

The *puc1* Cyclin Regulates the G1 Phase of the Fission Yeast Cell Cycle in Response to Cell Size

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Eukaryotic cells coordinate cell size with cell division by regulating the length of the G1 and G2 phases of the cell cycle. In fission yeast, the length of the G1 phase depends on a precise balance between levels of positive (*cig1*, *cig2*, *puc1*, and *cdc13* cyclins) and negative (*rum1* and *ste9*-APC) regulators of *cdc2*. Early in G1, cyclin proteolysis and *rum1* inhibition keep the *cdc2*/cyclin complexes inactive. At the end of G1, the balance is reversed and *cdc2*/cyclin activity down-regulates both *rum1* and the cyclin-degrading activity of the APC. Here we present data showing that the *puc1* cyclin, a close relative of the Cln cyclins in budding yeast, plays an important role in regulating the length of G1. Fission yeast cells lacking *cig1* and *cig2* have a cell cycle distribution similar to that of wild-type cells, with a short G1 and a long G2. However, when the *puc1*⁺ gene is deleted in this genetic background, the length of G1 is extended and these cells undergo S phase with a greater cell size than wild-type cells. This G1 delay is completely abolished in cells lacking *rum1*. *Cdc2/puc1* function may be important to down-regulate the *rum1* Cdk inhibitor at the end of G1.

INTRODUCTION

The fission yeast *Schizosaccharomyces pombe* provides a model system with which to study the relationship between cell cycle progression and cell size. In this organism, the size of individual cells can be easily determined because they are cylindrical and growth occurs by length extension (Mitchison, 1957). In common with other eukaryotes, progression through the cell cycle is regulated principally before the onset of S phase and the onset of mitosis. In both cases, a critical cell mass must be attained before progression occurs (Nurse, 1975; Nurse and Thuriaux, 1977; Nasmyth *et al.*, 1979; Sveiczer *et al.*, 1996). In rapidly growing wild-type cells, the mitotic size control is limiting, because cell division produces daughter cells with a mass already greater than the minimum required to initiate S phase. In these conditions, G1 is very short and the onset of S phase is regulated by its dependence on completion of the previous mitosis (Nurse *et al.*, 1976; Nurse and Thuriaux, 1977; Nasmyth *et al.*, 1979). In conditions of nutrient limitation, mitosis is initiated at a reduced cell size, producing small daughter cells that must delay the initiation of S phase until the critical mass is achieved (Fantès and Nurse, 1977; Nasmyth, 1979).

Cyclins and Cdk inhibitors play a key role in determining the timing of S phase and relating it to the achievement of a critical cell size. *Rum1* and *ste9/srw1* are negative regulators of *cdc2*/cyclin complexes in G1, because small cells lacking either *rum1* or *ste9/srw1* are unable to delay progression through G1, resulting in the initiation of S phase immediately after the completion of mitosis (Moreno and Nurse, 1994; Sveiczer *et al.*, 1996; Yamaguchi *et al.*, 1997; Kitamura *et al.*, 1998). *Rum1* is a Cdk inhibitor that is present during the G1 phase of the cell cycle and inhibits *cdc2*/cyclin kinase activity until the critical mass required to pass Start is achieved (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995; Labib *et al.*, 1995; Sveiczer *et al.*, 1996; Correa-Bordes *et al.*, 1997; Benito *et al.*, 1998). *Ste9/srw1* is a WD-repeat protein that is highly homologous to budding yeast *Hct1/Cdh1* (Schwab *et al.*, 1997; Visintin *et al.*, 1997) and *Drosophila fizzy*-related (Sgrist and Lehner, 1997) and is involved in the degradation of B cyclins at the end of mitosis and G1 (Yamaguchi *et al.*, 1997; Kitamura *et al.*, 1998). Therefore, as cells exit mitosis, cyclin degradation and the Cdk inhibitor *rum1* operate together to inactivate *cdc2*/cyclin complexes during G1. If one of these two mechanisms is missing, the G1 phase is much shorter than in wild-type cells.

Cdc2 associates with several cyclins (*puc1*, *cig1*, *cig2*, and *cdc13*) during the fission yeast cell cycle. *Cig1*, *cig2*, and *cdc13* are B-type cyclins, whereas *puc1* is more closely re-

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Table 1. *Schizosaccharomyces pombe* strains

Strain	Genotype	Source
P1	972 <i>h</i> ⁻	P. Nurse
S192	968 <i>h</i> ⁹⁰	P. Nurse
S522	<i>rum1Δ::ura4⁺ ura4-d18 h⁺</i>	S. Moreno
S694	<i>rum1Δ::kanMX h⁻</i>	This study
S432	<i>cig1Δ::ura4⁺ ura4-d18 h⁻</i>	A. Bueno
S349	<i>cig2Δ::ura4⁺ ura4-d18 h⁻</i>	H. Okayama
S346	<i>puc1Δ::ura4⁺ ura4-d18 h⁻</i>	S. Forsburg
S517	<i>cig1Δ::ura4⁺ cig2Δ::ura4⁺ ura4-d18 h⁻</i>	This study
S453	<i>cig1Δ::ura4⁺ puc1Δ::ura4⁺ ura4-d18 h⁺</i>	This study
S519	<i>cig2Δ::ura4⁺ puc1Δ::ura4⁺ ura4-d18 h⁻</i>	This study
S514	<i>cig1Δ::ura4⁺ cig2Δ::ura4⁺ puc1Δ::ura4⁺ ura4-d18 h⁺</i>	This study
S629	<i>cig1Δ::ura4⁺ cig2Δ::ura4⁺ puc1Δ::ura4⁺ ura4-d18 h⁹⁰</i>	This study
S695	<i>cig1Δ::ura4⁺ cig2Δ::ura4⁺ puc1Δ::ura4⁺ ura4-d18 rum1Δ::kanMX h⁺</i>	This study
P369	<i>wee1-50 h⁻</i>	P. Nurse
S132	<i>wee1-50 rum1Δ::ura4⁺ ura4-d18 leu1-32 h⁹⁰</i>	S. Moreno
S466	<i>wee1-50 cig1Δ::ura4⁺ ura4-d18 h⁻</i>	This study
S511	<i>wee1-50 cig2Δ::ura4⁺ ura4-d18 h⁻</i>	This study
S465	<i>wee1-50 puc1Δ::ura4⁺ ura4-d18 h⁻</i>	This study
S512	<i>wee1-50 cig1Δ::ura4⁺ cig2Δ::ura4⁺ ura4-d18 h⁻</i>	This study
S458	<i>wee1-50 cig1Δ::ura4⁺ puc1Δ::ura4⁺ ura4-d18 h⁻</i>	This study
S472	<i>wee1-50 cig2Δ::ura4⁺ puc1Δ::ura4⁺ ura4-d18 h⁺</i>	This study
S516	<i>wee1-50 cig1Δ::ura4⁺ cig2Δ::ura4⁺ puc1Δ::ura4⁺ ura4-d18 h⁻</i>	This study
S696	<i>wee1-50 cig1Δ::ura4⁺ cig2Δ::ura4⁺ puc1Δ::ura4⁺ ura4-d18 rum1Δ::kanMX h⁻</i>	This study

lated to *Saccharomyces cerevisiae* Cln cyclins (reviewed by Fisher and Nurse, 1995; Stern and Nurse, 1996). Although B cyclins are essential for entry into S phase and mitosis during the fission yeast cell cycle (Hayles *et al.*, 1994; Fisher and Nurse, 1996; Martín-Castellanos *et al.*, 1996; Mondesert *et al.*, 1996) and also promote G1 progression past Start (Obara-Ishihara and Okayama, 1994; Martín-Castellanos *et al.*, 1996), the role of putative G1 cyclins such as *puc1* remains unclear. In wild-type cells, *cdc2/cig2* regulates entry into S phase (Obara-Ishihara and Okayama, 1994; Martín-Castellanos *et al.*, 1996; Mondesert *et al.*, 1996) and *cdc2/cdc13* regulates entry into mitosis (Booher *et al.*, 1989; Moreno *et al.*, 1989). *Cdc2/cig1* also contributes to the onset of S phase (Fisher and Nurse, 1996; Mondesert *et al.*, 1996). The *S. pombe puc1⁺* gene was isolated as a cDNA that conferred a factor resistance to *S. cerevisiae* cells deficient in the G1 cyclin *Cln3*, and expression of *puc1⁺* rescues the lethal *cdc* phenotype caused by deletion of *CLN1*, *CLN2*, and *CLN3* (Forsburg and Nurse, 1991). Therefore, the fission yeast *puc1* protein fulfills all the roles of a G1 cyclin in budding yeast. However, previous reports have failed to provide evidence for a role for *puc1* during G1 in fission yeast (Forsburg and Nurse, 1994).

