p25\textsuperscript{rum1} promotes proteolysis of the mitotic B-cyclin p56\textsuperscript{cdc13} during G\textsubscript{1} of the fission yeast cell cycle

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The fission yeast *Schizosaccharomyces pombe* CDK inhibitor p25\textsuperscript{rum1} plays a major role in regulating cell cycle progression during G\textsubscript{1}. Here we show that p25\textsuperscript{rum1}, associates with the CDK p34\textsuperscript{cdc2}/p56\textsuperscript{cdc13} during G\textsubscript{1} in normally cycling cells and is required for the rapid proteolysis of p56\textsuperscript{cdc13}. In vitro binding data indicate that p25\textsuperscript{rum1} has specificity for the B-cyclin p56\textsuperscript{cdc13} component of the CDK and can bind the cyclin even in the absence of the cyclin destruction box. At the G\textsubscript{1}–S-phase transition, p25\textsuperscript{rum1} levels decrease and p56\textsuperscript{cdc13} levels increase. We also show that on release from a G\textsubscript{1} block, the rapid disappearance of p25\textsuperscript{rum1} requires the activity of the CDK p34\textsuperscript{cdc2}/cig1l and that this same CDK phosphorylates p25\textsuperscript{rum1} in vitro. We propose that the binding of p25\textsuperscript{rum1} to p56\textsuperscript{cdc13} promotes cyclin proteolysis during G\textsubscript{1}, with p25\textsuperscript{rum1} possibly acting as an adaptor protein, promoting transfer of p56\textsuperscript{cdc13} to the proteolytic machinery. At the G\textsubscript{1}–S-phase transition, p25\textsuperscript{rum1} becomes targeted for proteolysis by a mechanism which may involve p34\textsuperscript{cdc2}/cig1lp phosphorylation. As a consequence, at this point in the cell cycle p56\textsuperscript{cdc13} proteolysis is inhibited, leading to a rise in p56\textsuperscript{cdc13} levels in preparation for mitosis.

Keywords: CDK/cyclin proteolysis/G\textsubscript{1}–S-phase transition/p25\textsuperscript{rum1}/p34\textsuperscript{cdc2}/p56\textsuperscript{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 G\textsubscript{1} 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 G\textsubscript{1} and the CLBs acting during G\textsubscript{1}–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 G\textsubscript{1}, Cln2p first forms a complex with the E2 ubiquitin-conjugating enzyme Cdc34p, and then becomes ubiquitinated before degradation by the proteosome (Willems et al., 1996). Phosphorylation of Cln2p by the p34\textsuperscript{CDC28} 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 G\textsubscript{1} (Amon et al., 1994; Iminger et al., 1995). The CKI p40\textsuperscript{SIC1} inhibits the CLB-dependent CDKs in G\textsubscript{1} 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\textsuperscript{SIC1} by the CLN protein kinases allows an increase in CLB protein kinase to occur, leading to passage through G\textsubscript{1}. 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 G\textsubscript{1} into S-phase.

In the fission yeast these controls are less well understood. Early G\textsubscript{1} cells have low levels of mitotic p34\textsuperscript{cdc2}/p56\textsuperscript{cdc13} protein kinase activity and of p56\textsuperscript{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 G\textsubscript{1} cells (J.Hayles, unpublished results). The lack of a transcriptional control suggests that translational or proteolytic mechanisms may be more important in regulating p34\textsuperscript{cdc2} protein kinase activity in fission yeast G\textsubscript{1} cells. Of relevance to these mechanisms is the *rum1* gene which encodes the CKI p25\textsuperscript{rum1} (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995; Martin-Castellanos et al., 1996). This CKI specifically inhibits the p34\textsuperscript{cdc2}/p56\textsuperscript{cdc13} mitotic kinase and is present only in G\textsubscript{1} cells when it forms a complex with p34\textsuperscript{cdc2}/p56\textsuperscript{cdc13}. In the absence of the *rum1* gene, fission yeast cells blocked in G\textsubscript{1} are unable to keep p34\textsuperscript{cdc2}/p56\textsuperscript{cdc13} protein kinase levels low and, as a consequence, they can initiate mitosis without having undergone S-phase. These results indicate that p25\textsuperscript{rum1} plays a pivotal role in regulating the p34\textsuperscript{cdc2}/p56\textsuperscript{cdc13} protein kinase during G\textsubscript{1}.

