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The Srk1 Protein Kinase Is a Target for the Sty1 Stress-activated MAPK in Fission Yeast*

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Deborah A. Smith‡, W. Mark Toone§, Dongrong Chen§¶, Jürg Bähler¶, Nic Jones§, Brian A. Morgan‡, and Janet Quinn‡‖

From the ‡School of Biochemistry and Genetics, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, United Kingdom, the §Cancer Research-UK Cell Regulation Group, Paterson Institute for Cancer Research, Christie Hospital National Health Service Trust, Manchester M20 4BX, United Kingdom, and the ¶Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom

The fission yeast stress-activated Sty1/Spc1 MAPK pathway responds to a similar range of stresses as do the mammalian p38 and SAPK/JNK MAPK pathways. In addition, *sty1*$^-$ cells are sterile and exhibit a G_2 cell cycle delay, indicating additional roles of Sty1 in meiosis and cell cycle progression. To identify novel proteins involved in stress responses, a microarray analysis of the *Schizosaccharomyces pombe* genome was performed to find genes that are up-regulated following exposure to stress in a Sty1-dependent manner. One such gene identified, *srk1*$^+$ (Sty1-regulated kinase 1), encodes a putative serine/threonine kinase homologous to mammalian calmodulin kinases. At the C terminus of Srk1 is a putative MAPK binding motif similar to that in the p38 substrates, MAPK-activated protein kinases 2 and 3. Indeed, we find that Srk1 is present in a complex with the Sty1 MAPK and is directly phosphorylated by Sty1. Furthermore, upon stress, Srk1 translocates from the cytoplasm to the nucleus in a process that is dependent on the Sty1 MAPK. Finally, we show that Srk1 has a role in regulating meiosis in fission yeast; following nitrogen limitation, *srk1*$^-$ cells enter meiosis significantly faster than wild-type cells and overexpression of *srk1*$^+$ inhibits the nitrogen starvation-induced arrest in G_1.

Central to stress responses in eukaryotes are the stress-activated MAPK[1] signaling pathways, which relay signals from the membrane or the cytoplasm to the nucleus to elicit necessary changes in gene expression (1). Similar to the mammalian p38 and SAPK/JNK stress-activated MAPK signaling pathways, the Sty1 (Spc1) MAPK pathway in the fission yeast *Schizosaccharomyces pombe* responds to a wide range of stimuli including oxidative stress, osmotic stress, and heat stress, nutrient limitation, and heavy metal toxicity and to DNA damaging agents such as UV light (2–6). *sty1*$^-$ cells are sensitive to these stress conditions and are also profoundly sterile, as nitrogen limitation induced-activation of Sty1 is important for sexual development (7). Interestingly, *sty1*$^-$ cells also display a G_2 cell cycle delay, which is intensified by stress (2, 3, 8); more recently, Sty1 has been implicated in a mitotic checkpoint that ensures proper spindle orientation in response to damage to the actin cytoskeleton (9).

Sty1 is activated through phosphorylation on conserved tyrosine (Tyr-173) and threonine (Thr-171) residues by the MAPKK, Wis1, which in turn is activated through phosphorylation by two MAPKK kinases Wak1 (also known as Wis4 and Wik1) (6, 10, 11) and Win1 (12, 13). Activation of Sty1 is transient because of the action of the Pyp1 and Pyp2 phosphotyrosine-specific phosphatases that dephosphorylate and inactivate the MAPK (2, 4, 10, 14).

Recent studies have identified mechanisms involved in the relay of stress signals to the MAPK module. In particular, heat shock-induced activation of Sty1 is regulated via inhibition of the Pyp1 phosphatase (15), whereas oxidative stress signals are relayed to the Sty1 pathway through a "two-component" phosphorelay system, which comprises the peroxide-sensing histidine kinases Mak2 and Mak3, the phosphorelay protein Mpr1, and the response regulator protein Mcs4 (6, 11, 16, 17). The mechanisms by which cells sense and regulate Sty1 activation in response to other stresses such as osmotic stress, nutrient limitation, UV irradiation, or DNA damage are unknown.

Stress-dependent activation of Sty1 stimulates gene transcription via two bZip transcription factors, Pap1 and Atf1 (16, 18–23). Atf1 is phosphorylated by Sty1 in response to stress, and, although Pap1 does not appear to be a direct target of Sty1, H_2O_2-induced nuclear accumulation of Pap1 is impaired in a *sty1*$^-$ mutant at all but very low concentrations of H_2O_2 (7, 20–22, 24). Analysis of the phenotypes of *pap1*$^-$ and *atf1*$^-$ mutants has suggested that Pap1 controls the response to a range of drugs and heavy metals, and low level oxidative stress (18, 20, 22, 24), and that Atf1 is important in regulating the response to osmotic stress, nutrient deprivation, and high levels of oxidative stress (16, 19, 21, 23, 24). However, although there is considerable overlap in the phenotypes of the *pap1*$^-$ and *atf1*$^-$ mutants with the *sty1*$^-$ mutant, several phenotypes appear to be Sty1-specific including sensitivity to DNA damaging agents and the G_2 cell cycle delay. Hence, these data suggest that there are other downstream targets for Sty1.

In an attempt to identify novel stress regulatory proteins in fission yeast, a microarray analysis was performed to identify genes that are induced regulated following exposure to stress.[2] Genes encoding proteins with potential regulatory functions such as protein kinases were of particular interest. Here we

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‖ Supported by a Medical Research Council Career Development award. To whom correspondence should be addressed. Tel.: 44-191-2227444; Fax: 44-191-2227424; E-mail: janet.quinn@ncl.ac.uk.

[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; NTA, nitrilotriacetic acid; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKAP kinase, mitogen-activated protein kinase-activated protein kinase.

[2] D. Chen, W. M. Toone, J. Mata, R. Lynne, G. Burn, K. Kiuinen, A. Brazma, N. Jones, and J. Bähler, manuscript in preparation.

TABLE I
S. pombe strains used in this study (all strains are leu1–32 ura4-D18)

Strain	Genotype	Source or reference
972	*h*$^-$	Our stock
CHP428	*h*$^+$*his7–366 ade6–210*	Gift from Charlie Hoffman
CHP429	*h*$^-$*his7–366 ade6–216*	Gift from Charlie Hoffman
JM1160	*h*$^-$*sty1*::*ura4*$^+$	Ref. 2
JP76	*h*$^-$*srk1*::*ura4*$^+$	This study
JM1689	*h*$^-$*sty1(9myc):ura4*$^+$	Ref. 21
JP203	*h*$^-$*srk1(3Pk):ura4*$^+$	This study
JM1521	*h*$^+$*sty1(HA6His):ura4*$^+$	Ref. 13
JP229	*h*$^+$*srk1(3Pk):ura4*$^+$ *sty1(HA6His):ura4*$^+$	This study
JP116	*h*$^-$*srk1*::*ura4*$^+$ *sty1(HA6His):ura4*$^+$	This study
JP231	*h*$^-$*srk1*::*ura4*$^+$ *sty1(9myc):ura4*$^+$	This study
JM544	*h*$^-$*wis1*::*ura4*$^+$	Ref. 2
JP224	*h*$^+$*srk1(3Pk):ura4*$^+$ *sty1*::*ura4*$^+$	This study
JP226	*h*$^+$*srk1(3Pk):ura4*$^+$ *wis1*^{DD}*(12myc):ura4*$^+$	This study

describe the identification and characterization of *srk1*$^+$ (Sty1-regulated kinase 1), which encodes a putative serine/threonine kinase that is up-regulated following exposure to stress. This protein shows a high degree of homology to the Rck2 protein kinase in the budding yeast *Saccharomyces cerevisiae*, previously described as a suppressor of fission yeast checkpoint mutations (26). Recently, Rck2 has been shown to be a substrate for the stress-activated Hog1 MAPK in *S. cerevisiae* (27, 28). Here, we present evidence that Srk1 is a target for the Sty1 MAPK in fission yeast, and that the cellular localization of Srk1 is regulated by stress in a process that is dependent on the Sty1 MAPK. In addition, we find that Srk1 has a role in the Sty1-regulated process of sexual development.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—*S. pombe* strains (Table I) were grown at 30 °C in rich medium (YE5S) or in synthetic minimal medium (EMM2) as described previously (29, 30). To assess mating efficiency, *h*$^+$ and *h*$^-$ wild-type or *h*$^+$ and *h*$^-$ *srk1*$^-$ strains were incubated at 25 °C on EMM medium lacking a nitrogen source.

Microarray Analysis—Microarray analysis was performed as described elsewhere.[2] In brief, wild-type (972 *h*$^-$) or a congenic *sty1*$^-$ strain (972 *sty1*$^-$) were grown to mid-log (A_{600} 0.2) and subjected to an oxidative stress (0.5 mM H_2O_2), heat stress (sudden shift from 30 °C to 38 °C), or an osmotic stress (1 M sorbitol). Total RNA was extracted using a hot phenol method from 25-ml samples of culture taken before and 15 and 60 min after stress (31). Probe preparation and microarray construction were as described elsewhere.[2] Arrays were scanned using a GenePix 4000 scanner from Axon Instruments (Foster City, CA). All analysis was performed using GeneSpring data analysis software (Silicon Genetics, Redwood City, CA). All results are the average of at least two independent biological experiments.

RNA Analysis—Cells were grown to mid-log and untreated or stressed with H_2O_2 as indicated. RNA was extracted essentially as described in Ref. 31, and Northern blotting was performed with gene-specific probes (32). Hybridization conditions were as described in the GeneScreen protocol (NEN Life Sciences).

