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Sin1: an evolutionarily conserved component of the eukaryotic SAPK pathway

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The fission yeast Sty1/Spc1 mitogen-activated protein (MAP) kinase is a member of the eukaryotic stress-activated MAP kinase (SAPK) family. We have identified a protein, Sin1, that interacts with Sty1/Spc1 which is a member of a new evolutionarily conserved gene family. Cells lacking Sin1 display many, but not all, of the phenotypes of cells lacking the Sty1/Spc1 MAP kinase including sterility, multiple stress sensitivity and a cell-cycle delay. Sin1 is phosphorylated after stress but this is not Sty1/Spc1-dependent. Importantly, Sin1 is not required for activation of Sty1/Spc1 but is required for stress-dependent transcription via its substrate, Atf1. We find that in the absence of Sin1, Sty1/Spc1 appears to translocate to the nucleus but Atf1 is not fully phosphorylated and becomes unstable in response to environmental stress. Sin1 is also required for effective transcription via the AP-1 factor Pap1 but does not prevent its nuclear translocation. Remarkably chimaeric fusions of *sin1* with chicken *sin1* sequences rescue loss of *sin1* function. We conclude that Sin1 is a novel component of the eukaryotic SAPK pathway.
Keywords: cell cycle/environmental stress/MAP kinase/ *Schizosaccharomyces pombe*/transcription factor

Introduction

The mitogen-activated protein kinase (MAPK) signalling pathways are critical for the response of cells to changes in their external environment (Marshall, 1994; Herskowitz, 1995; Waskiewicz and Cooper, 1995; Treisman, 1996). They serve to transduce signals generated at the cell surface to the nucleus, where changes in gene expression result. In mammalian cells multiple distinct pathways have been identified, the best characterized of which leads to activation of the extracellular-signal-regulated kinases ERK1 and ERK2 in response to a variety of growth factors and mitogens, and which are involved in the control of cell proliferation and differentiation (Marshall, 1994). More 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; Freshney *et al*., 1994; Galcheva-Gargova *et al*., 1994; Han *et al*., 1994; Kyriakis *et al*., 1994; Lee *et al*., 1994; Rouse *et al*., 1994). These stress-activated MAP kinases (SAPKs) fall into two distinct classes based on sequence and are termed the c-Jun N-terminal kinase (JNK) and p38 kinases. Roles for the SAPKs have been demonstrated in the adaptive response of cells to stress and in T-cell activation, inflammation and ischaemic injury (Davies, 1994; Waskiewicz and Cooper, 1995). As such the SAPKs are receiving a great deal of attention as potential targets for novel therapeutics.

Since activation of MAPK pathways results in changes in gene expression, understanding how MAP kinases trigger transcriptional activation is a key issue. Attention has been focussed on the identification and characterization of transcription factors whose activity is modulated by MAPK-phosphorylation. In mammalian cells, a number of factors have now been identified that are phosphorylated by the SAPKs (Treisman, 1996). Elk-1, a member of the ternary complex factor (TCF) family of erythroblastosis virus E26 (ETS) domain proteins that synergizes with the serum response factor (SRF) factor to mediate activation of certain immediate early genes such as the c-*fos* gene, has been shown to be a target of both JNK and p38 (Gille *et al*., 1992, 1995; Marais *et al*., 1993; Whitmarsh *et al*., 1995; Zinck *et al*., 1995). The c-Jun factor is regulated by JNK (Hibi *et al*., 1993; Derijard *et al*., 1994; Kyriakis *et al*., 1994) but not by p38, whereas ATF2 is phosphorylated and regulated by both JNK (Gupta *et al*., 1995; Livingstone *et al*., 1995; van Dam *et al*., 1995) and p38 (Raingeaud *et al*., 1995). In all three cases phosphorylation results in an increase in the activation potential of the factor although the mechanisms that underlie these increases are not understood.

The molecular dissection of the SAPK pathways has been greatly aided by the identification of a similar pathway in the unicellular fission yeast *Schizosaccharomyces pombe*. The central elements of this pathway are the Sty1 MAP kinase (also known as Spc1 or Phh1), Wis1 MAPKK and Wak1 (also known as Wis4 or Wik1) and Win1 MAPKKKs (Warbrick and Fantes, 1991; Millar *et al*., 1995; Shiozaki and Russell, 1995; Kato *et al*., 1996; Shieh *et al*., 1997, 1998; Samejima *et al*., 1998). Cells lacking the Sty1 MAP kinase are sensitive to multiple environmental stresses, are unable to undergo sexual

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conjugation or differentiation and are delayed in the timing of mitotic initiation. Importantly, Sty1 is not only structurally related to the mammalian SAPKs but is activated by a similar range of environmental insults (Millar *et al*., 1995; Shiozaki and Russell, 1995; Degols *et al*., 1996, Degols and Russell, 1997; Shieh *et al*., 1997). Most notably Sty1 stimulates gene expression via the Atf1 and Pap1 transcription factors, homologues of human ATF2 and c-Jun, respectively; indicating that these pathways are likely to have derived from a common ancestor (Toda *et al*., 1991; Takeda *et al*., 1995; Kanoh *et al*., 1996; Shiozaki and Russell, 1996; Wilkinson *et al*., 1996; Gaits *et al*., 1998; Toone *et al*., 1998; Wilkinson and Millar, 1998). Nuclear translocation of activated Sty1 allows it to bind and phosphorylate Atf1, but the precise mechanism by which this phosphorylation induces transcriptional activation is not known (Shiozaki *et al*., 1996; Wilkinson *et al*., 1996). Sty1 also binds to Pap1 but, in this case, the mechanism of activation appears to be distinct. In contrast to Atf1 the bulk of Pap1 protein is cytoplasmic in unstressed cells and its translocation to the nucleus is controlled by Sty1 (Toone *et al*., 1998). The mechanism by which this occurs does not seem to involve phosphorylation since Pap1 is not a phosphorylation target of Sty1 *in vitro*. Nevertheless, Atf1 and Pap1 control the expression of a distinct but overlapping set of genes in response to Sty1 activation (reviewed in Wilkinson and Millar, 1998).

Although the Atf1 and Pap1 transcription factors are key components of the fission yeast SAPK pathway they are not the only targets for Sty1. In particular, cells lacking Sty1 are delayed in the timing of mitotic initiation, whereas cells lacking both Atf1 and Pap1 are not. In an effort to understand how the Sty1 MAPK pathway controls cell-cycle progression we have sought additional SAPK targets. Here we describe a new Sty1-interacting protein that is a key component of the fission yeast SAPK pathway and which is structurally and functionally conserved in all eukaryotes.

Results

Sin1 is a member of an evolutionarily conserved gene family

To identify new effectors of the fission yeast SAPK pathway, we undertook a two-hybrid screen in *Saccharomyces cerevisiae* for *S.pombe* proteins which would interact with Sty1. Amongst the isolates we identified were partial cDNAs that corresponded to three distinct gene products. These were termed Sin1 (eight isolates), Sin2 (five isolates) and Sin3 (three isolates) for Sty1 (or SAPK) interacting protein. We found that Sin2 is a member of a novel evolutionarily conserved gene family and will be described elsewhere (S.Tournier and J.B.A.Millar, unpublished data) and that Sin3 is identical to the Pyp2 MAP kinase phosphatase, which has previously been shown to interact with Sty1 (Millar *et al*., 1995). We have characterized Sin1 in this paper. The ability of Sin1 to interact with other components of the Sty1 pathway and unrelated proteins was tested by two-hybrid analysis. We found that the Sin1 induces expression of the β-galactosidase reporter gene only when co-transformed with a plasmid expressing the Sty1 MAPK and not the Wis1 MAPKK or Wak1/Wis4 MAPKKK (Table I). Sin1 also did not interact in this assay with unrelated MAPKs such as Pmk1 or unrelated clones such as Mcs2 or Mcs6, components of fission yeast CAK (data not shown). This suggests that the Sin1 protein interacts only with the MAPK and not other components of the Sty1 pathway.

