Fission Yeast Ste9, a Homolog of Hct1/Cdh1 and Fizzy-related, Is a Novel Negative Regulator of Cell Cycle Progression during G₁-Phase

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When proliferating fission yeast cells are exposed to nitrogen starvation, they initiate conjugation and differentiate into ascospores. Cell cycle arrest in the G₁-phase is one of the prerequisites for cell differentiation, because conjugation occurs only in the pre-Start G₁-phase. The role of *ste9*⁺ in the cell cycle progression was investigated. Ste9 is a WD-repeat protein that is highly homologous to Hct1/Cdh1 and Fizzy-related. The *ste9* mutants were sterile because they were defective in cell cycle arrest in the G₁-phase upon starvation. Sterility was partially suppressed by the mutation in *cig2* that encoded the major G₁/S cyclin. Although cells lacking Ste9 function grow normally, the *ste9* mutation was synthetically lethal with the *wee1* mutation. In the double mutants of *ste9 cdc10*^{ts}, cells arrested in G₁-phase at the restrictive temperature, but the level of mitotic cyclin (Cdc13) did not decrease. In these cells, abortive mitosis occurred from the pre-Start G₁-phase. Overexpression of Ste9 decreased the Cdc13 protein level and the H1-histone kinase activity. In these cells, mitosis was inhibited and an extra round of DNA replication occurred. Ste9 regulates G₁ progression possibly by controlling the amount of the mitotic cyclin in the G₁-phase.

INTRODUCTION

Each cell must duplicate its cellular constituents for proliferation. Two major events in this process are precise replication of the whole genome and accurate transmission of the duplicated genomes to two daughter cells. A typical eukaryotic cell cycle consists of four distinct phases, i.e., G₁, S, G₂, and M. Among these phases, G₁ is most important for strict regulation of cell proliferation in many types of cells. Before the specific duration in the G₁-phase called the 'restriction point' in mammalian cells or the 'Start' in yeast, a cell determines whether it enters the next cell cycle or ceases proliferation (Hartwell *et al.*, 1974; Nurse and Bissett, 1981; Pardee, 1989). To make this decision, cells in the G₁-phase monitor many types of environ-

mental and intracellular information such as the presence or absence of nutrients, completion of the events of the previous cycle, and cell size. When all requirements are fulfilled, the cells override the restraint for the restriction point/Start control and are committed to progression through G₁ to the S-phase. In contrast, when environment conditions, such as the absence of essential nutrients or the presence of negative growth factors, are unfavorable for cell proliferation, cells exit the cycling phase and enter into a quiescent G₀-phase.

Tumor cells have many characteristics that differ from those of normal cells. The most noticeable one is their uncontrolled proliferation, suggesting that cell cycle regulation is ruined in tumor cells (Sherr, 1996). Tumor cells override the restraint for the "restriction point" even under unfavorable conditions for growth. Indeed, many oncogenic proteins strongly induce cell proliferation. Conversely, most of the tumor suppressor genes encode proteins that restrain progression to the S-phase from G₁. Inactivation of these tumor sup-

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pressor genes leads to uncontrolled cell multiplication due to lack of the inhibition of G₁/S progression. Therefore, cell cycle regulation in the G₁-phase is very important to guarantee normal cell proliferation.

Fission yeast, *Schizosaccharomyces pombe*, has provided a useful model system with which to study these cell cycle controls (Nurse, 1990; Forsburg and Nurse, 1991; MacNeill and Fantes, 1995). There are three alternative fates for the proliferating fission yeast cells in response to environmental situation; they continue to proliferate, enter into a dormant stationary phase, or differentiate into ascospores (Egel, 1989; Su *et al.*, 1996). Upon nitrogen starvation, the cells arrest in the pre-Start G₁ period and withdraw from the cycling phase (Costello *et al.*, 1986). In homothallic (*h*⁹⁰) strains, haploid vegetative cells of opposite mating types conjugate to form a diploid zygote that subsequently undergoes meiosis and sporulation to differentiate into dormant ascospores. Alternatively, heterothallic cells (*h*⁺ or *h*⁻) exit from a proliferating phase and maintain quiescence (Costello *et al.*, 1986; Su *et al.*, 1996). Importantly, cells can initiate the conjugation process only in the pre-Start G₁-phase (Nurse and Bissett, 1981). Once the cells pass Start, they are committed to a new round of the mitotic cycle and never enter the developmental cycle until they complete mitosis and again arrest in the pre-Start G₁ period. Thus, in fission yeast as in other eukaryotes, the regulatory mechanism operates to block cell cycle progression in the G₁-phase in response to unfavorable environmental conditions.

The Rum1 protein encodes a critical regulator of the G₁-phase in fission yeast (Labib and Moreno, 1995). First, it acts as an inhibitor of the S-phase onset, delaying Start until a cell has attained a critical minimal mass. Second, Rum1 influences the dependence of Start and the S-phase upon completion of the mitosis of the previous cycle. Third, the Rum1 protein defines the cell as being in the pre-Start G₁ period and prevents such a cell from undergoing mitosis. As an inhibitor of cdk, Rum1 inhibits cdk activity of the Cdc2/Cdc13 complex strongly and the Cdc2/Cig2 complex weakly (Correa-Bordes and Nurse, 1995). The *rum1* gene is not essential for viability, and the *rum1* mutant grows normally. However, the *rum1* mutant cannot arrest its cell cycle in the pre-Start G₁-phase upon nitrogen starvation and is sterile (i.e., mating-deficient) (Moreno and Nurse, 1994).

Although Rum1 is an important regulator of G₁-phase progression, our knowledge about the cellular mechanism of G₁ arrest upon unfavorable environmental conditions is not enough. To identify other genes involved in this control, we have searched for mutants whose phenotype mimicked the *rum1* mutant. In this paper, we provide evidence that the *ste9* mutant exhibits defects indistinguishable from those of the *rum1* mutant. The Ste9 regulates the G₁-phase

progression possibly by down-regulating the amount of mitotic cyclin (Cdc13) during G₁-phase.

MATERIALS AND METHODS

General Techniques for Yeast

The *S. pombe* strains used in this study are listed in Table 1. Yeast cells were grown in YE (complete medium), EMM2, or SD (minimal medium). For mating and sporulation, MEA and SSA were used. For liquid culture, EMM2 and its nitrogen-free version (EMM2-N) were used. Standard methods for *S. pombe* were as described (Gutz *et al.*, 1974; Moreno *et al.*, 1991). Sterile mutants were crossed using protoplast fusion (Kitamura *et al.*, 1990). Transformation of *S. pombe* cells was done by the lithium acetate method (Okazaki *et al.*, 1990). Viability was monitored by plating and counting the number of colonies after 3–4 d of incubation. To discriminate haploid cells from diploid cells, the cells were plated onto YE medium containing phloxine B (Moreno *et al.*, 1991).