Disruption of srk1$^+$—A disruption cassette, comprising the *ura4*$^+$ gene flanked by 80 base pairs of DNA sequence corresponding to regions 5′ and 3′ of the *srk1*$^+$ open reading frame, was generated by PCR, and introduced into CHP429. Disruption of the *srk1*$^+$ gene was confirmed by PCR.

Chromosomal Tagging of srk1$^+$—A C-terminal 300 bp fragment of *srk1*$^+$ was ligated into pREP42Pk C (33), which had been digested with *Eco*RI to remove the *ars1* sequence (pRIP42Pk C). pRIP42Pk C allows the construction of protein fusions where the protein of interest is tagged at the C terminus with three copies of the Pk epitope. The pRIP42Pk C-Srk1 plasmid was linearized by digestion of the *Kpn*I site present in the *srk1*$^+$ sequence and introduced into CHP429. Chromosomal insertion of the C-terminal Pk tag was confirmed by PCR and DNA sequencing.

Plasmids—For expression in *S. pombe*, the *srk1*$^+$ gene was ligated into pREP41 (34), pREP41FLAG (kind gift from S. K. Whitehall) or pREP41HM N (33) to create pREP41-Srk1, pREP41FLAG-Srk1, or pREP41HM N-Srk1, respectively. For expression in *Escherichia coli*, the *srk1*$^+$ gene was amplified from a cDNA library and ligated into pET14 (Novagen). Mutagenesis of *srk1*$^+$ to create *srk1*^{K153A} was performed using overlapping PCR and confirmed by DNA sequencing.

Flow Cytometry—Cells were fixed in 70% ethanol at 0 °C. After resuspension in 50 mM sodium citrate buffer, cells were treated overnight at 37 °C with 0.1 mg/ml RNase A. Following this, cells were treated with 4 μg/ml propidium iodide for 2 h at room temperature to stain the DNA. Cells were then sonicated and analyzed on a BD PharMingen FACScan flow cytometer.

Expression and Purification of Recombinant Srk1—His_6-tagged Srk1^{K153A} was expressed in *E. coli* BL21 (DE3) cells (35) from pET14, and pellets were lysed in binding buffer (20 mM Tris, pH 7.9, 0.5 M NaCl, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride). His_6-tagged Srk1^{K153A} was purified using Ni^{2+}-NTA-agarose and, after washing with binding buffer containing 60 mM imidazole, was eluted with imidazole according to the instructions of the manufacturer (Qiagen).

Co-precipitation Assays—Chromosomally tagged *sty1–9myc* (JM1689) cells, containing either pREP41FLAG or pREP41FLAG-Srk1, were grown in minimal medium lacking thiamine for 20 h. Approximately 2×10^8 cells were harvested from unstressed cells, or following stress treatment as indicated, and snap frozen in liquid nitrogen. Pelleted cells were lysed into lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM imidazole, 0.1% Nonidet P-40, 50 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.07 TIU/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin). FLAG-tagged Srk1 was immunoprecipitated from extracts using anti-FLAG M2 affinity agarose (Sigma). The agarose beads were washed three times with lysis buffer and resuspended in SDS-loading buffer. Proteins were resolved on SDS-PAGE, and co-precipitation of Sty1–9myc was monitored by Western blotting using 9E10 monoclonal anti-Myc antibody (Sigma).

Cells carrying either or both chromosomally tagged *sty1–6HisHA* (6) and *srk1-Pk* were grown in rich medium and extracts prepared as above. Sty1–6HisHA was precipitated from the extracts using Ni^{2+}-NTA-agarose (Qiagen). The Ni^{2+}-NTA-agarose was washed five times in lysis buffer containing 20 mM imidazole. Proteins were resolved on SDS-PAGE, and co-precipitation of Srk1-Pk was assayed by Western blotting using an anti-Pk-TAG antibody (Serotec). In the co-precipitation experiment, phosphorylated Sty1 and total levels of Sty1 were determined as below.

Sty1 Phosphorylation Assays—Strains containing chromosomally tagged *sty1–6HisHA* (JM1521) (6) were grown to mid-log and incubated with 1 mM H_2O_2 for the times indicated. Detection of phosphorylated Sty1 was determined as described previously (2) by Western blotting with anti-phospho-p38 antibody (New England Biolabs). Total levels of Sty1 were determined with an anti-HA antibody (Sigma).

In Vivo Phosphorylation of Srk1—Phosphorylation of Srk1-Pk was monitored in wild-type (JP203) and *sty1*$^-$ (JP224) strains by Western blot analysis. Cells were collected before or after a 10-min exposure to 1 mM H_2O_2 and extracts prepared as before. Wild-type extracts were dephosphorylated by treatment with λ-phosphatase (400 units/μl, New England Biolabs) for 15 min at 37 °C. 100 μg of protein was analyzed by SDS-PAGE on 7.5% gels and Srk1-Pk visualized by Western blotting using an anti-Pk TAG antibody.

In Vitro Phosphorylation of Srk1—The immune-complex assay was performed as described previously (21) with the following modifications. Extracts were prepared from cells carrying chromosomally tagged *sty1–9myc* (JM1689), either before or after treatment with 1 mM H_2O_2 for 10 min. 300 μg of lysate was incubated with 3 μl of 9E10 monoclonal anti-Myc antibody for 1 h at 4 °C to which 20 μl of protein G-Sepharose (Amersham Biosciences) was added for an additional 1 h. The beads were collected and washed three times with lysis buffer (see above),

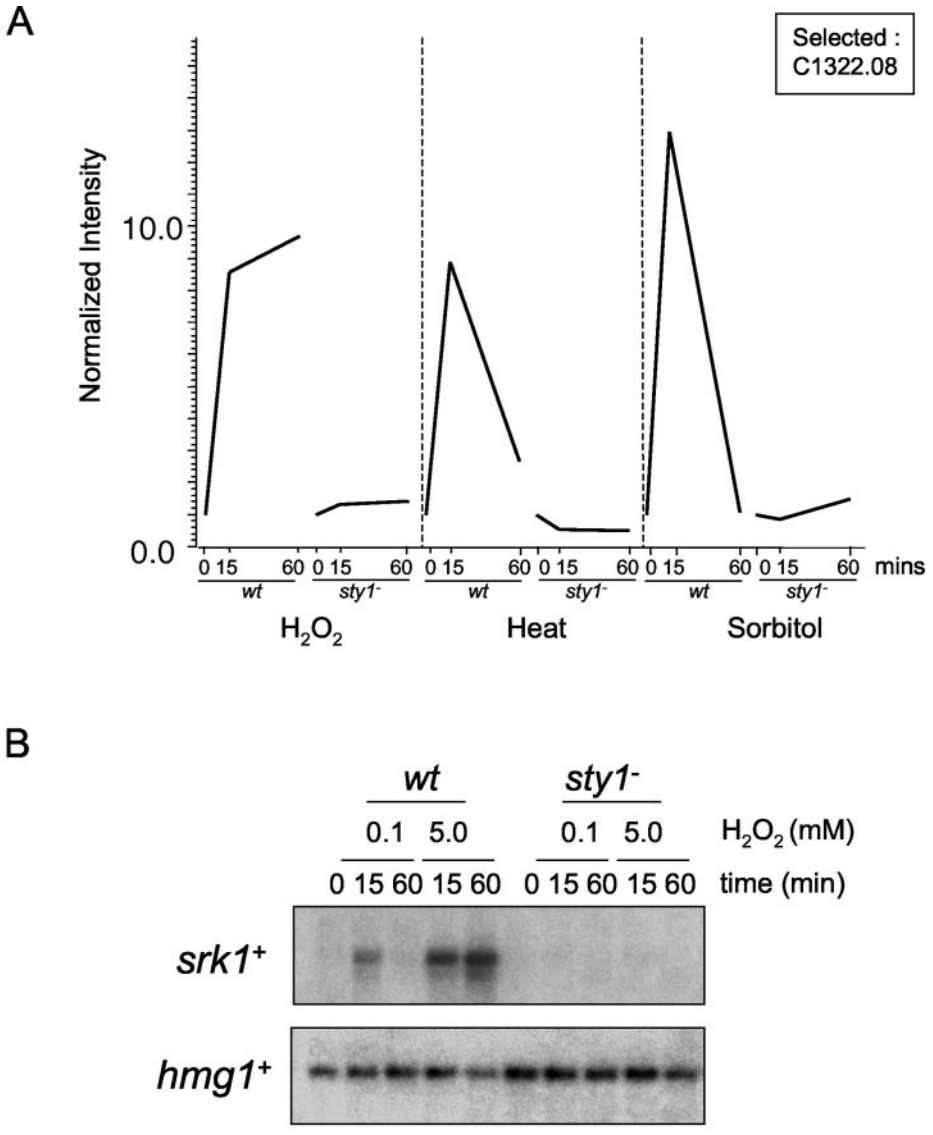

FIG. 1. ***srk1*$^+$ gene expression is induced in response to stress in a Sty1-dependent manner.** *A*, microarray data showing the expression of *srk1*$^+$ in wild-type and *sty1*$^-$ cells following treatment with H_2O_2 (0.5 mM), heat stress (sudden shift from 30 to 38 °C), or sorbitol (1.0 M). *B*, Northern blot analysis of RNA isolated from mid-log cultures of wild-type (*wt*), and *sty1*$^-$ mutant strains treated with both low (0.1 mM) and high (5 mM) concentrations of H_2O_2 for the times indicated with probes specific for *srk1*$^+$ or *hmg1*$^+$ (loading control).

then 5 μg of recombinant GST-Atf1 (21) or 6His-Srk1^{K153A}, immobilized on GSH-agarose or Ni^{2+}-NTA-agarose, respectively, was added and the beads washed once with kinase buffer (20 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 2 mM dithiothreitol, 20 μM ATP). The buffer was removed, and the beads were resuspended in 20 μl of kinase buffer to which 5 μCi of [γ-^{32}P]ATP was added. Kinase reactions were allowed to proceed for 30 min at 30 °C and stopped by the addition of SDS-sample buffer. Samples were analyzed by SDS-PAGE on 10% gels, transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA), and autoradiographed.

