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# *Schizosaccharomyces pombe skp1*$^+$ Encodes a Protein Kinase Related to Mammalian Glycogen Synthase Kinase 3 and Complements a *cdc14* Cytokinesis Mutant

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**We report the cloning of the *skp1*$^+$ gene, a *Schizosaccharomyces pombe* homolog of the glycogen synthase kinase 3 (GSK-3) family whose members in higher eukaryotes are involved in cell fate determination, nuclear signalling, and hormonal regulation. skp1 is 67% identical to mammalian GSK-3β and displays similar biochemical properties in vitro. Like GSK-3β, skp1 is phosphorylated on a conserved tyrosine residue, and this phosphorylation is required for efficient activity. skp1 is also phosphorylated at a serine which has been identified as S-335. Phosphorylation at this site is likely to inhibit its function. Unlike the mammalian enzyme, skp1 both tyrosine autophosphorylates in yeast cells and can phosphorylate other proteins on tyrosine in bacteria. The *skp1*$^+$ gene is not essential. However, cells with deletions in *skp1*$^+$ are sensitive to heat shock and exhibit defects in sporulation. Overexpression of wild-type *skp1*$^+$ specifically complements *cdc14-118*, one of several mutations causing a defect in cytokinesis. In addition, certain phosphorylation site mutants induce a delay or block in cytokinesis when overexpressed. Together, these data identify novel interactions of a fission yeast GSK-3 homolog with elements of the cytokinesis machinery.**

Glycogen synthase kinase 3 (GSK-3) is the mammalian member of a highly conserved family of protein serine/threonine kinases implicated in cell fate determination, nuclear signalling, and hormonal regulation. The protein kinase was first characterized as a regulator of glycogen synthase (13, 23, 48) but has since been shown to be identical to several other protein kinases such as tau protein kinase 1 (TPK1), multifunctional protein kinase (MFPK), and eIF-2 kinase (19, 46, 58). Molecular cloning of the enzyme revealed two related genes in mammals (termed α and β) which encode proteins highly related to a *Drosophila* gene termed *shaggy* (*sgg*) or *zeste-white3*, a component of the wingless signalling pathway (5, 54, 59). Mammalian GSK-3β rescues flies disrupted for *sgg*, indicating functional conservation between these proteins (47). In *Dictyostelium discoideum*, GSK-A has a clear role in cell fate decisions whereby disruption of the gene results in the differentiation of prestalk-B cells at the expense of prespore cells (21). More recently, injection of dominant negative GSK-3 into developing *Xenopus* embryos has shown that the kinase has a role in axial patterning (22, 41).

The cellular functions of the GSK-3 family are complex and poorly understood. Several putative substrates, including two subunits of protein phosphatase 1, inhibitor 2 (12) and G subunit (11), ATP citrate lyase (27), tau (19), and several nuclear proteins, including the c-Jun component of the AP-1 transcription factor (6, 38) and c-Myc (44), have been identified. In *Drosophila melanogaster*, the *armadillo* gene product (a β-catenin homolog) has been placed downstream of Shaggy by genetic analysis. Evidence for a role in signalling includes inactivation in mammals in response to insulin and genetic epistasis of *sgg* with the wingless and notch genes (5, 58). Further, in *Xenopus laevis*, injection of GSK-3β into developing embryos can rescue the Xwnt-8 phenotype, suggesting that it acts downstream of this gene in establishment of the embryonic dorsal axis (22, 41).

As an approach to investigating the physiological functions of this protein kinase family, we have exploited the high degree of amino acid conservation of the protein to isolate GSK-3 relatives from genetically tractable organisms. Recently, homologs have been identified in the slime mold *D. discoideum* (21), plants (2), and the budding yeast *Saccharomyces cerevisiae* (3, 45). The slime mold GSK-3, GSK-A, is necessary for correct cell fate determination (21), and the budding yeast enzyme is necessary for meiosis (45). Here, we describe the cloning, gene disruption, and characterization of a fission yeast GSK-3 relative termed skp1 (Shaggy kinase in *Schizosaccharomyces pombe*).

## MATERIALS AND METHODS

**Strains, growth media, and genetic methods.** The yeast strains used in this study are listed in Table 1. Media used to grow *S. pombe* cells and general genetic manipulations of *S. pombe* were as described elsewhere (36). Transformations were performed by electroporation (43). Cells were labeled with $^{32}P_i$ as detailed previously (17). DAPI (4′,6-diamidino-2-phenylindole) staining was performed as described elsewhere (36).

**Isolation of cDNA clones.** A random-primed probe was generated from a *Sac*I fragment of human GSK-3β and used to screen 80,000 colonies of an *S. pombe* plasmid library (pDB20). Hybridization conditions were 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaH_2PO_4$, and 1 mM EDTA [pH 7.7])–5× Denhardt's solution–30% formamide–1% sodium dodecyl sulfate (SDS)–100 μg of sonicated calf thymus DNA per ml at 42°C for 18 h. The filters were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 20 min and with 0.5× SSC–0.5% SDS twice for 30 min at 50°C and then exposed to film at −70°C for 18 h (with an intensifying screen). Seven positive clones were isolated and digested with *Hin*dIII, and the smaller fragments were subcloned into pBluescript. These were then sequenced with universal and reverse primers and shown to contain identical sequences. The largest cDNA (2.1 kb) was then subcloned as a *Not*I fragment into pBluescript and subjected to complete sequence analysis using an exonuclease III-nested deletion series.

**Isolation of genomic clones and gene deletion.** A fission yeast genomic library in the plasmid pUR19 (1) was plated at 30,000 colonies per filter on two 110-cm

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TABLE 1. Yeast strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| 972 | *h*$^-$ | Our stock |
| KGY246 | *h*$^-$ *leu1-32 ura4-D18 ade6-M210* | Our stock |
| KGY247 | *h*$^+$ *leu1-32 ura4-D18 ade6-M210* | Our stock |
| KGY248 | *h*$^-$ *leu1-32 ura4-D18 ade6-M216* | Our stock |
| KGY249 | *h*$^+$ *leu1-32 ura4-D18 ade6-M216* | Our stock |
| KGY137 | *h*$^-$/*h*$^+$ *leu1-32*/*leu1-32 ura4-D18*/*ura4-D18 ade6-M210*/*ade6-M216* | Our stock |
| KGY424 | *h*$^+$/*h*$^+$ *skp1*$^+$/*skp1*::*ura4*$^+$ *leu1-32*/*leu1-32 ura4-D18*/*ura4-D18 ade6-M210*/*ade6-M216* | This study |
| KGY428 | *h*$^+$/*h*$^{90}$ *skp1*$^+$/*skp1*::*ura4*$^+$ *leu1-32*/*leu1-32 ura4-D18*/*ura4-D18 ade6-M210*/*ade6-M216* | This study |
| KGY474 | *h*$^-$ *skp1*::*ura4*$^+$ *leu1-32 ura4-D18 ade6-M210* | This study |
| KGY478 | *h*$^+$ *skp1*::*ura4*$^+$ *leu1-32 ura4-D18 ade6-M216* | This study |
| KGY479 | *h*$^-$/*h*$^+$ *skp1*::*ura4*$^+$/*skp1*::*ura4*$^+$ *leu1-32*/*leu1-32 ura4-D18*/*ura4-D18 ade6-M210*/*ade6-M216* | This study |
| KGY603 | *h*$^-$ *skp1*::*ura4*$^+$ *ura4-D18* | This study |
| KGY604 | *h*$^+$ *skp1*::*ura4*$^+$ *ura4-D18* | This study |
| KGY5 | *h*$^{90}$ *skp1*::*ura4*$^+$ *ura4-D18 leu1-32 ade6-M210*/*216* | This study |
| KGY492 | *h*$^-$ *cdc3-124 leu1-32 ura4-D18 ade6-M216* | Our stock |
| KGY439 | *h*$^-$ *cdc4-8 leu1-32 ura4-D18* | Our stock |
| KGY320 | *h*$^-$ *cdc7-24 leu1-32 ura4-D18 ade6-M216* | Our stock |
| KGY657 | *h*$^+$ *cdc8-110 leu1-32 ura4-D18* | Our stock |
| KGY653 | *h*$^-$ *cdc11-123 leu1-32 ura4-D18 ade6-M210* | Our stock |
| KGY658 | *h*$^+$ *cdc12-112 leu1-32* | Our stock |
| KGY660 | *h*$^+$ *cdc14-118 leu1-32* | V. Simanis |
| KGY637 | *h*$^-$ *cdc15-136 ura4-D18 leu1-32 ade6-M210* | Our stock |

Hybond-N nylon filters (Amersham). To isolate *skp1*$^+$ genomic clones, colonies were replica plated and screened in duplicate with a random-primed [$\alpha$-$^{32}$P]dCTP-labeled probe corresponding to the most 5′ *Hin*dIII fragment of the *skp1*$^+$ cDNA. Hybridization conditions were the same as described below for Southern blotting. From nine genomic clones which contained all or a part of the *skp1*$^+$ gene, one which contained substantial amounts of 5′ and 3′ flanking sequences was chosen for further studies and was termed pKG230 (see Fig. 2A).