Molecular Cloning and Gene Disruption of *ste9*

A diploid strain DS9-1, harboring the nonsense *ste9*-B36 mutation homozygously, was transformed by the *S. pombe* genomic library and plated onto SSA. Two sporulating colonies were identified by staining with iodine vapor, and plasmids were recovered from both colonies. Restriction mapping revealed that two plasmids contained the overlapping genomic DNA. A 5-kilobase (kb) *Hind*III fragment (Figure 1A), which was sufficient for rescue of the *ste9* mutation, was integrated into a genome of the *ste9* mutant. The resultant stable integrant normally conjugated and sporulated as did wild-type cells. Tetrad analysis of this integrant revealed that the integrated *Hind*III fragment contained the *ste9*⁺ gene itself, not a multicopy suppressor. A *Sall*-*Hind*III fragment of 3.4 kb, which complemented the *ste9* mutation, was cloned into pAL-SK vector (kindly provided by Dr. K. Tanaka), and the resultant plasmid was named pAL(*ste9*). The nucleotide sequence of *ste9*⁺ has been submitted to the DDBJ/EMBL/GenBank databases under accession number AB001285.

Chromosomal disruption of the *ste9* gene was carried out by one-step gene disruption (Rothstein, 1983). The *Bcl*I-*Bcl*I fragment of 1.5 kb was replaced by the *S. pombe* *ura4*⁺ gene (Figure 1A; Grimm *et al.*, 1988). Although deleted construct covered a part of the 5'-untranslated sequence, we confirmed by 5'-rapid amplification of cDNA ends analysis that this region was transcribed to *ste9* mRNA. The resultant *Sall*-*Hind*III fragment containing the disrupted *ste9* allele was used to transform wild-type cells, and stable *Ura*⁺ transformants were obtained from haploid and diploid strains. Successful replacement of the wild-type gene by the disrupted allele was confirmed by genomic Southern analysis.

Construction of *nmt-ste9*⁺ Gene and *Ste9*-overexpressing Strain

The coding region of *ste9* was amplified by PCR using sense (TGAAGTCAGGGATCCTAACG) and antisense (GAGTGAATGGGATCCATTAC) oligonucleotide primers. To facilitate cloning, *Bam*HI sites (indicated by underlining) were introduced in front of the initiation codon and behind the termination codon. After digestion with *Bam*HI, the amplified DNA was inserted into the *Bam*HI site of pREP1 or pREP2, which contained the thiamine-repressible *nmt1* promoter (Maundrell, 1990, 1993). The strain harboring the integrated *nmt-ste9*⁺ gene was constructed as follows. One of the resultant plasmids, pREP2(*ste9*), was digested at the unique *Aat*I site in the *ura4*⁺ gene and transformed yeast cells to integrate the linearized plasmid into the genomic *ura4*-294 locus, after which stable *Ura*⁺ integrants were isolated. The cells were maintained in the presence of 5 µg/ml thiamine. To induce expression, the cells were inoculated into thiamine-free medium after extensive washing.

Flow Cytometry and Microscopy

Cells were cultured as described in the text. For flow cytometry, cells were collected, washed once with cold water, and then fixed with 70% ethanol and stored at 4°C. After digestion by RNase A and staining with propidium iodide, the cells were analyzed by FACS-Calibur (Becton-Dickinson, San Jose, CA). For microscopic observation, cells were fixed with 70% ethanol. After the cells had been washed with water, nuclei and septa were stained with DAPI and calcofluor, respectively, and inspected under a fluorescent microscope.

Immunoblotting

Cells were collected, washed once with water, resuspended in 100 µl of water, and then heated at 90°C for 5 min. Extracts were prepared as described (Masai *et al.*, 1995). Briefly, the cells were disrupted by vigorous vortexing with glass beads in SDS sample buffer containing 4 M urea. Sixty micrograms of protein per each lane were fractionated by SDS-PAGE (8% acrylamide gel), and then transferred to nitrocellulose paper. The blot was probed with anti-Cdc13 antibody or anti-Cdc2 antibody specific for the PSTAIRE region (kindly provided by Dr. M. Yanagida and Dr. M. Yamashita, respectively). Rum1 protein tagged by triple hemagglutinin epitopes was visualized with anti-HA monoclonal antibody (12CA5, Boehringer Mannheim, Indianapolis, IN).

Assay of H1-Histone Kinase Activity

Cell extracts were prepared in HB buffer (Moreno *et al.*, 1991) by vigorous vortexing with glass beads. Kinase reaction was performed in HB buffer with H1-histone and [γ -³²P]ATP for 15 min at 30°C. Reactions were terminated by adding an equal volume of 2× sample buffer. Samples were boiled for 5 min, run on a 12% polyacrylamide gel, and then quantified with BAS1000 (Fuji film).

RESULTS

The *ste9*⁺ Gene Encodes a Homolog of Hct1/Cdh1 and Fizzy-related

To look for genes responsible for the regulation of cell cycle progression in G₁-phase, especially for the cell cycle arrest, we examined whether the known sterile mutants exhibited the phenotype similar to that of the *rum1* mutant. As described below in detail, the *ste9* mutant conformed to this criterion and exhibited defects indistinguishable from those of the *rum1* mutant. The *ste9* mutant was identified previously (Leupold and Sipiczki, 1991), but its molecular characterization has not been attempted. To determine its coding product, the *ste9*⁺ gene was isolated by rescuing the sterility of its mutant. Sequencing revealed one uninterrupted large open reading frame composed of 556 amino acids (Figure 1A). A characteristic motif, called the WD-repeat, was found in its C-terminal half (Voorn and Ploegh, 1992; Neer *et al.*, 1994). In contrast, the N-terminal half showed no characteristic sequence. Homology searching revealed that Ste9 belonged to the subfamily of WD-repeat proteins including Cdc20 and Hct1/Cdh1 (*Saccharomyces cerevisiae*), Fizzy and Fizzy-related (*Drosophila melanogaster*), p55^{CDC} (human and rat), and Slp1 (*S. pombe*) (Sethi *et al.*, 1991; Weinstein *et al.*, 1994; Dawson *et al.*, 1995;

