A Mitotic Role for a Novel Fission Yeast Protein Kinase *dsk1* with Cell Cycle Stage Dependent Phosphorylation and Localization

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The fission yeast *dsk1*⁺ gene, a multicopy suppressor for cold-sensitive *dis1* mutants, encodes a novel 61-kd protein kinase. It is a phosphoprotein, and phosphoserine is the major phosphorylated amino acid. Hyperphosphorylation of *dsk1* causes a mobility shift, resulting in two *dsk1*-specific protein bands. The phosphorylation pattern is strikingly altered when cell cycle progression is delayed or arrested. The slowly migrating phosphorylated form is prominent in mitotically arrested cells, and the fast migrating form is enriched in interphase-arrested cells. *dsk1* is a protein kinase. It auto-phosphorylates as well as phosphorylates myelin basic protein (MBP). Phosphotyrosine as well as phosphoserine/threonine were found in autophosphorylation, but no tyrosine phosphorylation occurs when MBP was used as the substrate. The *dsk1* immunoprecipitates from mitotically arrested cells have a several-fold higher kinase activity than that from wild type. The haploid gene disruptant is viable, indicating that the *dsk1*⁺ gene is non-essential for viability. High dosage of *dsk1*⁺, however, strongly delays the G2/M progression. Immunofluorescence microscopy using anti-*dsk1* antibody shows that localization pattern of *dsk1* protein strikingly alters depending on cell cycle stages. In G2-arrested cells, *dsk1* locates in the cytoplasm, whereas in mitotically arrested cells, nuclear stain is intense. In wild-type cells, nuclear stain is seen only in mitotic cells. Hence *dsk1* protein may play an important role in mitotic control by altering cellular location, degree of phosphorylation and kinase activity. We discuss possible roles of *dsk1* kinase as an add-on regulator in mitosis.

INTRODUCTION

The fission yeast *Schizosaccharomyces pombe* cold-sensitive (cs) *dis1* mutants display a characteristic mitosis-defective phenotype. At the restrictive temperature, the chromosomes condense and the spindle forms and elongates but fails to separate the chromosomes (Ohkura *et al.*, 1988). *cdc2* kinase activity monitored as histone H1 kinase increases and remains high in *dis1* mutant at the restrictive temperature (Kinoshita *et al.*, 1991b), suggesting that *dis1* can enter mitosis but fail to exit from it. Another mitotic phenotype is that artificial linear minichromosomes are rapidly lost in *dis1* at the permissive temperature (Ohkura *et al.*, 1988). Two other classes of cs *dis* mutants, namely, *dis2* and *dis3*, which exhibit highly similar phenotypes, have been isolated (Ohkura *et al.*, 1988). Because any pair of double *dis* mutants are lethal at the permissive temperature, the

three *dis*⁺ genes might share an overlapping essential function. The *dis2*⁺ gene was isolated by transformation and found to encode a polypeptide highly similar to the catalytic subunit of mammalian type 1 protein phosphatase (Ohkura *et al.*, 1989). The *dis3*⁺ gene was also isolated by complementation of the mutant phenotype and found to be essential for viability and to code for a 110-kd protein enriched in the nucleus (Kinoshita *et al.*, 1991). The function of the *dis3* protein may also be implicated in protein dephosphorylation as multicopy plasmid carrying *dis3*⁺ suppresses the cs phenotype of a phosphatase *ppe1* deletion mutant (Shimanuki *et al.*, 1993). *dis3* protein has sequence similarity to the budding yeast *Saccharomyces cerevisiae* *SSD1/SRK1* (Sutton *et al.*, 1991; Wilson *et al.*, 1991), which appears to be related to the protein phosphatase *SIT4* and the protein kinase regulatory subunit *BCY1*.

We attempted to isolate the *dis1*⁺ gene by cloning genomic DNA fragments that complement the *cs* phenotype of *dis1-288*. However, we encountered several independent genomic DNA sequences that could suppress *cs dis1* alleles as multicopy suppressors (Takeuchi, unpublished observations). By nucleotide sequence determination, we found that one of the strongest suppressors among them encodes a protein kinase designated *dsk1* (*dis1*-suppressing protein kinase). We report here characterization of the *dsk1* protein kinase and provide evidence that *dsk1* kinase is implicated in a novel cell cycle control.

MATERIALS AND METHODS

Strains and Media

Haploid strains of *S. pombe* were used (Gutz *et al.*, 1974). *cs dis1-288* was previously described (Ohkura *et al.*, 1988). The following mutant strains were used, *nda3-KM311* (Hiraoka *et al.*, 1984), *nuc2-663* (Hirano *et al.*, 1988), *cdc25-22* (Russell and Nurse, 1986), *cdc2-33* (Nurse *et al.*, 1976), *cdc10-129* (Nurse *et al.*, 1976), and *cdc17-K42* (Nasmyth and Nurse, 1981). Rich yeast extract, peptone, dextrose growth medium (YPD) and synthetic EMM2 media were used.

Cloning of the *dsk1*⁺ Gene

S. pombe dis1-288 cells were transformed with a genomic library of *S. pombe* using pDB248⁺ vector with the *Saccharomyces cerevisiae* LEU2 as the marker gene (Beach and Nurse, 1981). The lithium acetate method (Ito *et al.*, 1983) was used for transformation of *S. pombe*. The plasmids recovered from Cs⁺ Leu⁺ transformants were subcloned to determine the minimal complementable genomic DNA fragment. Analyses by restriction mapping indicated that there are eight independent groups of genomic DNAs which can complement *cs dis1* mutants (Takeuchi, unpublished observations). One of those plasmids, pDS113, contains the *dsk1*⁺ gene. For construction of pDS113-9 (p(*dsk1*-sp)), a multicopy plasmid with the *dsk1*⁺ gene lacking the spacer region, pDS113-6 was cleaved by *Eco*T22I/*Hpa* I and blunt-ended ligated. For construction of pADH-*dsk1*, pTD184 was cleaved by *Tth*111I/*Eco*RV and resulting 1.6-kb fragment was blunt-end ligated into the *Sma* I site of the ADH promoter (Russell and Hall, 1983).

Southern, Northern, and Nucleotide Sequence Determination

Standard procedures for Southern and Northern hybridization (Maniatis *et al.*, 1982) were followed. Nucleotide sequence was determined by the dideoxy method (Yanisch-Perron *et al.*, 1985).

