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Spy1, a Histidine-Containing Phosphotransfer Signaling Protein, Regulates the Fission Yeast Cell Cycle through the Mcs4 Response Regulator

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Common histidine-to-aspartate (His-to-Asp) phosphorelay signaling systems involve three types of signaling components: a sensor His kinase, a response regulator, and a histidine-containing phosphotransfer (HPt) protein. In the fission yeast *Schizosaccharomyces pombe*, two response regulators, Mcs4 and Prr1, have been identified recently, and it was shown that they are involved in the signal transduction implicated in stress responses. Furthermore, Mcs4 appears to be involved in mitotic cell-cycle control. However, neither the HPt phosphotransmitter nor His kinase has been characterized in *S. pombe*. In this study, we identified a gene encoding an HPt phosphotransmitter, named Spy1 (*S. pombe* YPD1-like protein). The *spy1*$^+$ gene showed an ability to complement a mutational lesion of the *Saccharomyces cerevisiae YPD1* gene, which is involved in an osmosensing signal transduction. The result from yeast two-hybrid analysis indicated that Spy1 interacts with Mcs4. To gain insight into the function of Spy1, a series of genetic analyses were conducted. The results provided evidence that Spy1, together with Mcs4, plays a role in regulation of the G_2/M cell cycle progression. Spy1-deficient cells appear to be precocious in the entry to M phase. In the proposed model, Spy1 modulates Mcs4 in a negative manner, presumably through a direct His-to-Asp phosphorelay, operating upstream of the Sty1 mitogen-activated protein kinase cascade.

Common prokaryotic signal transduction mechanisms are generally referred to as "histidine-to-aspartate (His-to-Asp) phosphorelay systems" (or "two-component regulatory systems"). Such a His-to-Asp phosphorelay involves two or more of the common signal transducers, a sensor exhibiting histidine (His) kinase activity, a response regulator containing a phosphoaccepting receiver, and a histidine-containing phosphotransmitter (HPt) (4, 5,12, 23, 29, 39). To date, numerous instances of His-to-Asp phosphorelay systems, involved in a wide variety of adaptive responses to environmental stimuli, have been reported for many bacterial species. The His-to-Asp phosphorelay system was once thought to be restricted to prokaryotes. However, many instances have recently been reported for diverse eukaryotic species, including yeasts (17, 18), fungi (3), slime molds (7, 32, 41), and even higher plants (6, 13, 15, 40). Thus, the His-to-Asp phosphorelay is a paradigm of intracellular signal transduction through protein phosphorylation in both prokaryotes and eukaryotes.

In eukaryotes, the best-characterized His-to-Asp phosphorelay is the osmoresponsive signal transduction in the budding yeast *Saccharomyces cerevisiae* (20, 43). Together, the three components Sln1 (sensor His kinase), Ypd1 (HPt phosphotransmitter), and Ssk1 (response regulator) are involved in the His-to-Asp phosphorelay signaling pathway. A striking fact is that the yeast His-to-Asp phosphorelay pathway is directly linked to a eukaryotic mitogen-activated protein kinase (MAPK) signaling cascade (termed the HOG1 [high-osmolarity glycerol response]) cascade. The fission yeast *Schizosaccharomyces pombe* is an alternative model microorganism with which to gain an insight into how a bacterial type of signal transduction mechanism is integrated into a eukaryotic signal transduction cascade. Nonetheless, clarification of such a His-to-Asp phosphorelay system in *S. pombe* is at a very early stage.

In *S. pombe*, so far, two response regulators, named Prr1 and Mcs4, have been uncovered and characterized (8, 28, 33, 37). The Prr1 response regulator has a typical phosphoaccepting receiver domain, preceded by a mammalian heat shock factor-like DNA-binding domain. It was demonstrated that Prr1 is responsible for transcriptional regulation of some genes (e.g., *trr1*$^+$ and *ctt1*$^+$), which are induced by oxidative stress (28). The Mcs4 response regulator appears to be the counterpart (or homologue) of the *S. cerevisiae* Ssk1 response regulator, as judged by the fact that their amino acid sequences are very similar to each other and that Mcs4 functions immediately upstream of an *S. pombe* stress-activated MAPK cascade (8, 33, 37). This particular MAPK cascade has recently been characterized extensively, and it includes MAPK Sty1 (also known as Spc1 and Phh1) (16, 21, 35), MAPKK Wis1 (34), and MAPKKK Wak1 (also known as Wik1) (33, 37). The Sty1 MAPK cascade is considered to be analogous to the *S. cerevisiae* HOG1 MAPK cascade. In contrast to the HOG1 MAPK cascade, however, the Sty1 MAPK cascade is activated by multiple environmental stresses, including osmotic stress, oxidative stress, heat shock, and UV light (9, 10, 21, 33, 35, 36, 38). More interestingly, it is known that the Sty1 MAPK cascade links stress signaling with control of sexual differentiation in *S. pombe* (16, 36). Furthermore, the Sty1 MAPK cascade was suggested to somehow integrate stress sensing into control of mitosis (35). Thus, the Sty1 MAPK cascade appears to be crucial for linking stress sensing with two processes fundamental to all eukaryotes, namely, control of both mitosis and meiosis. In this context, it should be noted that the *mcs4*$^+$ gene was originally identified as a mutation which is capable of suppress-

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TABLE 1. Yeast strains and plasmids relevant to this study

