Regulation of the G₁ phase of the cell cycle by periodic stabilization and degradation of the p25rum1 CDK inhibitor

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Introduction

Eukaryotic cells co-ordinate cell growth with cell division at a point late in G₁ called Start in yeast and the restriction point in animal cells. Beyond this point, cells become committed to a new round of cell division (Hartwell et al., 1974; Pardee, 1974; Nurse, 1975). Genetic analysis in budding and fission yeast identified a single gene, CDC28/cdc2Δ, encoding the essential cyclin-dependent kinase (CDK) required not only for passage through Start but also to initiate mitosis (Nurse and Bisset, 1981; Piggott et al., 1982; Reed and Wittenberg, 1990). In the budding yeast Saccharomyces cerevisiae, nine different cyclins associate with Cdc28 (Nasmyth, 1993, 1996). Six B-type cyclins (Clb1–6) are required for S phase and mitosis and three G₁ cyclins (Cln1–3) are necessary for passage through Start. The three G₁ cyclins have different functions. Cln3 is needed to promote the activation of Start-dependent transcription factors (SBF and MBF), whereas Cln1 and Cln2 are required to trigger Start-regulated events such as acquisition of pheromone resistance, budding and spindle pole body duplication (Dirick et al., 1995; Stuart and Wittenberg, 1995). In the fission yeast Schizosaccharomyces pombe, four cyclins, puc1, cig1, cig2 and cdc13, can form complexes with cdc2 (reviewed in Fisher and Nurse, 1995). Cig1, cig2 and cdc13 are B-type cyclins that have been shown to play a role in regulating the cell cycle. Cig2 regulates the G₁–S transition (Obara-Ishihara and Okayama, 1994; Martin-Castellanos et al., 1996; Monderset et al., 1996), while cdc13 is the mitotic cyclin (Booher et al., 1989; Moreno et al., 1989). Cig1 has been argued to make a minor contribution to the onset of S phase, because cells deleted for cig2Δ and cdc13Δ can still undergo S phase but a triple deletion cig1Δ cig2Δ cdc13Δ blocks the cell cycle before the initiation of DNA replication (Fisher and Nurse, 1996; Monderset et al., 1996). The role of puc1 in the fission yeast mitotic cycle has not been clearly established (Forsburg and Nurse, 1991, 1994).

CDK inhibitors are negative regulators of CDK–cyclin complexes. Some CDK inhibitors, like budding yeast Far1, seem to respond to extracellular signals such as the presence of mating pheromones (Chang and Herskowitz, 1990; Peter et al., 1993; Peter and Herskowitz, 1994), but others, like budding yeast p40SIC1 (Donovan et al., 1994; Nugroho and Mendenhall, 1994; Schwoob et al., 1994; Schneider et al., 1996) or fission yeast p25rum1 (Moreno and Nurse, 1994), appear to be part of the intrinsic cell cycle machinery. While Far1 inhibits Cln-type cyclins, p40SIC1 and p25rum1 are specific inhibitors of B-type cyclins. P25rum1 inhibits the cdc2–cdc13 complex, preventing the activation of this mitotic complex in G₁ cells (Correa-Bordes and Nurse, 1995; Martin-Castellanos et al., 1996). P25rum1 is also an inhibitor of cig2-associated cdc2 kinase (Correa-Bordes and Nurse, 1995; Martin-Castellanos et al., 1996). It has been proposed that a transient inhibition of cdc2–cig2 complexes in G₁ is important in setting the minimum cell size required to pass Start (Labib and Moreno, 1996; Martin-Castellanos et al., 1996). Thus, p25rum1 plays a central role in the regulation of the fission yeast G₁ phase. It prevents the onset of mitosis in cells that have not initiated DNA replication and it determines the cell cycle timing of Start, maintaining cells in the pre-Start state until they have attained the minimal critical mass required to initiate the cell cycle.

Degradation of cyclins and CDK inhibitors is important for moving from one phase of the cell cycle to the next (reviewed by Deshaies, 1995). Degradation of mitotic cyclins depends on a sequence located near the amineterminus called the destruction box, which targets cyclins...
to the ubiquitin-dependent proteolytic pathway (Glotzer et al., 1991; Hershko et al., 1991). A multiprotein complex, called cyclosome or anaphase-promoting complex (APC), has been shown to contain the E3 ubiquitin–protein ligase activity that catalyses the ligation of multiple ubiquitin molecules to cyclins (Hershko et al., 1994; King et al., 1995; Sudakin et al., 1995). APC is a high molecular weight complex that contains the Cdc16, Cdc23 and Cdc27 protein members of the TPR family essential for the onset of anaphase (Iriniger et al., 1995; Tugendreich et al., 1995), highly conserved among eukaryotes (Hirano et al., 1988; O’Donnell et al., 1991; Mirabito and Morris, 1993; Samejima and Yanagida, 1994; King et al., 1995; Tugendreich et al., 1995). The fission yeast cut2 protein (a protein that might be involved in holding sister chromatids together until the onset of anaphase) also contains a destruction box that is recognized by APC in mitosis and destroyed through this proteolytic pathway (Funabiki et al., 1996).

Degradation of the mitotic cyclins occurs in an interval from anaphase until passage through Start or the restriction point in late G1 (Amon et al., 1994; Brandeis and Hunt, 1996). This prevents the accumulation of mitotic cyclins before the formation of the G1 complexes, and this period of low CDK activity is thought to be important for proper assembly of pre-initiation DNA replication complexes (Adachi and Laemmli, 1994; Dahmann et al., 1995; Nasmyth, 1996; Stern and Nurse, 1996; Wuarin and Nurse, 1996).

During the G1 phase, the right balance of cyclins and CDK inhibitors is necessary to produce a co-ordinate Start to the cell cycle. In budding yeast, G1 cyclins have very short half-lives and are destroyed as cells enter S phase. Degradation of the CDK inhibitor p40<sup>SC1</sup> is required for cells to initiate S phase (Schwob et al., 1994; Schneider et al., 1996). Proteolysis of G1 cyclins and of p40<sup>SC1</sup> also occurs through the ubiquitin-dependent proteolytic pathway and requires the participation of a ubiquitin–protein ligase complex, different from APC, formed by a multiprotein complex containing the product of the CDC34, CDC4, SKP1 and CDC53 genes (Schwob et al., 1994; Deshaies, 1995; Bai et al., 1996; Connelly and Hieter, 1996; Schneider et al., 1996; Willems et al., 1996; Verma et al., 1997). CDC34 encodes an E2 ubiquitin-conjugating enzyme (Goebl et al., 1988), CDC4 a protein with WD40 repeats (Yochem and Byers, 1987), SKP1 a protein that interacts with Cdc4 (Bai et al., 1996; Connelly and Hieter, 1996) and CDC53 a member of the cullin protein family conserved in yeast, Caenorhabditis elegans, Drosophila and humans (Kipreos et al., 1996; Mathias et al., 1996). Mutations in any of these genes lead to stabilization of p40<sup>SC1</sup> and G1 cyclins. G1 cyclins are unstable because they contain PEST sequences (Rogers et al., 1986). CDK phosphorylation of residues located near the PEST sequence seems to be the signal that targets Cln2 and Cln3 to degradation (Yaglom et al., 1995; Lanker et al., 1996).