The DNA construct used for the *skp1*$^+$ gene deletion was produced in three steps. First, the ~4.6-kb *Pst*I fragment which contains all of the 5′ flanking region, the coding region, and 502 bp of the 3′ flanking region (see Fig. 1A) was excised from pKG230 and ligated into *Pst*I-linearized pGEM5Z to create pKG24. Second, the majority of the *skp1*$^+$ protein coding region which is present in two ~400-bp *Hin*dIII fragments (see Fig. 2A) was removed from pKG24. It was replaced with a 1.8-kb *Hin*dIII fragment containing the *S. pombe* selectable marker, *ura4*$^+$, to create pKG194. Lastly, the *Pst*I fragment was excised from pKG194 and used to replace the original *Pst*I fragment in pKG230 to create pKG249. A 4.6-kb *skp1*::*ura4*$^+$ *Bgl*II fragment was isolated from pKG249 (see Fig. 2A) and used to transform the diploid *S. pombe* strain KGY137 (*ura4-D18*/*ura4-D18 leu1-32*/*leu1-32 ade6-M210*/*ade6-M216 h*$^-$/*h*$^+$) to uracil prototrophy. Stable Ura$^+$ integrants were isolated by replica plating to yeast extract agar five times at daily intervals and then back to selective conditions. A mutant in which one copy of the *skp1*$^+$ gene had been correctly replaced with the *ura4*$^+$ gene was identified by Southern blot analysis.

**Southern blot analysis.** Genomic DNA was prepared from diploid *S. pombe* strains by procedures detailed previously (36). For Southern hybridization analysis, ~0.5 μg of genomic DNA was digested overnight at 37°C, size fractionated on a 0.8% agarose gel, and transferred to a GeneScreen Plus membrane. The membrane was treated for 1 h in hybridization buffer (5× Denhardt's solution, 0.5% SDS, 5× SSPE, and 100 μg of hydrolyzed yeast RNA per ml) and then incubated in the same buffer with a random-primed [$\alpha$-$^{32}$P]dCTP-labeled probe for 16 h at 65°C. Following hybridization, the filters were washed twice at 65°C for 30 min in 0.2× SSPE–0.2% SDS at 65°C. Low-stringency hybridizations were performed in 25% formamide–6× SSC–5× Denhardt's solution–10% dextran sulfate–0.1% SDS–100 μg of RNA per ml at 37°C for 16 h, and the filters were washed in 2× SSC–0.2% SDS for 1 h at 50°C. Hybridizing bands were detected by autoradiography at −70°C with an intensifying screen.

**Bacterial and yeast expression.** An *Nde*I site was introduced at the initiating codon of the *skp1*$^+$ cDNA together with a *Bgl*II site after the stop codon by PCR amplification. DNA sequence analysis confirmed that mutations within the coding sequence had not been introduced by PCR. The *skp1*$^+$ open reading frame was then subcloned into the *Nde*I-*Bam*HI site of bacterial expression plasmid pRK171. pLysS bacteria were transformed with the pRK171-*skp1*$^+$ plasmid, and protein production was induced at mid-log growth phase by the addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). For antibody production, bacterial cells producing skp1 were lysed by brief sonication followed by boiling in 2× Laemmli sample buffer and the proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Bacterial skp1 protein was cut from the gel, electroeluted into 10 mM Tris-HCl (pH 7.5)–1 mM EDTA, and injected subcutaneously into rabbits.

For expression in yeast cells, the *Nde*I-*Bgl*II fragment of *skp1*$^+$ was subcloned into the *Nde*I and *Bam*HI sites of pREP1 so that expression was controlled by the *nmt1*$^+$ promoter (35). For low-level expression, cells were maintained in minimal medium with 5 μg of thiamine per ml. To increase the level of expression, cells were washed twice with minimal medium lacking thiamine, resuspended and grown in media lacking thiamine, and analyzed 18 to 20 h later.

**Western blotting and immunoprecipitations.** *S. pombe* cells were lysed in Nonidet P-40 (NP-40) buffer with mechanical shearing followed by boiling in SDS lysis buffer and dilution with NP-40 buffer (18). Cell lysates were clarified by centrifugation. For Western blotting (immunoblotting), protein extracts were resolved by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Blots were probed sequentially with anti-skp1 antibodies or antiphosphotyrosine antibodies (RC20; Transduction Labs) followed by peroxidase-conjugated secondary antibodies. Reactive proteins were visualized by chemiluminescence (ECL; Amersham). For immunoprecipitation of the protein, lysates were prepared as above and incubated at 4°C for 60 min with 4 μl of antiserum. Protein A-Sepharose was added for a further 30 min, and the immunocomplexes were recovered by centrifugation and washed extensively.

For extraction of native protein from bacteria, cells were freeze fractured in NP-40 buffer (1% NP-40, 150 mM NaCl, 4 mM $NaH_2PO_4$, 6 mM $Na_2HPO_4$, 2 mM EDTA, 50 mM NaF, 0.1 mM $Na_3VO_4$, 4 μg of leupeptin per ml) and sonicated and the debris was removed by centrifugation. Immunoprecipitations were carried out as described above.

**Phosphoamino acid analysis and tryptic peptide mapping.** $^{32}$P-labeled skp1 was subjected to partial acid hydrolysis while bound to the PDVF membrane (29), and the phosphoamino acids were separated in two dimensions by thin-layer electrophoresis at pHs 1.9 and 3.5 (9). For tryptic digestion, pieces of the PDVF membrane containing $^{32}$P-labeled skp1 were pretreated with methanol for 30 s and then incubated at 37°C for 30 min with 0.1% Tween 20 in 50 mM ammonium bicarbonate, pH 8.0. After three short washes with 50 mM ammonium bicarbonate, phosphopeptides were released from the membrane with two 2.5-h incubations at 37°C in 50 mM ammonium bicarbonate, pH 8.0, with 10 μg of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)–trypsin added for each incubation. After lyophilization, the phosphopeptides were separated in two dimensions with electrophoresis at pH 1.9 as detailed previously (7). Phosphopeptides were scraped from the thin-layer cellulose plates and assayed for phosphoamino acid composition or cleaved with other proteases as described elsewhere (7). Phosphoamino acids and tryptic phosphopeptides were visualized by autoradiography or with the use of a Molecular Dynamics PhosphorImager.

**Purification of skp1 from yeast cells.** skp1 protein was produced under control of the thiamine-repressible *nmt1* promoter from the multicopy pREP1 plasmid in the *skp1.d* strain (35). Cells were lysed in NP-40 buffer by mechanical shearing after 20 h of growth in the absence of thiamine, and the soluble fraction was retained after centrifugation. Total cell lysate was diluted 10-fold in TED buffer (20 mM Tris [pH 7.5], 1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol) and incubated with 4 ml of phosphocellulose resin (preequilibrated in TED buffer) for 1 h at 4°C. The phosphocellulose was washed four times with TED buffer, and then the protein was eluted by incubation with 4 ml of TED buffer containing 500 mM NaCl for 20 min at 4°C. The skp1 protein was then dialyzed against TED buffer, clarified, applied to a Mono S column, and eluted in a linear salt gradient.

skp1-containing fractions were detected by Western blot analysis, desalted into HED buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol) (10DG Econo column [Bio-Rad]), applied to a Mono Q column, and eluted in a linear salt gradient (1 M NaCl). The skp1-containing fraction was then dialyzed against TEG buffer (10 mM Tris [pH 7.5], 1 mM EDTA, 50% glycerol) and stored at −20°C.

**Protein kinase assays.** skp1 and GSK-3β phosphorylation of the phospho-GS peptide, protein phosphatase 1, inhibitor 2, c-Jun, and ATP citrate lyase was performed as described elsewhere (11, 26). Heparin, protein kinase inhibitor (PKI), and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] were included in all kinase reactions to inhibit trace contaminants of casein kinase II, protein kinase A, and $Ca^{2+}$-dependent protein kinases, respectively. Partially purified skp1 protein was added directly to the kinase reaction mixtures. Immunoprecipitated kinase was prepared as follows: *S. pombe* cells ($\sim 10^7$) were lysed by mechanical shearing in NP-40 buffer (1.5 ml) and clarified twice in a microcentrifuge. Anti-skp1 antibodies (5 μl) were added to the extract and incubated at 4°C for 1 h. Protein A-Sepharose (50 μl) was then added to the extract and incubated at 4°C for 30 min to recover the immunocomplexes. These were washed four times in 200 μl of NP-40 buffer prior to kinase reactions. Typically, 20 μl of the protein A immunocomplex was added to the kinase reaction mixtures. Phosphorylated substrates were resolved by SDS–12.5% PAGE and exposed to X-ray film at −70°C. The phospho-GS peptide was resolved by Tricine gel electrophoresis (50).

**In vitro mutagenesis.** The original *skp1*$^+$ cDNA was subcloned from pDB20 as a *Not*I fragment into the blunted *Eco*RI site of pSelect (Promega) for mutagenesis. Various oligonucleotides were synthesized to introduce point mutations within the *skp1*$^+$ cDNA, and mutations were made as described in the Altered Sites protocol (Promega). The following changes were made: Y-192→F, Y-192→E, S-335→A, and K-61→A. The *skp1* mutants were then subcloned into the bacterial expression plasmid pRK171 and expressed in pLysS bacteria or subcloned into pREP1 for expression in *S. pombe*.

**Nucleotide sequence accession number.** The accession number of the *skp1*$^+$ sequence is L29449.

## RESULTS

**Cloning of a GSK-3 homolog, *skp1*$^+$, from *S. pombe*.** An *S. pombe* cDNA library in a bacterium-yeast shuttle vector (15) was screened under reduced stringency conditions with a 1.2-kb *Sac*I fragment of human GSK-3β encompassing the entire open reading frame. Seven hybridizing colonies were isolated, and the recovered plasmids were found to contain overlapping fragments of the same cDNA. The DNA sequence of the largest clone (2.1 kb) was determined by using an exonuclease III deletion scheme. A genomic *skp1*$^+$ clone was subsequently isolated and analyzed by restriction mapping, and its DNA sequence was partially determined. It differed in the protein coding region only by the presence of a 48-bp intron located after nucleotide 15 of the open reading frame (data not shown). The first AUG codon of the cDNA initiates an open reading frame of 1,154 bp with the potential to encode a 387-amino-acid protein (predicted $M_r$, 44,100) (Fig. 1A). This predicted protein has all of the motifs expected for protein serine/threonine kinases (20) and 67% amino acid identity with the catalytic domain of human GSK-3β. The amino acid sequence of skp1 is 60% identical to that of ScGSK-3/MDS1, a budding-yeast homolog of GSK-3 (Fig. 1B) (3, 45). To ascertain the genomic location of the *skp1*$^+$ gene, the cDNA fragment was hybridized to contiguous bacteriophage and cosmid clones covering the entire *S. pombe* genome (24, 31). The *skp1*$^+$ gene was found within cosmid clone 16H3 and *Not*I fragment J of chromosome I.

