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Identification of Cdc37 as a Novel Regulator of the Stress-Responsive Mitogen-Activated Protein Kinase

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Eukaryotic cells utilize multiple mitogen-activated protein kinases (MAPKs) to transmit various extracellular stimuli to the nucleus. A subfamily of MAPKs that mediates environmental stress stimuli is also called stress-activated protein kinase (SAPK), which has crucial roles in cellular survival under stress conditions as well as inflammatory responses. Here we report that Cdc37, an evolutionarily conserved kinase-specific chaperone, is a positive regulator of Spc1 SAPK in the fission yeast *Schizosaccharomyces pombe*. Through a genetic screen, we have identified *cdc37* as a mutation that compromises signaling through Spc1 SAPK. The Cdc37 protein physically interacts with Spc1, and the *cdc37* mutation affects both the cellular level of the Spc1 protein and stress-induced Spc1 phosphorylation by Wis1 MAPK kinase (MAPKK). Consistently, expression of the stress response genes regulated by the Spc1 pathway is compromised in *cdc37* mutant cells. On the other hand, a mutation in Hsp90, which often cooperates with Cdc37 in chaperoning protein kinases, does not affect Spc1 SAPK. These results suggest that Spc1 SAPK is a novel client protein for the Cdc37 chaperone, and the Cdc37 function is important to maintain the stability of the Spc1 protein and to facilitate stress signaling from Wis1 MAPKK to Spc1 SAPK.

A mitogen-activated protein kinase (MAPK) cascade is a signaling module ubiquitous among eukaryotes that transmits extracellular stimuli to the nucleus. A MAPK cascade is composed of three conserved kinases, MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK), and signals are transmitted through sequential activation of the three kinases by phosphorylation; stimulus-activated MAPKKK phosphorylates MAPKK, which in turn phosphorylates and activates MAPK. Activated MAPK phosphorylates downstream effector proteins, such as transcription factors, and modulates their function (6, 20, 27, 41). A subfamily of MAPKs dedicated for transmitting environmental stress stimuli, such as high-osmolarity stress, oxidative stress, heat shock, and genotoxic agents, are also known as stress-activated protein kinases (SAPKs). Among those are Hog1 in budding yeast *Saccharomyces cerevisiae* (4), Spc1 (also known as StyI) in fission yeast *Schizosaccharomyces pombe* (29, 43), and mammalian p38 (19, 24). Genetic studies of yeast SAPKs indicate that SAPKs play key roles in cellular stress resistance (18, 34), and mammalian SAPKs are also implicated in inflammation and the response of cancer cells to cytotoxic treatments (23, 53).

The structures and functions of SAPK cascades are highly conserved throughout evolution; like the mammalian p38 pathway, the fission yeast Spc1 cascade, which consists of Wis4 and Win1 MAPKKKs, Wis1 MAPKK, and Spc1 MAPK, is activated by a wide range of stress, including osmostress, oxidative stress, and heat shock (34). Interestingly, the highly homologous Hog1 SAPK cascade in budding yeast is responsive mostly to high-osmolarity stress (42), although a recent report also indicates heat shock-induced activation of Hog1 (55). Upon environmental stress, fission yeast Spc1 phosphorylates and activates the Atf1 transcription factor (44, 54), a fission yeast ortholog of mammalian ATF2, which is also a key substrate of the mammalian SAPKs (17, 39). Atf1 is responsible for expression of a number of stress resistance genes, including those in osmoregulation and oxidative stress responses (9, 38, 44, 54). Because of the high similarity between the fission yeast and mammalian SAPK cascades, the genetic study of the Spc1 pathway provides an excellent opportunity to identify and analyze novel elements in stress signaling by SAPK cascades.

CDC37 was first identified in budding yeast as a gene essential for cell cycle progression in G_1 (13, 40), and structurally related proteins were subsequently identified in *Drosophila melanogaster* and mammalian cells (7, 8, 50). Although the function of Cdc37 was not apparent from its amino acid sequence, recent studies revealed that Cdc37 is a part of the chaperone complex required for the stability and/or activity of some protein kinases (reviewed in reference 21). Budding yeast Cdc28, a cyclin-dependent kinase (Cdk), shows significantly shorter half-lives in *cdc37* mutants (12). Cdc37 is also required for maintaining the oncogenic tyrosine kinase v-Src in a soluble, biologically active form when expressed in budding yeast (11). Mammalian Cdc37 was found as a 50-kDa protein that forms a complex with diverse protein kinases, such as v-Src, Raf, and Cdk (16, 37, 50), while no MAPK has been identified as a target of Cdc37. Complexes of Cdc37 with different protein kinases often contain a molecular chaperone, Hsp90, and it has been proposed that Cdc37 is a kinase-targeting subunit of Hsp90 (50). On the other hand, in vitro experiments using budding yeast Cdc37 suggest that Cdc37 itself has a Hsp90-like chaperone activity (22) and, therefore, Cdc37 may also function independently of Hsp90.

Here we report a genetic screen in fission yeast to identify novel components of the Spc1 SAPK cascade. We have identified *cdc37* as a mutation that inhibits the Spc1 function in response to the ectopic expression of activated Wis1 MAPKK.

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TABLE 1. *S. pombe* strains used in this study

Strain	Genotype[a]	Source or reference
PR109	*h*$^-$	Laboratory stock
CA6	*h*$^-$ *his7-366 ade6-M216*	Laboratory stock
CA7	*h*$^+$ *his7-366 ade6-M216*	Laboratory stock
CA14	*h*$^-$ *spc1*::*ura4*$^+$	Laboratory stock
CA39	*h*$^-$ *atf1*::*ura4*$^+$	Laboratory stock
CA76	*h*$^-$ *spc1*:*HA6H*(*ura4*$^+$)	Laboratory stock
CA178	*h*$^-$ *wis1*:*myc*(*ura4*$^+$)	14
CA211	*h*$^-$ *his7-366 ade6-M216 atf1*::*ura4*$^+$	Laboratory stock
CA212	*h*$^+$ *his7-366 ade6-M210 atf1*::*ura4*$^+$	Laboratory stock
CA1150	*h*$^+$ *his7-366*::*his7*$^+$:*spc1*$^+$ *ade6-M210*	This study
CA1388	*h*$^-$ *cdc37-681*	This study
CA1390	*h*$^-$ *spc1*:*HA6H*(*ura4*$^+$) *cdc37-681*	This study
CA1412	*h*$^+$ *his7-366 ade6-M210 cdc37-681*	This study
CA1417	*h*$^-$ *cdc37-681 atf1*::*ura4*$^+$	This study
CA1496	*h*$^+$ *swo1-26*	3
CA1450	*h*$^+$ *his7-366 wis1*:*myc*(*ura4*$^+$) *cdc37-681*	This study
CA1621	*h*$^-$ *cdc37*:*HA6H*(*ura4*$^+$)	This study
CA1623	*h*$^-$ *cdc37*:*GFP*(*ura4*$^+$)	This study
CA1653	*h*$^-$ *cdc37*:*HA6H*(*ura4*$^+$) *spc1*:*myc*(*ura4*$^+$)	This study
CA1725	*h*$^-$ *his7-366*::*his7*$^+$:*spc1*$^+$ *ade6-M216 atf1*::*ura4*$^+$	This study
CA1726	*h*$^+$ *his7-366*::*his7*$^+$:*spc1*$^+$ *ade6-M210 atf1*::*ura4*$^+$	This study
CA1727	*h*$^+$/$^-$ *his7-366*/*his7 ade6-M210*/*ade6-M216 cdc37*::*ura4*$^+$	This study

[a] All strains are *leu1-32 ura4-D18*.

The Cdc37 protein physically interacts with Spc1 MAPK, and the *cdc37* mutation brings about significant decreases in both the abundance and stress-induced phosphorylation of Spc1. Our study suggests that Cdc37 functions as a molecular chaperone to maintain the stability of Spc1 MAPK as well as to facilitate the phosphorylation of Spc1 by Wis1 MAPKK.