CDK–cyclin complexes not only play positive roles in the cell cycle, determining when cells initiate S phase or mitosis, but are also required to inhibit the initiation of a unscheduled cell cycle event. In fission yeast, inactivation of the mitotic cdc2–cdc13 complex causes cells to undergo repeated rounds of DNA replication without intervening mitoses (Broek et al., 1991; Hayles et al., 1994; Fisher and Nurse, 1996). Budding yeast mutants with low levels of Cdc28–Cln5 kinase re-replicate (Dahmann et al., 1996), Drosophila mutants in cyclin A also undergo re-replication (Sauer et al., 1995) and, in starfish oocytes, cyclin B suppresses DNA replication between meiosis I and meiosis II (Adachi and Laemmli, 1994; Picard et al., 1996). These results suggest that CDK–cyclin complexes play a dual role in the cell cycle. They are required to promote the initiation of S phase and mitosis and, in addition, in G2 they prevent extra rounds of DNA replication within the same cell cycle (Nurse, 1994; Nasmyth, 1996; Stern and Nurse, 1996). In fission yeast, high level expression of the rum1<sup>+</sup> gene blocks mitosis but allows repeated rounds of DNA replication (Moreno and Nurse, 1994). This result is explained because p25<sup>rum1</sup> strongly inhibits thecdc2–cdc13 complex leading to a phenotype which is similar to the cdc13<sup>+</sup> deletion (Hayles et al., 1994). Thus, transit from G2 to G1 in fission yeast occurs by inactivation of the cdc2–cdc13 kinase complex, either by destruction of the mitotic cdc13 cyclin in mitosis, or by production of high levels of the p25<sup>rum1</sup> CDK inhibitor. According to this idea, the presence of cdc2–cdc13 activity defines a cell in G2, and destruction of this complex in mitosis resets the cell to G1, allowing a new S phase (Nurse, 1994; Stern and Nurse, 1996). Therefore, G1 is defined as a period of low CDK–cyclin activity prior to the initiation of a new cell cycle.

Here we have studied the regulation of the p25<sup>rum1</sup> protein. We have found that p25<sup>rum1</sup> levels sharply oscillate during the fission yeast cell cycle. This oscillation is due mainly to changes in protein stability, p25<sup>rum1</sup> is stabilized in G1, a period where there is low CDK–cyclin activity, and is destroyed as cells enter S phase, when CDK–cyclin activity begins to rise. We have also found that phosphorylation of p25<sup>rum1</sup> by cdc2–cyclin complexes at the end of G1 is important for targeting p25<sup>rum1</sup> for degradation.

**Results**

**p25<sup>rum1</sup> level oscillates through the cell cycle**

In order to study the regulation of the rum1<sup>+</sup> gene through the cell cycle, we determined the levels of p25<sup>rum1</sup> and of rum1<sup>+</sup> mRNA in synchronous cultures. Rapidly growing wild-type fission yeast cells have a very short G1 and lack the pre-Start G1 interval. Since rum1<sup>+</sup> function is required in G1, we used the temperature-sensitive mutant wee1-50 where the pre-Start G1 interval is extended when incubated at the restrictive temperature of 36°C (Nurse, 1975) (Figure 1A). Cells of the wee1-50 strain were grown at 25°C, and a synchronous culture was made using an elutriator rotor. Small cells in early G2 were selected and incubated at 36°C. These cells proceeded synchronously into mitosis. Cell cycle position and the degree of synchrony were monitored by determining the mitotic index and the percentage of cells in G1 after flow cytometry analysis, as a function of time. Protein extracts were prepared every 20 min for two cell cycles, and p25<sup>rum1</sup> levels were measured by Western blotting using an anti-p25<sup>rum1</sup> affinity-purified polyclonal antibody. p25<sup>rum1</sup> levels were sharply periodic, rising to a peak 40 min after the shift as cells were undergoing anaphase and decreasing at 100 min when the cells were exiting G1 (Figure 1B). In
**Fig. 1.** Periodicity of p25\textsuperscript{rum1} and rum1\textsuperscript{+} mRNA levels during the cell cycle. p25\textsuperscript{rum1} protein and rum1\textsuperscript{+} mRNA levels were measured in a synchronous culture of the temperature-sensitive wee1-50 strain. (A) Wild-type or wee1-50 cells at 25°C have a very short G1. wee1-50 cells at 36°C enter mitosis with a reduced cell size and, as a consequence, they have an extended G1 to meet the minimal cell size requirement to pass Start. (B) A homogeneous population of cells in early G2 was selected by elutriation of the wee1-50 strain at 25°C. This culture was incubated for 20 min at 25°C and then shifted up to 36°C. Samples were taken every 20 min to determine cdc13 and rum1 protein levels, the percentage of G1 cells and the mitotic index. rum1 and cdc13 protein levels were measured by Western blot using affinity-purified R3 anti-rum1 polyclonal antibody and affinity-purified SP4 anti-cdc13 polyclonal antibody, respectively. The percentage of G1 cells was measured by flow cytometry and the mitotic index by counting the number of cells in anaphase after DAPI staining (see Materials and methods). p25\textsuperscript{rum1} levels increase as cells undergo anaphase and decrease as cells enter S-phase. In contrast, levels of cdc13 decrease in anaphase and increase at the end of G1. We used a rum1Δ (Δ) and purified p25\textsuperscript{rum1} (rum1) as negative and positive controls. (C) rum1\textsuperscript{+} mRNA levels determined in a similar experiment also oscillate during the cell cycle. The time of the peak of transcription was 40 min earlier than the time of the peak of the protein. We observed a 10-fold oscillation in the levels of protein compared with a 2- to 3-fold in the level of the transcript. The blot was probed with ura4\textsuperscript{+} as a loading control and with cdc18\textsuperscript{+} as a gene known to be transcribed in G1/S (Kelly et al., 1993). rum1\textsuperscript{+} mRNA levels were normalized using the ura4\textsuperscript{+} gene.

In the same experiment, p56\textsuperscript{cdc13} levels were exactly the opposite to those of p25\textsuperscript{rum1}. p56\textsuperscript{cdc13} levels dropped at the onset of anaphase and started to accumulate during the next S phase. This experiment shows that p25\textsuperscript{rum1} protein levels oscillate though the cell cycle and that the levels are maximal in G1, consistent with previous observations where p25\textsuperscript{rum1} was shown to accumulate in cells arrested in G1 but not in S phase or G2 (Correa-
Bordes and Nurse, 1995). rum1+ mRNA levels were also determined in a similar experiment and found to oscillate through the cell cycle with a peak of expression at the end of the G2 phase, 40–60 min earlier than the protein (Figure 1C). Levels of rum1+ mRNA only changed 2- to 3-fold (when normalized to the ura4+ mRNA) compared with a 10-fold oscillation in the protein levels, suggesting that additional post-transcriptional mechanisms are involved in regulating p25rum1 levels.