For the binding kinase assay, 300 μg of lysate isolated from wild-type (CHP429), *sty1*$^-$ (JM1160), or *wis1*$^-$ (JM544) strains, before and after treatment with 1 mM H_2O_2 for 10 min, was incubated with 5 μg of recombinant 6His-Srk1^{K153A} prebound to Ni^{2+}-NTA-agarose for 1 h at 4 °C. The beads were washed three times with lysis buffer, once with kinase buffer, and kinase reactions performed as described above.

Fluorescence Microscopy—Immunolocalization of 9myc-epitope tagged Sty1 and Pk-epitope tagged Srk1 was carried out essentially as described in Ref. 24. 10-ml samples of exponentially growing cells (A_{595} 0.5), untreated or treated with either 1 mM H_2O_2 or 0.6 M KCl for the indicated times, were collected and fixed in 3.7% formaldehyde. To examine Sty1-Myc and Srk1-Pk localization, cells were incubated overnight with a 1:1000 dilution of a monoclonal anti-Myc 9E10 antibody (Sigma) or with a 1:1000 dilution of an anti-Pk-TAG antibody, respectively. A 1:50 dilution of goat anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (Sigma) was added, and cells were incubated for 1 h at room temperature. Cells were then spread onto poly-L-lysine-coated slides and cover slips mounted onto slides using Vectashield mounting medium containing 1.5 mg/ml 4′-6-diamidino-2-phenylindole (Vector Laboratories). For fluorescence microscopy, 4′-6-diamidino-2-phenylindole and fluorescein isothiocyanate fluorescence was captured by exciting cells with 365-nm and 450–490-nm wavelengths, respectively, using a Zeiss Axioscope microscope, with a 63× oil immersion objective, and Axiovision imaging system.

RESULTS

srk1$^+$ *Expression Is Induced following Stress*—We identified *srk1*$^+$(c1322.08) during a microarray analysis designed to identify genes in the fission yeast genome, the expression of which changes following exposure to stress. As shown in Fig. 1*A*, *srk1*$^+$ mRNA is induced in response to a range of different stress stimuli. Exposure of wild-type cells to a heat shock or oxidative stress (0.5 mM H_2O_2) resulted in a 9–10 fold increase in *srk1*$^+$ levels, whereas osmotically stressing cells (1.0 M sorbitol) resulted in an ~13-fold induction in the levels of *srk1*$^+$. The stress-induced expression of *srk1*$^+$ is absolutely dependent on the stress-activated MAPK, Sty1. *srk1*$^+$ is one of only two genes encoding a putative kinase gene product to be up-regulated following stress. Northern blotting analysis was performed to confirm the microarray data. In agreement, *srk1*$^+$ expression is induced in a Sty1-dependent manner in response to both low and high concentrations of the oxidative stress agent, H_2O_2 (Fig. 1*B*).

The *srk1*$^+$ gene is located on chromosome III (cosmid c1322, accession no. AL035259) and encodes a putative serine-

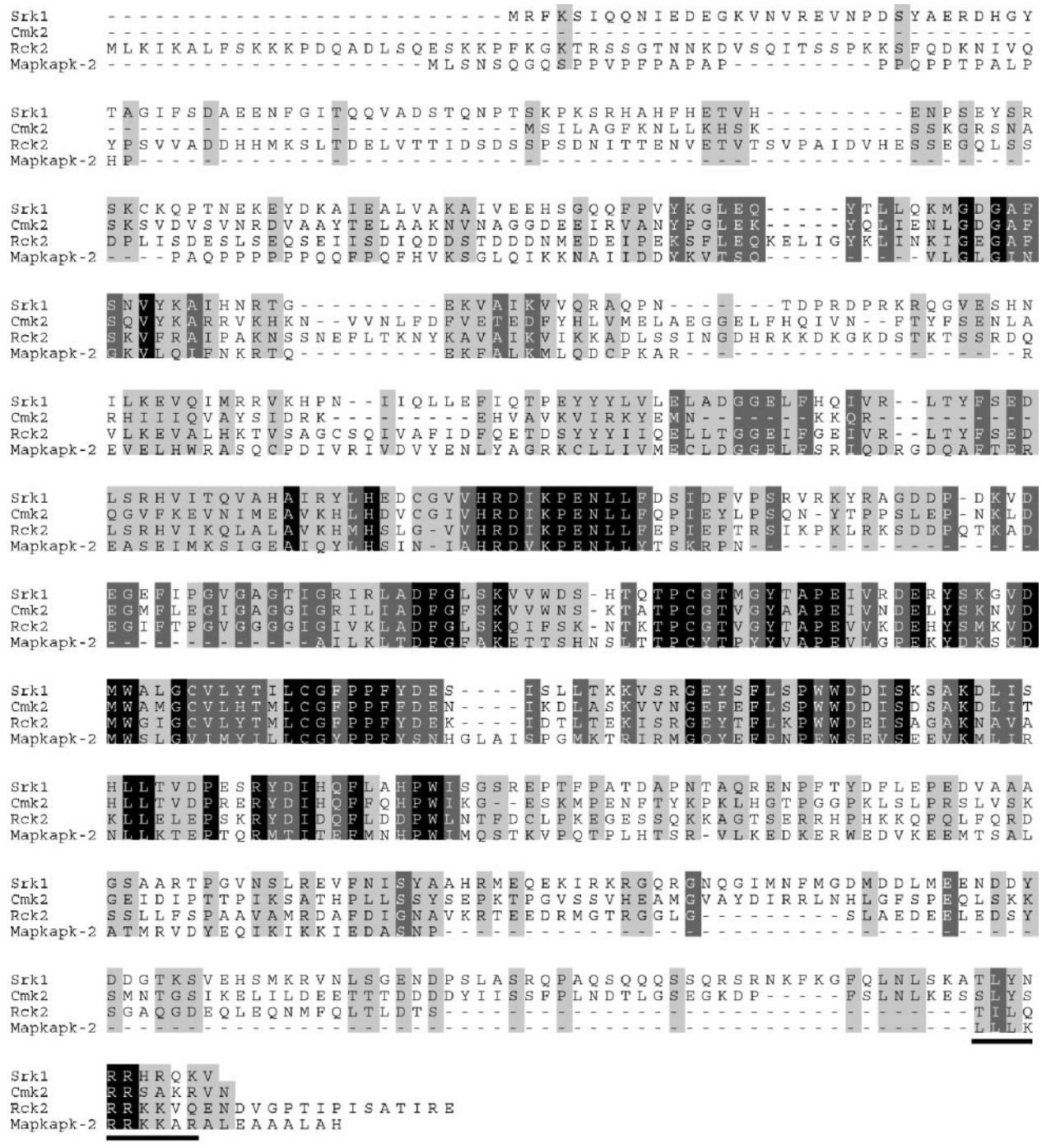

FIG. 2. **Sequence alignment.** The figure shows sequence comparison of Srk1 with fission yeast Cmk2, budding yeast Rck2, and mammalian MAPKAP kinase 2. Residues of Srk1 identical to those in any of the other proteins are indicated by *white* on *black letters*. *Dark gray* indicates identities of three of four residues. *Light gray* indicates identities of two of four residues. *Dashes* indicate single-residue gaps introduced to maximize the alignment. At the C terminus, the putative MAPK binding motif in Srk1, aligned with the MAPK binding motif in Rck2 and MAPKAP kinase 2, is *underlined*.

threonine kinase of 580 amino acids with a predicted molecular mass of 66 kDa. As shown in Fig. 2, a computer-based amino acid sequence comparison revealed that the greatest degree of amino acid sequence identity was shared with fission yeast Cmk2 (39%), and budding yeast Rck2/Clk1 (32%) (26). The kinase domains in all of these proteins share significant homology with the mammalian Ca^{2+}/calmodulin kinase superfamily, although the significance of this is unclear as extensive analysis of *S. cerevisiae* Rck2/Clk1 failed to uncover any regulation by calmodulin (36).

Significantly, Rck2 has been recently implicated in the stress response in *S. cerevisiae*, as Rck2 is a substrate for the osmo-stress-activated Hog1 MAPK (27, 28). Sequence alignment of Rck2 with MAPK-activated protein kinase (MAPKAP kinase) 2, which is phosphorylated by the mammalian p38 stress-activated MAPK, revealed the conservation of a MAPK-binding motif at the extreme C terminus (28). Comparison of Srk1, Rck2, and MAPKAP kinase 2 sequences revealed that a putative MAPK-binding motif is also present in the fission yeast Srk1 kinase (Fig. 2).

