A novel gene, *psp1*\(^{+}\), which functionally complements a temperature-sensitive mutant defective in cell cycle progression both in G\(_1\)/S and G\(_2\)/M has been isolated from the genomic and cDNA libraries of *Schizosaccharomyces pombe*. Disruption of this gene is lethal for cell growth at 30 °C indicating that it is an essential gene for vegetative cell growth. Western analysis of the protein by polyclonal antibody made from glutathione S-transferase-Psp1 fusion protein indicated that the Psp1 protein exists in two different molecular weight forms depending on the growth state of the cell. In *in vitro* experiments with a phosphatase showed that this difference is due to phosphorylation. The dephosphorylated form of the protein is dominant in actively growing cells whereas the phosphorylated form becomes the major species when cells enter the stationary phase. The Cdc2-Cdc13 complex is shown to phosphorylate the GST-Psp1 fusion protein in *in vitro*, and site-directed mutagenesis and phosphoamino acid analysis indicated that the serine residue at position 333 in the carboxyl-terminal region is required for phosphorylation. In situ fluorescein isothiocyanate-conjugated antibody staining showed that this protein tends to be localized to both ends of the cell upon entry into the stationary phase of cell growth. However, overexpression of the novel protein Psp1 in actively growing cells inhibits cell growth causing accumulation of DNA (4n or 8n). Thus we speculate that Psp1 can function at both G\(_1\)/S and G\(_2\)/M phases complementing the defect of the new mutant we have isolated. It is likely that Psp1 is required both for proper DNA replication and for the process of mitosis.

DNA replication (S) and mitosis (M) are the two major events in the eukaryotic cell division cycle and are proceeded by the two gap periods, G\(_1\) and G\(_2\), respectively. Cell cycle transition from G\(_1\) to S phase (G\(_1\)/S) and from G\(_2\) to mitosis (G\(_2\)/M) occurs in a strict sequence. Blocking S phase prevents onset of the subsequent mitosis, which would be lethal if chromosome replication had not been completed, and blocking mitosis prevents initiation of the subsequent S phase which would otherwise lead to increase in ploidy. Dependences of S phase and mitosis are examples of checkpoints that ensure orderly progression through the cell cycle. Many genes are thought to be involved in this process, and tight regulation of function or expression of the relevant genes is required for proper cell cycle progression. The checkpoint control at the two transition points, G\(_1\)/S and G\(_2\)/M, ensures either progression or blocking of cell cycle according to the state of the cell. Several regulators such as cyclin-dependent kinases (CDKs)\(^{1}\) function at these points (1, 2). CDK activity is subject to regulation by association with positive regulatory subunits known as cyclins, negative regulators known as CDK inhibitors (CKIs), and by phosphorylation (reviewed by Pines (3), Morgan (4), Elledge and Harper (5), and Harper and Elledge (6)). The balanced function of these factors controls CDK activity and serves to integrate signals intended to coordinate cell cycle transitions. The levels of these proteins are tightly regulated both transcriptionally and post-translationally in yeast and mammalian cells. Association with G\(_2\) cyclins such as cyclin B and cyclin D in budding yeast, is required at the first checkpoint G\(_1\)/S (7–12), while in multicellular eukaryotes, several different CDKs, including CDK1, CDK4, and CDK6, are involved at different stages of G\(_1\) and S phase (13–17). Different G\(_1\) cyclins such as CLN1, CLN2, and CLN3 of budding yeast or cyclin D and cyclin E of mammalian cells and S phase cyclins such as CLB5, CLB6, or cyclin E and cyclin A are associated with these CDKs to ensure kinase function of the CDKs (18–22) at G\(_1\) to S phase progression. The phosphorylation state of the CDK itself and proper timing of destruction of the cyclins are important for regulation of CDK activities (4). Thus checkpoint control at G\(_1\)/S by these multiple elements is important for the cell to commence DNA replication, to initiate the cell cycle, and to integrate the positive and negative signals of the cell cycle. At the second checkpoint, G\(_2\)/M, p34\(^{cdc2}\), the prototypic member of cyclin-dependent kinase, is known to be the major regulator both in yeast and mammalian cells. Association with G\(_2\) cyclins such as cyclin B is required to function as an active protein kinase and to induce active mitosis (23, 24). The phosphorylation state of Cdc2 directly affects its kinase function and cell cycle progression to mitosis (4, 25, 26). Cdc25 dephosphorylates Cdc2 at serine/threonine residues, and this activates the kinase function of the Cdc2-Cdc13 (cyclin B) complex (27–29). Meanwhile, phosphorylation of Cdc2 by Wee1 kinase results in inactivation of Cdc2 kinase function at G\(_2\)/M (30–33), and destruction of G\(_2\) cyclin by the ubiquitin-associated cyclosome complex signals

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\(^{1}\)The abbreviations used are: CDK, cyclin-dependent kinase; CKI, CDK inhibitor; EMM, Edinburgh minimal medium; MMP, phosphate-free minimal medium; EMS, ethyl methanesulfonate; kb, kilobase(s); FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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A Novel Protein, Psp1, Essential for Cell Cycle Progression of *Schizosaccharomyces pombe* Is Phosphorylated by Cdc2-Cdc13 upon Entry into G\(_0\)-like Stationary Phase of Cell Growth*\

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the cell to exit from mitosis and to prepare for the next round of G1 to S phase progression (34). The CKIs such as p21, p27, or p15, which have negative function to inhibit CDK activity (5, 35), counteract the cyclins and are required to prevent cell cycle progression when cells are damaged. Mutations in CKI genes cause unregulated cell cycle progression, and this results in abnormal cell growth. In particular, deregulation of START at G1/S may allow cell growth and division to become insensitive to external cues (36–40). This can be a consequence of either the aberrant expression of positive regulators, such as the cyclins, or the loss of negative regulators, such as the CKIs. Thus proper regulation of cyclin-CDK-CKI complex formation is critical for normal cell cycle progression.