**p25rum1 is polyubiquitinated and degraded by the proteasome pathway**

Protein degradation plays a crucial role in the regulation of the cell cycle. Destruction of mitotic cyclins controls exit from mitosis into G1 (Murray and Kirschner, 1989; Murray et al., 1989). In the budding yeast and animal cells, G1 cyclins and the CDK inhibitors p40 SIC1 and p27KIP1 are destroyed at the end of G1 by the ubiquitin-dependent proteasome pathway (Wittenberg et al., 1990; Tyers et al., 1992; Donovan et al., 1994; Schwoob et al., 1994; Deshaies et al., 1995; Pagano et al., 1995; Bai et al., 1996; Clurman et al., 1996; Willems et al., 1996; Won and Reed, 1996; Diehl et al., 1997). To establish whether p25rum1 is a substrate of the proteasome degradation pathway, we determined p25rum1 levels in the fission yeast temperature-sensitive mutant mts3-1, defective in subunit 14 of the 26S proteasome (Gordon et al., 1996). p25rum1 levels were measured in this mutant at 25°C and 2, 4, and 6 h after the shift to 36°C. p25rum1 protein was more abundant in the mts3-1 mutant at the restrictive temperature than in the wild-type strain (Figure 2, lane 9). These bands were absent both in wild-type and rum1+-deleted cells expressing His6-ubiquitin grown under identical conditions (Figure 2, lanes 7 and 8). This result clearly shows that p25rum1 is polyubiquitinated and degraded through the ubiquitin-dependent 26S proteasome pathway.

**Phosphorylation of p25rum1 is important for its stability**

In budding yeast, phosphorylation of several Cdc28-specific sites in the carboxy-terminus of the G1 cyclins Cln2 and Cln3 is important in promoting their degradation (Yaglom et al., 1995; Lanker et al., 1996). We have found in p25rum1 eight putative CDK phosphorylation sites containing the minimal consensus sequence of S/T-P (Figure 3A). Three of these sites, at positions 58, 62 and 212, correspond exactly to the full consensus cdc2 phosphorylation site of S/T-P-X/K/R (where X is any amino acid) (Moreno and Nurse, 1990). We have mutated all eight serines and threonines to alanine and expressed each mutant using the nmt1 promoter in S. pombe. Mutants rum1-A58 and rum1-A62 showed a phenotype consistent with hyperactivation or stabilization of p25rum1. In both cases, very few transformants were obtained even when we used the weakest modified nmt1 promoter (pREP81X) to transform yeast cells in the presence of thiamine, compared with a control pREP3X plasmid with the rum1+ gene (data not shown, see below). The transformants grew very slowly into small colonies containing many elongated cells with a phenotype similar to the phenotype of cells overexpressing the wild-type rum1+ gene (Moreno and Nurse, 1994). A double mutant rum1-A58A62 showed a more severe phenotype than each of the single mutants. Cells transformed with the rest of the alanine mutants (A5; A13A16A19; A110 and A212) showed a phenotype identical to cells expressing wild-type rum1+ plasmid control (data not shown).

Expression of the rum1+ gene driven by its own
promoter using the multicopy plasmid pIRT2 gives a fully wild-type phenotype, with no signs of cell elongation or diploidization. When we expressed the rum1-1A58A62 mutant allele driven by the rum1+ promoter using the pIRT2 plasmid, we obtained many small microcolonies and a few normal size colonies, all of which were integrants (Figure 3B). Flow cytometry analysis of these integrants showed a high frequency of diploids (Figure 3C).

The phenotype of the cells expressing the rum1-1A58A62 mutant could be explained either because the mutant protein is more active as a CDK inhibitor or because it is more stable, or both. To distinguish between these possibilities, we expressed wild-type rum1+ and mutant rum1-1A58A62 in Escherichia coli and purified both pro-
Stabilization of p25\textsuperscript{rum1} causes a cell cycle delay in G1

To confirm this hypothesis, we studied the stability of p25\textsuperscript{rum1} and p25\textsuperscript{rum1-A58A62} proteins through the cell cycle using the cdc10-129 mutant to synchronize cells in G1. This was investigated by integrating the wild-type rum1\textsuperscript{+} gene and the rum1\textsuperscript{-A58A62} mutant allele in a cdc10-129 rum1\textsuperscript{-} strain. Exponentially growing cultures of cdc10-129 rum1\textsuperscript{-} int-rum1\textsuperscript{+} and cdc10-129 rum1\textsuperscript{-} int-rum1-A58A62 were incubated for 4 h at 36°C and then released to 25°C. Wild-type p25\textsuperscript{rum1} disappeared by 90 min after the release as cells were entering S phase (Figure 5A and C). In contrast, the p25\textsuperscript{rum1-A58A62} levels were high and stable during the course of the experiment (Figure 5B). These levels of p25\textsuperscript{rum1-A58A62} were reduced compared with the levels required to block mitosis in cells overproducing rum1\textsuperscript{+} from the nmt1 promoter (Figure 5B, ovp). Interestingly, the cdc10-129 rum1\textsuperscript{-} strain with the integrated rum1-A58A62 allele underwent S phase and mitosis even in the presence of considerable amounts of p25\textsuperscript{rum1-A58A62} mutant protein (Figure 5B and C). Flow cytometry analysis showed that both strains underwent S phase after the release, though with a delay of ~15 min in the strain containing the rum1-A58A62 allele (Figure 5C). We also observed a delay of ~30 min in the accumulation of cdc13 cyclin in cells expressing rum1-A58A62 compared with the control, indicating that once cells initiate DNA replication the cdc13 protein levels increase independently of the presence or absence of p25\textsuperscript{rum1} protein.

If p25\textsuperscript{rum1} plays a role in determining the length of G1, stabilization of this protein should delay the onset of S phase. Flow cytometry analysis of haploid integrants of the rum1-A58A62 allele in the rum1 deletion (rum1\textsuperscript{-}) and in the cdc10-129 rum1\textsuperscript{-} strains grown at 25°C clearly revealed a G1 population (Figure 6A). To quantify this delay, we selected the smallest cells in the cdc10-129 rum1\textsuperscript{-} int-rum1-A58A62 culture, that were in G1, S and early G2 phases, by elutriation at 25°C. Half of these cells were incubated at 25°C and samples for flow cytometry analysis were taken every 20 min for 6 h (Figure 6B, left). All the G1 cells underwent S phase by 40 min. In the next cell cycle, these cells remained in G1 for ~90 min after cytokinesis. Considering that the cell cycle length in this experiment was 260 min, then the onset of S phase was delayed by as much as 0.3 of a cell cycle. In a synchronous culture of the cdc10-129 control strain at 25°C, we did not observe any delay in G1 (data not shown, see Moreno and Nurse 1994 Figure 4D). The other half of the cells were shifted at time 0 after elutriation to 36°C. Ninety percent of these cells underwent one complete round of cell division and then arrested at the cdc10 block point in G1 (Figure 6B, right). The remaining