***skp1*$^+$ gene deletion.** To determine the consequence of deleting *skp1*$^+$, a null allele of the *skp1*$^+$ gene was generated. The majority of the *skp1*$^+$ open reading frame was replaced with the *ura4*$^+$ gene such that only the first 42 codons and the last 63 codons of *skp1*$^+$ remained (Fig. 2A). A ~5.1-kb *Bgl*II restriction fragment containing the deletion construction (Fig. 2B) was used to transform a uracil auxotrophic diploid strain to uracil prototrophy, and three stable Ura$^+$ diploid colonies were isolated. Southern blot analysis of genomic DNA from these colonies revealed that the null allele had correctly substituted for one copy of the wild-type *skp1*$^+$ gene in just one case (data not shown and Fig. 2B, lane 2). The wild-type *skp1*$^+$ gene is contained within a ~4.2-kb *Bgl*II DNA fragment (Fig. 2B, lane 1). Two hybridizing bands were detected in the diploid heterozygous for the *skp1* null allele (Fig. 2B, lane 2). The lower band corresponded to the wild-type gene locus, and the upper band corresponded in size to the predicted addition of 914 bp to the *skp1*$^+$ gene locus had the null allele correctly replaced the wild-type gene.

To investigate the phenotype of an *skp1* null mutant, the *skp1*$^+$/*skp1*::*ura4*$^+$ diploid was induced to sporulate and the spores were plated onto rich medium. Colonies that formed were replica plated to agar plates lacking uracil to select for haploid cells harboring the *skp1* null allele. Approximately 50% of the colonies grew under these conditions, and Southern blot analysis confirmed that these haploid cells contained the *skp1* null mutation (Fig. 2B, lane 3, for example). *skp1* null mutants, hereafter referred to as *skp1.d*, grew well at 20, 25, 32, and 36°C under both rich and poor nutrient conditions. The length and shape of *skp1.d* were indistinguishable from those of wild-type cells (see Fig. 7), and doubling times in liquid media were not significantly different, demonstrating that *skp1*$^+$ is not an essential gene.

To gain some insight into the biochemical pathway in which skp1 participates, we tested the sensitivity of *skp1.d* cells to a number of environmental stresses. We found that *skp1.d* cells grew similarly to wild-type cells on plates containing 1.2 M sorbitol, low nitrogen (5 mM $NH_4$), low glucose (0.5%), and 8% glycerol rather than glucose as the carbon source. These data suggested that *skp1.d* was not osmotically sensitive nor sensitive to nutritional stresses. In addition, *skp1.d* cells readily survived UV and radiation damage (7a) and behaved like wild-type cells on plates containing 10 mM hydroxyurea. These data indicated that the checkpoint controls governing entry into mitosis were intact in *skp1.d* cells. However, *skp1.d* cells were quite sensitive to heat shock. The wild-type and *skp1.d* cells were grown at 29°C to mid-log phase and transferred directly to 44°C. Aliquots were removed at 30-min intervals, diluted, and plated at 29°C to score survival. The *skp1.d* cells lost viability considerably faster than wild-type cells (Fig. 3A). While ~45% of wild-type cells survived a 90-min heat shock, only ~3% of *skp1.d* cells remained viable.

A *skp1.d h*$^{90}$ strain was obtained to test whether *skp1*$^+$ might be involved in conjugation, meiosis, or sporulation. This strain was grown to mid-log phase in parallel with wild-type *h*$^{90}$ cells and transferred to media lacking nitrogen to induce sexual differentiation. The numbers of zygotes, asci, free spores, and unmated cells in each culture were determined microscopically. Under these conditions, there was no difference between the efficiencies of mating or sporulation of wild-type and *skp1.d* cells. However, a defect in sporulation was observed under different nutritional conditions. When a diploid strain homozygous for the *skp1* null mutation was grown to saturation and allowed to undergo meiosis and sporulation, sporulation occurred more slowly and to a substantially lesser extent compared with an isogenic wild-type diploid. While >70% of wild-type diploids underwent sporulation under these conditions, <40% of the *skp1.d* diploids sporulated. Furthermore, in a high percentage of *skp1.d* asci, spore morphology was abnormal (Fig. 3B). Sizeable spore-like bodies were found, and/or spores were smaller and appeared to be attached to one side of the ascus. Despite these abnormalities, many asci appeared normal and released spores when the ascus walls were digested. The spore viability of *skp1.d* cells was compared with that of the wild type and found not to differ significantly. Together, these data suggest that *skp1*$^+$ is not essential in

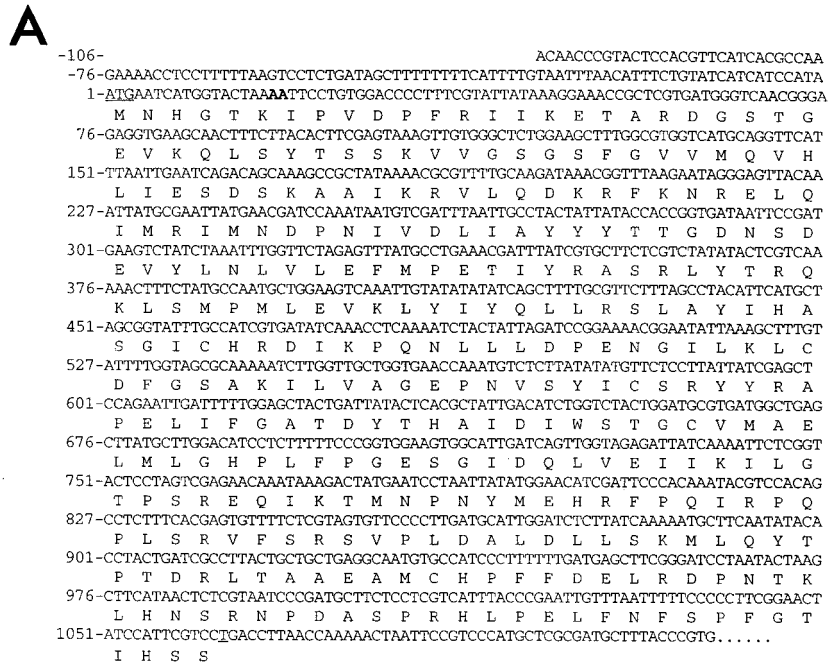



FIG. 1. *skp1*$^+$ cDNA and protein sequence. (A) The initiation codon and first base of the stop codon are underlined, and the bases flanking the single intron of 48 bp are boldfaced. The open reading frame encodes a protein of 387 amino acids, which is shown in single-letter code. (B) Protein sequence alignment of human GSK-3α and -β, *Drosophila* Shaggy, *S. cerevisiae* GSK-3/MDS1, and *S. pombe* skp1 proteins throughout the protein kinase domain. Identical amino acids are marked with asterisks, and conservative replacements are indicated with dots. The 11 serine/threonine kinase subdomains (20) are marked with roman numerals.

meiosis or sporulation but might be important in translating certain nutritional signals during the process of spore morphogenesis.

**skp1 displays GSK-3-like protein kinase activity.** To study the biochemical properties of the skp1 protein kinase, an antiserum was raised in rabbits by using full-length skp1 produced in *Escherichia coli* as the antigen. Translation of *skp1*$^+$ cRNA in rabbit reticulocyte lysates yielded a 42-kDa protein (data not shown), and the antiserum recognized a single protein of the same size in immunoblots of lysates from wild-type and *skp1.d* cells overproducing skp1 (Fig. 4A, lanes 1 and 3). This protein was not detected in lysates obtained from the *skp1.d* strain (Fig. 4A, lane 2). As shown by this immunoblotting experiment, the level of overproduction achieved in our experiments is approximately five times that of endogenous levels as measured by densitometry. A protein migrating in this position was also specifically immunoprecipitated by the anti-skp1 serum from *skp1.d* cells overproducing skp1 (Fig. 4A, lanes 4 and 6) but not from the *skp1.d* strain (Fig. 4A, lane 5). These data demonstrated that the antibody is specific for skp1.