Here we present data showing that the *puc1* cyclin plays an important role in G1. Fission yeast cells lacking the G1 cyclins *cig1*, *cig2*, and *puc1* have an extended G1 period that is abolished when the *rum1⁺* gene is deleted.

MATERIALS AND METHODS

Fission Yeast Strains and Methods

The *S. pombe* strains used in this study are listed in Table 1. Growth conditions and strain manipulations were as described by Moreno *et al.* (1991) and Fantes and Nurse (1977). The *cig1Δ::ura4⁺ ura4-d18 h⁻*, *cig2Δ::ura4⁺ ura4-d18 h⁻*, *puc1Δ::ura4⁺ ura4-d18 h⁻*, and

rum1Δ::ura4⁺ ura4-d18 leu1-32 ade6-M216 h⁻ strains have been described (Bueno *et al.*, 1991; Forsburg and Nurse, 1994; Moreno and Nurse, 1994; Obara-Ishihara and Okayama, 1994). Tetrad analysis was performed to construct double- and triple-cyclin mutants, and the identity of these mutants was confirmed by Southern blotting. For the quadruple *cig1Δ cig2Δ puc1Δ rum1Δ*, a *rum1* genomic clone in pTZ18R was digested with *NruI* and *SpeI* and a KanMX4 cassette was introduced. The resulting plasmid was digested with *NdeI*, and the purified fragment was used to transform the wild-type, the *cig1Δ cig2Δ puc1Δ*, and the *wee1-50 cig1Δ cig2Δ puc1Δ* strains. Transformants containing the *rum1⁺* gene deleted were selected in medium containing G418 and confirmed by Southern blotting.

Yeast transformation was carried out with the use of the lithium acetate transformation protocol (Moreno *et al.*, 1991). All experiments in liquid culture were carried out in minimal medium containing the required supplements, starting with a cell density of $2-4 \times 10^6$ cells/ml, corresponding to midexponential-phase growth.

Synchronous Cultures

h⁻ wee1-50 cig1Δ cig2Δ puc1Δ cells were grown at 25°C in minimal medium. Cells were synchronized at 25°C with the use of a JE-5.0 elutriation system (Beckman Instruments, Fullerton, CA) and then incubated at 25°C. Samples were taken every 20 min during two cell cycles for protein extracts and for flow cytometry analysis.

Flow Cytometry and Microscopy

About 10^7 cells were spun down, washed once with water, fixed in 70% ethanol, and processed for flow cytometry or DAPI staining, as described previously (Sazer and Sherwood, 1990; Moreno *et al.*, 1991). A Becton-Dickinson (Franklin Lakes, NJ) FACScan was used for flow cytometry. To estimate the proportion of G1 cells, we determined the percentage of cells with a DNA content less than a value midway between 1C and 2C. The mitotic index was determined by counting the percentage of anaphase cells (cells with two nuclei and without a septum) after DAPI staining.

Cell size measurements were made with the use of the forward light scatter (FSC) data of the FACS, considering 100 as the size of the wild type, *wee1-50* at 25°C, or *wee1-50* at 36°C.

Protein Extracts and Western Blots

Total protein extracts were prepared from 3×10^8 cells collected by centrifugation, washed in Stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN₃, pH 8.0), and resuspended in 25 μ l of RIPA buffer (10 mM sodium phosphate, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, 150 mM NaCl, pH 7.0) containing the following protease inhibitors: 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 100 μ M PMSF. Cells were boiled for 5 min and broken with the use of 750 mg of glass beads (0.4 mm; Sigma, St. Louis, MO) for 15 s in a Fast-Prep machine (Savant Instruments, Hollbrook, NY), and the crude extract was recovered by washing with 0.5 ml of RIPA buffer.

For Western blots, 100 μ g of total protein extract was run on a 14% SDS-PAGE gel, transferred to nitrocellulose, and probed with affinity-purified SP4 anti-cdc13 (1:250), PN24 anti-cdc2 (1:250), or R4 anti-rum1 (1:50) polyclonal antibodies. Goat anti-rabbit conjugated to HRP (1:3500) was used as a secondary antibody. Mouse TAT1 anti-tubulin mAbs (1:500) and goat anti-mouse conjugated to HRP (1:2000) as a secondary antibody was used to detect tubulin as a loading control. Immunoblots were developed with the use of the ECL kit (Amersham, Arlington Heights, IL).

Cdc2 kinase assays and rum1 inhibition assays were performed with the use of the protocols described by Benito *et al.* (1998). In the experiment shown in Figure 3B, extracts were immunoprecipitated with SP4 anti-cdc13 antibodies and assayed according to Benito *et al.* (1998).

Rum1 Inhibition Assays

Extracts from 3×10^8 cells prepared according to Benito *et al.* (1998) were spun at 4°C in a microfuge for 5 min, and the protein concentration was determined by the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Samples of 0.5 mg each were immunoprecipitated at 0°C for 1 h with the use of 2 μ l of C2 anti-cdc2, 2 μ l of 9830-U anti-cig1, or 2 μ l of anti-puc1 polyclonal antibodies. Thirty microliters of protein A-Sepharose was then added for 30 min at 4°C, and the immunoprecipitates were washed three times with 1 ml of homogenizing buffer (Moreno *et al.*, 1991). Immunoprecipitates (~20 μ l) were preincubated with different concentrations of purified rum1 protein for 5 min, diluted with 20 μ l of homogenizing buffer containing 50 μ M ATP, 0.5 mg/ml substrate histone H1 (Calbiochem, La Jolla, CA), and 40 μ Ci/ml [γ -³²P]ATP, and incubated at 25°C for 30 min. The reactions were stopped with 40 μ l of 2 \times SDS-PAGE sample buffer and denatured at 100°C for 5 min, and samples were run on a 14% SDS-PAGE gel. Phosphorylated proteins were detected by autoradiography.

RNA Preparation and Northern Blots

RNA from cells was prepared by glass bead lysis in the presence of phenol. RNA gels were run in the presence of formaldehyde, transferred to GeneScreen Plus (New England Nuclear, Boston, MA), and probed according to the manufacturer's instructions. Quantification of ³²P signals was performed with the use of a Fuji (Tokyo, Japan) PhosphorImager.