\textbf{Fig. 5.} p25\textsuperscript{rum1-A58A62} is stable throughout the cell cycle. Cultures of cdc10-129 rum1\textsuperscript{-} int::rum1\textsuperscript{+} (A) and cdc10-129 rum1\textsuperscript{-} int::rum1-A58A62 (B) were grown at 25°C to mid-exponential phase in minimal medium, shifted to 36°C for 4 h and then released at 25°C. Samples for Western blot and flow cytometry were taken before the shift to 36°C (ASN), 4 h after the shift to 36°C (\(t = 0\)) and every 30 min after the release at 25°C. Levels of p25\textsuperscript{rum1} and p56\textsuperscript{cdc13} were determined in both strains. (A) p25\textsuperscript{rum1} levels were high at the block point (\(t = 0\)) and disappeared just before the onset of S phase (\(t = 60\)). (B) p25\textsuperscript{rum1-A58A62} levels were high and constant throughout the experiment. p56\textsuperscript{cdc13} levels were undetectable at the cdc10-129 arrest point and then increased in both strains as cells underwent S phase. A delay of ~30 min in the accumulation of p56\textsuperscript{cdc13} in the onset of S phase was observed in the strain expressing rum1-A58A62 compared with the control strain. rum1\textsuperscript{-} (\(\Delta\)) and rum1\textsuperscript{+} overexpressor (OVP) strains were used as negative and positive controls. (C) The percentage of cells in G1 after the release to 25°C. Asn, asynchronous cells grown at 25°C. White bars, cdc10-129 rum1\textsuperscript{-} int::rum1\textsuperscript{+} cells. Black bars, cdc10-129 rum1\textsuperscript{-} int::rum1-A58A62 cells.
Expression of $\text{rum1-A58A62}$ mutant causes a cell cycle delay in G1. (A) Flow cytometry of $\text{rum1}^\Delta$ int::$\text{rum1}^+\text{A58A62}$, $\text{cdc10-129} \text{rum1}^\Delta$ int::$\text{rum1}^+\text{A58A62}$ haploid cells growing exponentially at 25°C in minimal medium. Since expression of $\text{rum1-A58A62}$ mutant allele induces a high degree of diploidization, for this experiment a fresh haploid pink colony was selected from a YES phloxin B plate and inoculated in minimal medium until early exponential phase. The DNA content was measured by FACS analysis. (B) A synchronous culture of the $\text{cdc10-129} \text{rum1}^\Delta$ int-rum1::A58A62 haploid strain was prepared at 25°C. Half of the culture was incubated at 25°C (left panel) and the other half at 36°C (right panel). The generation time of the cells growing at 25°C was 260 min as determined using the two samples shown with arrows with a similar FACS profile and separated by one generation time. The G1 delay of 90 min was estimated using the 260 and 170 min samples (in between 160 and 180 min), where ~50% of the cells have 1C and 50% 2C DNA content.

10% stayed in G1 for the rest of the experiment, indicating that at least some of the cells selected by elutriation were in pre-Start G1 when the cdc10 function was inactivated by the temperature shift.

**p25rum1 is phosphorylated in vivo**

From the previous experiments, we concluded that mutations in two putative cdc2 phosphorylation sites, T58 and T62, affect the stability of p25rum1. One possible hypothesis is that phosphorylation of p25rum1 by cdc2 on residues T58 and/or T62 could target p25rum1 for destruction at the end of G1. We have found that p25rum1 protein is an excellent substrate for the cdc2 protein kinase. Phosphorylation of p25rum1 in vitro by immunocomplexes of cdc2 induced a mobility shift from 34 to 36 kDa (Figure 7A). This shift in mobility was not observed when we used the mutant p25rum1-A58A62 as a substrate, suggesting that at least in vitro cdc2 was able to phosphorylate p25rum1 on residues T58 and T62. We next studied which of the cdc2 complexes were involved in this phosphorylation event. Immunoprecipitates of cdc13, cig2 and cig1 were assayed using p25rum1, p25rum1-A58A62 and histone H1 as substrates. We found that cdc2–cig1 complexes were able to phosphorylate p25rum1 as efficiently as histone H1 as substrates. We next studied which of the cdc2 complexes were involved in this phosphorylation event. Immunoprecipitates of cdc13, cig2 and cig1 were assayed using p25rum1, p25rum1-A58A62 and histone H1 as substrates. We found that cdc2–cig1 complexes were able to phosphorylate p25rum1 as efficiently as histone H1 and to induce a mobility shift in p25rum1 similar to the one induced by immunocomplexes of cdc2 (Figure 7A). Phosphorylation of p25rum1 by cdc2–cig2 and cdc2–cdc13 was detected only after very long exposures and seemed to occur at sites other than T58 and T62 (Figure 7A, see figure legend). Furthermore, immunocomplexes of cdc2 from cells deleted for cig1 gave a pattern of p25rum1 phosphorylation very similar to the one induced by cdc2–cig2 and cdc2–cdc13 (Figure 7A). This result suggests that cdc2–cig1 complexes, which are insensitive to p25rum1 inhibition (Figure 4B; Correa-Bordes and Nurse, 1995; Martín-Castellanos et al., 1996), are involved in the phosphorylation of p25rum1.

To determine if p25rum1 is phosphorylated in vivo, we immunoprecipitated p25rum1 from 32P-labelled cells. A faint 36 kDa band was detected from wild-type cells (Figure 7B, left panel). This band was absent in the $\text{rum1}^\Delta$ and appeared much stronger in cells overproducing $\text{rum1}^+$ (Figure 7B, left panel), suggesting that it should correspond to in vivo labelled p25rum1. Given that phosphorylation of p25rum1 seems to be the signal that triggers its degradation, it is expected that the half-life of phosphorylated p25rum1 would be very short. Hence we have to overexpress the protein to moderate levels to be able to obtain enough labelled protein to make the phosphopeptide maps. We used single copy integrants of wild-type rum1+ or the $\text{rum1-A58A62}$ mutant allele expressed from the weakest version of the nmt1 promoter (pREP81X). In these conditions, we found a 5-fold reduction in the phosphorylation of mutant p25rum1-A58A62 as compared with wild-type p25rum1 (Figure 7B, right panel; Figure 8A). In addition, there was a clear mobility shift in the phosphorylated wild-type p25rum1 compared with the mutant protein (Figure 7B, right panel; Figure 8A). The apparent size of in vivo phosphorylated p25rum1 was 2 kDa larger than p25rum1-A58A62, indicating that phosphorylation of p25rum1 in vivo was causing a shift in mobility identical to the one observed when p25rum1 was phosphorylated in vitro.
Regulation of rum1 degradation by phosphorylation