Collectively, the microarray data showing that *srk1*$^+$ is one of only two stress-induced kinase encoding genes in fission yeast,

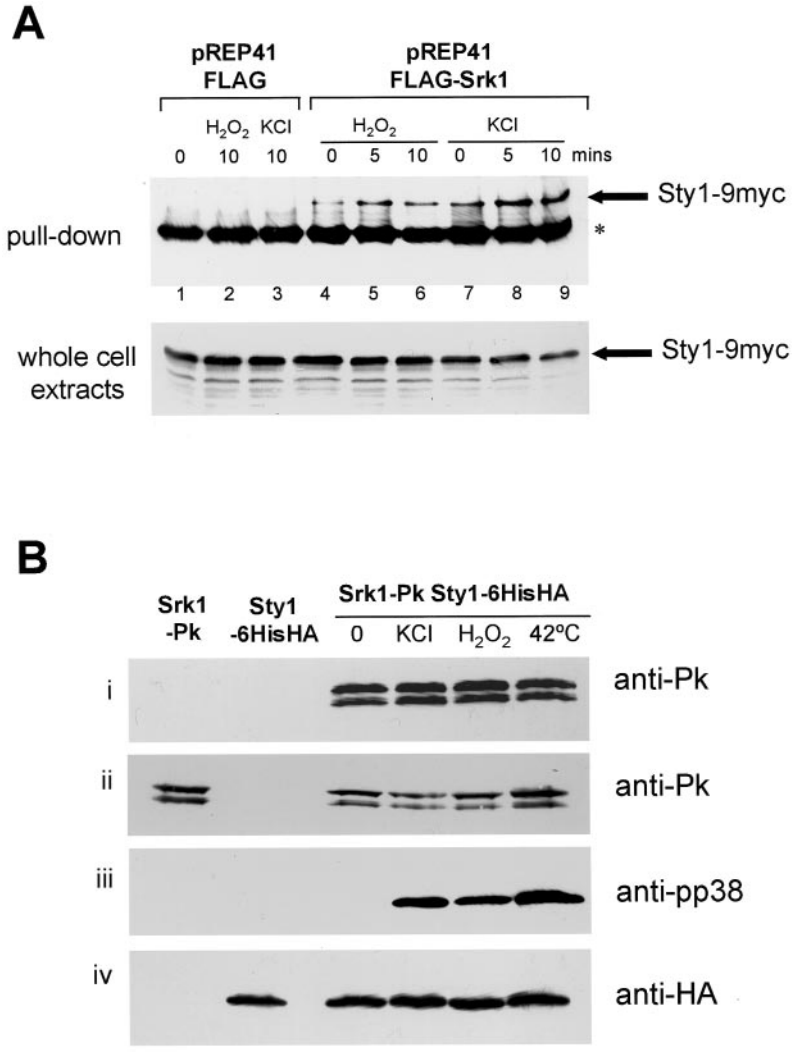

FIG. 3. **Srk1 interacts with the Sty1 MAPK *in vivo*.** *A*, Sty1 co-precipitates with Srk1. Extracts were prepared from mid-log cultures of cells (JM1689), expressing chromosomally tagged Sty1–9myc and containing either pREP41FLAG or pREP41FLAG-Srk1, before or after treatment with 1 mM H_2O_2 or 0.6 M KCl for the stated times. Proteins purified with anti-FLAG M2 antibody-coupled agarose were subjected to SDS-PAGE and the presence of Sty1 analyzed by Western blotting using a monoclonal anti-Myc antibody. Sty1–9myc was present only in precipitates from cells expressing pREP41FLAG-Srk1. The lower band seen (*) is the result of dissociation of the heavy chain from the immobilized anti-FLAG M2 antibody. Analysis of whole cell extracts (*lower panel*) shows that equal amounts of Sty1–9myc were present in all pull-down reactions. *B*, Srk1 co-precipitates with Sty1. Cells bearing a genomic copy of *sty1–6HisHA* or a genomic copy of *srk1-Pk*, or both tagged genes, were grown to mid-log phase and harvested before or after a 10-min exposure to 0.6 M KCl, 1 mM H_2O_2, or 42 °C as indicated. Whole cell extracts were prepared under native conditions and Sty1 purified using Ni^{2+}-NTA-agarose beads. After SDS-PAGE, the precipitates were analyzed by Western blotting. Srk1 was detected with anti-Pk epitope antibodies (*panel i*) and Sty1 with either anti-phospho-p38 antibodies (*anti-pp38*; that recognize the phosphorylated and therefore presumably active Sty1; *panel iii*) or anti-HA epitope antibodies (*panel iv*). Analysis of whole cell extracts shows that approximately equal amounts of Srk1-Pk were present in all pull-down reactions (*panel ii*).

together with the fact that Srk1 has a putative MAPK binding motif and that the *S. cerevisiae* homologue Rck2 is a component of the Hog1 stress-activated MAPK pathway, suggested that Srk1 may be linked with the activity of the Sty1 pathway.

Sty1 Interacts with the Srk1 Kinase—In *S. cerevisiae*, Rck2, a homologue of Srk1, binds to, and is a substrate for, the Hog1 MAPK (27, 28). To investigate whether the Srk1 kinase interacts with the Sty1 MAPK, Srk1 was N-terminally tagged with a FLAG epitope and expressed from the thiamine-repressible *nmt41* promoter in cells bearing a *sty1–9myc* allele (JM1689). Srk1 was immunoprecipitated from the cell using anti-FLAG antibody-coupled agarose beads, and co-precipitation of Sty1 was assayed by Western blotting using an anti-Myc antibody. Sty1–9myc co-precipitated with Srk1 in extracts prepared from unstressed cells and from cells that had been subjected to oxidative (1 mM H_2O_2) or osmotic (0.6 M KCl) stress (Fig. 3*A*, *lanes 4–9*). Sty1–9myc did not precipitate with anti-FLAG coupled agarose beads in the absence of FLAG-tagged Srk1 (Fig. 3*A*, *lanes 1–3*).

To ensure that the Srk1-Sty1 interaction shown above was not an artifact arising from overexpression of Srk1 from the *nmt* promoter, co-precipitation experiments were repeated using chromosomally tagged $srk1^+$ and $sty1^+$ alleles. A strain was constructed in which Sty1 was C-terminally tagged with six histidines and a hemagglutinin (HA) epitope and Srk1 was C-terminally tagged with three tandem Pk epitopes (JP229). Extracts were prepared from cells that were unstressed or that had been subjected to an osmotic (0.6 M KCl), oxidative (1 mM H_2O_2), or heat shock (42 °C) stress for 10 min. Complexes were purified on Ni^{2+}-NTA-agarose beads and visualized by Western blot using an anti-Pk antibody (Fig. 3*B*, *panel i*). Srk1-Pk specifically co-precipitated with tagged but not untagged Sty1. Approximately equal amounts of Srk1-Pk were present in all pull-down reactions, as shown by Western blotting of whole cell extracts (Fig. 3*B*, *panel ii*). Moreover, Srk1 interacted with Sty1 purified from unstressed cells as well as cells treated with a range of different stresses (Fig. 3*B*, *panel i*). The same blot was also probed with an anti-phospho-p38 antibody (*panel iii*) that recognizes only the active phosphorylated form of Sty1 (37), and, following stripping, with an anti-HA antibody to determine total amounts of Sty1 (*panel iv*). The anti-phospho-p38 Western blot shows that Sty1 was phosphorylated in the pull-down reactions using extracts prepared from stressed cells (*panel iii*), and the anti-HA Western blot confirms that equal amounts of Sty1 were present in the pull-down reactions. These results show that Srk1-Sty1 interact *in vivo* and that Srk1 can interact with both inactive (unphosphorylated) and active (phosphorylated) forms of Sty1.

Srk1 Is Not Required for Sty1 Activation or Nuclear Translocation—As Srk1 forms a complex with the Sty1 MAPK *in vivo*, we next asked whether Srk1 regulates Sty1 activation following stress. Wild-type cells (JM1521) and $srk1^-$ cells (JP116) containing a *sty1–6HisHA* allele were grown to mid-log phase and treated with 1 mM H_2O_2 to induce oxidative stress. Phosphorylation of Sty1 was monitored by Western blot using the anti-phospho-p38 antibody. No difference in the level or

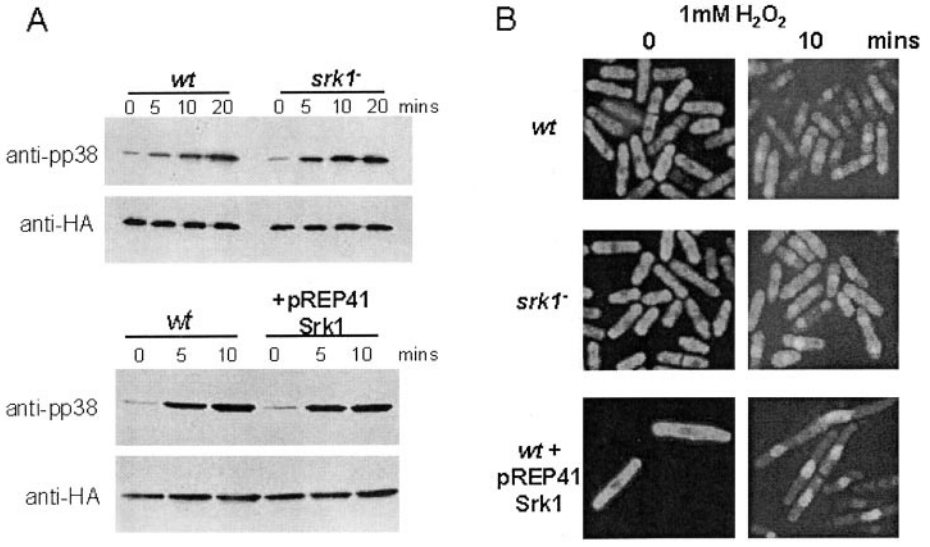

FIG. 4. **Srk1 is not required for Sty1 phosphorylation or nuclear translocation.** *A*, Sty1 phosphorylation is Srk1-independent. The panel shows a Western blot of Ni^{2+}-NTA-agarose-purified Sty1-6His-HA from wild-type (*wt*), *srk1*$^-$, or wild-type cells transformed with pREP41Srk1, following treatment with 1 mM H_2O_2 for the designated times, probed with an anti-phospho-p38 antibody (*anti-pp38*) or an anti-HA antibody (loading control). *B*, Sty1 nuclear translocation is Srk1-independent. Wild-type (*wt*), *srk1*$^-$ or *wt* cells transformed with pREP41Srk1, all carrying a genomic copy of *sty1–9myc*, were grown to mid-log and treated with 1 mM H_2O_2 for the times indicated. Cells were fixed and probed by indirect immunofluorescence for the presence of Sty1–9myc as described under "Experimental Procedures."