Even though the global cell cycle controls operating at the S (22, 41–45) and M phase transitions (46–48) have been extensively analyzed, unknown relevant factors still remain to be identified. Fission yeast Schizosaccharomyces pombe has been a useful model organism to study cell cycle control in eukaryotic cells. The relative ease of genetic manipulation of yeast has allowed detailed analysis of gene function in vivo, which has produced a paradigm for cell cycle control applicable to higher organisms. In an attempt to isolate factors involved in control of cell cycle progression, we searched for new cell cycle-related genes in S. pombe. In this paper we describe isolation of a novel gene, psp1+ (a gene encoding phosphoprotein at stationary phase of S. pombe cell), essential for progression of cell cycle. We used a strategy to isolate a new conditional mutant defective in cell cycle progression by chemical mutagenesis and to find a clone functionally complementing this mutant phenotype. Genetic and biochemical studies have permitted us to identify the function of the newly isolated psp1+ in S. pombe cells.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—The newly isolated temperature-sensitive (ts) mutant was derived from a haploid strain, ED665 (h+, ade6-M210, ura4-D18, leu1-32). For gene disruption analysis and back-crossing test, a S. pombe diploid strain, SP296 (h+, ade6-M210/ade6-M216, ura4-D18/urad18, leu1-32/leu1-32) and a haploid strain (h+, ade6-M216, ura4-D18, leu1-32/leu1-32) were used. For genetic complementation tests and comparative flow cytometric analysis, the known cell cycle mutants, cdc2−, cdc10−, cdc20−, and cdc22− were used. Ye-glucose (YPED) agar medium and Edinburgh minimal medium (EMM) were used as described by Gutz et al. (49). cDNA clones were also isolated by screening a genomic library prepared by ligating genomic DNA partially digested with Sau3AI into the BclI site of pBluescript vector (50).

**Isolation of Temperature-sensitive Mutants of S. pombe Defective in Cell Cycle Progression**—To identify a gene responsible for loss of cell cycle progression when mutated as shown in the mutant cyj92, a functional complementation test of the mutant with a S. pombe genomic library was carried out. The mutant cyj92, showing an elongated morphology and defect in cell cycle progression at 36 °C, was transformed with a S. pombe genomic library prepared by ligating genomic DNA partially digested with Sau3AI into the BclI site of pBluescript vector (50, 51). The transformed cells showing Leu+ colonies at 36 °C were first selected, replicated onto the plates containing phloxin B, and incubated at 36 °C. The pink colonies that grew up and showed wild type morphology at 36 °C were selected. Individual plasmid isolated from these transformants was retransformed to test its ability to suppress the elongated ts phenotype of cyj92 (50). Two such plasmids, containing 5.5-kb and 6.9-kb insert DNA, were isolated and characterized by restriction enzyme mapping and Southern hybridization. The 4.2-kb Sau3AI-PvuII fragment present in both of these plasmids and possessing the suppressive function was subcloned (Fig. 2a, pYJ4) and sequenced by the dyeoxy method of Sanger et al. (60) with USB Sequenase (U. S. Biochemical Corp.). cDNA clones were also isolated by screening a S. pombe cDNA library constructed in the λ-ZAP vector (61) with the labeled 1.5-kb PvuII-RsalHI fragment encoding most of the coding sequences. The DNA containing open reading frame sequences was designated as psp1+ (phosphoprotein of stationary phase of S. pombe). The disruption construct of the psp1 gene, with the urad4+ as the selectable marker, was made as follows (Fig. 2a, pYJ5). The PvuII-EcorV fragment containing most of the Psp1 coding sequences, including 5′-nontranslating region DNA, was deleted and replaced with the 1.8-kb urad4+ fragment. The resulting 5.5-kb BamHI-Apel fragment was used to transform a diploid strain SP286. Transplacement of the genomic psp1 gene with the urad4+ disrupted copy was confirmed by Southern analysis. The disrupted diploid strain was then sporulated according to the methods of Gutz et al. (49), and viability of the resulting meiotic products was analyzed (Fig. 2c).

**Flow Cytometric Analysis**—To determine the transcription start site of psp1+, the primer extension method was used (62). The antisense primer sequence that corresponds to the sequence from +150 to +169 (5′-GAGCAATTCTCATTATCATG-3′) of the amino-terminal coding re
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region was synthesized and labeled with T4 polynucleotide kinase (New England Biolabs) and γ-32P]ATP. The labeled primer was then hybridized with 5–10 μg of poly(A) RNAs isolated from the ED665 strain grown in EMM. Moloney murine leukemia virus reverse transcriptase (New England Biolabs) was added to the hybridization mixture and incubated at 37 °C for 30 min. The position of transcription start site was determined by comparing the size of the extended fragment with the sequenced DNA using the same primer.

Preparation of Membrane and Cytosolic Fractions—To determine the location of the Psp1 protein in S. pombe cells, membrane and cytosolic fractions of the cells were prepared (63). Cells were broken by the glass bead method (64) in 0.5 M Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM EGTA, 0.25 M sucrose, 20 μg/mL leupeptin/mL, and 10 μM phenylmethylsulfonil fluoride) and centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant was used as a cytosolic fraction. The pellet fraction was treated with 1% Triton X-100 for 30 min at 4 °C and used as a membrane fraction.

Production of GST-Psp1 Fusion Proteins—To prepare the protein product of the psp1 gene, coding region DNA of psp1 was fused to the isopropyl-1-thio-β-D-galactopyranoside-inducible glutathione S-transferase (GST) of fusion vector pGEX-3X. The GST-psp1 fusion constructs that encode different portions of Psp1 were prepared as follows (Fig. 4c, A). The DNA fragment containing the whole coding region (w), amino-terminal sequence 1–307 (Nm), or carboxyl-terminal sequence 323–408 (Cm2), and 338–408 (Cm3), bearing substitutions of serine residues at 327, 333, and 341 with alanine, respectively, were fused into the BamHI site of plasmid pGEX-3X. The DNA fragments encoding carboxyl-terminal sequences 323–408 (Cm1), 330–408 (Cm2), and 338–408 (Cm3), bearing substitutions of serine residues at 327, 333, and 341 with alanine, respectively, were fused into the same vector. Escherichia coli strain DH5α transformed with these fusion plasmids was grown in LB medium containing ampicillin, and production of GST-Psp1 fusion proteins was induced by growing cells in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C. Each GST-Psp1 fusion protein was purified on glutathione-agarose beads as described previously from E. coli crude cell extracts (65). For in vitro phosphorylation of Psp1 protein by Cdk2 kinase, GST and GST-Psp1 fusion proteins purified on glutathione-agarose beads were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0.