Fig. 7. p25rum1 is phosphorylated in vivo. (A) Wild-type and cig1-deleted cells were grown to mid-exponential phase in minimal medium. Extracts were prepared in HB buffer (see Materials and methods) and 2 mg of total protein was immunoprecipitated with anti-cdc2, anti-cig1, anti-cig2 and anti-cdc13 antibodies. Protein kinase activity was measured using p25rum1 (rum1), p25rum1-A58A62 (A58A62) and histone H1 (H1) as substrates. The phosphorylated products were separated in a long run 14% SDS–PAGE and exposed to autoradiography. cdc2 immunocomplexes could phosphorylate p25rum1 as efficiently as they could histone H1. p25rum1 phosphorylation induced a band shift from 34 to 36 kDa that was not observed in the mutant p25rum1-A58A62. cig1 immunocomplexes induced a similar band shift to cdc2. p25rum1 was a much poorer substrate than histone H1 for the cig2 and cdc13 immunocomplexes. The cdc2 and cig1 gels were exposed for 8 h and the cig2 and cdc13 gels for 40 h. (B) Extracts from rum1Δ-deleted cells (Δ), wild-type (wt) and cells overproducing rum1 (ovp) labelled in vivo with [32P]orthophosphate were immunoprecipitated with R4 anti-rum1 antibody and separated on an SDS–PAGE gel. Coomassie blue staining of purified p25rum1 was used as the 34 kDa molecular weight marker. Cells expressing rum1 or rum1-A58A62 from the weakest version of the nmt1 promoter (pREP81X) were labelled in vivo and immunoprecipitated with R4 anti-rum1 antibody (in vivo rum1, A58A62) and run on a long SDS–PAGE gel together with p25rum1 and p25rum1-A58A62 phosphorylated in vitro with cdc2 or cig1 immunocomplexes (in vitro rum1, A58A62). Similar shifts in mobility were seen in p25rum1 phosphorylated in vivo and in vitro.

by cdc2 or cig1 immunocomplexes (Figure 7B, right panel; Figure 8A).

Tryptic phosphopeptide maps of in vivo 32P-labelled wild-type p25rum1 showed two phosphopeptides (Figure 8B, phosphopeptides 1 and 2), while p25rum1-A58A62 showed only one (corresponding to phosphopeptide 2). Phosphopeptide 1, the major phosphopeptide present in wild-type p25rum1, was absent in the mutant p25rum1-A58A62, suggesting that this phosphopeptide might contain the residues T58 and T62. To confirm this, we performed two experiments. First, phoshoamino acid analysis of phosphopeptide 1 revealed phosphothreonine while phosphopeptide 2 contained phosphoserine. Second, we generated a rum1-S58S62 mutant allele where the two threonine residues (T58 and T62) were mutated to serine. In vivo 32P-labelled p25rum1-S58S62 also showed a shift in mobility similar to wild-type p25rum1 (Figure 8A). Tryptic phosphopeptide maps of in vivo 32P-labelled p25rum1-S58S62 also showed two main phosphopeptides with migration similar to phosphopeptides 1 and 2 in wild-type p25rum1 (Figure 8B). Phosphoamino acid analysis of phosphopeptide 1 in p25rum1-S58S62 revealed phosphoserine instead of the phosphothreonine in wild-type p25rum1. Taken together, these results clearly show that p25rum1 is phosphorylated in vivo on residues T58 and T62.

cdc2–cig1 influences the stability of p25rum1

To study the timing of activation of the different CDK–cyclin complexes through the fission yeast cell cycle, we measured the protein kinase activity associated with cdc13, cig2 and cig1 in synchronous cultures of the wee1-50 strain. Small cells in G2 were isolated at 25°C by elutriation and then incubated at 36°C. Samples were collected every 20 min for two cell cycles, and histone H1 kinase activity was determined in immunoprecipitates of cig2 and cig1, rum1 kinase activity was measured in immunoprecipitates...
Fig. 8. p25rum1 is phosphorylated in vivo at residues T58 and T62. (A) Cultures of cells containing integrated copies of rum1+, rum1-A58A62 and rum1-S58S62 in pREP81X were labelled with [32P]phosphate (see Materials and methods). Extracts were prepared and immunoprecipitated with anti-rum1 antibodies. (B) Tryptic phosphopeptides of p25rum1, p25rum1-A58A62, p25rum1-S58S62 and a mixture of the phosphopeptides of p25rum1 and p25rum1-A58A62. Two main phosphopeptides were observed in 32P-labelled p25rum1 (numbered 1 and 2). In p25rum1 and p25rum1-A58A62, phosphopeptide 1 contained phosphothreonine while phosphopeptide 2 contained phosphoserine. In p25rum1-S58S62, phosphopeptide 1 contained phosphoserine. Sample origins are indicated with a dot.

Discussion

The rum1+ gene encodes a CDK inhibitor important for regulating the G1 phase of the fission yeast cell cycle (Moreno and Nurse, 1994; Moreno et al., 1994; Correa-Bordes and Nurse, 1995; Labib et al., 1995; Jallepalli and Kelly, 1996; Labib and Moreno, 1996; Martin-Castellanos et al., 1996; Correa-Bordes et al., 1997). p25rum1 has two functions in G1: it determines the length of the pre-Start G1 period and prevents mitosis from happening in early G1 cells (Moreno and Nurse, 1994). Here we have presented evidence to explain these two effects of p25rum1. We have shown that p25rum1 oscillates through the cell cycle and is present in the cell from anaphase until the end of G1 (Figure 9). In fission yeast, not all the cdc13 cyclin is degraded at the end of mitosis (Hayles and Nurse, 1995; Creanor and Mitchison, 1996; Yamano et al., 1996; see also Figure 1B). This means that cells in early G1 contain significant levels of cdc13 that could associate with cdc2 to form an active complex and which could trigger premature DNA replication or a lethal mitosis with unreplicated DNA. Thus, the presence of p25rum1 oscillates through the cell cycle and is present in the cell from anaphase until the end of G1, inhibiting its protein kinase activity (Correa-Bordes and Nurse, 1995).

Low CDK–cyclin activity before S phase is important for resetting the chromatin to a state which is permissive for DNA replication (Dahmann et al., 1995; Nasmyth, 1996; Stern and Nurse, 1996; Wuarin and Nurse, 1996). It has been argued that dephosphorylation in G1 of one or several proteins that bind to the origin recognition complex (ORC), such as MCMs or cdc18/Cdc6, is necessary for proper assembly of the DNA replication initiation complexes (Dahmann et al., 1995; Nasmyth, 1996; Stern and Nurse, 1996; Wuarin and Nurse, 1996). In G1, low CDK activity and high protein phosphatase activity provide conditions in which these dephosphorylation reactions can take place. In fission yeast, p25rum1 plays an active role in down-regulating p25rum1 levels in vivo.
Regulation of rum1 degradation by phosphorylation

Fig. 9. Protein kinase activity of the different CDK–cyclin complexes through the fission yeast cell cycle. A synchronous population of wee1-50 cells was prepared at 25°C by elutriation, the culture was incubated for 20 min at 25°C and then shifted up to 36°C. Samples were taken every 20 min to determine cdc13-, cig2- and cig1-associated kinase activity, the percentage of G1 cells and the mitotic index. cdc13- and cig2-associated kinase activity was measured using histone H1 as substrate. cig1-associated kinase was determined using p25rum1 as substrate. cdc13–cdc2 and cig1–cdc2 protein kinase activity peaks in mitosis while cig2–cdc2 peaks in G1/S. The percentage of G1 cells was measured by flow cytometry and the mitotic index by counting the number of cells in anaphase after DAPI staining (see Materials and methods).