Table I. Two-hybrid analysis of protein interaction

Bait plasmid	Prey plasmid	β-galactosidase (A.U.)
pAS2	pACTII	2.1 ± 0.3
pAS2	pACTII-Sin1	2.3 ± 0.3
pAS2-Sty1	pACTII	2.1 ± 0.5
pAS2-Sty1	pACTII-Sin1	50.6 ± 1.3
pAS2-Wis1	pACTII	2.4 ± 0.1
pAS2-Wis1	pACTII-Sin1	2.5 ± 0.3
pAS2-Wak1	pACTII	1.7 ± 0.4
pAS2-Wak1	pACTII-Sin1	2.0 ± 0.6

Diploids from a cross of *S.cerevisiae* strains CG1945 and Y187 (Fromont-Racine *et al*., 1997) were co-transformed with either pAS2, pAS2-Sty1, pAS2-Wis1 or pAS2-Wak1 bait plasmids and either pACTII or pACT1-Sin1 prey plasmid and grown in the absence of leucine and tryptophan. Expression of LacZ was measured by assessing β-galactosidase activity in extracts as described in Materials and methods. Measurements are expressed in arbitrary units (A.U.) as the mean of three independent determinations (±SD).

A genomic clone bearing the full-length *sin1* gene was isolated (Figure 1A). Sequencing revealed an open reading frame (ORF) of 1953 bp, interrupted by a small intron located close to the termination codon, which encodes a protein with a predicted molecular weight of ~72 kDa. Database searches indicate that Sin1 does not contain any known structural motifs but is homologous to the gene product encoded by a partial human cDNA, JC310 (Figure 1B). The JC310 clone was isolated based on its ability to suppress the temperature sensitive defect of a RAS2^{Val19} mutation when overexpressed in *S.cerevisiae* (Colicelli *et al*., 1991). While this work was underway a full-length cDNA with 98% sequence homology to JC310 was isolated in a screen for segmentally expressed genes in the chicken hindbrain (Figure 1B; J.Christiansen and D. G.Wilkinson, manuscript in preparation). Two hypothetical proteins, one from *S.cerevisiae* and and the other from *Caenorhabditis elegans*, also show extensive homology to these sequences (H.Martin and J.Millar, data not shown). These and functional complementation studies described below indicate that Sin1 is a novel conserved protein.

Sin1 interacts with Sty1 and is phosphorylated in response to environmental stress

To determine whether Sin1 and Sty1 interact in fission yeast Sin1 was N-terminally tagged with three tandem haemagglutinin (HA) epitopes and expressed from the thiamine repressible *nmt41* promoter in fission yeast cells bearing Sty1 C-terminally tagged with six histidines (His) (Figure 2A). Complexes were purified on Ni^{2+}–NTA agarose beads and proteins visualized by Western blot. We found that Sin1 specifically co-precipitated with tagged but not untagged Sty1 (Figure 2A). We were also able to show that a bacterially produced glutathione *S*-transferase (GST)–Sin1 fusion protein effectively precipitates epitope-tagged Sty1 from cell lysates (data not shown).

We noted that Sin1 migrates in a diffuse manner on

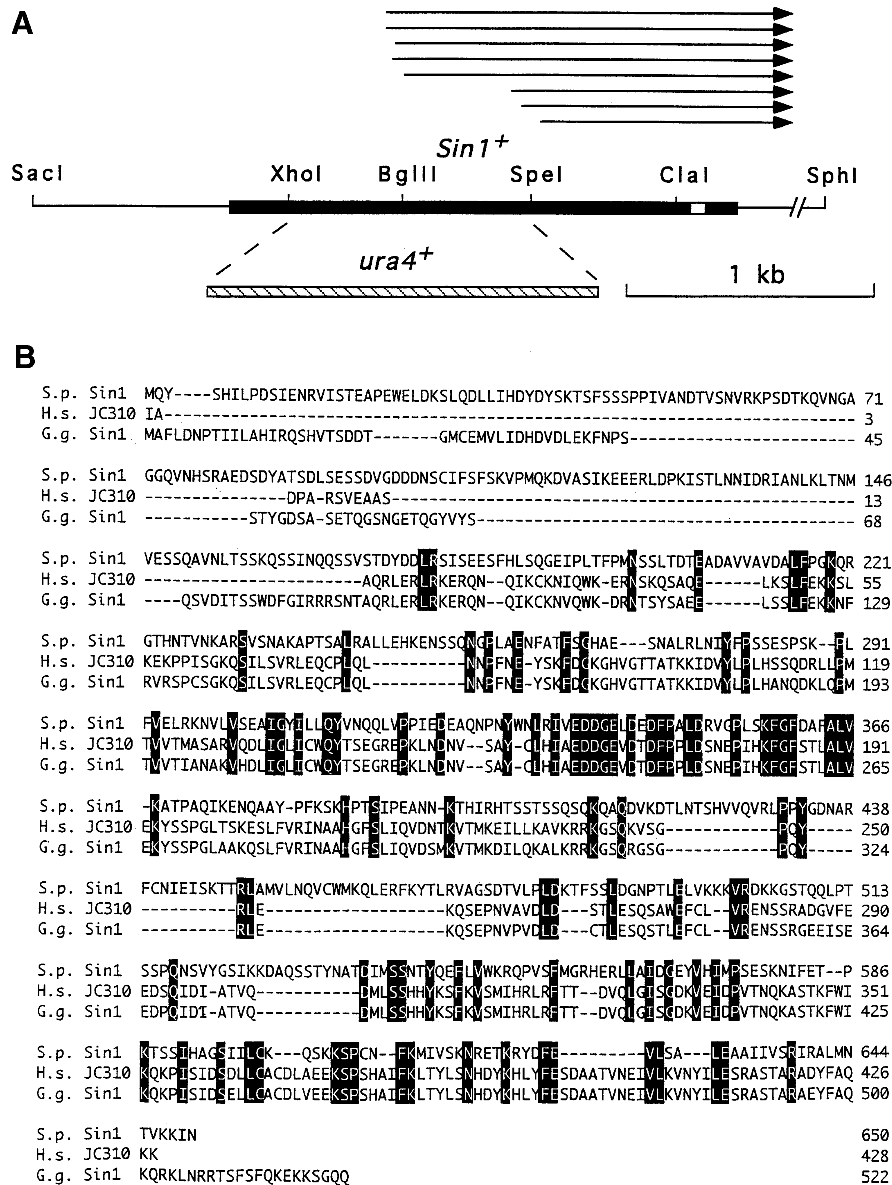

Fig. 1. Sin1 is a member of an evolutionarily conserved gene family. (**A**) Restriction map of the *sin1* genomic clone. The position of the ORF (closed bar) and intron (open bar) are shown. Arrows indicate the cDNAs isolated from the two-hybrid screen. The *ura4*$^+$ gene (hatched bar) was introduced between the *Xho*I and *Spe*I sites to generate pSin1::ura4. (**B**) Alignment of the complete fission yeast Sin1 amino acid sequence (S.p. Sin1) to the predicted sequence from a partial cDNA from humans (H.s. JC310) and a full cDNA sequence from chicken (G.g. Sin1). Boxes indicate identities amongst all three proteins. Homologies were generated using a MegAlign work package based on a Jotun Hein algorithm (DNASTAR). The nucleotide sequence of the fission yeast and chicken *sin1* genes will appear in the and DDBJ/EMBL/GenBank nucleotide sequence databases under the accession Nos AF155208 and AF153127, respectively.

SDS–PAGE, indicating that the protein may be subject to post-translational modifications, such as phosphorylation. In particular, Sin1 contains a number of potential serine–proline or threonine–proline motifs which may act as targets for phosphorylation, although only one of these conforms to a consensus MAP kinase site. To investigate whether Sin1 is a phosphoprotein *in vivo*, Sin1 was immunoprecipitated from log phase wild-type cells before being treated *in vitro* with alkaline phosphosphatase. Treatment with phosphatase caused Sin1 to migrate as a single non-diffuse band of high electrophoretic mobility, indicating that it is multiply-phosphorylated in unstressed cells (Figure 2B). Following treatment of wild-type cells with 0.5 M NaCl, slower migrating forms of Sin1 were observed which were also sensitive to treatment with phosphatase *in vitro*, indicating that Sin1 is hyperphosphorylated after stress (Figure 2B). Indeed hyperphosphorylation of Sin1 was also rapidly induced after treatment of cells with 1 mM H_2O_2, mild heat shock (to 42°C) or 5 min centrifugation, cellular stresses known to cause Sty1 activation (Figure 2C). To determine whether this phosphorylation was due to Sty1, epitope-tagged Sin1 was expressed in *Δsty1* cells. No change in the mobility of Sin1 on SDS–PAGE gels was observed in the absence of Sty1 before or after the cells were challenged with an osmotic stress, indicating that an alternative kinase(s) phosphorylates Sin1 (Figure 2D). In keeping with this interpretation, a bacterially produced, full-length GST–Sin1 fusion protein was not phosphorylated by Sty1 *in vitro* (data