Strain of plasmid	Genotype	Source
Strains		
S. pombe		
JY333	*h⁻ leu1-32 ade6-M216*	M. Yamamoto
JY741	*h⁻ leu1-32 ade6-M216 ura4-D18*	M. Yamamoto
JY746	*h⁺ leu1-32 ade6-M210 ura4-D18*	M. Yamamoto
Gp14	*h⁻ leu1-32 ade6-M216 ura4-D18 mcs4::ura4⁺*	G. Cottarel (8)
M132	*h⁻ leu1-32 cdc25-22*	H. Okayama
KI001	*h⁻ leu1-32 ade6-M216 ura4-D18 spy1::ura4⁺*	This study
KI002	*h⁻ leu1-32 ade6-M216 ura4-D18 spy1::ura4⁺ mcs4::ura4⁺*	This study
KI003	*h⁻ leu1-32 ura4-D18 spy1::ura4⁺ cdc25-22*	This study
KI004	*h⁻ leu1-32 ade6-M216 ura4-D18 spy1::ura4⁺ sty1::ura4⁺*	This study
JM1160	*h⁺ leu1-32 ade6-M216 ura4-D18 sty1::ura4⁺*	J. B. A. Millar
S. cerevisiae CUY1	*MATα ura3-52 leu2-3 his3-200 YPD1::LEU2*/pGB22 [pGB22;pRS413(*HIS3*) *GAL1p-PTP2*]	T. Suzuki (40)
Plasmids		
pREP1	*nmt1* promoter, *LEU2* marker	K. Maundrell
pKA013	*spy1⁺* gene under control of *nmt1* promoter	This study
pKA018	*spy1HQ* gene under control of *nmt1* promoter	This study
pCUY326	*ADH1* promoter, *URA3* marker	C. Ueguchi
pKA014	*spy1⁺* gene under control of *ADH1* promoter	This study
pKA019	*spy1HQ* gene under control of *ADH1* promoter	This study

ing the lethal phenotype (the so-called "mitotic catastrophe") caused by the *cdc2-3w* and *wee1-50* double mutations (24). These and other previous results supported the idea that, together with the Sty1 MAPK cascade, a His-to-Asp phosphorelay pathway involving Mcs4 plays a role in a presumed stress-responsive control of mitosis. However, such a putative His-to-Asp phosphorelay in *S. pombe* is entirely elusive, because neither His kinase nor the HPt phosphotransmitter has been characterized. In this study, an HPt phosphotransmitter of *S. pombe* was identified and characterized. This newly uncovered HPt phosphotransmitter, named Spy1, was suggested to play a role, together with Mcs4, in control of the timing of the mitotic initiation (or the G_2/M transition).

MATERIALS AND METHODS

Strains, plasmids, and media. The *S. pombe* strains and plasmids used in this study are listed in Table 1. These strains were grown either in YPD medium (1% yeast extract [Difco], 2% Polypeptone [Wako], 2% glucose) containing 10 μg of adenine per ml or in SD medium (0.67% yeast nitrogen base without amino acids [Difco], 2% glucose) supplemented with the necessary growth requirements in standard amounts. EMM minimal medium and MEA medium were also used (25).

Purification and phosphorylation of Spy1 and Mcs4. The *spy1⁺* or *mcs4⁺* coding sequence was placed under the T7 promoter on pET22b(+), which is an *Escherichia coli* expression vector (Novagen, Madison, Wis.). The appropriately constructed plasmid was transferred into *E. coli* BL21(DE3). The cells were grown in Luria-Bertani medium in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside). A cleared-cell lysate was obtained by use of an Aminco French pressure cell. This sample was applied to an Ni column with the rapid affinity purification pET His-Tag system supplied by Novagen. Other methods were those recommended by the supplier. The purified Spy1 protein (8 μg) was incubated with urea-treated *E. coli* cytoplasmic membrane (20 μg) at 37°C in the presence of 0.05 mM [γ-^{32}P]ATP (10,000 cpm pmol^{-1}), 200 mM KCl, and 5 mM $MgCl_2$. After incubation, the samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography. For phosphate transfer experiment, radioactively phosphorylated Spy1 protein was purified by means of gel filtration as described previously (1, 14). Other details were the same as those described previously (40).

Northern hybridization analysis. Northern hybridization analysis was carried out as described previously (2). Exponentially growing cells in YPD medium were collected and resuspended in fresh YPD medium containing 0.9 M KCl or 1 mM H_2O_2. A total RNA fraction was prepared from the cells at each time. After denaturation with formamide-formaldehyde, RNA (5 μg) was analyzed on a 1.4% agarose gel containing formaldehyde, followed by alkali blotting onto Hybond-N$^+$ membrane (Amersham International). Hybridization was carried out with ^{32}P-labelled probe, which specifically encompassed the *gpd1⁺*, *ctt1⁺*, or *leu1⁺* coding sequence, at 65°C for 2 h in Rapid-hyb buffer, as recommended by the supplier (Amersham International).