Insertion and Disruption of *dsk1*⁺

Integration of the genomic sequence into the chromosome by homologous recombination was done by the method of Rothstein (1983). Gene disruption of *dsk1*⁺ was performed with two different procedures, insertion and deletion. First, the *S. pombe ura4*⁺ gene (1.8 kb *Sac* I) was inserted at the essential *Sac* I site in a 6.5-kb *Hind*III fragment containing the *dsk1*⁺ gene (pDS113). The resulting plasmid pTD185 was digested with *Bgl*II and *Pvu* II, and the 4.3-kb fragment was used for transformation of a diploid (TP6D; the genotype, *h*⁺/*h*⁻ *leu1/leu1 ade6-210/ade6-216 his2/+ura4/ura4*). Stable Ura⁺ transformants were obtained and examined by tetrad analysis. Genomic Southern hybridization of heterozygous diploid, Ura⁺, and Ura⁻ haploid DNA produced restriction fragments (*Bgl*II/*Cl*a I cut) with expected sizes by insertion on the chromosome by homologous recombination. Second, a 2.5-kb *Bgl*III/*Pvu* II containing the *dsk1*⁺ gene was inserted into

the *Bam*HI/*Eco*RV site of Bluescript KS(+). The resulting plasmid pTD184 was digested with *Mlu* I/*Nde* I so that an internal portion (0.7 kb) of the *dsk1*⁺ coding region was deleted. Then the 1.8-kb *Sma* I *ura4*⁺ fragment was ligated into the blunt ends of pTD184. The resulting plasmid pTD184MNV was double-digested with *Cl*a I/*Not* I to obtain a 3.5-kb fragment containing the disrupted *dsk1*⁺ gene. The fragment was used for transformation of the same diploid described above. Genomic Southern hybridization of diploid and haploid DNA was done, and restriction bands with sizes expected for the disrupted gene were obtained. In both insertion and deletion mutants, the *ade6* marker was removed by crosses and the phenotype of resulting disruptants (the genotype; *h*⁻ *leu1 ura4 dsk1::ura4*⁺) was studied.

Induced Expression of the *dsk1*⁺ Gene under the *nmt1* Promoter

To express the *dsk1*⁺ gene under the promoter of *nmt1*⁺ gene (Maundrell, 1990), plasmid pREP1 (a kind gift of Dr. Maundrell) was digested with *Bam*HI and ligated to the 1.6-kb *Bam*HI fragment of pTD184TBVB that contains the *dsk1*⁺ coding region. The resulting plasmid pNM(184TBVB) was introduced into wild type (HM123; *h*⁻ *leu1*) or *cs dis1-288* mutant in the presence of thiamine. Transformants were first grown in minimal EMM2 medium containing 2 μ M thiamine. Exponentially growing cells were collected, washed three times, and transferred to EMM2 without thiamine. Derepression of the *nmt1*⁺ promoter takes roughly 10 h at 33°C in the absence of thiamine. To maintain cells at logarithmic phase, fresh EMM2 without thiamine was added at an appropriate interval.

Indirect Immunofluorescence Microscopy

The procedures for *S. pombe* (Hagan and Hyams, 1988; Hirano *et al.*, 1988) immunofluorescence were employed. Monoclonal antibody TAT-1 against *Trypanosoma brucei* α -tubulin (a kind gift of Dr. K. Gull, University of Manchester, UK) was used for staining microtubules in *S. pombe* (Woods *et al.*, 1989; Hagan and Yanagida, 1990). Cells were digested with 1.2 mg/ml Zymolyase 100 T (Seikagaku, Tokyo, Japan). Immunofluorescence microscopy using anti-*dsk1* antibodies was done as described below. Cells were fixed with methanol at -80°C (Booher *et al.*, 1989). Wild-type (HM123) cells carrying a vector (pSK248) or a multicopy plasmid with the *dsk1*⁺ gene (pDS113-6) were grown in the EMM2 medium at 33°C. Δ *dsk1* mutant cells carrying pSK248, pDS113-6, pDS113-8 (a COOH deletion), or pDS113-9 (a spacer deletion) were also grown in EMM2 at 33°C. *cdc25-22* and *nuc2-663* mutant cells carrying pSK248 or pDS113-6 were first grown in EMM2 at 26°C, then transferred to 36°C, and incubated for 4 h. Affinity purified anti-*dsk1* antibodies were prepared as described (Smith and Fisher, 1984). Anti-rabbit FITC antibodies (Cappel, Malvern, PA) were used as the second antibody.

Immunoblotting and Immunoprecipitation

The procedures of immunoblotting described by Towbin *et al.* (1979) were followed. The protein bands were electrophoretically transferred to nitrocellulose membrane after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting using anti-phosphotyrosine antibodies (PY20; ICN ImmunoBiologicals, Lisle, IL) was performed according to the procedures of Morrison *et al.* (1988). Affinity purification of anti-*dsk1* antibodies was as described in Ohkura *et al.* (1989). *S. pombe* extracts for immunoprecipitation were prepared as described (Shiozaki and Yanagida, unpublished observations). The amount of soluble protein used for immunoprecipitation was 6 mg. Immunoprecipitation was done as described in Harlow *et al.* (1988) with modifications appropriate for the extracts of *S. pombe* (Shiozaki and Yanagida, unpublished observations). To remove phosphate, immunoprecipitates was treated with potato acid phosphatase (PAP; grade II, Boehringer, Mannheim, Germany) with the procedures of Cooper and King (1986). PAP was purified by a MonoQ column (Pharmacia, Uppsala, Sweden) before use.

FACScan Analysis

A Becton-Dickinson FACScan (San Jose, CA) was used to monitor the cellular DNA contents. Procedures employed were similar to those described previously (Costello *et al.*, 1986). Cells ($1-5 \times 10^7$) were collected, washed twice with 1 ml distilled water, then resuspended in 3 ml distilled water. Ethanol (7 ml) was added with vigorous agitation, and cells were stored at 4°C for >12 h. After washing once with 5 ml of 50 mM sodium citrate (pH 7.0), and resuspension in 1 ml of the same buffer, RNase A (Sigma, St. Louis, MO; preboiled for 15 min) was added to a final concentration of 0.5 mg/ml. After a 2-h incubation at 37°C, propidium iodide (Sigma) was added (final 12.5 µg/ml), and the resulting stained cell suspensions were analyzed.

Preparation of Bacterial-made *dsk1* Proteins and Antisera

Two *dsk1* polypeptides, one deleting the NH₂-terminal 23 residues and another consisting of the spacer region, were made in *E. coli* using the T7 system (Studier *et al.*, 1986). First, the BamHI linker (10 mer, Takara) was inserted at the HindIII site of pUC118. Then, the resulting plasmid pUC118B was ligated, at the Xba I site, to the 0.4-kb EcoT221/*Mlu* I, which contained the spacer region of *dsk1*⁺. This plasmid pS67P was cut with BamHI, and the 0.4-kb fragment was inserted into the BamHI site of the expression vector pAR3039. The resulting plasmid pARDS18-1 produced a large quantity of polypeptide in *E. coli* BL21 (DE3) pLysS (Rosenberg *et al.*, 1987). The M.W. in SDS-PAGE was 22 kd (predicted size was 16 kd). This difference might be due to high content of Ser, Thr, and Pro in the spacer polypeptide. Second, pTD184 was digested with Tth111I and ligated to BamHI linker (10 mer). This plasmid was then digested with EcoRV and ligated to BamHI linker. The resulting plasmid pTD184TBVB was cleaved with BamHI, and the 1.6-kb BamHI fragment containing the greater part of the coding region of *dsk1*⁺ (deleting NH₂-terminal 23 amino acids and having 12 amino acids of C-terminal β -galactosidase) was obtained and inserted into the BamHI site of the expression vector pAR3039. The resulting plasmid pARDS18-3 produced a large amount of polypeptide (M.W., 60 kd) in *E. coli* BL21(DE3). These fusion polypeptides were purified by the procedures of Watt *et al.* (1985) and used as the antigen to immunize rabbits as described previously (Hirano *et al.*, 1988). *dsk1* protein was produced in *E. coli*. The inclusion body was isolated by centrifugation at 10 000 rpm for 10 min, and resulting pellets were washed by 50 mM tris(hydroxymethyl)aminomethane (Tris) HCl at pH 7.5 containing 0.1 M NaCl, 1 mM MgCl₂, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride (PMSF), solubilized by sonication in buffer A containing 2.5% SDS and 10% glycerol and passed through Ampure DT column (Amersham, Arlington Height, IL) in buffer A.

