p25^{rum1} promotes proteolysis of the mitotic B-cyclin p56^{cdc13} during G₁ of the fission yeast cell cycle

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The fission yeast *Schizosaccharomyces pombe* CDK inhibitor p25^{rum1} plays a major role in regulating cell cycle progression during G₁. Here we show that p25^{rum1} associates with the CDK p34^{cdc2}/p56^{cdc13} during G₁ in normally cycling cells and is required for the rapid proteolysis of p56^{cdc13}. *In vitro* binding data indicate that p25^{rum1} has specificity for the B-cyclin p56^{cdc13} component of the CDK and can bind the cyclin even in the absence of the cyclin destruction box. At the G₁–S-phase transition, p25^{rum1} levels decrease and p56^{cdc13} levels increase. We also show that on release from a G₁ block, the rapid disappearance of p25^{rum1} requires the activity of the CDK p34^{cdc2}/p56^{cdc13} and that this same CDK phosphorylates p25^{rum1} *in vitro*. We propose that the binding of p25^{rum1} to p56^{cdc13} promotes cyclin proteolysis during G₁, with p25^{rum1} possibly acting as an adaptor protein, promoting transfer of p56^{cdc13} to the proteolytic machinery. At the G₁–S-phase transition, p25^{rum1} becomes targeted for proteolysis by a mechanism which may involve p34^{cdc2}/p56^{cdc13} phosphorylation. As a consequence, at this point in the cell cycle p56^{cdc13} proteolysis is inhibited, leading to a rise of p56^{cdc13} levels in preparation for mitosis.

Keywords: CDK/cyclin proteolysis/G₁–S-phase transition/p25^{rum1}/p34^{cdc2}/p56^{cdc13}

Introduction

Cyclin-dependent kinases (CDKs) are important for controlling the major transitions during the cell cycle, including the onset of S-phase and mitosis. Their activities are tightly regulated by a combination of different mechanisms including transcription, phosphorylation, proteolysis and association with CDK inhibitors (CKIs) (Nasmyth, 1993; Nigg, 1995; Sherr and Roberts, 1995). These mechanisms ensure that CDK activities are kept low during those phases of the cell cycle when they are not required and only increase when they are needed to bring about cell cycle progression. An important example is the control of CDK activities during the G₁ phase. This is best understood in the budding yeast where the controls appear to involve transcriptional regulation, specific cyclin proteolysis,

phosphorylation and action of a CKI. Two types of cyclin are required for cell cycle progression in budding yeast, the CLNs acting during G₁ and the CLB acting during G₁–S and then again at mitosis (Nasmyth, 1993). There are transcriptional controls for both classes of cyclin gene which operate during the cell cycle and ensure that the cyclin proteins can only be made at the appropriate times (Amon *et al.*, 1993). In addition to these controls there are specific proteolytic mechanisms. For example, during late G₁, Cln2p first forms a complex with the E2 ubiquitin-conjugating enzyme Cdc34p, and then becomes ubiquitinated before degradation by the proteasome (Willems *et al.*, 1996). Phosphorylation of Cln2p by the p34^{cdc2} CDK is thought to activate proteolysis by promoting the association of Cln2p with Cdc34p (Lanker *et al.*, 1996; Willems *et al.*, 1996). The CLB cyclins are targeted for degradation at the end of mitosis by the anaphase-promoting complex (APC) and this proteolysis persists throughout G₁ (Amon *et al.*, 1994; Irniger *et al.*, 1995). The CKI p40^{SIC1} inhibits the CLB-dependent CDKs in G₁ and is thought to be targeted for ubiquitination by a process involving Cdc34p and CLN-dependent phosphorylation (Schwob *et al.*, 1994; Schneider *et al.*, 1996). The removal of p40^{SIC1} by the CLN protein kinases allows an increase in CLB protein kinase to occur, leading to passage through G₁. Thus, a combination of regulatory mechanisms control the appearance and disappearance of cyclins and their associated CDKs at the appropriate times during the cell cycle, resulting in an orderly progression through G₁ into S-phase.

In the fission yeast these controls are less well understood. Early G₁ cells have low levels of mitotic p34^{cdc2}/p56^{cdc13} protein kinase activity and of p56^{cdc13} (Hayles *et al.*, 1994; Correa-Bordes and Nurse, 1995). These low levels are not brought about by regulating transcription of the *cdc2* and *cdc13* genes, because these transcripts can be detected in G₁ cells (J.Hayles, unpublished results). The lack of a transcriptional control suggests that translational or proteolytic mechanisms may be more important in regulating p34^{cdc2} protein kinase activity in fission yeast G₁ cells. Of relevance to these mechanisms is the *rum1* gene which encodes the CKI p25^{rum1} (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995; Martin-Castellanos *et al.*, 1996). This CKI specifically inhibits the p34^{cdc2}/p56^{cdc13} mitotic kinase and is present only in G₁ cells when it forms a complex with p34^{cdc2}/p56^{cdc13}. In the absence of the *rum1* gene, fission yeast cells blocked in G₁ are unable to keep p34^{cdc2}/p56^{cdc13} protein kinase levels low and, as a consequence, they can initiate mitosis without having undergone S-phase. These results indicate that p25^{rum1} plays a pivotal role in regulating the p34^{cdc2}/p56^{cdc13} protein kinase during G₁.