Immunochromic Analysis—To generate polyclonal antibody against Psp1 protein, affinity-purified GST-Psp1(w) protein was treated with factor Xa enzyme and separated on SDS-polyacrylamide gel (65). The Psp1 protein band on the gel was eluted, mixed with Freund’s adjuvant solution, and used to inject a rabbit. After two more successive injections in 2-week intervals, serum was collected and used as a source of antibody for Western blot analysis and immunocytochemical experiments (74). For determining cellular localization of Psp1 protein, FITC-conjugated anti-rabbit IgG (Sigma, Catalog No. F7512) was used. Cells grown at log or stationary phase were collected, washed, and fixed with methanol (56, 66, 67). Antiserum prepared against Psp1 was mixed first with the fixed cell suspension and then treated with FITC-conjugated anti-rabbit IgG in a concentration of 1/50 dilution. Nuclei of the cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (56).

Characteristics of the newly isolated temperature-sensitive mutant cyj92 defective in cell cycle progression. a, cellular morphology of the mutant cyj92: wild type morphology at 23 °C and an elongated cell shape at the restrictive temperature 36 °C. b, flow cytometry analysis of cyj92 (3 and 4) together with G1-specific mutant cdc10− (1) and G2/M-specific mutant cdc2− (2). Panels 1 in 1–4, cells grown in YEPD medium at 23 °C, panels 2 in I–3, cells transferred to nitrogen-deficient EMM and incubated at 23 °C for 25 h; panel 2 in 4, cyj92 cells grown in YEPD at 36 °C for 8 h, panels 3 and 4 in 4–3, cells arrested at G1 phase (panels 2 in I–3) were transferred back to YEPD and incubated at 36 °C for 4–8 h; and panels 3 and 4 in 4, cyj92 cells containing the genomic clone pYJ1 which suppresses the elongated mutant phenotype was grown in EM at both 23 °C (panel 3) and 36 °C (panel 4). This showed that the mutant, cyj92, has a defect both in completion of S phase (panel 2 in 3) and in proper mitosis (panel 2 in 4). The genomic clone pYJ1 suppresses this defect and leads normal cell cycle progression (panel 4 in 4). The absissa indicates intensity of fluorescence per cell, and the ordinate indicates cell numbers. The vertical line at the right side corresponds to n (G2), c, back-cross test of the mutant. The mutant cyj92 was crossed to wild type ED668 cells, and the diploid was sporulated. The resulting tetrads were incubated at 23 °C first and replica-plated and incubated at 36 °C.

5′-CTTGTGCGAGGCACTTCTGGTCG-3′, m2, 5′-TGCCCTCTGTCGGGCA-AAAAT-3′, and m3, 5′-GCACATCTGGGCCCTGATCGC-3′ and antisense strands of m1, 5′-GCAAGAGTCCTGCGACAG-3′, m2, 5′-ATTCTTGGGGCAAGGAGGCC-3′, and m3, 5′-GGAACGACAGGGAAGATGTCG-3′. Also, the sense strand of the amino terminus, 5′-ATGCCTTGTGAACTCATTGCG-3′, and the antisense strand of the carboxyl terminus, 5′-TACCCGAGCCTGGTGATCTAC-3′ were synthesized. To introduce point mutation at the sequence encoding serine 327 (Ser-327–Ala), the two primer pairs, sense strand of amino terminus and antisense strand of m1 and sense strand of m1 and antisense strand of carboxyl terminus were used for the first PCR. Then the second PCR was carried out using the first PCR product as a template and sense

FIG. 1. Characteristics of the newly isolated temperature-sensitive mutant cyj92 defective in cell cycle progression. a, cellular morphology of the mutant cyj92: wild type morphology at 23 °C and an elongated cell shape at the restrictive temperature 36 °C. b, flow cytometry analysis of cyj92 (3 and 4) together with G1-specific mutant cdc10− (1) and G2/M-specific mutant cdc2− (2). Panels 1 in 1–4, cells grown in YEPD medium at 23 °C, panels 2 in I–3, cells transferred to nitrogen-deficient EMM and incubated at 23 °C for 25 h; panel 2 in 4, cyj92 cells grown in YEPD at 36 °C for 8 h, panels 3 and 4 in 4–3, cells arrested at G1 phase (panels 2 in I–3) were transferred back to YEPD and incubated at 36 °C for 4–8 h; and panels 3 and 4 in 4, cyj92 cells containing the genomic clone pYJ1 which suppresses the elongated mutant phenotype was grown in EM at both 23 °C (panel 3) and 36 °C (panel 4). This showed that the mutant, cyj92, has a defect both in completion of S phase (panel 2 in 3) and in proper mitosis (panel 2 in 4). The genomic clone pYJ1 suppresses this defect and leads normal cell cycle progression (panel 4 in 4). The absissa indicates intensity of fluorescence per cell, and the ordinate indicates cell numbers. The vertical line at the right side corresponds to n (G2), c, back-cross test of the mutant. The mutant cyj92 was crossed to wild type ED668 cells, and the diploid was sporulated. The resulting tetrads were incubated at 23 °C first and replica-plated and incubated at 36 °C.
strand sequence of amino terminus and antisense strand sequence of carboxyl terminus as the primers. Amplified DNA was then cloned into vector plasmid pTZ18U. The change in the sequence at residue 327 was confirmed by sequencing the amplified fragment (Fig. 4c, B). Mutations at Ser-333 and Ser-341 were introduced by similar PCR methods. The primer pairs of the sense strand of amino terminus and antisense strand of m2 or m3 were used for the first PCR and followed by the second PCR with the amino- and carboxyl-terminal sequence pairs. For overexpression of these mutated Psp1 in ED665, amplified DNAs were cloned into plasmid vector pREP1 containing the thiamine-inducible nmt1 promoter (p*nmt1-m1, p*nmt1-m2, and p*nmt1-m3).