Assay of Protein Kinase In Vitro and Phospho Amino Acid Analysis

In vivo labeling of *S. pombe* by ³²P-H₃PO₄ (1 mCi for 10 ml EMM1 for immunoprecipitation and subsequent autoradiography of protein bands; 10 mCi for 10 ml for phosphoamino acid analysis of the protein band) was done by the procedures previously described (Shiozaki and Yanagida, 1992). Immunoprecipitates produced by affinity-purified anti-*dsk1* antibodies were washed three times by buffer H (Booher *et al.*, 1989) and twice by the kinase assay buffer KAB [50 mM TrisHCl at pH 7.5 containing 10 mM MgCl₂, 1 mM dithiothreitol (DTT) and 1 mM PMSF]. The precipitates were suspended in 10 µl KAB, and the kinase activity was assayed with the substrate myelin basic protein (MBP) (0.5 mg/ml; bovine brain, Sigma) after the addition of 0.1 mM ATP, 10 µCi γ -³²P-ATP at 25°C for 15 min. The reaction was stopped by boiling the reaction mixture after adding an equal volume of SDS-PAGE sample buffer. The cleared supernatant after centrifugation was run in SDS-PAGE and examined by autoradiography. The ³²P-labeled band of *dsk1* protein was excised and electroeluted. After hydrolysis in 6N HCl at 110°C for 1.5 h, the sample

was analyzed by two dimensional thin layer chromatography (first, pH 1.9; second, pH 3.5; Cooper *et al.*, 1983).

RESULTS

The *dsk1*⁺ Gene Encodes a Putative Protein Kinase

We isolated the *dsk1*⁺ gene of *S. pombe* as a multicopy suppressor of the cs mutant *dis1-288* (Ohkura *et al.*, 1988). Transformants containing the *dsk1*⁺ gene on plasmid pDS113 grow normally at the restrictive temperature of 22°C (Figure 1A). Complementation was not allele specific, as the plasmid rescued other cs *dis1* alleles. Caffeine hypersensitivity of *dis1* mutants (Ohkura *et al.*, 1988) was also complemented by the plasmid.

Subcloning revealed that pDS113-6, containing a 2.5-kb *Pvu* II-*Bgl*II fragment, complements *dis1-288*. Nucleotide sequence determination (Figure 1B) indicated that the insert DNA contained 1.6-kb open reading frame (indicated by the arrow in Figure 1A). The predicted amino acid sequence (Figure 1B) consists of 544 residues (calculated M.W., 61,071). The immediate upstream region of the predicted initiation codon contained termination codons in the same reading frame. No intron consensus was found in the coding region. *Nde* I, *Sac* I, and *Mlu* I sites (Figure 1B) essential for complementation existed within the coding region. A database search uncovered similarity to protein kinases, although none of them was highly similar. The amino acid sequences of two protein kinases, budding yeast YAK1 (Garret *et al.*, 1989 and 1991) and mouse CLK/STY (Ben-David *et al.*, 1991; Howell *et al.*, 1991), are 33 and 27% identical, respectively, to *dsk1* (Figure 1C) in the kinase domains (Hanks *et al.*, 1988). *dsk1* contains a spacer sequence, consisting of 125 highly hydrophilic residues (18% Ser, 9% Thr, and 10% Pro). Genomic Southern hybridization using the *dsk1*⁺ coding region as a probe showed a set of bands with the expected sizes under nonstringent conditions, suggesting that the *dsk1*⁺ gene is unique in the genome.

The *S. pombe* ADH promoter (Russell and Hall, 1983) was ligated with the *dsk1*⁺ coding sequence at the *Sma* I site. The resulting plasmid pADH(*dsk1*) containing a truncation of the NH₂-terminal 23 amino acids, rescues *dis1-288*.

dsk1 Disruption Mutant is Viable

To characterize the phenotype of the *dsk1* null mutant, a gene disruption was made using two approaches (MATERIALS AND METHODS). To make an insertional mutation (Figure 2a), the *dsk1*⁺ gene was cleaved at the essential *Sac* I site, and the fragment containing the *S. pombe ura4*⁺ gene (Grimm *et al.*, 1988) was inserted. For the deletion, 0.7 kb of the coding region was deleted and replaced with the *ura4*⁺ gene (Figure 2B). Then, linear insertion or deletion fragments were used

Figure 1. Subcloning and sequence determination of the *dsk1*⁺ gene. (A) Plasmid pDS113 containing the *dsk1*⁺ gene (←) complements a *cs* mutant *dis1-288* (+). Derivatives of pDS113 were made and examined for their ability to complement *dis1-288*. pADH-*dsk1* represents the *dsk1* gene under control of the *S. pombe* ADH promoter (Russell and Hall, 1983). Restriction sites; G, *Bgl*II; H, *Hind*III; M, *Mlu*I; N, *Nde*I; P, *Pvu*II; S, *Sac*I. (B) Nucleotide sequence of the *dsk1*⁺ gene is shown with the predicted amino acid sequence. Relevant restriction sites are shown. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases with accession number D13447. (C) Amino acid sequence comparison among three protein kinases, *dsk1*, budding yeast *YAK1* (Garret *et al.*, 1989, 1991), and *CLK/STY* (Ben-David *et al.*, 1991; Howell *et al.*, 1991). Identical amino acids are indicated by the asterisks. The spacer amino acid sequence of *dsk1* is also shown. The dots, - and + represent serine or threonine, acidic, and basic amino acids, respectively.

for one-step gene replacement of the chromosomal *dsk1*⁺ gene in the diploid TP6D by homologous recombination (see MATERIALS AND METHODS). The resulting heterozygous Ura⁺ diploid transformants were sporulated, and, in both cases, were found to produce four viable spores (the Ura marker segregated 2⁺:2⁻). Hence the *dsk1*⁺ gene is non-essential for viability.

To confirm that the *dsk1*⁺ gene was actually disrupted on the chromosome, Southern blots of Ura⁺ diploid and haploid genomic DNA were probed with the *dsk1*⁺ gene (Figure 2C). Restriction fragments with the expected sizes for the disrupted gene were obtained in diploid

and haploid (see the details in the Figure legend). Tetrad analysis of a cross between Δ *dsk1* deletion and *cs dis1-288* showed that these two loci are not linked. The double mutant was viable and showed a phenotype similar to *dis1-288*. Therefore, the *dsk1*⁺ gene is a multicopy suppressor for *dis1* mutants. The *dsk1*⁺ gene was mapped in a 2 000 kb *Not*I fragment of chromosome II (Fan *et al.*, 1988). The *dis1* locus was allocated in the short arm in chromosome III (Takeuchi and Yanagida, unpublished data).

