Molecular Biology of the Cell
Vol. 9, 1309–1321, June 1998

Cyclin B Proteolysis and the Cyclin-dependent Kinase Inhibitor rum1p Are Required for Pheromone-induced G_1 Arrest in Fission Yeast

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Submitted October 8, 1997; Accepted March 10, 1998
Monitoring Editor: Ira Herskowitz

The blocking of G_1 progression by fission yeast pheromones requires inhibition of the cyclin-dependent kinase cdc2p associated with the B-cyclins cdc13p and cig2p. We show that cyclosome-mediated degradation of cdc13p and cig2p is necessary for down-regulation of B-cyclin–associated cdc2p kinase activity and for phermone-induced G_1 arrest. The cyclin-dependent kinase inhibitor rum1p is also required to maintain this G_1 arrest; it binds both cdc13p and cig2p and is specifically required for cdc13p proteolysis. We propose that rum1p acts as an adaptor targeting cdc13p for degradation by the cyclosome. In contrast, the cig2p–cdc2p kinase can be down-regulated, and the cyclin cig2p can be proteolyzed independently of rum1p. We suggest that pheromone signaling inhibits the cig2p–cdc2p kinase, bringing about a transient G_1 arrest. As a consequence, rum1p levels increase, thus inhibiting and inducing proteolysis of the cdc13p–cdc2p kinase; this is necessary to maintain G_1 arrest. We have also shown that pheromone-induced transcription occurs only in G_1 and is independent of rum1p.

INTRODUCTION

Entry into S-phase and mitosis in the eukaryotic cell cycle is controlled by the activation of cyclin-dependent kinases (CDKs). In the yeasts, both processes are initiated by a single CDK core enzyme encoded by *cdc2* in fission yeast and *CDC28* in budding yeast. Cdc2p and Cdc28p associate with mitotic B-type cyclins to initiate mitosis, cdc13p in fission yeast (Booher and Beach, 1988; Hagan *et al.*, 1988; Booher *et al.*, 1989; Moreno *et al.*, 1989), and Clb1–4p in budding yeast (Ghiara *et al.*, 1991; Surana *et al.*, 1991; Fitch *et al.*, 1992; Richardson *et al.*, 1992) and with S-phase B-cyclins to trigger S-phase, usually cig2p in fission yeast (Fisher and Nurse, 1996; Martin-Castellanos *et al.*, 1996; Mondesert *et al.*, 1996) and Clb5–6p in budding yeast (Epstein and Cross, 1992; Kühne and Linder, 1993; Schwob and Nasmyth, 1993; Schwob *et al.*, 1994). There is considerable overlap between mitotic and S-phase B-cyclins (Schwob *et al.*, 1994; Fisher and Nurse, 1996; Mondesert *et al.*, 1996), and in fission yeast a single cyclin cdc13p can bring about both S-phase and mitosis (Fisher and Nurse, 1996; Mondesert *et al.*, 1996). In budding yeast, activation of S-phase Clbp–Cdc28p protein kinase depends on the prior activation of Cdc28p associated with another class of G_1 cyclins, Cln1–3p.

The mechanisms ensuring the timely inactivation and activation of cyclin B–CDK in G_1 have been studied mainly in budding yeast. S-phase Clbp–Cdc28p protein kinase is up-regulated by three independent mechanisms, all of which involve Clnp-Cdc28p kinase activity. Clnp–Cdc28p protein kinase 1) activates transcription of *CLB* genes (Epstein and Cross, 1992; Schwob and Nasmyth, 1993) and 2) inactivates Clbp proteolysis (Amon *et al.*, 1994). The latter involves ubiquitin-mediated degradation of B-type cyclins, which requires the cyclosome (Sudakin *et al.*, 1995) or anaphase-promoting complex consisting of eight subunits, including Apc1p/bimEp/cut4p (Peters *et al.*, 1996; Yamashita *et al.*, 1996; Zachariae *et al.*, 1996), Cdc16p, Cdc23p, and Cdc27p (Irniger *et al.*, 1995; King

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et al., 1995; Tugendreich *et al.*, 1995). Cyclosome-mediated proteolysis is activated at the metaphase–anaphase transition, and its activity is maintained during early G_1 where it contributes to the prevention of a premature rise of Clbp–Cdc28p kinase activity (Irniger *et al.*, 1995). 3) Clnp–Cdc28p protein kinase phosphorylates the cyclin-dependent kinase inhibitor (CKI) Sic1p, targeting it for ubiquitin-mediated degradation via the ubiquitin-conjugating enzyme Cdc34p (Schwob *et al.*, 1994; Schneider *et al.*, 1996). Sic1p is present in early G_1 (Donovan *et al.*, 1994; Schwob *et al.*, 1994) and specifically inhibits Clbp–Cdc28p protein kinase activity (Mendenhall, 1993; Schwob *et al.*, 1994). Thus in budding yeast, down-regulation of Clbp-associated kinase is brought about by transcriptional, proteolytic, and CKI mechanisms that are relieved in late G_1 by Clnp–Cdc28p protein kinase activity. A second CKI in budding yeast, Far1p, directly inhibits the Clnp–Cdc28p protein kinase activity in response to pheromone and causes G_1 arrest (Chang and Herskowitz, 1990). Far1p is activated by the pheromone-dependent MAP kinase Fus3p, allowing Far1p to bind and inhibit the Clnp–Cdc28p protein kinase (Peter *et al.*, 1993; Peter and Herskowitz, 1994).

In fission yeast, the CKI encoded by the *rum1* gene plays a crucial role in regulating the cyclin B–CDK activity in G_1 (Moreno and Nurse, 1994). rum1p is a potent in vitro inhibitor of cdc2p associated with the mitotic B-type cyclin cdc13p (Correa-Bordes and Nurse, 1995; Jallepalli and Kelly, 1996) and also partly inhibits the in vitro kinase activity associated with the G_1 B-cyclin cig2p (Correa-Bordes and Nurse, 1995; Martin-Castellanos *et al.*, 1996). A *rum1*Δ mutant initiates mitosis from G_1 when S-phase is blocked (Moreno and Nurse, 1994). In these cells, the mitotic cdc13p–cdc2p kinase is activated prematurely (Correa-Bordes and Nurse, 1995), suggesting that one function of rum1p is to provide a safeguard that prevents mitosis from taking place in G_1 cells. rum1p is also required to extend G_1 during nitrogen starvation or in a *wee1* mutant background (Moreno and Nurse, 1994).

To better understand the mechanisms that control the activation of the G_1 cyclin B–cdc2p kinases in fission yeast, we have investigated the cell cycle controls that bring about pheromone-induced G_1 arrest (Davey and Nielsen, 1994; Imai and Yamamoto, 1994). We have shown previously that the fission yeast-mating pheromone P-factor blocks entry into S-phase by inhibiting both the cig2p- and cdc13p-associated cdc2p kinase activity in G_1 (Stern and Nurse, 1997). Here we show that *rum1*$^+$ is required for this pheromone-induced G_1 arrest. Our data establish that the cdc13p–cdc2p kinase is the main target for rum1p, whereas down-regulation of the cig2p-associated kinase activity can occur by another mechanism. Mutants in the cyclin B degradation machinery accumulate both cig2p and cdc13p and fail to arrest in G_1 in response to pheromones. Turnover of cdc13p requires rum1p, whereas cig2p turnover can occur in the absence of rum1p, suggesting that rum1p may act as an adaptor specifically targeting cdc13p for cyclosome-dependent degradation.