**Phosphatase Treatment and Phosphorylation of Psp1 Protein by the Cdc2-Cdc13 Complex**—To examine the phosphorylation state of Psp1 protein at different stages of cell growth, cellular proteins were treated

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**FIG. 2. Sequence of psp1** gene complementing the mutant phenotype of a novel ts2 mutant cyj92. a, map of genomic clones and their subclones complementing the mutant phenotype of cyj92 and a disruption clone. + indicates suppression of the mutant phenotype. b, nucleotide and deduced amino acid sequence of psp1. Lowercase and uppercase letters indicate genomic and cDNA sequences, respectively. The arrowheads indicate the start sites and the end of two cDNAs. The short intron sequence at the 5′-nontranslating region is absent in the cDNA clone 1. The potential Cdc2 substrate sequences, SPS32, SPS29, and SPS32 were underlined.

**c**, effect of disruption of psp1 on cell viability. Diploid SP286 strain containing psp1 gene disrupted with ura4 as evidenced by the Southern analysis of its chromosomal DNA (lane 2 in 1) produced two viable and two nonviable spores at 30 °C on tetrad analysis (2). These sequence data are available from GenBank under accession number L36906 and were deposited in August, 1994.
in vitro with phosphatase and analyzed. Cells were grown to log or stationary phase, collected, and broken in phosphatase buffer (50 mM Tris-HCl, pH 7.8, 5 mM dithiothreitol, 2 mM MgCl₂, 100 μg/ml bovine serum albumin) by the glass beads method (50). After removing the glass beads by centrifugation at 500 g for 4 min, the supernatant was solubilized in phosphatase buffer containing 1% Triton X-100. 5–10 μl of supernatant, solubilized pellet fraction, or whole cell extract was mixed with phosphatase and incubated at 30°C for 30 min in the presence of 100 μCi of ortho-[32P]ATP. The resulting protein was then resolved in SDS-PAGE and electrotransferred to a nitrocellulose paper for Western analysis with the polyclonal antiserum to Psp1 (final 1/40 dilute) and 50 μg/ml of protein A-agarose (Sigma). The DNA content was 4n (Fig. 1a, panels 1 and 2). A known G1/S mutant, cyj92, showed a normal cell cycle arrest at G1 after nitrogen starvation (Fig. 1a, panel 2, a and c). Cells were grown to late log phase (580 g/ml histone H1 (Boehringer Mannheim) was used (73). In vivo labeling of the protein was carried out with ortho-[32P]PPhosphate according to the procedure of Moreno et al. (50). Cells were first grown to late log phase (A₅₄₀ 2.5–3.0) in 5–10 ml of low phosphate medium (MMP plus 1 mM phosphate) and then transferred to the same volume of fresh MMP containing 1 mM ortho-[32P]PPhosphate (Amersham, Catalog No. PBS13). After incubation at 30°C for 5–6 h, cell extracts were prepared from the harvested cells as described above. For immunoprecipitation, 800–1,000 μl aliquots of the cell extracts were mixed with 20 μl of antisera of Psp1 (final 1/40 dilute) and 50 μl of protein A-agarose (Sigma). The immunoprecipitate was analyzed on SDS-PAGE and by autoradiography (74).

Phosphoamino Acid Analysis.—To determine whether the phosphorylated amino acid is serine as expected for phosphorylation by the Cdc2-Cdc13 complex, phosphoamino acid analysis of Psp1 was carried out. The protein labeled in vivo with ortho-[32P]PPhosphate was immunoprecipitated with Psp1 antibody and separated by SDS-PAGE. At the same time, GST-Psp1 protein phosphorylated in vitro by the Cdc2-Cdc13 complex with γ-[32P]ATP was also separated by SDS-PAGE. After being transferred to a polyvinylidene difluoride membrane (Millipore) and localized by autoradiography, the phosphorylated Psp1 band was excised and subjected to phosphoamino acid analysis (64, 75). The protein on the membrane was eluted and hydrolyzed in 6 N HCl for 1 h at 110°C. After being dried in a Speed Vac evaporator, the sample was dissolved in 6–10 μl of water containing 100 μCi each of phosphoserine, phosphothreonine, and phosphotyrosine. The sample was subjected to two-dimensional thin layer electrophoresis (HTLE 7000 apparatus, CBS Scientific). The first dimension was electrophoresed for 20 min at 1.5 kV in pH 1.9 buffer (50 ml of 88% formic acid, 156 ml of glacial acetic acid, and 1794 ml of water). The second dimension was electrophoresed for 16 min at 1.3 kV in pH 3.5 buffer (100 ml of glacial acetic acid, 50 ml of pyridine, and 1880 ml of water). [32P]-Labeled individual phosphoamino acids were identified by aligning the phosphoamino acid markers stained with ninhydrin to the signals obtained by autoradiography.