RESULTS

Identification of *puc1* as a G1 Cyclin

We decided to investigate the role of *puc1* in the fission yeast cell cycle by constructing strains lacking different combinations of *puc1*, *cig1*, and *cig2*. The three possible double mutant combinations and the triple mutant of *puc1*⁺, *cig1*⁺, and *cig2*⁺ genes were constructed by tetrad dissection in

wild-type and *wee1-50* backgrounds. All mutant strains were viable, supporting previous observations indicating that the only essential cyclin in fission yeast is *cdc13* (Fisher and Nurse, 1996; Mondesert *et al.*, 1996). Cultures of these mutants were grown in minimal medium at 25°C and analyzed by flow cytometry (Figure 1A). The *cig2* Δ *puc1* Δ double mutant and the *cig1* Δ *cig2* Δ *puc1* Δ triple mutants showed G1 populations of 6 and 17%, respectively (Figure 1, A and C). These populations increased to 20 and 40%, respectively, in a *wee1-50* background at the permissive temperature of 25°C (Figure 1, A and C). The *wee1* tyrosine kinase phosphorylates *cdc2*-Y15 and thereby delays mitosis until cells reach a critical size (reviewed by Nurse, 1990). The size of cells carrying a *wee1-50* mutation is normal at 25°C, but at 35°C the cells divide to a reduced size and the G1 phase is consequently extended until the minimal size needed to enter S phase has been achieved (Nurse, 1975). The phenotypes of the mutants were more dramatic when the *wee1-50* cells were incubated at the restrictive temperature of 35°C (Figure 1, B and C). The G1 delay is not due to an advancement into mitosis, as in the *wee* mutant, because all of the mutants were of equal size or larger than the corresponding control cells (wild type, *wee1-50* at 25°C, and *wee1-50* at 35°C) (Figure 1, B and D). Indeed, the size of the *wee1-50* *cig1* Δ *cig2* Δ *puc1* Δ cells at 35°C was similar to that of wild-type cells (Figure 1B). In the more extreme case, the triple mutant *cig1* Δ *cig2* Δ *puc1* Δ , cells were on average 15% larger than wild-type cells (Figure 1D). These results clearly indicate that *puc1*, like *cig1* and *cig2* cyclins, plays an active role in promoting progression through G1 in the fission yeast cell cycle.

Cells Lacking *cig1*, *cig2*, and *puc1* Are Hyperfertile

Next, we examined the behavior of the triple mutant *cig1* Δ *cig2* Δ *puc1* Δ in response to nitrogen starvation. Wild-type cells when starved for nitrogen undergo two divisions before arresting in G1. As shown in Figure 2A, accumulation of a high proportion of cells in G1 is observed 6 h after the shift to medium without nitrogen, which accounts for approximately two generations at 25°C. In the same experiment, the triple mutant *cig1* Δ *cig2* Δ *puc1* Δ underwent cell cycle arrest in G1 after a single cell division. In this mutant, >90% of the population was in G1 by 3 h and 100% was in G1 by 4 h after the shift to minimal medium without nitrogen (Figure 2A). When we used homothallic *h*⁹⁰ strains for this experiment, ~25% of the cells in the triple mutant *cig1* Δ *cig2* Δ *puc1* Δ had already started to conjugate after 4 h in minimal medium without nitrogen (Figure 2B). Under these conditions, wild-type cells have not yet started to conjugate. This result is consistent with the triple mutant strain being hyperfertile, as has been described previously for the *cig2*⁺ deletion (Obara-Ishihara and Okayama, 1994).

Rum1 Accumulates in the *cig1* Δ *cig2* Δ *puc1* Δ Mutant

When wild-type cells are starved for nitrogen, *rum1* protein accumulates (Figure 2C) (Kitamura *et al.*, 1998). Low levels of *rum1* protein are detectable 1 h after the shift to medium without nitrogen, and high levels are detectable after 6 h. In cells deleted for *cig1*⁺, *cig2*⁺, and *puc1*⁺, *rum1* protein is detectable even in exponentially growing cells (Figure 2C)

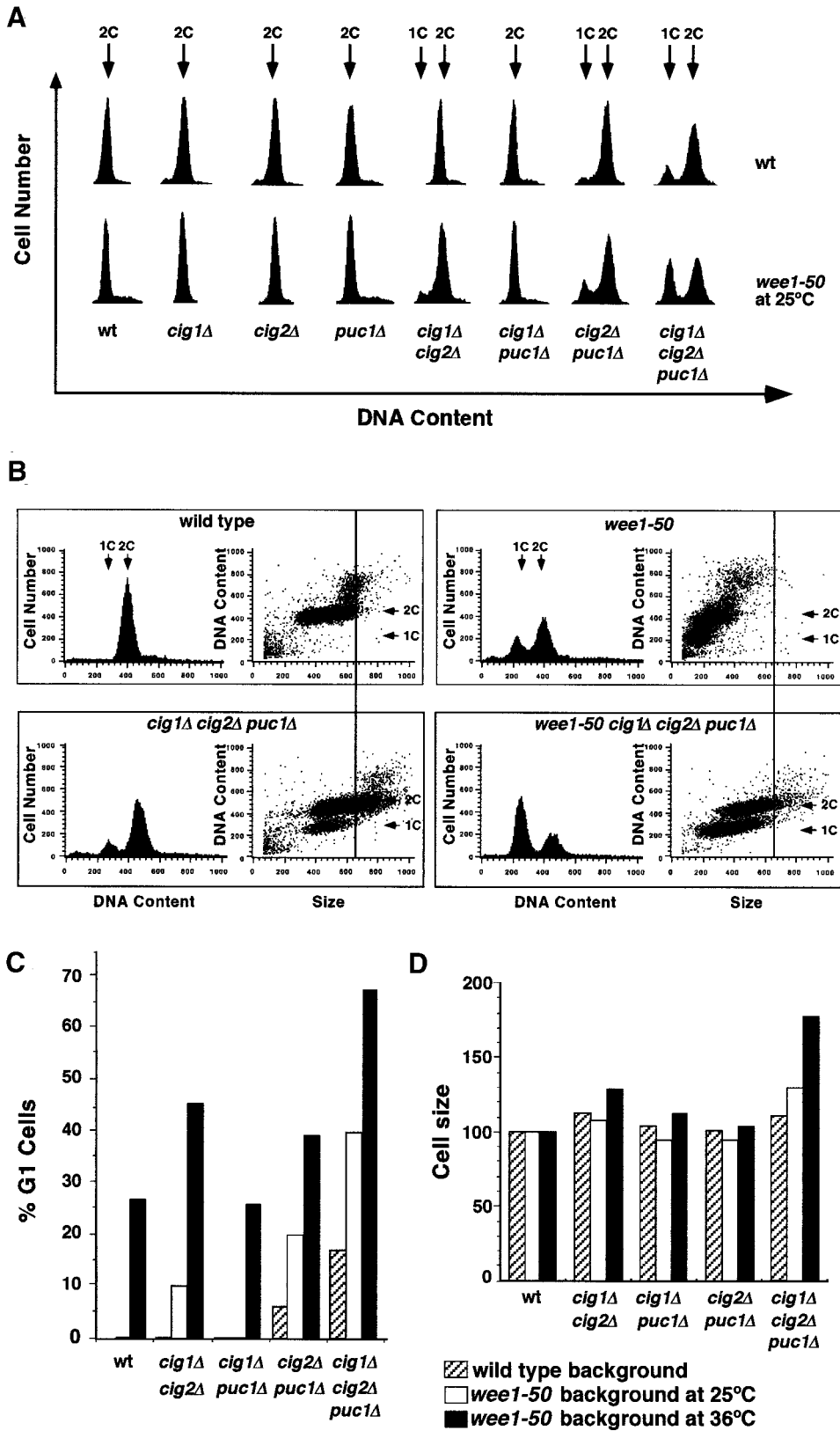


Figure 1. Deletion of *cig1*⁺, *cig2*⁺, and *puc1*⁺ causes a cell cycle delay in G1. (A) Flow cytometry analysis of the wild type and *cig1*, *cig2*, and *puc1* single, double, and triple mutants in wild-type and *wee1-50* backgrounds at 25°C. (B) Flow cytometry analysis of wild type, *cig1*Δ *cig2*Δ *puc1*Δ, *wee1-50*, and *wee1-50* *cig1*Δ *cig2*Δ *puc1*Δ at 35°C. For each strain, there is a histogram (left) and a dot-plot (right) representation of the data. The vertical lines in the dot plots correspond to the biggest cells in the wild-type culture. (C) Quantification of the data shown in A and B to indicate the percentage of cells in G1 in the different cyclin mutants. (D) Cell size of the different cyclin mutants in arbitrary units considering 100 as the size of the wild type, *wee1-50* at 25°C, or *wee1-50* at 36°C. Cells were grown in minimal medium to midexponential phase.