MATERIALS AND METHODS

Fission Yeast Strains and Methods

The following strains were constructed: *h*$^-$*cdc22-M45cyr1*Δ*::LEU2 sxa2*Δ*::ura4*$^+$*leu1–32ura4-D18*; *h*$^-$*rum1-HAcyr1*Δ*::LEU2sxa2*Δ*::ura4*$^+$ *leu1–32ura4-D18ade6–704his3-D1*; *h*$^-$*cyr1*Δ*::LEU2sxa2*Δ*::ura4*$^+$*:: REP6Xrum1leu1–32ura4-D18ade6–704*; *h*$^-$*rum1*Δ*::his3*$^+$*cyr1*Δ*:: LEU2sxa2*Δ*::ura4*$^+$*leu1–32ura4-D18his3-D1ade6–704*; *h*$^-$*cdc10–129rum1*Δ*::his3*$^+$*cyr1*Δ*::LEU2sxa2*Δ*::ura4*$^+$*leu1–32ura4-D18his3-D1ade6–704*; *h*$^-$*rum1*Δ*::his3*$^+$*cig2*Δ*::ura4*$^+$*cyr1*Δ*::LEU2sxa2*Δ*:: ura4*$^+$*leu1–32ura4-D18his3-D1ade6–704*; *h*$^-$*nuc2–663 cyr1*Δ*::ura4*$^+$ *sxa2*Δ*::ura4*$^+$*leu1–32ura4-D18ade6–704*.

Strains were constructed using random spore analysis. Candidate colonies with the appropriate selectable markers and mutations were tested for formation of conjugation tubes on agar plates containing 3 μg/ml P-factor. Only *h*$^-$*cyr1*Δ*sxa2*Δ mutant cells will respond to P-factor and grow conjugation tubes. *rum1*Δ*:his3*$^+$ and *nuc2*^{ts} mutants were crossed into the *h*$^-$*cyr1*Δ*sxa2*Δ background in the presence of a *nuc2* plasmid or *rum1* plasmid, respectively. The plasmids were lost after selection of the *h*$^-$*rum1*Δ*cyr1*Δ*sxa2*Δ and *h*$^-$*nuc2–663cyr1*Δ*sxa2*Δ triple mutants using marker selection or temperature-sensitive phenotype and response to P-factor on agar plates or both. For the construction of the *cig2*Δ*rum1*Δ*cyr1*Δ*sxa2*Δ, quadruple mutant colonies were tested by PCR for the absence of *cig2*$^+$. A pheromone-responsive strain carrying an N-terminal hemagglutinin peptide (HA)-tagged *rum1* at the *rum1* locus (Correa-Bordes and Nurse, 1995) was selected on the basis of an increased size of a *rum1-HA* PCR product compared with the *rum1* wild-type allele.

Media and growth conditions were as described by Moreno *et al.* (1991). Physiological experiments with P-factor, flow cytometric analysis (FACS), cell number, and cell size measurements were as described by Stern and Nurse (1997).

*Construction of a rum1*Δ*::his3*$^+$ *Mutant Strain*

A 1.9-kb *Spe*I fragment containing the whole *rum1*$^+$ open reading frame was removed from a 6.1-kb genomic *rum1*$^+$ clone in pTZ18R and replaced by blunt-end ligation with a 1.8-kb *Eco*RV–*Dra*I fragment of the *his3*$^+$ gene. The linearized 6.1-kb *rum1*Δ*::his3*$^+$ deletion construct was transformed into a *his3-D1* strain, and a stable his prototroph colony was isolated. Southern blotting established that the integration had taken place at the *rum1*$^+$ locus. The *rum1*Δ*::his3*$^+$ mutant was sterile and synthetically lethal with a *cdc10.129* allele like the previously described *rum1::ura4*$^+$ strain (Moreno and Nurse, 1994). Both phenotypes were rescued in the presence of a *rum1*$^+$-containing plasmid. A single copy of the *Eco*RV–*Dra*I *his3*$^+$ fragment does not fully rescue the his3-D1 deletion in liquid culture. We therefore supplemented the medium with histidine for physiological experiments.

RNA Preparation and Northern Blot

RNA was prepared by glass bead lysis in the presence of phenol and SDS and was subsequently separated using a formaldehyde gel. Ten micrograms as measured by OD_{260} were loaded in each track. Probes for blotting were prepared by random oligo priming with [^{32}P]dATP using a Prime-It kit (Stratagene, La Jolla, CA).

Cloning of mat1-Mm and fus1

A 210-bp fragment of the mat1-Mm gene was amplified from genomic DNA by PCR using the following primers: CATATG-CATTTGTATAGCAT and AATAATGTCAGCAGAAGACC. The resulting PCR fragment was cloned into the REP5 vector using the *Nde*I and *Bam*HI sites in the primers. A 1.1-kb fragment of the *fus1* gene was amplified in a similar manner using the following primers: CGGGATCCGGGGTACTCAAGTGTTACGTCTGG and CGGGATC-CAGCTGCTTTAGCCGTTTAGAAGG. The resulting PCR fragment was cloned into pKS$^+$ using the *Bam*HI sites in the primers.

Protein Kinase Assays and Immunoprecipitations

Kinase assays were performed as described by Stern and Nurse (1997). Cig2p-associated cdc2p kinase activity was immunoprecipitated from 3.8 mg (Figure 3A) and 2.5 mg (Figure 5B) of soluble extract with 10 μl of anti-cig2p polyclonal serum MOC8 (Stern and Nurse, 1997). Cdc13p-associated kinase activity was immunoprecipitated with 10 μl of anti-cdc13p serum SP4 (Moreno *et al.*, 1989) from 1 mg (Figure 3A) and 2.5 mg (Figure 5B) of soluble extract.

For cyclin immunoprecipitations (Figure 3B), 6 mg of soluble extract were incubated for 15 min at 4°C with 20 μl of polyclonal rabbit anti-cig2p serum (MOC8), with polyclonal rabbit anti-cdc13p serum (SP4), or with the respective preimmune serum. Rum1-HAp was immunoprecipitated for 30 min at 4°C from 9.5 mg of soluble extract (Figure 3C) with 30 μl of 12CA5 mAbs coupled to AffiGel beads (Bio-Rad, Richmond, CA; 4.3 mg/ml).

The immunoprecipitations were washed three times with 1 ml of HB buffer, boiled in 1× SDS sample buffer, separated on 10% SDS-PAGE, and Western blotted. The filters were probed for rum1HAp (Figure 3B) with the 12CA5 mAb (1:500 dilution), for cig2p (Figure 3C) with affinity-purified rabbit anti-cig2p antibody MOC6 (1:2000), and for cdc13p with affinity-purified rabbit anti-cdc13p SP4 (1:1000) (Figure 3C).

Western Blot

For Western blotting, 1–4 × 10^8 cells were harvested by centrifugation, washed once with ice-cold STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3, pH 8.0), resuspended in 50–100 μl of HB buffer, and boiled for 4 min. Glass beads were added to the meniscus, and cells were broken by vortexing on an IWAKI TWM 4836 microtube mixer (Iwaki Glass, Ikuta, Japan) for 2–5 min. Extract (50 μg) was separated on 10% SDS-PAGE (Laemmli, 1970) and transferred to ECL nitrocellulose or an Immobilon-P membrane (Millipore, Bedford, MA), and the protein of interest was detected using ECL (Amersham, Arlington Heights, IL). Dilutions of the antibodies were 1:1000 (Figure 5C) and 1:2000 (Figure 3C) for the anti-cig2p affinity-purified polyclonal antibody, 1:1000 for rabbit anti-cdc13p antibodies (SP4), 1:1000 for rabbit anti-rum1p antibodies (Correa-Bordes and Nurse, 1995), and 1:50,000 for the anti-α-tubulin monoclonal antibody (T5168; Sigma, St. Louis, MO).

In Vivo ^{35}S-Methionine Labeling

Cells were grown in minimal medium with glutamate (1 g/l) as a nitrogen source, to an OD_{595} of 0.5 (6 × 10^6 cells/ml) in the presence or absence of P-factor. Cells (10 ml) were incubated with 600 μCi of ^{35}S-methionine (Amersham Promix) for 10 min. After labeling, cells were harvested and washed with 10 ml of cold STOP buffer, resuspended in 50 μl of HB buffer, and broken with 1 ml of glass bead for 1 min. After cell breakage, the crude extract was recovered with 1 ml of cold HB buffer. Cell debris was removed by a 5-min spin in a microcentrifuge, and rum1p was isolated by immunoprecipitation with 10 μl of rum1p antiserum.