Plasmid construction. For construction of pKA013 (named pREP1-Spy1 in Fig. 7), in which the *spy1⁺* gene is controlled under the *nmt1* promoter, the coding sequence of *spy1⁺* was PCR amplified with the primers (5′-TTCTAAC ATATGAGTGTATATCGTGATAACATG and 5′-GGGGATCCAAAGGCT AGGTACTTTGAC). After digestion with *Nde*I and *Bam*HI, the fragment was cloned in the same site of pREP1 (19). For construct pKA018, which is identical to pKA013 except that the His-221 of Spy1 was replaced with Gln, site-directed mutagenesis was carried out with the oligonucleotide 5′-pGATCCTTTAAGGA ATTGCCCCAACGAGGAAAGC. For *S. cerevisiae* complementation analysis, two plasmids (2 μm origin, *URA3* marker) were constructed, and named pKA014 and pKA019, respectively. pKA014 carries the *S. pombe spy1⁺* gene, which was placed under the *S. cerevisiae ADH1* promoter (named pSpy1 in Fig. 2), whereas pKA019 carries the His-221-to-Gln mutation in the *spy1* gene (named pSpy1HQ in Fig. 2). For two-hybrid analysis, three plasmids, pKA027, pKA015, and pKA030, were constructed. To construct pKA027, the *mcs4⁺* gene was PCR amplified with primers 5′-ATGAATTCCATATGCGCATTTGGTTTAAAAAAG and 5′-GCT AGTCGACTCGACCGCGAAAACGGC. After digestion with *Eco*RI and *Bam*HI, the fragment encoding *mcs4⁺* was cloned in the same site of pGBT9. pKA015 was constructed as follows. An *Nde*I-*Bam*HI fragment carrying the *spy1⁺* gene was isolated from pKA013, treated with T4 DNA polymerase, and then cloned into an *Sma*I site of pGAD424. pKA030 was identical to pKA015, except that the His-221 of Spy1 was replaced with Gln.

Two-hybrid analysis. The kit used for two-hybrid analysis (MATCHMAKER; Clontech) was obtained through Toyobo Co. The kit included the vectors pGBT9, providing the GAL4 DNA-binding domain (*TRP1* marker), and pGAD424, providing the GAL4 activation domain (*LEU2* marker). Analysis was carried out according to the manual by using strain HF7c as a host.

Gene disruption. For *spy1* disruption, 4,684 bp of an *Sph*I-*Bam*HI fragment carrying the *spy1⁺* gene was amplified by PCR with the appropriate primers and cloned on pUC18 to construct plasmid pHAI 207 (Fig. 1A). Then the *Mun*I and *Xho*I region in the *spy1* open reading frame (ORF) was replaced with a *ura4⁺* cassette and used for linear transformation after *Vsp*I digestion (Fig. 1A). Stable Ura4$^+$ transformants were selected for a diploid strain (JY741 × JY746), and then the *spy1::ura4⁺* construct on the chromosome was confirmed by Southern hybridization. After sporulation, Ura$^+$ haploid segregants were analyzed.

RESULTS

Identification of the *spy1⁺* gene that encodes an HPt phosphotransmitter. An extensive inspection of the current genome sequence database for *S. pombe* revealed the occurrence of a gene encoding a protein highly homologous to the *S. cerevisiae* Ypd1 protein. As shown in Fig. 1, this gene was found as an ORF in cosmid c725 from chromosome II (gene name,

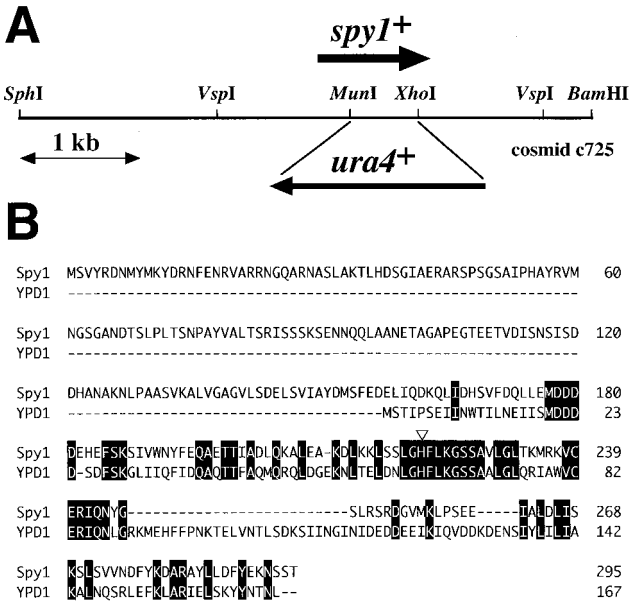

FIG. 1. The *spy1*$^+$ gene encodes an HPt phosphotransmitter. (A) Schematic representation of the *S. pombe* chromosomal region encompassing the *spy1*$^+$ gene. This region is carried in the c725 cosmid, whose entire nucleotide sequence has been determined (GenBank accession no. AL034352). In this study, an *spy1*Δ strain was constructed by inserting the *ura4*$^+$ marker, as shown schematically. (B) The deduced amino acid sequence of Spy1 was aligned with that of the budding yeast Ypd1 protein. The open triangle indicates the presumed phosphorylation site (histidine). The amino acids identical in Spy1 and Ypd1 are highlighted.

SPBC725.02; GenBank accession no. AL034352). This putative gene, named *spy1*$^+$ (*S. pombe YPD1* homologue), specifies an uninterrupted ORF encoding a protein of 295 amino acids. Its C-terminal region, consisting of about 140 amino acids, has 39% identity to that of Ypd1 (Fig. 1B). In particular, their amino acid sequences around the presumed phosphorylation sites (His-221 in Spy1) are particularly conserved (Fig. 1B). Spy1 has an extension at its N terminus (to the amino acid position of 157), which is absent in Ypd1.