Table 1. *S. pombe* strains

Strain	Genotype
L972	<i>h</i> ^{-s}
C766-1A	<i>h</i> ⁹⁰ <i>leu1</i>
KJ100-8B	<i>h</i> ⁹⁰ <i>ste9::ura4</i> ⁺ <i>ura4</i> -D18
KJ32-11B	<i>h</i> ⁹⁰ <i>ste9</i> -B36 <i>leu1</i>
KJ153-1A	<i>h</i> ⁹⁰ <i>rum1::ura4</i> ⁺ <i>leu1</i> <i>ura4</i> -D18
KJ218-5C	<i>h</i> ⁹⁰ <i>ste9::ura4</i> ⁺ <i>cig2::ura4</i> ⁺ <i>ade6</i> -M210 <i>leu1</i> <i>ura4</i> -D18
KJ57-4C	<i>h</i> ⁹⁰ <i>ste9</i> -B36 <i>cig2</i> -S18 <i>leu1</i> <i>ura4</i>
KJ192-3A	<i>h</i> ⁹⁰ <i>ste9</i> -59 <i>cig2::ura4</i> ⁺ <i>leu1</i> <i>ura4</i> -D18
KJ109-5A	<i>h</i> ⁹⁰ <i>wee1</i> -50 <i>cig2::ura4</i> ⁺ <i>ade6</i> -M216 <i>leu1</i> <i>ura4</i> -D18
KJ111-1A	<i>h</i> ⁹⁰ <i>ste9::ura4</i> ⁺ <i>wee1</i> -50 <i>cig2::ura4</i> ⁺ <i>ade6</i> -M216 <i>leu1</i> <i>ura4</i>
KJ152-9A	<i>h</i> ⁹⁰ <i>rum1::ura4</i> ⁺ <i>wee1</i> -50 <i>ade6</i> -M210 <i>leu1</i> <i>ura4</i>
KJ32-2A	<i>h</i> ⁹⁰ <i>cdc10</i> -129 <i>leu1</i>
KJ32-1A	<i>h</i> ⁹⁰ <i>ste9</i> -B36 <i>cdc10</i> -129 <i>leu1</i>
KJ143-1A	<i>h</i> ⁹⁰ <i>rad17d::ura4</i> ⁺ <i>leu1</i> <i>ura4</i> -D18
6-695	<i>h</i> ⁹⁰ <i>ura4</i> -294::int[<i>ura4</i> ⁺ , <i>nmt-ste9</i> ⁺] <i>leu1</i>
KJ141-8A	<i>h</i> ^{-s} <i>cdc2</i> -M26
KJ124-3A	<i>h</i> ^{-s} <i>cdc2</i> -M26 <i>ste9::ura4</i> ⁺ <i>ura4</i>
KJ155-1A	<i>h</i> ^{-s} <i>cdc2</i> -M26 <i>chk1::ura4</i> ⁺ <i>ura4</i>
KJ238-7B	<i>h</i> ⁹⁰ <i>ste9</i> -B36 <i>cig2::ura4</i> ⁺ <i>rum1</i> ⁺ -3HA <i>ura4</i> -D18 <i>leu1</i>
KJ218-2B	<i>h</i> ^{-s} <i>ste9::ura4</i> ⁺ <i>cdc10</i> -V50 <i>ura4</i> -D18 <i>leu1</i>
Sp224	<i>h</i> ⁹⁰ <i>rum1::ura4</i> ⁺ <i>cdc10</i> -129 <i>ura4</i> -D18 <i>leu1</i> -32
DS9-1	<i>h</i> ⁹⁰ <i>ste9</i> -B36 <i>his1</i> -102 <i>ade6</i> -M216 + <i>leu1</i>
	<i>h</i> ^{+s} <i>ste9</i> -B36 + <i>ade6</i> -M216 <i>ura4</i> -D18 <i>leu1</i>

Matsumoto, 1997; Schwab *et al.*, 1997; Sigrist and Lehner, 1997; Visintin *et al.*, 1997). All proteins contain seven WD-repeats at the C-terminal region, and these WD-repeat domains of these proteins are highly conserved. In addition, several proteins of unknown function identified by genome sequencing project are also homologous, such as a hypothetical protein on the *S. pombe* chromosome II (genomic cosmid clone 1198, accession number U33008) and the ZK1307.6 (*C. elegans*). Among these proteins, Ste9 has the highest homology with the Hct1/Cdh1 (Figure 1B) and Fizzy-related (our unpublished results). As described below, mutants of these three genes exhibit similar phenotype.

The chromosomal *ste9*⁺ gene was disrupted (Figure 1A). Diploid cells harboring one wild-type and one disrupted *ste9* allele were subjected to tetrad analysis. Although a slight reduction in the *ste9*⁻ spore's viability was observed, the *ste9*-disrupted cells grew normally with a generation time comparable to that of wild-type cultures under a standard culture condition. They exhibited neither temperature- nor cold-sensitive growth. However, the *ste9* disruptant failed to conjugate in nitrogen-free sporulation medium. Furthermore, diploid cells harboring the *ste9* null allele homozygously scarcely sporulated, and sporulation was also defective in the heteroallelic diploid cells harboring Δ *ste9* and *ste9*-B36. Thus, disrupted *ste9* allele lacked its function, and *ste9*⁺ was dispensable for proliferation but essential for cell differentiation, i.e., mating and subsequent sporulation. We used both

Figure 1. Molecular cloning and characterization of *ste9*⁺. (A) Restriction enzyme map of *ste9*⁺. The top shaded box indicates the genomic region around *ste9*⁺. The arrow indicates the Ste9 open reading frame. Each WD-repeat is indicated by the black box. Restriction sites: H, *Hind*III; S, *Sall*; Bc, *Bcl*I; C, *Cla*I; Xh, *Xho*I; Pv, *Pvu*II. The second line shows the disrupted *ste9* allele. The thin lines in the lower part indicate various truncated genomic fragments. Their ability to rescue the *ste9* mutation is indicated by (+) and (-). The nucleotide sequence of *ste9*⁺ has been submitted to the DDBJ/EMBL/GenBank databases under accession number AB001285. (B) Alignments of Ste9 and Hct1/Cdh1. Identical amino acids between two proteins are indicated by vertical lines (|). Residues of similar property are indicated by asterisks (*).

nonsense (*ste9*-B36) and disrupted alleles for the experiments described below.

The *ste9*⁺ Gene Is Essential for G₁ Arrest upon Starvation

To understand the physiological role of Ste9, cell division in a nitrogen-free medium was monitored because *ste9* mutant was sterile. Exponentially proliferating cells were transferred to a nitrogen-free medium. At each time indicated, a portion of the culture was taken and inspected microscopically. Profile of changes in percentage of septated cells was similar between wild-type and the *ste9* mutant strains (Figure 2A). The total cell number increased 3.8 times for the wild-type strain and 4.4 times for the *ste9* mutant, indicating that both strains passed through mitosis twice before they ceased to proliferate. We and others previously isolated several sterile mutants that rapidly lost viability after nutritional starvation (Kitamura *et al.*, 1990; Devoti *et al.*, 1991; Warbrick and Fantes, 1991; Takeda *et al.*, 1995). To test whether the *ste9* mutants normally responded to starvation, viability in long-term culture was monitored. Viability of wild-type and *ste9* cells cultured in nitrogen-free medium for 9 d was 82.7% and 68.2%, respectively. Viable cells were somewhat decreased in prolonged culture in the *ste9* mutant, but viability was still fairly high. Microscopic observation revealed that both wild-type and *ste9* cells were small and spherical under the starved condition, indicating that both strains successfully entered into the stationary phase. Many fission yeast genes responsible for sexual development are transcriptionally induced in response to nitrogen starvation depending on the Ste11 transcription factor (Sugimoto *et al.*, 1991). *mei2* is one such gene, and its transcription occurs in a mating-type or pheromone signaling-independent manner (Shimoda *et al.*, 1987). Northern analysis revealed that transcription of *mei2* was strongly induced by nitrogen starvation in *ste9* cells as well as in wild-type cells (our unpublished results).

The above data indicate that the *ste9* mutant is competent to respond to nitrogen starvation. Nevertheless, *ste9* mutants are completely sterile. In wild-type cells, starvation of nitrogen induces cell cycle arrest in the G₁-phase (Costello *et al.*, 1986). This is one of the prerequisites for fission yeast cells to commit to the developmental pathway and initiate conjugation (Nurse and Bissett, 1981). We next examined whether the *ste9* mutant arrested in the G₁-phase in response to nitrogen limitation. DNA content of cells cultured in nitrogen-free medium was analyzed by flow cytometry (Figure 2B). In *S. pombe*, most of the actively proliferating cells are in the G₂-phase, and a newborn cell has already completed DNA synthesis after cytokinesis. Consistently, the majority of cells in the exponential culture had a 2C DNA content in both wild-type and *ste9* strains. Upon nitrogen starvation, cells with a 1C DNA content gradually accumulated in the wild-type strain, indicating that most cells arrested in

the G₁-phase as they ceased to proliferate. In contrast, *ste9* strain showed only a minor G₁ peak and remained as cells with the 2C DNA content after 8 h (Figure 2B), or even after 24 h (our unpublished results). Consistent with the previous report (Moreno and Nurse, 1994), the control *rum1* strain was also defective in the starvation induced-G₁ arrest. We conclude that the *ste9* mutant is defective in cell cycle arrest in the G₁-phase in response to nitrogen starvation, while they are competent to enter the stationary phase from the G₂-phase. Therefore, the primary cause of the sterility in the *ste9* mutant is its failure to arrest in G₁.