RESULTS

CDK Inhibitor rum1p Is Required for Pheromone-induced G_1 Arrest

The mating pheromone P-factor brings about G_1 arrest by inhibiting the cdc2p protein kinase activity (Stern and Nurse, 1997). Given that the CKI rum1p is present during G_1 and is required for pheromone-induced conjugation (Moreno and Nurse, 1994), it is possible that rum1p may have an analogous role to budding yeast Far1p in bringing about pheromone-induced G_1 arrest (Imai and Yamamoto, 1994). To test this possibility, we crossed a *rum1*Δ into a *cyr1*Δ*sxa2*Δ background. This genetic background is required to observe P-factor–induced G_1 arrest in exponentially growing cells. Elimination of the adenylate cyclase *cyr1*$^+$ gene leads to constitutive expression of nutritionally controlled genes, including components of the pheromone signal transduction cascade (Maeda *et al.*, 1990; Kawamukai *et al.*, 1991; Sugimoto, 1991); *sxa2*$^+$ encodes a P-factor–degrading protease (Imai and Yamamoto, 1992; Ladds *et al.*, 1996). Control *h*$^-$*rum1*$^+$*cyr1*Δ*sxa2*Δ cells were completely arrested in G_1 6 h after addition of P-factor (Figure 1A). In contrast, addition of P-factor to *h*$^-$*rum1*Δ*cyr1*Δ*sxa2*Δ cells failed to arrest them in G_1 (Figure 1A), and the cells continued to divide like untreated cells (Figure 1B). Similar results were obtained with *h*$^+$*rum1*Δ*cyr1*Δ, which did not arrest in G_1 in response to the mating pheromone M-factor (our unpublished results). We conclude that *rum1* is required for pheromone-induced G_1 arrest.

To investigate whether ectopic expression of *rum1* rescues the G_1 arrest defect, we integrated a REP6Xrum1 plasmid with the *rum1* cDNA under the control of the thiamine-repressible *nmt* promoter (Maundrell, 1993) into a *rum1*Δ strain. In this strain, pheromone-induced G_1 arrest was restored even when the promoter was switched off in the presence of thiamine (Figure 1C), indicating that low-level ectopic expression of *rum1* is sufficient to rescue the G_1 arrest defect.

rum1p is barely detectable in exponentially growing cells. If rum1p has a physiological role in bringing about G_1 arrest in response to pheromone, then rum1p levels need to increase after pheromone addition to cells. As expected, rum1p levels increased rapidly and became maximal within 2–3 h (Figure 2A, left panel) after pheromone addition to cells. Previous work has shown that rum1p levels increase when cells are arrested in G_1 (Correa-Bordes and Nurse, 1995). Therefore, the increase in rum1p levels after pheromone addition could be an effect of the G_1 arrest induced by pheromone. To investigate this further, P-factor was added to cells arrested in G_2 using a cdc25^{ts} (*cdc25–22*) mutant. These G_2-arrested cells are capable of responding to pheromone (Stern and Nurse, 1997) but

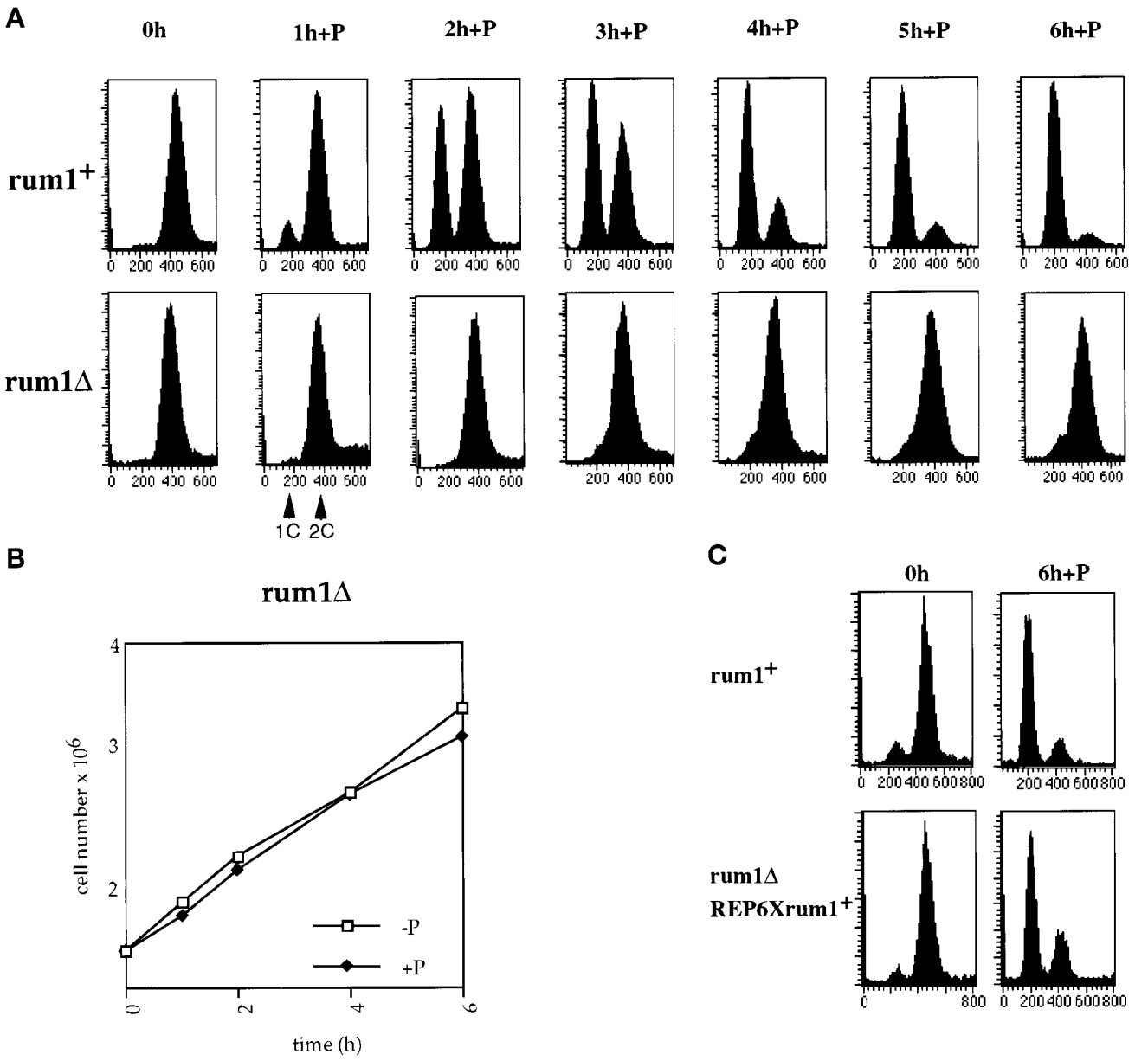

Figure 1. The *rum1*Δ mutant does not arrest in G_1 in response to pheromone. (A) FACS analysis of *cyr1*Δ*sxa2*Δ and *rum1*Δ*cyr1*Δ*sxa2*Δ strains exposed to P-factor at 25°C. (B) Growth curve of *rum1*Δ*cyr1*Δ*sxa2*Δ in the absence and presence of P-factor. (C) FACS analysis of a *cyr1*Δ*sxa2*Δ and a *rum1*Δ*cyr1*Δ*sxa2*Δ*::REP6Xrum1*$^+$integrant exposed to P-factor for 6 h in medium containing thiamine.

did not accumulate rum1p (Figure 2A, middle panel), suggesting that cells need to be in G_1 for rum1p to be induced. Furthermore, the addition of P-factor to nitrogen-starved cells already blocked in G_1 did not lead to a further increase of rum1p levels (Figure 2B), indicating that pheromone addition to these G_1 cells had no further direct effect on rum1p induction. This suggests that pheromone may not directly induce rum1p accumulation but, rather, that rum1p is induced after pheromone addition as an indirect consequence of cells arresting in G_1. We conclude that the primary role for rum1p may be in maintaining G_1 arrest after pheromone addition rather than in bringing about the initial G_1 arrest.