Given these observations we have investigated the role of p25^{rum1} in G₁ regulation further. In the present study, we show that the low levels of p56^{cdc13} during G₁ are

Fig. 1. The rate of p56^{cdc13} translation in G₁ is not affected by *rum1*. Cells of *cdc10-129* and *cdc10-129 rum1Δ* strains were pulse-labelled with [³⁵S]methionine for 55 min at 25°C or 30 min after 3.5 h at 36°C. Immunoprecipitates using anti-p56^{cdc13} antibodies [lanes marked (+)] and control pre-immune antibodies [lanes marked (-)] were analysed by SDS-PAGE followed by fluorography.

brought about by p25^{rum1} promoting rapid proteolysis of p56^{cdc13}. p25^{rum1} associates *in vitro* with the p56^{cdc13} cyclin moiety, suggesting that p25^{rum1} may act as an adaptor protein which targets p56^{cdc13} for degradation. p25^{rum1} disappears upon onset of S-phase by a mechanism which may involve p34^{cdc2}/cig1p phosphorylation, and as a consequence p56^{cdc13} levels rise in preparation for mitosis. This process allows an orderly progression through G₁ of the fission yeast cell cycle.

Results

p25^{rum1} maintains high p56^{cdc13} turnover

We have previously shown that the level of the B-cyclin p56^{cdc13} is much reduced in early G₁ cells of fission yeast (Hayles *et al.*, 1994; Correa-Bordes and Nurse, 1995). To investigate whether p25^{rum1} influences p56^{cdc13} levels during G₁, we have monitored the effects of p25^{rum1} on the rates of p56^{cdc13} translation and turnover. The rate of translation of p56^{cdc13} was measured in normally cycling cells and in G₁-arrested cells, using the strains *cdc10-129* and *cdc10-129 rum1Δ* growing at 25°C, and 3.5 h after shift to 36°C. Cells were pulse-labelled with [³⁵S]methionine for 30 min and immunoprecipitations performed using anti-p56^{cdc13} antibodies [lanes marked (+) in Figure 1] and control pre-immune antibodies [lanes marked (-) in Figure 1]. Comparison of the (+) and (-) lanes showed that the anti-p56^{cdc13} antibodies specifically immunoprecipitated a protein of the correct molecular weight for p56^{cdc13}. The rate of translation of p56^{cdc13} at 25°C in cycling cells which were predominantly in G₂ was compared with that in cells arrested in G₁ by shifting to 36°C (Figure 1, compare lanes 2 and 4). After correcting for the rate of cell labelling using the unspecific upper band shown in Figure 1 as the control, the rate of incorporation into p56^{cdc13} was found to be slightly reduced at 36°C to 80% of the value at 25°C. This result indicates that the rate of p56^{cdc13} translation is approximately similar in G₁ and G₂ cells, consistent with the fact that *cdc13* transcript levels are constant throughout the cell cycle (J. Hayles, unpublished results). The rates of p56^{cdc13} translation were also similar in cycling and G₁-arrested cells, whether the *rum1* gene was present (Figure 1, lanes 2 and 4) or absent (Figure 1, lanes 6 and 8). After correcting for cell labelling as before, the rate of incorporation into p56^{cdc13} was slightly increased at 36°C to 140% of the value at 25°C. Therefore, despite the fact that p56^{cdc13} is at a much higher

Fig. 2. p25^{rum1} is required for rapid proteolysis of p56^{cdc13} in G₁. (A) The *cdc13*⁺ gene controlled by the *nmt1* promoter was expressed in a *cdc10-129 cdc13Δ* strain in the presence or absence of *rum1*. The cells of both strains were arrested in G₁ at 36°C for 2.5 h, and the *nmt* promoter switched off by adding thiamine. As a control the same experiment was performed at 25°C. (B) Abundance of the p56^{cdc13} in the G₁ extracts was determined by densitometry after immunoblotting with anti-p56^{cdc13} antibodies.

level in G₂ cells compared with G₁ cells, there is no significant difference in the rate of synthesis of the protein between the two stages of the cell cycle.