MATERIALS AND METHODS

Yeast strains and general techniques. *S. pombe* strains used in this study are listed in Table 1. Growth media and basic techniques for *S. pombe* have been described previously (2, 30). *S. pombe* cells were grown in YES yeast extract medium and EMM2 synthetic medium.

Integration of the *spc1*$^+$ gene at the *his7* locus. The 2.9-kb *Eco*RI-*Spe*I genomic DNA fragment containing the *spc1*$^+$ gene was cloned into the *Not*I site of pBluescript II (Stratagene), to which the *his7*$^+$ marker gene was inserted at the *Sma*I site. The resultant plasmid was used to transform a wild-type (CA7) *S. pombe* strain. Stable His$^+$ transformants were selected, and the integration of the plasmid construct at the *his7* locus was verified by Southern hybridization analysis. The integrated *spc1*$^+$ gene was able to suppress the osmosensitivity and the temperature sensitivity of the Δ*spc1* strain (data not shown).

Isolation of mutants resistant to Wis1 overexpression. Strains CA1725 and CA1726 were transformed with the pREP1-WIS1DD-HA6H plasmid, which expresses Wis1DD (46), a constitutive active mutant of Wis1 MAPKK under the control of the thiamine-repressible *nmt1* promoter (28). The transformants were grown in liquid EMM2 lacking thiamine (EMM2−T) at 30°C for ~16 h and plated onto EMM2−T at the concentration of $\sim 2 \times 10^7$ cells per plate. After incubation at 30°C for 7 days, growing colonies were picked up and streaked to confirm their growth on EMM2−T. Resultant colonies were subjected to anti-hemagglutinin (anti-HA) immunoblotting to select mutants that had not lost the ability of Wis1 overexpression.

Cloning of the *cdc37*$^+$ gene. A temperature-sensitive strain, CA1388, isolated from the above screen was transformed with an *S. pombe* genomic library and plated onto EMM2 medium. After incubation at 25°C for 7 days, transformants were replica plated onto YES medium and incubated at 37°C for 1 day. The transformants were further replica plated onto fresh YES medium, which was followed by incubation at 37°C for 2 days. Forty-one out of ~80,000 transformants showed colony formation after the second replica plating at 37°C. DNA sequencing and PCR analyses of the recovered plasmids from all of the *ts*$^+$ colonies showed that they all contained the *cdc37*$^+$ gene.

A mutation in the *cdc37* gene in the isolated mutants was further confirmed by determining the mutation site. Wild-type and mutant *cdc37* gene sequences were PCR amplified using genomic DNA from wild-type and the *ts* mutant strains as templates. In all the mutant sequences, the 1,285th T in the *cdc37*$^+$ open reading frame was mutated to C, resulting in the replacement of Leu-285 in the Cdc37 protein with proline.

Construction of *cdc37* null mutant, *cdc37*:*HA6H*, and *cdc37*:*GFP* strains. For *cdc37* gene disruption, 0.5-kb sequences immediately upstream and downstream of the *cdc37*$^+$ open reading frame were amplified by PCR with pairs of primers (SpCdC37disrpN5, GGCGTGGGAAGTAATAGAGT, and SpCdC37disrpN3, TCGCCCTATAGTGAGTCGTACTCGAAGCAAATTATAATTTCAA, for the upstream sequence; SpCdC37disrpC5, CCAGCTTTTGTTCCCTTTAGTA TTTTTCTGCTTACGGGTGTG, and SpCdC37disrpC3, AATGACTATGTAC CTCACTAC, for the downstream sequence). The 1.8-kb *Hin*dIII fragment of the *ura4*$^+$ marker gene was amplified by PCR using the aforementioned 0.5-kb fragments upstream and downstream of *cdc37*$^+$ as primers. The resultant PCR product, a *ura4*$^+$ fragment flanked with the genomic sequences adjacent to *cdc37*$^+$, was used to transform a diploid strain constructed by mating strains CA6 and CA7. Stable Ura$^+$ transformants were isolated, and the disruption of one of the *cdc37* loci was confirmed by Southern hybridization experiments, which were followed by tetrad analysis.

For constructing strains in which the chromosomal *cdc37*$^+$ gene is tagged with the sequences encoding two copies of the HA epitope followed by six consecutive histidines (HA6H) (45) or the green fluorescent protein (GFP), a *Not*I site was introduced by PCR at the 3′ end of the *cdc37*$^+$ sequence. The resultant PCR fragment was digested at the *Hpa*I site within the *cdc37*$^+$ sequence and the 3′-end *Not*I site, and the resultant 1.8-kb *Hpa*I-*Not*I fragment was cloned to construct pBluescript-ΔNCdc37-HA_2His_6 and pBluescript-ΔNCdc37-GFP with the *ura4*$^+$ marker gene. These plasmids were used to transform a wild-type (PR109) strain, and the integration of the plasmids at the *cdc37*$^+$ locus was confirmed by Southern blotting analysis.

Immunoblotting of Spc1 and Wis1 in crude lysate. For Western blotting analyses, *S. pombe* cell lysate was prepared as described previously (52). Cells grown to the mid-log phase were harvested by filtration in the presence of 10% trichloroacetic acid (TCA). Harvested cells were then suspended in 10% TCA and vortexed vigorously with 0.5-mm-diameter glass beads for 5 min at 4°C. After breakage, cell suspensions were centrifuged at 800 × *g* for 10 min, and the supernatant was discarded. The remaining pellets were suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer containing 0.5 M Tris-HCl (pH 8.0) and boiled for 5 min, followed by centrifugation at 16,100 × *g* for 15 min. The solubilized proteins in the supernatant were subjected to immunoblotting with rabbit polyclonal anti-Spc1 antibodies and mouse monoclonal anti-myc antibodies (9E10; BAbCO).

Stress treatments of *S. pombe* cells. Treatments of *S. pombe* cultures with high-osmolarity stress and oxidative stress have been described previously (47). The phosphorylation state of Spc1 in stressed cells was monitored by immunoblotting using anti-phospho-p38 antibodies (Cell Signaling Technology Inc.) that recognize active Spc1 phosphorylated on both Thr-171 and Tyr-173 (45). Northern hybridization analyses of *gpd1*+, *pyp2*+, *ctt1*+, and *leu1* have been described previously (32, 44).

Immunoprecipitation. Immunoprecipitation was performed following the procedure described previously (33). Anti-myc rabbit polyclonal antibodies (A14; Santa Cruz Biotech.) conjugated to protein A-Sepharose (Pharmacia Biotech) were used to precipitate the Spc1-myc or Wis1-myc proteins from the lysate, which was followed by immunoblotting with mouse monoclonal anti-myc antibodies (9E10; BAbCO), mouse monoclonal anti-HA antibodies (12CA5; Boehringer Mannheim), or rabbit polyclonal anti-Spc1 antibodies.

Fluorescence microscopy. To examine the cellular localization of the Cdc37 protein, the *cdc37*:*GFP* strain (CA1623) grown to the early mid-log phase in YES medium was observed with an Eclipse E600 microscope (Nikon) equipped with a 100× objective lens and a digital charge-coupled device camera (Hamamatsu). Chromosomal DNA was stained with Hoechst 33342 (Sigma) as described previously (5). Images were captured by using the Openlab software (Improvision) and transferred to Adobe Photoshop (Adobe Systems) for figure preparation.