kinetics of Sty1-phosphorylation was seen in *srk1*$^-$ cells compared with wild-type cells (Fig. 4*A*). Additionally, no effect on the stability of Sty1 was seen in *srk1*$^-$ cells. Similar results were obtained following treatment with either a heat shock (42 °C) or osmotic stress (0.6 M KCl) (data not shown). We also examined whether expression of *srk1*$^+$ from the *nmt41* promoter had any affect on Sty1 activation or protein stability. Similar to the deletion of the *srk1*$^+$ gene, overexpression of Srk1 in wild-type cells (JM1521) had no affect on Sty1 activation, or protein stability, in response to oxidative stress (1 mM H_2O_2), osmotic stress (0.6 M KCl), or heat shock (42 °C) (Fig. 4*A*, and data not shown).

Styl is cytoplasmic in unstressed cells and translocates to the nucleus in response to stress (37). To assess whether Srk1 may be involved in this process, Sty1 tagged with 9myc epitopes was localized by indirect immunofluorescence in either a wild-type (JM1689) or a *srk1*$^-$ strain (JP231), or in a wild-type strain carrying pREP41-Srk1. In all these strains, Sty1 was found to be cytoplasmic in unstressed cells and concentrated in the nucleus after a 10-min exposure to 1 mM H_2O_2 (Fig. 4*B*). Similarly, Sty1 localization in response to a heat shock or osmotic stress was not affected by *srk1*$^+$ deletion or overexpression (data not shown). However, we did note that overexpression of *srk1*$^+$ from the *nmt41* promoter results in cells that are elongated at division compared with wild-type cells (see below).

Collectively, these results show that Srk1 does not regulate Sty1 activation. This, together with the fact that Hog1 phosphorylates Rck2 in budding yeast, raised the possibility that Srk1 acts downstream of the Sty1 MAPK and may be a direct substrate for the kinase.

Srk1 Is a Stress-induced Phosphoprotein—To investigate whether Srk1 is phosphorylated *in vivo*, the mobility of chromosomally tagged Srk1-Pk was analyzed by PAGE. Western analysis showed that, following stress, slower migrating forms of Srk1 are evident (Fig. 5*A*, compare *lanes 1* and *2*). It is highly likely that these slower migrating forms of Srk1 are due to phosphorylation, as these species are lost following treatment with λ-protein phosphatase (Fig. 5*A*, *lanes 5* and *6*). Furthermore, the phosphorylation of Srk1 correlated with the activation of Sty1 (Fig. 5*B*). To further investigate whether Sty1 is involved in the stress-induced phosphorylation of Srk1, the mobility of Srk1-Pk from *sty1*$^-$ cells was examined. However, the levels of Srk1 protein are significantly reduced in *sty1*$^-$ cells, in agreement with the microarray and Northern analysis that revealed that *srk1*$^+$ expression is Sty1-dependent (Fig. 1). Nonetheless, a long exposure of the Western blot failed to

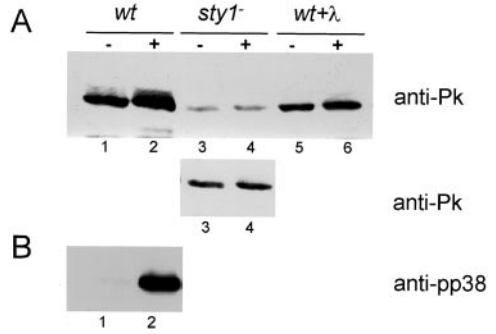

FIG. 5. **Srk1 is a phosphorylated *in vivo* following stress.** *A*, *sty1–6HisHA* (*wt*) or *sty1*$^-$ cells carrying a genomic copy of *srk1*$^+$ tagged with a Pk-epitope were grown to mid-log. Extracts were made from cultures either untreated (−) or stressed with 1 mM H_2O_2 for 10 min (+). Srk1 was detected by Western blot analysis using an anti-Pk antibody. The phosphorylation state of Srk1 was monitored by the appearance of slower migrating forms. Cell extracts from wild-type cells were treated with λ-phosphatase (*wt*+λ). Because of the low level of expression of Srk1 in the *sty1*$^-$ strain, the blot was exposed to film for longer to aid visualization of the mobility of Srk1 (*lower panel*). *B*, activation of Sty1 was monitored in the wild-type strain, using the same extracts, by Western blot analysis using an anti-phospho-p38 antibody (*anti-pp38*).

reveal any stress-induced slower migrating forms of Srk1 in the *sty1*$^-$ mutant (Fig. 5*A*, *lanes 3* and *4*, *lower panel*). This result suggests that stress-induced phosphorylation of Srk1 requires the Sty1 MAPK and is consistent with the hypothesis that Srk1 may be a direct downstream target of Sty1.

Srk1 Is a Substrate of the Sty1 MAPK—Purified recombinant Srk1 was tested as a potential substrate in Sty1-specific kinase assays. However, as wild-type Srk1 was found to have significant autophosphorylation activity, a kinase-inactive mutant of Srk1 ($Srk1^{K153A}$) was generated for these experiments. In the first assay, Sty1–9myc was immunoprecipitated from yeast extracts prepared from unstressed cells or cells that had been treated with 1 mM H_2O_2 for 10 min. These precipitates were incubated with either recombinant $Srk1^{K153A}$ or recombinant Atf1, a known Sty1 substrate (7, 21). As shown in Fig. 6*A*, phosphate incorporation into both Atf1 (*lanes 1* and *2*) and Srk1 (*lanes 3* and *4*) was significantly increased upon incubation with Sty1–9myc immunoprecipitated from cells exposed to oxidative stress. Similar results were obtained using extracts from cells exposed to oxidative stress and heat shock (data not shown). Analysis of the level of phosphate incorporation into Atf1 and Srk1 revealed that ~10-fold less phosphate was in-

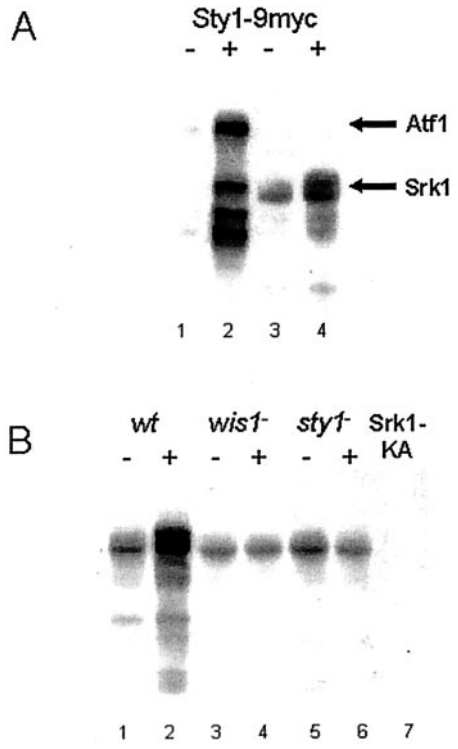

FIG. 6. **Srk1 is phosphorylated by Sty1 kinase *in vitro*.** *A*, cells expressing chromosomally tagged Sty1–9myc were grown to mid-log and aliquots harvested before (−) and after (+) a 10-min treatment with 1 mM H_2O_2. Extracts were prepared, Sty1 protein immunoprecipitated, and the immune complexes tested for kinase activity using recombinant GST-Atf1 (*lanes 1* and *2*) or 6His-Srk1^{K153A} (*lanes 3* and *4*) as substrates. The phosphorylation of GST-Atf1 and 6His-Srk1^{K153A} was assessed by SDS-PAGE and autoradiography. *B*, extracts were prepared from wild-type (*wt*), *sty1*$^-$, and *wis1*$^-$ cells that had been harvested before (−) or after (+) a 10 min treatment with 1 mM H_2O_2, and incubated with recombinant 6His-Srk1^{K153A} prebound to Ni^{2+}-NTA-agarose beads for 1 h at 4 °C, as described under "Experimental Procedures." The beads were extensively washed and a kinase reaction assay allowed to proceed for 30 min at 30 °C. The phosphorylation of 6His-Srk1^{K153A} was assessed by SDS-PAGE and autoradiography.

corporated into Srk1. The reason for this is unknown, but it may be relevant that there are more potential Sty1-phosphorylation sites in Atf1 compared with Srk1.