It was demonstrated that the Cig2 cyclin was a major partner of the Cdc2 kinase in the G₁/S-phase in fission yeast (Fisher and Nurse, 1996; Martín-Castellanos *et al.*, 1996; Mondesert *et al.*, 1996). Cells harboring the deleted *cig2* allele are viable but exhibit a delay in progression through G₁ to the S-phase in some situations, e.g., small cells generated by the *wee* mutations or nitrogen starvation. We tested the possibility of whether a decrease in the G₁/S cdk activity by inactivation of Cig2 restored mating ability in the *ste9* mutant. This was indeed the case; mating defect was suppressed by the mutation in *cig2*. Efficiency of the suppression varied depending on the allele of *ste9* mutation (Table 2): 6% in the double disruptant of $\Delta ste9 \Delta cig2$, 17% in the *ste9-B36 cig2-S18*, and ~50% in the *ste9-59 Δcig2*. The *ste9-59* allele encodes a partially functional protein because mating is blocked but sporulation is hardly affected by this mutation (Kitamura and Tsujimoto, unpublished). We also investigated the effect of inactivation of other cyclin genes including *puc1* (Forsburg and Nurse, 1994), *cig1* (Bueno *et al.*, 1991) or *pas1* (Tanaka and Okayama, personal communication). However, the sterility of the *ste9* strain was not rescued by the disruption of these cyclin genes, indicating that the relationship between *cig2* and *ste9* was specific. These data strongly support the previous conclusion that the cause of the sterility in the *ste9* mutant is its G₁-arrest defect. We noticed that about 10% of cells formed azygotic asci in the $\Delta ste9$ and *ste9-B36* strains in combination with the *cig2* mutation. Unexpectedly, these azygotic asci were produced from haploid cells without diploidization by the preceding mating. This haploid meiosis was also observed in the *rum1 cig2* strain (Kitamura, unpublished). The reason for this aberrant meiosis is currently under investigation.

ste9 and *wee1* Mutations Cause Synthetic Lethality

Cell cycle regulation in the G₁-phase is obviously defective in the *ste9* mutants, although cells completely lacking the Ste9 function still grow in a healthy manner in the rapidly proliferating phase. In rapidly proliferating wild-type cells, the size of two daughter cells born in the previous cell cycle is already beyond the

Figure 2. The *ste9* mutants cannot arrest in the G₁-phase upon nitrogen starvation. (A) Changes in cell number (open symbols) and percent of septated cells (filled symbols) of cells cultured in EMM2-N. Wild-type (L972, circle) and $\Delta ste9$ (KJ100-8C, square) strains were used. (B) Wild-type (L972, left) or $\Delta ste9$ (KJ100-8C, center) strains were cultured in EMM2-N for the duration indicated and analyzed by flow cytometry. The $\Delta rum1$ (KJ153-1A, right) is a control for the G₁ arrest-defective strain.

critical size required for passing the Start restraint. Consequently, the length of the G₁-phase is very short and the pre-Start G₁ period is virtually lacking in actively proliferating cells (Nurse, 1975; Nurse and Thuriaux, 1977; Nasmyth, 1979). A protein kinase en-

Table 2. Suppression of the mating and meiosis deficiency in the *ste9* strains by the *cig2* mutation

Relevant genotype	Frequency (%)		
	ZA	AA	V
$\Delta ste9 \Delta cig2$	6.1 $\pm$ 0.8	10.6 $\pm$ 1.0	83.4 $\pm$ 1.8
<i>ste9-B36 cig2-S18</i>	17.1 $\pm$ 1.2	11.4 $\pm$ 0.6	71.6 $\pm$ 1.7
<i>ste9-59 $\Delta cig2$</i>	48.9 $\pm$ 3.7	0.8 $\pm$ 0.1	50.4 $\pm$ 3.7
<i>ste9*</i>	<0.2	<0.2	>99.6

All strains are homothallic mating type (*h⁹⁰*). Each strain was cultured on MEA for 2 d at 28°C. Data are the average of four to six independent countings and expressed as mean $\pm$ SE. At least 500 cells were examined in each counting. ZA, Zygotes and zygotic asci; AA, aberrant azygotic asci derived from haploid cells; V, vegetative cells. Construction and characterization of $\Delta ste9$ allele is described in the text. *ste9-B36* is a nonsense allele isolated by Leupold and Sipiczki (1991). *ste9-59* is a novel allele encoding partially active protein (our unpublished result, see text). *ste9** represents all three *ste9* alleles described above. The *cig2-S18* mutation was identified as an extragenic suppressor of the sterility of *ste9-B36* strain. This *cig2* mutation inactivates the protein because it cannot complement the deleted *cig2* allele (Kitamura, unpublished).

coded by *wee1*⁺ negatively regulates G₂/M-phase transition by phosphorylating Cdc2 (McGowan and Russell, 1993). Cells harboring the deleted *wee1* allele are viable, but timing of the initiation of mitosis is advanced. As a result, half-size cells are generated after cell division (Russell and Nurse, 1987). In these small cells, the G₁ period must be lengthened until the minimum size required for overriding the Start-restraint is attained. We next examined the role of Ste9 in such small cells. For this purpose, a temperature-sensitive *wee1-50* allele (Nurse, 1975) was used. Both parental *ste9* and the *wee1-50* single mutant could grow at 35°C. In contrast, growth of the *ste9 wee1-50* double mutant was temperature-sensitive (see below). This indicates that *ste9*⁺ was dispensable in an otherwise wild-type background but indispensable in the absence of the *wee1*-function. The *mik1*⁺ gene encodes a protein kinase whose function overlaps with Wee1, but disruption of the *mik1* gene does not confer any significant phenotype (Lundgren *et al.*, 1991). Accordingly, the $\Delta ste9 \Delta mik1$ double disruptant was viable, and the phenotype of this double disruptant was identical with that of the parental $\Delta ste9$ cells.