To examine whether or not Spy1 can function as an HPt phosphotransmitter in a His-to-Asp phosphorelay, a complementation experiment employing a *ypd1*Δ mutant of *S. cerevisiae* was carried out (Fig. 2). As previously known, disruption of the *YPD1* gene results in lethality, presumably due to an excessive phosphorylation (or activation) of the HOG1 MAPK (30). Such a hyperphosphorylation event in the *ypd1*Δ cells can be eliminated by overproduction of the Ptp2 protein tyrosine phosphatase. Therefore, the *ypd1*Δ cells carrying a plasmid harboring a galactose-inducible *PTP2* gene (designated as *Pgal-PTP2*) can grow on SC medium in which the glucose was replaced with galactose, while they cannot grow on standard SC medium (Fig. 2; +vector) (30). When the *spy1*$^+$ gene of *S. pombe* was introduced into such *ypd1*Δ cells, the transformed cells were capable of growing on the SC medium, as in the case of the cells carrying the plasmid-borne *YPD1* gene (Fig. 2; +pSpy1 and +pYpd1, respectively). A mutant *spy1* gene encoding the Spy1 protein having an amino acid substitution (His-221 to Gln) at the presumed phosphorylation site showed no ability to do so (Fig. 2; +pSpy1HQ). These results demonstrated that Spy1 is capable of functioning as an HPt phosphotransmitter.

Spy1 interacts with Mcs4. It is known that Ypd1 functions as an intermediate of phosphorelay towards the downstream target, Ssk1, in *S. cerevisiae* (30). By analogy, it was assumed for

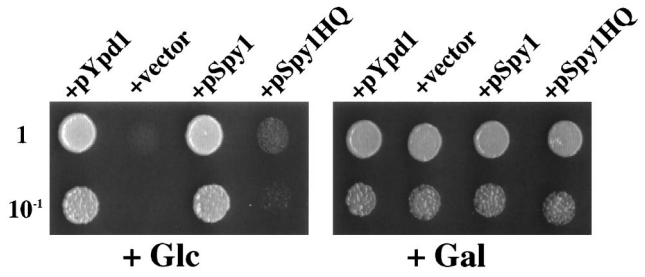

FIG. 2. The *spy1*$^+$ gene is able to complement the mutational lesion of the *YPD1* gene of *S. cerevisiae*. The *ypd1*Δ mutant carrying a plasmid harboring a composite *PTP2* gene (P_{GAL1}-*PTP2*) cannot grow on SC medium supplemented by glucose, while it can grow on SC medium supplemented with galactose (see the text) (+vector). Plasmid pKA014 carrying the recombinant *spy1*$^+$ gene (designated as pSpy1) was transferred into the *ypd1*Δ mutant, and then the viability of the transformed cells (about 2×10^2 and 2×10^3) was examined by spotting on galactose-synthetic complete (SC) medium (Gal) or glucose-SC medium (Glc). The cells were incubated for 3 days at 30°C. The same analyses were carried out for the plasmids carrying the mutant *spy1* gene (designated as pSpy1HQ) as well as the budding yeast *YPD1* gene (designated as pYpd1).

S. pombe that Spy1 most likely functions together with Mcs4, which appears to be the homologue of Ssk1. To examine this, yeast two-hybrid analyses were adopted by using Mcs4 and Spy1 as bait and prey, respectively (Fig. 3). A positive result was obtained, as judged by both the β-galactosidase and histidine-auxotrophy assays, suggesting that Spy1 and Mcs4 interact with each other. Interestingly, the mutant Spy1HQ protein also showed such an interaction. To analyze whether Spy1 specifically interacts with Mcs4, another *S. pombe* response regulator, Prr1, was also used as bait in the two-hybrid analysis. However, Prr1 fused to the DNA-binding domain of Gal4 showed evident activity by itself. Accordingly, thus far, we have not been able to address this.

Spy1 undergoes phosphorylation and transfers its phosphate to Mcs4. It is crucial to ask the question of whether or not Spy1 is capable of undergoing phosphorylation at the putative phosphoaccepting histidine site (His-221). This could be assessed by employing the *E. coli* cytoplasmic membrane that contains the overproduced ArcB hybrid sensor His kinase (40). When some heterologous HPt phosphotransmitters were incubated with the *E. coli* cytoplasmic membrane in the presence of ATP, they can acquire a phosphoryl group at a certain histidine residue, as previously demonstrated for the higher plant (*Arabidopsis*) HPt phosphotransmitters (40). This artificial in vitro phosphorylation system was used to show that Spy1 has the ability to undergo phosphorylation. The Spy1 protein was purified, together with its mutant derivative, which has the His-to-Gln substitution at the position of 221 (Fig. 4A). These

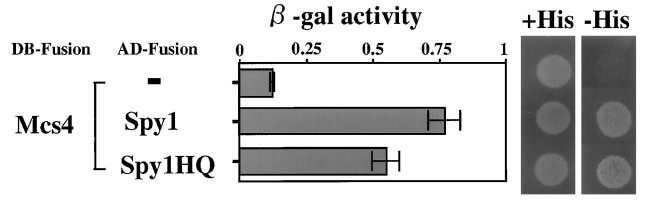

FIG. 3. Yeast two-hybrid analysis. Two-hybrid analysis was carried out with the combination indicated. The results are shown as β-galactosidase activity and histidine auxotrophy (+His or −His). In this experiment, it should be noted that neither AD-Spy1 nor AD-Spy1HQ alone showed significant β-galactosidase activity (0.13 and 0.18 U, respectively) (data not shown). DB-Fusion and AD-Fusion indicate proteins which are fused with the GAL4 DNA-binding domain and GAL4 activation domain, respectively.