This conclusion suggests that the differences in p56^{cdc13} levels observed in the presence and absence of *rum1* are due to changes in p56^{cdc13} turnover. To test this directly, we attempted to perform the appropriate pulse-chase experiments but were unable to perform the chase using normal physiological growth conditions. As an alternative, we estimated p56^{cdc13} turnover by following p56^{cdc13} levels after switching off *cdc13* transcription. *cdc13* was expressed using the medium-level *nmt* repressible promoter of pREP41 stably integrated into the *cdc10-129 cdc13Δ* and *cdc10-129 cdc13Δ rum1Δ* strains. The cells of both strains were arrested largely in G₁ by shifting to 36°C for 2.5 h, the *nmt* promoter switched off by adding thiamine, and the levels of p56^{cdc13} monitored by Western blot analysis. It can be seen from Figure 2 that p56^{cdc13} is proteolysed at a higher rate in G₁ cells when p25^{rum1} is present; p56^{cdc13} has a half-life of ~10 min in the presence of *rum1* and 30 min in the absence of *rum1*. No difference in the kinetics of p56^{cdc13} proteolysis was observed after switching off *cdc13* transcription at 25°C, where exponentially growing cells are mainly in G₂ (Figure 2A). The increase in half-life of p56^{cdc13} from 10 min to 30 min that occurs when *rum1* is deleted explains why the rate of incorporation into p56^{cdc13} changes to 140% of control in the absence of *rum1* compared with 80% of control in the presence of *rum1*. If the rate of p56^{cdc13} translation in both situations were approximately similar, then decreased

Fig. 3. p25^{rum1} is not required for down-regulation of p42^{cut2} in G₁. *cdc10-129* and *cdc10-129 rum1Δ* strains were grown in minimal medium at 25°C and then shifted to 36°C for 4 h. Samples were taken to make total extracts as described. Proteins (25 mg) from these extracts were Western blotted and probed with anti-p56^{cdc13} and anti-p42^{cut2} antibodies. Total extracts of exponentially growing *cut2HA+* cells were used as a control for anti-p42^{cut2} antibodies.

turnover in the absence of *rum1* would result in a higher level of detected incorporation compared with cells containing *rum1*.

The increase in p56^{cdc13} stability in *rum1Δ* cells could be explained by p25^{rum1} being specifically required for rapid proteolysis of the mitotic cyclin in G₁, or could be due to a rise in mitotic kinase activity caused by the absence of p25^{rum1} resulting in cells moving from G₁ to some state later in the cell cycle. To investigate this further, we have monitored the levels of p42^{cut2} in G₁ in the presence or absence of *rum1*. p42^{cut2} is degraded in anaphase and remains unstable during G₁ (Funabiki *et al.*, 1996a), and it has been reported that cells arrested using a *cdc10^{ts}* mutant have reduced levels of p56^{cdc13} and p42^{cut2} (Funabiki *et al.*, 1996b). Therefore, p42^{cut2} turnover acts as a marker for a cell being in G₁. We found that p56^{cdc13} was undetectable and p42^{cut2} levels were reduced 2- to 3-fold in a *cdc10-129* mutant (Figure 3, lane 2). When *rum1* was deleted, a similar drop in p42^{cut2} levels was still observed but in contrast p56^{cdc13} remained at a high level (Figure 3, lane 4). Extracts from exponentially growing *cut2HA+* cells were used to confirm that the anti-cut2 antibodies were detecting p42^{cut2} (Figure 3, lane 5). This result shows that G₁ down-regulation of p42^{cut2} still occurs in the double mutant *cdc10-129 rum1Δ* at the restrictive temperature, indicating that p25^{rum1} is required specifically for p56^{cdc13} proteolysis in G₁, but is not required for p42^{cut2} proteolysis. This suggests that the effects of p25^{rum1} on p56^{cdc13} proteolysis are likely to be specific and are not due to cells moving from G₁ to a later state in the cell cycle. In the absence of p25^{rum1}, proteolysis still occurs but the rate is reduced to a level which is insufficient to counteract the continuing translation of p56^{cdc13}. As a consequence, the level of p56^{cdc13} remains high in G₁ cells lacking p25^{rum1}.

p25^{rum1} binds p56^{cdc13} in vitro

The above results indicate that p25^{rum1} promotes p56^{cdc13} turnover in G₁-arrested cells. Our earlier work showed that p25^{rum1} associates directly with p56^{cdc13} in cells arrested in G₁ (Correa-Bordes and Nurse, 1995). To investigate this process of association further, we have examined the ability of p25^{rum1} to form complexes with

Fig. 4. p25^{rum1} has *in vitro* binding specificity for p56^{cdc13}. *In vitro*-translated [³⁵S]methionine-labelled products of the indicated cyclins and p34^{cdc2} were incubated with (lanes +) or without (lanes -) p25^{rum1}. Immunocomplexes obtained using anti-p25^{rum1} antibodies were separated by SDS-PAGE followed by fluorography.