Because the sterility of the *ste9* mutant was suppressed by the *cig2* mutation, we examined whether the lethality of *ste9 wee1* strain was also suppressed by simultaneous inactivation of *cig2*. However, temperature-sensitive lethality was not suppressed by the *cig2* mutation, and the triple mutant also exhibited a growth defect at 35°C (Figures 3A and 7C). The growth profile of this triple mutant at the restrictive temperature was investigated in detail. Figure 3A shows the change in viability at the restrictive temper-

ature. Control *wee1-50* cells continued to proliferate and the number of viable cells exponentially increased. In marked contrast, *wee1-50* cells lacking the Ste9 function began to die after 2 h. These cells did not cease cell division; the total cell number increased (our unpublished results). The DNA contents of these cells were analyzed by flow cytometry (Figure 3B). As the heat-labile Wee1 protein was inactivated, the length of the G₁ period elongated. Consistently, the 1C peak corresponding to the G₁ population was clearly seen in the control *wee1-50 cig2* cells. In contrast, no apparent 1C peak was observed in the triple mutant of *ste9 wee1-50 cig2*. To emphasize 1C peak clearly, we used the *cig2*-disrupted strain for the experiment shown in Figure 3, but the *ste9 wee1-50* double mutants also gave similar results. DAPI staining of these cells revealed that cells became small and executed aberrant septation, which led to the asymmetric nuclear segregation. These data are interpreted as follows: the *wee1 ste9* double mutant could not delay DNA synthesis until the restraint of size control was cleared and could enter the S-phase immediately after previous mitosis even in the cells too small to initiate DNA synthesis. Repeats of this uncontrolled cell cycle progression led to cell death. Consistent with this hypothesis, the addition of hydroxyurea (HU), an inhibitor of DNA synthesis, at 2 h after a temperature increase, completely prevented subsequent cell death (Figure 3A).

ste9 Mutants Enter the M-Phase from the Pre-Start G₁-Phase

As described above, *ste9*⁺ is essential for the proper regulation of cell cycle progression in the G₁-phase. The Cdc10 protein, in combination with Res1 or Res2, functions as a transcription factor for the genes that are essential for transition from the G₁ to the S-phase (Lowndes *et al.*, 1992; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994). At the restrictive temperature, *cdc10^{ts}* mutants arrest in the pre-Start period of the G₁-phase (Nurse *et al.*, 1976; Nurse and Bissett, 1981). We found that the *ste9 cdc10^{ts}* double mutant rapidly lost viability compared with the *cdc10^{ts}* single mutant at the restrictive temperature (36°C). Even at the semipermissive temperature (32°C), the double mutant was inviable, whereas the control *cdc10^{ts}* mutant formed colonies (Figure 4A). The control *ste9* strain grew normally at all temperatures examined, indicating that the *cdc10^{ts}* cells were extremely sensitive to the lack of the Ste9 function. Analysis of the DNA content of the cells at 30°C revealed that the apparent 1C peak corresponding to the G₁ population was seen in the *cdc10^{ts}* strain, but no such population was found in the *ste9 cdc10^{ts}* strain (Figure 4B). The DNA profiles of both strains at 32°C were also quite different, in that appearance of the

cells in the G₁/early S-phase was remarkably delayed in the double mutants (Figure 4B). These observations led to the idea that Start-promoting activity is intense in the *ste9* mutant (see DISCUSSION).

The phenotype of the *ste9 cdc10^{ts}* double mutant at the restrictive temperature was examined in more detail. Asynchronous populations of *cdc10* or the double mutant of *ste9 cdc10* at the permissive temperature were shifted to 36°C. At the indicated time, a portion of the culture was taken for microscopic observation and flow cytometry. In both cultures, septated cells decreased after 1 h and then increased at the restrictive temperature (Figure 4C). In the *cdc10* strain, septated cells decreased again by 4 h. On the contrary, 10–20% of the population still had septa in the *ste9 cdc10* strain, indicating that cell division might continue even at the restrictive temperature. Figure 4D shows the DNA profile of these cells at 36°C. Both *cdc10* and *ste9 cdc10* cells arrested with a 1C DNA content by 4 h. After 6 h, the major peak shifted to the right, but these shifted peaks did not correspond to the 2C population. These shifts may be an artifact due to cell elongation, and the DNA profile did not alter by preventing DNA synthesis using HU (Figure 4D). We concluded that both single and double mutants arrested in the G₁-phase at restrictive temperatures. DAPI staining of the *ste9 cdc10* strain revealed that the nucleus was bisected by the septum at the restrictive temperature (Figure 4E). The appearance of these cells exhibiting a so-called “cut” phenotype indicated that septum formation was uncoupled with completion of DNA replication or nuclear separation. In the DNA profile of the double mutant, a small peak with less than a 1C DNA content was observed at 6 h after a temperature increase (Figure 4D, indicated by a downward arrow). This minor peak was seen even in the presence of HU but was never seen in the *cdc10* single mutant. This peak might represent dead cells produced by the aberrant mitosis in the double mutant.

These observations strongly suggest that the *ste9 cdc10* mutant is defective in some checkpoint process. To date, two related, but distinct, DNA structure checkpoint mechanisms that prevent premature mitosis were discovered in fission yeast (Carr, 1995; Stewart and Enoch, 1996). One is the S-phase-mitosis (S/M) checkpoint, which responds to changes in the state of DNA replication. Another is the DNA damage checkpoint, which monitors the state of DNA damage and searches for lesions in the newly replicated DNA, such as incomplete ligation. We tested whether the *ste9* mutant was defective in these checkpoint mechanisms. However, *ste9* cells survived in the presence of HU or methylmethane sulfonate as did wild-type cells (our unpublished results). In addition, wild-type and *ste9* strains have the same sensitivity to UV light, indicating that Ste9 is apparently dispensable for the replication- and the damage-checkpoint functions.

Figure 3. Synthetic lethality between *ste9* and *wee1* mutations. (A) Viability for *wee1-50 Δcig2* (KJ109-5A, open square), $\Delta ste9 wee1-50 \Delta cig2$ (KJ111-1A, filled circle), and $\Delta ste9 wee1-50 \Delta cig2$ cells in the presence of HU (open triangle). Log-phase cultures at 23°C were shifted to 35°C at 0 h. HU was added after 2 h of incubation at a final concentration of 12 mM. (B) DNA content of *wee1-50 Δcig2* (left) and $\Delta ste9 wee1-50 \Delta cig2$ (right) at the restrictive temperature. Cells were shifted to 35°C at 0 h.

Figure 4. The *ste9* mutant initiates mitosis from the G_1 -phase in the absence of S-phase. In all experiments, KJ32-2A (*cdc10*–129) and KJ32-1A (*ste9*–B36 *cdc10*–129) were used. (A) The double mutant of *ste9 cdc10* cannot grow even at the semipermissive temperature for *cdc10*^{ts} (32°C). Cells of *cdc10* (left) or *ste9 cdc10* (right) were cultured for 3 d at 32°C and 37°C. (B) Flow cytometric analysis of *cdc10* (left) or *ste9 cdc10* (right) at the semipermissive temperature. (C) Changes of the septation index. Asynchronous culture of *cdc10* (open square) or *ste9 cdc10* (filled circle) at the permissive temperature was shifted to 36°C. At the time indicated, cells were stained with calcofluor, and septated and nonseptated cells were counted. (D) DNA content of *cdc10* (left) and *ste9 cdc10* (right) mutants at 36°C. HU was added at the same time as the cells were shifted to 36°C. Addition of HU at 2 h at the restrictive temperature resulted in the same DNA profile (our unpublished observations). (E) “Cut” phenotype in the *ste9 cdc10* double mutant at the restrictive temperature. Cells were incubated at 36°C for 6 h and stained with DAPI and calcofluor. (F) Lethal mitosis in the *ste9 cdc10* double mutant occurred in the absence of S-phase. HU (final concentration 12 mM) was added to the cells of *cdc10* (open bar) and *ste9 cdc10* (black bar), which were then incubated at 28°C or 36°C for 4 h. Aliquots were plated onto YE and incubated at 25°C, and the number of colonies was counted after 3 d. Viability is expressed as the percent ratio of colony numbers of cells after 4 h of incubation relative to that of untreated cells at the beginning of the experiments (0 h). Sensitivity to HU of the *cdc10* single mutant at 28°C was not examined, but the *cdc10* cells were viable at 36°C in the presence of HU.