The increase of rum1p levels in cells blocked in G_1 by P-factor still occurred in cells expressing a constant low level of *rum1* from the *nmt* promotor (Figure 2A, right panel). This suggests that rum1p upregulation involves primarily posttranscriptional mechanisms. Increased *rum1* transcription might

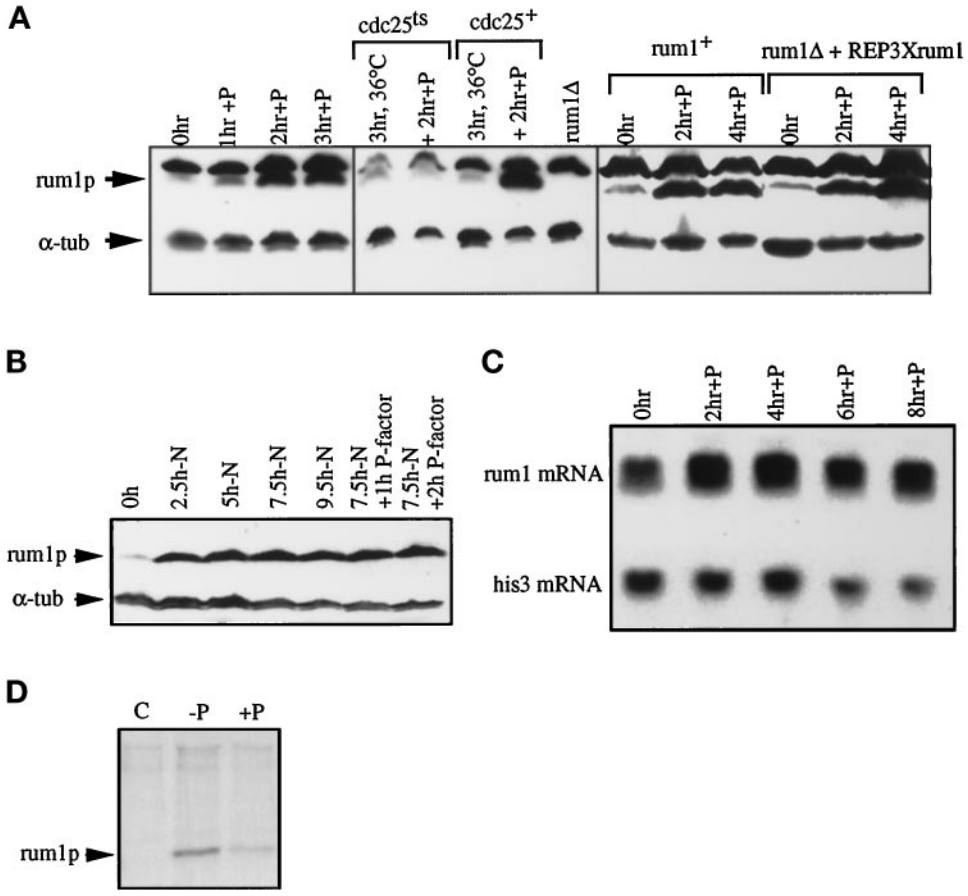

Figure 2. rum1p is induced in G_1 in pheromone. (A) Western blot of extracts from P-factor–treated cells probed with anti-rum1p and control anti-α-tubulin antibodies. A lane with extracts from isogenic rum1Δ cells demonstrates that the slower migrating band cross-reacting with anti-rum1p is nonspecific. A *cyr1*Δ*sxa2*Δ strain exposed to P-factor for 3 h at 25°C (left panel), *cdc25–22cyr1*Δ*sxa2*Δ and *cyr1*Δ*sxa2*Δ strains incubated at 36°C for 3 h and exposed to P-factor for 2 h at 36°C (middle panel), and a *rum1*Δ*cyr1*Δ*sxa2*Δ*::REP6Xrum1*$^+$ integrant and a *rum1*$^+$ control exposed to P-factor at 25°C for 4 h (right panel). (B) *Cyr1*Δ*sxa2*Δ cells were nitrogen starved, and after 7.5 h, P-factor was added to half the culture. Extracts were Western blotted and probed with anti-rum1p and anti-α-tubulin antibodies. This anti-rum1p antibody shows no nonspecific cross-reacting band. (C) Northern blot of RNA samples from a *cyr1*Δ*sxa2*Δ strain exposed to P-factor for 8 h at 29°C, probed for *rum1*$^+$ and *his3*$^+$ mRNA. (D) Protein extracts from ^{35}S-methionine pulse-labeled *cyr1*Δ*sxa2*Δ cells before (C, −P) or after 3 h exposure to P-factor (+P), immunoprecipitated with preimmune serum (C), or anti-rum1p antibody (−P and +P). Because there was considerably more ^{35}S-methionine–labeled protein in the −P extract than in the +P extract, the rum1p-specific band was quantified and normalized with respect to an unspecific band. With this quantification, 120 relative units of ^{35}S were incorporated in the absence of pheromone, compared with 97 relative units in the presence of pheromone.

contribute, however, to the increased level of rum1p in pheromone because *rum1* transcript levels increased ~1.6-fold after P-factor addition (Figure 2C). The posttranscriptional mechanism probably involves changes in rum1p turnover, as pulse labeling of cells with ^{35}S-methionine for 10 min showed that the levels of rum1p translation were not increased in pheromone-treated cells (Figure 2D). This conclusion is supported by the recent observation that rum1p accumulates in proteasome mutants that are defective in ubiquitin-mediated proteolysis (Benito *et al.*, 1998).

Cdc13p-associated cdc2p Kinase Is Deregulated in a rum1 Mutant

Next we investigated further the effects of rum1p on the cdc2p kinase during G_1 cell cycle arrest in pheromone. In vitro, rum1p inhibits the cdc13p-associated kinase and to a lesser extent the cig2p-associated kinase (Correa-Bordes and Nurse, 1995). Both cyclins can promote S-phase (Fisher and Nurse, 1996; Mondesert *et al.*, 1996), and so the cdc2p kinase activity associated with both cyclins needs to be inhibited to bring about and maintain G_1 arrest. Thus, the failure of *rum1*Δ cells to undergo pheromone-induced G_1 arrest could be due to a lack of inhi-