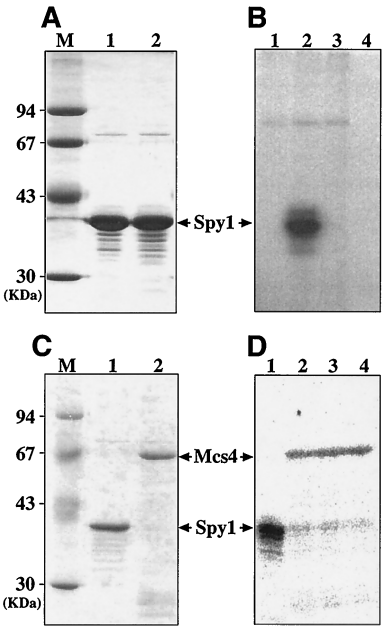

FIG. 4. In vitro phosphorylation of Spy1 and Mcs4. (A) Both histidine-tagged Spy1 (lane 1) and Spy1HQ (lane 2) proteins were purified as described in Materials and Methods. They were analyzed by SDS-PAGE followed by staining with Coomassie brilliant blue (16 μg each). Molecular mass markers are shown in lane M. (B) The purified Spy1 protein was incubated with the *E. coli* membrane in the presence of [γ-^{32}P]ATP for 30 min, as also described in Materials and Methods. The samples were analyzed by SDS-PAGE followed by autoradiography (lane 1, membrane alone; lane 2, membrane plus Spy1; lane 3, membrane plus Spy1HQ; and lane 4, Spy1 alone). (C) The histidine-tagged Mcs4 (lane 2) was purified and analyzed by SDS-PAGE, followed by staining with Coomassie brilliant blue. Spy1 protein used for the phosphate transfer experiment was also indicated (lane 1). (D) Autoradiogram showing phosphotransfer between phospho-Spy1 and Mcs4. ^{32}P-labelled phospho-Spy1 was purified as described previously (lane 1) (1, 14) and then was incubated with the purified Mcs4 protein at 16°C. Aliquots were removed 0.5 min (lane 2), 1 min (lane 3), and 5 min (lane 4) later and analyzed by SDS-PAGE.

purified proteins were incubated with the *E. coli* cytoplasmic membrane in the presence of [γ-^{32}P]ATP under the in vitro conditions established previously (40). The results showed that the wild-type Spy1 protein is capable of undergoing phosphorylation in a manner catalyzed by the *E. coli* cytoplasmic membrane, while the mutant protein is not (Fig. 4B).

Next, the phosphorylated Spy1 was purified and incubated with purified Mcs4 protein. As shown in Fig. 4D, the phosphoryl group on Spy1 was apparently transferred to Mcs4. From these biochemical data, it was suggested that the Spy1 protein has the ability to acquire a phosphoryl group at the histidine site at position 221 and transfer its phosphate to Mcs4.

Function of Spy1 in stress response. The results presented above are compatible with the idea that Spy1 and Mcs4 together constitute a His-Asp phosphorelay pathway in *S. pombe*. To address this genetically, we attempted to construct an *spy1* deletion mutant by creating an *spy1::ura4*$^+$ allele on the chromosome (Fig. 1A); the *Vsp*I DNA segment encompassing the *spy1::ura4*$^+$ construct was integrated into a diploid strain via homologous recombination. Tetrad dissection of asci from heterozygous diploids gave rise to four viable spores on germination that showed a 2:2 segregation of uracil auxotrophs to uracil prototrophs (data not shown). This indicated that the *spy1*$^+$ gene is not essential for growth under standard growth conditions. This is in a sharp contrast to the fact that the *ypd1*Δ mutant is lethal in *S. cerevisiae* (30). The natures of Spy1-deficient cells were then characterized in comparison with those of Mcs4-deficient cells.

It is known that the *gpd1*$^+$ gene encoding glycerol-3-phosphate dehydrogenase is induced by osmotic stress (e.g., 0.9 M KCl in medium) in a manner dependent on Mcs4 as well as the Sty1 MAPK cascade (2, 33, 36, 42). The *ctt1*$^+$ gene encoding catalase is also under the same regulatory circuit—in this case, in response to oxidative stress (e.g., H_2O_2 treatment) (33). It was reported that Mcs4-deficient cells are osmosensitive for growth, although not as severely as Sty1-deficient cells (33, 37). Based on these results, the expression of *gpd1*$^+$ and *ctt1*$^+$ in Spy1-deficient cells was examined by Northern hybridization, after the cells had been subjected to either osmotic or oxidative stresses (Fig. 5). In Mcs4-deficient cells, the osmoinducible expression of *gpd1*$^+$ and the H_2O_2-inducible expression of *ctt1*$^+$ were markedly impaired, as previously reported (33). However, the regulatory profiles of *gpd1*$^+$ and *ctt1*$^+$ were not significantly altered in the *spy1*Δ background, compared with those in the wild-type background. Furthermore, Spy1-deficient cells grew well on SD agar plates containing either 1 M KCl or 2 mM H_2O_2 (data not shown). Therefore, no evidence was obtained that implicated Spy1 in the stress-responsive signal transduction pathway, as far as the osmotic and oxidative stress responses were concerned.