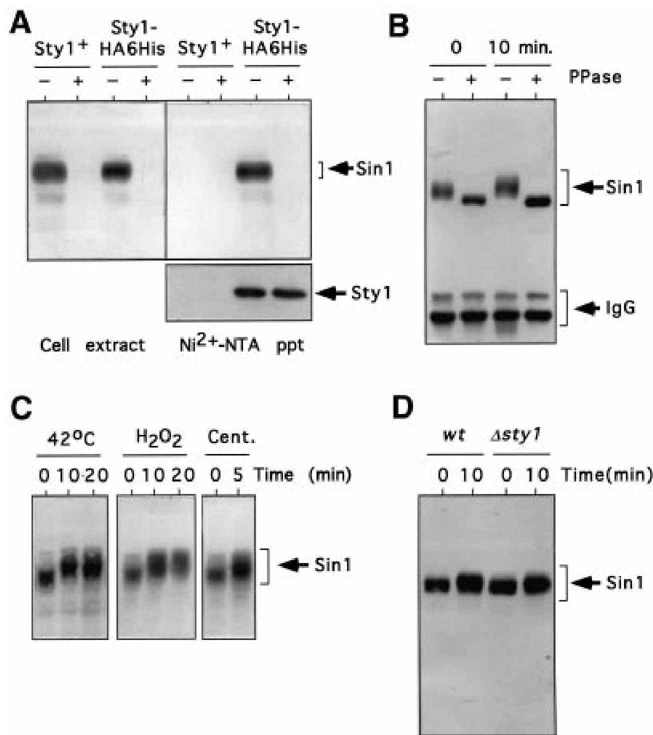

Fig. 2. Sin1 associates with Sty1 and is a stress-induced phosphoprotein. (**A**) Sin1 interacts with Sty1 *in vivo*. Wild-type (JM 1059) or tagged Sty1(6His HA) cells (JM1521) were transformed with pREP41-3HA-Sin1 and grown to log-phase in minimal medium lacking leucine at 30°C either in the absence (–) or presence (+) of 10 µg/ml thiamine. Cell extracts from these cells were made following centrifugation and bead lysis. Proteins were precipitated on Ni^{2+}–NTA beads and the presence of the HA epitope determined by Western blot (Ni^{2+}–NTA). As a control 50 µg of total cell extract was probed directly for the presence of the HA epitope (Cell extract). (**B**) Sin1 is a phosphoprotein. Wild-type (wt) (JM 1059) were transformed with pREP41-3HA-Sin1 and grown to log-phase in minimal medium lacking leucine. Cultures was then either incubated in the same medium either untreated (0 min) or with 0.5 M NaCl for 10 min (10 min). Cells were harvested and the Sin1 protein immunoprecipitated from cell lysates with a monoclonal anibody to the HA epitope (12CA5). Immunoprecipitates were washed and then incubated at 30°C in phosphatase buffer for 20 min either in the presence (+) or absence (–) of 10 units alkaline phosphatase (PPase). Proteins were then separated by SDS–PAGE and probed for the presence of the HA epitope as in (A). (**C**) Phosphorylation of Sin1 is increased after stress. Wild-type cells expressing pREP41-3HA-Sin1 as above were grown in minimal medium lacking leucine and then either heat shocked (42°C) or incubated in the presence of an oxidative stress (1 mM H_2O_2) or centrifuged at 4000 r.p.m. (Cent.) for the times indicated. Cells were harvested and extracts made. Twenty micrograms of total cell protein was probed for the presence of Sin1 by Western blot as in (A). (**D**) Sin1 is not phosphorylated by Sty1. Wild-type (JM 1059) or *Δsty1* (JM 1160) cells transformed with pREP41-3HA-Sin1 and grown to log-phase in minimal medium lacking leucine before being incubated in the same medium containing 0.5 M NaCl for times indicated. The Sin1 protein was detected by Western blot as in (A).

not shown). These results indicate that Sin1 binds the Sty1 MAP kinase and is the target of one or more stress-activated kinases *in vivo*.

Cells lacking Sin1 are delayed in the timing of mitotic initiation

The Sty1 MAP kinase pathway is required for several functions in fission yeast including maintaining the correct timing of mitotic initiation, for onset of cellular differentiation and for protection of the cells under adverse conditions. We reasoned that disrupting the function of new components of this pathway would effect one or more of these processes. To investigate the cellular function of Sin1 we replaced internal *sin1* sequences with the *ura4*$^+$ gene (Figure 1A) and integrated the disrupt into a sporulating diploid strain. Tetrad dissection of asci from heterozygous diploids gave rise to four viable spores, which on germination showed a 2:2 segregation of uracil auxotrophs to uracil prototrophs (data not shown), indicating that *sin1* is a non-essential gene. Cells lacking Sty1, but not either of the Atf1 or Pap1 transcription factors, divide at a greater cell size than wild-type (Figure 3A; Millar *et al*., 1995, Shiozaki and Russell, 1995). Importantly, *Δsin1* cells were also found to be elongated at division with respect to wild-type dividing at 20.2 ± 1.4 µm indicating that, like Sty1, Sin1 plays a role in the timing of mitotic initiation (Figure 3A). In rich medium we noted that *Δsin1* cells also appear to be somewhat wider than wild-type cells, the reason for which is unknown.

Sin1 is required for sexual conjugation and differentiation

The Sty1 kinase and the Atf1 transcription factor, but not Pap1, are required for the onset of sexual conjugation and differentiation (Shiozaki and Russell, 1996; Wilkinson *et al*., 1996). Cells lacking these proteins mate with only 5–10% efficiency of the wild-type. Homothallic strains bearing a deletion in either Sty1 or Sin1 were grown to stationary phase for 48 h in minimal medium lacking a nitrogen source and the number of cells that had undergone sexual conjugation and meiosis was assessed. Cells lacking functional Sin1 display a complete mating defect which could be fully restored by reintroduction of a Sin1 genomic clone but not control plasmid (Table II). It should be noted that this defect was more profound than in cells lacking Sty1 and that the mating defect of *Δsty1* cells could not be restored by overexpressing *sin1* (Table II). These results indicate Sin1 is also absolutely required for initiation of sexual differentiation.

Cells lacking Sin1 are sensitive to multiple environmental stresses

The Sty1 MAP kinase pathway is required for the survival of cells to multiple environmental insults. To examine the role of Sin1 in the stress response, *Δsin1* cells or cells lacking various components of the Sty1 MAP kinase pathway were grown on rich medium either at normal temperature (30°C), high temperature (37°C) or in the presence or absence of an osmotic stress (1 M KCl) or oxidative stress (50 µM $CdSO_4$). Importantly, as with cells lacking either Sty1 or Atf1, *Δsin1* cells were unable to grow under conditions of osmotic stress (Figure 3B). In addition *Δsin1* cells, like *Δsty1* cells, were also temperature sensitive, a phenotype not displayed by cells lacking either Atf1 or Pap1 or both (Figure 3B). However in contrast to *Δsty1* cells, *Δsin1* cells are able to proliferate in the presence of 50 µm $CdSO_4$ (Figure 3B) or 1 mM *t*-butyl hydrogen peroxide (data not shown). Thus Sin1 is required for many, but not all, of the processes controlled by Sty1 MAP kinase, in particular those controlled by Atf1, supporting the notion that Sin1 is an authentic component of the fission yeast SAPK pathway.