p56^{cdc13} *in vitro*. We first tested whether p25^{rum1} could bind either p56^{cdc13} alone or p56^{cdc13} complexed with p34^{cdc2}. Both p34^{cdc2} and p56^{cdc13} were translated *in vitro* using [³⁵S]methionine and the Promega-TNT system. They were then tested separately and after mixing to determine whether they could form complexes with p25^{rum1}. This was carried out by adding purified bacterially produced p25^{rum1} to the *in vitro* translation mixes for 30 min, followed by immunoprecipitation with anti-p25^{rum1} antibodies. In Figure 4, the labelled proteins produced by *in vitro* translation are shown by the lanes marked 'Input', while the immunoprecipitations with anti-p25^{rum1} and control antibodies are shown respectively as (+) and (-). It can be seen that p25^{rum1} could form specific complexes with p56^{cdc13} and with p34^{cdc2}/p56^{cdc13} complexes but not with p34^{cdc2} alone. We next tested whether the destruction box which is required for B-cyclin proteolysis in mitosis was required for p25^{rum1} binding. The *cdc13Δ90* mutant lacks this destruction box, but the mutant protein could still form an *in vitro* complex with p25^{rum1} (Figure 4). Finally, we tested if p25^{rum1} could form *in vitro* complexes with the B-cyclins encoded by *cig1* and *cig2* or with complexes of these cyclins with p34^{cdc2}. No specific p25^{rum1} association was detected with these cyclins, either alone or in association with p34^{cdc2}.

We conclude that p25^{rum1} can become stably associated either with free p56^{cdc13} or with p56^{cdc13} complexed with p34^{cdc2} and that this association does not require the cyclin destruction box. In addition, the association appears to be specific to the B-cyclin p56^{cdc13} because there is no detectable association with other B-cyclins encoded by *cig1* and *cig2*, at least *in vitro*. These results suggest that p25^{rum1} specifically associates either with free p56^{cdc13} or with p56^{cdc13}/p34^{cdc2} complexes present in G₁ cells and that these associations may play a role in targeting p56^{cdc13} for proteolysis during this phase of the cell cycle.

p25^{rum1} association with p56^{cdc13}/p34^{cdc2} during the cell cycle

If p25^{rum1} targets p56^{cdc13} for proteolysis when cells are arrested in G₁, then p25^{rum1} association with p56^{cdc13} or

Fig. 5. p25^{rum1}-associated kinase is p34^{cdc2} dependent. p25^{rum1} (lanes 1–6) and p34^{cdc2} (lanes 7–10) were immunoprecipitated from extracts of the indicated strains grown at 25°C and assayed for H1-histone kinase activity at either 25 or 40°C. p25^{rum1} immunoprecipitates from wild-type cells were assayed for H1-histone kinase activity in the absence (lane 1) or presence of 2 nM p25^{rum1} (lane 2).

with p56^{cdc13}/p34^{cdc2} complexes might be expected to vary during the normal cell cycle, peaking in G₁. We attempted to test this possibility by immunoprecipitations using anti-p25^{rum1} antibodies followed by Western blotting, but the limited amounts of material available from selection synchronous cultures prevented this approach from being successful. As an alternative, we monitored whether p25^{rum1} was associated with p56^{cdc13}/p34^{cdc2} protein kinase activity, because enzymatic assays can be more sensitive than Western blotting. In a first experiment, anti-p25^{rum1} antibodies were used to immunoprecipitate from extracts prepared from asynchronously growing wild-type, *rum1Δ* and the temperature-sensitive *cdc2-33* strains. An H1-histone kinase activity was detected in the extracts from the wild-type cells but not from the *rum1Δ* cells grown at 25°C (Figure 5, lanes 3 and 4). This activity entirely disappeared when assayed at 40°C in the extract prepared from the *cdc2-33* strain, but was still present in the wild-type extract at 40°C (Figure 5, lanes 5 and 6). Because the p34^{cdc2} H1-histone kinase activity is temperature-sensitive in this strain—as shown by assays performed with immunoprecipitates prepared using anti-p34^{cdc2} antibodies (Figure 5, lanes 7–10)—we conclude that the H1-histone kinase activity associated with p25^{rum1} is due to p34^{cdc2}. This protein kinase activity was shown to be derived mostly from p56^{cdc13}/p34^{cdc2} complexes, by exploiting the fact that these complexes are very sensitive to the addition of bacterially produced p25^{rum1} to the *in vitro* protein kinase assays, while complexes of the *cig2* cyclin are only partially sensitive and complexes of the *cig1* cyclin are completely insensitive to p25^{rum1} (Correa-Bordes and Nurse, 1995). Addition of p25^{rum1} to the anti-p25^{rum1} immunoprecipitates completely inhibited the H1-histone kinase activity, establishing that p56^{cdc13} is the major B-cyclin involved in the active complexes (Figure 5, lanes 1 and 2). These results indicate that p25^{rum1} immunoprecipitates contain active p56^{cdc13}/p34^{cdc2} protein kinase activity. This activity can be inhibited by the addition of further p25^{rum1} *in vitro*, behaviour similar to that observed with p21^{cip1} immunoprecipitates from mammalian cells which contain CDK protein kinase activity inhibited by the addition of further p21^{cip1} (Zhang *et al.*, 1994). Possibly the complexes between p25^{rum1} and p56^{cdc13}/p34^{cdc2} formed in the immunoprecipitates become dissociated under the conditions of the *in vitro* protein kinase assay, leading to restoration of enzymatic activity.

