSTY1-4 was not similar to a number of previously identified mitotic regulators, including *cdc25* (Russell and Nurse 1986), *wee1* (Russell and Nurse 1987a), *nim1* (Russell and Nurse 1987b), *wis1* (Warbrick and Fantes 1991), *cdc2* (Durkacz et al. 1986), or *cdc13* (Fig. 3A; Hagan et al. 1988). The complementing activity of pSTY1-4 was localized by transposon mutagenesis and subcloning analysis (Fig. 3B; Sedgewick and Morgan 1994). Inactivating transposon insertions were used as templates for sequencing, which revealed a single open reading frame (ORF) of 1047 bp capable of encoding a polypeptide of 349 amino acids. The predicted protein sequence was analyzed against current

Figure 2. Plasmid complementation of *sty1* and *sty2* mutants. (A) *sty1-1* and *sty2-1* mutants are temperature sensitive. Wild-type strain 972 (top left segment), *sty1-1* cells (top right segment), and *sty2-1* cells (bottom segment) were grown on YEPD and then streaked onto YEPD plates and allowed to grow for 3 days either at 30°C (left plate) or 36.5°C (right plate). (B,C) Plasmid complementation of *sty2-1* and *sty1-1* mutants. *sty2-1 ura4-D18* cells (B) or *sty1-1 ura4-D18* cells (C) were transformed either with the control plasmid pUR19 (Cont.), pSTY1-4, a derivative of pUR19 containing 5.2 kb of genomic sequence (pSTY1), or a plasmid expressing the *wis1* ORF behind the thiamine-repressible *nmt1* promoter pREP42-*wis1* and the *ura4*⁺-selectable marker (pWIS1). Transformants were grown and streaked on minimal medium lacking thiamine and uracil and growth of the cells monitored after 3 days at either 30°C (left) or 36.5°C (right).

data bases and found to be related most closely to a sub-family of MAP kinases that have been implicated in osmoregulation in budding yeast and stress-activated pathways in human cells (Brewster et al. 1993; Kyriakis et al. 1994; Han et al. 1994; Lee et al. 1994). Specifically, the Sty1 protein kinase was found to be related most closely to HOG1 from budding yeast (86% identical) and 57% identical to CSBP1 MAP kinase from human cells (Fig. 4A; Lee et al. 1994) and somewhat more distantly related (between 40% and 43% identical) to a family of four stress-activated protein kinases (SAPKs) from rat cells (Kyriakis et al. 1994). All members of the MAP kinase superfamily so far described are activated by dual phosphorylation on two closely spaced residues that are located at residues Thr-171 and Tyr-173 in the Sty1 pro-

Table 2. *Strains used in this study*

Strain no.	Genotype	Reference/source
PR 109	<i>leu1-32 ura4-D18 h⁻</i>	P. Russell (The Scripps Research Institute, San Diego, CA)
JM 1093	<i>leu1-32 ura4-D18 his1-102 h⁻</i>	this study
GL 193	<i>leu1-32 ura4-D18 ade6-704 h⁻</i>	P. Russell
GL 196	<i>leu1-32 ura4-D18 cdc25-22 h⁺</i>	Russell and Nurse (1986)
PR 170	<i>leu1-32 ura4-D18 wee1-50 h⁺</i>	Russell and Nurse (1987a)
GL 190	<i>leu1-32 ura4-D18 wee1-50 cdc25::ura4 h⁺</i>	Russell and Nurse (1986)
PR 253	<i>leu1-32 ura4-D18 pyp1::ura4 h⁻</i>	Millar et al. (1992b)
JM 1032	<i>leu1-32 ura4-D18 ade6-704 pyp2::LEU2 h⁻</i>	this study
JM 1023	<i>leu1-32 ura4-D18 NMT-pyp1(ura4) h⁺</i>	this study
JM 1036	<i>leu1-32 ura4-D18 ade6-704 NMT-pyp1(ura4) pyp2::LEU2 h⁻</i>	this study
JM 1042	<i>leu1-32 ura4-D18 his1-102 NMT-pyp1(ura4) pyp2::LEU2 h⁺</i>	this study
JM 1144	<i>leu1-32 ura4-D18 sty1-1 h⁻</i>	this study
JM 1162	<i>leu1-32 ura4-D18 sty2-1 h⁻</i>	this study
JM 1206	<i>leu1-32 ura4-D18 sty1-1 pyp1::ura4 pyp2::LEU2</i>	this study
JM 1207	<i>leu1-32 ura4-D18 sty2-1 pyp1::ura4 pyp2::LEU2</i>	this study
JM 1099	<i>leu1-32 ura4-D18 adh:pyp1(ura4) pyp1⁺ h⁻</i>	this study
ED 890	<i>leu1-32 ura4-D18 ade6-M210 wis1::LEU2 h⁻</i>	Warbrick and Fantes (1991)
JM 544	<i>leu1-32 ura4-D18 wis1::ura4 h⁻</i>	this study
JM 1160	<i>leu1-32 ura4-D18 ade6-216 sty1::ura4 h⁻</i>	this study

tein. Notably, the residue separating these phosphorylation sites appears to be conserved among members of the MAP kinase subfamilies. In human Erk1 and Erk2 and budding yeast FUS3 and KSS1, this sequence is Thr-Glu-Tyr, whereas in members of the SAP kinase family and JNK1, it is Thr-Pro-Tyr; in fission yeast Sty1, budding yeast HOG1, human CSBP1, and murine p38, it is Thr-Gly-Tyr. A phylogenetic tree compiled by sequence comparison to a number of stress-activated MAP kinases reveals that Sty1 belongs to the latter subfamily of MAP kinases (Fig. 4B).