E **F**

Figure 4E–F (legend on facing page).

It is possible that lethal mitosis in the *cdc10 ste9* strain is caused by incomplete *cdc10* arrest and premature entry into mitosis. As described, HU effectively blocks cell cycle progression in the *ste9* cells because the replication checkpoint is operating. If G₁ arrest by the *cdc10* mutation is incomplete, it is expected that lethal mitosis is prevented in the presence of HU because of its inhibition of S-phase progression. As shown in Figure 4F, addition of HU to the *ste9 cdc10* double mutant did not prevent cell death at the restrictive temperature, indicating that lethal mitosis in the *cdc10 ste9* cells was not due to leakiness of cell cycle arrest. Therefore in the *ste9 cdc10* strain, DNA synthesis was bypassed, and the cells entered mitosis from the pre-Start G₁ period.

The amount of Cdc13 protein, a major mitotic B-type cyclin in *S. pombe*, culminates in the late G₂/M and is

very low in the G₁-phase due to selective degradation by the proteasome (Hayles *et al.*, 1994; Yamano *et al.*, 1996). Strong activation of the Cdc2/Cdc13 complex in G₁-arrested cells leads to mitosis in the absence of DNA synthesis (Hayles *et al.*, 1994; Yamano *et al.*, 1996). Because similar premature mitosis occurs in the G₁-arrested *ste9 cdc10* cells, we quantified the level of the Cdc13 protein at the restrictive temperature. In the *cdc10* single mutant, Cdc13 almost disappeared by 4 h at 36°C as the cells arrested in the G₁-phase (Figure 5). In contrast, Cdc13 did not decrease in the *ste9 cdc10* double mutant (Figure 5), although the cells largely arrested in G₁ by 4 h at 36°C (Figure 4D). This persistence of Cdc13 in *ste9 cdc10* cells, at least in part, seems relevant to the premature mitosis. The same premature entry into M-phase from pre-Start G₁ occurs also in the *rum1 cdc10* double mutant (Moreno and Nurse, 1994). These data indicate that both *ste9* and *rum1* are essential to prevent mitosis from the pre-Start G₁ period.

Overexpression of Ste9 Inhibits Mitosis and Induces an Extra Round of DNA Replication

Our observation that Cdc13 persisted in the G₁-arrested *ste9* cells (Figure 5) suggests an intriguing possibility that Ste9 down-regulates the amount of Cdc13 protein. To confirm this assumption, the Cdc13 level in Ste9-overexpressing cells was quantified with a strain harboring the integrated *nmt-ste9*⁺ gene. In the presence of thiamine, cells exponentially proliferated (Figure 6B), and the Cdc13 protein level did not significantly change throughout the incubation (Figure 6A). In marked contrast, Cdc13 decreased when the expression of Ste9 was induced in the thiamine-free medium. Consistently, activity of total H1-histone kinase was greatly reduced in these cells.

In the Ste9-overexpressing culture, increase in cell number stopped after 12 h of induction (Figure 6B). Ste9 overexpression requires a 12-h induction because the *nmt1* promoter takes that long to induce (Maundrell, 1990). Therefore, cessation of proliferation coincided well with the kinetics of induction. Concomitant with the end of cell number increase, cells became elongated and exhibited a low septation index (Figure 6C). Less than 1% of cells contained two nuclei and exhibited mitotic figures after 14 h. In these cells, onset of the S-phase was slightly delayed, as evidenced by the appearance of the 1C peak, after which the population with a 4C DNA content corresponding to diploid cells increased (Figure 6D). The possibility that overexpression of Ste9 led to asymmetric segregation of nuclear DNA was excluded because no cells of “cut” phenotype or missegregation of the nuclei were seen during incubation. We conclude that Ste9 plays a role in the regulation of Cdc13 cyclin level during G₁. Down-regulation of Cdc13 and the resultant

Figure 5. Cdc13, a mitotic B-cyclin, is not decreased in the G_1 -arrested *ste9* mutant. Extracts were prepared from *cdc10* (lanes 1–4) or *ste9 cdc10* (lanes 5–8) strains. Cells were cultured at 36°C for 0 h (lanes 1 and 5), 2 h (lanes 2 and 6), 3 h (lanes 3 and 7), and 4 h (lanes 4 and 8). Western blotting was carried out using anti-Cdc13 antibody (upper panel) or anti-PSTAIRE antibody for the loading control (lower panel). The location of Cdc13 is indicated by the arrow. The lower bands (indicated by asterisk) are unknown protein cross-reacted with the anti-Cdc13 antibody.

reduction of mitotic cdk activity should lead to prevention of mitosis and the induction of an extra round of DNA replication, as demonstrated by Hayles *et al.* (1994). These features are quite similar to those of the Rum1-overexpressing strain (Moreno and Nurse, 1994).

In fission yeast, the state of the Cdc2 kinase determines whether cells are in G_1 or G_2 (O'Connell and Nurse, 1994). When *cdc2^{ts}* mutants were subjected to heat shock to destroy the heat-labile Cdc2 protein, haploid cells in the G_2 -phase were reset to the pre-Start G_1 -phase. If these cells are again shifted back to permissive temperature, they rereplicate their genome and initiate a new round of cell cycle as diploid cells (Broek *et al.*, 1991). We explored the possibility that *cdc2* inactivation caused diploidization in the *ste9* mutant. As shown in Figure 6E, *cdc2^{ts}* single mutants indeed gave rise to diploid cells, whereas the double mutant of *ste9 cdc2^{ts}* remained as a haploid. Thus, functional inactivation of *ste9* completely prevents this diploidization, as reported for the *rum1* mutant (Labib *et al.*, 1995). Interestingly, similar abnormality was reported in *Drosophila*. Loss of Fizzy-related, a homolog of Ste9, inhibits endoreduplication in G_2 cells, which normally occurs in the developing embryo (Sigrist and Lehner, 1997). Because DNA replication requires G_1 cdk activity, Ste9 should be necessary to maintain the Cdc2 kinase in a pre-Start form.

Chk1 was also reported to be involved in some aspect of G_1 regulation (Carr *et al.*, 1995). We tested whether rereplication was also blocked in the double mutant of the *chk1 cdc2^{ts}* strain. However, the *chk1 cdc2^{ts}* strain effectively rereplicated their DNA and became diploid as well as the control *cdc2^{ts}* strain (Figure 6E). If Chk1 functions as a regulator of G_1 , its role is quite different from that of Ste9.