Having established that p25^{rum1} immunoprecipitates contain p56^{cdc13}/p34^{cdc2} protein kinase activity, extracts were prepared from different time points of a synchronous

Fig. 6. Oscillation of p25^{rum1}-associated p34^{cdc2} kinase during the cell cycle. Small wild-type cells in early G₂ were selected by elutriation and reinoculated in minimal medium at 32°C, and samples taken for determination of septation index and H1-histone kinase activity present in p25^{rum1} immunoprecipitates.

culture and immunoprecipitations performed using the anti-p25^{rum1} antibodies (Figure 6). These antibodies detected a peak in H1-histone kinase activity 20 min before the peak of septation, timing which corresponded to the period of the cell cycle when cells were traversing through G₁. These data are consistent with p25^{rum1} becoming associated with p56^{cdc13}/p34^{cdc2} protein kinase activity during the G₁ phase of the cell cycle, and we propose that this association is an early step in the process which leads to p56^{cdc13} proteolysis. As a consequence, the p56^{cdc13}/p34^{cdc2} protein kinase activity is kept at a low level during this phase of the cell cycle, preventing inappropriate entry into S-phase or mitosis.

As cells exit G₁ and proceed through S-phase, p56^{cdc13}/p34^{cdc2} protein kinase levels begin to rise. This rise is associated with a drop in p25^{rum1} levels, as shown in Figure 7B where temperature-sensitive *cdc10-V50* cells have been released from a G₁ block. Cells were arrested in G₁ by incubation for 4 h at 36°C and were then shifted down to 25°C. Within 60 min of shift-down, cells had entered S-phase, and by 90 min S-phase was largely complete (Figure 7A). Western blotting showed that p25^{rum1} had disappeared from cells by 60 min and by this time p56^{cdc13} had also begun to accumulate (Figure 7B). This result suggests that the disappearance of p25^{rum1} at the end of G₁ is important in allowing p56^{cdc13} levels to accumulate, and thus the reduction of p25^{rum1} levels at the end of G₁ may be an important regulatory mechanism of cell cycle progression.

***cig1p*-associated p34^{cdc2} kinase phosphorylates p25^{rum1}**

Phosphorylation has been implicated in targeting a number of proteins for proteolysis, and p25^{rum1} has several consensus phosphorylation sites for the p34^{cdc2} protein kinase (Moreno and Nurse, 1994). Therefore we decided to investigate whether p34^{cdc2} phosphorylation of p25^{rum1} might have a role in reducing the level of p25^{rum1} as cells exit G₁. Firstly, we tested whether p25^{rum1} was an *in vitro* substrate for the p34^{cdc2} protein kinase. Immunoprecipitates from a wild-type extract were prepared using antibodies against p34^{cdc2} and the three B-cyclins encoded by *cdc13*, *cig1* and *cig2* (Hagan *et al.*, 1988; Booher *et al.*, 1989; Moreno *et al.*, 1989; Bueno *et al.*, 1991; Obara-

Fig. 7. The drop of p25^{rum1} levels at the onset of S-phase depends on cig1p/p34^{cdc2} activity. (A) FACS analysis of G₁ block and release experiments of indicated strains. Cells were arrested in G₁ by incubation for 4 h at 36°C and shifted down to 25°C. (B) Western blots from total extracts of the experiments described above were probed with anti-p25^{rum1}, anti-cig2p and anti-p56^{cdc13} antibodies.

Ishihara and Okayama, 1994). p25^{rum1} kinase activity was detected with both the anti-p34^{cdc2} and anti-cig1p antibodies, but not with the other two (Figure 8). This result indicates that p25^{rum1} can be phosphorylated *in vitro* by the p34^{cdc2} protein kinase activity associated with the *cig1*-encoded B-cyclin, but not with the other two B-cyclins.

We next investigated whether the *cig1* gene had any *in vivo* effects on p25^{rum1} levels. These experiments were carried out in a *cdc10-V50* strain in which *cig1* had been deleted, together with control cells in which *cig2* was deleted. In *cig1Δ* cells, p25^{rum1} was still present at high levels 90 min after release from the *cdc10-V50*-imposed G₁ block, 60 min longer than controls (Figure 7B), indicating that *cig1* is required for the rapid turnover of p25^{rum1} that occurs when cells are released from a G₁ block. Therefore the direct phosphorylation of p25^{rum1} by the cig1p-associated p34^{cdc2} protein kinase may play a role in regulating p25^{rum1} turnover on progression from G₁ to S-phase. The *cig1Δ* cells were also delayed from entering S-phase on release from the *cdc10-V50* block by ~30–45 min compared with *cdc10-V50* control (Figure 7A). In contrast, *cdc10-V50 cig2Δ* showed no significant differences in either p25^{rum1} or S-phase entry compared with *cdc10-V50* (Figure 7A and B).