Haploid cells of the *dsk1* deletion mutant (*h⁻ leu1 ura4 dsk1::ura4⁺*; designated Δ *dsk1*) germinated normally and grew with a generation time of 3 h in a synthetic

Figure 2. Gene disruption of the *dsk1*⁺ gene. (A) Insertional disruption. The linear *Pvu* II-*Bgl* III fragment containing the *dsk1*⁺ gene was cleaved at *Sac* I, the *ura4*⁺ marker gene was inserted, and *S. pombe* cells were transformed. The disrupted gene was integrated into the chromosome of diploid TP6D (*h*⁺/*h*⁻ *his2*⁺/*leu1*/*leu1* *ura4*/*ura4* *ade6-210/ade6-216*) by homologous recombination using one-step gene replacement. (B) Deletion. The 0.7-kb coding region of *dsk1*⁺ was deleted and *ura4*⁺ gene was inserted. The linearized DNA was integrated on chromosome. Resulting heterozygous *Ura*⁺ diploid transformants were sporulated, and, in both cases, four viable spores were produced. (C) Genomic Southern hybridization probed with the coding region *Pvu* II-*Bgl* III fragment as a probe. All genomic DNAs were digested with *Cla* I-*Bgl* III. Lanes 1 and 3, diploid TP6D (*h*⁺/*h*⁻ *leu1*/*leu1* *ura4*/*ura4* *his2*⁺/*ade6-210/ade6-216*); lane 2, for the heterozygous *Ura*⁺ diploid with the insertional disruption of *dsk1*⁺ gene, an additional 4.8-kb band was present; lane 4, for the heterozygous diploid with the deletion of the *dsk1*⁺ gene, an additional 4.1-kb band was seen; lane 5, a wild-type haploid; lane 6, a haploid insertional disruptant; lane 7, a haploid deletion of the *dsk1*⁺ gene. All genomic DNAs were digested with *Call* I/*Bgl* III. Restriction fragments with the expected sizes for the disrupted gene were obtained. (D) Fluorescence micrographs of DAPI-stained *S. pombe* cells; left, wild type; right, Δ *dsk1* deletion mutants. Bar indicates 10 μ m.

medium EMM2, somewhat slower than the wild type in EMM2 (2.5 h) at 33°C. The average cell size of Δ *dsk1* was identical to that of wild type. DAPI-stained wild type and Δ *dsk1* cells are shown in Figure 2D.

dsk1⁺ Gene Produces Two Closely Migrating ~60-kd Proteins

To identify the *dsk1* polypeptide in fission yeast cell extracts, rabbit anti-*dsk1* sera were raised (MATERIALS AND METHODS). Two polypeptides were produced in *E. coli*; a 60-kd polypeptide, deleting the NH₂-terminus and a 16-kd polypeptide consisting of the highly hydrophilic spacer region. Antisera made were used for identification of *dsk1* protein by immunoblotting. *S. pombe* cell extracts were prepared, run in SDS-PAGE and were immunoblotted by using affinity-purified anti-*dsk1* antibodies. We found that the *dsk1* polypeptide existed as two closely migrating bands (indicated by the 2 lines in Figure 3A; apparent M.W.s, 61 and 63 kd). The lower band was more intense than the upper in wild-type cells exponentially growing in the rich YPD and the synthetic EMM2 media (lane 4). Although there is a nonspecific band, these two bands were completely absent in Δ *dsk1* mutant cell extracts (lane 1). The level of 61- and 63-kd band intensities increased in deletion or wild-type cells carrying a multicopy plasmid (pDS113 or pDS113-6) with the *dsk1*⁺ gene (lanes 2, 3, and 5). Hence these two bands appeared to be produced by the *dsk1*⁺ gene. The same immunoblot bands were obtained by using anti-*dsk1* antibodies against the polypeptide containing the spacer region, indicating that the spacer was actually present in the *dsk1* polypeptide.

dsk1 is a Phosphoprotein and Its Phosphorylation is Altered in Various Cell-Cycle Mutants

The relative intensities of the two bands were found to be strikingly altered in different mutant extracts. Three examples of mutants used are shown in Figure 3B with the wild-type control at 20 and 36°C. The slowly migrating 63-kd band was prominent in the extracts of mitotically blocked *dis1-288* and *nda3-311*, whereas the fast migrating 61-kd band was prominent in G2-blocked *cdc25-22* mutant cells. The upper band was hardly visible in *cdc25-22* arrested cells. The same pattern to *cdc25-22* was obtained with the *cdc2-33* arrested cells.

We found that the mobility difference of *dsk1* bands shown above was due to protein phosphorylation. Immunoprecipitation by anti-*dsk1* antibodies was done by using wild-type extracts, incubated in the presence or the absence of potato acid phosphatase (PAP), run in SDS-PAGE and immunoblotted with the use of the same antibodies. The slowly migrating 63-kd band completely disappeared after PAP digestion (indicated by +; no treatment indicated by - in Figure 3C). Therefore, the retarded *dsk1* band in SDS-PAGE is due to protein phosphorylation.

Figure 3. Immunochemical characterization and phospho-labeling of dsk1 polypeptide. (A) Wild-type and Δ dsk1 deletion mutant cell extracts were run in SDS-PAGE and immunoblotted by using anti-dsk1 antibodies. Lanes 1–3, Δ dsk1 cells carrying the vector plasmid, multicopy plasmids with the *dsk1*⁺ gene, pDS113, and pDS113-6. Lanes 4 and 5, wild-type cells carrying the vector plasmid and pDS113-6. The dsk1 protein bands that were absent in the deletion mutants are indicated by the two short lines. A nonspecific band was produced that was present in all the extracts. The positions of molecular weight markers are shown. (B) Wild-type and mutant cell extracts were run in SDS-PAGE and immunoblotted with the use of anti-dsk1 antibodies. *dis1* and *nda3*, mitotically arrested *dis1-288* and *nda3-311* cells at 20°C for 8 h (the generation time of wild type at 20°C is 5–6 h); *cdc25*, G2-blocked *cdc25-22* cells at 36°C for 4 h. wt, 20°C and wt, 36°C, wild-type cells treated in the same way. The slowly migrating band was much enhanced in *dis1* and *nda3*, whereas the fast migrating band was prominent in *cdc25* cells. (C) Immunoprecipitated dsk1 protein from wild-type cells was untreated (–) or treated (+) with potato acid phosphatase (PAP), run in SDS-PAGE and immunoblotted by using anti-dsk1 antibodies. The slowly migrating band disappeared after PAP treatment. (D) Δ dsk1 deletion, wild type (wt) and *nuc2* strains were first grown at 26°C then transferred to 36°C (the restrictive temperature for *nuc2*) in EMM1 medium containing ³²P-H₃PO₄, and ³²P labeled extracts were prepared. Immunoprecipitates by anti-dsk1 antibodies were run in SDS-PAGE followed by immunoblotting with anti-dsk1 antibodies (left) or autoradiography (right). Labeled bands were electroeluted from the gel, hydrolyzed, and analyzed by thin layer chromatography. The phosphoserine spot is intense, whereas phosphothreonine is very faint. Phosphotyrosine was not detected.