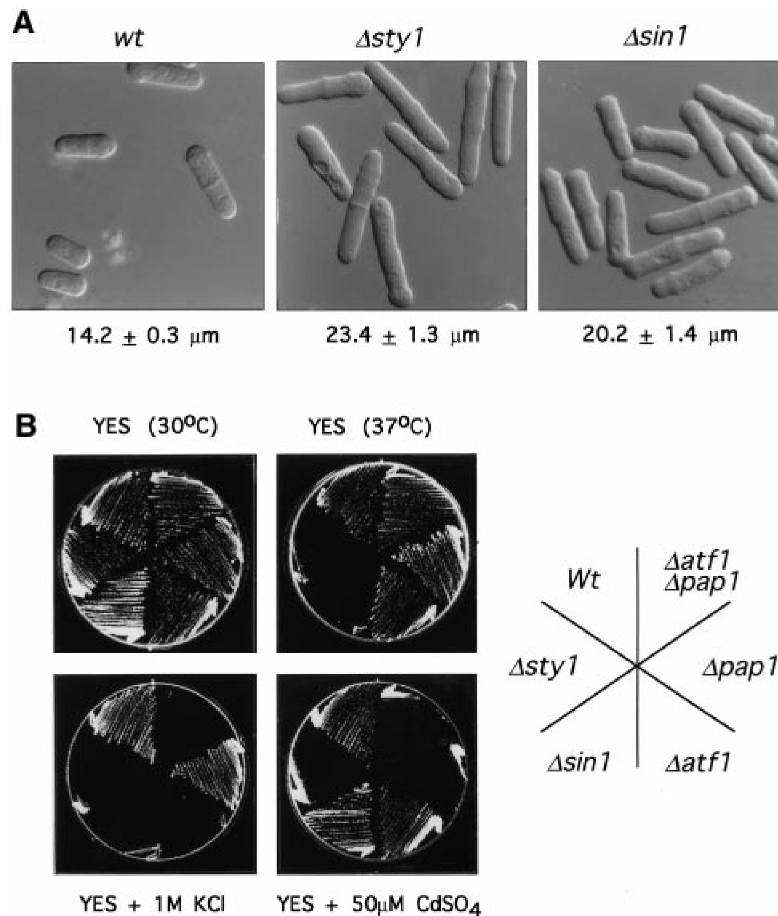

Fig. 3. Cells lacking Sin1 are delayed at the G_2/M transition and sensitive to environmental stress. (**A**) *Δsin1* cells are elongated at division. Wild-type (wt) (JM 1059), *sty1::ura4* (*Δsty1*) (JM 1160) or *sin1::ura4* (*Δsin1*) (JM 1797) cells were grown to log-phase in minimal medium and photographed using Normarski optics. Cell size at cell division was measured using a Nikon filar eyepiece drum micrometer at 1200× magnification. (**B**) *Δsin1* cells are stress sensitive. Wild-type (wt) (JM 1059), *sty1::ura4* (*Δsty1*) (JM 1160), *sin1::ura4* (*Δsin1*) (JM 1797), *atf1::ura4* (*Δatf1*) (JM 1529), *pap1::ura4* (*Δpap1*) (TP 103–3C) or *atf1::ura4 pap1::ura4* (*Δatf1 Δpap1*) (JM 1733) cells were grown on yeast extract and supplement (YES) at 30°C and then streaked to the same medium at 30°C (top left plate) or to YES at 37°C (top right), to the same medium containing 1 M KCl at 30°C (bottom left plate), or YES containing 50 μM $CdSO_4$ (bottom right plate) and incubated for two days.

Table II. Induction of sexual conjugation and differentiation

Strain	Plasmid	Mating efficiency (%)
wt h^{90}	pIRT2	41.5
wt h^{90}	pIRT2-Sin1	43.8
Δsty1 h^{90}	pIRT2	3.8
Δsty1 h^{90}	pIRT2-Sin1	3.6
Δsin1 h^{90}	pIRT2	0.0
Δsin1 h^{90}	pIRT2-Sin1	39.4

Homothallic cultures of wild-type (wt) (JY878), *sty1::ura4* (*Δsty*1) (1264) or *sin1::ura4* (*Δsin1*) (JM820) cells were transformed with either pIRT2 or pIRT2-Sin1, grown to log-phase in liquid EMM and then transferred to the same medium lacking a nitrogen source (NH_4Cl) for two days. Mating efficiency was assessed microscopically.

Sin1 is required for Atf1-dependent gene transcription

Induction of a number of genes in response to stress including glycerol-3-phosphate dehydrogenase (*gpd1*), the Pyp2 tyrosine-specific MAP kinase phosphatase (*pyp2*), thioredoxin reductase (*trr1*) and catalase (*ctt1*) are entirely dependent on the Sty1 MAP kinase (Millar *et al*., 1995, Degols *et al*., 1996; Wilkinson *et al*., 1996; Toone *et al*., 1998). Some of these (including *pyp2* and *gpd1*) are targets of the Atf1 transcription factor alone, whereas others are either dependent solely on Pap1 (e.g. *trr1*) or on both Atf1 and Pap1 (e.g. *ctt1*). In the latter case, the requirement for Atf1 and Pap1 is dependent on the nature of the inductive stress (Toone *et al*., 1998; Wilkinson and Millar, 1998). Since Sin1 is required for proliferation under certain adverse conditions, we next investigated the role of Sin1 in rapid activation of gene expression following the exposure of cells to multiple stresses. In response to either an osmotic shock (0.9 M KCl), oxidative stress (1 mM hydrogen peroxide) or a mild heat shock (42°C) expression of *pyp2* and *gpd1* was virtually absent in *Δsin1* cells, although some residual induction of *gpd1* occurred in response to osmotic stress (Figure 4). Loss of Sin1 did not, however, effect the expression of *cdc2*, or other unrelated genes, implying that Sin1 does not control general transcription (Figure 4; data not shown). These findings indicate that Sin1 is required for rapid stress-induced expression of genes under the control of the Wis1–Sty1–Atf1 pathway.

Sin1 is not required for Sty1 activation

The previous results establish Sin1 as a component of a signal transduction cascade leading to stress-activated gene expression. Since Sty1 is required for this response,

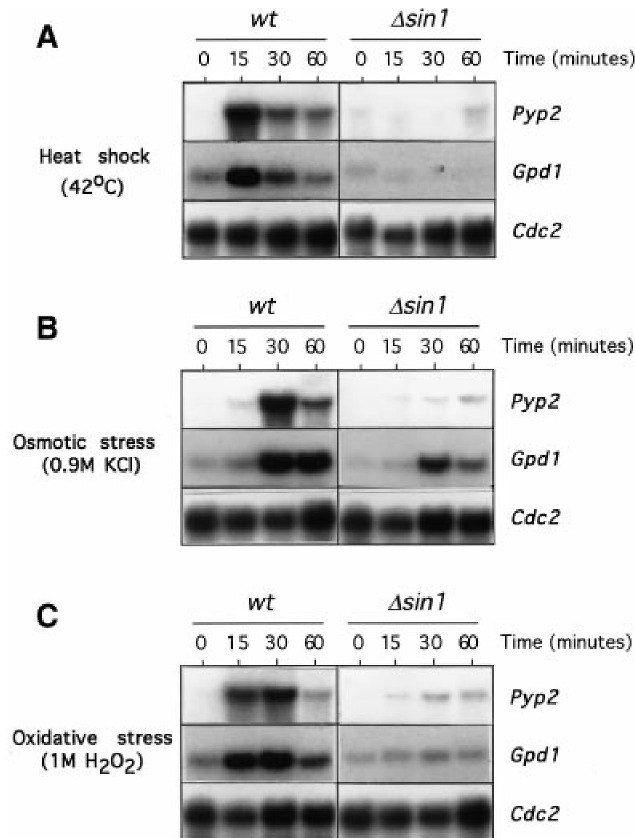

Fig. 4. Sin1 is required for Atf1-dependent gene transcription. Log-phase cultures growing in YES at 30°C of either wild-type (wt) (JM1059) or *Δsin1* cells (JM1797) cells were either shifted to 42°C (**A**) or incubated in the same medium containing 0.9 M KCl (**B**) or 1 mM H_2O_2 (**C**) at 30°C for the times indicated. Total RNA was extracted and equal quantities were separated by electrophoresis. Northern blots were then sequentially probed using DNA specific to the *pyp2*, *gpd1* and *cdc2* genes.

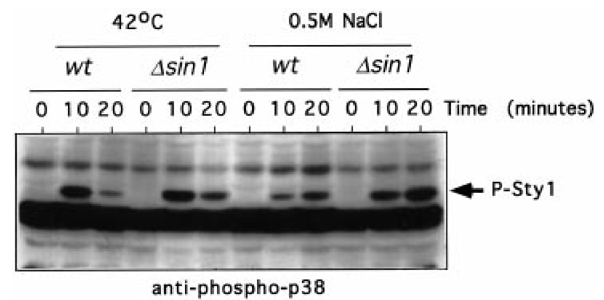

Fig. 5. Sin1 is not required for Sty1 phosphorylation. Effect of Sin1 on Sty1 activation. Log phase cultures of wild-type (wt) (JM1521) or *Δsin1* (1849) cells bearing an integrated and epitope-tagged version of Sty1 growing in YES medium at 30°C were incubated in the same medium containing either 0.5 M NaCl or shifted to 42°C for the times indicated. Approximately 2×10^8 cells were harvested at each time point, lysed under denaturing conditions and the Sty1 protein precipitated using Ni^{2+}–NTA agarose. Precipitates were probed by Western blot for the presence of phosphorylated MAP kinase (α-p38).