Two further points can be made from these experiments.

Fig. 8. cig1p/p34^{cdc2} complex phosphorylates p25^{rum1} *in vitro*. p34^{cdc2} associated with different cyclins was immunoprecipitated from wild-type extracts and assayed for protein kinase activity using p25^{rum1} as a substrate.

Firstly, the rapidity with which p56^{cdc13} appears after release from the G₁ block suggests that inactivation of the p56^{cdc13} proteolytic machinery, possibly involving *cig1*, occurs very rapidly following activation of the *cdc10* function. Secondly, deleting *cig2* had no effect on the timing of S-phase onset in the *cdc10-V50* G₁ block and release experiments. In these experiments, the cells are larger than normal and it is possible that in the enlarged cells formed in these circumstances, the *cig2*-associated p34^{cdc2} protein kinase is no longer limiting for G₁ progression (Fisher and Nurse, 1996).

Discussion

The fission yeast B-cyclin p56^{cdc13}/p34^{cdc2} protein kinase is able to bring about the onset of mitosis in G₁ cells (Hayles *et al.*, 1994) and therefore needs to be kept under tight control to avoid a catastrophic segregation of unreplicated chromosomes. We have previously shown that the CDK inhibitor p25^{rum1} is crucially involved in this control and that it acts as a potent inhibitor of p56^{cdc13}/p34^{cdc2} protein kinase activity (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995). In the present work we have demonstrated that p25^{rum1} is required for rapid proteolysis of p56^{cdc13} in G₁ cells and that the regulation of p25^{rum1} during the cell cycle may involve the p34^{cdc2} protein kinase associated with the B-cyclin cig1p. Specifically, we have made the following observations: (i) In G₁-arrested cells, p56^{cdc13} disappears rapidly and this disappearance requires *rum1*. Another protein proteolysed in G₁, p42^{cut2} (Funabiki *et al.*, 1996a), does not require *rum1* for turnover. (ii) The rate of translation of p56^{cdc13} in G₁ cells is the same in the presence or absence of the *rum1* gene, but the rate of p56^{cdc13} proteolysis is increased in the presence of *rum1*. (iii) p25^{rum1} binds specifically *in vitro* either to p56^{cdc13} or to the p56^{cdc13}/p34^{cdc2} complex, even when the cyclin destruction box is lacking. (iv) p25^{rum1} associates with the p56^{cdc13}/p34^{cdc2} protein kinase during G₁ of the normal cell cycle. (v) The cig1p/p34^{cdc2} protein kinase phosphorylates p25^{rum1} *in vitro* and, in the absence of cig1p, the disappearance of p25^{rum1} is delayed when cells are released from a G₁ block.

From these observations we conclude that an important role for p25^{rum1} in cell cycle regulation is to associate with p56^{cdc13} or p56^{cdc13}/p34^{cdc2} complexes in G₁ cells and to promote p56^{cdc13} proteolysis. We imagine that, as well as acting as a direct inhibitor of p56^{cdc13}/p34^{cdc2} protein kinase activity, p25^{rum1} has a second role as an adaptor promoting transfer of p56^{cdc13} to the proteolytic machinery. Given that p56^{cdc13} translation is not reduced in G₁ cells, the p25^{rum1}-mediated promotion of p56^{cdc13}

proteolysis must be a major mechanism preventing the accumulation of active p56^{cdc13}/p34^{cdc2} protein kinase activity in G₁ cells. This is of considerable importance because p34^{cdc2} is not inhibited by Y15 phosphorylation during G₁ (Hayles and Nurse, 1995) and, as a consequence, p56^{cdc13}/p34^{cdc2} protein kinase activity could appear in G₁ cells and bring about an inappropriate entry into S-phase or a catastrophic entry into mitosis. Proteolysis of p56^{cdc13} can still occur in cells lacking *rum1*, and is clearly sufficient to allow exit from mitosis. However, the rate of proteolysis is insufficient to reduce the levels of p56^{cdc13} during the G₁ phase of the cell cycle. p25^{rum1} may act as an adaptor, with the binding of p25^{rum1} to p56^{cdc13} enhancing the transfer of cyclin to the *nuc2/cut9* proteasome machinery (Hirano *et al.*, 1988; Samejima and Yanagida, 1994). Such a mechanism would share characteristics with other proteolytic processes where association with a specific adaptor protein targets a substrate protein for rapid turnover via the proteasome. For example, the E6 protein binds p53 and targets it for ubiquitination, and antizyme targets ornithine decarboxylase to the proteasome (Ciechanover, 1994). p25^{rum1} appears to have an effect on p56^{cdc13} proteolysis only in G₁ cells and not during mitotic exit. Perhaps there are differences in the cyclin proteolytic mechanisms at these two different stages of the cell cycle with p25^{rum1} only having a significant targeting role during G₁.