Functional Relationship between Ste9 and Rum1

As described, various phenotypes of the *ste9* mutant are indistinguishable from those of the *rum1* mutant (Moreno and Nurse, 1994). The similarity of phenotypes in these mutants suggests that the roles of both proteins may be functionally related, although their primary structures are not homologous. We investigated the functional relationship between *ste9* and *rum1*. First, the amount of Rum1 protein in the *ste9* mutant was monitored. As reported earlier, Rum1 protein was very low in the asynchronous, actively proliferating cells. Upon nitrogen starvation, which induces G_1 arrest in wild-type strain, considerable level of Rum1 protein was detected (Figure 7A). In contrast, such accumulation did not occur even in the *ste9 cig2* cells (Figure 7A). Northern analysis revealed that the level of *rum1* mRNA was induced in the same extent in both strains upon starvation (our unpublished results). This might simply reflect the fact that the *ste9* mutant cannot arrest in the G_1 -phase (Figure 2B) and Rum1 protein is unstable except in the G_1 -phase (Correa-Bordes and Nurse, 1995). However, sterility was partially suppressed by the *cig2* mutation (Table 2), and the G_1 -arrest defect was partially recovered in the *ste9 cig2* cells (Figure 7B). Therefore, Ste9 might be actively involved in the production and/or stabilization of Rum1.

Next, the effect of the multicopy *ste9⁺* or *rum1⁺* gene on the suppression of the mutant phenotype was investigated. Sterility of both mutants could not be mutually suppressed by the introduction of the other gene. However, the synthetic lethality of *ste9 wee1–50* cells at the restrictive temperature was weakly suppressed by the plasmid-borne *rum1⁺* gene (Figure 7C). Conversely, *rum1 wee1–50* double mutant was also viable in the presence of the *ste9⁺*-plasmid. Furthermore, growth defect in the *ste9 cdc10* cells at the semi-permissive temperature was effectively suppressed by the *rum1⁺*-plasmid (Figure 7D). Inviability of *rum1 cdc10* cells was also suppressed by the *ste9⁺*-plasmid. These data indicate that the functions of Ste9 and Rum1 are mutually interrelated. However, both proteins do not work in a simple linear pathway, and their functions might be complementary and partly overlapping.

DISCUSSION

In this study, we demonstrated that the *ste9⁺* gene was essential for proper regulation of cell cycle progression in the G_1 -phase. First, the *ste9* mutant was sterile because of the failure in G_1 arrest upon nitrogen starvation. Cell cycle arrest in the pre-Start G_1 period is essential to enter the developmental pathway, and the sterility was partially suppressed by the inactivation of *cig2*, which encoded a major G_1 /S cyclin in *S. pombe*.

Figure 6. Overexpression of Ste9 prevents mitosis and induces re-replication of the genome. (A) Changes in the Cdc13 level and the H1-histone kinase activity during Ste9-overexpression. Cells harboring the integrated *mnt-ste9⁺* gene were cultured to mid-log phase in the presence of thiamine. At 0 h, the cells were washed and then reinoculated into fresh medium without thiamine (ON) or with thiamine (OFF). Cell extracts were prepared from the cultures at the times indicated. Note that induction from the *mnt* promoter requires more than 10 h (Maundrell, 1990). Immunoblotting was carried out by anti-Cdc13 antibody or by anti-PSTAIRE antibody (upper two panels). Arrow and asterisk indicate the locations of Cdc13 and cross-reacted proteins, respectively. H1-histone kinase activities of uninduced (OFF) and induced (ON) cultures were also measured (lower panel). (B) Overexpression of Ste9 inhibits cell division. Cell numbers of induced (ON) and uninduced (OFF) cultures were counted at the indicated time. (C) Changes in septation index in the same cultures described in panel B. (D) Changes in the DNA content during Ste9 overexpression. Samples were taken at the indicated time for flow cytometry. The DNA profiles of the uninduced culture at each time were the same as that of 0 h. (E) The *ste9* mutation prevents re-replication of DNA due to heat inactivation of the temperature-sensitive Cdc2 protein. Log-phase cells were shifted to EMM2-N and cultured at 36°C for 6 h. Cells were plated at 0 h (open bar) and at 6 h (black bar) onto YE medium containing phloxine B. Numbers of red colonies (diploid cells) and pink colonies (haploid cells) were counted, and the percent of diploid cells was calculated. Strains: *cdc2*-M26 (KJ141-8A); Δ *ste9 cdc2*-M26 (KJ124-3A); Δ *chk1 cdc2*-M26 (KJ155-1A).

Second, Ste9 was indispensable for the growth of the *wee1* cells, which had to lengthen the pre-Start G₁ period to restrain DNA synthesis until the critical size to override Start control was attained. Third, Ste9 is

essential to prevent premature entry into mitosis from the pre-Start G₁ period in the absence of DNA replication. Furthermore, Ste9 might be required for maintenance of the Cdc2 kinase in a pre-Start form, as

suggested by the fact that overexpression of Ste9 induced rereplication of the genome due to reduction of the mitotic kinase activity of the Cdc13/Cdc2 complex, and rereplication in the *cdc2^{ts}* strain was prevented by the *ste9* mutation.

The *ste9* gene is identical to the recently identified *srw1* (Yamaguchi *et al.*, 1997). The Ste9 is evolutionary highly conserved and is a homolog of Hct1/Cdh1 of budding yeast and Fizzy-related (Fzr) of higher organisms. The *hct1/cdh1* mutant is viable, but mitotic Clb2 cyclin is highly stabilized and is not efficiently degraded in the mutant (Schwab *et al.*, 1997; Visintin *et al.*, 1997). Similarly, *Drosophila* Fzr is required for down-regulation of cyclins A, B, and B3 during G₁ when the embryonic epidermal cell stops proliferation after mitosis 16 (Sigrist and Lehner, 1997). In good agreement with these reports, mitotic Cdc13 cyclin remains in the *ste9* mutant arrested in G₁. Therefore, all of the Ste9, Hct1/Cdt1, and Fzr are supposed to be involved in the regulation of the amount of mitotic cyclin during G₁, although no biochemical function of these proteins has yet been demonstrated. Budding yeast Cdc20, a representative protein of this WD-protein family, is essential for the degradation of Pds1 and for entry into anaphase but apparently dispensable for Clb2 degradation (Visintin *et al.*, 1997). It is proposed that Hct1 and Cdc20 might target distinct, but overlapping, sets of proteins for degradation, perhaps by recruiting these substrates to cyclosome/anaphase-promoting complex for ubiquitination (Cohen-Fix and Koshland, 1997; Hoyt, 1997). In fission yeast, Slp1 might be a homolog of Cdc20. Although the primary structures of WD-repeat domain are highly conserved between Ste9 and Slp1, the amino-terminal regions beyond WD-repeat domain are not as homologous. Both proteins exert their own role through the function of the N-terminal region. It is noteworthy if the WD-repeat regions of both proteins interact with a common protein, because the WD-repeat is involved in protein-protein interaction (Sondek *et al.*, 1996). Although genetic interaction is demonstrated between Cdc20 and Hct1, we did not observe such interaction between Ste9 and Slp1; double mutants of $\Delta ste9 slp1-362$ did not show synthetic phenotype, and the growth defect of *slp1-362* was not suppressed by the *ste9⁺*-plasmid (our unpublished results). The roles of Ste9, Slp1, and the uncharacterized WD-repeat proteins of this family found by the genome-sequencing project must be further investigated.