Table III. Strains used in this study

Strain No.	Genotype	Reference /source
CH 428	*ade6-M210 his7-366* h^+	C.Hoffman
CH 429	*ade6-M216 his7-366* h^-	C.Hoffman
JM 1160	*ade6-216 sty1::ura4* h^-	Millar *et al*., 1995
JM 1529	*his7-366 atf1::ura4* h^+	this study
TP 103–3C	*pap1::ura4* h^-	Toone *et al*., 1998
JM 1733	*his7-366 atf1::ura4 pap1::ura4* h^+	this study
JM 1797	*ade6-M216 his7-366 sin1::ura4* h^+	this study
JY 878	*ade6-M216* h^{90}	David Hughes
JM 1264	*ade6-M216 sty1::ura4* h^{90}	Shieh *et al*., 1997
JM 1820	*ade6-M210 his7-366 sin1::ura4* h^{90}	this study
KS 1479	*atf1* (*HA6His*)*:ura4* h^-	Shiozaki *et al*., 1997
JM 1852	*atf1* (*HA6His*)*:ura4 sin1::ura4*	this study
JM 1521	*ade6-M210 his7-366 sty1* (*HA6His*)*:ura4* h^+	Shieh *et al*., 1997
JM 1849	*ade6-M210 his7-366 sty1* (*HA6His*)*:ura4 sin1::ura4*	this study
HM 1689	*ade6-M216 his7-366 sty1* (*9myc*)*:ura4* h^-	this study
JM 1809	*ade6-M216 his7-366 sty1* (*9myc*)*:ura4 sin1::ura4* this study	

All strains were *leu1-32 ura4-D18* unless otherwise stated.

we next asked whether Sin1 is required for Sty1 activation. Wild-type or *Δsin1* cells bearing a His_6-tagged *sty1* allele were grown to log-phase in rich medium and then challenged to either a heat shock (42°C) or osmotic stress (0.5 M NaCl). Phosphorylation of Sty1 was monitored by Western blot using an antibody that recognizes only the phosphorylated, and by inference activated, form of Sty1 (Gaits *et al*., 1998). No difference in the degree or kinetics of Sty1 phosphorylation could be observed either in the presence or absence of Sin1 (Figure 5). However, in this and other experiments we noted that dephosphorylation of Sty1 was delayed, probably due to the lack of induction of the Pyp2 MAP kinase phosphatase. Since Sin1 does not appear to interact with other components of the Sty1 pathway and is not required for Sty1 activation it is unlikely to be part of the pre-activated MAPK complex.

Sin1 is required for full phosphorylation of Atf1 in the nucleus

The Sty1 MAP kinase is cytoplasmic in unstressed cells and translocates to the nucleus only in response to stress. Transport to the nucleus requires dual phosphorylation but not activity of the MAP kinase (Gaits *et al*., 1998). To assess whether Sin1 may be required for this process, a *sty1-9myc* allele was generated and Sty1 was localized in either a wild-type or *Δsin1* background by indirect immunofluorescence. In both wild-type and *Δsin1* cells, Sty1 was found to be localized throughout the cytoplasm in unstressed cells but concentrated in the nucleus (as assessed by co-incidence with DAPi staining) after 10 min stimulation with an osmotic stress (0.5 M NaCl) (Figure 6A). However, in three independent experiments the intensity of nuclear Sty1 staining in *Δsin1* cells was found to be ~50% that of wild-type (Figure 6A). These results indicate that Sin1 is not absolutely required for nuclear translocation of the Sty1 MAP kinase, but may facilitate the transport process.

We have previously shown that Sty1 associates with Atf1 *in vivo* and is required for its phosphorylation after stress (Shiozaki and Russell, 1996; Wilkinson *et al*., 1996). Although not formally demonstrated for Atf1, phosphorylation of ATF2 by the JNK or p38 MAP kinase leads to its transcriptional activation. To analyse the role of Sin1 in Atf1-mediated transcriptional activation, phosphorylation of Atf1 was assessed in the absence of Sin1. For these experiments, a chromosomal version of *atf1* was used that expresses Atf1 tagged with His_6 and

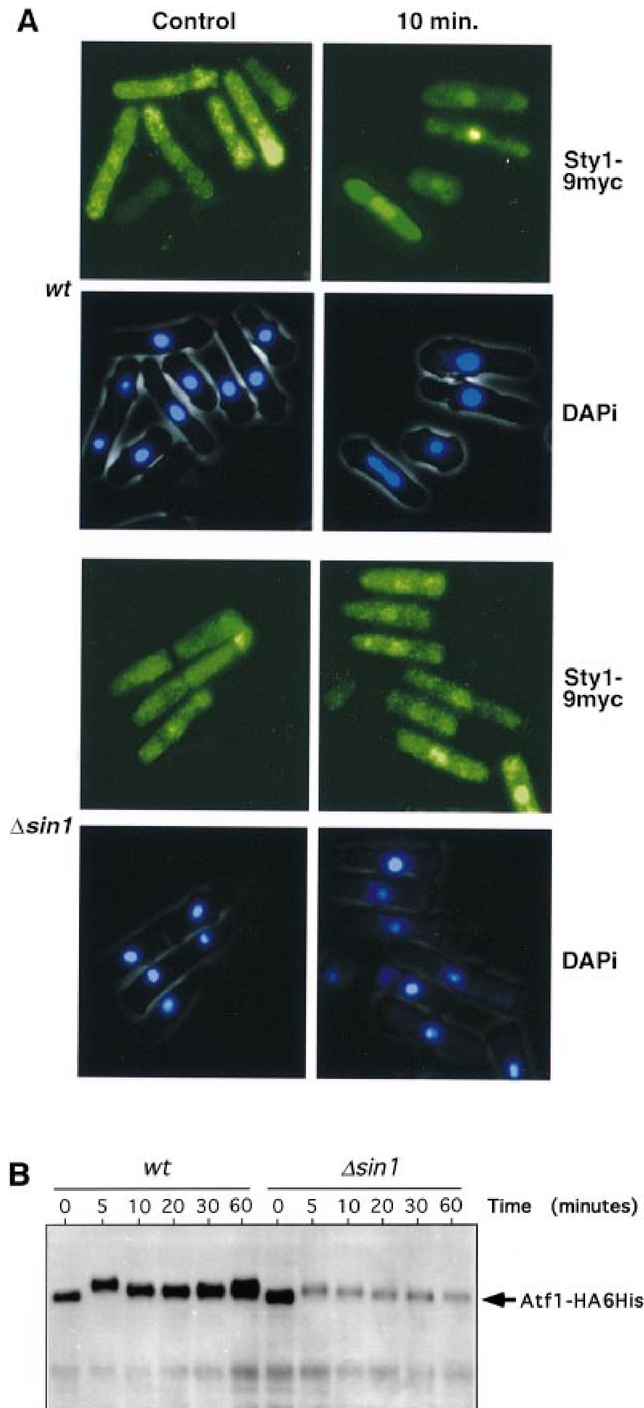

Fig. 6. Effect of Sin1 on translocation of Sty1 and phosphorylation of Atf1. (**A**) Log phase cultures of wild-type (wt) (HM 1689) or *Δsin1* (JM 1809) cells bearing an integrated and nine myc epitope-tagged version of Sty1 growing in YES medium at 30°C (Control) were incubated in the same medium containing 0.5 M NaCl for the times indicated. Cells were fixed and probed by indirect immunofluorescence for the presence of the myc epitope (Sty1-9myc) or nuclei (DAPi) as described in Materials and methods. (**B**) Log phase cultures of wild-type (wt) (KS 1479) or *Δsin1* (JM 1852) cells both bearing an integrated and C-terminally tagged version of Atf1 containing His_6 and two HA epitopes growing in YES medium at 30°C were incubated in the same medium containing 0.5 M NaCl for the times indicated. Approximately 2×10^8 cells were harvested at each time point, lysed under denaturing conditions and the Atf1 protein precipitated using Ni^{2+}–NTA agarose. Precipitates were probed by Western blot for the presence of the HA-epitope tag (α-HA).

the HA epitope (Shiozaki and Russell, 1996). Previous results have shown that this shift is due to phosphorylation and is entirely dependent on the Sty1 MAP kinase (Shiozaki and Russell, 1996). Treatment of wild-type cells with osmotic stress induces a rapid phosphorylation of Atf1 *in vivo* which is retained for up to an hour, as previously observed. The level of Atf1 protein also increases at later times, probably because Atf1 stimulates its own expression (Degols and Russell, 1997). In the absence of Sin1, Atf1 undergoes a rapid stress-dependent phosphorylation, the extent of which is notably less than that observed in wild-type cells (Figure 6B). This is consistent with our observation that a proportion of Sty1 does enter the nucleus. More strikingly, the level of Atf1 protein rapidly decreases and no increase in the level of the protein is observed after prolonged incubation (Figure 6B). We presume this latter result is because *Δsin1* cells do not support Atf1-dependent transcription. By indirect immunofluorescence Atf1 protein is still observed in the nucleus in the absence of Sin1 but to a some what lesser intensity (data not shown). The steady-state level and full stress-induced phosphorylation of Atf1 were restored when an episomal plasmid expressing *sin1*, but not empty vector, was reintroduced to *Δsin1* cells (data not shown). These results suggest that Sin1 is required both for full phosphorylation and stability of Atf1 after stimulation of cells by environmental stress. This is likely to account for the requirement for Sin1 in Atf1-dependent gene transcription.