RESULTS

Isolation of mutants resistant to the overexpression of Wis1 MAPKK. Aiming to identify new components of the SAPK cascade, we performed a genetic screen in *S. pombe*, the rationale of which is illustrated in Fig. 1. Overexpression of Wis1

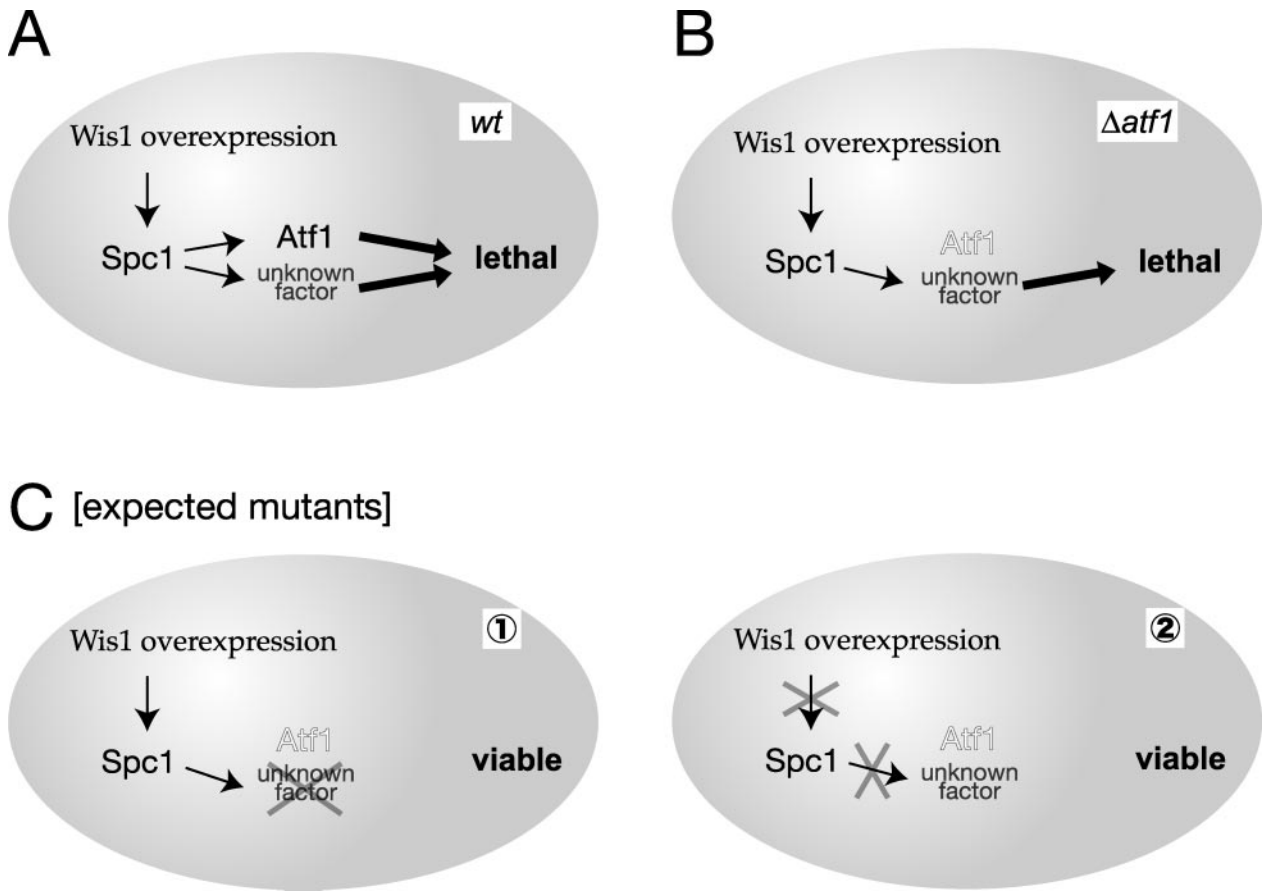


FIG. 1. Strategy of the genetic screen. (A) In wild-type cells, Wis1 overexpression leads to hyperactivation of Spc1, which causes cellular lethality through Atf1 and an unknown factor. (B) In Δ*atf1* mutant cells, Spc1 activated by Wis1 overexpression brings about cellular lethality through an unknown factor. (C) The toxicity of Wis1 overexpression is expected to be suppressed in mutants defective in (i) the unknown target of Spc1 MAPK or (ii) factors required for activity and/or function of Spc1 MAPK.

MAPKK results in a lethal phenotype accompanied by cell lysis, partly due to a defect in cellular osmoregulation (43, 44). This lethal phenotype is caused by deregulated hyperactivation of Spc1 MAPK and is suppressed by the *spc1* null (Δ*spc1*) mutation. However, Wis1 overexpression brings about a severe growth defect even in the absence of Atf1, the only known target transcription factor for Spc1, although the cell lysis phenotype is suppressed by the Δ*atf1* mutation (Fig. 1B) (44). This observation implies that hyperactivated Spc1 MAPK phosphorylates an unknown factor in addition to Atf1, leading to a growth defect. Therefore, we screened for mutations that rescue the lethality of Wis1 overexpression in Δ*atf1* strains (Fig. 1C), because they would include mutations in (i) the unknown factor downstream of Spc1, or (ii) novel factors required for activation or function of Spc1 MAPK.

Three classes of unwanted mutants could be isolated in the suppressor mutation screen described above. First, mutants defective in transcription from the Wis1 overexpression plasmid would be isolated. We used HA epitope-tagged Wis1 for overexpression and eliminated this class of mutants by measuring the Wis1-HA protein level by anti-HA immunoblotting. Second, mutations in upstream components that positively regulate Wis1, such as Wis4 and Win1 MAPKKKs, would weaken the toxicity of Wis1 overexpression. To eliminate this possibility, we overexpressed the constitutively active form of Wis1, Wis1DD, in which the MAPKKK phosphorylation sites in Wis1 are substituted with aspartic acid residues that mimic phosphorylation (46). Third, as mentioned above, *spc1* mutations completely repress the lethality by Wis1 overexpression (43). To avoid isolating *spc1* mutations, we used strains in which an additional copy of the $spc1^+$ gene was integrated in the genome, as it is unlikely that two copies of $spc1^+$ are mutated at the same time when we screen for spontaneous mutations.

Out of $\sim 10^9$ cells plated, about 1,000 viable colonies appeared spontaneously under the Wis1 overexpression condition. When 184 isolates were examined by anti-HA immunoblotting, 61 of them were confirmed for the overexpression of Wis1DD-HA. Subsequent genetic analyses showed that a mutant, named *sws1-681* (suppressor of Wis1 overexpression), exhibited a temperature-sensitive (*ts*) growth phenotype (see Fig. 3B) in addition to the resistance to Wis1 overexpression. This report focuses on the detailed study of the *sws1* mutation.

Atf1-dependent gene expression is defective in the *sws1* mutant. As described above, the *sws1* mutation may represent an unknown, Atf1-independent branch downstream of Spc1 MAPK or a novel factor required for activation and/or function of Spc1 (Fig. 1C). In order to distinguish these two possibilities, we examined whether the *sws1-681* mutation