RESULTS

Characteristics of a Novel Temperature-sensitive (ts⁻) Mutant Defective in Cell Cycle Progression and a Gene Complementing This ts⁻ Phenotype.—Through EMS mutagenesis of S. pombe cells, several new temperature-sensitive mutants defective in cell cycle progression were obtained (52). Morphological analysis of one of the mutants, cyj92, showed a normal cell shape at 23°C but showed an elongated cellular morphology at 36°C (Fig. 1b). When cyj92 cells were grown in rich medium at 23°C, most of the cells were rounded and cell cycle progression stopped at S phase (Fig. 1c, panels 1 and 2). A known G1/S mutant, cyj92, showed cell cycle arrest at G1 after nitrogen starvation (Fig. 1b, 4, panel 1). When they were shifted to a restrictive temperature 36°C, DNA content increased and in the majority of cells the DNA content was 4n (Fig. 1a, panels 1 and 2). But when they were shifted to a restrictive temperature 36°C, DNA content increased and in the majority of cells the DNA content was 4n (Fig. 1b, 4, panel 2) indicating blockage of cell cycle after G2 phase. When the mutant cells were grown in nitrogen-deficient medium at 23°C instead, cell growth was arrested at G1 phase as shown in wild type cells (Fig. 1b, 3, panel 2). However, if these G1-arrested cyj92 cells were shifted to YEPlD medium and incubated at 36°C, unlike wild type cells, they could not progress completely to G2 phase, and cell cycle progression stopped at S phase (Fig. 1a, 3, panels 3 and 4). A known G1/S mutant, cdc10⁻, and G/M mutant, cdc2⁻, showed cell cycle arrest at G1 after nitrogen starvation at permissive temperature 23°C also (Fig. 1b, 1 and 2, panels 2) when they were tested simultaneously with cyj92. They

FIG. 2—continued
showed their mutant phenotypes at the restrictive temperature (36 °C); that is, blockage of cell cycle progression to G2/S phase and after G2 phase, respectively, as expected (Fig. 1b, 1 and 2, panels 3 and 4). Back-cross of the mutant strain cyj92 to wild type strain ED668 revealed 2^-2^ segregation of the mutant phenotype at 36 °C which indicates that the mutation shown in cyj92 is a single gene mutation (Fig. 1c). Genetic complementation tests of cyj92 with known cdc mutants of G1 and S such as cdc10^-, cdc20^- , or cdc22^- and G2/M phase mutants cdc2^- and wee1^- showed that the mutation in strain cyj92 was not allelic with any of them. Thus we concluded that the mutant we had isolated is a new ts^- mutant possessing a defect in cell cycle progression after the G2 phase at restrictive conditions and also at G1/S phase when cells pass the G2 phase normally. It is likely that once the mutant cells progressed to G2 phase, the mutation in cyj92 can cause blockage of further progression to M or cytokinesis. If mutant cells were in G1, this progression through the next S phase was also adversely affected. It is alternatively possible that the mutation carried in strain cyj92 causes blockage of cell cycle progression both at G1/S and G2/M.

Functional complementation of this mutant with a S. pombe genomic library identified the two overlapping clones of 8.5 kb and 6.9 kb (pYJ1 and pYJ2). Subcloning and retransformation data confirmed that the 4.2-kb PvuII-Stau3A fragment (pYJ4) was sufficient to suppress the ts^- phenotype (Fig. 2a). cyj92 mutant cells harboring plasmids pYJ1 or pYJ4 showed normal cell morphology at 36 °C, and their DNA content analysis indicated normal cell cycle progression at the restrictive temperature, 36 °C. Increased ploidy seen in cyj92 mutant cells (Fig. 1b, 4, panel 2) also disappeared in the transformed cells (Fig. 1b, 4, panel 4); transformants were mainly 2n like the wild type. Sequence analysis of this genomic DNA revealed an open reading frame sequence of 408 amino acids (Fig. 2b). Meanwhile, sequence analysis of several cDNA clones indicated the presence of two different transcripts depending on whether or not a short intron at the 5^-terminus was processed. As shown in Fig. 2b, one cDNA clone, which started at -90 (clone 1) did not contain sequences between -57 and -2 (intron). However, the other cDNA clone, which started at -88 (clone 2) contained these sequences. Primer extension experiments using mRNA isolated from cells grown in EMM also revealed two transcript start sites correlating with the two cDNA clones that differed by the presence or absence of the intron sequence (data not shown). Hydropathy plot analysis of the deduced amino acid sequence of psp1^+^- showed that it is very hydrophobic and contains a putative transmembrane domain at its carboxyl terminus (amino acids 353–369). Sequence homology comparison of this protein showed that no known protein sequences in the GenBank™ data base exhibited high homology with it. Thus we concluded this gene to be a newly identified gene and designated it psp1^+^- (phosphoprotein of stationary phase of S. pombe). Sporulation and tetrad analysis of a diploid strain containing one copy of psp1 transplanted with ura4^- (Fig. 2a, pYJ5) showed 2^-2^- segregation for cell viability at 30 °C (Fig. 2c). All viable spores were Ura^- indicating that psp1 is essential for vegetative cell growth at 30 °C.

Expression of Psp1—Northern analysis of total RNA isolated from the wild type cells grown in EMM showed a 1.3-kb transcript, and its level did not fluctuate much throughout the cell cycle. The level of psp1 transcript in the synchronized cells released from the S phase arrest was almost same as that in the cells progressing through G2 and M phase (data not shown). The level of Psp1 protein detected by Western analysis in the same synchronized cells was also constant throughout the cell cycle (data not shown). Thus, in actively growing cells the amount of Psp1 protein as well as its transcript is likely to be constant throughout the cell cycle. When the location of Psp1 protein in cells was examined by Western analysis using fractionated cell extracts and Psp1 antibody, Psp1 protein was found to be in the pellet fraction rather than in the soluble fraction (Fig. 3a). In addition, two different molecular weight forms of Psp1 protein were detected, and the ratio of these two forms differed depending on the phase of cell growth. In actively growing log phase cells (Fig. 3a, M-1, and Fig. 3b, 1–5), the lower molecular weight protein band was the major form. However, as soon as cells enter stationary phase, the higher molecular weight species became predominant (Fig. 3a, M-2, and Fig. 3b, 6–8). This result suggests that Psp1 protein is modified to a higher molecular weight form upon entry into G0-like stationary phase.