To determine the relationship between the *sty1-1* mutation and the novel MAP kinase encoded by the pSTY1-4 genomic clone, the ORF of pSTY1-4 was interrupted by the *ura4⁺* gene (Fig. 3C). The resulting plasmid pSTY1::ura4 was unable to rescue a *sty1-1* mutant. pSTY1::ura4 was linearized with *EcoRI* and stably integrated into a *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M216/ade6-M210 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

Figure 3. Restriction map and disruption of *sty1* genomic clone. (A) Restriction map of the insert of pSTY1-4. (Hatched region) The position of the ORF; (arrow) the direction of the ORF, with the arrowhead at the 3' end of the gene. Intervals of 1 kb are marked under the map. (B) Location of inactivating TnHIS3 transposon insertions and restriction site-based subclones of the pSTY1-4 insert. Functional complementation was tested by transformation into a *sty1-1 ura4-D18* strain and the transformants assayed for growth at the restrictive temperature of 36.5°C. (C) Map of pSTY1::ura4 construction.

A

Sty1	MA---E---FIRTQIFGTCEFTITRYSDLPQIGMGAFGLVCSAKDQLTGMNVAVKKIMKPFSTP	58
HOG1	MTNEE---FIRTQIEGTVEITNRYNDLNPVGMGAFLVCSATDITLTSQPAIKKIMKPFSTA	61
CSBP1	MS---QERPTFYRQELNKITWEVPERYQNLSPVGSAGYSGVCAAFDTKTGLRVAVKKLSRPFQSI	62
Sty1	VLAKRTYRELKLLKHLRHNELISLSDF-----ISPEEDIYFVTELLGDLHRLTTSRPLETQFI	118
HOG1	VLAKRTYRELKLLKHLRHNELICLQDIF-----LSPLEDIYFVTELLGDLHRLTTSRPLEKQFV	121
CSBP1	IHKRTYRELRLKHKHKNHENVIGLLDVEITPARSLEEFNDVYLVLHLMGADLNNIVKCKQLTDDHV	127
Sty1	QYFLYQILRGLKIVHSAGVIHRDLKPSNINENCDLKICDFGLARIQDPQMTGYVSTRYYRAPE	183
HOG1	QYFLYQILRGLKYVHSAGVIHRDLKPSNINENCDLKICDFGLARIQDPQMTGYVSTRYYRAPE	186
CSBP1	QFLIYQILRGLKITHSADITHRLKPSNLAVNEDCELKILDFGLARHTDDEMTGYVAIRWYRAPE	192
Sty1	IMLTWQKYNVEVDIWSAGCIFAEMIEGKPLFPGRDHVNOFSITTELLGTPPMETVETICSKNTLR	248
HOG1	IMLTWQKYDVEVDIWSAGCIFAEMIEGKPLFPGRDHVHQSITTELLGSPPKDVINTICSENTLR	251
CSBP1	IMLNMHYNQTVDIWSVGCIMAEELLTGRTLFPGTDHINQLQQIMRLTGTPPAYLINRMPSEARN	257
Sty1	FVQSLPQKEKVPFAEKFNADPDALDLLEKMLVFDPRKRITSAADALAHNYLAPYHDPDDEPVADP	313
HOG1	EVTSLPHRDPITPFSERFKITVEPDVLDLEKMLVEDPKRITAADALAHNYSAPYHDPDDEPVADA	316
CSBP1	YIQSLTQMPKMNFAVFIIGANPLAVDLLEKMLVLDSDKRITAAQALAHAYFAQYHDPDDEPVADP	322
Sty1	VFDWSEQNDLPVETWKMVMYSEVLSFHN---M-----DNEELQSE	349
HOG1	KEDWHFNADLPVDITWRVMYSEILDHFK---IGGSDGQIDISATFDDQVAAATAAAQAQAQAQ	378
CSBP1	-YDQSEESRDLLIDENKSLTDEVTSEVPPLD-----QEEMES	360
Sty1	AQVQLNMAAHSHNGAGTTGNDHSDIAGGKNGQRSCSCK	349
HOG1		416
CSBP1		360

B

Figure 4. Sty1 shares homology to osmoregulated MAP kinases. (A) Alignment of the complete Sty1 amino acid sequence to that of budding yeast HOG1 and human CSBP1 MAP kinases. Boxes indicate identities among all three proteins. (B) A phylogenetic tree of amino acid sequence comparison of fission yeast Sty1 to rat SAPK α II, rat SAPK β , rat SAPK γ (Kyriakis et al. 1994), human JNK1 (Dérizard et al. 1994), budding yeast HOG1 (Brewster et al. 1993), human CSBP1 (Lee et al. 1994), and mouse p38 (Han et al. 1994). Homologies and phylogenetic trees were generated using a MegAlign work package based on a Jotun Hein algorithm (DNASTAR). The nucleotide sequence of the *sty1* gene will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number X89262.

shown). All uracil prototrophs were found to be temperature sensitive and elongated relative to wild type to the same extent as the *sty1-1* mutation. Random spore analysis of a cross between these uracil prototrophs and *sty1-1* cells revealed that of 3000 spores analyzed no wild-type recombinants could be isolated. We conclude that Δ *sty1* cells are viable and that *sty1-1* cells harbor a null mutant in a novel MAP kinase. Double mutant Δ *wis1* Δ *sty1* cells were found to be indistinguishable from the single mutants alone, suggesting that the Sty1 MAP kinase lies on the same signal transduction pathway as the Wis1 MAP kinase kinase.