Function of Spy1 in control of the mitotic cell cycle. Mcs4 appears to be involved, not only in the stress-responsive signaling, but also in a signaling circuitry of mitotic control, as demonstrated previously (8, 33, 37). The main engine of the mitotic G_2/M transition in *S. pombe* consists of the Cdc13 (cyclin B)/Cdc2 kinase, the Wee1/Mik1 tyrosine kinases, and the Cdc25 phosphatase (11). As documented previously, certain *S. pombe* mutants (e.g., *cdc25-22*) that are delayed in the timing of the mitotic G_2/M transition divide at a cell length longer than the wild-type cells, whereas other mutants (e.g., *wee1-50*) resulting in an advancement of the G_2/M transition divide at a shorter cell length (11, 22, 31). Based on such hallmarks of mutational lesions of the mitotic control, it was previously reported that Mcs4-deficient cells are delayed in the

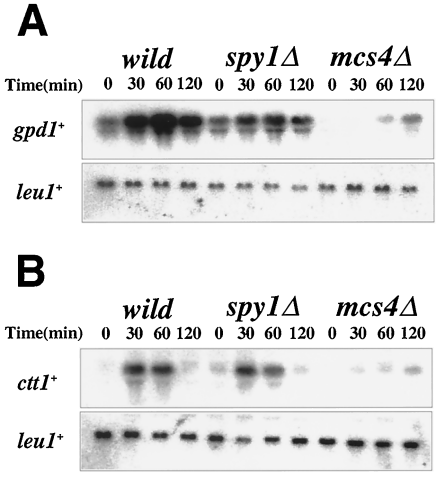

FIG. 5. Spy1-deficient strain shows normal stress responses. (A) Northern hybridization showing osmotic induction of *gpd1*$^+$ mRNA in the *spy1*Δ mutant. RNA was prepared before and after the addition of 0.9 M KCl at the indicated time and hybridized with *gpd1*$^+$ probe. The same filter was also hybridized with the *leu1*$^+$ probe for the control of the loading amount. (B) Northern hybridization showing oxidative induction of *ctt1*$^+$ mRNA in the *spy1*Δ mutant. RNA was prepared before and after the addition of 1 mM H_2O_2 at the indicated time and hybridized with *ctt1*$^+$ probe. The same filter was also hybridized with *leu1*$^+$ probe for control of the loading amount.

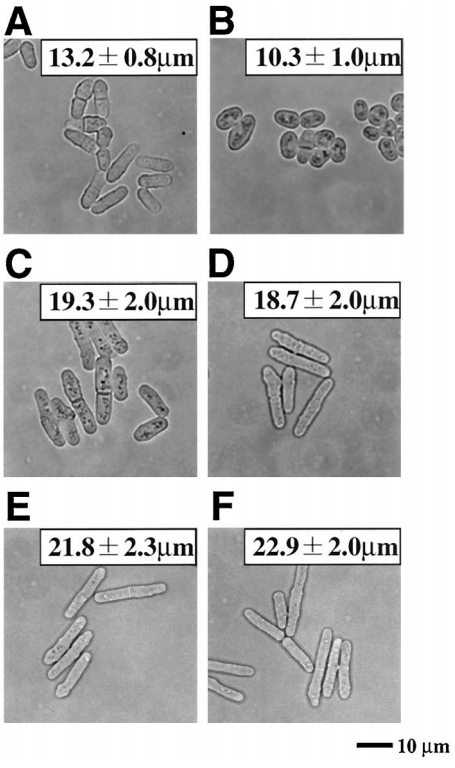

FIG. 6. Initiation of mitosis is accelerated in the *spy1*Δ mutant. (A) Wild type. (B) *spy1*Δ. (C) *mcs4*Δ. (D) *mcs4*Δ *spy1*Δ. (E) *sty1*Δ. (F) *sty1*Δ *spy1*Δ. Each of indicated cell types growing exponentially in EMM medium at 30°C was photographed. The average cell size of septated cells was determined from 20 individuals (± standard deviation).

timing of the G_2/M transition, thereby exhibiting an elongated cell size, as confirmed in Fig. 6C (8, 33, 37). Spy1-deficient cells were then assessed in terms of this particular aspect.

To assess the timing of cell division, Spy1-deficient cells were grown in a given medium, and then, at the exponential growth phase, they were observed under a phase-contrast microscope. The average cell length at division (i.e., length of septated cell) was statistically measured (Fig. 6B). As mentioned above, Mcs4-deficient cells were elongated (Fig. 6C; 19.3 ± 2.0 μm) and were significantly longer than wild-type cells (Fig. 6A; 13.2 ± 0.8 μm). This is quite consistent with the previous result, which suggested that the *mcs4*Δ mutant is delayed in the timing of the G_2/M transition (8, 33, 37). In sharp contrast, it was found that Spy1-deficient cells had an ovoid morphology, which was significantly shorter (Fig. 6B; 10.3 ± 1.0 μm) than the wild type. Such a short ovoid morphology is indicative of precocious entry into the M phase. It should be noted that this morphological change of the *spy1*Δ mutant was suppressed by introducing the plasmid-borne *spy1*$^+$ gene, but not by the mutant *spy1-HQ* gene (data not shown).

To conduct critical epistatic analyses, we constructed an *mcs4*Δ and *spy1*Δ double mutant, and also constructed an *sty1*Δ and *spy1*Δ double mutant. As mentioned above, Mcs4 was considered to function upstream of the Sty1 MAPK. The *sty1*Δ single mutant showing an elongated cell size is also impaired in control of the G_2/M transition (Fig. 6E), as well documented previously (21, 35). Here the *mcs4*Δ and *spy1*Δ double mutant showed a cell size (18.7 ± 2.0 μm) very similar to that of the *mcs4*Δ single mutant (Fig. 6C and D). The *sty1*Δ and *spy1*Δ double mutant also showed an elongated cell size (22.9 ± 2.0 μm) very similar to that of the *sty1*Δ single mutant (Fig. 6E and F). These results of epistatic analyses strongly suggest that Spy1 functions upstream of Mcs4 and Sty1 in a presumed linear signaling pathway.