To directly demonstrate that the dsk1 protein is phosphorylated, *S. pombe* cells were in vivo ³²P-labeled, and immunoprecipitation was performed by using anti-dsk1 antibodies (MATERIALS AND METHODS). Cells from three strains, Δ dsk1, wild type, and *nuc2-663* were grown at 26°C, then transferred to 36°C (the restrictive temperature for *nuc2*) and incubated in the presence of ³²P-H₃PO₄ for 5 h. Cell extracts were made and immunoprecipitated by anti-dsk1 antibodies followed by SDS-PAGE and autoradiography. The left panel in Figure 3D shows the immunoblot pattern by anti-dsk1. The slowly migrating band was prominent in *nuc2* cells which were blocked in mitosis at metaphase (Hirano *et al.*, 1988), and thus the ³²P-labeled upper dsk1 band in the *nuc2*-mutant was enhanced (right panel). As a con-

trol, no labeled band was obtained from Δ dsk1 cells. Thus dsk1 is a phosphoprotein, and the retarded dsk1 protein band represents a hyperphosphorylated form. The fast migrating band in the wild type might be weakly phosphorylated, judging from the presence of a faint 61-kd band in autoradiography. We attempted to employ *cdc25* mutant for the ³²P-labeling experiment of dsk1, but *cdc25-22* poorly grew in the phosphate-deficient EMM1 medium used for labeling at the permissive temperature and did not produce the arrested mutant phenotype at the restrictive temperature.

To determine which amino acid was phosphorylated, the ³²P-labeled dsk1 band was excised from the acrylamide gel, hydrolyzed, and run in a thin layer chromatography (MATERIALS AND METHODS). An intense radioactive spot at the position of phosphoserine and a faint one at phosphothreonine were obtained. No radioactivity was detected at the position of phosphotyrosine.

Cell-Cycle Stage Specific Shifting of dsk1 Protein Band

We addressed the question of whether phosphorylation of dsk1 protein is cell-cycle stage specific, that is, if the relative band intensities of the two dsk1 bands alter in G2 or M phase of the normal wild-type cell cycle. To answer this question, a wild-type synchronous culture was made, and the dsk1 protein band pattern was monitored in the course of the cell cycle by immunoblot by using anti-dsk1 antibodies (Figure 4A). Haploid wild-type cells grown in rich YPD medium were run in a Beckman elutriator rotor, and small early G2 cells were collected and grown at 33°C. A highly synchronous increase in the mitotic index (Cell plate index; CPI) was obtained (Figure 4A). The relative intensity of the slowly migrating dsk1 band was apparently high in mitotic cells (bottom), and weaker in other stages. These changes, however, are subtle and not conclusive. The further work is needed.

Then, *ts cdc25-22* mutant cells grown at the permissive temperature (26°C) were transferred and incubated at the restrictive temperature (36°C) for 4 h (Moreno *et al.*, 1989). As expected, the fast migrating band intensity increased (Figure 4B). Cells were then released to the permissive temperature, 26°C, and a rapid increase of the upper band intensity was observed (Figure 4B).

If *cs nda3-311* cells grown at the permissive temperature (33°C) were transferred to the restrictive temperature (20°C) for 8 h; the slowly migrating band intensity was enhanced (Figure 4C). However, upon release to the permissive temperature, the faster migrating band intensity increased. These results indicate that the departure from the wild-type dsk1 band pattern can be caused by a cell cycle block. We examined other cell cycle mutants, namely G1-arrested *cdc10* (Nurse *et al.*, 1976), S-phase arrested *cdc17* (Nasmyth *et al.*, 1981),

Figure 4. Band intensity ratios of dsk1 protein in wild type and mutants. (A) Exponentially growing wild-type cells were separated in an elutriator rotor to select early G2 cells for a synchronous culture at 33°C in rich YPD medium. Cells with a septum were counted (cell plate index, CPI). Cells were taken every 10 min, run in SDS-PAGE, and immunoblotted by using anti-dsk1 antibodies. The relative upper-band intensity seems to be high in mitosis at the time of high CPI. (B) *cdc25-22* mutant cells grown in EMM2 at 26°C were transferred to the restrictive temperature (36°C) and incubated for 4 h, followed by release to 26°C (→). Cells were taken at the indicated time (min), run in SDS-PAGE, and immunoblotted using anti-dsk1 antibodies. The fast migrating dsk1 band was predominant at 36°C for 4 h, but after the release, both fast migrating and slowly migrating bands were seen. (C) *nda3-311* mutant cells grown at 33°C in YPD were shifted to the restrictive temperature (20°C) and incubated for 8 h, followed by the release to the permissive temperature, 33°C (→). Cells were taken at the indicated time (min), run in SDS-PAGE, and immunoblotted using anti-dsk1 antibodies. The slowly migrating band was enhanced at 20°C for 8 h, but after the release both slowly migrating and fast migrating bands were seen.

and M-arrested *nuc2* (Hirano *et al.*, 1988), and found that only the fast migrating band was observed in the first two mutants, whereas the slowly migrating band was much enhanced in *nuc2* cells. The slowly migrating band becomes the principal band in M phase arrested mutant cells.

Protein Ser/Thr and Tyr Autophosphorylation In Vitro by dsk1

To examine whether the *dsk1*⁺ gene product has protein kinase activity, dsk1 protein immunoprecipitated by anti-dsk1 antibodies was assayed for kinase activity. We found that dsk1 protein made in both *S. pombe* and *E. coli* is autophosphorylated in vitro (Figure 5). Four cell extracts, namely, deletion ($\Delta dsk1$), wild type (wt), wild type transformed with the multicopy plasmid *pdsk1*⁺ (*/pdsk1*), and a constitutive overexpression plasmid (*/pADH(dsk1)*), which produced the N-terminal truncated protein (described above), were prepared and immunoprecipitated by anti-dsk1 antibodies. Resulting precipitates were incubated with γ -³²P-ATP at 25°C for 15 min in the presence of 10 mM MgCl₂

and run in SDS-PAGE. As shown in Figure 5A (left; immunoblot using anti-dsk1 and ³²P-autoradiography of the same samples), the dsk1 protein was phosphorylated in vitro. The activity was roughly proportional to the amount of dsk1 protein present. The phosphorylated dsk1 band was extracted, hydrolyzed, and the phosphorylated amino acids were analyzed by thin layer chromatography. Phosphorylated Ser and Tyr were detected (right panel, Figure 5A). Phosphorylated threonine was negligible. Tyrosine phosphorylation was unexpected since no tyrosine phosphorylation was detected for the in vivo labeled dsk1 protein (Figure 3D).

Subsequently, bacterial-made dsk1 protein was employed for assaying autophosphorylation. Bacterial made dsk1 protein was purified, solubilized (MATE-

Figure 5. Autophosphorylation of dsk1 protein in vitro. (A) Immunoprecipitates of *S. pombe* cell extracts by anti-dsk1 antibodies were collected and incubated in the presence of γ -³²P-ATP. They were run in SDS-PAGE, immunoblotted using anti-dsk1 antibodies (top left), and autoradiographed (bottom left). $\Delta dsk1$, mutant cells with deletion of the *dsk1*⁺ gene; wt, wild-type cells; */pdsk1*, wild-type cells carrying a multicopy plasmid with the *dsk1*⁺ gene; */pADH(dsk1)*, $\Delta dsk1$ cells carrying a plasmid *pADH(dsk1)*, which is a high-expression plasmid for the *dsk1*⁺ gene. ³²P-labeled dsk1 protein was electroeluted from the wild-type band, hydrolyzed, run in a thin layer for analysis of phosphoamino acid content. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. (B) Bacterial-made dsk1 protein (indicated by *bdsk1*) was incubated with or without calf thymus histone H1 (H1) in the presence of γ -³²P-ATP and 10 mM MgCl₂. Samples were run in SDS-PAGE, stained with coomassie blue (indicated by CB, left) and autoradiographed (³²P, middle). Labeled dsk1 protein band was analyzed for phosphoamino acid content (right).