Osmotic stress causes tyrosine phosphorylation of Sty1 and reduces cell size at division

The striking homology of Sty1 to the HOG1 MAP kinase from *S. cerevisiae* and of Wis1 to the HOG4 MAP kinase prompted us to examine whether Δ *sty1* cells and Δ *wis1* cells were sensitive to osmotic stress. Neither Δ *sty1* nor Δ *wis1* mutants were able to proliferate in the presence of 1.5 M sorbitol or 0.8 M KCl (Fig. 5A), and arrested as highly elongated, branched, and septated cells (data not shown). Wild-type cells continued to proliferate under these conditions (Fig. 5A). These results confirm the previous observation that Δ *wis1* cells are sensitive to high salt conditions (Shiozaki and Russell 1995) and demonstrate that both *sty1* and *wis1* are critically required for the cellular response to osmotic stress in fission yeast.

We wished to examine whether Sty1 bears the hallmark of MAP kinases of being phosphorylated on tyro-

sine residues by the action of a MAP kinase kinase. Because initial attempts to identify the Sty1 protein in whole cell extracts were unsuccessful, an episomal plasmid ectopically expressing a carboxy-terminally tagged version of Sty1 from a partially defective thiamine-repressible *nmt1* promoter was constructed. Log-phase cultures bearing this plasmid were subjected to osmotic stress and the Sty1 protein affinity precipitated. The phosphorylation state of Sty1 was assessed by Western blot using a monoclonal antibody to phosphotyrosine. Figure 5B demonstrates that in wild-type cells, the level of phosphotyrosine on the Sty1 protein increases rapidly after exposure to 0.9 M KCl. Duplicate samples probed using a monoclonal antibody to the hemagglutinin (HA) epitope tag showed that the level of protein did not change through the course of the experiment (Fig. 5B). In all experiments, phosphotyrosine was detectable on the Sty1 protein, even in minimal medium. Significantly, this basal level of phosphotyrosine was absent in Δ *wis1* cells, and no induction of tyrosine phosphorylation on the Sty1 protein was observed even on prolonged exposure to osmotic stress (Fig. 5B). These results strongly suggest that Wis1 is the MAP kinase kinase that tyrosine phosphorylates and activates the Sty1 MAP kinase in vivo.

From our observation that *wis1* and *sty1* are required for the cellular response to osmotic stress, we hypothesized that cell size at division may be regulated by external osmolarity. To test this prediction, wild-type cells were grown to mid-log phase in rich medium either alone or in the presence of 1 M sorbitol and cell size at division measured. Although no difference in growth

Figure 5. Sty1 and Wis1 are members of an osmosensing MAP kinase cascade. (A) Sty1 and Wis1 are required for resistance to osmotic stress. Wild type, *sty1::ura4* and *wis1::LEU2* cells were grown on YEPD plates and then streaked onto either YEPD medium alone (top left), YEPD containing 1.5 M Sorbitol (top right), and YEPD containing 0.8 M KCl (bottom left) and incubated for 3 days at 30°C. (Bottom right) The location of each strain on the plates. (B) Osmotic stress induces tyrosine phosphorylation of Sty1. The following strains, *leu1-32 ura4-D18* (WT); *wis1::ura4 leu1-32 ura4-D18* ($\Delta WIS1$); *adh:pyp1(ura4) leu1-32 ura4-D18* cells (*ADH-PYP1*), were transformed with pREP41-*sty1*(HA-6His) and grown at 30°C to mid-log phase in minimal medium lacking leucine and in the absence of thiamine to induce overexpression of epitope-tagged *sty1* from the thiamine-repressible *nmt1* promoter. Cells were then incubated in the same medium containing 0.9 M KCl for the times indicated. Approximately 2×10^8 cells were harvested and lysed at each time point, and the Sty1 protein precipitated using Ni^{2+} -NTA agarose. Precipitates were probed by Western blot for the presence of phosphotyrosine (α -pTyr) or HA epitope tag (α -HA).

rate was observed, wild-type cells divide at $11.2 \pm 1.1 \mu\text{m}$ in the presence of sorbitol compared with $14.3 \pm 1.3 \mu\text{m}$ in rich medium alone. In contrast, $\Delta wis1$ cells were unable to undergo a reduction in cell size at division in response to a mild osmotic stress, dividing at $18.6 \pm 1.8 \mu\text{m}$ in rich medium and $18.4 \pm 2.6 \mu\text{m}$ in rich medium containing 1 M sorbitol. These results are consistent

with the conclusion that the *sty1* MAP kinase pathway controls cell size at division in response to osmotic stress. It was noted, however, that cell size at division of $\Delta wis1$ cells is reduced on rich medium as compared with minimal medium, as observed previously (Warbrick and Fantes 1991).

Pyp2 MAP kinase phosphatase is induced by osmotic stress

The role of *pyp1* and *pyp2* in the regulation of the *sty1* MAP kinase pathway was examined. Overexpression of *pyp1* was found to abolish the increase in phosphotyrosine content on the Sty1 protein induced by osmotic stress (Fig. 5B), raising the possibility that Sty1 may be a direct substrate for Pyp1 and Pyp2 PTPases. Initial experiments showed that neither the *pyp1* nor *pyp2* mRNAs are periodically expressed in the cell cycle (data not shown). However, we observed that the *pyp2* mRNA, although low in normal medium, increases dramatically following exposure to 0.9 M KCl, reaching a maximum after ~ 1 hr and declining to basal levels by 3 hr (Fig. 6A). In contrast, neither *cdc2* mRNA (Fig. 6A) nor *pyp1* mRNA was altered during the time course of this experiment (data not shown). Similar results were obtained when cells were exposed to medium containing 1.2 M sorbitol (data not shown). Importantly, no induction of *pyp2* mRNA was observed in a $\Delta wis1$ strain (Fig. 6A) or in a *sty1-1* mutant (M. Wilkinson and J. Millar, unpubl.), indicating that the *sty1* MAP kinase pathway is required for induction of *pyp2* mRNA in response to osmotic stress.