in this process by reducing the activity of cdc2–cyclinB complexes in G1. Taking this into consideration, the cell cycle can be divided into two periods. The first period extends from anaphase until the end of G1, and is characterized by high protein phosphatase activity and low cdc2–cyclin B activity. The second period starts at the end of G1, when cdc2–cyclin B activity increases and the cells proceed to S phase and subsequently to mitosis. The alternation of these two periods allows dephosphorylation of cdc2–cyclinB substrates in G1 before S phase, and their phosphorylation in S phase, G2 and mitosis. These two periods could not co-exist. Protein phosphatases and CDK–cyclin complexes negatively regulate each other. Dephosphorylation of cdc2 on T167 by protein phosphatases leads to its inactivation (Gould et al., 1991), and phosphorylation by CDK–cyclin complexes on T316 inactivates fission yeast and human protein phosphatase 1 (PP1) (Dohadwala et al., 1994; Yamano et al., 1994). In early G1, when levels of the p25rum1 CDK inhibitor are high, the effect of protein phosphatases predominates. As the cell grows during G1, cig1, cig2 and cdc13 cyclins begin to accumulate and CDK activity eventually predominates at the end of G1. These two biochemical states coincide in time with the pre-Start and post-Start periods, Start being the point where the switch occurs.

Phosphorylation of p25rum1 by cdc2–cyclin complexes is important in regulating p25rum1 stability. We have found that p25rum1 is phosphorylated in vivo and that rum1 mutants in two CDK phosphorylation sites, T58 and T62, show a gain-of-function phenotype. Cells bearing an integrated single copy of these mutant alleles expressed from the rum1 promoter showed a high frequency of diploidization and a long delay in G1, especially in combination with the cdc10-129 mutant at 25°C. These phenotypes are not the result of the mutant proteins

Fig. 10. p25rum1 is stabilized in cells lacking the cig1+ gene. The rum1+ gene was overexpressed from the thiamine-repressible umtl promoter (Maundrell, 1989), in wild-type (●) and cig1A (○) cells containing an integrated copy of pREP3X(sup3-3) rum1+. Cells were grown to mid-exponential phase at 25°C in minimal medium containing 5 μg/ml thiamine, washed three times in minimal medium and resuspended in fresh medium lacking thiamine, at a density calculated to produce 4×10^6 cells/ml after 16 h, when expression from the umtl promoter peaks. Thiamine was then added to the cultures to repress the umtl promoter and samples for making RNA and protein were taken at the indicated times. p25rum1 levels were determined by Western blot using affinity-purified R4 anti-p25rum1 antibody and rum1+ mRNA by Northern blot. Equal loading for immunoblots and Northern blots was confirmed by detection of p34cdc2 and ethidium bromide staining of rRNA. Levels of p25rum1 were quantified by densitometric analysis.
being more powerful inhibitors than the wild-type protein. Rather, they are more stable and do not oscillate through the cell cycle. The diploidization phenotype induced by these mutant alleles could be explained if stabilization of the mutant protein in G2 occasionally reduces the level of the cdc2–cdc13 activity below the threshold level required to prevent initiation of another round of S phase within the same cell cycle (Hayles et al., 1994). Thus, p25rum1, which is normally present only in G1, induces extra rounds of S phase when present in G2.

Of the three cdc2–cyclin B complexes described in fission yeast, cdc2–cdc13 is the mitotic kinase while cdc2–cig2 is involved in the onset of S phase (Booher et al., 1989; Moreno et al., 1996; Fisher and Nurse, 1996; Martin-Castellanos et al., 1996; Monderst et al., 1996). The cdc2–cig1 complex might also contribute to the onset of S phase because a cdc13Δ cig2Δ double mutant can still undergo S phase while the cdc13Δ cig2Δ cig1Δ triple mutant cannot (Fisher and Nurse, 1996). p25rum1 is an inhibitor of cdc2–cdc13 and cdc2–cig2 complexes but it does not inhibit cdc2–cig1 complexes (Figure 4B; Correa-Bordes and Nurse, 1995; Martin-Castellanos et al., 1996). Here we present evidence suggesting that p25rum1 may be a substrate for the cdc2–cig1 protein kinase. First, cdc2–cig1 is able to phosphorylate p25rum1 efficiently and to induce a band shift similar to that observed in vitro with immunocomplexes of cdc2. The size of this band corresponds exactly to the size of p25rum1 phosphorylated in vitro. In contrast, p25rum1 is a poor substrate for cdc2–cdc13 and cdc2–cig2 complexes. Second, p25rum1 is stabilized in cells lacking cig1, suggesting that p25rum1 is a substrate for cig1-associated kinase and that this phosphorylation might be the signal that targets p25rum1 for degradation. Deletion of cig1 specifically increases the half-life of p25rum1, suggesting that cdc2–cig1 may be the principal trigger of p25rum1 instability. This is consistent with the fact that cdc2–cig1 kinase activity accumulates during G2 phase and G2, when p25rum1 levels are low. We propose that one of the functions of cdc2–cig1 complexes is to relieve the effect of p25rum1 inhibition over cdc2–cig2 and cdc2–cdc13 complexes, ensuring that p25rum1 is destroyed by the time cells undergo DNA replication and is absent in S phase and G2. However, in addition to cdc2–cig1, there must be other cdc2–cyclin complexes able to phosphorylate p25rum1 because in cells lacking cig1, p25rum1 is still unstable and these cells do not show the phenotypes that we observed in the rum1-1 A58Δ162 mutant. We currently are addressing this point by constructing multiple deletions of fission yeast cyclins.

We have also shown that p25rum1 is stabilized in the mts3-1 mutant defective in subunit 14 of the 26S proteosome (Gordon et al., 1996), suggesting that degradation of p25rum1 occurs through the proteosome-dependent proteolytic pathway. Further support for this idea comes from the recent finding that p25rum1 is stabilized in the fission yeast pop1-1 mutant, defective in a protein homologue of the budding yeast Cdc4 (Kominami and Toda, 1997). As in S. cerevisiae, the fission yeast Cdc4 homologue could be part of a complex involved in the ubiquitination and degradation of multiple targets at the end of G1, including G1 cyclins and the CDK inhibitor p25rum1. Proteolysis of other CDK inhibitors such as p40SIC1 and p27KIP1 is mediated by the ubiquitin-dependent proteolytic pathway (Schwob et al., 1994; Pagano et al., 1995; Verma et al., 1997). Interestingly, pop1 mutant cells like rum1-1 A58Δ162 cells diploidized at high frequency (Kominami and Toda, 1997). Degradation of inhibitors is a recurrent theme in biology (Murray, 1995). In the immune system, the 1xB-α family of inhibitors prevents nuclear localization of the transcriptional activator NF-κB (Thanos and Maniatis, 1995). Extracellular signals including tumour necrosis factor-α (TNF-α), lipopolysaccharide (LPS) and interleukin-1 (IL-1) induce rapid degradation of 1xB-α (Pahl and Bauerle, 1996). Degradation of 1xB-α is preceded by phosphorylation on two serine residues, S32 and S36, that convert this protein into an efficient substrate for the protein ubiquitination machinery (Brown et al., 1995; Chen et al., 1995; Traenckner et al., 1995). In this regard, it is interesting that the two putative phosphorylation sites in p25rum1 are located in the amino-terminus region and are also separated by four amino acids.