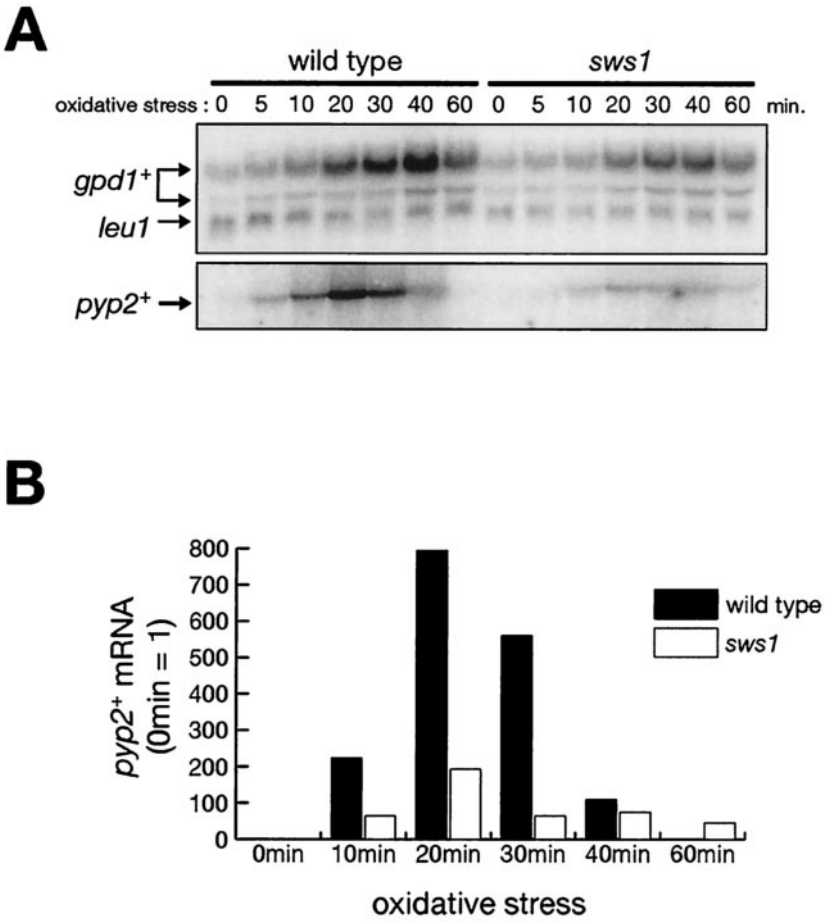


FIG. 2. The *sws1* mutant is defective in the expression of the stress-response genes regulated by Atf1. (A) Wild-type (PR109) and *sws1-681* (CA1388) strains were grown to the mid-log phase at 30°C in YES medium and treated with oxidative stress induced by 0.3 mM H_2O_2. Aliquots of cells were taken at the indicated times, and total RNA was extracted for the Northern blot analysis of $gpd1^+$ and $pyp2^+$ mRNA. The *leu1* probe served as a loading control. (B) The levels of $pyp2^+$ mRNA in the experiment shown in panel A were quantified and normalized by the *leu1* mRNA levels using the Storm system (Molecular Dynamics). Numbers are in arbitrary units.

affects the activity of Atf1 in stress-induced gene expression. Three genes of which transcription is induced by the Spc1-Atf1 pathway upon stress were studied by Northern blotting analyses: $gpd1^+$(encoding glycerol-3-phosphate dehydrogenase), $pyp2^+$(encoding tyrosine-specific phosphatase, which dephosphorylates and inactivates Spc1 MAPK), and $ctt1^+$ (encoding catalase, an enzyme which decomposes hydrogen peroxide) (10, 32, 44, 54). In wild-type cells, $gpd1^+$ mRNA was induced within 10 min of oxidative stress by 0.3 mM H_2O_2 and reached the maximum level at 40 min (Fig. 2A). On the other hand, in the *sws1-681* mutant, the level of $gpd1^+$ mRNA was reduced to approximately 50% of wild type, while the kinetics of induction was similar to that in wild-type cells. The effect of the *sws1* mutation was more obvious in the $pyp2^+$ expression. In the wild-type strain, $pyp2^+$ expression was detected at 5 min after exposure to the stress, with maximum induction at 20 min. In the *sws1* mutant, $pyp2^+$ expression upon stress was significantly compromised, and the maximum level of $pyp2^+$ mRNA was approximately 25% of that in wild-type cells (Fig. 2). We observed that the stress-induced expression of $ctt1^+$ was also defective in *sws1-681* cells (data not shown). These results indicate that expression of the Atf1-dependent genes is compromised in the *sws1-681* mutant.

The conclusion above implies that the *sws1* mutation represents a factor required for activation and/or function of Spc1 MAPK, rather than an Atf1-independent pathway downstream of Spc1. Although the *sws1-681* mutation was originally isolated in the Δ*atf1* background, this model predicts that, like *spc1* mutations, the *sws1* mutation suppresses the phenotypes of Wis1 MAPKK overexpression even in the presence of $atf1^+$. As expected, the *sws1-681* $atf1^+$ strain formed viable colonies even when Wis1 was overexpressed (Fig. 3A). Taken altogether, these results imply that the *sws1* mutant is defective in the activation and/or function of Spc1 MAPK.

***sws1* is allelic to $cdc37^+$.** In addition to the resistance to Wis1 overexpression, *sws1-681* cells exhibited a *ts* growth phenotype, and they stopped dividing at temperatures above 36°C (Fig. 3B). Heterozygous diploids constructed by mating wild-type and *sws1-681* strains showed neither of the phenotypes, indicating that *sws1-681* is a recessive mutation (data not shown). In order to identify the $sws1^+$ gene, haploid *sws1-681* cells were transformed with an *S. pombe* genomic library, and the plasmid

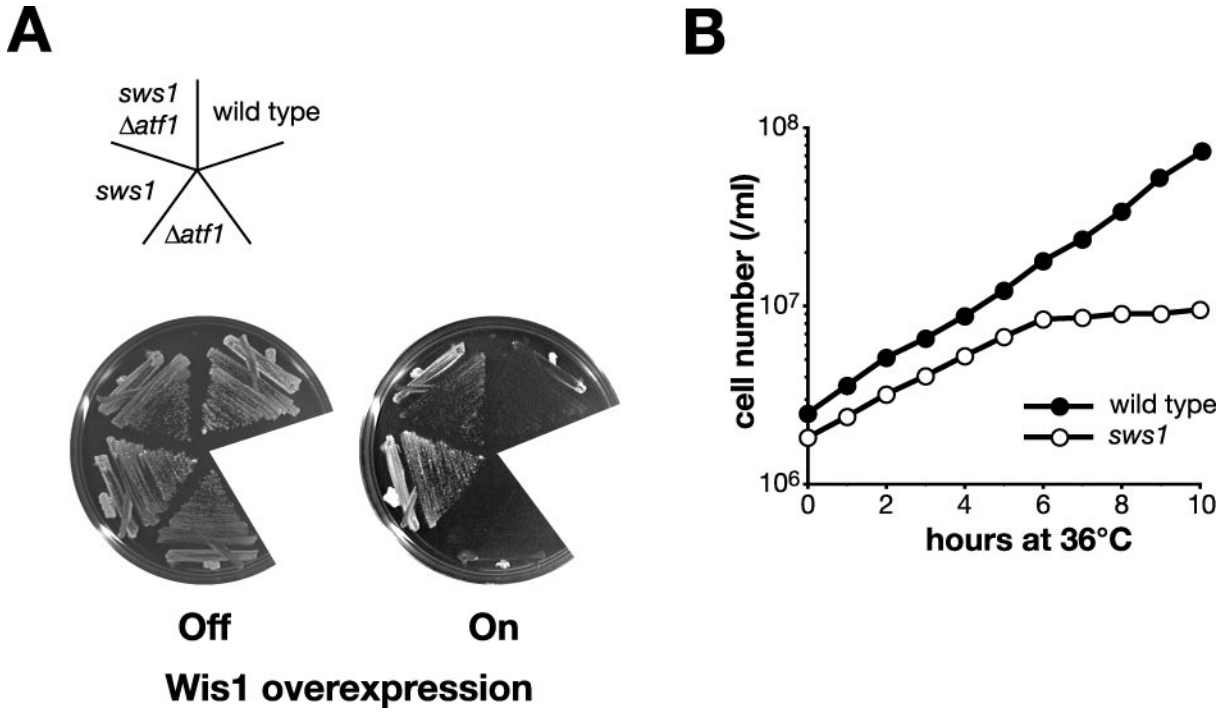


FIG. 3. (A) The *sws1* mutation suppresses the lethality of Wis1 overexpression even in the presence of Atf1. Wild-type (PR109), Δ*atf1* (CA211), *sws1-681* (CA1388), and *sws1-681* Δ*atf1* (CA1417) strains were transformed with the pREP1-WIS1DD plasmid, which expresses the constitutively active mutant form of Wis1 under the regulation of the thiamine-repressible *nmt1* promoter. Transformants were streaked on EMM2 with (Off) or without (On) thiamine and grown for 3 days at 30°C. The *sws1-681* mutant grew as well as the *sws1-681* Δ*atf1* double mutant even when Wis1 was overexpressed. (B) Temperature-sensitive growth of the *sws1-681* mutant. Wild-type (PR109) and *sws1-681* (CA1388) strains were grown to the early log phase at 30°C in YES medium. At time zero, the cultures were shifted to 36°C and the cell number was monitored along the time course using a Coulter counter (Beckman Coulter).