Altogether from the results shown in Fig. 6, one can reasonably propose the following scenario. Spy1, together with Mcs4, is involved in a signaling pathway for mitotic control of the cell cycle in *S. pombe*. Mcs4 is a positive regulator for progression of the G_2/M transition, whereas Spy1 functions upstream of Mcs4 as a negative regulator for Mcs4 through the presumed His-to-Asp phosphorelay, which operates upstream of the Sty1 MAPK cascade.

A link between Spy1 and the mechanism underlying control of the G_2/M transition. If the *spy1*$^+$ gene is indeed involved in a signal transduction pathway that somehow regulates the mitotic cell cycle (particularly, the G_2/M transition), one can expect that the *spy1*Δ mutation should display a genetic interaction with the well-documented main controller of the G_2/M transition [i.e., the Cdc13 (cyclin B)/Cdc2 kinase, the Wee1/Mik1 tyrosine kinases, and the Cdc25 phosphatase]. To address this issue, we employed the well-known *cdc25-22* mutant and constructed its double mutant with *spy1*Δ. They were characterized in terms of the cell length at division, as explained above (Fig. 7). The temperature-sensitive *cdc25-22* mutant showed an elongated cell size even at 30°C (Fig. 7A). (The size was about twice that of the wild type). This mitotic lesion in *cdc25-22* was clearly affected by introduction of the *spy1*Δ mutation, as judged by the fact that the *cdc25-22* and *spy1*Δ double mutant showed a shorter cell size. Furthermore, when the temperature sensitivity for growth of *cdc25-22* on the SD agar plate at 34°C was examined, it was found that the *spy1*Δ mutation served as an extragenic suppressor for growth, at least partially (Fig. 7B). This notion was further supported by the results of appropriate control experiments, in which the *spy1*$^+$ gene was reintroduced into the double mutant. The

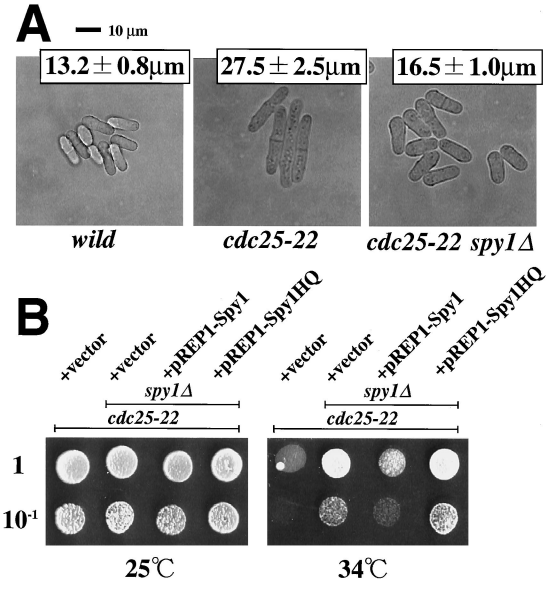

FIG. 7. *spy1*Δ mutation can function as a suppressor for *cdc25-22* mutation. (A) The indicated cell types growing exponentially in EMM medium at 30°C were photographed. The average cell size of septated cells was determined from 20 individuals (± standard deviation). The *cdc25-22* mutant and *cdc25-22 spy1*Δ double mutant which carry each of the plasmids indicated were spotted onto EMM plates at the proper dilution. Cells were grown at 25 or 34°C for 3 days and photographed.

results also revealed the functional importance of the His-221 phosphorylation site of Spy1. However, the temperature-sensitive phenotype of *cdc25-22* was not suppressed at 37°C by introduction of the *spy1*Δ mutation (data not shown). In any event, the observed genetic interactions between the *cdc25* and *spy1* mutations is compatible with the idea that Spy1 plays a role in the signaling pathway of the mitotic control per se.

DISCUSSION

The idea that there is a link between a His-to-Asp phosphorelay system and cell cycle control in *S. pombe* first came from an intriguing finding obtained by three groups (8, 33, 37). They collectively showed that the *mcs4*$^+$ gene, which was originally identified as a suppressor of the mitotic catastrophe phenotype of a *cdc2-3w wee1-50* double mutant (24), encodes a response regulator, named Mcs4, which acts upstream of the Wak1 (or Wik1)-Wis1-Sty1 (or Spc1) stress-activated MAPK cascade. Based on these findings, it was considered that a His-to-Asp phosphorelay involving the Mcs4 response regulator is part of a sensor system for multiple environmental signals that modulates the timing of entry into mitosis by regulating the Wak1 (Wik1)-Wis1-Sty1 (Spc1) MAPK cascade. Nevertheless, clarification of the presumed His-to-Asp phosphorelay system is at a very early stage. Altogether the results in this study showed that the Spy1 HPt phosphotransmitter functions upstream of the Mcs4 response regulator and most likely regulates Mcs4 function in a negative manner through a direct His-to-Asp phosphorelay. Consequently, Spy1-deficient cells enter mitosis precociously, thereby resulting in a short and ovoid cell morphology. It may also be worth mentioning that such a phenotype of *spy1*Δ is exaggerated when grown on a minimal medium, but is less evident on a rich medium (data not shown).