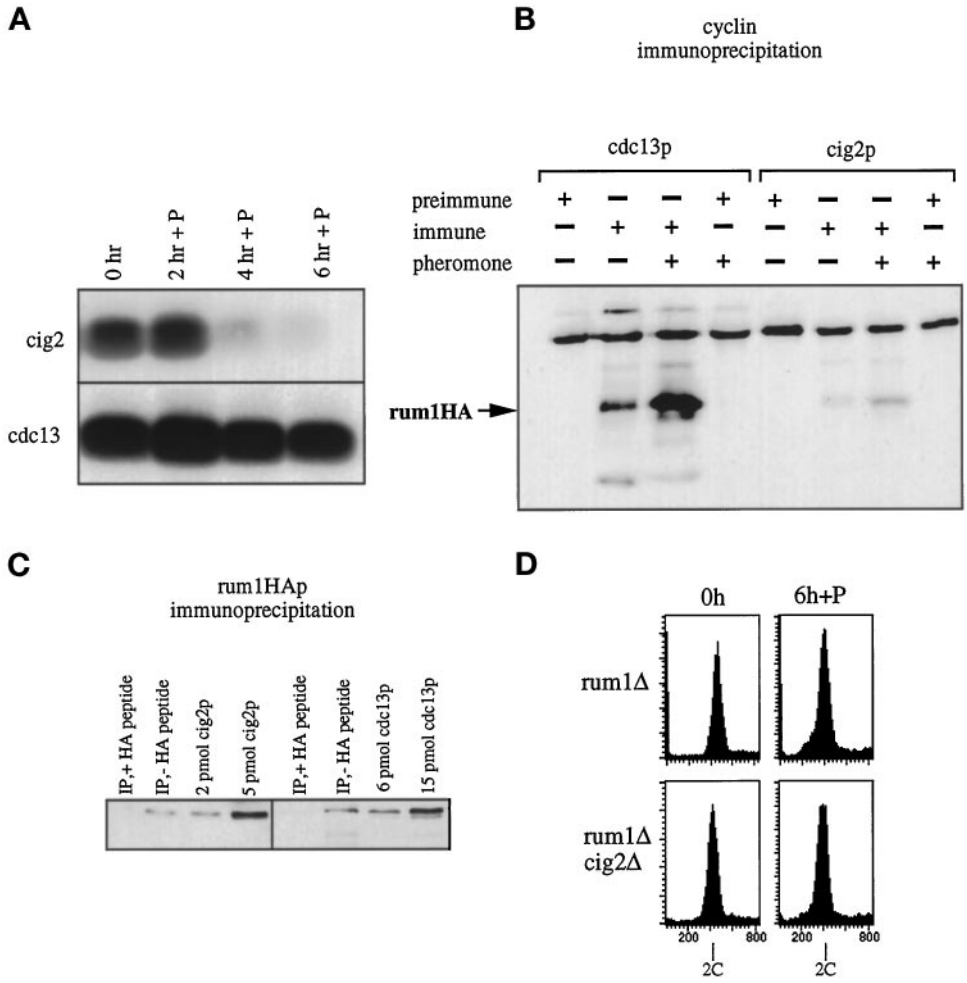

Figure 3. rum1p binds to and inhibits the cdc13p–cdc2p kinase in P-factor. (A) cig2p- and cdc13p-associated H1 kinase activities in a *rum1*Δ*cyr1*Δ*sxa2*Δ after exposure to P-factor at 25°C. (B) Immunoprecipitations from a *rum1-HAcyr1*Δ*sxa2*Δ strain with anti-cdc13p and anti-cig2p antibodies before and 3 h after P-factor addition, Western blotted, and probed with anti-HA-antibody. (C) Immunoprecipitations from a *rum1-HAcyr1*Δ*sxa2*Δ strain with anti-HA antibodies 3 h after P-factor addition, Western blotted, and probed with antibodies raised against cig2p (left panel) and cdc13p (right panel). Extracts (2 and 5 μl) containing the indicated amounts of cig2p and cdc13p were loaded to quantify the coimmunoprecipitated cyclins. (D) FACS analysis of h^-*rum1*Δ*cyr1*Δ*sxa2*Δ, h^-*cig2*Δ*rum1*Δ*cyr1*Δ*sxa2*Δ mutants incubated in P-factor for 6 h at 25°C.

bition of either the cig2p- or cdc13p-associated cdc2p protein kinase activity. We monitored both kinase activities in a *rum1*Δ mutant after addition of P-factor. The cig2p-associated activity still responded to P-factor falling to a low level within 4 h, whereas the cdc13p-associated activity remained high (Figure 3A). This indicates that the rum1p inhibitor is required to inhibit cdc13p-associated kinase activity but not to inhibit cig2p-associated kinase activity.

We confirmed that the inappropriate entry into S-phase in pheromone-treated *rum1*Δ cells does not require *cig2* by using a *cig2*Δ*rum1*Δ*cyr1*Δ*sxa2*Δ quadruple mutant. We found that a *cig2*Δ background did not restore the ability of a *rum1*Δ to G_1 arrest in response to P-factor (Figure 3D), indicating that *cig2* is not required in a *rum1*Δ mutant to overcome the G_1 block. Because cdc13p is the major B-type cyclin compensating for the loss of *cig2*, these results indicate that the premature onset of S-phase observed in pheromone-treated *rum1*Δ cells results from deregulation of the cdc13p-associated kinase activity rather than the cig2p-associated kinase activity.

Given these results, we used a *rum1-HAcyr1*Δ*sxa2*Δ strain to test whether rum1p physically associated with cdc13p after pheromone addition. Immunoprecipitations of cdc13p and cig2p before and after addition of pheromone were analyzed by Western blotting with anti-HA antibodies. rum1p was found associated with cdc13p, and this association was increased after 3 h in P-factor (Figure 3B). rum1p was also found

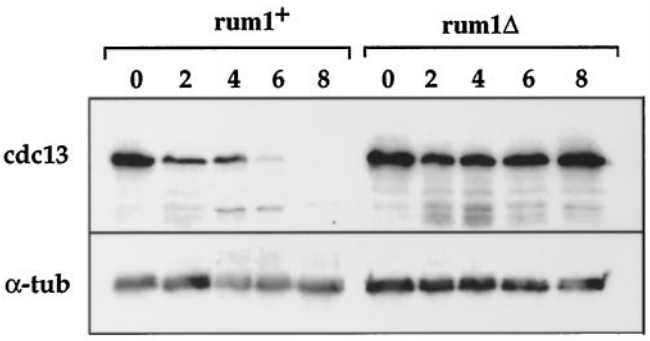

Figure 4. rum1p is required for cdc13p degradation in pheromone. Cdc13p and α-tubulin protein levels in *rum1*$^+$*cyr1*Δ*sxa2*Δ and *rum1*Δ*cyr1*Δ*sxa2*Δ strains after addition of P-factor.

associated with cig2p, although the amount detected was lower (Figure 3B). In a reciprocal experiment, rum1HAp immunoprecipitation was found to coprecipitate 2 pmol of cig2p and 6 pmol of cdc13p (Figure 3C). Similar results were obtained by immunoprecipitations of rum1p from a *cig2-HAcyr1*Δ*sxa2*Δ strain (our unpublished results). The different amounts of cig2p and cdc13p in rum1p precipitations are similar to the different cyclin B concentrations in the cell, because the level of cdc13p is about three times that of cig2p (Figure 3C and our unpublished results). The increased amount of cdc13p associated with rum1p is consistent with rum1p having an effect on the cdc13p-associated cdc2p protein kinase.

rum1p Is Required for Cyclosome-dependent Proteolysis of cdc13p during Pheromone-induced G_1 Arrest

We also found that rum1p is required for cdc13p proteolysis during the response to pheromone. We monitored cdc13p levels after P-factor addition in *rum1*$^+$ cells and found that they became reduced within 2 h of P-factor addition and were very low by 6 h after addition (Figure 4). This drop in level contributed to the observed inhibition of cdc13p-associated kinase activity. In *rum1*Δ cells, cdc13p levels remained completely constant (Figure 4). *Cdc13* transcript levels were unchanged after addition of P-factor to *rum1*$^+$ and *rum1*Δ cells, indicating that transcriptional control does not contribute to regulation of cdc13p in pheromone (our unpublished observations). These results establish that rum1p is required for the reduction in cdc13p levels observed after pheromone addition.

Given this result, we investigated whether cyclosome-mediated cyclin B degradation was required for pheromone-induced G_1 arrest. The *nuc2*$^+$ gene encodes a component of the cyclosome and is homologous to the budding yeast *CDC27* gene (Hirano *et al.*, 1990; Goebl and Yanagida, 1991). When pheromone was added to the temperature-sensitive *nuc2–663* mutant at the permissive temperature of 25°C, only 10% of cells arrested in G_1, demonstrating that complete *nuc2*$^+$ activity is required to bring about G_1 arrest (Figure 5A). This effect was completely reversed by *nuc2*$^+$ expression from a plasmid (Figure 5A). Cdc13p level and associated kinase activities remained high (Figure 5, B and C, bottom panels), indicating that the *nuc2* gene product and thus the cyclosome are required for pheromone-induced proteolysis of cdc13p. We have shown previously that nondegradable cdc13p prevents pheromone-induced G_1 arrest (Stern and Nurse, 1997). Together these data suggest that in pheromone-treated cells cdc13p undergoes proteolysis by a mechanism that requires both rum1p and the cyclosome. This proteolysis keeps cdc13p-associated kinase activity low, allowing G_1 arrest to be maintained in the presence of pheromone.