Sin1 controls Pap1-dependent gene transcription but not nuclear entry

To investigate the role of Sin1 in Pap1-dependent transcription the induction of the *ctt1* and *trr1* genes was investigated in cells lacking Sin1. In response to either heat shock or oxidative stress, induction of catalase (*ctt1*) and thioredoxin reductase (*trr1*) is dependent on the Pap1 transcription factor. As the results in Figure 7 indicate, rapid induction of *ctt1* after either heat shock or oxidative stress was severely compromised in the absence of Sin1, although the message eventually accumulated to near wild-type levels after prolonged exposure (Figure 7A and B). Induction of *trr1* under the same conditions was virtually abolished (Figure 7A and B). Previous results have suggested that Sty1 controls transcriptional activation of Pap1 by controlling its nuclear translocation (Toone *et al*., 1998). In contrast, we find that loss of Sin1 does not block the nuclear accumulation of ectopically expressed GFP–pap1 fusion protein (Figure 7C). These results indicate that Sin1 is also required for the induction of both Pap1-dependent gene transcription in fission yeast, by a mechanism that is distinct from control of Pap1 nuclear entry.

Sin1 is functionally conserved

Since many of the components of the fission yeast SAPK pathway are conserved in metazoans, we next tested whether the structural homologues of Sin1 from either mammalian or avian origin could substitute for Sin1 in fission yeast. Various full-length, truncated or fusion proteins between fission yeast and chicken *sin1* sequences were constructed and cloned behind the thiamine repressible *nmt1* promoter. The ability of these constructs

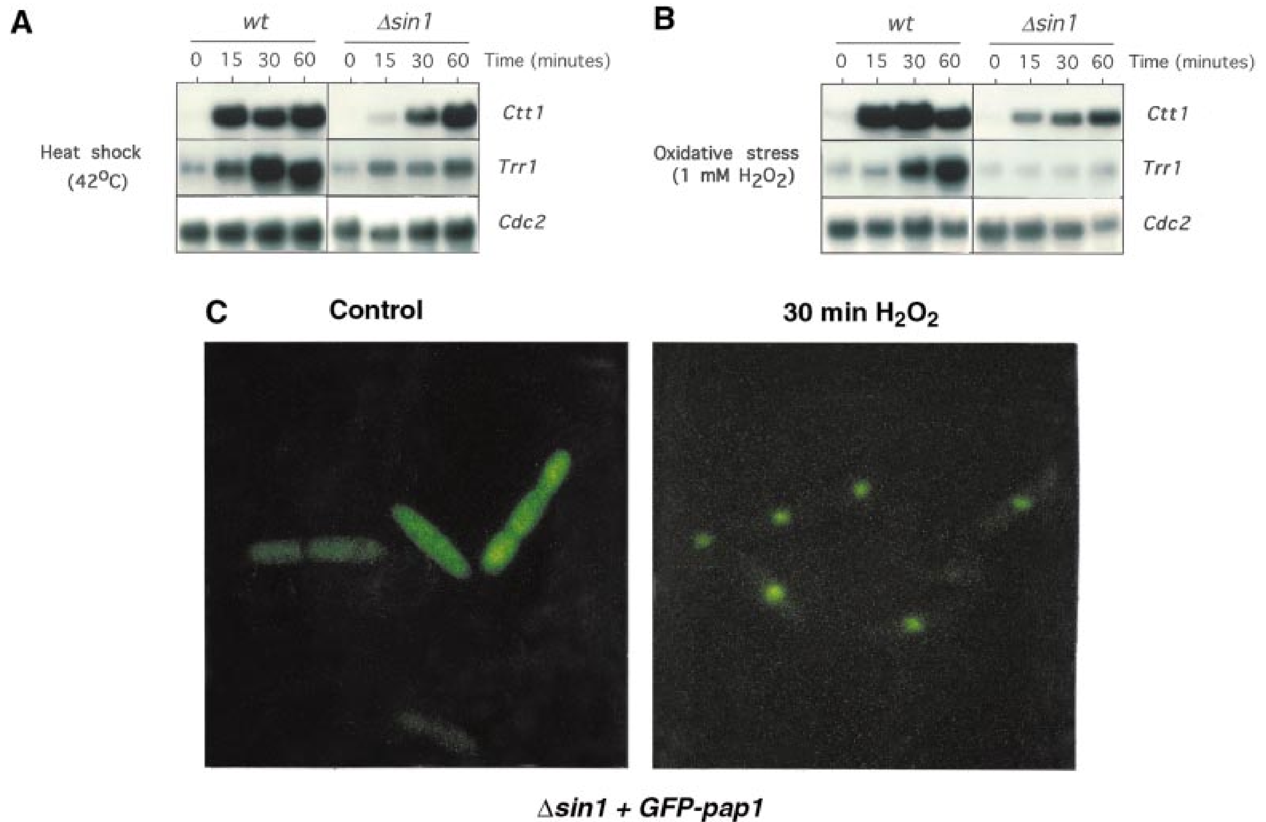

Fig. 7. Sin1 controls Pap1-dependent gene transcription but not its nuclear translocation. Sin1 is required for rapid Pap1-dependent transcription. Log-phase cultures growing in YES at 30°C of either wild-type (JM1059) or *Δsin1* cells (JM1797) cells were incubated in the same medium containing either (**A**) 1 mM H_2O_2 or (**B**) shifted to 42°C for the times indicated. Total RNA was extracted and equal quantities were separated by electrophoresis. Northern blots were then sequentially probed using DNA specific to the *ctt1*, *trr1* and *cdc2* genes. (**C**) Sin1 is not required for Pap1 nuclear translocation. *Δsin1* cells (JM1797) transformed with pREP41-GFP–pap1 (Toone *et al*., 1998) were grown to log-phase in minimal medium lacking thiamine and leucine. Localization of Pap1 was assessed by direct immunofluorescence as detailed in Materials and methods before (Control) and after addition of 1 mM H_2O_2 for 30 min (30 min).

to suppress the temperature sensitivity of *Δsin1* cells was then assessed. Whereas the full-length fission yeast gene [*S.pombe* Sin1(1–650)] could rescue the defect of *Δsin1* cells at high temperature, a construct expressing only the first 486 amino acids [*S.pombe* Sin1 (1–486)] could not, indicating that the C-terminal 164 amino acids are essential for Sin1 function *in vivo* (Figure 8). Surprisingly, fusion of the first 486 amino acids of the *S.pombe* Sin1 to the C-terminal 182 amino acids of the chicken Sin1 sequence restored the ability of *Δsin1* cells to grow at high temperature, indicating that the C-terminal domain of fission yeast and chicken sequences are functionally related (Figure 8). Notably we isolated a clone by the two-hybrid screen that expresses only the C-terminal 244 amino acids of Sin1, suggesting that this region is the presumptive binding domain for Sty1. Full-length chicken Sin1 was also able to suppress the temperature sensitivity of a *Δsin1* strain, although in this case the rescue was very poor. We note that the fission yeast sequence contains additional amino acids between residues 419 and 490 that are not represented in either human or chicken sequences, which may be important for functional rescue. Together these data suggest that Sin1 is a member of a new class of evolutionarily conserved signal transducer.