A second phosphorylation assay was performed based on the fact that Srk1 binds to the Sty1 MAPK. Extracts were prepared from wild-type cells (CHP429), and cells containing a deletion of the *sty1*$^+$ gene (JM1160) or *wis1*$^+$ (JM544) gene, which encodes the MAPKK, both before and after treatment with 1 mM H_2O_2 for 10 min. These extracts were incubated with recombinant His_6-tagged Srk1 coupled to Ni^{2+}-NTA-agarose, following which the Ni^{2+}-NTA-agarose was subjected to several washes, and phosphorylation of bound Srk1 assayed by incubating with [γ-^{32}P]ATP. Stimulation of Srk1 phosphorylation was only seen following incubation with extracts prepared from wild-type cells following stress (Fig. 6*B*, *lane 2*). Deletion of either the *sty1*$^+$ or the *wis1*$^+$ gene resulted in no detectable stress-induced phosphorylation of Srk1. Hence, these data suggest that Sty1 activation is essential for Srk1 phosphorylation. Interestingly, there is a basal level of phosphorylation of Srk1 that occurs in a Sty1-independent manner. This is not due to residual kinase activity in the Srk1^{K153A} mutant, as incubation of recombinant Srk1^{K153A} alone with [γ-^{32}P]ATP resulted in no phosphorylation (Fig. 6*B*, *lane 7*).

Deletion of the srk1$^+$ *Gene Partially Suppresses the Lethality Associated with Overexpression of the MAPKK Wis1*—Overexpression of *wis1*$^+$ from the thiamine-repressible *nmt1* promoter results in lethal cell lysis. However, previous work has shown that deletion of downstream components of the Sty1 pathway, such as Sty1 (2, 3), suppresses this lethality. Our data suggest that Srk1 is a downstream target of Sty1; hence, we tested whether deletion of *srk1*$^+$ would prevent cell death resulting from Wis1 overexpression. Wild-type (CHP429), *srk1*$^-$ (JP76), and *sty1*$^-$ (JM1160) cells were transformed with pREP1Wis1, in which Wis1 expression is under the control of the thiamine-repressible *nmt1* promoter. Upon thiamine release, wild-type cells carrying pREP1Wis1 failed to grow, but cells deleted for *sty1*$^+$ grew normally (Fig. 7). *srk1*$^-$ cells overexpressing Wis1 were viable, but slower growing than *sty1*$^-$ cells under the same conditions (Fig. 7). This demonstrates that deletion of *srk1*$^+$ partially suppresses the lethality caused by Wis1 overexpression, in contrast to deletion of *sty1*$^+$, which completely suppresses this lethality.

Srk1 Translocates to the Nucleus Following Stress—The cellular location of Srk1 was investigated by indirect immunofluorescence using the chromosomally tagged *srk1-Pk* (JP203) strain (Fig. 8*A*). Under non-stressed conditions, Srk1 was found to be cytoplasmic and visibly excluded from the nucleus. However, following treatment with an osmotic stress (0.6 M KCl), Srk1 translocates to the nucleus with nuclear staining maximal at 10 min. The nuclear localization of Srk1 is transient, as Srk1 is visibly excluded from the nucleus 30 min following stress. We also found that Srk1 translocates to the nucleus with similar kinetics in response to oxidative stress or heat shock (data not shown).

To investigate whether Sty1 is involved in regulating the cellular localization of Srk1, the *srk1-Pk* allele was introduced into a *sty1*$^-$ background (JP224). However, as shown previously (Fig. 5*A*), Srk1 protein levels are significantly reduced in *sty1*$^-$ cells and consequently much weaker immunofluorescence signals were obtained. Nonetheless, no stress-induced nuclear translocation of Srk1 was detected in the *sty1*$^-$ strain (Fig. 8*B*), which suggests that Sty1-mediated phosphorylation of Srk1 may be important for this mechanism. Moreover, we noted that the localization of Srk1 appears to be more uniform in *sty1*$^-$ cells, with only a slight indication of nuclear exclusion in some cells.

To investigate the localization of Srk1 under conditions where Sty1 is constitutively active, the *srk1-Pk* allele was introduced in a strain (JP226) carrying a constitutively active *wis1*^{DD} allele (38). In the *wis1*^{DD} strain, the two phosphorylation sites on the MAPKK Wis1, Ser-469 and Thr-473, which are targeted by the upstream MAPKK kinases, are mutated to aspartic acid to mimic hyperphosphorylation. Consequently, the Wis1^{DD} protein activates Sty1 even in the absence of stress. However, Srk1 failed to translocate to the nucleus in the *wis1*^{DD} mutant following osmotic stress (Fig. 8*C*, *upper panel*). This was contrary to our initial predictions that Srk1 might be constitutively nuclear in a strain in which Sty1 was constitutively active. However, it has been previously reported that, although the basal level of phosphorylated Sty1 is increased in a *wis1*^{DD} mutant, the level of Sty1 activation following stress is much lower than in wild-type cells (38). Therefore, we assayed the levels of active Sty1 in wild-type and *wis1*^{DD} cells, by preparing extracts and performing Srk1 phosphorylation assays as described earlier (Fig. 6*B*). In agreement, the level of Srk1 phosphorylation was significantly higher when incubated with extracts prepared from wild-type cells subjected to stress than with extracts prepared from the *wis1*^{DD} mutant (Fig. 8*C*, *lower panel*). These results strongly suggest that phosphorylation of Srk1 is a key regulatory factor in the stress-induced nuclear translocation of Srk1.

Deletion of srk1$^+$ *Accelerates Meiosis*—To investigate the

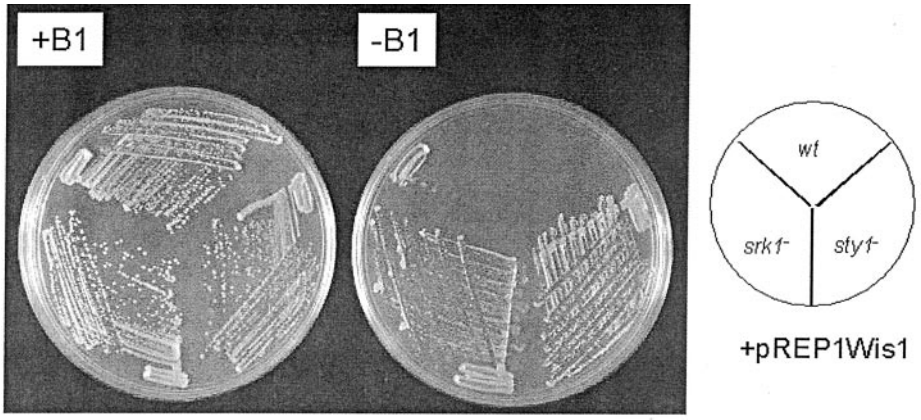

FIG. 7. **Deletion of the *srk1*$^+$ gene partially suppresses the lethality caused by overexpression of Wis1.** pREP1Wis1 was transformed into wild-type (*wt*), *sty1*$^-$, and *srk1*$^-$ cells. Expression of *wis1*$^+$ was repressed in the presence (+*B1*), and induced in the absence, of thiamine (−*B1*).

cellular role of Srk1, *srk1*$^-$ cells were tested for sensitivity to an extensive range of stress conditions and drugs. The *srk1*$^-$ strain did not display obvious sensitivity to any of the stress conditions tested (data not shown). It may be relevant in this respect that deletion of the *RCK2* gene, the *srk1*$^+$ homologue, in budding yeast, did not result in osmosensitivity, even though Rck2 is a substrate for the osmo-sensing Hog1 MAPK (27, 28).

Previous work has implicated Rck2 as an inhibitor of meiosis in budding yeast (39); hence, it was possible that Srk1 has a similar role in fission yeast. In *S. pombe*, nitrogen starvation stimulates a program of sexual development in which cells arrest in G_1, conjugate with partners of the opposite mating type and then undergo meiosis. To test the role of Srk1 in meiosis, *h*$^+$ *srk1*$^-$ and *h*$^-$ *srk1*$^-$ haploids were crossed to yield homozygous diploids. Following a 24-h incubation on sporulation medium that lacks a nitrogen source, ~30% of the diploids had conjugated and sporulated to give tetrads with 4 mature spores. Under the same conditions, only 2% of an isogenic wild-type cross had undergone conjugation and sporulation (Fig. 9*A*). This demonstrates that the *srk1*$^-$/*srk1*$^-$ homozygous diploid enters meiosis much more rapidly than a wild-type diploid, suggesting that Srk1 is an inhibitor of meiosis in fission yeast.

Overexpression of Srk1 Delays Mitotic Initiation—In addition to the analysis of phenotypes associated with deletion of the *srk1*$^+$ gene, the effects of expression of *srk1*$^+$ from the thiamine-repressible *nmt41* promoter were examined. Following thiamine release, cells carrying pREP41-Srk1 grow more slowly and are much more elongated at cell division in comparison to wild-type cells (Fig. 9*B*). A wide range of cell lengths were observed in cells carrying pREP41-Srk1, presumably because of the range of plasmid copy numbers within the population of cells. However, lengths greater than 30 μm were routinely seen compared with ~14 μm for wild-type cells (CHP429). Overexpression of homologous proteins, namely *S. cerevisiae* Rck2 (26), or a constitutively active form of mouse CaMKII (41), has also previously been shown to induce a G_2 delay in *S. pombe*.