RIALS AND METHODS) and incubated at 25°C for 15 min in the presence of γ - 32 P-ATP and histone H1 (Coomassie-blue stained protein bands are shown in left panel of Figure 5B). Bacterial-made dsk1 protein was efficiently autophosphorylated (middle panel), but histone H1 mixed with dsk1 protein was not. Then, 32 P-labeled dsk1 protein band was excised from the gel and extracted, hydrolyzed and analyzed by thin layer chromatography. As shown in the right panel, phosphoserine is intense. In addition, the 32 P-labeled spots corresponding to phosphotyrosine and phosphothreonine were found. The reason why the level of phosphotyrosine was low in bacterial made dsk1 (bdsk1) was not understood. Anti-phosphotyrosine antibodies detected the bdsk1 band before autophosphorylation in vitro suggesting that bdsk1 was already tyrosine phosphorylated. No mobility shift was found in autophosphorylated dsk1 proteins, possibly because the shift could be caused by phosphorylation at a specific site(s) in vivo.

Phosphorylation of Myelin Basic Protein In Vitro by dsk1

The results described above strongly suggested that dsk1 is a protein kinase, but a substrate trans-acted by dsk1 had to be found for establishing dsk1 as a kinase. By using bacterial dsk1 protein we looked for a substrate on which dsk1 is capable of phosphorylating in vitro. We were unable to show that caseine, histone H1, H2A, enolase, or phosphorylase b was phosphorylated by dsk1. Myelin basic protein (MBP), however, was phosphorylated by bacterial as well as fission yeast dsk1. Wild-type *S. pombe* cell extracts were immunoprecipitated by anti-dsk1, and resulting immunoprecipitates were incubated with MBP (final concentration; 0.5 mg/ml) in the presence of γ - 32 P-ATP for 0–20 min (Figure 6A). Both dsk1 and MBP were phosphorylated. Because no radioactivity was found for Δ dsk1 deletion mutants after incubation for 20 min, the kinase activity detected for MBP was dependent on the presence of dsk1 protein.

Phosphorylated MBP after 15-min incubation with dsk1 was hydrolyzed, and phosphoamino acid content was analyzed. As shown in Figure 6B, phosphoserine and a trace of threonine were detected. No phosphotyrosine was found. These results indicate that dsk1 is a protein ser/thr kinase and may have a relatively narrow substrate specificity.

G2/M Progression was Delayed by Overexpression of dsk1⁺

We examined whether the overexpression of dsk1⁺ would produce any phenotype. First, the dsk1⁺ gene was introduced under the control of an *S. pombe* inducible promoter *nmt1*⁺ (Maundrell, 1990) (MATERIALS AND METHODS). In the absence of thiamine the *nmt1*⁺ was expressed but not in the presence of thiamine. Wild-type cells carrying a plasmid with the *NMT*-

Figure 6. Phosphorylation of MBP protein by dsk1 in vitro. (A) *S. pombe* wild type (WT) and *dsk1* deletion mutant (Δ dsk1) cell extracts were immunoprecipitated by anti-dsk1 antibodies, mixed with myelin basic protein (MBP, 0.5 mg/ml), and incubated in the presence of γ - 32 P-ATP for 0–20 min. Both dsk1 and MBP proteins were phosphorylated at a similar rate by immunoprecipitates of wild type, but no phosphorylation took place by that of deletion mutant even after 20 min. (B) Phosphorylated MBP protein was analyzed by thin-layer chromatography after hydrolysis. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

dsk1⁺ gene produced normal colonies in the presence of thiamine (streaked at left, +T in Figure 7A) but minute ones in the absence of thiamine (streaked at left, –T). Wild-type cells carrying the vector plasmid produced normal colonies under both conditions. Therefore the overexpression of *dsk1*⁺ is harmful for normal cell growth.

The level of overexpressed dsk1 protein was measured. Wild-type cells carrying plasmid with the *NMT-dsk1*⁺ were first grown with thiamine and then transferred to the medium without thiamine and incubated for 18 h. As shown in immunoblot of cell extracts using anti-dsk1 (Figure 7B from left, 0, 8, 10, 12, 14, 16, and 18 h), the *dsk1*⁺ gene begins to overexpress at 10 h; a large excess of dsk1 protein is present after 12 h. In parallel with this overexpression, the cell number increase is arrested, and the average size of cells increased (Figure 7, C and D). The cell size after 13 h in the absence of thiamine was approximately two- to three-fold longer than in the presence of thiamine. The frequency of the singly nucleated cells increased (80% at 0 h, 90% at 12 h, and 98% at 14 h). Simultaneously the mitotic index decreased from 12% at 0 h to 1% at 14 h. Immunofluorescence microscopy using anti-tubulin antibody TAT-1 (Woods *et al.*, 1989) showed that elongated cells contained mostly cytoplasmic microtubules (right panel, Figure 5D; DAPI stain of the same cells at left) reminiscent of the interphase cells in *S. pombe* (Hagan and Hyams, 1988). Mitotic cells displaying the spindle were only infrequently (<2%) found in *dsk1*-overexpressed cells at 14 h. Second, the constitutive overexpression

Figure 7. Delay of cell cycle progression by overexpression of the *dsk1*⁺ gene. (A) Two strains streaked on plates in the presence of thiamine (+T) or the absence of thiamine (–T). Right, wild-type cells carrying the vector plasmid for *nmt1* promoter. Left, wild-type cells

by the ADH promoter was examined by using pADH(*dsk1*) plasmid. The degree of ADH-*dsk1* overexpression is less than that of the NMT-*dsk1* so that a milder overexpression phenotype was expected. Wild-type cells carrying the ADH(*dsk1*) plasmid produced were elongated (~1.4 times of the wild type average cell length) containing the single nucleus (Figure 7E, indicated by ADH) with the G2 DNA content. However, normal sized colonies were made.

The DNA content of NMT-*dsk1* overexpressing cells were measured by FACSscan analysis, as shown in Figure 8 with the control of *cdc10* mutant that arrests in G1. Wild-type cells carrying plasmid pNMT(*dsk1*) were incubated with the EMM2 medium lacking thiamine. Cells with the G2 DNA content were abundant at 14–16 h in the absence of thiamine. These results indicated that the *dsk1* overexpressing cells were much delayed in the progression from G2 to M.