Using this antiserum, the presence of the skp1 protein was monitored by immunoblotting during its partial purification through Mono Q and Mono S ion-exchange chromatographies. The partially purified enzyme was tested for its substrate specificity relative to that of GSK-3β. GSK-3 will phosphorylate an oligopeptide derived from glycogen synthase only after prior phosphorylation by casein kinase II (phospho-GS peptide) (25). Like GSK-3β, the partially purified skp1 protein pre-

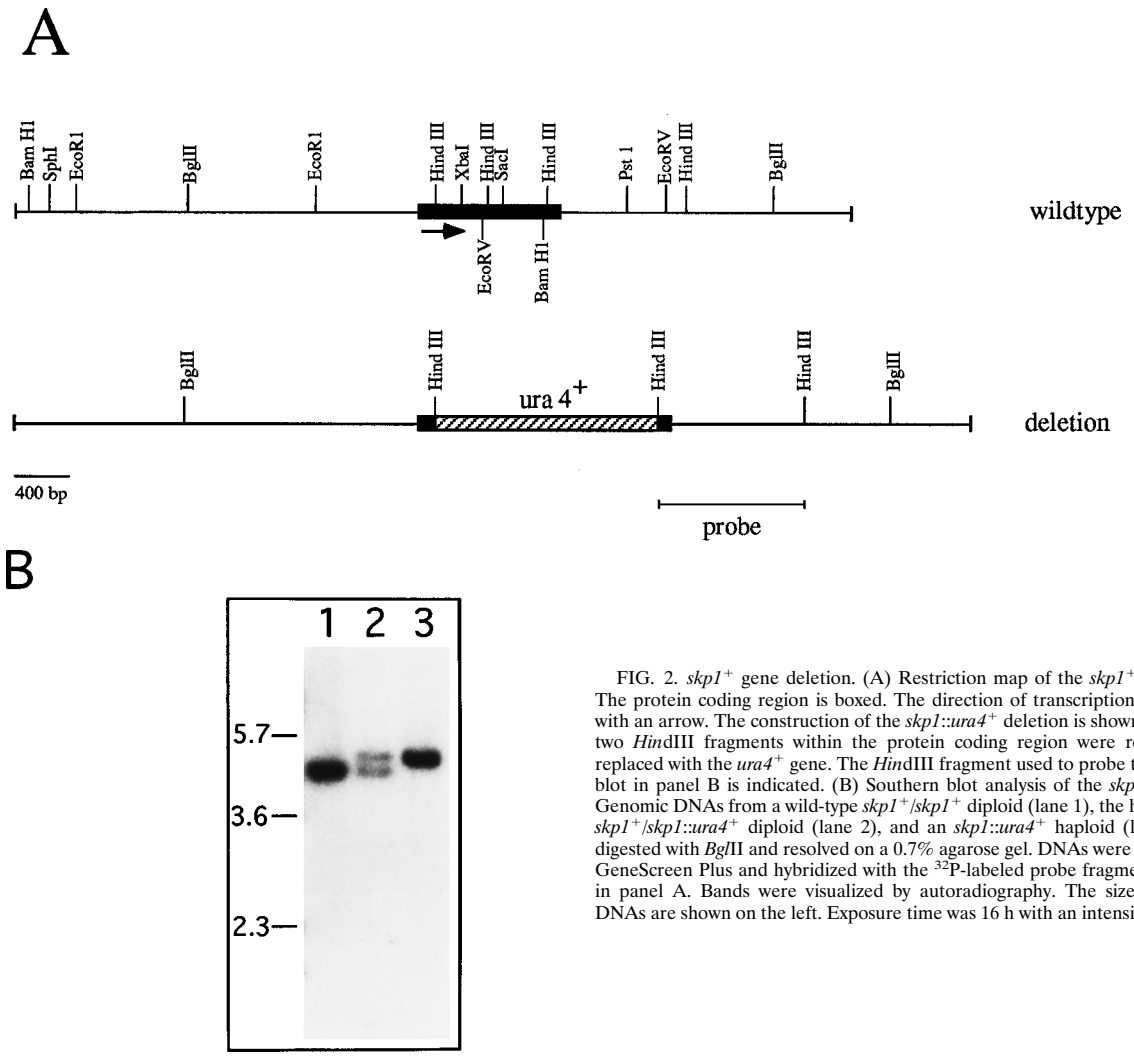



FIG. 2. *skp1*$^+$ gene deletion. (A) Restriction map of the *skp1*$^+$ gene locus. The protein coding region is boxed. The direction of transcription is indicated with an arrow. The construction of the *skp1*::*ura4*$^+$ deletion is shown below. The two *Hin*dIII fragments within the protein coding region were removed and replaced with the *ura4*$^+$ gene. The *Hin*dIII fragment used to probe the Southern blot in panel B is indicated. (B) Southern blot analysis of the *skp1*$^+$ deletion. Genomic DNAs from a wild-type *skp1*$^+$/*skp1*$^+$ diploid (lane 1), the heterozygous *skp1*$^+$/*skp1*::*ura4*$^+$ diploid (lane 2), and an *skp1*::*ura4*$^+$ haploid (lane 3) were digested with *Bgl*II and resolved on a 0.7% agarose gel. DNAs were blotted onto GeneScreen Plus and hybridized with the $^{32}$P-labeled probe fragment indicated in panel A. Bands were visualized by autoradiography. The sizes of marker DNAs are shown on the left. Exposure time was 16 h with an intensifying screen.

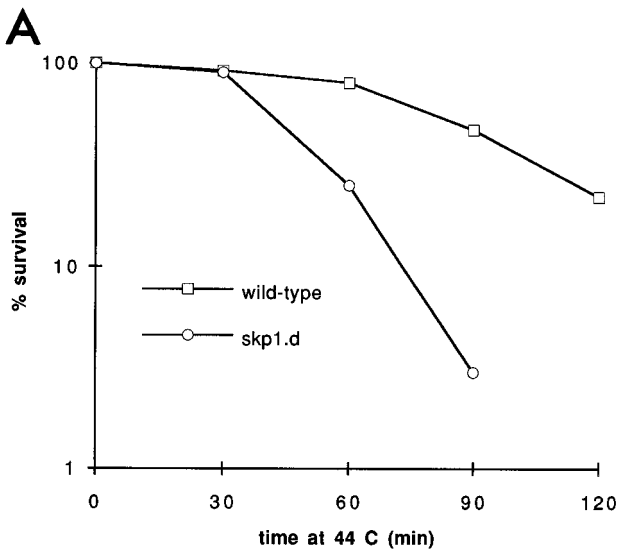
ferred this substrate over other conventional exogenous protein kinase substrates, and peptide kinase activity was absolutely dependent upon prior phosphorylation of the GS-1 peptide by casein kinase II (Table 2).

skp1 immunoprecipitates were also analyzed for protein kinase activity. skp1 immunoprecipitates from wild-type *S. pombe* cells contained a protein kinase activity capable of phosphorylating phospho-GS (Fig. 4B). Preimmune sera did not precipitate this kinase activity, and immunocomplexes from *skp1.d* also lacked this kinase activity. Elevated phospho-GS peptide kinase activity was recovered from *S. pombe* cells overproducing skp1. By all of the above criteria, then, skp1 exhibits a protein kinase activity very similar to that of its mammalian homolog, GSK-3.

**skp1 is phosphorylated on Y-192.** Mammalian GSK-3β is normally phosphorylated at a single tyrosine, residue 216 (39). The sequence around this residue is highly conserved among all GSK-3-like proteins, including skp1 (Fig. 5A). To determine whether skp1 was also phosphorylated on tyrosine, skp1 immunoprecipitates were immunoblotted with antiphosphotyrosine antibodies (Fig. 5B). A 42-kDa protein that was absent in *skp1.d* was detected in skp1 immunoprecipitates from wild-type cells. Overproduction of skp1 in *skp1.d* cells resulted in an enhancement of the 42-kDa protein detected by the antiphosphotyrosine antibodies, and this protein was not detected in immunoprecipitates from *skp1.d* cells overproducing an skp1 mutant in which the conserved tyrosine site, Y-192, was substituted by phenylalanine (Y192F).

To confirm that Y-192 was the single site of tyrosine phosphorylation in skp1, *skp1.d* strains overproducing either wild-type or Y192F Skp1 protein were labeled with $^{32}P_i$ and skp1 was immunoprecipitated from protein lysates. A 42-kDa phosphate-labeled protein was present in both strains but absent from the *skp1.d* strain (Fig. 5C). Phosphoamino acid analyses revealed that the wild-type protein contained phosphotyrosine and phosphoserine. As expected, the Y192F mutant contained only phosphoserine (Fig. 5D). A partial acid hydrolysis product, marked X, which migrates farther than phosphotyrosine at pH 1.9 (Fig. 5D) is an unknown derivative of phosphotyrosine, since rehydrolysis in acid yielded only phosphotyrosine (data not shown). The results of further phosphopeptide mapping experiments were also consistent with Y-192 being the site of tyrosine phosphorylation (data not shown). Thus, skp1 is phosphorylated on a single tyrosine, Y-192, in *S. pombe* cells.

**Tyrosine phosphorylation is important for skp1 activity.** We have previously shown that tyrosine phosphorylation at the site



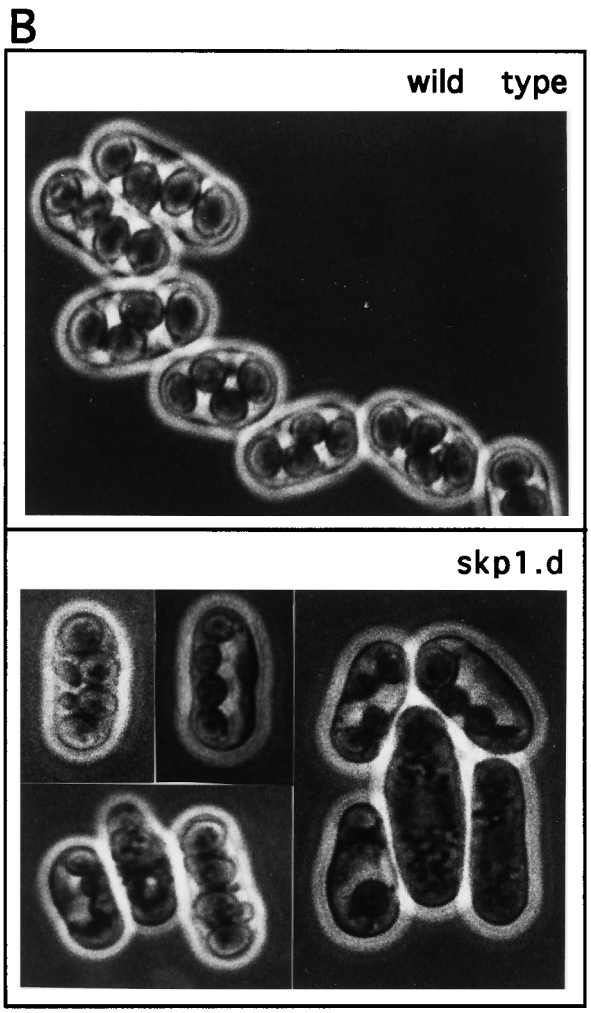




FIG. 3. Phenotypic defects of *skp1.d* cells. (A) Wild-type or *skp1.d* cells were grown to mid-log phase at 29°C in rich media and transferred to 44°C. An aliquot was taken at the indicated times, diluted, and plated in triplicate at 29°C. Survival was scored as colony formation after 5 days of incubation. (B) Wild-type diploids and *skp1*::*ura4*$^+$/*skp1*::*ura4*$^+$ diploids were grown to saturation in rich media. Sporulation was observed over the next 3 days, and typical examples of asci were photographed.