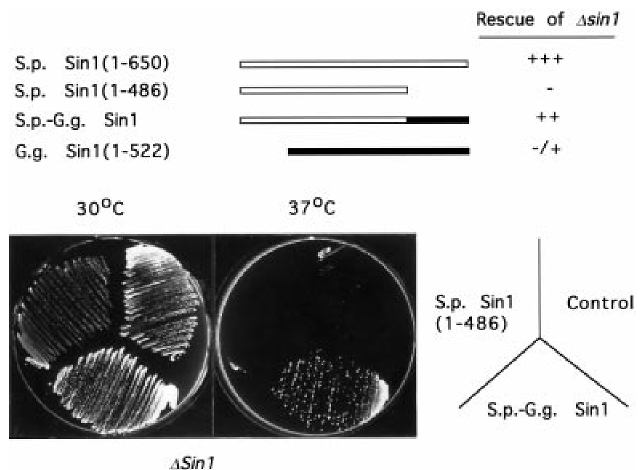

Fig. 8. Functional conservation of Sin1. Diagrammatic representation of full-length and chimaeric fusion proteins from *S.pombe* and *Gallus gallus sin1* sequences. *Δsin1* cells (JM 1852) were transformed with either pREP41 (Control), pREP41-Sin1(*Δ*C) expressing only the first 486 amino acids of fission yeast Sin1 [S.p. Sin1 (1–486)] or pREP41-Sin1 (S.p.–G.g.) expressing a chimaeric fusion protein between fission yeast and chicken sequences (S.p.–G.g. Sin1). Cells were grown on EMM plates lacking leucine at 30°C and then streaked onto the same plates at either 30 or 37°C for 2 days.

Discussion

Here we have identified Sin1 as a new component of the fission yeast stress-activated Sty1 MAP kinase pathway. This conclusion is based on the following observations. First, Sin1 binds specifically to the Sty1 MAP kinase *in vivo* and is phosphorylated after environmental stress. Secondly, cells lacking Sin1 display many of the phenotypes of cells lacking Sty1 including stress sensitivity, sterility and cell-cycle delay. Lastly, Sin1 is required for the induction of a number of genes including *pyp2*, *gpd1*, *ctt1* and *trr1* which are under the control of both the Sty1 MAP kinase and its targets, the Atf1 and Pap1 transcription factors.

Although stress-activated and Sty1-dependent gene transcription is defective in the absence of Sin1, activation of Sty1 kinase, as judged by appearance of the doubly phosphorylated form of Sty1, appears to be normal. This distinguishes Sin1 from recently identified regulators of MAP kinases in mammalian cells such as JIP-1 or MP1, which act as scaffold proteins that support the integrity of the pre-activation MAPK complex (Dickens *et al*., 1997; Schaeffer *et al*., 1998; Whitmarsh *et al*., 1998). Indeed Sin1 does not interact with other components of the Sty1 MAP kinase pathway, including the Wis1 MAPKK and Wak1 MAPKKK. Rather, it places Sin1 function between MAP kinase activation and stimulation of gene transcription. Since Sty1, like many MAP kinases, is cytoplasmic in unstimulated cells and translocates to the nucleus after activation, we considered the possibility that Sin1 may be required for translocation of the doubly phosphorylated MAP kinase to the nucleus. Indirect immunocytochemical analysis using an integrated epitope-tagged Sty1 suggests that this is not the case. This observation is consistent with the fact that, in the absence of Sin1, Sty1 is still able to phosphorylate constitutively nuclear Atf1. However, neither the intensity of nuclear accumulation nor Sty1-dependent Atf1 phosphorylation are as pronounced as in wild-type cells. This suggests that Sin1 may facilitate, but is not absolutely required for, the transport process. It will be highly informative, in this regard, to identify additional proteins that bind Sin1, its requirements for association with Sty1 and its cellular location before and after challenge by environmental stress. These experiments are now underway.

We observe that Atf1 is not only under-phosphorylated in the absence of Sin1 but rapidly degraded. Importantly, recent evidence from mammalian cells has implicated protein stability of SAPK targets as a means of controlling stress-dependent stimulation of gene transcription (Fuchs *et al*., 1998). The ability of JNK to phosphorylate the c-Jun transcription factor requires its association with the delta domain, which is positioned between amino acids 30–57 in the N-terminus. Association of JNK to this domain also controls the proteolytic degradation of c-Jun through the 26S proteasome, although, importantly in this case, JNK does not need to be active as a kinase (Treier *et al*., 1994; Fuchs *et al*., 1996; Musti *et al*., 1997). This lead researchers to find that JNK also targets ATF2 for ubiquitination by a similar process (Fuchs *et al*., 1997). Although a number of phosphorylation sites for Sty1 have been identified on the Atf1 protein, the function of these phosphorylations is not understood (N.Jones, personal communication). It is conceivable that deregulated entry of Sty1 to the nucleus may lead to incomplete Atf1 phosphorylation and subsequent degradation. Alternatively, Sin1 may function to protect phosphorylated Atf1 from ubiquitination. We are currently attempting to distinguish between these possibilities.

Our results indicate that Sin1 is also partly required for Pap1-dependent transcription. The regulation of Pap1 is distinct from that of Atf1, in that Pap1 is not an *in vitro* target for Sty1 MAP kinase (Toone *et al*., 1998). Instead translocation of cytoplasmic Pap1 to the nucleus is thought to be the primary mechanism by which Pap1-dependent transcription is controlled by Sty1. One caveat to this is that translocation of Pap1 appears to be too slow to fully account for the rapid induction of gene expression (Toone *et al*., 1998). We find that in the absence of Sin1, rapid induction of Pap1-dependent transcription of *ctt1* is defective, but this eventually recovers after prolonged incubation as Pap1 is translocated to the nucleus. It is conceivable that a small nuclear pool of Pap1 is rapidly activated by Sty1 after exposure to stress and subsequently reinforced by a Sin1-independent bulk import of Pap1 from the cytoplasm at later times. Regardless, the eventual increase in *ctt1* expression explains why *sin1*-deletes are able to proliferate under conditions of prolonged exposure to oxidative stresses such as heavy metals and hydrogen peroxide. It is important to point out that cells lacking Sin1, like *Δsty1* cells, are both temperature sensitive and delayed in the timing of mitotic initiation whereas cells lacking both Atf1 and Pap1 are not. This suggests that Sin1 controls processes in fission yeast other than those regulated by Atf1 and Pap1, including control of cell-cycle progression. The lack of known structural motifs in the Sin1 protein at present prevents further speculation as to its function. Importantly, however, Sin1 itself is a phosphoprotein that is hyperphosphorylated after environmental stress but not a substrate of Sty1, suggesting Sin1 may act at a focal point between the Sty1 pathway and other stress-activated kinases.

In conclusion, here we have presented data which establishes Sin1 as a component of the stress-activated Sty1/Spc1 MAP kinase pathway in fission yeast. Since the fission yeast Sty1 pathway displays many of the characteristics of the JNK and p38 MAP kinase pathways in mammals, a prediction from our results is that Sin1 homologues play a central role in stress-activated gene transcription by SAPKs in other species. Future work will be aimed at testing this hypothesis.

Materials and methods

Media and general techniques

Media and genetic methods for studying fission yeast have been reviewed (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× magnification. Transformations were regularly performed by electroporation (Prentice, 1991) using a Bio-Rad Gene Pulser^{TM}.

Assessment of mating efficiency

Homothallic (h^{90}) cells were grown to log-phase for two days in liquid Edinburgh minimal medium (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 spore-containing asci or cells undergoing sexual conjugation.

Two-hybrid screening and β-galactosidase assays

The full-length *sty1* ORF was fused to the 3′ end of the GAL4 DNA-binding domain by digesting pREP41-sty1(HA6His) (Millar *et al*., 1995) with *Nde*I and *Bam*HI and cloning the fragment into the *Nde*I and *Bam*HI sites of pAS2ΔΔ-BC to construct pAS2ΔΔ-BC-sty1. An *S.pombe* cDNA library (Durfee *et al*., 1993) was screened using an improved mating strategy (Fromont-Racine *et al*., 1997). We obtained 32 clones, positive for *HIS3* and *LacZ* expression from 2×10^6 diploid colonies. Interacting proteins were identified by automated DNA sequencing. Quantification of interactions was performed by assessing β-galactosidase activity in cell extracts as previously described (Buck *et al*., 1995).

Identification and sequencing of fission yeast Sin1

An *Xho*I fragment from pACT-Sin1 containing a partial *sin1* cDNA was used to probe an *S.pombe* pURB1 genomic library (Barbet *et al*., 1992). Two positive clones with overlapping inserts were isolated and designated pURB1-Sin1-1 and pURB1-Sin1-2. Sequencing of the partial cDNAs from pACT-Sin1 and the two genomic clones revealed an ORF of 1953 bp, interrupted by a small intron located close to the termination codon. Dideoxy sequencing was performed using a T7 sequencing kit from Pharmacia.

