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 (Fantes 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 (Sigrist 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-
related 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) 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 (Booher et al., 1996; Mondesert et al., 1996). The *cdc2/cig2* gene is deleted. 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., 1996). The resulting plasmid was digested with *NdeI* and the purified fragment was used to transform the wild-type, the *cig1Δ cig2Δ puc1Δ*, and the *weel-50 cig1Δ cig2Δ puc1Δ* strains. Transformants containing the *rum1Δ* 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 × 10^6 cells/ml, corresponding to midexponential phase.

### Synchronous Cultures

*h*^-^*weel-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 10^5 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) FACSscan 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.

### 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 KanMX 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 *weel-50 cig1Δ cig2Δ puc1Δ* strains. Transformants containing the *rum1Δ* gene deleted were selected in medium containing G418 and confirmed by Southern blotting.

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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 $3 \times 10^8$ cells collected by centrifugation, washed in Stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN$_3$, pH 8.0), and resuspended in 25 μl 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 μg/ml leupeptin, 10 μg/ml aprotonin, and 100 μM 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, Holbrook, NY), and the crude extract was recovered by washing with 0.5 ml of RIPA buffer.

For Western blots, 100 μg of total protein extract was run on a 14% SDS-PAGE gel, transferred to nitrocellulose, and probed with affinity-purified SP4 anti-cdc13 antibodies and assayed according to Benito et al. (1991), with the use of the protocols described by Benito et al. (1996; 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 cig1Δ puc1Δ double mutant and the cig1Δ cig2Δ puc1Δ 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 phosphorylatescdc2-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 wee1 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 cig1Δ cig2Δ puc1Δ cells at 35°C was similar to that of wild-type cells (Figure 1B). In the more extreme case, the triple mutant cig1Δ cig2Δ puc1Δ, 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.

**Rum1 Inhibition Assays**

Extracts from $3 \times 10^8$ 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 μl of C2 anti-cdc2, 2 μl of 9830-U anti-cig1, or 2 μl 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 μl) were preincubated with different concentrations of purified rum1 protein for 5 min, with diluted 20 μl of homogenizing buffer containing 50 μM ATP, 0.5 mg/ml substrate histone H1 (Calbiochem, La Jolla, CA), and 40 μCi/ml [$^{32}$P]ATP, and incubated at 25°C for 30 min. The reactions were stopped with 40 μl of 2X 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 $^{32}$P 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 cig1Δ puc1Δ double mutant and the cig1Δ cig2Δ puc1Δ 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 wee1 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 cig1Δ cig2Δ puc1Δ cells at 35°C was similar to that of wild-type cells (Figure 1B). In the more extreme case, the triple mutant cig1Δ cig2Δ puc1Δ, 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 cig1Δ cig2Δ puc1Δ 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 cig1Δ cig2Δ puc1Δ 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 h90 strains for this experiment, ~25% of the cells in the triple mutant cig1Δ cig2Δ puc1Δ 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 cig1Δ cig2Δ puc1Δ 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, cig1Δ cig2Δ puc1Δ, wee1-50, and wee1-50 cig1Δ cig2Δ puc1Δ 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.
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 SCFpop1/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 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 synchronized 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
Rum1 protein persists for longer in the \( \text{cig1} \Delta \text{cig2} \Delta \text{puc1} \Delta \) mutant than in the wild type. Wild-type and \( \text{cig1} \Delta \text{cig2} \Delta \text{puc1} \Delta \) 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 \( \text{cig1} \Delta \text{cig2} \Delta \text{puc1} \Delta \) 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 \( \text{cig1} \Delta \text{cig2} \Delta \text{puc1} \Delta \) mutant cells during the release from nitrogen starvation.
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 weel-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, weel-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 weel-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.

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**Figure 4. Rum1 protein levels oscillate throughout the cell cycle in cells lacking cig1, cig2, and puc1. weel-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.**
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 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 (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 the double mutants (Figure 7). Once again, in the quadruple mutant *cig1Δ cig2Δ puc1Δ rum1Δ*, the G1 population was not observed (Figure 7). This

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**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*ΔA58A62 (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*mut1* 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*ΔA58A62. 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.

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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, Sveiczer 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 zygot es 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), suggesting 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 1A 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 puc1Δ 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 SCFP6op1 and is degraded (Kominami and Toda, 1997; Benito et al., 1998). In addition, we have found that cdc2/
pucl 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 pucl 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 pucl1Δ 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 pucl1Δ 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 pucl1 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 pucl1, 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 pucl1, 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 pucl1 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), pucl1 is likely to allow G1 progression but cannot promote entry into S phase. Only B-cyclins can do this, and pucl1 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 pucl1Δ 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 pucl1Δ 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; Sveiczer 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/pucl (Cdc28/Cln) activity is to phosphorylate rum1 (Sic1), which is the signal that triggers its ubiquitination and degradation by the SCFp30/p30 (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 pucl 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 pucl 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

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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 rum1Δ 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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