The Cyclosome Mediates cig2p Degradation in the Pheromone-induced G_1 Arrest

The B-cyclins cig2p and cdc13p are redundant for promoting entry into S-phase, and so the cdc2p kinase activity associated with both cyclins needs to be inhibited to bring about G_1 arrest in pheromone. Therefore we investigated the mechanism by which pheromone inhibits cig2p-associated kinase activity by monitoring cig2p levels. Cig2p levels and associated kinase activity decreased in pheromone-treated *nuc2*$^+$ cells and reached low levels 4 h after P-factor addition (Figure 5, B and C, wt). In the *nuc2–663* mutant, cig2p levels and cig2p-associated kinase activities remained high (Figure 5, B and C, top panels). This contrasts to the situation in a *rum1*Δ, where the cig2p-associated kinase activity was down-regulated in response to pheromone (Figure 3A). Cig2p levels were also down-regulated in a *rum1*Δ after addition of P-factor (Figure 5C). This was not due to reduced *cig2* transcription, which was maintained at a constant level after addition of P-factor to *rum1*Δ cells (our unpublished observations). Thus, *rum1*Δ and *nuc2–663* mutants are similar in that they fail to arrest in G_1 in response to pheromone but differ in cig2p turnover, which can occur in a *rum1*Δ but not a *nuc2–663* mutant. We conclude that cig2p-associated kinase activity is down-regulated in pheromone by cyclosome-induced cig2p proteolysis, but unlike the situation with cdc13p, this proteolysis does not require rum1p.

Effects of rum1 on Pheromone-induced Transcription

The experiments described above identify a role for rum1p in maintaining G_1 arrest after pheromone addition. We next investigated whether lack of *rum1* also affects the pheromone-induced transcription using the mating type gene *mat1-Mm*, which is specifically in-

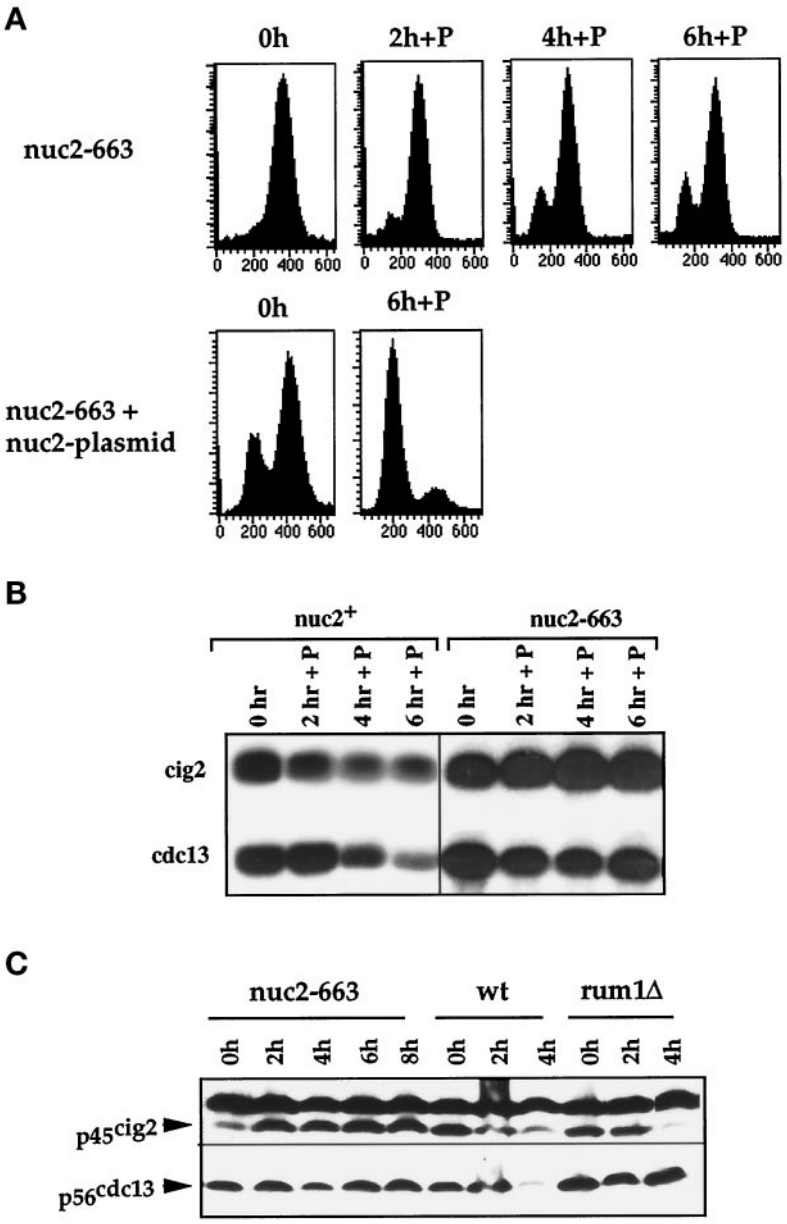

Figure 5. A mutant defective in the cyclosome/APC fails to arrest in G_1 and does not down-regulate B-cyclin protein levels and associated kinase activities. (A) FACS analysis of *nuc2–663cyr1*Δ*sxa2*Δ and *nuc2–663cyr1*Δ*sxa2*Δ containing a *nuc2*$^+$ plasmid exposed to P-factor at 25°C. 1C peak seen at 0 h in *nuc2–663* with *nuc2* plasmid is due to plasmid loss. (B) Cig2p- and cdc13p-associated H1 kinase activities in a *nuc2*$^+$ and a *nuc2–663* mutant after P-factor addition at 25°C. (C) Cig2p and cdc13p levels in *nuc2–663cyr1*Δ*sxa2*Δ (*nuc2–663*), *cyr1*Δ*sxa2*Δ (wt) and *rum1*Δ*cyr1*Δ*sxa2*Δ (*rum1*Δ) cells after exposure to P-factor. A nonspecific, 50-kDa protein cross-reacted with cig2p antibodies at a 1:1000 dilution and served as a loading control.

duced by P-factor (Willer *et al.*, 1995). Figure 6A shows that *mat1-Mm* transcript was induced in a *cyr1*Δ*sxa2*Δ after addition of P-factor. In a *rum1*Δ*cyr1*Δ*sxa2*Δ triple mutant, *mat1-Mm* transcripts were still induced by P-factor, but to a much lower level (Figure 6A).

This reduction could have been either because *rum1* was directly required for activation of pheromone-dependent transcription or because full induction of pheromone-dependent genes required G_1 arrest, which was defective in the *rum1* mutant. To distinguish between these two explanations, we assessed the expression of P-factor–induced transcription at various stages in the cell cycle. A *cyr1*Δ*sxa2*Δ strain was arrested in G_1 using the temperature-sensitive *cdc10–129* and *cdc2-M26* mutants or in early S-phase using hydroxyurea and the temperature-sensitive *cdc22-M46* mutant. The cultures were shifted to 25°C after cell cycle arrest and incubated in P-factor for 90 min. Samples for RNA preparation were taken at the beginning and the end of the P-factor treatment, and the transcript levels of two P-factor–dependent genes, *mat1-Mm* (Willer *et al.*, 1995) and *fus1* (Petersen *et al.*, 1995), were assessed. Both genes were induced by P-factor in G_1-arrested *cdc10–129* and *cdc2-M26* mutant cells, but little induction was observed in cells released from the S-phase blocks (Figure 6B). To test