Given these observations we have investigated the role of p25\textsuperscript{rum1} in G\textsubscript{1} regulation further. In the present study, we show that the low levels of p56\textsuperscript{cdc13} during G\textsubscript{1} are
with [35S]methionine for 55 min at 25°C or 30 min after 3.5 h at 36°C. Immunoprecipitates using anti-p56cdc13 antibodies [lanes marked (+)] and control pre-immune antibodies [lanes marked (–)] were analysed by SDS-PAGE followed by fluorography.

brought about by p25rum1 promoting rapid proteolysis of p56cdc13. p25rum1 associates in vitro with the p56cdc13 cyclin moiety, suggesting that p25rum1 may act as an adaptor protein which targets p56cdc13 for degradation. p25rum1 disappears upon onset of S-phase by a mechanism which may involve p34cdc2/cig1p phosphorylation, and as a consequence p56cdc13 levels rise in preparation for mitosis. This process allows an orderly progression through G1 of the fission yeast cell cycle.

Results

p25rum1 maintains high p56cdc13 turnover

We have previously shown that the level of the B-cyclin p56cdc13 is much reduced in early G1 cells of fission yeast (Hayles et al., 1994; Correa-Bordes and Nurse, 1995). To investigate whether p25rum1 influences p56cdc13 levels during G1, we have monitored the effects of p25rum1 on the rates of p56cdc13 translation and turnover. The rate of translation of p56cdc13 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-p56cdc13 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-p56cdc13 antibodies specifically immunoprecipitated a protein of the correct molecular weight for p56cdc13. The rate of translation of p56cdc13 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 p56cdc13 was found to be slightly reduced at 36°C to 80% of the value at 25°C. This result indicates that the rate of p56cdc13 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 p56cdc13 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 p56cdc13 was slightly increased at 36°C to 140% of the value at 25°C. Therefore, despite the fact that p56cdc13 is at a much higher 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 p56cdc13 levels observed in the presence and absence of rum1 are due to changes in p56cdc13 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 p56cdc13 turnover by following p56cdc13 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 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 p56cdc13 in the G1 extracts was determined by densitometry after immunoblotting with anti-p56cdc13 antibodies.

If the rate of p56cdc13 translation in both situations were approximately similar, then decreased
p25rum1 promotes B-cyclin proteolysis during G1

The increase in p56cdc13 stability in rum1Δ cells could be explained by p25rum1 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 p25rum1 resulting in cells moving from G1 to some state later in the cell cycle. To investigate this further, we have monitored the levels of p42cut2 in G1 in the presence or absence of rum1. p42cut2 is degraded in anaphase and remains unstable during G1 (Funabiki et al., 1996a), and it has been reported that cells arrested using a cdc10 mutant have reduced levels of p56cdc13 and p42cut2 (Funabiki et al., 1996b). Therefore, p42cut2 turnover acts as a marker for a cell being in G1. We found that p56cdc13 was undetectable and p42cut2 levels were reduced 2- to 3-fold in a cdc10-129 mutant (Figure 3, lane 2). When rum1 was deleted, a similar drop in p42cut2 levels was still observed but in contrast p56cdc13 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 p42cut2 (Figure 3, lane 5). This result shows that G1 down-regulation of p42cut2 still occurs in the double mutant cdc10-129 rum1Δ at the restrictive temperature, indicating that p25rum1 is required specifically for p56cdc13 proteolysis in G1, but is not required for p42cut2 proteolysis. This suggests that the effects of p25rum1 on p56cdc13 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 p25rum1, proteolysis still occurs but the rate is reduced to a level which is insufficient to counteract the continuing translation of p56cdc13. As a consequence, the level of p56cdc13 remains high in G1 cells lacking p25rum1.