clones that complement the *sws1 ts* phenotype were isolated. Among ~80,000 transformants screened, 41 of them showed growth at 37°C, and all the plasmids recovered from the ts^+ colonies were found to contain the $cdc37^+$ gene (GenBank accession number AJ132376). As shown in Fig. 4A, one of the plasmids isolated from the library, pDB248-CDC37, which contains $cdc37^+$ and an adjacent open reading frame, *SPBC9B6.09c*, suppressed the *sws1 ts* phenotype. We subcloned the open reading frame of $cdc37^+$ into the *S. pombe* expression vector pREP1 (28), and the resultant plasmid, pREP1-CDC37, was also capable of suppressing the *ts* phenotype of *sws1* cells (Fig. 4A), indicating the complementation of *sws1-681* by the $cdc37^+$ gene. In addition, a 1-bp substitution that changes Leu-285 of the Cdc37 protein to proline was found in the *cdc37* gene cloned from the *sws1-681* mutant (Fig. 4B). Replacement of this mutated sequence with the wild-type $cdc37^+$ sequence by homologous recombination rescued the *sws1 ts* phenotype (data not shown). Taken together, we concluded that $sws1^+$ is identical to $cdc37^+$ and, hereafter, we refer to *sws1-681* as *cdc37-681*.

Fission yeast $cdc37^+$ encodes a 466-amino-acid, 53-kDa protein with a significant sequence similarity to orthologs in humans, mice, chickens, flies, worms, and budding yeasts. To investigate the cellular function of fission yeast Cdc37, we performed a gene disruption experiment. The entire open reading frame of one of the $cdc37^+$ genes in wild-type diploid cells was replaced with the $ura4^+$ marker gene by homologous recombination (see Materials and Methods). Sporulation of the resultant heterozygous diploid followed by tetrad analysis revealed that each tetrad produced two viable segregants of the *wis4* mutant and two inviable segregants, indicating that the $cdc37^+$ gene is essential for cellular viability. Most of the mutant *cdc37* haploid segregants divided several times after germination to form microcolonies of very short cells with one or two elongated cells (Fig. 4C). Thus, Cdc37 has functions essential for vegetative cell growth of *S. pombe*, which contrasts with the fact that the Spc1 MAPK cascade is dispensable for cell viability in the absence of environmental stress (29, 43).

Both the amount of and the stress-induced phosphorylation of Spc1 are reduced in the *cdc37* mutant. Cdc37 is important for the stability and/or activity of several kinases, such as v-Src, Raf, and Cdk (see introduction). Therefore, it is possible that Cdc37 may also regulate Spc1 MAPK or Wis1 MAPKK in fission yeast, although no MAPK or MAPKK has been reported to require Cdc37 for the kinase function. To examine this possibility, we compared wild-type and *cdc37* mutant cells for the amounts of Spc1 and Wis1 as well as Spc1 activation in response to stress. The cell lysate was prepared from *wis1:myc* and *cdc37-681 wis1:myc* strains, in which chromosomal $wis1^+$ is tagged with the sequence encoding the *myc* epitope (14), and the protein levels of Spc1 and Wis1 were evaluated by anti-Spc1 and anti-myc immunoblotting, respectively (Fig. 5A). In the *cdc37* mutant, the amount of Spc1 protein was reduced to 30 to 50% of that in wild-type cells, whereas the level of Wis1 MAPKK showed little difference between the two strains. Northern blotting experiments showed that the $spc1^+$ mRNA

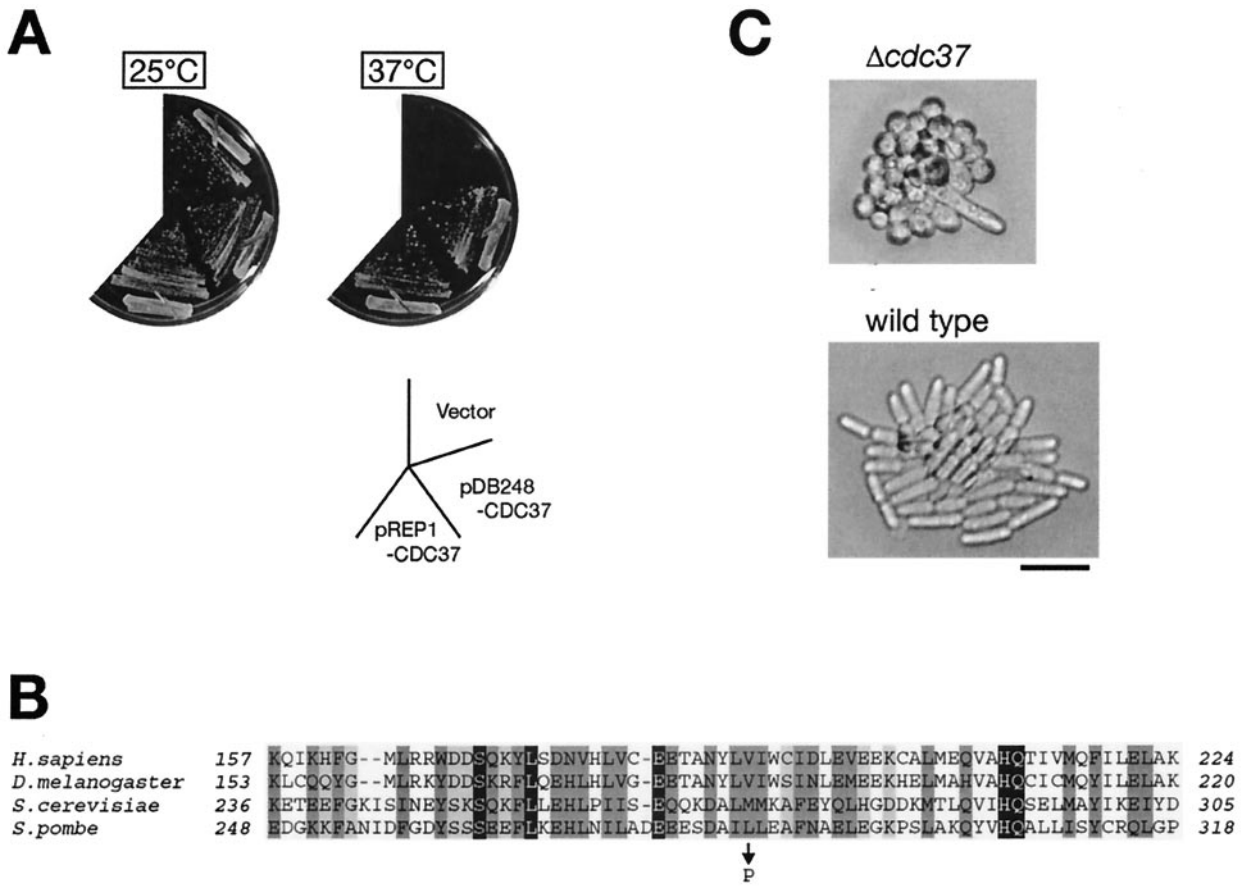


FIG. 4. *sws1* is allelic to fission yeast *cdc37*$^+$. (A) The temperature-sensitive (*ts*) growth phenotype of the *sws1-681* mutant is suppressed by the *cdc37*$^+$ gene. A *sws1-681* strain (CA1388) was transformed with the empty vector, pDB248-CDC37, or pREP1-CDC37 plasmids, and the transformants were incubated at the permissive (25°C) or restrictive (37°C) temperature for the *sws1-681* mutant. The pDB248-CDC37 plasmid was obtained from the *S. pombe* genomic library for its ability to complement the *sws1-681 ts* phenotype, and it contains the *cdc37*$^+$ gene and an adjacent gene, SPBC9B6.09c. The pREP1-CDC37 expression plasmid contains only the open reading frame of *cdc37*$^+$ under the regulation of the *nmt1* promoter. (B) Leu-285 of Cdc37 is changed to proline in the *sws1-681* mutant. The mutation site and the surrounding sequences of *S. pombe* Cdc37 are aligned with the corresponding regions of Cdc37 orthologs in the human (*Homo sapiens*), fruit fly (*D. melanogaster*), and budding yeast (*S. cerevisiae*) sequences by a multiple alignment program, ClustalW (http://clustalw.genome.ad.jp/). (C) Heterozygous diploid cells of *cdc37*$^+$/Δ*cdc37* were sporulated, and the spores dissected on a YES plate were incubated at 25°C for 3 days. Δ*cdc37* mutant cells show a growth arrest phenotype with abnormally short cell lengths. Bar, 10 μm.