Overexpression of Srk1 Causes Defective G_1 Arrest—The observation that deletion of *srk1*$^+$ accelerates meiosis suggests that Srk1 may be involved in the response to nitrogen limitation. We therefore investigated whether overexpression of *srk1*$^+$ from the *nmt41* promoter in heterothallic cells affects nitrogen starvation-induced G_1 arrest (Fig. 9*C*). FACScan analysis of the DNA content of wild-type (CHP429), *sty1*$^-$ (JM1160), and wild-type cells carrying pREP41Srk1 was performed before and after nitrogen starvation. Before nitrogen starvation all these strains showed a single peak corresponding to a 2C DNA content, consistent with the fact that the majority of the fission yeast cell cycle is spent in G_2 (data not shown). Following nitrogen starvation, the majority of wild-type cells arrested in G_1 with a 1C DNA content (Fig. 9*C*). In contrast, less than 50% of wild-type cells carrying pREP41Srk1 arrested in G_1 following nitrogen limitation. The fact that not all cells were defective in the G_1 arrest is probably due to the range of plasmid copy numbers within the cell population. In agreement with previous work, cells lacking the Sty1 MAPK fail to arrest in G_1 following nitrogen limitation (2, 7, 40). These data, taken together with the observation that *srk1*$^-$ cells exhibit a meiotic defect, suggest that one of the cellular roles of Srk1 is to regulate the response to nitrogen limitation.

DISCUSSION

Although much is known about the core components of the Sty1 MAPK pathway, there is still much to learn regarding how stress signals are relayed to and from the MAPK module. Here, we describe the identification of a putative serine/threonine protein kinase, Srk1, as a novel component of the fission yeast Sty1 stress-activated MAPK pathway. A microarray analysis of the *S. pombe* genome revealed that expression of *srk1*$^+$ is up-regulated in response to multiple stresses and that this induction required the Sty1 MAPK. Significantly, the microarray analysis revealed Srk1 to be one of only two putative kinases whose genes are induced in a stress-dependent manner.[2] Biochemical analysis of Srk1 has revealed that this kinase is a stress-induced phosphoprotein, which forms a stable complex with both inactive and phosphorylated forms of Sty1. Furthermore, Srk1 is a substrate for the Sty1 MAPK, and Sty1 activation is required for the stress-dependent increase in Srk1 phosphorylation. We also found that the cellular localization of Srk1 is regulated by multiple stresses in a Sty1-dependent manner. Collectively, these results demonstrate that Sty1 regulates Srk1 by transcriptional and post-translational mechanisms.

Srk1 shares a high degree of homology with Rck2 from budding yeast, which has been shown to be a direct substrate for the osmotic stress-sensing Hog1 MAPK (27, 28). Surprisingly, although Rck2 binds to, and is phosphorylated by the Hog1 MAPK, *rck2*$^-$ cells are not sensitive to osmotic stress. However, deletion of the *RCK2* gene suppresses the cell lethality associated with constitutive activation of the Hog1 MAPK, which strongly suggests that Rck2 is a specialized effector of the Hog1 pathway (27, 28). Similarly, deletion of the *srk1*$^+$ gene in *S. pombe* suppresses the lethality associated with hyperactivation of the Sty1 pathway, because of overexpression of the MAPKK Wis1, which further supports the hypothesis that Srk1 is a target of the Sty1 MAPK. Significantly, however, we noted that deletion of *srk1*$^+$ only partially suppressed the lethality caused by overexpression of Wis1, whereas deletion of *sty1*$^+$ completely suppresses this lethality. This may be explained in terms of organization and function of components of the Sty1

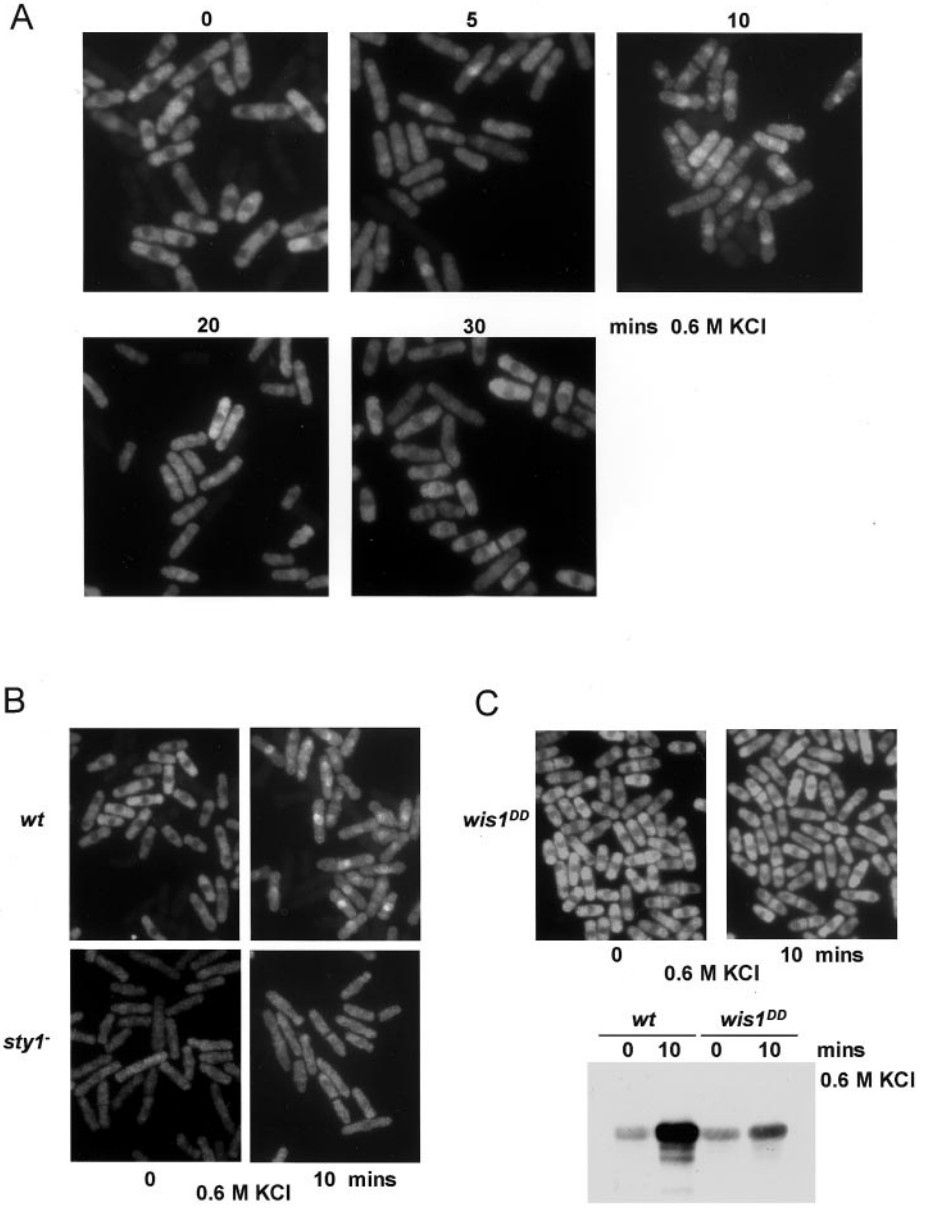

FIG. 8. **Srk1 translocates to the nucleus following stress.** *A*, the cellular localization of Srk1 was examined by immunofluorescence microscopy in wild-type cells carrying chromosomally tagged *srk1-Pk* before stress or following exposure to 0.6 M KCl for 5, 10, 20, and 30 min. *B*, localization of Srk1 in wild-type and *sty1*$^-$ cells before or after treatment with 0.6 M KCl for 10 min. *C*, localization of Srk1 in *wis1*^{DD} cells, before or after treatment with 0.6 M KCl for 10 min (*upper panel*). Extracts prepared from the same cells were incubated with recombinant 6His-Srk1^{K153A} prebound to Ni^{2+}-NTA-agarose beads for 1 h at 4 °C, as described under "Experimental Procedures." The beads were extensively washed and a kinase reaction assay allowed to proceed for 30 min at 30 °C. The phosphorylation of 6His-Srk1^{K153A} was assessed by SDS-PAGE and autoradiography.

MAPK pathway. Sty1 is the only known protein to be regulated by Wis1. The fact that deletion of *sty1*$^+$ completely suppresses the lethality caused by Wis1 overexpression strongly suggests that this lethality is due to hyperactivation of the Sty1 kinase. However, in contrast to Sty1 being the only Wis1 target, there are several Sty1 substrates. This work has revealed that, in addition to the transcription factor Atf1, Sty1 also phosphorylates the Srk1 protein kinase. Moreover, it is likely that other substrates for Sty1 exist, as several phenotypes, including sensitivity to DNA damaging agents and the G_2 cell cycle delay, remain Sty1-specific. Inactivation of a single Sty1 substrate, such as Srk1, would not be expected to completely suppress the lethality caused by Wis1 overexpression. Indeed, deletion of all genes encoding Sty1 substrates may be needed to completely suppress the Wis1 overexpression phenotype.

In contrast to the Hog1 pathway in budding yeast, the fission yeast Sty1 pathway responds to a diverse range of environmental stimuli. Here, we show that, in response to multiple stresses, activated Sty1 phosphorylates the Srk1 kinase and Srk1 translocates to the nucleus. Intriguingly, similar to the *rck2* mutant, deletion of the *srk1*$^+$ gene does not result in any obvious stress sensitivity. However, the Sty1 pathway is also important for sexual development in fission yeast. In cultures of homothallic wild-type cells, nitrogen starvation promotes sexual development in which cells arrest in G_1, conjugate with partners of the opposite mating type, and then undergo meiosis. Nitrogen limitation induces activation of Sty1, and Sty1 is required for *ste11*$^+$ transcription, which is critical for meiosis to proceed (7). Here we have found that the Sty1 substrate, Srk1, is also involved in regulating sexual development. However, deletion of *srk1*, in contrast to *sty1*$^-$ cells, results in cells that have a hyperactivated meiotic program, which suggests that Srk1 functions as an inhibitor of meiosis.