p25rum1 binds p56cdc13 in vitro

The above results indicate that p25rum1 promotes p56cdc13 turnover in G1-arrested cells. Our earlier work showed that p25rum1 associates directly with p56cdc13 in cells arrested in G1 (Correa-Bordes and Nurse, 1995). To investigate this process of association further, we have examined the ability of p25rum1 to form complexes with p56cdc13 in vitro. We first tested whether p25rum1 could bind either p56cdc13 alone or p56cdc13 complexed with p34cdc2. Both p34cdc2 and p56cdc13 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 p25rum1. This was carried out by adding purified bacterially produced p25rum1 to the in vitro translation mixes for 30 min, followed by immunoprecipitation with anti-p25rum1 antibodies. In Figure 4, the labelled proteins produced by in vitro translation are shown by the lanes marked 'Input', while the immunoprecipitations with anti-p25rum1 and control antibodies are shown respectively as (+) and (–). It can be seen that p25rum1 could form specific complexes with p56cdc13 and with p34cdc2/p56cdc13 complexes but not with p34cdc2 alone. We next tested whether the destruction box which is required for B-cyclin proteolysis in mitosis was required for p25rum1 binding. The cdc13Δ90 mutant lacks this destruction box, but the mutant protein could still form an in vitro complex with p25rum1 (Figure 4). Finally, we tested if p25rum1 could form in vitro complexes with the B-cyclins encoded by cig1 and cig2 or with complexes of these cyclins with p34cdc2. No specific p25rum1 association was detected with these cyclins, either alone or in association with p34cdc2.

We conclude that p25rum1 can become stably associated either with free p56cdc13 or with p56cdc13 complexed with p34cdc2 and that this association does not require the cyclin destruction box. In addition, the association appears to be specific to the B-cyclin p56cdc13 because there is no detectable association with other B-cyclins encoded by cig1 and cig2, at least in vitro. These results suggest that p25rum1 specifically associates either with free p56cdc13 or with p56cdc13/p34cdc2 complexes present in G1 cells and that these associations may play a role in targeting p56cdc13 for proteolysis during this phase of the cell cycle.

p25rum1 association with p56cdc13/p34cdc2 during the cell cycle

If p25rum1 targets p56cdc13 for proteolysis when cells are arrested in G1, then p25rum1 association with p56cdc13 or...
with p56cdc13/p34cdc2 complexes might be expected to vary during the normal cell cycle, peaking in G1. We attempted to test this possibility by immunoprecipitations using anti-p25rum1 antibodies followed by Western blotting, but the limited amounts of material available from selection synchronic cultures prevented this approach from being successful. As an alternative, we monitored whether p25rum1 was associated with p56cdc13/p34cdc2 protein kinase activity, because enzymatic assays can be more sensitive than Western blotting. In a first experiment, anti-p25rum1 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 p34cdc2 H1-histone kinase activity is temperature-sensitive in this strain—as shown by assays performed with immunoprecipitates prepared using anti-p34cdc2 antibodies (Figure 5, lanes 7–10)—we conclude that the H1-histone kinase activity associated with p25rum1 is due to p34cdc2. This protein kinase activity was shown to be derived mostly from p56cdc13/p34cdc2 complexes, by exploiting the fact that these complexes are very sensitive to the addition of bacterially produced p25rum1 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 p25rum1 (Correa-Bordes and Nurse, 1995). Addition of p25rum1 to the anti-p25rum1 immunoprecipitates completely inhibited the H1-histone kinase activity, establishing that p56cdc13 is the major B-cyclin involved in the active complexes (Figure 5, lanes 1 and 2). These results indicate that p25rum1 immunoprecipitates contain active p56cdc13/p34cdc2 protein kinase activity. This activity can be inhibited by the addition of further p25rum1 in vitro, behaviour similar to that observed with p21cip1 immunoprecipitates from mammalian cells which contain CDK protein kinase activity inhibited by the addition of further p21cip1 (Zhang et al., 1994). Possibly the complexes between p25rum1 and p56cdc13/p34cdc2 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 p25rum1 immunoprecipitates contain p56cdc13/p34cdc2 protein kinase activity, extracts were prepared from different time points of a synchronous culture and immunoprecipitations performed using the anti-p25rum1 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 p25rum1 becoming associated with p56cdc13/p34cdc2 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 p56cdc13 proteolysis. As a consequence, the p56cdc13/p34cdc2 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, p56cdc13/p34cdc2 protein kinase levels begin to rise. This rise is associated with a drop in p25rum1 levels, as shown in Figure 6. Western blotting showed that p25rum1 had disappeared from cells by 60 min and by this time p56cdc13 had also begun to accumulate. This result suggests that the disappearance of p25rum1 at the end of G1 is important in allowing p56cdc13 levels to accumulate, and thus the reduction of p25rum1 levels at the end of G1 may be an important regulatory mechanism of cell cycle progression.