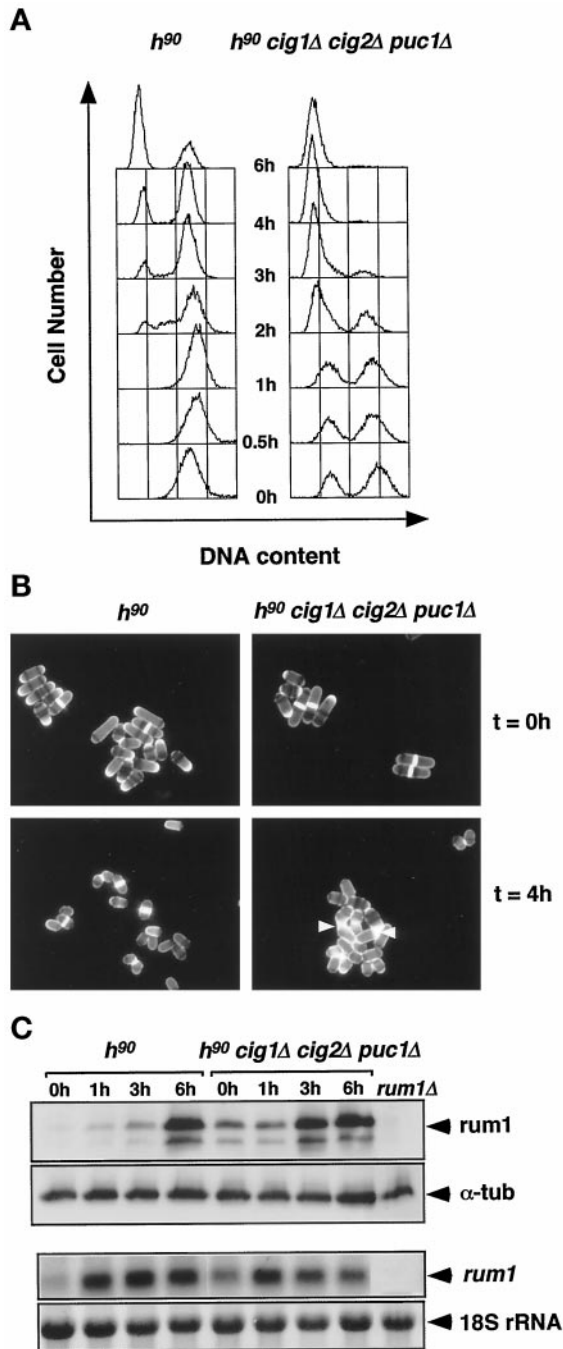


Figure 2. The *cig1Δ cig2Δ puc1Δ* mutant arrest in G1 after a single round of cell division in nitrogen-free medium. Wild-type and *cig1Δ cig2Δ puc1Δ* mutant cells grown at 25°C in minimal medium were centrifuged, washed four times in minimal medium without nitrogen, and resuspended in minimal medium without nitrogen at 25°C. Samples were taken for FACS analysis and protein and RNA preparation at the indicated times. (A) Flow cytometry analysis of *h⁹⁰* wild type and *h⁹⁰ cig1Δ cig2Δ puc1Δ* mutants after nitrogen starvation at 25°C. (B) DAPI and calcofluor staining of cells before (t = 0h) and 4 h after (t = 4h) nitrogen starvation. Arrowheads show cells undergoing conjugation. (C) Rum1 protein and mRNA levels. Anti-tubulin antibodies and 18S rRNA were used as protein- and RNA-loading controls.

and begins to accumulate to high levels after 3 h in minimal medium lacking nitrogen (Figure 2C). This accumulation of rum1 protein is due to posttranscriptional mechanisms, because no significant difference was detected in the levels of mRNA in the wild type versus the triple mutant (Figure 2C). The fact that rum1 levels are higher and accumulate earlier in the triple mutant than in wild-type cells may explain why these cells are delayed in G1 and why they arrest more readily in G1.

Rum1 Is Still Degraded in cig1Δ cig2Δ puc1Δ Cells

Rum1 protein is unstable during most of the cell cycle; it becomes stabilized from anaphase until the end of G1 (Correa-Bordes and Nurse, 1995; Benito *et al.*, 1998). Phosphorylation of rum1 by cdc2/cyclin complexes at residues T58 and T62 is the signal that targets its degradation through the SCF^{POP1/POP2} ubiquitin-dependent proteolytic pathway (Kominami and Toda, 1997; Benito *et al.*, 1998; Jallepalli *et al.*, 1998; Kominami *et al.*, 1998). Mutation of one or both of these residues to alanine causes stabilization of rum1 and induces a cell cycle delay in G1 (Benito *et al.*, 1998). To test whether rum1 protein is still degraded in the triple mutant *cig1Δ cig2Δ puc1Δ* as cells progress into S phase, we performed two experiments. First, the wild-type strain and the *cig1Δ cig2Δ puc1Δ* mutant strain were nitrogen starved for 8 h at 25°C, and nitrogen was then added back so that cells would resume growth. In both wild-type and *cig1Δ cig2Δ puc1Δ* cells, rum1 protein levels increased as the cells arrested in G1 (Figure 3A, compare +N and time 0). When nitrogen was added to the cultures, rum1 levels decreased as cells progressed through S phase (Figure 3, A and C). The decrease in rum1 levels occurred 2–3 h after the release in the wild-type strain and 3–5 h after the release in the triple mutant (Figure 3A). This experiment indicates that rum1 is still degraded in the absence of *cig1*, *cig2*, and *puc1*.

The timing of cdc13 protein accumulation was delayed only slightly in the triple mutant compared with the wild-type control (Figure 3A). There was also a lower level of cdc2/cdc13 kinase activity in cells arrested in G1 and a small delay in the activation of this kinase complex after the addition of nitrogen to the triple mutant compared with wild-type cells (Figure 3B). Interestingly, cells arrested in G1 in the *cig1Δ cig2Δ puc1Δ* triple mutant were approximately twice the size of wild-type cells (Figure 3C, time 0). Even with this larger cell size, the triple mutant *cig1Δ cig2Δ puc1Δ* has to grow to approximately 2.5 times the size of the wild type before it undergoes S phase (Figure 3C). This result clearly shows that in the triple cyclin mutant the G1/S transition is delayed and the cell size at which these cells undergo S phase increases. This is in good agreement with the prediction made recently by Novak *et al.* (1998) with the use of a mathematical model of the fission yeast cell cycle (see Table 3 in that paper).

To confirm by an independent method that the rum1 levels decrease as cells undergo S phase in the absence of *cig1*, *cig2*, and *puc1*, we determined the levels of rum1 in synchronous cultures of the triple mutant generated by centrifugal elutriation. When a *wee1-50 cig1Δ cig2Δ puc1Δ* mutant is grown in minimal medium at 25°C, 40% of the population is in G1 (Figure 1, A and C, and Figure 4A, Async.). Small cells in early G1 were selected by elutriation and incubated at 25°C for one cell cycle. Cell cycle position was