The phenotype of the *ste9* mutant is indistinguishable from that of the *rum1* mutant (Moreno and Nurse, 1994). Rum1 is a potent cdk inhibitor but it is noteworthy that the Rum1 promotes proteolysis of the Cdc13 cyclin during G₁-phase (Correa-Bordes *et al.*, 1997). We also showed that both functional inactivation and overexpression of Ste9 significantly affected the level of Cdc13 protein. Therefore, Ste9 would also

be involved in proteolysis of some important cell cycle regulators, one of the candidates being Cdc13 cyclin. Because sterility of *ste9* mutants was suppressed by the *cig2* mutation, this cyclin might be also a possible target. Inactivation of all three known B-cyclins in fission yeast (Cig1, Cig2, and Cdc13) results in lethality, and such cells arrest before DNA replication (Fisher and Nurse, 1996). Because overexpression of Ste9 induces rereplication, at least one B-cyclin functioning at the onset of DNA replication should be resistant to the effect of Ste9. High level expression of budding yeast Hct1 induces the rapid and selective disappearance of the mitotic Clb2 cyclin, probably by proteolysis, but the level of Clb5, an S-phase cyclin, does not change (Schwab *et al.*, 1997).

In general, proteolysis of mitotic B-cyclin persists throughout G₁ (Amon *et al.*, 1994; Brandeis and Hunt, 1996). Both Ste9 and Rum1 have pivotal roles in decreasing levels and activities of the Cdc2/Cdc13 complex during G₁. In the absence of this control, fission yeast cells in G₁ can initiate mitosis without having undergone DNA replication. Proteolysis is indispensable for normal cell cycle progression (King *et al.*, 1996), and expression of undegradable Cdc13 resulted in the cell cycle arrest at anaphase (Yamano *et al.*, 1996). The fact that *ste9⁺* and *rum1⁺* are dispensable for normal growth is curious because Cdc13 protein remains in the *ste9* or *rum1* mutant arrested in G₁ (Moreno and Nurse, 1994; this study). In budding yeast, *HCT1* is a nonessential gene, but viability of the *hct1* mutant depends on Sic1, a Clb-specific cdk inhibitor. Therefore, defects of cyclin degradation in *hct1* cells is compensated by the activity of Sic1. Obviously, cdk activity must be inactivated upon exit from mitosis to G₁; therefore, exit from mitosis is guaranteed either by degradation of mitotic cyclin or by the activity of cdk inhibitor. Like Hct1/Cdt1 and Sic1 in budding yeast, Ste9 and Rum1 execute an essential and overlapping function during G₁. Surprisingly, a double disruptant of *ste9 rum1* is still viable (Kitamura, unpublished). There are several possible explanations for the viability of the *ste9 rum1* cells. 1) Both proteins have an effect on the Cdc13 proteolysis only in the G₁-phase and not during mitotic exit. 2) Function of Ste9 overlaps that of other WD-repeat protein(s) belonging to the same Cdc20 subfamily. 3) cdk activity is inactivated by a mechanism other than cyclin degradation and inhibition by Rum1. Phosphorylation of Cdc2 at tyrosine 15 might be responsible for this inhibition of cdk activity because both *ste9* and *rum1* are synthetically lethal with the *wee1* mutation. However, it is demonstrated that this inhibitory tyrosine residue is dephosphorylated in pre-Start G₁ and becomes phosphorylated only after cells enter late G₁ (Hayles and Nurse, 1995). In *Drosophila*, the roughex (*rux*) protein plays an analogous role to those of Ste9 and Rum1. Rux is required to establish G₁-phase in the

Figure 7. Functional relationship between Ste9 and Rum1. (A) Changes of Rum1 protein level after starvation. The *ste9-B36 Δcig2 rum1⁺-3HA* strain (KJ238-7B) was transformed with a vector or the *ste9⁺*-plasmid. Both strains cultured in EMM2 were shifted to nitrogen-free medium. At the indicated time, samples were taken for immunoblotting. (B) DNA content of *ste9⁺* or *ste9⁻* cells in the absence of *cig2* function. Samples from the same cultures described in panel A were analyzed by flow cytometry. (C) Mutual suppression of synthetic lethality by either *rum1⁺*- or *ste9⁺*-plasmid. Cells of $\Delta ste9 wee1-50 \Delta cig2$ (KJ111-1A, upper panel) or $\Delta rum1 wee1-50$ (KJ152-9A, lower panel) were transformed with the vector, pAL(*ste9*), and pREP1(*rum1*). Each transformant was streaked onto EMM2 plates containing 5 μ M thiamine and incubated for 3 d at 34.5°C. (D) Suppression of the synthetic lethality at semipermissive temperature for *cdc10* cells. Cells of $\Delta ste9 cdc10-V50$ (KJ218-2B) were transformed with the vector, pAL(*ste9*), and pAL(*rum1*). Transformants were incubated at 30°C for 3 d (upper panel). Cells of $\Delta rum1 cdc10-129$ (Sp224) were transformed with the same plasmids, and then incubated at 32°C for 3 d (lower panel).

developing eye (Thomas *et al.*, 1994). In the *rux* mutant, cells accumulate Cyclin A in early G₁ and enter S-phase prematurely. This phenotype is suppressed by the down-regulation of Cyclin A. Furthermore, overexpression of Rux protein induces degradation of Cyclin A and inhibits S-phase entry caused by Cyclin A accumulation (Sprenger *et al.*, 1997; Thomas *et al.*, 1997). Thus *rux* shares several features with *ste9* and

rum1; all proteins function as a negative regulator of G₁ progression. Also in fission yeast, such unidentified protein might be operating to inhibit mitotic cdk activity upon exit from mitosis. The important question of how fission yeast cells inhibit cdk activity during G₁ remains to be answered.

Uncontrolled Start-promoting activity would lead to disturbance of cell cycle regulation, as in the case of

cancer cells. As demonstrated in cell cycle arrest by mating pheromone (Stern and Nurse, 1997), proper regulation of the G₁ cdk activity should also be important in the G₁ arrest upon nutritional starvation. The following observations lead to the idea that Start-promoting activity is deregulated in the *ste9* mutant. First, both wild-type and *ste9* strains divide twice under nutritional starvation but only the *ste9* mutant cannot arrest in the pre-Start period and initiates an additional round of DNA synthesis. Second, cells lacking *ste9*⁺ might initiate a new round of DNA replication as soon as the previous mitosis is completed, regardless of whether the cells attain the critical size. This leads to cell death in the *wee1*⁻ background because cell mass is lost in every cell division as occurs in the *rum1 wee1* strain (Moreno and Nurse, 1994; Sveiczer *et al.*, 1996). Third, the population in G₁ at the semipermissive temperature is considerably less in the *ste9 cdc10* double mutant than in the control *cdc10* strain. Because inhibition of the ubiquitin-dependent proteolytic activity overcomes Start arrest and induces DNA synthesis in fission yeast (Yamano *et al.*, 1996), it is possible that deregulation of the Start-promoting activity is related to the proteolytic defect. In this aspect, it is intriguing that the G₁ arrest induced by mating pheromone exposure is overridden in the budding yeast *hct1* mutant (Schwab *et al.*, 1997). This defect is suppressed by the deletion of the *CLB2* gene. In the epidermis of *Drosophila* Fzr-deficient embryos, extra division preceded by DNA replication occurs after mitosis 16 instead of arresting in G₁ (Sigrist and Lehner, 1997). This also might be due to the defect of cyclin degradation. Even the mitotic B-cyclin can induce DNA replication (Amon *et al.*, 1994; Irniger *et al.*, 1995; Fisher and Nurse, 1996); therefore, these cyclins escaping from degradation would be responsible for the promotion of the progression from G₁ to S-phase.

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