cig1p-associated p34cdc2 kinase phosphorylates p25rum1

Phosphorylation has been implicated in targeting a number of proteins for proteolysis, and p25rum1 has several consensus phosphorylation sites for the p34cdc2 protein kinase (Moreno and Nurse, 1994). Therefore we decided to investigate whether p34cdc2 phosphorylation of p25rum1 might have a role in reducing the level of p25rum1 as cells exit G1. Firstly, we tested whether p25rum1 was an in vitro substrate for the p34cdc2 protein kinase. Immunoprecipitates from a wild-type extract were prepared using antibodies against p34cdc2 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-
Ishihara and Okayama, 1994). p25rum1 kinase activity was detected with both the anti-p34cdc2 and anti-cig1p antibodies, but not with the other two (Figure 8). This result indicates that p25rum1 can be phosphorylated in vitro by the p34cdc2 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 p25rum1 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, p25rum1 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 p25rum1 that occurs when cells are released from a G1 block. Therefore the direct phosphorylation of p25rum1 by the cig1p-associated p34cdc2 protein kinase may play a role in regulating p25rum1 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 p25rum1 or S-phase entry compared with cdc10-V50 (Figure 7A and B).

Two further points can be made from these experiments.

Firstly, the rapidity with which p56cdc13 appears after release from the G1 block suggests that inactivation of the p56cdc13 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 p34cdc2 protein kinase is no longer limiting for G1 progression (Fisher and Nurse, 1996).

Discussion

The fission yeast B-cyclin p56cdc13/p34cdc2 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 p25rum1 is crucially involved in this control and that it acts as a potent inhibitor of p56cdc13/p34cdc2 protein kinase activity (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995). In the present work we have demonstrated that p25rum1 is required for rapid proteolysis of p56cdc13 in G1 cells and that the regulation of p25rum1 during the cell cycle may involve the p34cdc2 protein kinase associated with the B-cyclin cig1p.

Specifically, we have made the following observations: (i) in G1-arrested cells, p56cdc13 disappears rapidly and this disappearance requires rum1. Another protein proteolysed in G1, p42cut2 (Funabiki et al., 1996a), does not require rum1 for turnover. (ii) The rate of translation of p56cdc13 in G1 cells is the same in the presence or absence of the rum1 gene, but the rate of p56cdc13 proteolysis is increased in the presence of rum1. (iii) p25rum1 binds specifically in vitro either to p56cdc13 or to the p56cdc13/p34cdc2 complex, even when the cyclin destruction box is lacking. (iv) p25rum1 associates with the p56cdc13/p34cdc2 protein kinase during G1 of the normal cell cycle. (v) The cig1p/p34cdc2 protein kinase phosphorylates p25rum1 in vitro and, in the absence of cig1p, the disappearance of p25rum1 is delayed when cells are released from a G1 block.