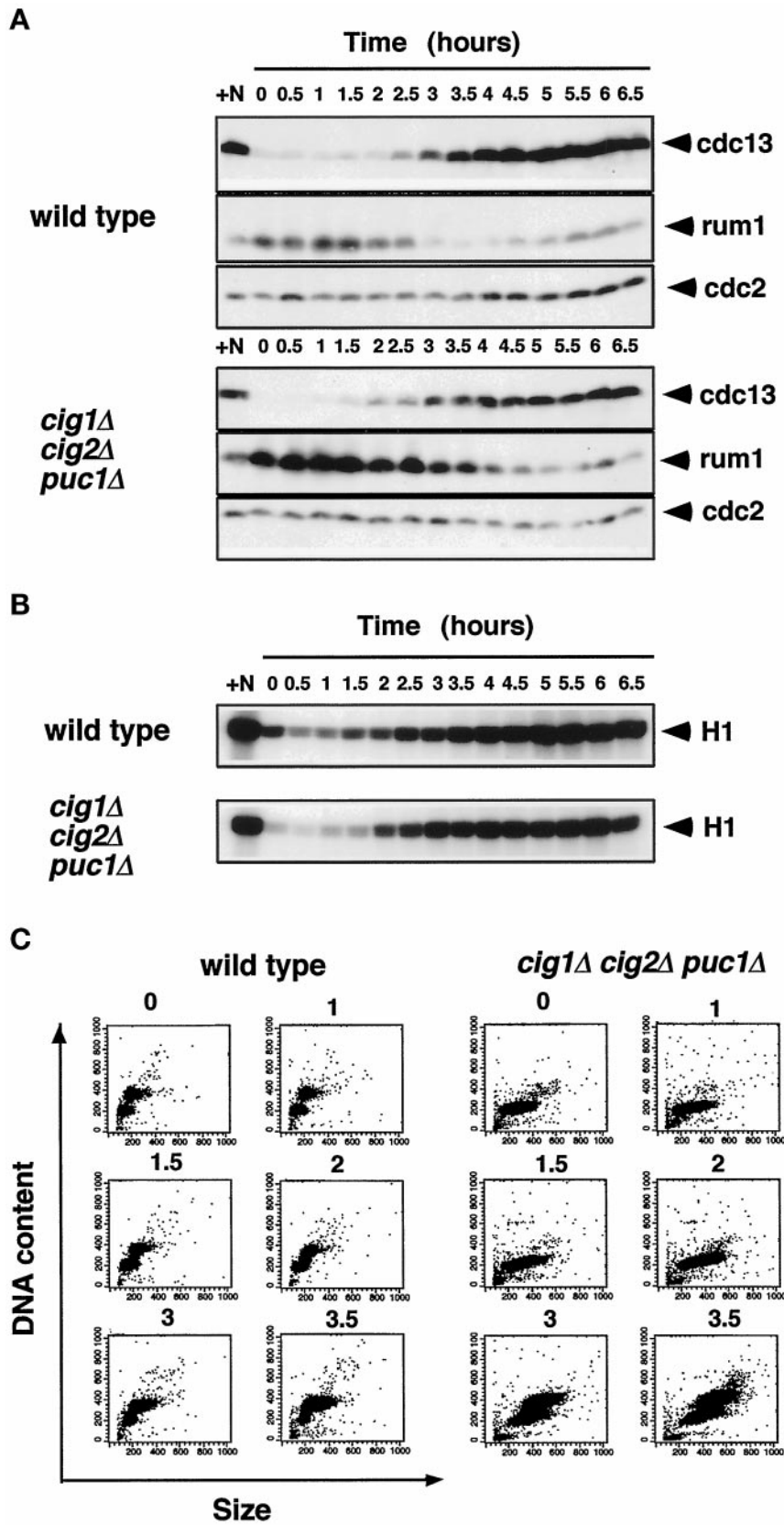


Figure 3. Rum1 protein persists for longer in the *cig1Δ cig2Δ puc1Δ* mutant than in the wild type. Wild-type and *cig1Δ cig2Δ puc1Δ* mutant cells were nitrogen starved for 8 h, and then nitrogen was added back to the culture. Samples were taken for protein extracts and flow cytometry at the indicated times. (A) Cdc13, rum1, and cdc2 protein levels in wild-type and *cig1Δ cig2Δ puc1Δ* cells. +N corresponds to cells growing in minimal medium. (B) Cdc2/cdc13 kinase assays. Cdc2/cdc13 complexes were immunoprecipitated with anti-cdc13 antibodies and assayed with the use of histone H1 as substrate. (C) Flow cytometry analysis of wild-type and *cig1Δ cig2Δ puc1Δ* mutant cells during the release from nitrogen starvation.

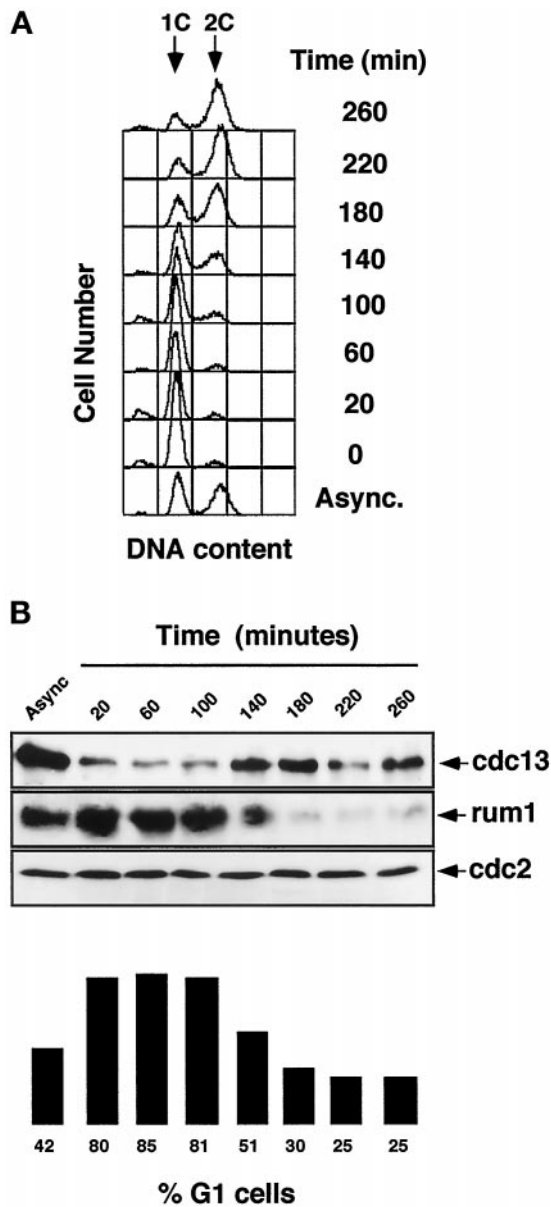


Figure 4. Rum1 protein levels oscillate throughout the cell cycle in cells lacking *cig1*, *cig2*, and *puc1*. *wee1-50 cig1Δ cig2Δ puc1Δ* mutant cells growing in minimal medium at 25°C (Async.) were synchronized by elutriation. A homogenous G1 population was selected and incubated at 25°C. Samples were taken every 40 min for protein extracts and FACS analysis. (A) Flow cytometry analysis. (B) Cdc13, rum1, and cdc2 protein levels throughout the cell cycle.

determined by flow cytometry (Figure 4A). Protein extracts were prepared every 40 min, and rum1, cdc13, and cdc2 protein levels were measured by Western blotting with the use of anti-rum1, anti-cdc13, and anti-cdc2 affinity-purified polyclonal antibodies (Figure 4B). Rum1 protein levels were high in G1 cells and decreased as cells entered S phase (Figure 4, A and B). It took >100 min for these cells to initiate S phase after the elutriation. In the same experiment, cdc13

levels were exactly the opposite of those of rum1. Cdc13 levels were low in G1 cells and increased at ~140 min at the onset of DNA replication (Figure 4, A and B). These two experiments confirm that in the absence of *cig1*, *cig2*, and *puc1* cyclins, rum1 is still down-regulated during S phase and G2, suggesting that another kinase is able to phosphorylate and promote the degradation of rum1.