The nitrogen limitation-induced G_1 cell cycle arrest is not a unique feature of the sexual development program, as heterothallic cells starved for nitrogen arrest in G_1. However, overexpression of Srk1 in a wild-type heterothallic strain results in cells that fail to arrest in G_1 following nitrogen starvation. This is also seen in *sty1*$^-$ cells and is consistent with Srk1 having a role in inhibiting the response to nitrogen limitation. Taken together, our data suggest that, although Sty1 regulates the phosphorylation and nuclear translocation of Srk1 in a manner that is independent of the nature of the stress, the Sty1 regulation of Srk1 may have a specific role in

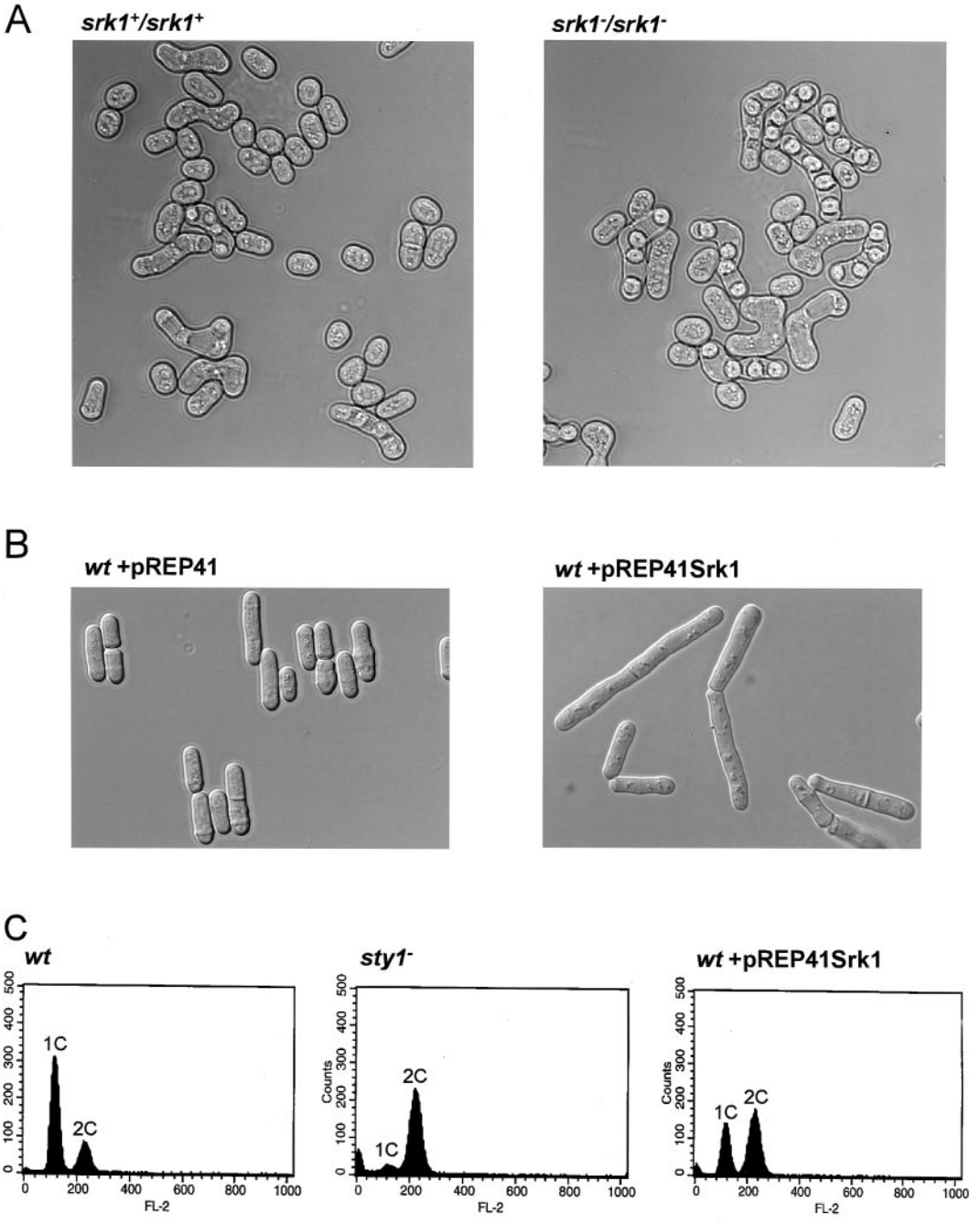

FIG. 9. **Phenotypes associated with deletion or overexpression of *srk1*$^+$.** *A*, deletion of *srk1* accelerates meiosis. h^+ and h^- wild-type (*srk1*$^+$) or h^+ and h^- *srk1*$^-$ strains were incubated for 24 h on EMM medium lacking a nitrogen source and photographed using Nomarski optics. *B*, overexpression of *srk1*$^+$ causes a G_2 cell cycle delay. Wild-type (*wt*) cells transformed with either pREP41 or pREP41Srk1 were grown to mid-log phase in minimal medium and photographed using Nomarski optics. *C*, overexpression of *srk1*$^+$ prevents arrest in G_1 following nitrogen limitation. FACScan analysis of DNA content in wild-type (*wt*), *sty1*$^-$ cells and in *wt* cells transformed with pREP41Srk1, following nitrogen starvation.

the response to nitrogen limitation and consequently meiosis. Further experimentation will be directed at dissecting the role of Srk1 in sexual development in fission yeast.

We have also found that overexpression of *srk1*$^+$ results in cells that are delayed in the timing of mitotic initiation and divide at a longer cell size than wild-type cells. Previous studies have shown that overexpression of related proteins, namely *S. cerevisiae* Rck2 (26), or a constitutively active form of mouse CaMKII (41), also induces a G_2 delay in *S. pombe*. As *sty1*$^-$ cells also exhibit a delay in the timing of mitotic initiation (2, 3, 8), it was possible that Srk1 may be involved in this cell cycle function of Sty1. However, cells deleted for *srk1*$^-$ show no obvious mitotic defects (data not shown), which suggests that the G_2 delay arising from overexpression of the kinase may be an artifact resulting from non-physiological levels of Srk1.

The stress-activated MAPK pathways are highly evolutionarily conserved (1). This is highlighted by the fact that both p38 and SAPK/JNK can partially substitute for Hog1 in mediating the osmotic stress response in *S. cerevisiae* (42, 43). It is well established that mammalian stress-activated kinases phosphorylate substrates in addition to transcription factors (44). For example, targets of p38 include MAPKAP kinases 2 and 3 (45, 46), and the MAPK-interacting kinase Mnk1 (47). The identification of the Rck2 kinase as a substrate for the budding yeast Hog1 MAPK (27, 28), together with this work showing that the Sty1 MAPK is required for phosphorylation of the Srk1 kinase,

further emphasizes the high degree of conservation that exists between the mammalian and yeast stress-activated MAPK pathways. This conservation is exemplified by the sequence similarity between budding yeast Rck2, fission yeast Srk1, and mammalian MAPKAP kinases (this work and Ref. 28).

Localization studies revealed that mammalian MAPKAP kinase 2 normally resides in the nucleus but following stress rapidly translocates to the cytoplasm upon phosphorylation by p38 (25, 48). The p38-mediated phosphorylation is believed to increase the accessibility of a nuclear export signal (25). Analysis of the predicted protein sequence of Srk1 revealed the presence of several potential nuclear localization sequences.[3] Intriguingly, however, the cellular localization of Srk1 is very different to that seen with MAPKAP 2. Under non-stressed conditions Srk1 resides in the cytoplasm, but following stress quickly moves to the nucleus. Similar to that seen in higher cells, however, the cellular localization of Srk1 appears to be regulated by Sty1, as stress-induced nuclear accumulation of Srk1 does not occur in *sty1*$^-$ cells. Moreover, the localization of Srk1 appears to be more uniform throughout the cell, which raises the possibility that Sty1 may act to anchor Srk1 in the cytoplasm under non-stressed conditions. Interpretation of these data, however, must be tempered by the fact that Srk1 protein levels are significantly reduced in a *sty1*$^-$ mutant. We also found that Srk1 fails to translocate to the nucleus following stress in cells carrying a constitutively active *wis1*^{DD} allele. The Wis1^{DD} protein activates Sty1 even in the absence of stress; hence, we may have expected Srk1 to be constitutively nuclear in the *wis1*^{DD} strain. However, previous work has shown that the level of active Sty1 is much lower in *wis1*^{DD} than wild-type cells (38). Consistent with this we found that the level of Srk1 phosphorylation is significantly lower in the *wis1*^{DD} strain than wild-type cells following stress. These results strongly suggest that phosphorylation by Sty1 is important for the stress-induced nuclear translocation of Srk1.

The nuclear accumulation of Srk1 following stress raises the possibility that targets for this kinase exist in the nucleus. It is currently unknown whether the budding yeast homologue of Srk1, Rck2, translocates to the nucleus following stress. However, a recent study has identified a cytoplasmic protein, translation elongation factor E2-F, as a substrate for the Rck2 kinase (28). Whether Rck2 and Srk1 phosphorylate the same nuclear or cytoplasmic substrates remains to be seen, but clearly the identification of downstream targets of Srk1 will further our understanding of the cellular role of Srk1 in fission yeast.

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[3] J. Quinn, unpublished observations.

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