Localization of this protein in situ by FITC-conjugated anti-rabbit IgG and Psp1 antibody demonstrated that Psp1 protein is present throughout the whole cell in the actively growing stage (Fig. 3c, 1 and 3), whereas it is localized to each end of the cell at stationary phase (Fig. 3c, 6 and 8). We suggest that when cells are actively growing, Psp1 protein function may be re-
Phosphoamino acid analysis of Psp1 protein labeled in vitro indicates phosphoserine detected by autoradiography; Tnmt1-psp1 by SDS-PAGE and autoradiography. The phosphorylation of Psp1 protein with the Cdc2-Cdc13 kinase complex was specific.

Fig. 4. Phosphorylation state of Psp1. a, phosphatase treatment of Psp1 protein in vitro. Proteins in the membrane (M) and total fraction (T) of the cells from log and stationary phase (sta) were treated in vitro with λ-phosphatase (PPase), separated on SDS-PAGE gels, and analyzed by Western blotting with Psp1 antibody. – and + indicate the untreated and phosphatase-treated proteins, respectively. b, in vitro phosphorylation of Psp1 with the Cdc2-Cdc13 kinase complex. The GST-Psp1 fusion protein purified from E. coli was mixed with the mitotic Cdc2-Cdc13 complex in the presence of \( \gamma^{32}\)P[ATP and analyzed by SDS-PAGE and autoradiography. The bands in lanes 1–3 showed the phosphorylated proteins, and those in lanes 4–6 are the same amount of the proteins as in lanes 1–3 stained with Coomassie Blue. c, identification of the serine residue phosphorylated in vitro by Cdc2-Cdc13 (A) and in vivo (B). Three serine residues (327, 333, and 341) at the potential Cdc2 substrate sites in the carboxyl-terminal region of Psp1 protein were mutated to alanine and tested for phosphorylation by Cdc2-Cdc13. Lanes 1–6 in A showed the phosphorylated part of the GST-Psp1 fusion protein. The whole Psp1 protein (w), carboxyl-terminal protein (Ct), and carboxyl-terminal protein mutated at serine 327 (Cm1) showed phosphorylation by Cdc2, but the carboxyl-terminal protein mutated at serine 333 (Cm2, lane 5) did not show phosphorylation by Cdc2. Lanes 0–4 in B showed the phosphorylated Psp1 protein produced in vivo from the pnmnt1-psp1 plasmid. The entire psp1 gene mutated at the base encoding serine residue was fused to pnt1 vector and was expressed in wild type ED665 cells. The cells grown to late log phase in the absence of thiamine were labeled in vivo as described under “Experimental Procedures.” Cell extracts were prepared and immunoprecipitated with Psp1 antibody and analyzed on SDS-PAGE gels. The open arrowhead indicates the protein produced from the chromosomal copy of the psp1+ gene, and the filled arrowhead indicates that from ptmnt1-psp1. The asterisk indicates the mutation point. d, phosphoamino acid analysis of Psp1 protein labeled in vitro by Cdc2 (left) and in vivo in the presence of ortho\(^{32}\)P[phosphate (right). S indicates phosphoserine detected by autoradiography; T and Y correspond to the positions of phosphothreonine and phosphotyrosine, respectively, detected by ninhydrin staining as shown in 2. e, direct interaction of Psp1 with Cdc2 kinase shown by the yeast two-hybrid method. psp1+ fused to the Gal4 binding domain sequence (pGBT9) and cdc2+ fused to the Gal4 activation domain sequence (pGAD) were introduced into yeast S. cerevisiae strain SFY526, and expression of the lacZ gene in yeast was examined. 1 and 2, cells containing pGBT9-psp1 and pGAD-cdc2 alone, respectively; 3, cells containing both pGBT9-psp1 and pGAD-cdc2; 4, cells containing pCL1 of the whole Gal4 gene.
When we substituted each serine residue at 327, 333, and 341 in the carboxyl-terminal region with alanine (Cm1, Cm2, and Cm3) and examined the phosphorylation state of the truncated Psp1 protein by Cdc2-Cdc13 in vitro, the serine at position 333 was the only critical one for phosphorylation by Cdc2 kinase (Fig. 4c, A-lanes 4–6). Experiments with in vivo labeled protein also indicated that the serine residue at 333 is required for phosphorylation (Fig. 4c, B). PCR-mediated site-directed mutagenesis of psp1 generated a base change at the sequences encoding serines 327, 333, or 341, respectively, and resulted in their being changed to alanine. When this mutated full-length psp1 was introduced in wild type cells as pnm1-psp1 fusions (pnm1-m1, pnm1-m2, pnm1-m3) (Fig. 4c, B, m1, m2, m3) and the phosphorylation state of the mutated Psp1 by in vivo phosphate labeling at stationary phase was examined, serine 333 was shown to be essential for phosphorylation in stationary phase. The cells containing the unmutated pnm1-psp1 (w) produced more phosphorylated Psp1 than cells containing only the chromosomal copy of psp1 (lanes 1 and 0). This indicated that the Psp1 produced from the overexpression plasmid pnm1-psp1 is phosphorylated like that in the chromosome. However, when the cells containing pnm1-m2 (mutation at serine 333) was used, in vivo phosphate labeling of the Psp1 greatly decreased (lane 3). This decrease was not observed when pnm1-m1 and pnm1-m3 (containing mutations at 327 and 341, respectively) were analyzed instead (lanes 2 and 4). This confirms the importance of serine 333 for phosphorylation in vivo. Phosphoamino acid analysis of the Psp1 protein phosphorylated both in vivo and in vitro confirmed that phosphorylation actually occurred at a serine residue (Fig. 4d).

To examine whether phosphorylation of Psp1 by Cdc2 occurred through direct binding of Cdc2 to Psp1, a yeast two-hybrid system was employed. When the plasmids containing Gal4 binding domain-cdc2 and Gal4 activation domain-psp1 were introduced into a S. cerevisiae strain SFY526 and β-galactosidase production was examined, β-galactosidase production was observed. This indicates interaction between Psp1 and Cdc2 (Fig. 4e). The level of β-galactosidase produced was not as great as that from GAL4 itself (lanes 3 and 4). However there is strong indication that Cdc2 and Psp1 interact with each other.