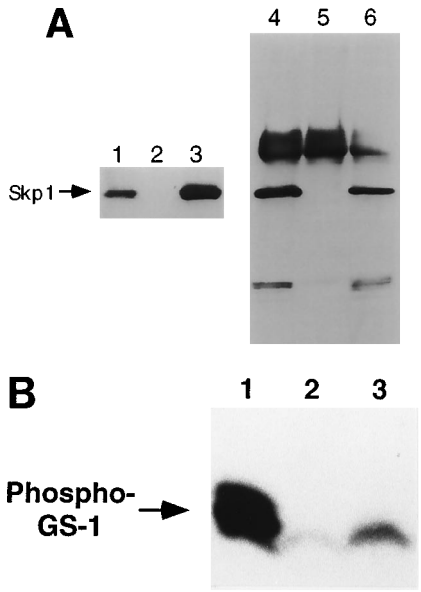



FIG. 4. Production and activity of skp1. (A) *S. pombe* lysates (lanes 1 to 3) and skp1 immunoprecipitates (lanes 4 to 6) were resolved by SDS-PAGE and transferred to a PVDF membrane. skp1 proteins were detected by ECL after incubation with anti-skp1 antibodies. Lane 1, wild-type 972; lanes 2 and 5, *skp1.d*; lanes 3 and 4, *skp1.d* containing pREP1skp1 grown in the absence of thiamine; lane 6, *skp1.d* containing pREPskp1Y192F grown in the absence of thiamine. (B) Phospho-GS1 peptide kinase assay. Lane 1, skp1 was immunoprecipitated from *skp1.d* containing pREP1skp1 grown in the absence of thiamine; lane 2, *skp1.d*; lane 3, *skp1.d* containing pREPskp1Y192F grown in the absence of thiamine and incubated with the phospho-GS1 peptide plus [$\alpha$-$^{32}$P]ATP. The peptide was resolved on a Tricine gel, and its phosphorylation was detected by autoradiography.

homologous to Y-192 is required for efficient GSK-3 activity (25). To determine whether skp1 activity was subject to similar regulation, an immunoprecipitate of the Y192F mutant protein from *skp1.d* cells was assayed with the phospho-GS-1 peptide in parallel with wild-type immunoprecipitated skp1 (Fig. 4B). Peptide kinase activity was detected at greatly reduced levels in this immunoprecipitate, thereby demonstrating that tyrosine phosphorylation is required for significant skp1 activity.

**skp1 is phosphorylated on serine 335.** In phosphotryptic peptide maps of skp1 from $^{32}$P-labeled cells, several phosphopeptides were detected (Fig. 6A). Most of these phosphopeptides (no. 2 to 6) contained phosphotyrosine only upon partial acid hydrolysis, and they were missing in the Y192F mutant protein (data not shown). We concluded from these data that peptides 2 to 6 were derived from phosphorylation at Y-192. In contrast, phosphopeptide 1 was found to contain phosphoserine (Fig. 6B). To begin identifying this serine phosphorylation site, peptide 1 was subjected to further proteolysis

TABLE 2. Substrate specificity of skp1

| Substrate | Rate of phosphorylation[a] | |
|---|---|---|
| | GSK-3β | skp1 |
| Phospho-GS peptide | 100.0 | 100.0 |
| Unphospho-GS peptide | 0.0 | 0.0 |
| Glycogen synthase | 3.3 | 4.5 |
| Myelin basic protein | 10.7 | 7.3 |
| ATP-citrate lyase | 6.3 | 8.5 |

[a] Relative to that of the phospho-GS peptide.

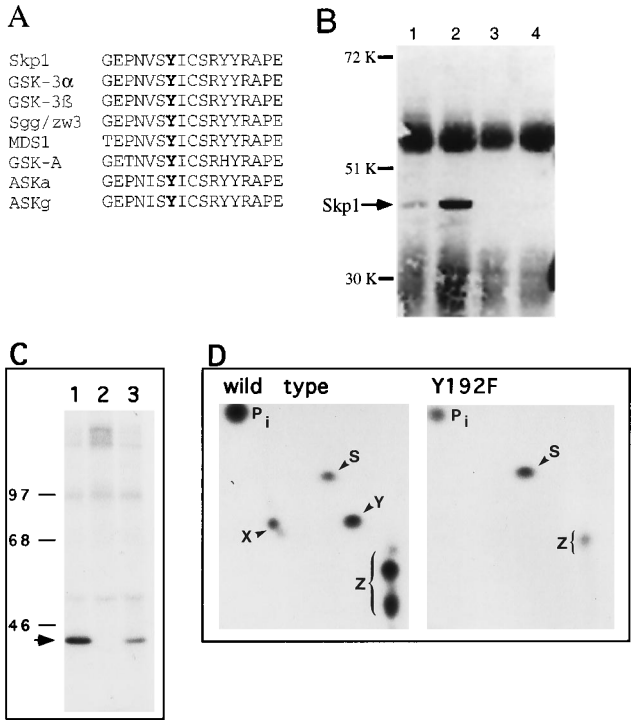



FIG. 5. Tyrosine phosphorylation of skp1. (A) Comparison of amino acid sequences of various GSK-3 homologs from yeasts, mammals, flies, and plants surrounding the phosphorylated tyrosine of GSK-3β. The conserved tyrosine is boldfaced. (B) skp1 protein was immunoprecipitated from *S. pombe* cell lysates by the addition of skp1 antibodies and incubation with protein A-Sepharose. Samples were resolved by SDS-PAGE, transferred to a PVDF membrane, incubated with antiphosphotyrosine antibodies (RC20), and visualized by chemiluminescence. Lane 1, wild-type cells; lane 2, *skp1.d* overexpressing wild-type *skp1*$^+$; lane 3, *skp1.d* overexpressing Y192F; lane 4, *skp1.d*. (C) *skp1.d* cells containing pREP plasmids expressing no *skp1* (lane 2), *skp1*$^+$ (lane 1), or Y192F (lane 3) were grown in 5 μg of thiamine per ml and labeled with $^{32}P_i$. skp1 proteins were isolated by incubation with anti-skp1 antibodies followed by protein A-Sepharose and visualized after resolution on SDS-PAGE and transfer to a PVDF membrane by autoradiography. The positions of molecular mass standards are given in kilodaltons, and skp1 proteins are indicated with an arrowhead. (D) The skp1 proteins in panel C were subjected to partial acid hydrolysis, and the resultant phosphoamino acids were separated by two-dimensional thin-layer electrophoresis and detected by autoradiography. S, phosphoserine; Y, phosphotyrosine; X and Z, partial acid hydrolysis products.

with a variety of site-specific proteases and its mobility on cellulose thin-layer plates was analyzed (data not shown). By using this information, five candidate serines were mutated to alanine residues individually, and the mutant proteins were produced in the *skp1.d* strain. Only one of these, S335A, lacked phosphopeptide 1 when isolated from $^{32}P$-labeled cells (Fig. 6C). This strongly indicated that S-335 was the major site of serine phosphorylation in skp1. The S335A protein still contained a small amount of phosphoserine (data not shown), indicating that at least one other serine is phosphorylated at low stoichiometry.

To determine whether the S335A protein had protein kinase activity, it was produced in *skp1.d* cells, immunoprecipitated, and assayed with the phospho-GS peptide. The S335A mutant protein was able to phosphorylate this peptide, indicating that phosphorylation at S-335 is not essential for skp1 activity (data not shown). It remains to be seen whether phosphorylation of this site inhibits skp1 protein kinase activity.

**skp1 is a dual-specificity kinase.** We have shown above that skp1 is phosphorylated on Y-192. To test whether this occurred via autophosphorylation, skp1 was produced in bacteria. Immunoprecipitates of skp1 from these cells reacted with phosphotyrosine-specific antibodies (Fig. 7A). No phosphotyrosine signal was detected upon production of a kinase-dead mutant (K61A) or the Y192E mutant, in agreement with the notion that skp1 can autophosphorylate on Y-192 (Fig. 7A). To examine whether tyrosine autophosphorylation also occurred in *S. pombe*, the phosphoamino acid content of the K61A mutant was examined following production in the *skp1.d* strain. This strain background eliminated the possibility of transphosphorylation of the K61A mutant by endogenous skp1. Phosphoamino acid analysis of the mutant immunoprecipitated from $^{32}P$-labeled cells revealed the presence of phosphoserine but no phosphotyrosine (Fig. 7B). Phosphotryptic peptide mapping indicated that serine phosphorylation occurred on S-335 (data not shown). Together, these data suggest that skp1 autophosphorylates on Y-192 and is phosphorylated on S-335 by a distinct protein kinase.

To test whether skp1 could phosphorylate other proteins on tyrosine, an antiphosphotyrosine immunoblot was performed on bacterial lysates from cells producing wild-type and kinase-dead (K61A) skp1 proteins (Fig. 7C). Several bacterial proteins which were not detected in cells containing kinase-dead protein were phosphorylated on tyrosine in the cells making skp1 protein. Further, incubation of baculovirus-expressed mammalian GSK-3β (which has a low stoichiometry of tyrosine phosphorylation) with wild-type skp1 and ATP increased the activity of GSK-3, presumably by skp1-induced tyrosine phosphorylation (data not shown).