These results prompted us to examine whether the tyrosine phosphorylation state of Sty1 was influenced by *pyp2* on prolonged exposure to osmotic stress. In a wild-type strain, the Sty1 protein was found to be maximally phosphorylated between 15 and 30 min after exposure to 0.9 M KCl and declined to basal levels after ~ 1 hr (Fig. 6B). Notably, the time course of tyrosine dephosphorylation coincides well with the time course of *pyp2* mRNA induction (Fig. 6A). To examine whether this dephosphorylation was influenced by *pyp2*, epitope-tagged Sty1 was introduced into a $\Delta pyp2$ strain. In these cells the percentage increase in phosphotyrosine on the Sty1 protein was only 68% in three independent experiments after 15 min of incubation with 0.9 M KCl compared to 485% observed in a wild-type strain. In contrast to wild type, Sty1 remained tyrosine phosphorylated on prolonged exposure to osmotic stress in $\Delta pyp2$ cells (Fig. 6B). Little difference in the time course of phosphorylation and dephosphorylation was observed in a $\Delta pyp1$ strain relative to wild type (data not shown). These results suggest that Pyp1 and Pyp2 perform distinct roles in the tyrosine dephosphorylation of Sty1 and that Pyp2 acts in a feedback pathway to tyrosine dephosphorylate Sty1.

To determine whether the Pyp2 protein could directly influence the phosphorylation state of Sty1 in vitro, tyrosine-phosphorylated Sty1 was incubated in the presence of a glutathione S-transferase (GST)-Pyp2 fusion

Figure 6. Pyp2 is a MAP kinase phosphatase. (A) Pyp2 mRNA is induced by osmotic stress. Log-phase cultures of either wild-type (WT) or $\Delta wis1$ cells were incubated in YEPD containing 0.9 M KCl for the times indicated. Total RNA was extracted and probed using DNA specific to the *pyp2* or *cdc2* genes. (B) Effect of *pyp2* on tyrosine phosphorylation of Sty1. Log-phase cultures of *leu1-32 ura4-D18* (WT), or *pyp2::ura4 leu1-32 ura4-D18* ($\Delta Pyp2$) cells transformed with pREP41-styl(HA-6His) were incubated in 0.9 M KCl for the times indicated. Sty1 was isolated by Ni^{2+} -NTA affinity precipitation and probed by Western blot for the presence of phosphotyrosine (α-PTyr) or HA epitope tag (α-HA). (C) Pyp2 PTPase dephosphorylates Sty1 in vitro. Tyrosine-phosphorylated Sty1 protein was isolated by affinity precipitation from osmotically stressed wild-type cells expressing epitope-tagged Sty1. Beads were incubated either in the presence (+) or absence (−) of 2 mM orthovanadate and either no protein (Control) or 10 μg of GST-Pyp2 fusion protein or 10 μg of GST-Pyp3 fusion protein for 30 min at 30°C. Sty1 was then probed by Western blot for the presence of phosphotyrosine (α-PTyr) or HA epitope tag (α-HA). (D) Pyp2 associates with the Sty1 MAP kinase. Cell lysates containing epitope-tagged Sty1 were incubated in the presence of no protein (Control), 10 μg of GST-Pyp2 fusion protein, or 10 μg of GST-Pyp3 fusion protein for 1 hr at 4°C. After this time proteins were precipitated on glutathione-agarose beads, washed three times with lysis buffer, and probed for the presence of Sty1 using a monoclonal antibody to the HA epitope tag.

protein containing the carboxy-terminal catalytic 303 amino acids of the Pyp2 protein (Millar et al. 1992b). As the results in Figure 6C demonstrate, GST-Pyp2 was able to reduce the level of phosphotyrosine on Sty1 by

~90%. Dephosphorylation by GST-Pyp2 was greatly inhibited by vanadate, a general PTPase inhibitor (Fig. 6C). During these experiments we noted that the Pyp2 PTPase forms a weak but specific association with Sty1. Lysates containing epitope-tagged Sty1 were incubated in the presence of either GST-Pyp2 or an equivalent concentration of a functionally unrelated PTPase fusion protein, GST-Pyp3 (Millar et al. 1992a). The results in Figure 6D illustrate that epitope-tagged Sty1 specifically coprecipitates with GST-Pyp2, whereas no association with GST-Pyp3 was observed. Together, these data indicate that Pyp2 is a MAP kinase phosphatase that acts in a feedback loop to inactivate Sty1 following exposure to osmotic stress (Fig. 7).