was not affected by the *cdc37-681* mutation (Fig. 5B), and Cdc37 may affect the stability of the Spc1 protein, as previously reported for other kinases (12, 50). On the other hand, while Cdc37 is known to function together with Hsp90 in the chaperoning of Raf1, Cdk, and other kinases, we found that Spc1 MAPK was not affected by a defect in Hsp90. *S. pombe* has only one Hsp90 gene, of which mutation, *swo1-26*, brings about the destabilization of the protein kinase Wee1 in cell cycle regulation (3); Swo1 Hsp90 binds to the Wee1 kinase and probably functions as a molecular chaperone. As shown in Fig. 5C, the protein level of Spc1 was affected by the *cdc37* mutation but not by *swo1-26*, implying that Cdc37 regulates Spc1 MAPK independently of Hsp90.

In order to examine whether *cdc37* affects the stress-induced activation of Spc1 MAPK, phosphorylation of Spc1 was monitored in wild-type and *cdc37-681* mutant strains exposed to high osmolarity and oxidative stress. In these strains, the chromosomal *spc1*$^+$ gene was tagged with the sequence encoding the HA epitope followed by six consecutive histidine residues (HA6H), so that Spc1 was easily purified by Ni-nitrilotriacetic acid beads and analyzed by immunoblotting with anti-HA antibodies as well as antibodies that cross-react with the phosphorylated, active form of Spc1 (45). As shown in Fig. 6A, stress-induced phosphorylation of Spc1 dramatically decreased in the *cdc37* mutant. Quantification of Spc1 phosphorylation followed by normalization with the amount of Spc1 protein indicated that the level of Spc1 phosphorylation upon oxidative stress in *cdc37-681* was only 20% of that in wild-type cells (Fig. 6A, lower panel). A significant reduction in Spc1 phosphorylation was also detected in *cdc37-681* cells exposed to high-osmolarity stress (data not shown). Consistently, immunofluorescence microscopy with anti-Spc1 antibodies showed that osmostress-induced nuclear accumulation of Spc1, which is dependent on Spc1 phosphorylation (14), was also significantly compromised in *cdc37-681* cells (data not shown).

Wis1 has a MAPK-docking sequence, and the interaction between Wis1 and Spc1 contributes to the efficient phosphorylation of Spc1 by Wis1 (31). Since the *cdc37* mutant showed

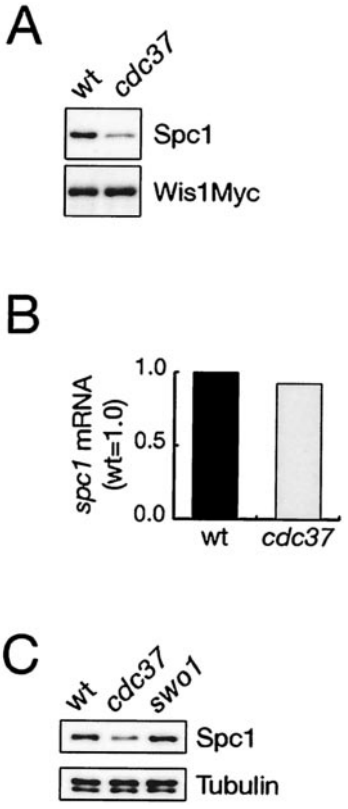


FIG. 5. The cellular level of Spc1 MAPK, but not Wis1 MAPKK, is reduced in the *cdc37* mutant. (A) Wild-type (CA178) and *cdc37-681* (CA1450) strains, in which chromosomal *wis1*$^+$ is tagged with the sequence encoding the *myc* epitope, were grown to the mid-log phase at 30°C in YES medium, and their cell lysate was subjected to immunoblotting with anti-Spc1 and anti-myc antibodies to examine the protein levels of Spc1 and Wis1myc, respectively. (B) Wild-type (PR109) and *cdc37-681* (CA1388) cells exponentially growing in YES medium at 30°C were harvested and subjected to a Northern hybridization analysis to quantify the *spc1*$^+$ mRNA levels. The results were quantified and normalized by the *leu1* mRNA levels using the Storm system (Molecular Dynamics). (C) The cellular levels of Spc1 MAPK in wild-type (PR109), *cdc37-681* (CA1388) and *swo1-26* (CA1496) strains were examined by anti-Spc1 immunoblotting as described for panel A. The protein level of Spc1 is not affected by the Hsp90 mutation, *swo1-26*. Immunoblotting with anti-tubulin antibodies (lower panel) showed that the amounts of the cell lysate loaded to each lane were comparable.

reduced phosphorylation of Spc1 MAPK by Wis1 MAPKK, we examined the physical interaction between Wis1 and Spc1 in the *cdc37* mutant by coprecipitation experiments. When Wis1 was immunoprecipitated with anti-myc antibodies from the cell lysate of a *wis1*:*myc* strain, copurified Spc1 was detected by anti-Spc1 immunoblotting (Fig. 6B). On the other hand, very little Spc1 was coprecipitated with Wis1 from a *wis1*:*myc cdc37-681* strain, while a detectable level of Spc1 was present in the cell lysate (Fig. 6B, bottom panel). Thus, the physical interaction between Wis1 MAPKK and Spc1 MAPK seems to be reduced in *cdc37-681*, and this defect is likely to contribute to the decrease in Spc1 phosphorylation in this mutant.

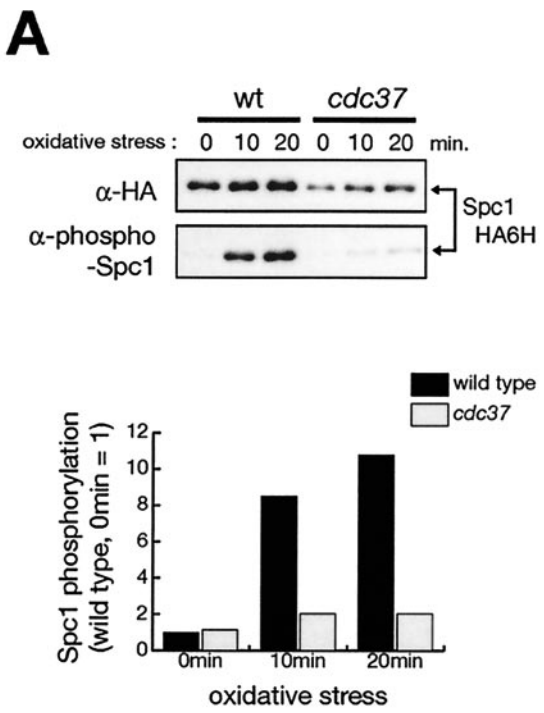
These results suggest that Cdc37 plays a significant role in the stable expression of the Spc1 protein and its interaction with Wis1, which are important for stress signaling from Wis1 MAPKK to Spc1 MAPK.