**Overexpression of *skp1*$^+$ rescues *cdc14-118*.** Overexpression of *skp1*$^+$ did not alter the phenotype of the wild-type cells (data not shown) or *skp1.d* cells (Fig. 8). Overexpression of the Y192F mutant had no apparent effect either. In contrast, mutation of Y-192 to glutamic acid (Y192E), a mutation made in an effort to mimic constitutive tyrosine phosphorylation, resulted in a protein which delayed cytokinesis when overproduced in *skp1.d* cells. Cells with two or more nuclei accumu-

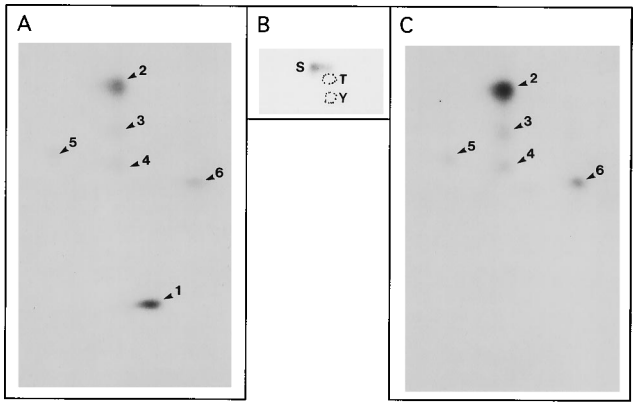



FIG. 6. skp1 is phosphorylated on S-335. *skp1.d* cells producing wild-type skp1 or the S335A mutants protein were labeled with $^{32}P_i$, and skp1 proteins were isolated by immunoprecipitation. The immunoprecipitates were resolved by SDS-PAGE and transferred to a PDVF membrane, and skp1 proteins were detected by autoradiography. Membrane pieces containing labeled skp1 were digested with trypsin, and the phosphopeptides were separated in two dimensions on cellulose thin-layer plates by electrophoresis at pH 1.9 and ascending chromatography. (A) Phosphopeptide map of wild-type skp1. (B) Phosphopeptide 1 was scraped from the thin-layer plate, eluted from the cellulose, and partially hydrolyzed in acid. The resultant phosphoamino acids were resolved by two-dimensional electrophoresis. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. The positions of the phosphothreonine and phosphotyrosine standards have been demarcated with dots. (C) Phosphopeptide map of the S335A mutant.

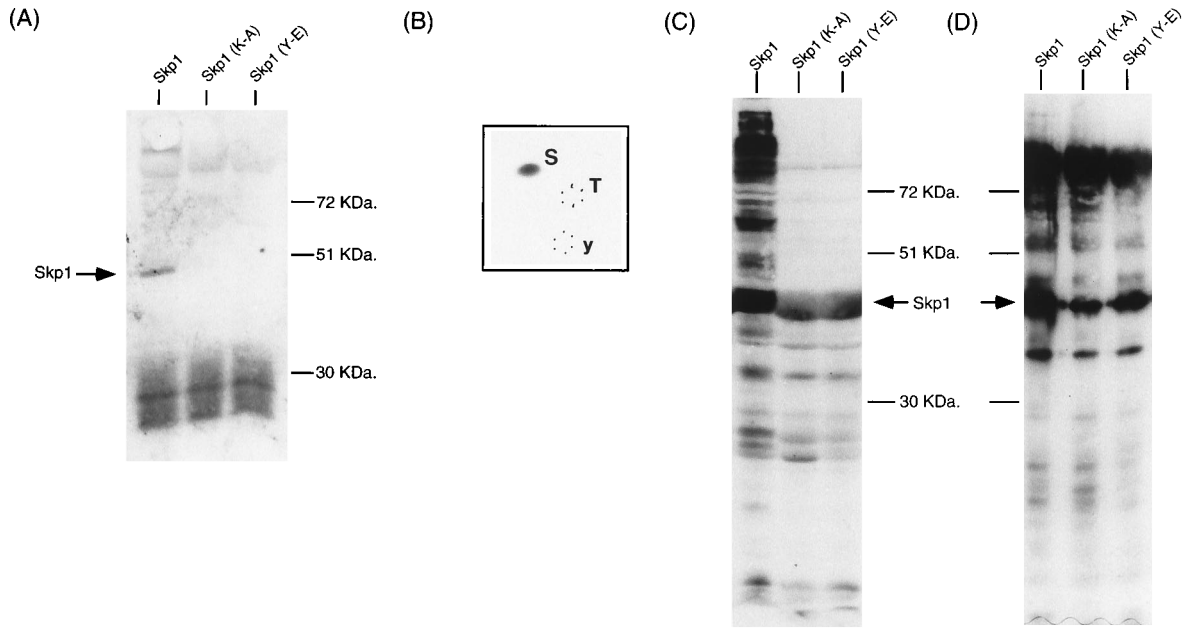



FIG. 7. Tyrosine kinase activity of skp1. (A) Antiphosphotyrosine immunoblot (RC20) of skp1 and mutants produced in bacteria. Skp1 (K-A), K61A; Skp1 (Y-E), Y192E. (B) Phosphoamino acid analysis of protein immunoprecipitated from $^{32}$P-labeled *skp1.d* overexpressing K61A. (C and D) Antiphosphotyrosine (RC20) and anti-skp1 immunoblots, respectively, of total cell lysates from bacteria producing skp1 kinase-dead protein (K61A [Skp (K-A)]) or the Y192E mutant protein [Skp (Y-E)].

lated in the absence of septation, although the culture continued to increase in cell number. Another interesting phenotype was observed when the S335A mutant was overexpressed in *skp1.d* cells. Again, cells were delayed during septation, but in this instance the chromatin was condensed in a large percentage of cells. Additionally, the cells formed septa, and their diameter was increased (Fig. 8). These phenotypes were also observed when Y192E and S335A were overexpressed in wild-type cells, but they were much less penetrant and had no significant effect on the growth of the cells in liquid or solid media (data not shown). Interestingly, the Y192E mutant did not display in vitro kinase activity when immunoprecipitated from *S. pombe* (data not shown), nor did it increase tyrosine phosphorylation when produced in bacteria (Fig. 7C).

Given the septation defects generated by overexpression of the Y192E and S335A mutants, a potential relationship between *skp1*$^+$ and the known cytokinesis mutants of *S. pombe* (*cdc3*, *-4*, *-7*, *-8*, *-11*, *-12*, *-14*, and *-15*) (39) was explored. We first tested whether *skp1*$^+$ could rescue any of the *cdc3-124*, *cdc4-8*, *cdc7-24*, *cdc8-110*, *cdc11-123*, *cdc12-112*, *cdc14-118*, or *cdc15-136* temperature-sensitive strains. Wild-type *skp1*$^+$ and the Y192F, Y192E, K61A, and S335A mutants were transformed into each of the cytokinesis mutants in parallel with a vector control and expressed at low and high levels under the control of the *nmt1* promoter. We found that *skp1*$^+$ could indeed complement one of the mutations, *cdc14-118*, whereas the *skp1* mutants were unable to do so (Fig. 9A). Complementation of *cdc14-118* by wild-type skp1 occurred only when it was overexpressed (Fig. 9A), and growth was rescued only to 32°C. Genomic clones of *skp1*$^+$ were unable to rescue *cdc14-118* cells again, suggesting that *skp1*$^+$ must be constitutively overexpressed for this effect to be observed (data not shown). *cdc14-118* cells overexpressing *skp1*$^+$ at 32°C were shorter and rounder than wild-type cells, leading to the formation of small rounded colonies (Fig. 9B).

At 25°C, *cdc14-118* cells overproducing *skp1*$^+$ were even shorter and rounder than at 32°C and formed colonies considerably more slowly than cells overexpressing the kinase-dead mutant (K61A) or cells containing the control pREP1 vector (Fig. 9C). Cells expressing the Y192F mutant formed colonies at intermediate rates. Cells expressing the S335A mutant grew extremely slowly and in fact contained cells which were mostly lysed (Fig. 9C and data not shown). Overexpression of the Y192E mutant in *cdc14-118* at 25°C resulted in a cdc$^-$ phenotype and prevented any colony formation (Fig. 9D). Overexpression of the Y192E mutant did not result in a cdc$^-$ phenotype in any of the other seven cytokinesis mutants (data not shown), again suggesting a specific interaction between *skp1*$^+$ and *cdc14*$^+$.

## DISCUSSION

We have isolated an *S. pombe* gene highly related to mammalian GSK-3 termed *skp1*$^+$. GSK-3-related genes have been identified in all eukaryotes examined to date, including mammals, flies, *D. discoideum, S. cerevisiae*, plants, and now *S. pombe* (2, 3, 21, 45, 53; also, this study). In comparison with human GSK-3β, the proteins encoded by these genes exhibit a greater than 65% amino acid identity within their catalytic domains. This high degree of GSK-3 sequence conservation between species implies a fundamental role for this kinase in cellular function, a function which presumably has been conserved throughout evolution. In support of this, mammalian GSK-3 can functionally substitute for the *Drosophila* homolog *shaggy/zeste-white3* (47). However, despite the mutant phenotypes associated with loss of this activity in a variety of organisms, the exact role of GSK-3 enzymes in cellular function is presently unknown.