Discussion

Protein modification by tyrosine phosphorylation, although relatively common in the control of proliferation, differentiation, and intercellular communication between mammalian cells, is rare in yeast. Only recently has the discovery of a number of tyrosine phosphatases in fission yeast revealed an important role for tyrosine phosphorylation in cellular growth control of unicellular eucaryotes. This study was initiated to uncover the mechanism of action of two tyrosine-specific phos-

Figure 7. Osmotic control in fission yeast. This model depicts the genetic and biochemical interactions described in this paper. Osmotic stress induces activation of Wis1 by an as-yet-unidentified MAP kinase kinase kinase (or MEKK). This leads to dual phosphorylation of Sty1 on residues Thr-171 and Tyr-173 to induce its activation. Activation of Sty1 leads to an advancement in the timing of mitosis and a reduction in cell size at division. We propose a feedback loop by which activation of Sty1 leads to accumulation of the *pyp2* mRNA presumably through the action of an unidentified transcription factor, which in turn leads to dephosphorylation of Sty1 and its inactivation. The phosphatases that dephosphorylate Thr-171 on the Sty1 protein have not been identified.

phatases, *pyp1* and *pyp2*, shown previously to act as inhibitors of mitotic initiation in fission yeast and to share an essential overlapping function (Millar et al. 1992b; Ottilie et al. 1992). We examined the mechanism underlying this loss of viability by isolating spontaneous mutations that could bypass the requirement for *pyp1* and *pyp2*.

Sty1 and Wis1 are required for the cellular response to osmotic stress

We have isolated mutations in two complementation groups that define a MAP kinase signaling pathway controlling cell size at division in fission yeast. We have identified the central members of this pathway as the Wis1 MAP kinase kinase and Sty1 MAP kinase. We present evidence that the Pyp1 and Pyp2 PTPases function in opposition to the Wis1 MAP kinase kinase to control tyrosine phosphorylation and, presumably, activity of the Sty1 MAP kinase. Sequence analysis reveals that Sty1 is closely related to a subfamily of MAP kinases that have been implicated in osmoregulation in both budding yeast and mammalian cells (Brewster et al. 1993; Han et al. 1994; Lee et al. 1994). We demonstrate that the *sty1* and *wis1* genes are themselves required for osmoregulation in fission yeast by virtue of the observations that, first, cells bearing deletions of either gene are sensitive to a number of independent osmotic stresses, and second, Sty1 is tyrosine phosphorylated following osmotic stress in a manner dependent on the *wis1* MAP kinase kinase. Thus, Sty1 is a structural and functional homolog of budding yeast HOG1, and Wis1 is the MAP kinase kinase that tyrosine phosphorylates and activates Sty1 in vivo. Because no prior assumptions were made as to the function of *pyp1* and *pyp2*, we note that it was fortuitous that the suppression screen in this study was performed in the presence of 1 M sorbitol in which *sty1*[−] and *wis1*[−] mutants are viable, and not in the presence of 1.5 M sorbitol, conditions under which they are unable to continue proliferating.

Loss of pyp1 and pyp2 causes hyperactivation of Sty1

Simultaneous inactivation of *pyp1* and *pyp2* causes aberrant cellular morphology and growth arrest. Several lines of evidence suggest that this is because of hyperactivation of the Sty1 MAP kinase. First, the synthetic lethality of a $\Delta pyp1 \Delta pyp2$ double mutant is suppressed by a mutation in either the *sty1* or *wis1* genes. Second, strong overexpression of the *wis1* MAP kinase kinase from the thiamine-repressible *nmt1* promoter is lethal and exhibits a terminal phenotype indistinguishable from the conditional *NMT-pyp1 $\Delta pyp2$* cells, an effect that is not observed in a *sty1-1* mutant or in a strain constitutively overexpressing the *pyp1* PTPase (J.-C. Shieh and J.B.A. Millar, unpubl.). The mechanism by which hyperactivation of the Sty1 kinase causes a loss of normal cell morphology and growth arrest is uncertain at present. Hyperactivation of Sty1 may lead to a lethal

increase in intracellular osmolarity such that cells eventually die by lysis. Alternatively, the Sty1 MAP kinase pathway may directly control cell polarity. We are attempting to distinguish between these possibilities. Recent evidence that correct positioning of the actin cytoskeleton in *S. cerevisiae* after osmotic stress requires the activity of the HOG1 MAP kinase supports the latter model (Brewster and Gustin 1994). Intriguingly, activation of a close homolog of Sty1 in rat PC12 cells leads to phosphorylation of Hsp27, a small heat shock protein implicated in actin filament stability (Lavoie et al. 1993; Rouse et al. 1994).

Distinct roles for the Pyp1 and Pyp2 MAP kinase phosphatases

The genetic data in this paper strongly suggest that both the Pyp1 and Pyp2 PTPases inhibit the *sty1* MAP kinase pathway. This conclusion is supported by the observation that both the Pyp1 and Pyp2 PTPase influence the tyrosine phosphorylation state of Sty1 in vivo and that the Pyp2 PTPase associates with and tyrosine dephosphorylates Sty1 in vitro. In particular, we observe that the dephosphorylation of Sty1 on prolonged exposure to osmotic stress is partially defective in a $\Delta pyp2$ strain. We find that the *pyp2* mRNA is induced strongly under these conditions, indicating that Pyp2 acts in a feedback loop to dephosphorylate Sty1. Notably, the mRNAs for a number of dual-specificity MAP kinase phosphatases are induced by extracellular stimulation, including human MKP1, CL100, PAC1, and budding yeast MSG5, although the mechanism of induction is not known (Keyse and Emslie 1992; Sun et al. 1993; Doi et al. 1994; Ward et al. 1994). We demonstrate that both *wis1* and *sty1* are required for induction of *pyp2* mRNA, presumably via the action of an as-yet-unidentified transcription factor. Notably, some dephosphorylation of Sty1 was observed consistently in a $\Delta pyp2$ strain indicating the existence for other mechanisms of Sty1 inactivation. In contrast to *pyp2*, the *pyp1* mRNA is not induced by osmotic stress indicating that Pyp1 and Pyp2 PTPases perform distinct roles as MAP kinase phosphatases for Sty1. The precise role of Pyp1 in Sty1 dephosphorylation is under further investigation.