Increased dsk1 Protein Kinase Activity in M-phase Arrested Cells

To determine whether *dsk1* kinase might have altered activity in G2- and M-phase arrested cells, we assayed the phosphorylation activity in immunoprecipitates by anti-*dsk1* by using MBP as the substrate. Extracts were prepared from *cdc25*-arrested (36°C for 4 h) and *nuc2*-arrested (36°C for 4 h) cells with the control extract of exponentially growing wild-type cells (36°C for 4 h). As shown in Figure 9 (top; indicated by Western), the fast and slowly migrating bands, respectively, in *cdc25*- and *nuc2*-arrested cells were prominent by immunoblot using anti-*dsk1*. The amount of ³²P-labeled MBP protein (bottom; indicated by ³²P) was much higher in the M-arrested *dsk1* than those of wild type and G2-arrested *dsk1*. Quantitative estimation of three independent experiments by the imaging analyzer Fuji BAS2000 showed a three- to seven-fold increase for *dsk1* activity in the M-arrested cells in comparison with the wild-type *dsk1*. It is curious that ³²P-labeling of MBP and *dsk1* by *cdc25*-arrested *dsk1* is higher than that by wild-type *dsk1*. This difference was reproducible in three

carrying plasmid with the *dsk1*⁺ gene under control of the *nmt1* promoter. Overexpressed cells poorly grew and produced tiny colonies after 2 days. (B) Wild-type cells carrying the NMT1-*dsk1* plasmid grown in the presence of thiamine were transferred to a culture without thiamine and taken at intervals, run in SDS-PAGE, and immunoblotted using anti-*dsk1* antibodies. Cells were taken at 0, 8, 10, 12, 14, 16, and 18 h. (C) Fluorescence micrographs of DAPI-stained wild-type cells carrying the NMT-*dsk1*⁺ plasmid incubated in the presence of thiamine (+T) or the absence (–T) for 13 h. The *dsk1*-overexpressed cells (–T) were highly elongated. (D) An immunofluorescence micrograph of the same cells at 18 h in the absence of thiamine using anti-tubulin antibody TAT1 (right) and DAPI to visualize DNA (left). (E) Fluorescence micrographs of DAPI-stained wild-type cells carrying the ADH vector (left) or the ADH-*dsk1*⁺ plasmid (right). Cells with the ADH-*dsk1*⁺ plasmid are elongated. Bar indicates 10 μm.

Figure 8. FACS analysis of *dsk1*-overexpressing cells. Wild-type cells carrying plasmid pNMT(*dsk1*) were incubated in the synthetic medium EMM2 lacking thiamine. Cells overexpressing *dsk1* protein were collected at 12, 14, and 16 h after the depletion of thiamine. A slight shift of the peak positions is due to the elongation of cells. Cells with the G2 DNA content (2C) were observed. As a control, *cdc10* cells were incubated at the restrictive temperature. Cells with the G1 DNA (1C) were produced after 4 h.

independent experiments, but the reason for this is unclear.

Immunofluorescent Localization of *dsk1* Protein in Wild-Type, M- or G2-Arrested Mutant Cells

To determine whether *dsk1* protein alters its cellular localization during normal cell cycle or in arrested mutant cells, immunofluorescence microscopy using affinity-purified anti-*dsk1* antibodies (MATERIALS AND METHODS) were performed. Anti-*dsk1* immunofluorescence of wild-type cells was found to be weak so that a multicopy plasmid with the *dsk1*⁺ gene (pDS113-6) was introduced into wild-type cells. As shown in Figure 10A (indicated by Wt/p(*dsk1*)), cytoplasmic immunofluorescence (right) was prominent in many cells. However, mitotic cells (indicated by the arrowheads in DAPI stain, left) displayed intense nuclear immunofluorescence in addition to cytoplasmic stain. Preimmune serum did not show immunofluorescence under the same condition. In Δ *dsk1* deletion mutant cells, immunofluorescence using anti-*dsk1* was hardly detected.

Then M-arrested *nuc2* mutant cells carrying p(*dsk1*) incubated at the restrictive temperature for 4 h (MATERIALS AND METHODS) were employed and the results are shown in Figure 10B. Intense nuclear immunofluorescence was found in the majority of cells (70%) in addition to cytoplasmic stain. This value is

similar to the frequency of *nuc2* mutant cells displaying the M-arrested phenotype.

G2-arrested *cdc25*-mutant cells carrying p(*dsk1*) were incubated at the restrictive temperature for 4 h. Cytoplasmic immunofluorescence was prominent in all the cells examined (Figure 10C). Nuclear fluorescence was hardly visible. These results strongly suggested that localization of *dsk1*, at least its part, is altered dependent upon the cell cycle stages. Nuclear localization is visible in wild-type mitotic or mitotically arrested mutant cells. Basically the same results were obtained using *cdc2-33* extract.

We have undertaken to identify a domain of *dsk1* protein responsible for nuclear localization. A series of plasmids truncating different parts of *dsk1* are made and introduced into *dsk1*-deletion mutant cells. It was found that mutant *dsk1* protein deleting the spacer region was enriched in the nucleus (Figure 10D).

DISCUSSION

We report in the present paper a novel protein kinase *dsk1* from fission yeast. The open reading frame encodes a 544 amino acid protein, having consensus kinase sequences, but no strong resemblance to any kinase so far known in available databases. It has a 125 amino acid spacer sequence between the kinase domains VI and VII, which contains abundant hydrophilic residues and possibly implicated in cellular localization. A feature of *dsk1* is that two protein bands were produced in SDS-PAGE by the *dsk1*⁺ gene, and that their band intensity ratio was strikingly altered in G2- or M-phase arrested mutants. The fast migrating band (apparent M.W., 61 kd) was enhanced in G2-arrested *cdc2* or *cdc25* mutant cells, whereas the slowly migrating band (63 kd) was intensified in M-phase arrested *dis1*, *nda3*, and

Figure 9. Phosphorylation of *dsk1* and MBP proteins by *dsk1* from wild-type, G2- and M-arrested mutant cells. Extracts of growing wild type, arrested *cdc25-22* and *nuc2-663* incubated at 36°C for 4 h were immunoprecipitated by anti-*dsk1* antibodies, mixed with MBP (0.5 mg/ml), and incubated at 25°C for 15 min in the presence of γ -³²P-ATP, followed by SDS-PAGE and autoradiography. Top panel, immunoblot using anti-*dsk1* antibodies (indicated by Western). The bottom and top bands, respectively, were prominent for G2-arrested *cdc25* and M-arrested *nuc2* cells. Bottom panel, autoradiography for *dsk1* and MBP bands (indicated by ³²P).

Figure 10. Immunofluorescence localization of *dsk1* protein in wild-type, M- and G2-arrested mutant cells using affinity-purified anti-*dsk1* antibodies. (A) Wild-type fission yeast cells carrying a multicopy plasmid with the *dsk1*⁺ gene (indicated by Wt/p(*dsk1*)) were stained by affinity-purified anti-*dsk1* antibodies. Left, DAPI stain. Cells indicated by the arrowheads represent mitotic cells showing the separating chromosomes. Right, anti-*dsk1* stain of the same cells. Cytoplasmic immunofluorescence was prominent in many cells except those mitotically anaphase cells displaying the nuclear immunofluorescence in addition to cytoplasmic stain. Wild-type cells carrying the vector plasmid displayed immunofluorescence with a similar, but less intense, distribution. (B) Temperature-sensitive *nuc2* mutant cells carrying p(*dsk1*) were incubated at the restrictive temperature for 4 h. Left, DAPI stain. Right, anti-*dsk1* antibody stain. In most cells, intense nuclear immunofluorescence was found in addition to cytoplasmic stain. (C) Temperature-sensitive *cdc25*-mutant cells carrying p(*dsk1*) were incubated at the restrictive temperature for 4 h. Left, DAPI stain. Right, anti-*dsk1* antibody stain. Cytoplasmic immunofluorescence was prominent, whereas nuclear fluorescence was negligible. (D) *dsk1*-deletion mutant cells carrying a multicopy plasmid with the *dsk1*⁺ gene lacking the spacer region were grown at 33°C. Intense nuclear stain was observed in addition to cytoplasmic fluorescence. Bar indicates 10 μ m.

nuc2 mutant cells. The slowly migrating band was enhanced only in M-arrested cells, whereas the fast migrating band became prominent not only in G2 but also in G1- or S-phase arrested mutants.