Is p25rum1 function likely to be conserved in other eukaryotes? p25rum1 is a CDK inhibitor that has specificity for S phase and mitotic cdc2–cyclin kinases. This is similar to the situation in budding yeast, where p40SIC1 has been shown to inhibit specifically Cdc28–Clb-associated kinases, preventing the initiation of S phase and mitosis during G1 (Schwob et al., 1994; Nasmyth, 1996). Like p25rum1, p40SIC1 is stabilized during anaphase and is degraded at the end of G1 through the Cdc4–Cdc34–Cdc53 ubiquitin-dependent proteolytic pathway (Donovan et al., 1994; Schwob et al., 1994). Degradation of p40SIC1 is also thought to depend on Cdc28 phosphorylation by the Cdc28–Cln kinases (Schwob et al., 1994; Schneider et al., 1996). In addition, there is sequence similarity in the CDK inhibitory domain between p40SIC1 and p25rum1, and expression of SIC1 rescues a deletion of rum1+ in fission yeast (A. Sánchez, I. González, M. Arellano and S. Moreno, submitted). Overproduction of SIC1 in fission yeast also induces re-replication (Jallepalli and Kelly, 1996; our unpublished results), suggesting that p40SIC1 and p25rum1 inhibit a similar set of CDK–cyclin complexes in fission yeast. None of the CDK inhibitors described so far in animal cells has a specificity for the Cdc2–cyclin B kinase (Sherr and Roberts, 1995; Elledge et al., 1991), and involves deletion of rum1-1 A58Δ162 strain was described by Bueno et al. (1991), and involves deletion of the entire open reading frame (ORF). The cig2Δ::ura4+ ura4-D18 h+ strain was described by Obara-Ishihara and Okayama (1994), and involves deletion of the N-terminal 80% of the protein. The rum1Δ::ura4+ ura4-D18 leu2-32 ade6-M216 h+ strain was described by Moreno and Nurse (1994), and involves deletion of the entire ORF. Since deletion of rum1+ causes sterility, all the crosses involving a deletion of rum1+ were done by transforming with pREP3X-rum1+, so that rum1+ is expressed from a plasmid, and the double mutants were checked subsequently to ensure that the plasmid had been lost. Tetrad analysis was performed to construct double mutants, and the identity of

### Materials and methods

#### Fission yeast strains and methods

Strains used in this study are congenic with the 972 h+ strain of S. pombe and are listed in Table I. Growth conditions and strain manipulations were as described by Moreno et al. (1991). The cig2Δ::ura4+ ura4-D18 h+ strain was described by Bueno et al. (1991), and involves deletion of the entire open reading frame (ORF). The cig2Δ::ura4+ ura4-D18 h+ strain was described by Obara-Ishihara and Okayama (1994), and involves deletion of the N-terminal 80% of the protein. The rum1Δ::ura4+ ura4-D18 leu2-32 ade6-M216 h+ strain was described by Moreno and Nurse (1994), and involves deletion of the entire ORF. Since deletion of rum1+ causes sterility, all the crosses involving a deletion of rum1+ were done by transforming with pREP3X-rum1+, so that rum1+ is expressed from a plasmid, and the double mutants were checked subsequently to ensure that the plasmid had been lost. Tetrad analysis was performed to construct double mutants, and the identity of
Table I. Schizosaccharomyces pombe strains

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<td>P.Nurse</td>
</tr>
<tr>
<td>P43</td>
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</table>

these mutants was confirmed by Southern blotting. Yeast transformation was carried out using the lithium acetate transformation protocol (Moreno et al., 1991).

All experiments in liquid culture were carried out in minimal medium (EMM) containing the required supplements, starting with a cell density of 2-4×10⁶ cells/ml, corresponding to mid-exponential phase growth. Temperature shift experiments were carried out using a water bath at 36°C.

To induce expression from the nmt1 promoter, cells were grown to mid-exponential phase in EMM containing 5 μg/ml thiamine, then spun down and washed four times with minimal medium, before resuspending in minimal medium lacking thiamine at a density calculated to produce 4×10⁶ cells/ml at the time of peak expression from the nmt1 promoter.

Plasmids

Plasmids are listed in Table II. A 3.8 kb PstI–BamHI fragment containing the rum1¹ gene was cloned into pT7Z8R. This plasmid was used to obtain the different rum1 mutants by site-directed mutagenesis using the MutA-gene phagemid in vitro mutagenesis kit (Bio-Rad) and the following oligonucleotides: T5A, 5'-CATAGTTGGTGCGGTGAAAGT-3', T13A T16A S19A, 5'-CCCCAGGAGCTTCCGGGAGCAGGACACAA-3', T58A, 5'-GTITTTAGAGCGGTGAAAGT-3', T62A, 5'-GCTTTTGGGACGCTTACGAGGT-3', T105A, 5'-GGAGAGAGGAGGCTTCTTCTC-3', S212A, 5'-GATT- TTGTTGTTGCGGTGATAAGC-3', T58A T62A, 5'-GCTTTTTGGGACGCTTACGAGGT-3', T58A T62A, 5'-GCTTTTGGGACGCTTACGAGGT-3'.

The underlined nucleotides contain the mutated sequence. All the mutants were sequenced after the mutagenesis. The 3.8 kb PstI–BamHI fragments were then subcloned into the pTR2 vector. To make the constructions in pREP-3X and the pREP-81X, DNA from the different amplified plasmids was modified by PCR using the high-fidelity Pwo polymerase (Boehringer Mannheim) and the following oligomers, 18mer 5'-oligonucleotide 5'-CCGGCTGCAAGCACAA-3', where the underlined sequence contains an Xhol site, and a 28mer 3’ oligonucleotide with the sequence 5'-GGCCATTITATTTACAGGATCAGACAA-3', where the underlined sequence contains a BamHI site. The 700 bp PCR products were digested with Xhol and BamHI and subcloned into pREP-3X and pREP-81X. The pREP1-His6-ubiquitin plasmid was a gift from Dr Horiyuki Yamano.

Table II. Plasmid list

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RNA preparation and Northern blots

RNA from elutriated cells was prepared by glass beads lysis in the presence of phenol. RNA gels were run in the presence of formaldehyde, transferred to GeneScreen Plus (NEN, Dupont) and probed according to the manufacturer’s instructions. Quantification of 32P signals was performed using a Fuji PhosphorImager.

Protein extracts and Western blots

Total protein extracts were prepared from 3×10⁸ 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 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, 10 μg/ml pepstatin, 10 μg/ml soybean trypsin inhibitor, 100 μM 1-chloro-3-tosylamido-7-amino-3-heptanone (TLCK), 100 μM N-tosyl-l-phenylalanine chloromethyl ketone (TPCK), 100 μM phenylmethylsulfonyl fluoride (PMSF),

Synchronous cultures

wee1-50 h– cells were grown at 25°C in EMM. Cells were synchronized at 25°C using a JE-5.0 elutriation system (Beckman Instruments, Inc.) and then shifted to 36°C, resulting in entry into mitosis at a reduced cell size. Samples were taken every 20 min during two cell cycles for making RNA or protein extracts and for flow cytometry analysis.
Detection of p25rum1–His6-ubiquitin conjugates

Quantification of ECL signals was performed by densitometric analysis. Immunoblots were developed using the ECL kit (Amersham). Goat anti-rabbit antibody affinity-purified SP4 anti-cdc13 (1:250), PN24 anti-cdc2 (1:250) or R3 (or R4) anti-rum1 polyclonal antibodies. Antibody. Immunoblots were developed using the ECL kit (Amersham). Quantification of ECL signals was performed by densitometric analysis.