Because Pyp1 and Pyp2 PTPases are specific for tyrosine residues, these data also suggest that two distinct classes of MAP kinase phosphatase act to dephosphorylate residues Thr-171 and Tyr-173 in the Sty1 protein. Pertinent to this, mutations that bypass the lethal combination of the deletion of two type 2C phosphatases *ptc1* and *ptc3* in fission yeast have been found to reside in the *wis1* MAP kinase kinase and a second locus, *spc1* (Shiozaki and Russell 1995). Sequence comparison revealed that *spc1* and *sty1* are the same gene, indicating a role for type 2C serine/threonine phosphatases in the inactivation of this pathway (K. Shiozaki and P. Russell, pers. comm.). Similarly, Maeda and colleagues have demonstrated that overexpression of a tyrosine-specific phosphatase, PTP2, or two serine/threonine-specific type 2C phosphatases, *PTC1* and *PTC3*, reverse

hyperactivation of the *HOG1* MAP kinase pathway caused by inactivation of the *SLN1* gene (see below); however the targets of these phosphatases have not been established (Maeda et al. 1994). Further experimentation will be required to determine the nature of the phosphatases regulating Thr-173 phosphorylation on the Sty1 protein.

An osmosensing MAP kinase pathway controlling cell size at division

The *pyp1* and *pyp2* PTPases have been implicated in the control of mitotic initiation in fission yeast on the basis of their effects on cell size at division (Millar et al. 1992b; Otilie et al. 1992). In this study we demonstrate that *pyp1* and *pyp2* mediate control of cell size at division by dephosphorylating Sty1 and act in opposition to the *wis1* MAP kinase kinase. Notably the *wis1* gene was initially described as a dose-dependent initiator of mitosis (Warbrick and Fantes 1991). We show that loss of *sty1* delays the timing of mitotic initiation. We demonstrate further that *wis1* and *sty1* are required for the cellular response to osmotic stress and that chronic exposure to osmotic stress causes a reduction in cell size at division. These results strongly support the conclusion that the *sty1* MAP kinase pathway regulates cell size at division in response to changes in external osmolarity, thus revealing a potentially vital link between the detection of environmental stress and control of the cell cycle.

Physiological and genetic studies indicate that the initiation of mitosis in fission yeast requires attainment of a critical cell mass (Nurse 1975; Fantes and Nurse 1977). Thus, mutations that alter the timing of mitosis uncouple size control from cell cycle progression. In principle, the *sty1* MAP kinase pathway may also alter mass control over the initiation of mitosis by impinging on the activity of the *cdc2* kinase. An alternative explanation is that osmotic stress and activation of the *sty1* MAP kinase pathway do not alter cell size control governing the initiation of mitosis but lead to an increase in intracellular density, for example, by an increase in intracellular glycerol concentration. To maintain mass control over the initiation of mitosis, cells undergo a reduction in cell volume. Because *S. pombe* cells grow by length extension this would be expressed as a reduction in cell size at division. Further experimentation will be required to determine which of these hypotheses is correct and to elucidate the molecular basis underlying this response. It has been known for some time that exposure of human cells to anisotonic media initiates a response that regulates cellular volume (Grinstein and Foskett 1990). Furthermore, it is now recognized that osmotic stress activates a number of MAP kinases including JNK1 and p38 in mammalian cells that share extensive sequence homology to Sty1 (Galcheva-Gargova et al. 1994; Han et al. 1994), raising the possibility that the mechanisms controlling eucaryotic cell size may be evolutionarily conserved.

A role for the Sty1 MAP kinase pathway in stationary phase?

Previous data have demonstrated that mutants in the *wis1* MAP kinase kinase rapidly lose viability in stationary phase suggesting that this pathway is influenced by nutritional stimuli (Warbrick and Fantes 1991). Cells overexpressing the *pyp1* PTPase and Δ *sty1* cells exhibit the same phenotype, indicating that the *sty1* MAP kinase may be activated on exit from the cell cycle (J.B.A. Millar, unpubl.). The nature of the signal that might activate the pathway upon entry into stationary phase is at present unclear, though we believe this is unlikely to be attributable to changes in external osmolarity of the medium. Unlike *sty1* and *wis1*, no defect in cell size at division, long-term survival in stationary phase or sensitivity to growth at high temperature has been ascribed to budding yeast bearing mutations in the *HOG1* and *HOG4* gene (Brewster et al. 1993). Furthermore, *wis1* does not complement a *HOG4* mutation nor vice versa, suggesting a possible evolutionary divergence between these two yeasts. It is intriguing that the mouse p38 MAP kinase is activated not only by osmotic stress but also by ultraviolet light, heat shock, bacterial endotoxic lipopolysaccharide (LPS), and stress related pro-inflammatory cytokines such as tumor necrosis factor α (TNF α) and interleukin-1 (IL-1) (Freshney et al. 1994; Han et al. 1994; Rouse et al. 1994; Dérjard et al. 1995), demonstrating that more than one stimulus can modulate the activity of this pathway in metazoa. It will be of great interest to determine the range of extracellular signals, other than osmotic stress, that control the *sty1* MAP kinase pathway in fission yeast and to what extent this pathway is evolutionarily conserved.