Cdc37 physically interacts with Spc1 MAPK in vivo. The Cdc37 protein binds to protein kinases, such as v-Src and Cdk, as a molecular chaperone important for the stability and/or activity of those kinases. Results described above suggest that Cdc37 is also important for the stable expression and function of Spc1 MAPK in *S. pombe*, implying a role for Cdc37 as a molecular chaperone for this SAPK. In order to examine whether Cdc37 interacts physically with Spc1, we tested the copurification of Cdc37 with Spc1 from the cell lysate. Spc1 was isolated by anti-myc immunoprecipitation from a *spc1*:*myc cdc37*:*HA6H* strain, in which chromosomal *spc1*$^+$ and *cdc37*$^+$ are tagged with the sequences encoding the *myc* epitope and HA6H, respectively. As shown in Fig. 7, anti-HA immunoblotting detected Cdc37HA6H coprecipitating with Spc1myc, while Cdc37HA6H was not detectable in the immunoprecipitates from a control strain expressing untagged Spc1 (Fig. 7, *spc1*$^+$). Similar experiments using the lysate prepared from cells exposed to high osmolarity and oxidative stress were also performed; anti-myc antibodies precipitated phosphorylated Spc1myc and a slightly reduced amount of Cdc37HA6H, while the protein level of Cdc37 in the cell lysate showed little change before and after stresses (Fig. 7, bottom panel). Thus, Cdc37 physically interacts with Spc1 in vivo, a result consistent with the notion that Cdc37 functions as a molecular chaperone for Spc1 MAPK.

Cellular localization of the Cdc37 protein. In response to stress, both Spc1 MAPK and Wis1 MAPKK show dynamic changes in their cellular localization (14, 15, 31). Under normal growth conditions, Spc1 is found throughout the cell, while Wis1 is found exclusively in the cytoplasm due to its nuclear export signal sequence. Once cells are exposed to osmostress, both proteins are translocated into the nucleus within a few minutes. Because of the detected interaction between Cdc37 and Spc1, the cellular localization of Cdc37 was studied both in the presence and absence of stress. We constructed an *S. pombe* strain in which the chromosomal *cdc37*$^+$ gene was tagged with the sequence encoding green fluorescent protein (GFP). The resultant *cdc37*:*GFP* strain showed no apparent growth defect at different temperatures tested (data not shown), indicating that the Cdc37GFP fusion protein is functional. Fluorescence microscopy of Cdc37GFP in living cells showed that the Cdc37 protein was located throughout the cell, with prominent localization in the chromatin region of the nucleus (Fig. 8A, upper panel, and B); within the chromatin region, one or two bright dots of Cdc37GFP signal were consistently observed (Fig. 8A, upper panel, and B). The cytoplasmic staining of Cdc37GFP was somewhat uneven, probably due to subcellular compartments in the cytoplasm. In contrast to Spc1 and Wis1, we did not observe a dramatic change in the localization of Cdc37 when cells were treated by high-osmolarity stress (Fig. 8A, lower panel), although the Cdc37GFP signal in the chromatin region became less marked.

DISCUSSION

Previous studies strongly suggest that Cdc37 is an evolutionarily conserved molecular chaperone specific for protein kinases (21), and its functions are essential for cell growth in both budding yeast (40) and fission yeast (this study). Cdc37 is expressed at high levels in some cancer cells (51), and the



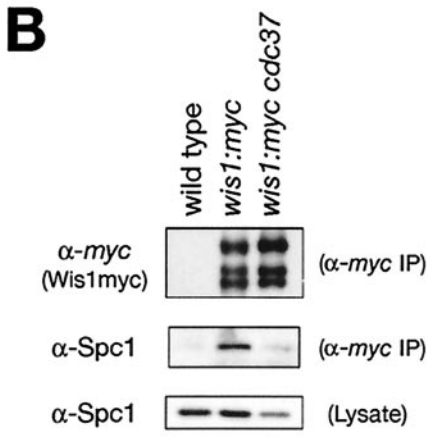


FIG. 6. Stress-induced phosphorylation of Spc1 MAPK is impaired in the *cdc37* mutant. (A) Wild-type (CA76) and *cdc37-681* (CA1390) strains carrying the *spc1*:*HA6H* allele were grown to the mid-log phase at 30°C in YES medium and treated with oxidative stress induced by 0.3 mM H_2O_2. Aliquots of cells were harvested at the indicated times, and the Spc1HA6H protein was purified by Ni-nitrilotriacetic acid chromatography followed by immunoblotting with anti-phospho-p38 MAPK to detect phosphorylated Spc1 as well as with anti-HA antibodies. Signals of anti-phospho-p38 antibodies were quantified by the Storm system (Molecular Dynamics) and plotted after normalization to signals of anti-HA antibodies. (B) The physical interaction between Spc1 MAPK and Wis1 MAPKK is compromised in the *cdc37* mutant. Anti-myc immunoprecipitation was performed with the cell lysate from wild-type (PR109), *wis1:myc* (CA178), and *wis1:myc cdc37-681* (CA1450) strains, and the precipitates were analyzed by immunoblotting with anti-myc antibodies (top panel) to detect Wis1myc and anti-Spc1 antibodies (middle panel). The protein levels of Spc1 in the cell lysate used in this experiment were also measured by anti-Spc1 immunoblotting (bottom panel).

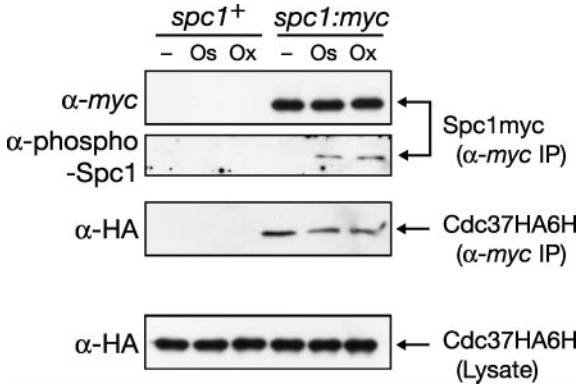


FIG. 7. Cdc37 physically interacts with Spc1 MAPK. *spc1*$^+$ (CA1621) and *spc1:myc* (CA1653) strains, in which chromosomal *cdc37*$^+$ is tagged with the sequence encoding the HA6H tag, were grown to the mid-log phase at 30°C in YES medium. Aliquots of cells were harvested before (−) or after treatments with either high-osmolarity stress induced by 0.6 M KCl (Os) or oxidative stress induced by 0.3 mM H_2O_2 (Ox). Crude lysate was prepared, and the Spc1myc protein was precipitated with protein A-Sepharose beads conjugated with anti-myc antibodies, followed by immunoblotting with anti-myc, anti-phospho-p38, and anti-HA antibodies. The protein levels of Cdc37HA6H in the crude lysate used in this experiment were also examined by anti-HA immunoblotting (bottom panel). Cdc37HA6H was coprecipitated with Spc1myc but not from the lysate of *spc1*$^+$ cells.

ectopic overexpression of Cdc37 in mice promotes cellular transformation (49). However, only a limited number of protein kinases have been demonstrated as clients for the Cdc37 chaperone. In this study, we identified Cdc37 as a positive regulator of the fission yeast Spc1, a member of the evolutionarily conserved stress-activated MAPK subfamily.

In the present study, we have obtained genetic and biochemical data suggesting that Cdc37 plays an important role in the SAPK pathway in *S. pombe*. First, *cdc37* was identified as a mutation that suppresses aberrant Spc1 signaling induced by overexpression of Wis1 MAPKK. Second, Cdc37 forms a complex with Spc1 in vivo, and in the *cdc37* mutant the protein level of Spc1, but not the *spc1*$^+$ mRNA, is reduced. Third, the interaction of Spc1 with Wis1 MAPKK is compromised in the *cdc37* mutant, and stress-induced phosphorylation of Spc1 by Wis1 is significantly reduced. Consistently, the expression of *gpd1*$^+$, *ctt1*$^+$, and *pyp2*$^+$ genes, which is induced upon stress by activated Spc1 through the Atf1 transcription factor, is also compromised in the *cdc37* mutant. These results support the notion that Spc1 MAPK is a novel target for the Cdc37 chaperone. Interaction with Cdc37 may stabilize the Spc1 protein and maintain Spc1 in a properly folded state competent for the interaction with Wis1 MAPKK.