Cdc2/puc1 Kinase Can Phosphorylate rum1 and Is Insensitive to rum1 Inhibition

Cdc2/cig1 and *cdc2/puc1* kinase complexes can efficiently phosphorylate rum1 in vitro at residues T58 and T62 (Benito *et al.*, 1998) (Figure 5A). Phosphorylation of rum1 by immunocomplexes of cdc2, *cig1*, or *puc1* induced a mobility shift from 34 to 36 kDa (Benito *et al.*, 1998) (Figure 5A). This shift in mobility was not observed when we used the mutant rum1-A58A62, which lacks the T58 and T62 cdc2 phosphorylation sites, as a substrate. *Cdc2/cig1* and *cdc2/puc1* kinase complexes were also resistant to rum1 inhibition. Different amounts of purified rum1 protein were added to cdc13, *cig2*, *cig1*, and *puc1* immunoprecipitates, and protein kinase activity was assayed with the use of histone H1 as a substrate (Figure 5B). Rum1 was able to inhibit the cdc13 and almost completely inhibited the *cig2*-associated H1 kinase activity at a concentration of 10 nM. In contrast, *cdc2/cig1* kinase and *cdc2/puc1* activity were not significantly inhibited (Benito *et al.*, 1998) (Figure 5B). These results suggest that *cdc2/cig1* and *cdc2/puc1* complexes, which are insensitive to rum1 inhibition, may be involved in the phosphorylation of rum1.

Deletion of *rum1*⁺ Suppresses the *cig1Δ cig2Δ puc1Δ* Mutant Phenotype

Phosphorylation of rum1 by *cdc2/cyclin* complexes at residues T58 and T62 targets the protein for degradation (Benito *et al.*, 1998). This relieves the effect of rum1 inhibition over *cdc2/cig2* and *cdc2/cdc13* and ensures that rum1 is absent in S phase and G2. High levels of rum1 protein in the triple mutant *cig1Δ cig2Δ puc1Δ* could inhibit *cdc2/cdc13* kinase activity and, as a consequence, cause a delay in the G1 phase of the cell cycle. To investigate if the reason for the G1 delay in these cells was the presence of high levels of rum1, we deleted the *rum1*⁺ gene in *cig1Δ cig2Δ puc1Δ* and in *wee1-50 cig1Δ cig2Δ puc1Δ*. As shown in Figure 6, deletion of *rum1*⁺ completely abolished the G1 population. The quadruple mutant *cig1Δ cig2Δ puc1Δ rum1Δ* behaves essentially like *rum1Δ*. These cells were wild type in size and sterile, like *rum1Δ* (data not shown) (Moreno and Nurse, 1994). In addition, *wee1-50 cig1Δ cig2Δ puc1Δ rum1Δ* cells at 25°C did not show any cells in G1 (Figure 6), and these cells died at 36°C with a phenotype identical to that of *wee1-50 rum1Δ*, consisting of very small cells unable to coordinate cell size with the cell cycle (Moreno and Nurse, 1994; Sveiczer *et al.*, 1996). These results indicate that rum1 protein prevents premature entry into S phase in cells lacking *cig1*, *cig2*, and *puc1*, presumably by inhibiting the *cdc2/cdc13* kinase activity and causing the delay in G1.

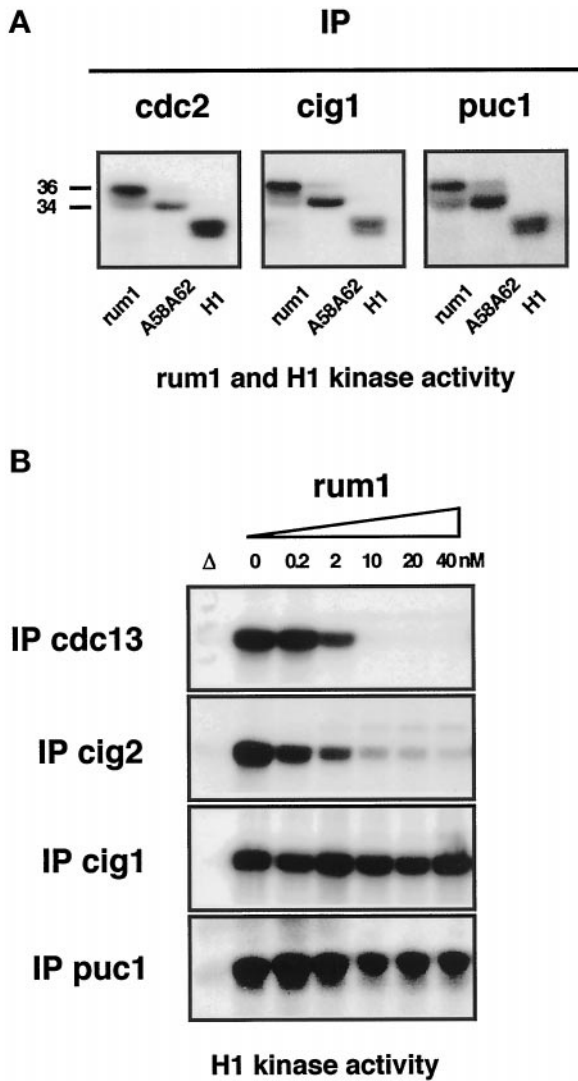


Figure 5. The cdc2/puc1 kinase can efficiently phosphorylate rum1 in vitro and is not inhibited by rum1. (A) Wild-type cells were grown to midexponential phase in minimal medium. Two milligrams of total protein extracts were immunoprecipitated with anti-cdc2, anti-cig1, and anti-puc1 antibodies. Protein kinase activity was measured with the use of rum1, rum1-A58A62 (A58A62), and histone H1 (H1) as substrates. The phosphorylated products were separated by 14% SDS-PAGE and exposed to autoradiography. Cdc2 immunocomplexes could phosphorylate p25^{rum1} as efficiently as they could phosphorylate histone H1. Rum1 phosphorylation induced a band shift from 34 to 36 kDa that was not observed in the mutant rum1-A58A62. Cig1 and puc1 immunocomplexes induced a similar band shift to cdc2. (B) Wild-type fission yeast extracts were immunoprecipitated with anti-cdc13, anti-cig2, anti-cig1, and anti-puc1 antibodies. The immunoprecipitates were preincubated with different concentrations of rum1 protein and then assayed for histone H1 kinase activity. As negative controls (Δ), extracts of *cig1Δ*, *cig2Δ*, and *puc1Δ* were immunoprecipitated with anti-cig1, anti-cig2, and anti-puc1 antibodies and assayed for histone H1 kinase activity. IP, immunoprecipitate.

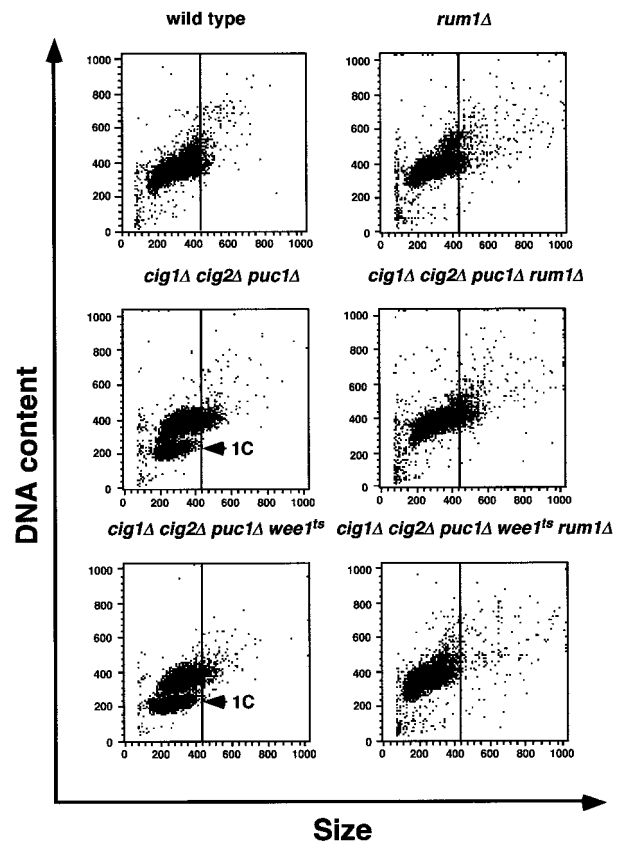


Figure 6. Deletion of the *rum1⁺* gene suppresses the G1-delay phenotype of *cig1Δ cig2Δ puc1Δ* mutant cells. The indicated strains were grown in minimal medium to midexponential phase at 25°C. Samples were taken to determine the cell cycle distribution by flow cytometry.