The fission yeast cell cycle is controlled at two major points: in G_1 at entry into S phase (initiation of DNA replication) and in G_2 at the initiation of mitosis (G_2/M transition). Genetic and physiological studies have revealed that the timing of both transitions requires attainment of a critical cell size (27). The Sty1 MAPK gene was identified as a gene that affects cell size at division. Sty1-deficient cells show an elongated cell morphology, due to the delay of mitosis (21, 35). Sty1 appears to influence, directly or indirectly, the activity of the Cdc2-Cdc13 (cyclin B) cell cycle control machinery by an as yet unknown mechanism that is most likely independent of both the Wee1 tyrosine kinase and Cdc25 protein phosphatase (35). It was previously suggested that the Mcs4 response regulator controls the timing of mitotic initiation by running this Sty1-dependent mechanism in a positive manner (33, 37). Here we showed that Spy1 regulates such a function of Mcs4 in a negative manner. Our genetic results are fully consistent with these views, because the *spy1* mutation showing a precocious entry into mitosis is not evident in both the *mcs4*Δ and *sty1*Δ backgrounds, but it clearly affects the phenotype of the *cdc25-22* mutant. Mcs4 was previously suggested to control the timing of mitosis also through an additional Sty1-independent pathway (33). In this context, our result is indicative that Spy1 functions through a Sty1-dependent pathway. In any case, our results in this study further highlight an important role for the Spy1-Mcs4 phosphorelay system in coordinated cell cycle progression in response to environmental stimuli. Nevertheless, the underlying molecular mechanism through which the presumed Spy1-to-Mcs4 phosphorelay mediates an effect upon the cell cycle control machinery is not clear and appears to be complex, as discussed further below.

It should also be mentioned briefly that Mcs4 is important not only for mitotic control, as mentioned above, but also for osmotic and oxidative stress responses that are dependent on the Sty1 MAPK and the Atf1 and Pap1 bZIP transcriptional factors, as indeed confirmed in this study (Fig. 5) (20, 33, 37). Our results from Spy1-deficient cells did not support the view that Spy1 is also implicated, through Mcs4, in such transcriptional regulation of the *gpd1*$^+$ and *ctt1*$^+$ genes in response to osmotic and oxidative stresses. In any case, the fact that we could not detect any sign of the presumed up-regulation of *gpd1*$^+$ and *ctt1*$^+$ genes in the *spy1*Δ mutant may suggest the occurrence of another *spy1*$^+$-like HPt phosphotransmitter in *S. pombe*. Clarification of this interesting issue must also await further studies. It should also be mentioned that Nguyen et al. recently characterized the function of the *S. pombe mpr1*$^+$ gene, which is identical to *spy1*$^+$, with special emphasis on oxidative stress response (26). They showed that, in their *mpr1*Δ cells, an elevated level of Sty1 phosphorylation did not increase further in response to oxidative stress, whereas the Sty1 phosphorylation in the wild-type cells was markedly induced. It was also shown that Mpr1 binds to Mcs4 in response to oxidative stress. Based on these findings, they proposed the model that oxidative stress stimuli are transmitted by a His-to-Asp (Mpr1/Spy1 to Mcs4) phosphorelay to the Sty1 MAPK cascade. However, they showed that the expression of *ctt1*$^+$ mRNA can be induced normally in *mpr1*Δ cells, as we also have demonstrated (Fig. 5). From the physiological viewpoint, we would like to argue that Spy1 (or Mpr1) is not involved crucially, if at all, in the oxidative stress response, as far as the induction of the *ctt1*$^+$ gene is concerned. Thus, the physiological importance of the *spy1*$^+$-dependent modulation of the Sty1 phosphorylation in response to oxidative stress should be addressed carefully.

The molecular mechanism by which the presumed His-to-Asp phosphorelay involving Spy1 and Mcs4 operates in *S. pombe* is not yet clear. In *S. cerevisiae*, a multistep His-to-Asp phosphorelay that consists of Sln1, Ypd1, and Ssk1 directly regulates the Ssk2/Ssk22 MAPKKKs in osmosensing (20, 43). The currently proposed model is that Sln1 His kinase phosphorylates Ypd1, which in turn negatively regulates Ssk1 through a phosphotransfer. The nonphosphorylated form of Ssk1 functions as a positive regulator of Ssk2/Ssk22. If a homologous phosphorelay operates in *S. pombe*, then, an Sln1-like His kinase is expected to exist. An inspection of the fission yeast databases revealed the presence of (at least) three proteins encoded by typical His kinase genes: sensor 1 with 2,344 amino acids, GenBank accession no. AL031543 (*SPCC74.06*); sensor 2 with 2,310 amino acids, GenBank accession no. Z98978 (*SPAC27E2.09*); and sensor 3 with 1,639 amino acids, GenBank accession no. AL157734 (*SPAC1834*). Each of these predicted proteins, like Sln1, has a typical His kinase domain followed by a receiver domain. Two of them most likely correspond to each of those described previously and named Mak1 and Mak2 (20, 33). In any case, this fact suggests that the situation with regard to the His-to-Asp phosphorelay in the fission yeast is more complex than that in the budding yeast. This is consistent with the fact that the budding yeast HOG1 MAPK cascade appears to be activated only by osmotic stress, while the fission yeast Sty1 MAPK cascade is implicated in a wide range of stress responses (20). In any event, clarification of a link between these multiple His kinases and the Spy1-Mcs4 components is entirely elusive, and experiments along these lines are under way in our laboratory.

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