Some studies strongly suggest that the function of Cdc37 is to target the Hsp90 chaperone machinery to protein kinases by interacting with both Hsp90 and kinases (16, 50). In addition, mutational inactivation of Cdc37 and Hsp90 similarly affect the stability and/or function of v-Src and Ste11 MAPKKK in budding yeast (1, 11, 26, 56) and the *sevenless* receptor tyrosine kinase pathway in *Drosophila* (7), indicating the cooperative action of Cdc37 and Hsp90. The genome sequence of *S. pombe* contains only one Hsp90 gene, *swo1*$^+$ (3); Swo1 binds to a protein kinase, Wee1, and the Wee1 protein is destabilized in the *swo1-26* mutant, suggesting the chaperone function of the

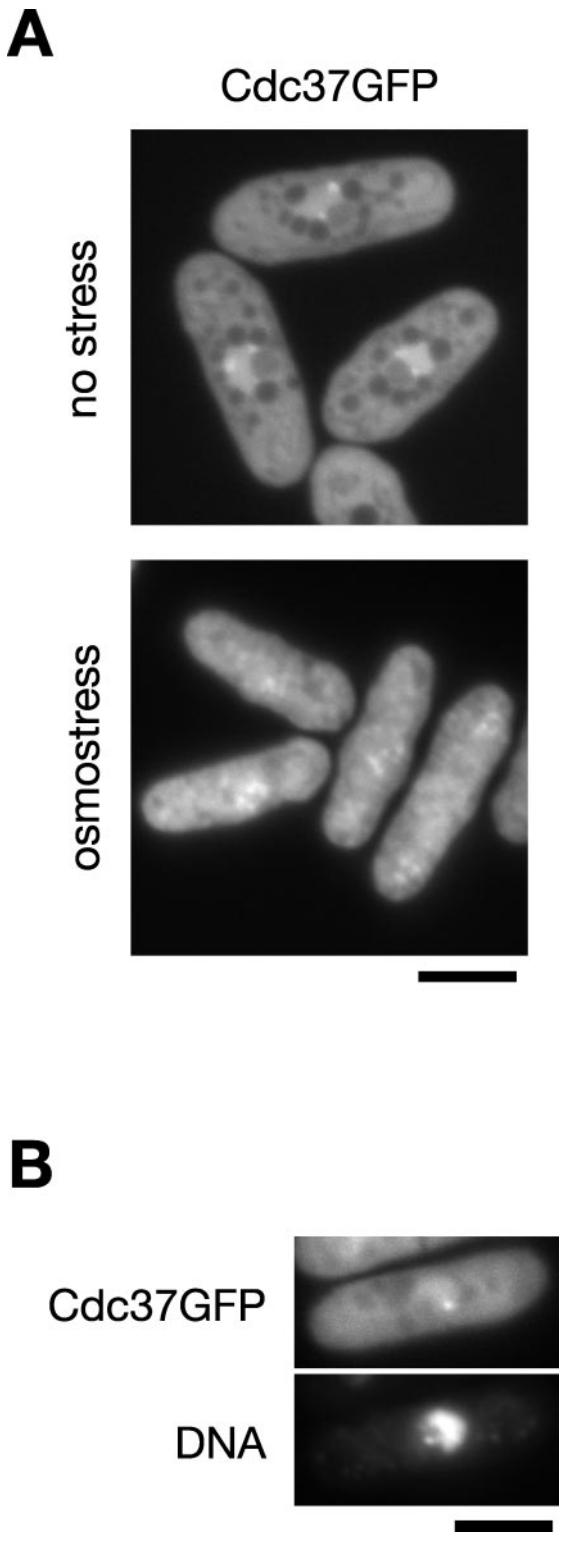


FIG. 8. Cellular localization of Cdc37 in *S. pombe*. (A) Strain CA1623, of which the chromosomal *cdc37*$^+$ gene was tagged with the sequence encoding GFP, was grown to the early log phase and observed by fluorescence microscopy in the presence (lower panel) or absence (upper panel) of high-osmolarity stress induced by 0.6 M KCl. (B) Chromosomal DNA in strain CA1623 was stained with Hoechst 33342, and cells were incubated in YES liquid medium. After 2 h of incubation, cells were observed with fluorescence microscopy for Cdc37GFP and DNA. Bar, 5 μm.

Swo1 Hsp90 for the Wee1 kinase. In contrast, no apparent defect in the stability and activation of Spc1 MAPK was observed in the *swo1-26* mutant and, therefore, the Hsp90 function does not appear to be important for Spc1. Interestingly, an Hsp90-like chaperone activity of the Cdc37 protein has been detected in vitro, and Cdc37 is able to perform the chaperone function independently of Hsp90 at least when overexpressed in budding yeast (22, 25). It is possible that Cdc37 and Hsp90 have some distinct functions in vivo through the regulation of different protein kinases.

Although the amount of active Spc1 MAPK is dramatically reduced in the *cdc37-681* mutant, the mutant cells do not show apparent growth defects under environmental stresses; *cdc37-681* cells are not sensitive to the high osmolarity of 1 M KCl, and the viability of the mutant cells exposed to oxidative stress by H_2O_2 is comparable to that of wild-type cells (data not shown). Consistently, in the *cdc37-681* mutant, Spc1-dependent phosphorylation of Atf1 is detectable (data not shown), and the stress response genes regulated by the Spc1-Atf1 pathway are induced upon stress, although the induction levels of those genes are lower than those in wild-type cells (Fig. 7). Thus, the remaining activity of Spc1 in the *cdc37-681* mutant may be sufficient for the cellular survival of stress at least under the conditions tested. The stress sensitivity is also not obvious in the Wis4 MAPKKK null mutant, which is significantly compromised for Spc1 activation (45), and the full activation of the Spc1 pathway does not seem to be necessary for survival under the experimental stress conditions.

Whereas the Spc1 MAPK pathway is not essential unless cells are exposed to environmental stress, *cdc37*$^+$ is absolutely required for cell viability and the Δ*cdc37* mutant is lethal. Therefore, Cdc37 must have functions other than regulating the Spc1 pathway. In budding yeast (12), flies (7), and mammals (36, 50), Cdc37 is important for the activity and/or stability of Cdk, and it is possible that the lethal phenotype of Δ*cdc37* in *S. pombe* is caused by the loss of functional Cdc2, an essential Cdk in the fission yeast cell cycle. However, in contrast to *cdc2* mutants that show a highly elongated cell morphology caused by cell cycle arrest (35), most Δ*cdc37* cells stop dividing with short cell length, and *cdc37-681* cells also do not show a *cdc* phenotype at the restrictive temperature (data not shown). Thus, the lethal phenotype of the *cdc37* mutants cannot be explained solely by inactivation of Cdc2, and the Cdc37 targets essential for cell growth in fission yeast remain to be identified.

In summary, we have identified Cdc37 as a novel regulator of Spc1 MAPK. Although the chaperone function of Cdc37 has been described for MAPKKKs, Raf in higher eukaryotes (16, 48), and Ste11 in budding yeast (1), this is the first report that a MAPK requires Cdc37. Because of the high conservation of SAPKs between fission yeast and mammalian cells, it will be of interest to examine whether human p38 MAPKs are also clients of Cdc37. In addition to *cdc37*$^+$, we have isolated another locus, named *sws2* in the genetic screen described in this report, and the characterization of *sws2* may also identify a novel, evolutionarily conserved regulator of SAPKs.

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