We have shown that proteolysis of another protein p42^{cut2} during G₁ is not affected by the absence of *rum1*, suggesting that the effect of p25^{rum1} on p56^{cdc13} proteolysis is direct and specific. However, we cannot exclude the possibility that there may also be some indirect effects of p25^{rum1} working through the p34^{cdc2} mitotic kinase, the activity of which will increase in the absence of *rum1*.

On entry into S-phase, p56^{cdc13} levels begin to increase in preparation for the onset of mitosis. The timing of this increase is correlated with a decrease in p25^{rum1} levels. Our results suggest that the *cig1p*/p34^{cdc2} protein kinase may have a role in this mechanism. In the absence of *cig1*, the decrease in p25^{rum1} levels is much delayed as cells exit G₁ after release from a block imposed using a *cdc10^s* mutant. The protein kinase *cig1p*/p34^{cdc2} can phosphorylate p25^{rum1} *in vitro*, unlike the p56^{cdc13}/p34^{cdc2} or *cig2p*/p34^{cdc2} protein kinases. We speculate that, at the G₁–S-phase boundary, *cig1p* can promote p25^{rum1} proteolysis as a consequence of the *cig1p*/p34^{cdc2} protein kinase phosphorylating p25^{rum1}. The subsequent reduction in p25^{rum1} levels allows p56^{cdc13} to accumulate, preparing cells for the onset of mitosis. There may also be some effect on the timing of S-phase which is somewhat delayed in cells lacking *cig1* after release from a G₁ block. The mechanism regulating the timing of the *cig1*-dependent p25^{rum1} proteolysis is not understood, but is likely to involve *cdc10*-dependent transcription, given that p25^{rum1} proteolysis occurs very rapidly after *cdc10* function is returned to cells.

This work has emphasized the importance of cell cycle-regulated B-cyclin and CDK inhibitor proteolysis and phosphorylation for controlling G₁ progression in fission yeast. There are some general similarities with the controls acting in the budding yeast. In this organism, B-cyclin proteolysis is activated at mitosis and persists in G₁ until cells pass Start just before S-phase (Amon *et al.*, 1994).

This B-cyclin destruction requires the Cdc6p, Cdc23p and Cdc27p proteins, all components of the E3-ubiquitin protein ligase proteasome complex, the activity of which is regulated by phosphorylation (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995). The CLN cyclins are also required in G₁ to promote onset of S-phase, and Cln2p degradation is induced by p34^{CDC28} CDK-dependent phosphorylation. It is likely that Cdc53p is a component of a ubiquitin–protein ligase complex that targets phosphorylated Cln2p for degradation by the proteasome pathway (Lanker *et al.*, 1996; Willems *et al.*, 1996). There is also selective proteolysis of the CDK inhibitor p40^{SIC1}; phosphorylation of the inhibitor by the Cln/Cdc28 protein kinase may target it for ubiquitination by the Cdc34/Cdc53/Cdc4 pathway (Donovan *et al.*, 1994; Schwob *et al.*, 1994; Schneider *et al.*, 1996).

Although there are clear general similarities between the two yeasts, there are differences in detail. For example, a CLN CDK phosphorylates the CDK inhibitor p40^{SIC1} in budding yeast while a B-cyclin CDK phosphorylates the CDK inhibitor p25^{rum1} in fission yeast. Also, p40^{SIC1} inhibits the B-cyclins which normally act in G₁ in budding yeast while p25^{rum1} inhibits the B-cyclin which normally acts in G₂ in fission yeast. However, the general picture which emerges from these studies is that there are global changes during the cell cycle in patterns of both specific proteolysis and CDK phosphorylation which are important for regulating cell cycle progression. For example, it appears that in both yeasts proteolysis of mitotic B-cyclins is active during G₁, and CDK-mediated phosphorylation of a CDK inhibitor at the end of G₁ leads to proteolysis of the inhibitor. The latter mechanism could contribute to a sharp on/off switch allowing an irreversible transition from G₁ to S-phase. Further work should help to identify the more general rules applying to all cell cycles and this should assist the understanding of cell cycle controls operating in other more complex cells.

Materials and methods

Fission yeast strains and methods

The following strains were used: 972h⁺; *cdc10-V50*, *ura4Δ18*, h⁺; *cdc10-V50*, *cig1Δ::ura4⁺*, *ura4Δ18*, h⁺; *cdc10-V50*, *cig2Δ::ura4⁺*, *ura4Δ18*, h⁺; *cdc10-129*, *cdc13Δ::ura4⁺*, *ura4Δ18*, *leu1-32*, *RIP45 cdc13⁺* integrant h⁺; *cdc10-129*, *cdc13Δ::ura4⁺*, *rum1Δ::ura4⁺*, *ura4Δ18*, *leu1-32*, *RIP45 cdc13⁺* integrant h⁺; and *cut-364::cut2+HA::LEU2*, *leu1-32* h⁺.