Growth-inhibitory Effect of Overexpressed Psp1 Protein on Actively Growing S. pombe Cells—Since a gene disruption study indicated that psp1 is essential for cell growth (Fig. 2c), we examined the multicopy gene dosage effect of this essential protein on cell growth. This was done by introducing multicopy plasmid pnm1-psp1 into wild type cells and measuring their growth rate in the presence and absence of thiamine. A wild type culture grown to log phase in the presence of thiamine, which represses the nmt1 promoter function, was split in half.

mid were first grown in EMM in the presence of thiamine, and Psp1 expression was induced by transferring the cells to EMM devoid of thiamine. The closed circles indicate the growth curve of the cells grown continuously in EMM containing thiamine (uninduced), and the closed squares indicate that of the cells grown in thiamine-less EMM (induced). The arrow in a indicates the point where the thiamine induction actually started, approximately 12 h after transfer to the thiamine-less medium. The growth curves in b were from the cells that were transferred to fresh medium to a lower cell density at the point where thiamine induction actually started in a. The bands in the inset showed the amount of Psp1 proteins detected by Western analysis of the proteins in the uninduced (A and B) and induced cells (a and b). c, DNA content and shape of Psp1 overexpressed cells. The cells from each point in the growth curves in b were stained with DAPI or analyzed flow cytometrically. 1 and 2, DAPI-stained cells from uninduced (1) and Psp1-induced (2) cultures; 3, flow cytometrically measured DNAs in uninduced (white) and induced (black) cells. The numbers in the panels correspond to the points in the growth curve in b: 0 (1), 7.5 (2), 10.5 (3), 13.5 (4), and 30 h (5) after transfer to the fresh medium.
we continued incubating one half at 30 °C in the same medium as before. The other half was transferred to medium devoid of thiamine which induces nmt1 promoter function. With the onset of induction psp1 at 12 h after transfer to thiamine-less medium, growth of the cells in thiamine-less medium became slower than those in thiamine-containing medium (Fig. 5a). When cells grown for 12 h in the above conditions were passaged into fresh medium at a lower cell density, the growth difference between the two cultures was greater in early log phase than that in late log phase (Fig. 5b). Cells overexpressing Psp1 protein in early log phase, as evidenced by Western analysis of the total protein with Psp1 antibody (Fig. 5b, inset, A and a), showed half the growth rate of the uninduced cells (Fig. 5b, A and a). However differences in growth rate, caused by psp1 overexpression, decreased as cells entered stationary phase (Fig. 5b, B and b). The protein produced in the cells from pnmnt1-psp1 1 log phase was mainly the low molecular weight form whereas that in stationary phase was the phosphorylated high molecular weight form (Fig. 5b, inset a and b) as for that produced from the chromosomal copy of psp1+ (Fig. 5b, inset A and B). The cells with retarded growth following overexpression of Psp1 also exhibited an altered cellular morphology (Fig. 5c). These cells rounded up and DAPI staining of nuclear DNA revealed that the area occupied by nuclear DNA enlarged, that is, the compactness of the nuclear DNA was lost (Fig. 5c, 1, panels 3 and 4). Flow cytometric observation of DNA content in the Psp1 overexpressed cells indicated doubled or tripled DNA contents (Fig. 5c, 1, panels 2–4). The changes in DNA content and cellular morphology were maximum at 13.5 h after transfer to fresh medium (4n or 8n, Fig. 5c, 1, panel 4). Growth rate at this hour was about one-fourth of that in uninduced cells (Fig. 5b, 4). However, by 24 h after transfer to fresh media, the growth difference became relatively small, and DNA content of the induced cells tended to return to amounts found in uninduced cells (2n or 4n). After 30 h, at which time cells entered the stationary phase (Fig. 5b, 5), DNA content and morphology of the cells overexpressing Psp1 were similar to those of the cells containing only one copy of the psp1 gene (Fig. 5c, 3, panel 5). Thus maximal growth inhibition and changes in DNA content on overproducing Psp1 was observed in the actively growing cells rather than in the slow growing cells. However, the doubling time of the cells in which Psp1 was overexpressed was prolonged.

**DISCUSSION**

Temperature-sensitive mutants of *S. pombe* defective in normal cell cycle progression provide a convenient system to identify the elements involved in cell cycle regulation. Random mutagenesis of *S. pombe* cells enabled one to isolate such a mutant. Using the same mutagenic approach as Nurse et al. (51), we isolated one mutant, cyj92, which possesses an elongated cellular morphology similar to G1/S mutants of Nurse et al. (51) at 36 °C. Comparative DNA content analysis of the mutant in rich and nitrogen-starved media indicated that the mutation in cyj92 hindered progression of *S. pombe* cell from G1 to S phase and perhaps also from G2 to M phase. The terminal phenotype of the mutation is accumulation of abnormal DNA which is either incapable of completing DNA replication (Fig. 1b, 3) or overreplicated in the absence of separation into daughter cells (Fig. 1b, 4). It is likely that the mutation causes both blocking of S phase which leads to the prevention of onset of the subsequent mitosis and a defect in mitosis which prevents reinitiation of the subsequent S phase and leads to an increase in ploidy.