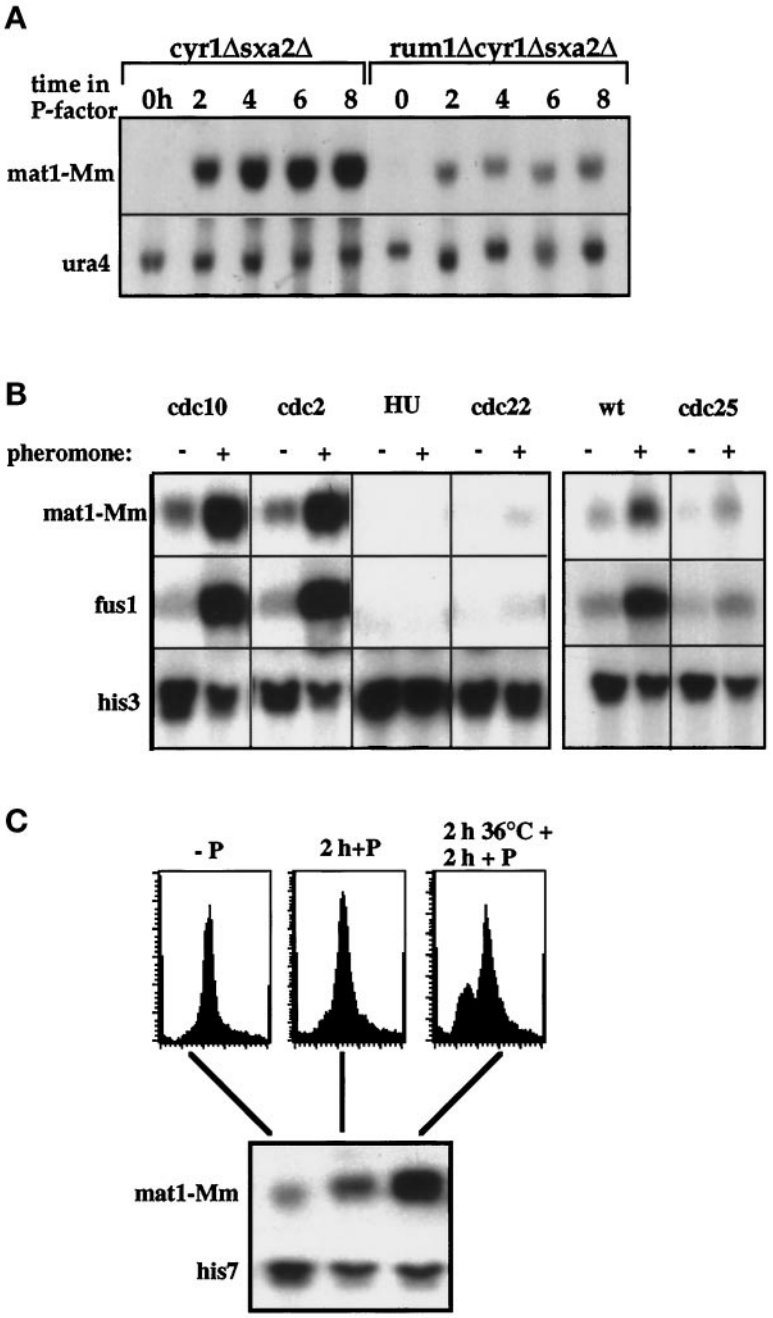

Figure 6. Expression of pheromone-dependent genes is restricted to G_1. (A) Northern blot of RNA samples from *cyr1Δsxa2Δ* and *rum1Δcyr1Δsxa2Δ* strains after addition of P-factor probed for *mat1-Mm* and for *ura4* as a loading control. (B) *Cdc10–129cyr1Δsxa2Δ* and *cdc22-M45cyr1Δsxa2Δ* strains arrested in G_1 or S, respectively, after 6 h at 36°C; *cdc2-M26cyr1Δsxa2Δ* strain after nitrogen starvation for 20 h followed by 5 h at 36.5°C in nitrogen; *cyr1Δsxa2Δ* cells after 6 h in hydroxyurea. All strains were released from the block and incubated in P-factor for 90 min. *Cyr1Δsxa2Δ* and *cdc25–22cyr1Δsxa2Δ* strains were incubated at 36°C for 4 h; P-factor was added for 90 min at 36°C. Northern blots were probed for the P-factor–inducible genes *mat1-Mm* and *fus1* and for *his3* as a loading control. (C) A *rum1Δ*cdc10–129cyr1Δ*sxa2Δ* mutant was exposed to P-factor with or without a 2-h preincubation at 36°C. DNA content analysis (top panel) and Northern blot probed with *mat1-Mm*- and *his7*-specific DNA (bottom panel).

pheromone-dependent transcription in G_2 cells, a *cdc25–22cyr1Δsxa2Δ* strain was arrested in G_2 for 4 h at 36°C and kept at the restrictive temperature during the subsequent 90 min exposure to P-factor. Although *fus1* and *mat1-Mm* transcripts were induced in a *cdc25*$^+$ control strain, induction was severely reduced in *cdc25–22* mutant cells. These results indicate that P-factor can induce expression of pheromone-dependent genes in the G_1 phase of the cell cycle, but expression is much reduced at later stages of the cell cycle. Therefore the low level of *mat1-Mm* expression in *rum1Δ* cells is likely to be caused by the failure of this strain to arrest in G_1.

To test this conclusion, a *cdc10–129rum1Δcyr1Δsxa2Δ* quadruple mutant was exposed to P-factor after a 2 h incubation at 36°C. P-factor induced a higher level of *mat1-Mm* transcript in cells that were prearrested in G_1 than in cells without the 36°C preincubation (Figure 6C). This result indicates that the level of *mat1-Mm* transcript

in a *rum1*Δ is reduced because of the shortened G_1 and can be elevated by prearresting *rum1*Δ cells in G_1. We conclude that pheromone can induce transcription only in G_1-arrested cells and that the effects of *rum1* on pheromone-induced transcription are because rum1p is required to maintain cells in G_1 for that induction to take place.

DISCUSSION

In this article we have investigated the effects of the CKI rum1p and cyclosome-dependent cyclin B degradation on pheromone-induced inhibition of the CDK cdc2p. Our major observations are as follows: 1) cyclosome-mediated degradation of cig2p and cdc13p is essential for down-regulation of cyclin B–cdc2p kinase activity during pheromone-induced G_1 arrest; 2) rum1p is required to maintain this G_1 arrest and specifically inhibits the cdc13p-cdc2p kinase; 3) rum1p mediates cdc13p turnover, whereas cig2p turnover can occur in a *rum1*-independent manner, indicating that rum1p is specifically required for cdc13p degradation by the cyclosome; and 4) pheromone-induced transcription requires cells to be in G_1 and is independent of rum1p.

Proteolysis of both cig2p and cdc13p B-cyclins in pheromone was shown to require the cyclosome by the lack of proteolysis in cells defective for the nuc2p cyclosome subunit (Figure 5). Thus cyclosome-mediated degradation of these B-cyclins is an important mechanism for pheromone-induced G_1 arrest. The maintenance of cyclosome activity during pheromone-induced G_1 arrest may involve cAMP. The cyclosome is stabilized by low cAMP levels, and mutants in *cut4*, the fission yeast Apc1/BimE cyclosome subunit, are sensitive to high levels of cAMP (Yamashita *et al.*, 1996). Pheromone response requires low levels of cAMP, and this could act in part by maintaining the cyclosome activity required to bring about cig2p and cdc13p proteolysis.

The CKI rum1p is also required for cdc13p cyclin B proteolysis and for down-regulation of cdc13p–cdc2p CDK activity. Levels of cdc13p and cdc13p–cdc2p CDK activity remain high in pheromone-treated *rum1*Δ cells, and rum1p physically interacts with cdc13p (Figure 3). This effect on proteolysis is specific because cig2p cyclin degradation does not require rum1p, even though rum1p can associate with cig2p (Figure 3). The fact that cig2p proteolysis still occurs in *rum1*Δ cells in a cyclosome-dependent manner indicates that the failure to turn over cdc13p is not simply due to the *rum1*Δ cells proceeding to a later stage in the cell cycle when the cyclosome is inactive. These data corroborate recent results that suggest that rum1p is required for cdc13p degradation in G_1 cells arrested at the cdc10 block point (Correa-Bordes *et al.*, 1997). We propose that rum1p in pheromone-treated cells acts as an adaptor protein specifically targeting cdc13p for degradation by the cyclosome during G_1 and thus maintaining G_1 arrest. rum1p is not required for the cdc13p proteolysis occurring at mitotic exit but may be necessary for inhibiting and degrading the cdc13p kinase during G_1. In contrast, rum1p is not required for cig2p proteolysis, suggesting either that no adapter protein is necessary or that one still has to be identified. Similar to *rum1*Δ, mutants in the *srw1*$^+$ gene specifically stabilize cdc13p but not cig2p (Yamaguchi *et al.*, 1998). *rum1*$^+$ and *srw1*$^+$ might act together to target cdc13p for degradation.