The activity of MAP kinase kinases is stimulated via phosphorylation by a MAP kinase kinase (for review, see Herskowitz 1995; Marshall 1994). In the genetic suppression screen performed in this study, only two complementation groups were identified that were viable in the absence of thiamine. This may indicate a functional redundancy in the action of upstream regulators of *wis1* or that mutations in such genes are lethal. Notably a set of mutants, which were lethal in the absence of thiamine, were isolated in the same screen and may include mutants in such upstream regulators. These are now the subject of further investigation. In budding yeast, a two-component system consisting of the *SLN1* and *SSK1* genes has been shown to regulate the HOG1 MAP kinase (Maeda et al. 1994), raising the exciting possibility that a similar system may control the *sty1* MAP kinase in fission yeast and possibly higher eukaryotes.

In conclusion, we have identified a conserved MAP kinase pathway in fission yeast that coordinates the cellular response to environmental stress with control of cell size at division. Recently, it has become apparent that stress-activated MAP kinase pathways may play an important role in a variety of physiological and pathological responses in mammals, including inflammation and tumor suppression. The closest human homolog to Sty1, CSBP1, has been shown to be the target of a group of

synthetic drugs that not only suppress accumulation of the stress related pro-inflammatory cytokines, TNF α and IL-1, but also act as potent therapeutic agents in a number of animal models of chronic and acute inflammation (Lee et al. 1994). We contend that further genetic dissection of the *sty1* MAP kinase pathway will advance our understanding of the mechanisms governing the cellular response to environmental stress, and provide an ideal model system with which to study these novel MAP kinase pathways in human cells.

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.

Construction of NMT-pyp1(*ura4*) and pyp2::LEU2 alleles

The *pyp1* ORF was cloned by PCR amplification from an *S. pombe* cDNA library. The 5' oligonucleotide, CCGGATC-CATATGAATTTTCAAACGGTTCAAA, incorporating *Bam*HI and *Nde*I sites (shown italicized) hybridized to sequences surrounding the ATG initiation codon, whereas the 3' oligonucleotide, CCGGATCCTCATGTATAAAACCGGAAATGAAC, incorporating a *Bam*HI site (shown italicized) hybridized to sequences surrounding the TGA termination codon. PCR amplification generated a 1.7-kb fragment that was cleaved with *Nde*I and *Bam*HI and cloned into the *Nde*I and *Bam*HI sites of pREP82 (Maundrell 1993) containing the thiamine-repressible *nmt1* promoter to form pREP82-pyp1. A 1.2-kb fragment containing the *ars1* autonomously replicating element was excised by digestion with *Eco*RI followed by self-ligation. The carboxy-terminal half of *pyp1* containing the PTPase catalytic domain was removed by digestion with *Sac*I to drop out a 692-bp fragment followed by self-ligation to form pRIP82-pyp1(T). The NMT-pyp1 strain was created by linearizing pRIP82-pyp1(T) using the unique *Sal*I site in *pyp1* and introduced into a *leu1-32 ura4-D18 h⁻* strain by lithium acetate transformation onto minimal medium lacking uracil. Stable integrants were identified and verified by Southern blot hybridization.

To create the *pyp2::LEU2* allele a 5.1-kb fragment from pGEM-3Z-pyp2 (Millar et al. 1992b) was excised with *Eco*RI and ligated into the *Eco*RI site of pBluescript to form pBSSK-pyp2. A 2.2-kb fragment containing *LEU2* was excised from pREP1 with *Hind*III, blunt ended with the DNA polymerase I, and ligated into pBSSK-pyp2 that had been digested with *Hpa*I and *Nru*I to excise a 1515-bp fragment containing much of the *pyp2* ORF to form pBSSK-pyp2::LEU2. The disrupted *pyp2* sequence was excised from pBSSK-pyp2::LEU2 with *Pst*I and *Hind*III and introduced into a *leu1-32 ura4-D18 ade6-704 h⁻* strain using lithium acetate transformation onto minimal media plates lacking leucine. Stable integrants were isolated and confirmed by Southern blot hybridization.

Isolation of spontaneous suppressors of NMT-pyp1

Two strains, JM1036 and JM1042, bearing the NMT-pyp1 Δ pyp2 alleles were grown to stationary phase in minimal me-

dium. Approximately 10⁷ cells in two independent screens were plated onto 20 YEPD plates containing 1 M sorbitol and 10 μ M of thiamine and allowed to incubate for 3 days at 30°C.

Isolation of the *sty1* genomic clone

A genomic library, pURB1 (Barbet et al. 1992), was introduced into *sty1-1 leu1-32 ura4-D18 h⁻* strain by electroporation and plated on medium lacking uracil. A total of 55,000 transformants were screened. Transformants were replica plated twice to 36.5°C. Six complementing colonies were identified. Plasmids from these six individual colonies were transformed into DH5 α . Three independent plasmids related by restriction map were identified and contained overlapping sequences. pSTY1-1 and pSTY1-5 contain a 11.5-kb insert. pSTY1-2 contains a 7.5-kb insert. pSTY1-3, pSTY1-4, and pSTY1-6 contain a 5.2-kb insert. pSTY1-4 was used for further analysis.

Localization and sequencing of the *sty1* gene

Simultaneous localization and sequencing was performed by transposon mutagenesis of the pSTY1-4 genomic clone using TnHIS3 as described previously (Sedgewick and Morgan 1994). Complementing activity was tested by transformation into a *sty1-1 ura4-D18* strain and assayed for growth at 36.5°C. Dideoxy sequencing was performed using a T7 sequencing kit from Pharmacia using primers described previously to the 5' and 3' ends of the transposon.