The characteristics of the mutant phenotype enabled us to isolate a novel gene, psp1+1, which suppresses the elongated mutant phenotype of cyj92 at 36 °C and made cell cycle progression normal. The finding that psp1 gene function is required for normal cell growth at 30 °C suggests that this gene is essential for vegetative growth. However, the fact that its overexpression in the actively growing cells (log phase cells) is detrimental to cell growth indicates that excessive functioning of this gene also causes a defect in normal cell growth. From the flow cytometric DNA analysis data we speculate that this is due either to accumulation of abnormal DNA or to inhibition of production of the elements necessary for normal cell division. Since we were not able to observe the accumulation of undivided cells, that is, the cells attached together without septum, we consider that overproduction of Psp1 protein does not affect the cytokinesis process itself but rather affects the normal process of DNA replication. The accumulation of the 2n or 4n DNA content in the actively growing cells by overproduction of Psp1 protein suggests that the signal for completion of DNA replication may not be transferred correctly to the elements working in G2 or M. Alternatively, it may be possible that overexpression of psp1 may lead to bypass or delay of the mitosis process and thereby allow continued replication of DNA. The fact that Psp1 protein is required for normal DNA replication but excessive function of this protein causes delay of the cell cycle suggests that this Psp1 may act at two different points in the cell cycle. Identification of target molecules with which Psp1 protein interacts will be necessary to elucidate the detailed function of this protein. The finding that the inhibitory effect on cell growth becomes less severe upon entry into the stationary phase indicated that this protein is in its active form in actively growing cells but is inactivated when cell growth slows down. However, we do not know whether inactivation of this protein is a prerequisite for entry into the stationary phase or conversely entry into stationary phase leads to inactivation of this protein.

The results that Psp1 is a phosphoprotein and that its dephosphorylated form is the major one in actively growing cells indicated that the function of this protein depends on phosphorylation. If this protein functions as a positive element, required in the cell cycle progression, the dephosphorylated form is active in actively growing cells and the phosphorylated Psp1, which is the major form in stationary phase cells, is inactive. However, if Psp1 functions as a negative element, the dephosphorylated form is inactive in actively growing cells, and the phosphorylated form is its active form in the stationary phase. Although we don't know where in the cell cycle Psp1 acts, the result that this protein is phosphorylated by the Cdc2-Cdc13 complex in vitro suggests that this protein could be one of the substrates for the Cdc2-Cdc13 complex and hence Cdc2 kinase may be associated with its function.

The carboxy-terminal domain of this novel protein is phosphorylated at serine 333 by Cdc2 kinase, and alteration of this serine to alanine abolishes phosphorylation by Cdc2 kinase in vitro. However, in vivo we were not able to detect a defect in phosphorylation in the cdc2 mutant at the restrictive temperature. Because it showed the mutant phenotype within 4–5 h after being shifted to restrictive temperature, this is not long.
enough for cdcl2- cells to enter the stationary phase to show its kinase function. It is only in the stationary phase of the cell growth that Psp1 protein is phosphorylated and cdcl2- cells die after 4–5 h at the restrictive temperature long before we were able to detect the defect in cdcl2 kinase function in the mutant. Thus it was not possible to determine whether mutation in cdcl2 kinase affected the function of Psp1 in actively growing cells. We consider that phosphorylation of Psp1 protein upon entering the stationary phase is one of the major events for slowing cell growth, and cdcl2 kinase controls this process. The yeast two-hybrid method confirmed that Cdc2 and Psp1 interact directly in vivo. Thus we can suggest that when cell cycle progresses actively in log phase, Psp1 protein is in its dephosphorylated state, and Cdc2 function is not required. However, when cell cycle progression slows at the stationary phase, Cdc2 kinase phosphorylates Psp1 protein, and the phosphorylated form of Psp1 facilitates entry into the G0 phase of the cell cycle or inactivates elements required for active cell growth. Cdc2 protein function is required for modification of Psp1 protein at this stage and there must be phosphatase(s), which removes the phosphate from Psp1 protein when cells begin active growth. We did not find yet what gene product is involved in dephosphorylation of Psp1 upon entry into active cell growth cycle. It will be interesting to find a specific phosphatase for Psp1, and function of this phosphatase may be a key regulatory element for activation of Psp1 protein function.

Localization of this Psp1 toward each tip of the cell upon entry into the stationary phase may have the property to form protein complexes in the cells for his critical reading of this manuscript. The cdcl2 gene is a multicopy suppressor for mutation in the cdcl2 gene. The cdcl2 gene is a multicopy suppressor for mutation in the cdcl2 gene. Therefore it was not possible to determine whether mutation in cdcl2 kinase affected the function of Psp1 in actively growing cells. We consider that phosphorylation of Psp1 protein upon entering the stationary phase is one of the major events for slowing cell growth, and cdcl2 kinase controls this process. The yeast two-hybrid method confirmed that Cdc2 and Psp1 interact directly in vivo. Thus we can suggest that when cell cycle progresses actively in log phase, Psp1 protein is in its dephosphorylated state, and Cdc2 function is not required. However, when cell cycle progression slows at the stationary phase, Cdc2 kinase phosphorylates Psp1 protein, and the phosphorylated form of Psp1 facilitates entry into the G0 phase of the cell cycle or inactivates elements required for active cell growth. Cdc2 protein function is required for modification of Psp1 protein at this stage and there must be phosphatase(s), which removes the phosphate from Psp1 protein when cells begin active growth. We did not find yet what gene product is involved in dephosphorylation of Psp1 upon entry into active cell growth cycle. It will be interesting to find a specific phosphatase for Psp1, and function of this phosphatase may be a key regulatory element for activation of Psp1 protein function.

Localization of this Psp1 toward each tip of the cell upon entry into the stationary phase may have the property to form protein complexes in the cells leading to translocation of the protein in a certain compartment of cells and the dephosphorylation of Psp1 may exist in monomeric form and is localized throughout cells as an active monomeric form. The following model for Psp1 protein function can be drawn (Fig. 6). Psp1 protein is activated by dephosphorylation with a phosphatase when the cells enter G1 to S phase of the cell cycle and is inactivated by phosphorylation with Cdc2 kinase upon entry into the stationary or G0 phase. It is an essential protein for cell growth, and regulation of this protein function by phosphorylation could be the key feature in cell cycle progression from G1 to S. In addition to Cdc2 protein, the other element(s) which interact directly with this Psp1 protein should exist, and finding this element(s) will be essential for further characterization of Psp1 protein function.

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Addendum—While we were preparing this manuscript, Ishii et al. (77) reported the sds23 gene which has the same DNA sequence as the p1′ gene. The sds23 gene is a multicopy suppressor for mutation in p1p and 20 S cyclosine/anaphase promoting complex.