Cell Cycle Distribution under Nitrogen-limiting Conditions

S. pombe cells have a very short G1 under normal laboratory growth conditions. We used the nitrogen-limiting growth media described by Fantes and Nurse (1977) to study the cell cycle distribution of the different strains constructed in this work. Cells were grown to midexponential phase at 25°C in minimal medium containing 20 mM NH₄Cl supplemented with 0.5% yeast extract (medium 3 as described by Fantes and Nurse, 1977) and shifted to minimal medium containing 20 mM L-proline instead of NH₄Cl as nitrogen a source (medium 6 as described by Fantes and Nurse, 1977). This nutritional shift-down experiment resets the G2/M size control, and as a consequence, cells are advanced into mitosis and cell division (Fantes and Nurse, 1977). In these conditions, wild-type cells have an elongated G1 (Fantes and Nurse, 1977; Rhind and Russell, 1998; Carlson *et al.*, 1999) (Figure 7). This G1 population was absent in cells deleted for the *rum1⁺* gene and was more prominent in *cig2Δ* and *puc1Δ* single and double mutants (Figure 7). In the triple mutant *cig1Δ cig2Δ puc1Δ*, the percentage of cells in G1 increased to 80%. Once again, in the quadruple mutant *cig1Δ cig2Δ puc1Δ rum1Δ*, the G1 population was not observed (Figure 7). This

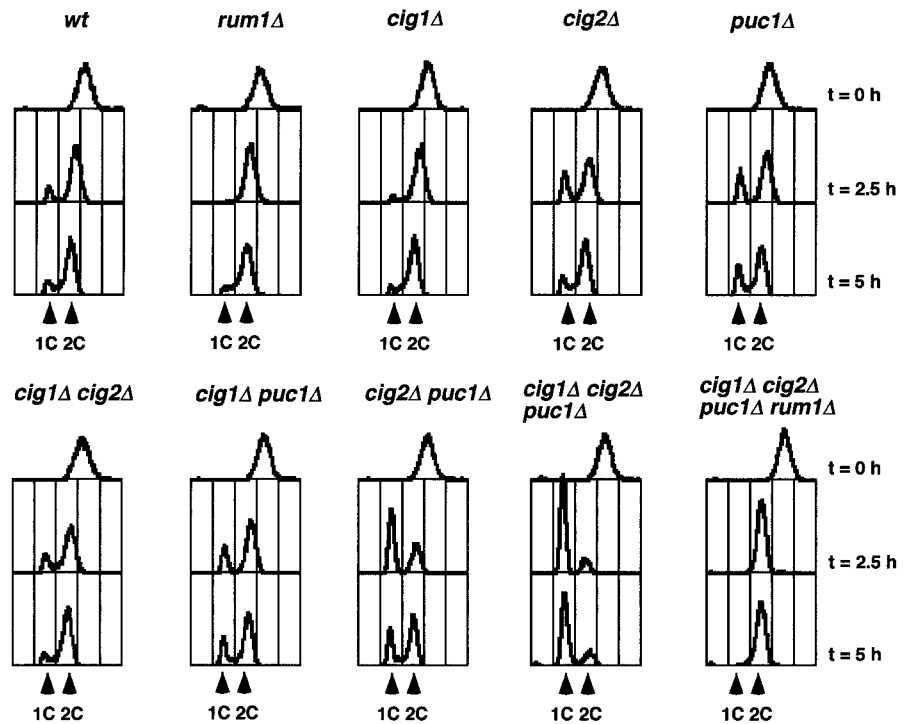


Figure 7. Cells lacking *rum1* do not delay progression through G1 upon nutritional shift-down experiments. Asynchronous cultures of the indicated strains were grown at 25°C in minimal medium containing 20 mM NH_4Cl supplemented with 0.5% yeast extract and then shifted to minimal medium containing 20 mM L-proline instead of NH_4Cl as a nitrogen source. A small population of G1 cells was detected in the wild type and the *cig1*⁺ deletion upon the shift. This population increased in cells deleted for *cig2*⁺ and *puc1*⁺. In the triple mutant *cig1Δ cig2Δ puc1Δ*, the percentage of cells in G1 increased to 80%. Deletion of *rum1*⁺ completely abolished this G1 population.

experiment suggests that the role of *puc1* and *cig2* in promoting G1 progression becomes more important when cells are growing under poor nutritional conditions and that even in the absence of the three cyclins (*cig1*, *cig2*, and *puc1*), *rum1* is essential to down-regulate the *cdc2/cdc13* kinase activity in G1.

DISCUSSION

There are two points in the cell cycle at which fission yeast coordinates cell size with the cell cycle. The first one operates at the end of G1 (G1/S size control) and the second operates at the end of G2 (G2/M size control or mitotic size control) (Fantes, 1977; Fantes and Nurse, 1977; Nurse and Thuriaux, 1977). More recently, Sveiczler *et al.*, (1996) proposed that the G2/M size control consists of a sizing mechanism (which normally is achieved in mid G2) and a timing mechanism (which is achieved at the end of G2). In wild-type cells, the mitotic size control is operational but the G1/S control is cryptic, because cells that complete mitosis are larger than the critical size for this control. In this report, we describe a fission yeast mutant in which the G1/S transition is delayed and the main point in the cell cycle at which coordination of size and division occurs is at the end of G1.

We have found that fission yeast cells lacking the three cyclins *cig1*, *cig2*, and *puc1* are perfectly viable; they mate to form zygotes that can undergo meiosis and sporulation. The resulting spores can germinate to give rise to colonies. These cells are 15% larger than wild-type cells, they are severely delayed in the G1 phase of the cell cycle, and they are hyperfertile. The latter phenotype has already been described for cells deleted for the *cig2*⁺ gene (Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994), suggest-

ing that *cdc2/cig2* may act as a negative regulator of mating. When cells of the triple mutant *cig1Δ cig2Δ puc1Δ* are starved for nitrogen, they complete the cell cycle they are in, accumulate in the G1 phase of the subsequent cell cycle, and almost immediately initiate conjugation. Wild-type cells normally divide twice before they arrest in G1. Because there is very little cell growth in minimal medium lacking nitrogen, cells of the triple mutant arrest in G1 with approximately twice the size of wild-type cells (Figures 2A and 3C). Upon refeeding with nitrogen, the triple cyclin mutant initiated S phase with more than twice the size of wild-type cells.

Fission yeast cells lacking *cig1* and *cig2* do not show a significant delay in G1 (Fisher and Nurse, 1996; Mondesert *et al.*, 1996) (Figure 1). Deletion of *puc1*⁺ in this genetic background generates a considerable G1 delay. This is the first demonstration of a role for *puc1* cyclin in G1. A previous report has failed to show that *puc1* functions in the mitotic cell cycle (Forsburg and Nurse, 1994). This conclusion was drawn because the *puc1Δ* single mutant and the double mutant with *cig1Δ* do not show any mitotic phenotypes. In this report, we have shown that *puc1* is required during G1 in cells lacking *cig1* and *cig2*. Therefore, we believe that *puc1* functions as a G1-specific cyclin analogous to budding yeast Cln cyclins and animal cell D-type cyclins (Nasmyth, 1993, 1996; Sherr, 1993). The *cdc2/puc1* kinase complex may act as a G1 kinase, probably phosphorylating and inactivating *rum1* and *ste9*-APC. Indeed, we have data showing that *cdc2/puc1* can efficiently phosphorylate *rum1* in vitro at residues T58 and T62 (Figure 5A), which are the two sites that are phosphorylated in vivo before *rum1* is recognized by SCF^{POP1} and is degraded (Kominami and Toda, 1997; Benito *et al.*, 1998). In addition, we have found that *cdc2/*

puc1 kinase activity is resistant to *rum1* inhibition (Figure 5B), showing that it is highly suited to act as a link between the achievement of a critical cell size and the release of other *cdc2/cyclin* complexes from *rum1* inhibition. If *puc1* is missing, then *cdc2/cdc13* must phosphorylate *rum1* itself. Because *cdc2/cdc13* complexes are inhibited by *rum1* in G1, the G1/S transition is delayed and the cell size at which these cells undergo S phase increases. As mentioned above, the triple mutant division size is 15% larger than that of the wild type. This means that in *cig1Δ cig2Δ puc1Δ* cells, the coordination of size and the cell cycle occurs at the end of G1 and that the minimal size requirement for the G1/S transition is larger than the size requirement for G2/M. Hence, these cells spend very little time in G2 and probably have a cryptic G2/M size control.