Published procedures were used for growth and classical genetic manipulations (Moreno *et al.*, 1991). The deletions of *cig1*, *cig2* and *cdc13* have been published previously (Bueno *et al.*, 1991; Obara-Ishihara and Okayama, 1994; Fisher and Nurse, 1996). Flow-cytometric analysis of DNA content was performed on a Becton-Dickinson FACS scan using propidium iodide staining of cells as described in Sazer and Sherwood (1990). *cdc13* switch-off experiments were performed as follows: cells were grown in minimal medium without thiamine at 25°C to mid-exponential phase, 400 ml of cells (2×10⁶ cells/ml) were shifted up to 36°C for 2.5 h and the promoter was switched off by adding thiamine (5 mg/ml) to the growth medium. Samples (50 ml) were taken every 15 min for a further 75 min, cells were harvested by centrifugation and extracts were made as described below. Synchronous cultures were prepared by centrifugal elution. Minimal MEM media were used for liquid cultures.

In vitro binding assay

The coding region of the *cdc2* gene and B-type cyclins genes (*cdc13*, *cig1* and *cig2*) were subcloned into a T7 expression vector. These constructs (1 mg) were translated *in vitro* using the TNT-coupled

reticulocyte lysate system (Promega) in a final volume of 50 ml. Five ml of each [³⁵S]methionine-translated reaction was incubated with (20 ng) or without purified bacterially produced p25^{rum1} in 20 ml of HB buffer for 30 min at room temperature. After incubation, 280 ml of HB buffer was added and immunoprecipitation performed using anti-rum1 antibodies. The immunoprecipitates were washed four times with the binding buffer and immunocomplexes separated by SDS-PAGE followed by fluorography.

In vivo [³⁵S]methionine labelling

Cells were grown in minimal medium (MEM), with glutamate (1 g/l) as a nitrogen source, to an OD₅₉₅ of 0.2 (4 × 10⁶ cells/ml). To label the proteins with ³⁵S, 10 ml of cells were incubated with 1 mCi of [³⁵S]methionine (Amersham Promix) for 30 (in the case of G₁-arrested cells at 36°C) or 55 min (for exponentially growing cells at 25°C). After labelling, cells were harvested and washed with cold STOP buffer and resuspended in 20 ml of cold HB buffer. Five hundred ml of glass beads were added to each sample and cells were broken by vortexing vigorously for three periods, each of 30 s. After the cell breakage, the crude extracts were recovered by washing the glass beads with 600 ml of cold HB buffer. Cell debris was removed by a 5 min spin in a microcentrifuge and p56^{cdc13} was isolated from the soluble extract by immunoprecipitation with anti-cdc13 antibodies (SP4).

Protein extracts, Western blots and protein kinase assay

Protein extracts for immunoblotting were prepared as described previously (Correa-Bordes and Nurse, 1995). For the Western blot, 20–25 mg of total protein extracts from each sample were electrophoresed using a 10% SDS-PAGE and transferred to Hybond Immobilon (Amersham) or nitrocellulose membranes; immunodetection used polyclonal antisera to p56^{cdc13}, p25^{rum1}, p46^{cig2}, p42^{cut2} and monoclonal α-tubulin antibodies (Sigma). Blots were quantified by densitometric scanning using an LKB scanner and were corrected for loading using α-tubulin loading controls. Protein extracts for kinase assay were prepared as described (Moreno *et al.*, 1991). The different cyclin-associated p34^{cdc2} kinase activities were immunoprecipitated from 400 μg of soluble extracts using polyclonal antisera to p34^{cdc2} (C2), p56^{cdc13} (SP4), cig1p (Basi and Draetta, 1995) and p46^{cig2}. The rum1-associated H1 kinase activity was immunoprecipitated with polyclonal antisera to p25^{rum1}. Antisera were incubated with the extracts on ice for 1 h, 10 ml of 50% (v/v) protein A-Sepharose Cl4B (Pharmacia) was added to the samples incubated with agitation at 4°C for 30 min. Immunocomplexes were harvested in a microcentrifuge at 2000 r.p.m. for 5 s and washed three times in HB buffer. The supernatant was removed and 20 ml of reaction buffer added. For H1-histone kinase assays, the reaction buffer was 200 mM [γ-³²P]ATP (40 mCi/ml; Amersham), 1 mg/ml H1-histone (Calbiochem) in HB; and for rum1 kinase assays it was 200 mM [γ-³²P]ATP (40 mCi/ml), 0.2 mg/ml p25^{rum1} in HB. In both cases, the reactions were incubated at 30°C for 20 min. The temperature-sensitive p34^{cdc2} assays were performed at 25 or 40°C (5 min pre-incubation of the immunoprecipitated at the required temperature was performed before addition of the substrate). Reactions were stopped by adding an equal volume of 2× sample buffer and boiled for 5 min. Samples were run on a 12% polyacrylamide gel. Phosphorylated H1-histone was detected by autoradiography and quantitated on a Molecular Dynamics PhosphorImager using ImageQuant software.

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