The initial G_1 arrest brought about by pheromone is likely to involve inhibition of the cig2p–cdc2p protein kinase by a mechanism that is independent of rum1p, although the molecular mechanism underlying pheromone signaling and the inhibition and proteolysis of the cig2p–cdc2p protein kinase remain to be elucidated. We imagine that these mechanisms bring about a transient G_1 arrest but that this cannot be maintained without further inhibition of the cdc13p–cdc2p protein kinase, because the latter can substitute for cig2p–cdc2p in bringing about S-phase (Fisher and Nurse 1996; Stern and Nurse, 1996). The transient G_1 arrest leads to a rise in rum1p levels that in turn prevents cdc13p–cdc2p protein kinase activity from increasing.

rum1p may also be able to inhibit cig2p–cdc2p activity at least temporarily, as suggested previously (Martin-Castellanos *et al.*, 1996), given that cig2p and rum1p physically interact; however, cig2p is unlikely to be an important long-term target of rum1p given that a *cig2*Δ does not rescue the G_1 arrest defect of a *rum1*Δ. The fact that a *cig2*Δ can rescue the sterility of a *rum1*Δ may be because conjugation and sporulation require both a pheromone and a starvation signal, and starvation-induced G_1 arrest is partially restored in a *cig2*Δ (Martin-Castellanos *et al.*, 1996).

The *rum1*Δ phenotype in pheromone is superficially reminiscent of the pheromone response of *far1* mutants in budding yeast. Although both *rum1* and *FAR1* encode CKIs that are essential for pheromone-induced G_1 arrest, there are important differences between their activities. Far1p inhibits the Cdc28p activity associated with the G_1 Clnp cyclins (Peter and Herskowitz, 1994), whereas rum1p specifically inhibits cdc2p associated with the mitotic B-cyclin cdc13p (Correa-Bordes and Nurse, 1995). Far1p is required exclusively for the pheromone response and is only active as a CDK inhibitor after phosphorylation by the pheromone-dependent MAP kinase Fus3p (Peter *et al.*, 1993). In contrast, the *rum1* function is not confined to pheromone response, being required in other situations with a prolonged G_1 phase, such as the extended G_1 in a *wee1–50* mutant or after nitrogen starvation (Moreno and Nurse, 1994), and in cells arrested in G_1 by a *cdc10.129* block (Correa-Bordes and Nurse, 1995). Also there is no evidence that rum1p needs an MAP

kinase-dependent phosphorylation event for activation. Bacterially produced rum1p is fully active as an inhibitor of cdc13p–cdc2p kinase (Correa-Bordes and Nurse, 1995), and a truncated *rum1* lacking all putative MAP kinase phosphorylation sites is able to rescue the sterility of a *rum1*Δ (Stern and Nurse, unpublished observations). rum1p has more in common with the second budding yeast CKI, Sic1p. Both are induced in G_1 and inhibit cyclin B-associated CDK to prevent premature onset of S-phase. However, despite these similarities, there is only very limited sequence homology between Sic1p and rum1p. The phenotypic consequences of loss of *rum1* and *SIC1* are also different, because *SIC1* is not required for sexual differentiation.

In this study we also found that pheromone induces transcription of the pheromone-dependent genes *mat1-Mm* and *fus1* only in G_1 cells (Figure 6). The cell cycle regulation of pheromone-dependent transcription might help restrict conjugation to the G_1 phase of the cell cycle. Yeast cells cannot conjugate when arrested in G_2, and mutants such as *rum1*Δ and *nuc2–663* that fail to arrest in G_1 under mating conditions are sterile (Moreno and Nurse, 1994; Kumada *et al.*, 1995). The failure to express pheromone-dependent genes later in the cell cycle could be due to reduced pheromone signaling or because a component of the transcriptional apparatus can only be activated in G_1. A possible candidate is the transcription factor ste11p (Sugimoto *et al.*, 1991), which is required for both nitrogen starvation and pheromone-induced transcription (Aono *et al.*, 1994; Petersen *et al.*, 1995). Pheromone-dependent transcription is also cell cycle regulated in budding yeast (Oehlen and Cross, 1994). The expression profile of pheromone-dependent transcripts is controlled by the activity of the G_1 CDK activity, Clnp–Cdc28p (Oehlen and Cross, 1994). Transcript levels are high in early G_1 and in S and G_2 when Clnp–Cdc28p protein kinase activity is low, and they dip in late G_1 when Clnp–Cdc28p protein kinase activity is high. Fission yeast may use a similar mechanism with cyclin B–cdc2p kinase activity, which is present from late G_1 until the end of mitosis, to restrict pheromone-induced transcription to G_1.

Fission yeast appears to use quick and reversible CKI action with irreversible cyclin turnover to inhibit B-cyclin kinases and maintain pheromone-induced G_1 arrest. A combination of CKI-mediated inhibition and proteolysis also controls the Clbp-associated kinase in budding yeast. Overexpression of nondegradable mitotic Clb2p can overcome pheromone-induced G_1 arrest (Amon *et al.*, 1994), and mutants in the cyclin B- and CDK-specific CKI Sic1p undergo premature S-phase after expression of nondegradable Clb5p in early G_1 cells (Schwob *et al.*, 1994). A recent study shows that cyclosome mutants in budding yeast are defective in pheromone-induced G_1 arrest similar to fission yeast *nuc2* mutants (Irniger and Nasmyth, 1997). Precocious S-phase in cyclosome mutants can be rescued by ectopic expression of Sic1p (Irniger and Nasmyth, 1997), indicating that CKI and cyclin proteolysis cooperate in G_1 regulation as in fission yeast. A major difference with fission yeast is that budding yeast secures a low cyclin B–associated kinase in early G_1 by both transcriptional and posttranscriptional mechanisms. In fission yeast *cdc13*$^+$ and *cig2*$^+$ transcription are not down-regulated in G_1 (Correa-Bordes and Nurse, 1995; Stern and Nurse, 1997), leaving the posttranscriptional mechanisms of cyclin B degradation and the CKI rum1p as the sole control of cyclin B-associated kinase in G_1. Posttranscriptional control using CKIs and regulation of cyclin B turnover may be the more generally used mechanism to control cyclin B–CDKs in G_1, with a transcriptional control providing a more robust control system.

It will be important to determine whether both CKIs and cyclosome-mediated proteolysis are involved in down-regulating cyclin B–CDKs or the related cyclin A–CDK in G_1 in higher eukaryotes. Cyclin A-associated CDKs have been implicated in the control of both S-phase (Girard *et al.*, 1991; Pagano *et al.*, 1992) and mitosis (Lehner and O'Farrell, 1989; Minshull *et al.*, 1989) in the Metazoa, and it may be crucial to tightly control its activity during G_1. Loss of the *Drosophila* gene *fizzy-related*, which is involved in degradation of A and B cyclins, results in cells failing to exit the cell cycle in G_1, suggesting that down-regulation of mitotic cyclins in G_1 might be equally important in higher eukaryotes as in yeast (Sigrist and Lehner, 1997). The *Drosophila roughex* (*rux*) gene also controls cyclin A kinase activity in G_1 (Gönczy *et al.*, 1994; Thomas *et al.*, 1994; Dong *et al.*, 1997). Like the *rum1*Δ mutant, *rux* mutant cells fail to arrest in G_1, and they enter S-phase prematurely, with elevated cyclin A-associated kinase activity (Thomas *et al.*, 1994; Sprenger *et al.*, 1997; Thomas *et al.*, 1997). *rux* may have a task similar to that of *rum1* in fission yeast or *SIC1* in budding yeast by preventing cyclin A from activating S-phase in early G_1.

ACKNOWLEDGMENTS

We thank Jacky Hayles, Benjamin Baum, Patrick Zarzov, and Takashi Toda for critical comments and suggestions, J. Correa-Bordes for his assistance concerning kinase assays and rum1p biochemistry, Takashi Toda for providing a *nuc2–663* strain, and all members of the ICRF Cell Cycle Laboratory for their help and support. This work was funded by ICRF. B.S. was also supported by a Boehringer Ingelheim Fonds fellowship.

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