In *D. melanogaster*, there appears to be a single GSK-3-related protein encoded by the *shaggy/zeste-white3* gene. Disruption of *shaggy* results in gross organizational abnormalities

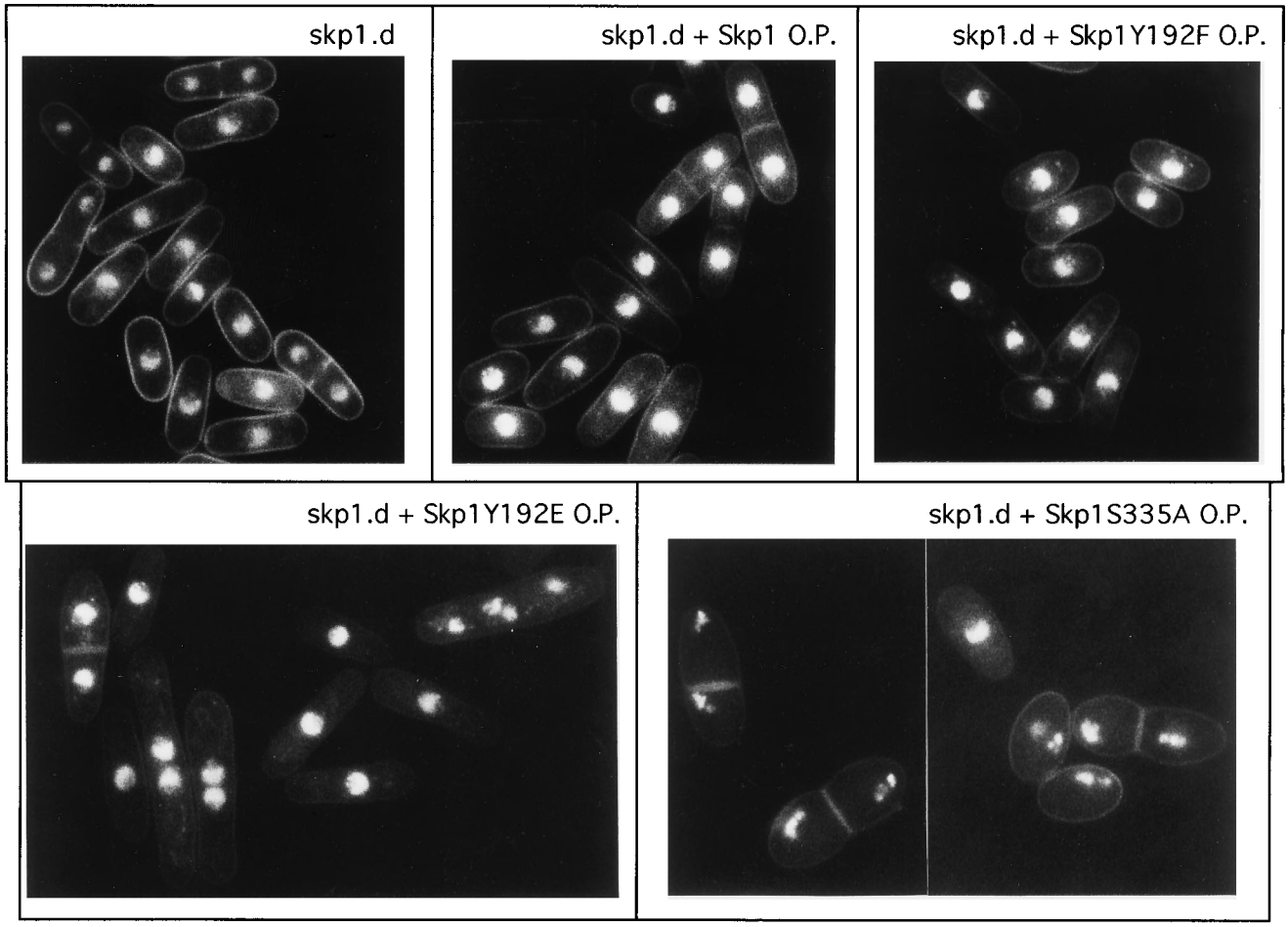
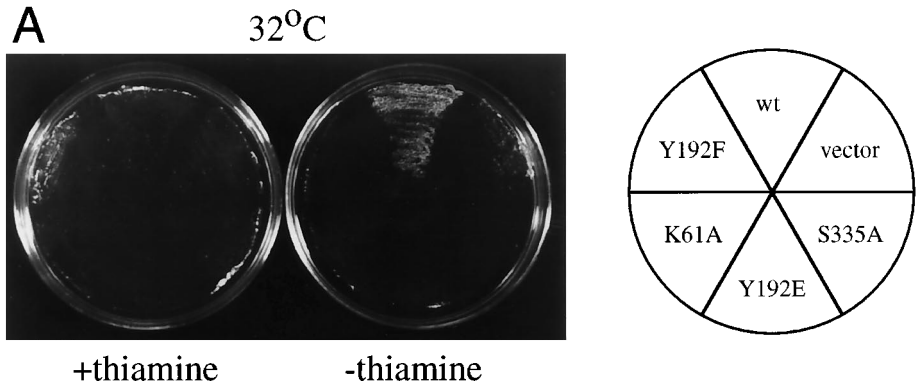


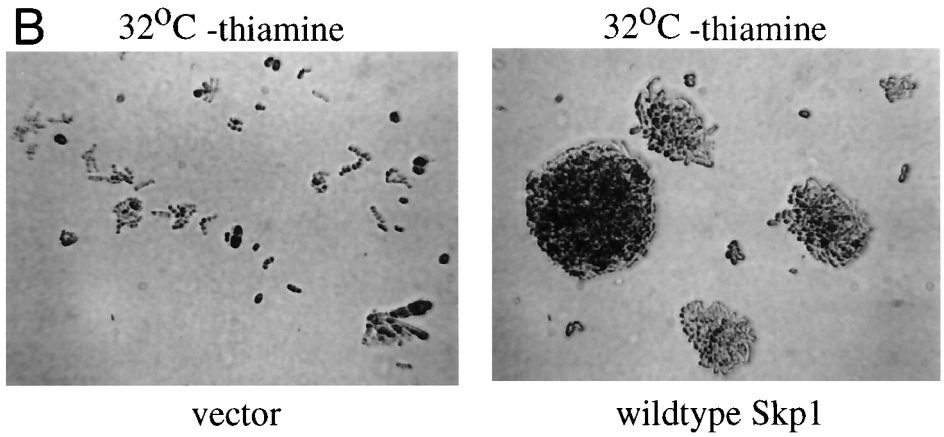

FIG. 8. Phenotypes of skp1 mutants. *skp1.d* cells were transformed with a control plasmid or pREP1 plasmids encoding the indicated skp1 proteins. Transformants were grown in the absence of thiamine for 20 h to allow high-level expression, fixed, and stained with DAPI. DAPI binds DNA and also binds to the cell wall and septa. O.P., overproduction.

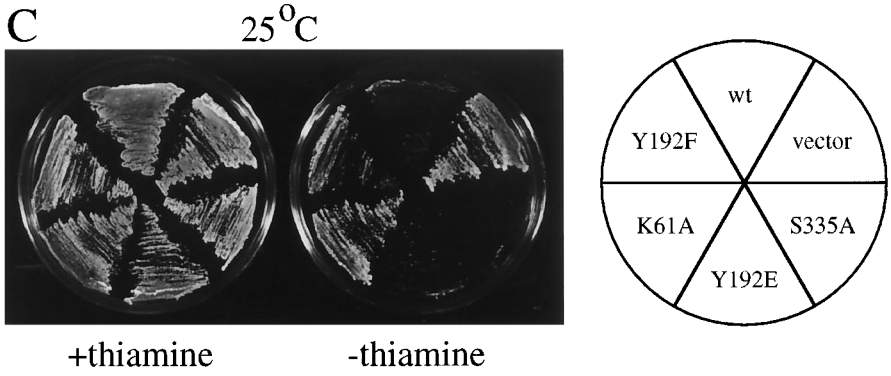
and larval death. However, generation of islands of disruptant cells by mitotic recombination has allowed detailed analysis of one phenotype within the context of a viable animal (4, 34, 40, 42). In these chimeras, cells become refractile to differentiation signals, resulting in their adoption of a default neural phenotype, which in the wing blade generates innervated bristles. In this system, the kinase-null cells are viable and can divide. Their defect appears to be at the level of response to environmental cues which can result in a switching of cell fate (4, 34, 40, 42). The single detectable *Dictyostelium* homolog (GSK-A), is dispensible for vegetative growth (21). However, disruption of the kinase has a profound effect on differentiation in response to nutrient deprivation. In the absence of the kinase, prestalk cells are formed at the expense of prespore cells, giving rise to a stalkogenous phenotype (21). This switch in cell fate cannot be reversed by addition of exogenous cyclic AMP (cAMP) (required for prespore cell differentiation), suggesting that GSK-A acts downstream of a cAMP receptor in the specification of prestalk versus prespore cell fate. Thus, similarly to *shaggy*, GSK-A has a role in cell fate determination.

Unlike the situations described above, there is not an obvious defect in the life cycle of *S. pombe* cells lacking *skp1*$^+$. *skp1*$^+$ is not required for vegetative growth nor for conjugation, meiosis, or sporulation when cells are starved for nitrogen. However, *skp1.d* diploid cells are defective for sporulation when simply grown to saturation, a condition more likely encountered outside the laboratory. Interestingly, the defects observed in spore maturation were very similar to those reported for *S. cerevisiae* cells lacking a related protein kinase gene, *MCK1* (37). In both instances, spore maturation occurred very slowly. Another phenotype associated with the loss of *skp1*$^+$ was an increased sensitivity to heat shock. More work will be required to determine if *skp1*$^+$ is required for induced thermotolerance, the ability of yeast cells to resist high temperatures by brief pretreatment at moderate temperature, and whether *skp1.d* cells are defective in inducing heat shock genes or in activating particular mitogen-activated protein (MAP) kinase cascades, such as MPK1 of *Saccharomyces cerevisiae* (28), required for thermotolerance. Another mutant exhibiting an increased sensitivity to heat shock is the null allele of the *S. pombe ptc1* gene, which encodes one of three protein phosphatase 2C isozymes (52). We are currently examining whether there is an interaction between these enzymes and skp1.