Construction of *sty1::ura4* allele

An *Eco*RI fragment from pSTY1-4 was subcloned into the *Eco*RI site of pBluescript to form pBSSK-STY1. Plasmid pBSSK-STY1 was digested with *Nru*I and a 1.6-kb fragment containing the *ura4⁺* gene ligated into this site after digestion of pREP1 with *Hind*III followed by blunt ending using the large fragment of DNA polymerase I to form pBSSK-STY1::ura4. pBSSK-sty1::ura4 was linearized with *Eco*RI 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 a *sty1*-specific probe. Colonies bearing a disruption of *sty1* 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 *sty1* by genomic Southern blot analysis.

Overexpression of *sty1*

The *sty1* cDNA was amplified from the genomic clone pSTY1-4 by PCR amplification. The 5' oligonucleotide, ATATCTA-GAGAATTCATATGGCAGAATTTATTCGTACACAA, incorporating *Xba*I, *Eco*RI, and *Nde*I sites (shown italicized), hybridized to sequences surrounding the ATG initiation codon, whereas the 3' oligonucleotide, TATGCGGCCGCGGGATTG-CAGTTCATTATCCA, incorporating a *Not*I site (shown italicized), hybridized to sequences prior to the TGA termination codon. PCR amplification generated a 1072-nucleotide fragment that was cleaved with *Nde*I and *Not*I and cloned into pREP41 (HA-6His) (J. Millar, unpubl.) to form pREP41-sty1 (HA-6His), which expresses *sty1* under the control of a partially crippled version of the *nmt1* thiamine-repressible promoter (Basi et al. 1993). pREP41-sty1(HA-6His) was used to transform strains bearing the *leu1-32* mutation and leucine prototrophs selected. The phenotype of overexpression was deter-

mined after at least 48 hr growth in the absence of thiamine. The pREP41–sty1(HA–6His) plasmid was found to rescue the temperature sensitivity of a *sty1-1 leu1-32 ura4-D18* strain when grown in the absence of thiamine.

Detection of Sty1 protein

The Sty1 protein was partially purified from cells containing the pREP41–sty1(HA–6His) plasmid (see above), which expresses a fusion protein of Sty1 to a HA peptide epitope and a six histidine carboxy-terminal tail. Briefly, pelleted 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 benzamide, 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 Ni^{2+} -NTA beads (Qiagen). 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 monoclonal antibody to phosphotyrosine (4G10, UBI). Detection was performed using peroxidase-conjugated anti-mouse IgGs (Amersham, UK) and chemiluminescence visualization (ECL, Amersham) according to the manufacturer's instructions.

Phosphatase assay

GST–Pyp2 and GST–Pyp3 fusion proteins were made in *Escherichia coli* as described previously (Millar et al. 1992a,b). Phosphatase assays were performed in phosphatase buffer (1 mM EDTA, 2 mM DTT, 200 mM imidazole at pH 7.2) as described previously (Millar et al. 1991).

Construction of *wis1::ura4* allele

The *wis1* gene was cloned by PCR amplification from an *S. pombe* cDNA library. The 5' oligonucleotide, CCCCTCGAG-CATATGTCTAGATCTTCTCCAAATAATCAACCC, incorporating *XhoI*, *NdeI*, *XbaI*, and *BglII* sites (shown italicized), hybridized to sequences surrounding the ATG initiation codon, whereas the 3' oligonucleotide, CACTGCAGCGGCCGCT-TCTTTTTCACCTTTC, incorporating a *PstI* and *NotI* site (shown italicized), hybridized to sequences surrounding the TGA termination codon. PCR amplification generated a 1846-nucleotide fragment that was cleaved with *PstI* and *XhoI* and cloned into the *PstI* and *XhoI* sites in pBluescript to form pBSSK–*wis1*. This plasmid was cleaved with *HindIII*, and a 1.66-kb *HindIII* fragment containing the *S. pombe ura4*⁺ gene was ligated into this site to form pBSSK–*wis1::ura4*. Plasmid pBSSK–*wis1::ura4* was cleaved with *PstI* and *XhoI* and used to transform a *leu1-32 ura4-D18 h*[−] strain. Stable uracil prototrophs were selected. Confirmation of the *wis1::ura4* disruption was determined by PCR amplification using the oligonucleotides described above.

Overexpression of *wis1*

The *wis1* ORF was amplified by PCR using the oligonucleotides described above and cleaved with *NdeI* and *NotI* and ligated into the *NdeI* and *NotI* sites of pREP42 (HA–6His) (J. Millar, unpubl.) to form pREP42–*wis1* (HA–6His). This plasmid was used to transform strains bearing the *ura4-D18* mutation and uracil prototrophs selected. The phenotype of overexpression was determined after at least 48 hr growth in the absence of thiamine.

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 ml of TE, of which one-fifth was digested with *HindIII* and subjected to electrophoresis and Southern blot hybridization. A *BglII* fragment isolated from the pSTY1-4 gene containing *sty1* sequence was radioactively labeled and used as a probe. To isolate RNA, *S. pombe* cells were cultured in YEPD to exponentially growing phase. Approximately 10 μ g of total RNA was isolated and resolved by agarose gel electrophoresis and transferred to nitrocellulose for hybridization as described previously (Aves et al. 1985). A 1-kb *EcoRV* fragment from pGEM-3Z–pyp2 was used to probe for *pyp2* mRNA. A 1-kb *NdeI*–*BamHI* fragment from pREP1–*cdc2* (HA–6His) was used to probe for *cdc2* mRNA.

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