Protein extracts and kinase assays

For kinase assays, extracts from 3×10^6 cells were made using HB buffer (Neco et al. 1989, 1991). Cell extracts were spun at 4°C in a microfuge for 5 min, and the protein concentration determined by the BCA protein assay reagent (Pierce). Samples of 0.5 μg (in v/v 1:1000 h’-synchronous culture) and 1.5 μg (in vitro phosphorylation assays) were immunoprecipitated at 0°C for 1 h, using 2 μl of SP4 anti-cdc13, 2 μl of anti-cig2, 2 μl of 9830-U anti-cig1 or 2 μl of C2 anti-cdc2 polyclonal antibodies. Thirty μl of protein A-Sepharose was then added for 30 min at 4°C and the immunoprecipitates washed three times with 1 ml of HB buffer. Immunoprecipitates (∼20 μl) were resuspended in 20 μl of HB containing 50 μM ATP, 0.5 mg/ml substract histone H1 (Calbiochem) (or 0.5 mg/ml of p25rum1 or p25rum1-AS58A62) and 40 μCi/ml [γ-32P]ATP, and incubated at 25°C for 30 min. The reactions were started with 40 μl of 2× SDS-PAGE sample buffer, denatured at 100°C for 5 min and samples were run on a 14% SDS-polyacrylamide gel. Phosphorylated proteins were detected by autoradiography.

Phospholabelling of p25rum1

We used the procedures described by Gould and Nurse (1989) and Den Hertog et al. (1995) with minor modifications. Cells were grown for 12 h in phosphate-free minimal medium containing 50 μM NaH₂PO₄ and appropriate supplements to a density of 5×10⁶ cells/ml. Five ml cultures were spun down, resuspended in 5 ml of fresh medium and 1.2 μCi of [γ-32P]orthophosphate was added. To label cells that were overexpressing p25rum1, p25rum1-AS58A62 or p25rum1-S552, S553 and S549 containing integrated copies of rum1⁺, rum1⁻S58A62 and rum1⁻S58S62 in prePS1X were grown to mid-log phase in the presence of μg/ml thiamine, washed five times in phosphate-free minimal medium lacking thiamine, and inoculated into fresh medium. After 18 h of growth at 32°C in the absence of thiamine, the cells were pelleted by centrifugation and resuspended in 20 ml of phosphate-free minimal medium containing 50 μM NaH₂PO₄ and 5 μCi of [γ-32P]orthophosphate at a concentration of 6×10⁶ cells/ml in the absence of thiamine. In all the experiments, the cells were labelled for 4 h at 32°C.

Cell lysates were prepared as described above and were immediately made to 1% SDS, 1 mM dithiothreitol and boiled for 5 min. The extracts were then diluted 10-fold in RIPA buffer lacking SDS, and centrifuged for 15 min at 4°C at 10 000 g. Immunoprecipitation was performed for 1 h on ice using 4 μl of anti-rum1 (R4) serum followed by incubation with protein A-Sepharose (Gould and Nurse, 1989; Den Haese et al., 1995). Immunoprecipitates were boiled for 3 min in SDS–PAGE sample buffer, resolved on 14% SDS–polyacrylamide gels and transferred to Immobilon-P membrane (Millipore). Bands were visualized after autoradiography using Kodak Biomax films at ~70°C with intensifying screens.

Phosphopeptide mapping and phosphoamino acid analysis

Phosphopeptide maps were accomplished as described previously by Boyle et al. (1991) and Van der Geer and Hunter (1994). 13P-Labeled bands were cut from the immobilon-P membrane and trypsinized in 200 μl of DM sodium bicarbonate pH 7.3 containing 15 μg of trypsin-TPCK (Worthington Biochemical Corp.). After 12 h at 37°C, 15 μg of trypsin was added and digested for another 4 h at 37°C. Phosphopeptides were separated on TLC plates by electrophoresis at pH 1.9 (formic acid:acetic acid:water; 50:15:74) for 60 min at 1 kV, followed by ascending chromatography in n-butanol-pyrindine-acetic acid:water (75:50:15:60). Individual phosphopeptides were recovered as described by Van der Geer and Hunter (1994), eluted in buffer at pH 1.9 and evaporated.

To determine the phosphoamino acid composition, the phosphopeptide was dissolved in 6 M HCl and incubated for 60 min at 110°C. The hydrolys products were separated by electrophoresis in two dimensions on TLC plates at pH 1.9 and 3.5 as described by Boyle et al. (1991). Peptides and phosphoamino acids were visualized by autoradiography on Kodak Biomax film at ~70°C with intensifying screens.

Purification of p25rum1 from E.coli

A 0.7 kb Nde–BamHI DNA fragment containing the rum1⁺ or rum1⁻AS58A62 ORF was cloned into pRK171 vector and transformed into the E.coli strain BDL21(DE3). Three hundred ml of a culture in LB containing 50 μg/ml ampicillin was grown at 37°C until OD600 = 0.7 and induced with 0.4 μM isopropyl-β-thiogalactopyranoside (IPTG) for 4 h. The cells were harvested by centrifugation, resuspended in 25 ml of ice-cold lysis buffer (25 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.3% Triton X-100) and sonicated on ice 10 times for 10 s at medium power. The lysate was centrifuged for 20 min at 10 000 r.p.m. at 4°C and the supernatant containing p25 rum1 soluble protein was loaded twice onto an anion exchanger Q-Sephrose (Sigma) column equilibrated with buffer A (50 mM Tris–HCl pH 7.5, 50 mM NaCl). The column was washed with buffer A. The flow-through and the wash were mixed and loaded into a cation exchanger S-Sepharose (Pharmacia) column equilibrated with buffer A. The protein was eluted from the column with a 50–500 mM NaCl salt gradient in 50 mM Tris–HCl pH 7.5. The fractions containing p25 rum1 were pooled and dialysed twice against distilled water. Protein concentration was determined by BCA protein assay reagent (Pierce), and 100 μg aliquots were lyophilized and stored at ~20°C.

p25 rum1 inhibition assays

Purified p25 rum1 or p25 rum1-AS58A62 was added to the immunoprecipitates before the addition of the kinase assay buffer. The histone H1 kinase reactions were carried out in the same conditions as described above.

Flow cytometry and macroscopy

About 10⁶ cells were spun down, washed once with water, fixed in 70% ethanol and processed for flow cytometry or 4′,6′-diamidino-2-phenylindole (DAPI) staining, as described previously (Sazer and Sherwood, 1990; Moreno et al., 1991). A Becton-Dickinson 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 septum) after DAPI staining.

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References


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