From these observations we conclude that an important role for p25rum1 in cell cycle regulation is to associate with p56cdc13 or p56cdc13/p34cdc2 complexes in G1 cells and to promote p56cdc13 proteolysis. We imagine that, as well as acting as a direct inhibitor of p56cdc13/p34cdc2 protein kinase activity, p25rum1 has a second role as an adaptor promoting transfer of p56cdc13 to the proteolytic machinery. Given that p56cdc13 translation is not reduced in G1 cells, the p25rum1-mediated promotion of p56cdc13

Fig. 8. cig1p/p34cdc2 complex phosphorylates p25rum1 in vitro. p34cdc2 associated with different cyclins was immunoprecipitated from wild-type extracts and assayed for protein kinase activity using p25rum1 as a substrate.
proteolysis must be a major mechanism preventing the accumulation of active p56cdc13/p34cdc2 protein kinase activity in G1 cells. This is of considerable importance because p34cdc2 is not inhibited by Y15 phosphorylation during G1 (Hayles and Nurse, 1995) and, as a consequence, p56cdc13/p34cdc2 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 p56cdc13 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 p56cdc13 during the G1 phase of the cell cycle. p25rum1 may act as an adaptor, with the binding of p25rum1 to p56cdc13 enhancing the transfer of cyclin to the nuc2/cut9 proteosome 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 proteosome. For example, the E6 protein binds p53 and targets it for ubiquitination, and antizyme targets ornithine decarboxylase to the proteosome (Ciechanover, 1994). p25rum1 appears to have an effect on p56cdc13 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 p25rum1 only having a significant targeting role during G1.

We have shown that proteolysis of another protein p42cig2 during G1 is not affected by the absence of rum1, suggesting that the effect of p25rum1 on p56cdc13 proteolysis is direct and specific. However, we cannot exclude the possibility that there may also be some indirect effects of p25rum1 working through the p34cdc2 mitotic kinase, the activity of which will increase in the absence of rum1.

On entry into S-phase, p56cdc13 levels begin to increase in preparation for the onset of mitosis. The timing of this increase is correlated with a decrease in p25rum1 levels. Our results suggest that the cig1p/p34cdc2 protein kinase may have a role in this mechanism. In the absence of cig1, the decrease in p25rum1 levels is much delayed as cells exit G1 after release from a block imposed using a cdc1010 mutant. The protein kinase cig1p/p34cdc2 can phosphorylate p25rum1 in vitro, unlike the p56cdc13/p34cdc2 or cig2p/p34cdc2 protein kinases. We speculate that, at the G1–S-phase boundary, cig1p can promote p25rum1 proteolysis as a consequence of the cig1p/p34cdc2 protein kinase phosphorylating p25rum1. The subsequent reduction in p25rum1 levels allows p56cdc13 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 p25rum1 proteolysis is not understood, but is likely to involve cdc10-dependent transcription, given that p25rum1 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 (Irninger et al., 1995; King et al., 1995; Sadakin et al., 1995). The CLN cyclins are also required in G1 to promote onset of S-phase, and Cln2p degradation is induced by p34cdc28 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 proteosome pathway (Lanker et al., 1996). There is also selective proteolysis of the CDK inhibitor p40SIC1: 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 p40SIC1 in budding yeast while a B-cyclin CDK phosphorylates the CDK inhibitor p25rum1 in fission yeast. Also, p40SIC1 inhibits the B-cyclins which normally act in G1. In budding yeast while p25rum1 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: 9727; cdc10-V50, ura4Δ18, h; cdc10-V50, cig1Δ::ura4Δ7, ura4Δ18, h; cdc10-V50, cig2Δ::ura4Δ7, ura4Δ18, h; cdc10-129, cdc13Δ::ura4Δ7, leu1-32, RIP5 cdc1313 integrant h; cdc10-129, cdc13::ura4Δ7, ura4Δ18, leu1-32, RIP5 cdc1313 integrant h; and cut3Δ4Δ::cut2Δ4Δ::H4::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 (Buono 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 (2×10^8 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\textsubscript{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


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