The lack of a significant phenotype for *skp1.d* can be explained in two ways. The first possibility is that skp1 does not have an important role in the life cycle of *S. pombe*. Given the critical role of its homologs in other eukaryotes, this seems unlikely. The other possibility is that there is another gene(s) which encodes a functionally redundant kinase. Several lines of evidence indicate that this is the case. First, another *S. pombe*


A
32°C
wt
Y192F
vector
K61A
S335A
Y192E
+thiamine
-thiamine


B
32°C -thiamine
32°C -thiamine
vector
wildtype Skp1


C
25°C
wt
Y192F
vector
K61A
S335A
Y192E
+thiamine
-thiamine

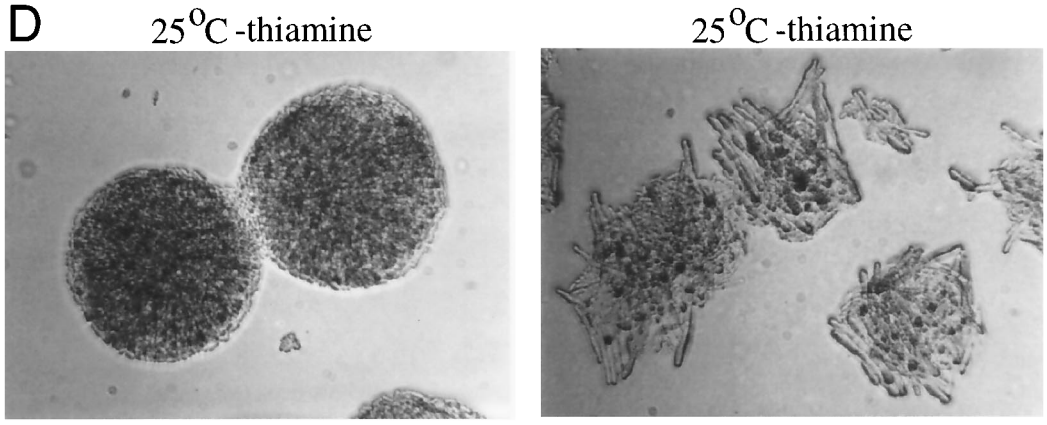

D
25°C -thiamine
25°C -thiamine
vector
Y192E

FIG. 9. skp1 rescues *cdc14-118*. The pREP1 plasmid and pREP1 plasmids containing wild-type (wt), Y192F, Y192E, S335A, or K61A *skp1* were transformed into the *cdc14-118* mutant, and colonies were allowed to form at 25°C in the presence of thiamine. Colonies from these six transformations were then streaked onto a single plate at 25°C in the presence of thiamine. Colonies were replica plated to thiamine-containing plates or plates lacking thiamine and incubated at 32°C. Plates were photographed after 4 days of incubation (A). Cells containing either a vector control or pREP1skp1 were also streaked directly from 25°C to plates lacking thiamine and incubated at 32°C for 2 days prior to photography of representative streaks (B). The original plate was also replica plated with or without thiamine and incubated at 25°C for 4 days prior to photography (C). Colonies containing a vector control or overexpressing the Y192E mutant were photographed after 3 days of incubation at 25°C (D).

gene which encodes a protein with significant sequence similarity to skp1 has been identified (8). Additionally, we have identified several DNA fragments from the *skp1.d* strain which hybridize to an *skp1*$^+$ probe, indicating that there could be more than one other related gene (data not shown). Secondly, kinase activity toward the GS peptide is still detected in *skp1.d* cells, albeit at low levels (data not shown). This result provides strong evidence that a redundant kinase activity exists.

We have shown that skp1 has a substrate specificity in vitro which is indistinguishable from that of GSK-3β. In addition, we have demonstrated that at least one mechanism of skp1 regulation is similar to its mammalian counterpart. Tyrosine phosphorylation at residue 216 is required for GSK-3β activity in vitro and in vivo (25). We have shown that skp1 is phosphorylated at the homologous residue, Y-192, and that this phosphorylation appears to be essential for significant activity in vitro and in vivo as well. skp1, therefore, joins the small group of protein kinases known to be regulated by tyrosine phosphorylation in yeast cells.

Phosphorylation of the homologous tyrosine residue in the MAP kinases and stress-activated protein kinases is similarly required for their activity (reviewed in reference 30). In these cases, however, the MAP kinases are phosphorylated and activated by distinct, dual-specificity kinases (MEK and SEK, respectively) (30). In contrast to these situations, we have shown that the phosphorylation of Y-192 in skp1 is due to autophosphorylation. Bacterially produced skp1 protein autophosphorylates on tyrosine, whereas its kinase-dead counterpart does not, and kinase-dead skp1 produced in *S. pombe* does not contain phosphorylated tyrosine, although it does become phosphorylated on serine. Wang et al. (57) have shown previously that bacterially expressed GSK-3β could autophosphorylate on serine, threonine, and tyrosine residues and that these autophosphorylation events had contrasting effects on kinase activity. However, it is not clear whether these effects have any relevance to the regulation of mammalian GSK-3 in vivo.

Despite the biochemical similarities with mammalian GSK-3, there is at least one property of skp1 that is distinct. We have found that skp1 is able to phosphorylate other proteins on tyrosine when produced in bacteria. Thus, unlike its mammalian counterparts, skp1 is a dual-specificity kinase similar to MEK and SEK (49, 60). Of note, the dual-specificity kinases SEK, MEK, and skp1 all phosphorylate the same conserved tyrosine in kinase subdomain VIII.

In addition to the phosphorylation of Y-192, we have shown that skp1 is phosphorylated at one other major site, and both biochemical studies and mutagenesis studies have indicated that this site is serine 335. This residue lies outside the protein kinase domain and is not conserved among GSK-3 homologs. S-335 is found in the sequence A<u>S</u>PR, suggesting that it is phosphorylated by a proline-directed kinase, perhaps of the CDK or MAP kinase families (16, 30). Since it becomes phosphorylated in the kinase-dead mutant, it is unlikely due to autophosphorylation. We suspect that phosphorylation of S-335 is inhibitory to skp1 protein kinase activity on the basis of the biological properties of the S335A mutant (discussed below). Inhibition by serine phosphorylation has been reported for the mammalian enzyme as well, although the site is quite distinct. Both p70 S6 kinase and p85 Rsk-1 can phosphorylate GSK-3α and -3β on serines 21 and 9, respectively, in vitro and cause their inactivation (55), although only p85 Rsk-1 can regulate GSK-3β activity in vivo (56).

We observed that overexpression of two *skp1* phosphorylation site mutants in *skp1.d* cells caused a delay during cytokinesis. Each of the mutants behaved quite differently. The Y192E mutant was made in an attempt to mimic constitutive tyrosine phosphorylation, and it delayed formation of F-actin contractile rings (data not shown) and septa. This phenotype is very similar to the terminal phenotype of the four so-called early-septation mutants of *S. pombe*, containing the *cdc7*, *cdc11*, *cdc14*, and *cdc15* mutations (33, 39). The S335A mutant delayed cell cycle progression at a later stage. F-actin rings (data not shown) and septa formed, but the cells were nevertheless delayed in separation. These observations prompted us to examine the possibility that skp1 interacted with one or more of the eight known cytokinesis genes, which, in addition to the four mentioned above, include *cdc3*, *cdc4*, *cdc8*, and *cdc12* (39). We were able to show that the overproduction of wild-type skp1 did rescue one of these, *cdc14-118*. The kinase-dead mutant and the phosphorylation site mutants were unable to do so. The inability of the Y192F mutant to rescue *cdc14-118* cells demonstrates the requirement of tyrosine phosphorylation for significant skp1 activity.

*cdc14*$^+$ encodes a 28-kDa protein of unknown function (14). While deletion of *cdc14* is lethal and results in the inability of cells to form septa, overexpression results in a $G_2$ block, suggesting that cdc14 might be important for both the initiation and the completion of mitosis (14). Genetic analysis has provided evidence that the products of the *cdc7*, *cdc11*, *cdc14*, and *cdc16* genes interact to regulate the formation of the septum and to coordinate septation with nuclear division (32). Our finding that *skp1* has a specific interaction with *cdc14* suggests that it too may have a role to play in the coordination of cell cycle events.

Overexpression of *skp1*$^+$ caused *cdc14-118* cells to become short and round at 25°C, exhibiting a morphology indistinguishable from that of *skp1.d* cells overexpressing the S335A mutant. Overexpression of the S335A mutant in *cdc14-118* at 25°C caused even more rounding and eventual cell lysis. For this reason, we speculate that the S335A protein is an unrestrained version of the wild-type protein and that phosphorylation of S335A serves to inhibit skp1 kinase activity. This can be tested in future experiments by comparing the specific activities of the wild-type and S335A proteins isolated from *S. pombe*.

In contrast to the effect of the S335A mutant, overexpression of the Y192E mutant caused a cdc$^-$ phenotype in *cdc14-118* cells at 25°C. This protein does not have detectable in vitro protein kinase activity, so we conclude that it is behaving as a dominant negative mutant. The ability of the Y192E mutant to cause a lethal block in cell cycle progression is specific to the *cdc14* mutant, as it did not occur in 10 other cdc mutants (data not shown). We speculate that the Y192E protein is binding

and titrating away some factor that is limiting in the absence of wild-type *cdc14* function. It will be interesting to determine in future experiments whether cdc14 is that limiting factor or whether cdc14 and skp1 interact with a common protein.

In *S. cerevisiae*, a GSK-3 homolog termed MDS1/ScGSK3 was identified by sequence similarity to GSK-3 (3) and independently by its ability to rescue the cold sensitivity of an *MCK1* null mutant (45). As mentioned above, *MCK1* encodes a related protein kinase (10, 37, 51). MDS1 is not required for vegetative growth but is required for some undetermined step in meiosis (45). It will be interesting to determine whether in fact skp1 and MDS1 are functionally interchangeable and whether their seemingly different roles are in fact related.

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