We provide evidence that the existence of these two adjacent bands are probably due to difference in protein phosphorylation. The slowly migrating band disappeared after digestion with potato acid phosphatase. Hence *dsk1* is a phosphoprotein. The *dsk1* protein band derived from in vivo labeled cells by ^{32}P - H_3PO_4 and immunoprecipitation by anti-*dsk1* antibodies was found to be ^{32}P -labeled. The degree of phospho-labeling was much increased in *dsk1* derived from M-phase arrested cells, suggesting that at least the slowly migrating *dsk1* band is phosphorylated and that the band shift is due to phosphorylation/dephosphorylation. Phosphoamino acid analysis showed that serine is the major phosphorylated residue but that weak threonine phosphorylation is also found. Therefore, a protein serine/threonine kinase(s) or phosphatase(s) responsible for phosphorylation or dephosphorylation of *dsk1* may exist. Autophosphorylation of *dsk1* in vivo is also possible.

We demonstrated that *dsk1* was a protein kinase that phosphorylated MBP in vitro using bacterial-made *dsk1* protein as well as immunoprecipitated *S. pombe* *dsk1*. Serine and threonine were the major and the minor phosphorylated amino acids, respectively, so that *dsk1* is probably a protein ser/thr kinase. No phosphotyrosine was detected in in vitro phosphorylated MBP. The same result was obtained using anti-phosphotyrosine (unpublished data). Curiously, however, tyrosine as well as serine and threonine residues of *dsk1* were autophosphorylated using bacterial or fission yeast *dsk1* proteins. Hence *dsk1* might belong to a group of protein ser/thr kinases which can autophosphorylate tyrosine in vitro but perhaps can not phosphorylate tyrosine in vivo (reviewed in Lindberg *et al.*, 1992). It remains to be seen, however, whether a yet unidentified substrate of *dsk1* might be tyrosine phosphorylated because MBP is the only substrate so far known among those examined for transphosphorylation by *dsk1*.

Cells without *dsk1* kinase could grow with a small reduction in the growth rate (the generation time of 3 vs. 2.5 h for Δ *dsk1* deletion mutant compared with the wild type, respectively). Therefore the *dsk1*⁺ is non-essential for viability. No abnormality in conjugation or sporulation has been found in a diploid homozygous for the *dsk1* deletion. The *dsk1*⁺ gene was originally isolated as a multicopy suppressor for a *dis1* mutant so that the overexpressed gene product was thought to have a positive effect for the exit from mitosis (see Figure 11A). The *cs* phenotype of the *dis1* mutant is the block of chromosome segregation in spite of spindle formation and elongation (Ohkura *et al.*, 1988). The failure to exit from mitosis is consistent with the fact that *cdc2* kinase is activated and remains high in *cs dis1* at the restrictive temperature (Kinoshita *et al.*, 1991). The *dis1*⁺ gene has

Figure 11. A mitotic role of *dsk1* protein kinase. (A) Overexpression of protein kinase *dsk1* may negatively and positively act on the entry into and the exit from M phase, respectively. (B) In the wild-type interphase or the G2-arrested *cdc25* mutant cells, *dsk1* protein is enriched in the cytoplasm. In the wild-type mitotic or the M-arrested *nuc2* mutant cells, *dsk1* protein is seen in the nucleus as well as in the cytoplasm. Δ *dsk1* deletion mutant cells carrying plasmid with the *dsk1*⁺ gene deleting the spacer region, truncated *dsk1* protein lose the property of stage-specific localization, being enriched in the nucleus of both interphase and mitotic cells.

been cloned, sequenced, and its predicted amino acid sequence does not resemble any known protein kinase or phosphatase catalytic subunits (Kurooka, unpublished observations). Whether *dis1* protein is phosphorylated and whether its phosphorylation is controlled by *dsk1* kinase will be the subject of future investigation.

A distinct phenotype was obtained by the overexpression of *dsk1*⁺ using the inducible promoter of the *nmt1*⁺ gene. *dsk1*-overexpressed cells were highly elongated with a single nucleus and a G2-phase cytoplasmic microtubule array with the G2 DNA content. This is reminiscent of *cdc2*- or *cdc25*-like G2 blocked mutant phenotypes. Because small colonies were able to form and the nuclear division took place later time in *dsk1*-overexpressing cells, the progression of G2/M was not blocked but rather delayed under *dsk1* overexpression. This result suggests that overexpressed *dsk1* kinase is inhibitory to mitotic entry (Figure 11A). Combined with the fact that overexpressed *dsk1* suppressed the *cs dis1* mutant, we speculate a function for the *dsk1* kinase; abundant *dsk1* kinase accelerates the exit when cells are in M-phase and also delays the entry into mitosis when cells are in G2. Consistently, overexpression of *dsk1*⁺ by the ADH promoter that is milder than that induced by the *nmt1* promoter causes cell elongation with the single nucleus with the G2 DNA content and can suppress the *cs* phenotype of *dis1* mutants. Hence overexpressed *dsk1* may act on cells moving out of M phase. This hypothesis is intriguing in regard with our

finding that *dsk1* protein was enriched in the nucleus in mitotically arrested mutant and wild-type mitotic cells but cytoplasmic-enriched in cells at other cell-cycle stages (Figure 11B). *dsk1* protein may execute a nuclear function for the completion of mitosis in the wild-type cell cycle. By strong overexpression, cytoplasmic *dsk1* kinase might become too high for cells to normally enter mitosis. By mild overexpression, the mitotic defect in *cs dis1* could be rescued. A correlation exists between nuclear location and hyper-phosphorylation of *dsk1*. It is unknown how *dsk1* protein locates in the nucleus. A protein containing the nuclear location signal may associate with the phosphorylated form of *dsk1* protein. Alternatively, phosphorylated *dsk1* itself is capable to targets into the nucleus. A model is that the spacer region is phosphorylated, and the unphosphorylated spacer region negatively regulates the nuclear location of *dsk1*. This explains the phenomenon that *dsk1* deleting the spacer region targets the nucleus in both mitotic and interphase cells.

A hypothesis for the physiological role is that *dsk1* kinase acts on the exit from mitosis by moving into the nucleus. There may exist another kinase that shares an essential function with *dsk1*. The *dsk1* kinase may be an added-on regulator for mitosis. When the initiation or completion of M phase is disturbed, it may tune up mitosis. *dsk1* kinase is altered in activity and location by phosphorylation so as to facilitate the progression of mitosis. Identification of the substrates of *dsk1* kinase in vivo is of significant interest.

The discovery of *dsk1* kinase has shed light on a novel protein phosphorylation system in mitosis. This system became particularly apparent when the cell cycle was delayed or arrested in mutant cells. Identification of the kinase or the phosphatase for *dsk1* as well as the substrate proteins of *dsk1* is crucial to understand the regulation of *dsk1* kinase and its implication in cell-cycle control.

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