The *rum1* protein is more abundant in the triple cyclin mutant than in wild-type cells (Figure 2C). This is similar to the situation in cells expressing a nondegradable *rum1-A58A62* mutant (Benito *et al.*, 1998). In this strain, *rum1* levels are high and constant throughout the cell cycle (Benito *et al.*, 1998). As a consequence, cells expressing *rum1-A58A62* suffer a delay in G1. The fact that in the *cig1Δ cig2Δ puc1Δ* mutant *rum1* protein still oscillates during the cell cycle suggests that it is still targeted by phosphorylation for degradation from the end of G1 until mitosis. At present, the kinases responsible for targeting *rum1* for degradation in the absence of *cig1*, *cig2*, and *puc1* remain to be identified. Although *cdc2/cdc13* may phosphorylate *rum1* once it becomes activated in late G1, it is also possible that another *cdc2/cyclin* activity remains to be identified, one that relieves *rum1* inhibition of *cdc2/cdc13* at the end of G1. In a fission yeast cell lacking *puc1*, *cig1*, and *cig2*, it is possible that another such G1 cyclin eventually accumulates, one that is resistant to *rum1* inhibition and so is able to relieve *rum1* inhibition of *cdc2/cdc13*. Whether such a cyclin exists, and whether its transcription is normally promoted by *puc1*, remain to be determined. Alternatively, *cdc2/cdc13* may eventually be able to overcome *rum1* and *ste9-APC* inhibition in the absence of any other CDK activity (see Novak *et al.*, 1998, for a mathematical model). This would be similar to *cdk2/cycE* and *p27* in animal cells, in which *p27* is both an inhibitor and a substrate of *cdk2/cycE* (Sheaff *et al.*, 1997).

There is one important difference between *puc1* and the other cyclins. Whereas *cig1*, *cig2*, and *cdc13* may regulate *rum1/ste9* and promote S phase (probably by triggering the firing of origins of replication), *puc1* is likely to allow G1 progression but cannot promote entry into S phase. Only B-cyclins can do this, and *puc1* does not cause S-phase entry in the *cig1Δ cig2Δ cdc13Δ* mutant (Fisher and Nurse, 1996; Mondesert *et al.*, 1996).

Is the accumulation of the *rum1* Cdk inhibitor the main cause for the delay in G1? To test this idea, we generated a strain lacking the three cyclins plus *rum1*. In this quadruple mutant (*cig1Δ cig2Δ puc1Δ rum1Δ*), the *cdc2/cdc13* kinase complex should be more active and the G1/S transition should be controlled by the interaction between *cdc2/cdc13* and *ste9-APC*. Cells of the quadruple mutant have a phenotype very similar to that of *rum1Δ* cells (Moreno and Nurse, 1994). They are wild type in size, unable to arrest in G1 when starved for nitrogen, and sterile. A quintuple *cig1Δ cig2Δ puc1Δ rum1Δ wee1-50* mutant showed a phenotype virtually identical to that of *rum1Δ wee1-50*, consisting of cells of

wild-type size at 25°C and very small cells at the restrictive temperature of 35°C, with no size control in either G1 or G2 (Moreno and Nurse, 1994; Sveiczzer *et al.*, 1996). There is a situation similar to this in the budding yeast *S. cerevisiae*, in which cells deleted for *CLN1*, *CLN2*, and *CLN3* arrest the cell cycle at the end of G1. Deletion of the Cdk inhibitor *Sic1* rescues the lethal phenotype of this strain (Schneider *et al.*, 1996; Tyers, 1996). *Sic1* in budding yeast is a functional homologue of fission yeast *rum1* (Sánchez-Díaz *et al.*, 1998). In addition, cells deleted for *SIC1* are partially resistant to mating pheromone (Tyers, 1996). We believe that the control of the length of G1 in fission and budding yeast is more similar than previously thought. In both yeasts, entry into S phase requires the activity of at least one S-phase-promoting *cdc2 (Cdc28)/B-type cyclin kinase* complex. These complexes are assembled in G1, but they are initially inactive as a result of the presence of high levels of the Cdk inhibitor *rum1 (Sic1)*. At the G1/S transition, *rum1 (Sic1)* is degraded and the liberated *cdc2 (Cdc28)/B* cyclin kinase complexes induce DNA synthesis. The main role of *cdc2/puc1 (Cdc28/Cln)* activity is to phosphorylate *rum1 (Sic1)*, which is the signal that triggers its ubiquitination and degradation by the SCF^{POP1/POP2} (SCF^{Cdc4})-proteasome pathway (see Hoyt, 1997, for a review). However, the situation might not be absolutely identical for the two yeasts because there are other functions of the *Cdc28/Cln* cyclins, such as the regulation of SBF- and MBF-dependent transcription (Tyers *et al.*, 1993; Dirick *et al.*, 1995; Stuart and Wittenberg, 1995; Levine *et al.*, 1996), which in fission yeast have been shown to be independent of *cdc2* activity (Baum *et al.*, 1997).

Physiological Implications

Why do fission yeast cells need *cig1*, *cig2*, and *puc1* cyclins and the Cdk inhibitor *rum1* if they are perfectly viable without them, at least under laboratory growth conditions? We can imagine two possible explanations. First, *S. pombe* is normally a haploid organism. Haploid cells are vulnerable during the G1 phase of the cell cycle because they do not have a homologous chromosome with which to repair possible damage in the DNA. By shortening G1 and controlling the cell cycle at G2/M, fission yeast seems to have solved this problem (see Nasmyth *et al.*, 1991; Rhind and Russell, 1998, for a similar discussion). For this reason, the presence of *cig1*, *cig2*, and *puc1* in G1 will contribute to minimizing the time that they spend in G1. This may be particularly important when fission yeast cells are growing under poor nutritional conditions (Figure 7) (Rhind and Russell, 1998; Carlson *et al.*, 1999), which is likely to be a very frequent situation in nature. A second possibility is that fission yeast cells depend on mating for survival. Yeasts of the genus *Schizosaccharomyces* are homothallic (Leupold, 1950; Egel, 1989), which means that they undergo frequent switching of mating type to generate a mixture of *h⁻* and *h⁺* cells (Egel, 1989). Under favorable conditions, fission yeast cells reproduce asexually by means of the mitotic cell cycle. When they experience starvation, they arrest in G1 and the mating process begins by the formation of zygotes that normally undergo meiosis and sporulation to give four spore asci. Haploid spores remain dormant until they encounter favorable growth conditions. Therefore, it seems logical that fission yeast cells need a system to carefully time the start of sexual development, when nutrients become limiting. If cells

conjugate while nutrients are still available, they will proliferate to a lesser degree. This is the case for a *cig1Δ cig2Δ puc1Δ* triple mutant that is derepressed for mating. If cells do not undergo conjugation even after complete nutrient depletion, as is the case for the *rum1⁺* deletion, they lose the ability to survive adverse conditions by forming spores. The presence of a control system involving positive (*cig1*, *cig2*, and *puc1*) and negative (*rum1* and *ste9*-APC) regulators of G1 progression may constitute a sophisticated mechanism by which the optimal time for conjugation is determined.

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