Overexpression and tagging of fission yeast Sin1

The plasmid pURB1-Sin1-1 was digested with *Sac*I and *Sph*I and the insert cloned into pIRT2 to form pIRT2-Sin1. The *sin1* ORF was amplified from the genomic clone pIRT2-Sin1 by PCR amplification. The 5′ oligonucleotide TTA*CCATGG*AT*CATATG*CAATATTCTCAT-ATATACTTC (Sin1-N1) incorporating *Nco*I and *Nde*I sites (shown italicized) hybridized to sequences surrounding the ATG initiation codon, whereas the 3′ oligonucleotide ACAT*GGATCC*ACCTATGTATTCATA-GAA (Sin1-REPC2) incorporating a *Bam*HI site (shown italicized) hybridized to sequences 3′ to the TGA termination codon. The PCR product was cleaved with *Nde*I and *Bam*HI and cloned into pREP41-3HA to form pREP41-3HA-Sin1 which expresses a 3HA-tagged *sin1* gene under the control of a partially defective version of the *nmt1* thiamine repressible promoter (Basi *et al*., 1993). The same fragment was cloned into pREP41 to form pREP41-Sin1. A truncated clone of Sin1 expressing only the first 486 amino acids of Sin1 was constructed in a similar manner by PCR using the 5′ oligonucleotide Sin1-N1 and the 3′ oligonucleotide TCTTATGGATCCTTAACTAAATGTTTTAT-CAAGTGG (Sin1-ΔC). The resulting 1.5 kb fragment was digested with *Nde*I and *Bam*HI and cloned into pREP41 to form pREP41-Sin1(ΔC). Plasmids were used to transformed strains bearing the *leu1-32* mutation and leucine prototrophs selected. Stable overexpression was reached after at least 48 h growth in the absence of thiamine.

Disruption of fission yeast Sin1 sequences

The plasmid pCRII-ura4 was digested with *Xho*I and *Spe*I to release a 1.6 kb fragment that was cloned into pIRT2-Sin1 which had been digested with *Xho*I and *Spe*I to form *pIRT2-Sin1::ura4*. *pIRT2-Sin1::ura4* was digested with *Sac*I and *Sph*I and transformed into a *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M216/ade6-M210* h^+/h^- diploid strain. Sporulation and tetrad dissection of stable heterozygous diploids produced *ura4*$^+$ haploids, demonstrating that *sin1* is not an essential gene. Uracil prototrophs contained a disruption of the s*in1* gene as verified by PCR and Southern hybridization (data not shown).

Identification and sequencing of chicken Sin1

Chicken Sin1 was isolated from a cDNA library constructed from stage 10–12 (Hamburger and Hamilton, 1992) chicken embryo hindbrains using the SuperscriptII System for cDNA Synthesis and Plasmid Cloning (Gibco-BRL) (J.Christiansen and D.G.Wilkinson, in preparation). Random clones were then chosen and used to make antisense RNA probes for use in whole-mount *in situ* hybridizations (Xu and Wilkinson, 1998). Sin1 was identified as being segmentally expressed in the hindbrain and was sequenced from both the 5′ and 3′ ends using the M13-reverse and M13-universal primers in conjunction with a Big Dye terminator kit and a 377 automated DNA Sequencer (Applied Biosystems). Sequences were analysed for putative ORFs using the MacVector 5.1 package (Oxford Molecular). Nucleic acid and protein sequence similarity searches were performed using the gapped BLAST algorithm (Altschul *et al*., 1997) accessed via the internet at http://www.ncbi.nlm.nih.gov.

Overexpression of chicken Sin1 and chimeric Sin1 fusion proteins

The chicken Sin1 cDNA was amplified from pSPORT1-Sin1 (G.g.) using by PCR using the 5′ oligonucleotide TACCTA*CATATG*GCTTT-CCTGGACAACCC (Sin1-GGF) incorporating an *Nde*I site (shown italicized) which hybridized to sequences surrounding the ATG initiation codon, whereas the 3′ oligonucleotide TTAATC*GGATCC*TCACT-GCTGTCCCGATTTCTT (Sin1-GGC) incorporating a *Bam*HI site (shown italicized) hybridized to sequences 3′ to the TGA termination codon. The PCR product was cleaved with *Nde*I and *Bam*HI and cloned into pREP41 to form pREP41-Sin1(G.g.). A chimaeric fusion protein of the first 486 amino acids of the fission yeast Sin1 with the last 186 amino acids of chicken Sin1 was constructed by combined PCR using the 5′ oligonucleotides Sin1-N1 and the 3′ oligonucleotide CTGGCTCT-CCAAGGTACTAAATGTTTTATCAAGTGG (Sin1-CP2) and the 5′ oligonucleotide CCACTTGATAAAACATTTAGTACCTTGGAG-AGCCAG (Sin1-CP1) in combination with the 3′ oligonucleotide Sin1-GGC. Products from each reaction were then combined and a second round of amplification performed using the oligonucleotides Sin1-N1 and Sin1-GGC. The resulting product was cleaved with *Nde*I and *Bam*HI and cloned into pREP41 to form pREP41-Sin1 (S.p.-G.g.). Plasmids were used to transformed strains bearing the *leu1-32* mutation and leucine prototrophs selected. Stable overexpression was reached after at least 48 h growth in the absence of thiamine.

Immunocytochemical analysis of Sty1 location

A tandem 9 myc epitope was amplified by PCR from the plasmid pC3280 (a gift of K.Nasmyth) using the 5′ oligonucleotide GAAAAA-GG*GCGGCCGC*ATGGTTCAC and the 3′ oligonucleotide ATATA-TAT*GCGGCCGC*CTTATGTCGGCATATTCGAG bearing *Not*I sites (italicized). The resulting PCR fragment was cloned into pBSSK-Ura4-Sty1(6HisHA) (Shieh *et al*., 1998) that had been digested with *Not*I to form pBSSK-Ura4-Sty1(9myc). pBSSK-Ura4-Sty1(9myc) was linearized with *Pac*I and the resulting fragment transformed into wild-type *S.pombe* cells bearing the *ura4-D18* auxotrophic marker. Stable integration of the tagged *sty1* gene at the genomic *sty1* locus was confirmed by Southern blot analysis and PCR. Indirect immunofluorescence microscopy and DAPi staining were performed by methanol fixation as previously described (Alfa *et al*., 1993). A monoclonal antibody to the myc epitope (9E10) and a rhodamine-conjugated antimouse antibody were used to detect Sty1 location.

Fluorescence microscopy

Indirect fluorescence microscopy was carried out on an Olympus IX70 inverted microscope with phase contrast and DIC optics and observed using a Photometrics CH350L liquid cooled CCD camera and Deltavision deconvolution software. Images were manipulated using PhotoShop. Preparation and detection of GFP fluorescence was performed essentially as previously described (Toone *et al*., 1998). Fluorescence was observed on a Zeiss Axiophot and images were captured on standard 35 mm film.

Detection of activated Sty1 protein

The Sty1 protein was partially purified from cells expressing Sty1 fused to a HA peptide epitope and a His_6 C-terminal tail. Briefly, pelleted cells were lysed into denaturing lysis buffer [1% nonident P40 (NP40), 6 M GuHCl pH 8.0] 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 a polyclonal antibody to the phosphorylated form of p38 (New England Biolabs). Detection was performed using a peroxidase-conjugated anti-mouse IgGs (Amersham, UK) and chemiluminescence visualization (ECL, Amersham, UK) according to the manufacturer's instructions.

Association of Sty1 and Sin1 in vivo

Wild-type cells or cells expressing Sty1 fused to a HA peptide epitope and a His_6 C-terminal tail were transformed with pREP41-3HA-Sin1. Pelleted cells were lysed into lysis buffer (0.05% NP40, 50 mM NaCl, 50 mM NaF, 10% glycerol, 2 mM Na-orthovanadate, 10 mM β-mercaptoethanol, 10 μg/ml aprotonin, 10 μg/ml benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 50 mM Tris–HCl pH 7.4) and proteins partially purified by affinity precipitation on Ni^{2+}–NTA beads (Quiagen). Precipitated proteins were resolved by SDS–PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were probed with a monoclonal antibody to the HA epitope (12CA5). Detection was performed as above.

DNA and RNA isolation and hybridization

Schizosaccharomyces pombe cells were cultured in YES medium (0.5% yeast extract, 2% glucose, 50 mg/l adenine) to stationary phase. Chromo-

somal 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 hybridization. To isolate RNA, *S.pombe* cells were cultured in YES to exponentially growing phase. Approximately 10 μg of total RNA was isolated and resolved by agarose gel electrophoresis before transfer to nitrocellulose for hybridization as previously described (Shieh *et al*., 1997). Probes for *pyp2*, *cdc2*, *ctt1*, *trr1* and *gpd1* were as previously described (Wilkinson *et al*., 1996; Toone *et al*., 1998).

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