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B-type cyclins regulate G_1 progression in fission yeast in opposition to the $p25^{rum1}$ cdk inhibitor

Cristina Martín-Castellanos, Karim Labib[1] and Sergio Moreno[2]

Instituto de Microbiología-Bioquímica, Departamento de Microbiología y Genética, CSIC/Universidad de Salamanca, Edificio Departamental, Campus Miguel de Unamuno, 37007 Salamanca, Spain

[1]Present address: Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK

[2]Corresponding author

The onset of S phase in fission yeast is regulated at Start, the point of commitment to the mitotic cell cycle. The $p34^{cdc2}$ kinase is essential for G_1 progression past Start, but until now its regulation has been poorly understood. Here we show that the cig2/cyc17 B-type cyclin has an important role in G_1 progression, and demonstrate that $p34^{cdc2}$ kinase activity is periodically associated with cig2 in G_1. Cells lacking cig2 are defective in G_1 progression, and this is particularly clear in small cells that must regulate Start with respect to cell size. We also find that the cig1 B-type cyclin can promote G_1 progression. Whilst $p25^{rum1}$ can inhibit cig2/cdc2 activity *in vitro*, and may transiently inhibit this complex *in vivo*, cig1 is regulated independently of $p25^{rum1}$. Since cig1/cdc2 kinase activity peaks in mitotic cells, and decreases after mitosis with similiar kinetics to cdc13-associated kinase activity, we suggest that cig2 is likely to be the principal fission yeast G_1 cyclin. cig2 protein levels accumulate in G_1 cells, and we propose that $p25^{rum1}$ may transiently inhibit cig2-associated $p34^{cdc2}$ activity until the critical cell size required for Start is reached.

Keywords: cdk inhibitor/cig2/G_1 cyclins/rum1/*Schizosaccharomyces pombe*

Introduction

The fission yeast *Schizosaccharomyces pombe* provides a model system with which to study the relationship between cell cycle progression and cell growth. In common with other eukaryotes, progression through the cell cycle is regulated principally before the onset of S phase and the onset of mitosis, and in both cases a critical cell mass must be attained before progression occurs (Nurse, 1975; Nurse and Thuriaux, 1977; Nasmyth *et al.*, 1979). In rapidly growing cells the mitotic control is limiting, since cell division produces daughter cells with a mass already greater than the minimum required to initiate S phase. In these conditions, G_1 is very short and the onset of S phase is regulated by its dependency upon completion of the previous mitosis (Nurse *et al.*, 1976; Nurse and Thuriaux, 1977; Nasmyth *et al.*, 1979). In conditions of nutrient limitation, mitosis is initiated at a reduced cell size (Fantes and Nurse, 1977), producing small daughter cells that must delay the initiation of S phase until the critical mass is achieved. Whilst the onset of mitosis involves activation of the $p34^{cdc2}$ protein kinase (Booher *et al.*, 1989; Moreno *et al.*, 1989), a mechanism conserved amongst eukaryotes (reviewed by Nurse, 1990), the onset of S phase remains poorly understood.

The controls regulating S phase initiation operate through Start, the point of commitment to the mitotic cell cycle (Hartwell, 1974). A cell can only undertake other developmental options such as the sexual cycle and meiosis from pre-Start G_1 in poor growth conditions where the critical mass needed to pass Start and enter S phase cannot be achieved. In order to understand the onset of S phase, we therefore need to understand the molecular basis of Start, and what determines its cell cycle timing. In fission yeast, the products of the *cdc2*, *cdc10* and *res1/sct1* genes are essential for progression past Start (Nurse and Bissett, 1981; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993). The *cdc10* and *res1/sct1* gene products form a transcriptional complex essential for the expression of genes required for S phase, indicating that the activation of such cell cycle-regulated transcription forms an important aspect of Start (Lowndes *et al.*, 1992; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Kelly *et al.*, 1993). The role of $p34^{cdc2}$ at Start remains unclear, although the $p85^{cdc10}$-containing transcriptional complex does not bind DNA in the absence of *cdc2* function (Reymond *et al.*, 1993). The general features of Start are conserved in budding yeast, where the $p34^{CDC28}$ kinase and transcriptional activation of S phase genes are again central elements (reviewed by Nasmyth, 1993).

What determines the timing of Start and relates it to the achievement of a critical cell mass? The *rum1* gene is likely to have a key role in these controls, since small cells lacking *rum1* are unable to delay progression past Start, resulting in the initiation of S phase immediately after the completion of mitosis (Moreno and Nurse, 1994). *rum1* function is required to maintain a cell in the pre-Start G_1 interval, and overexpression of the *rum1* gene product re-sets a G_2 cell back to pre-Start G_1. A model for *rum1* function suggests that it acts to maintain $p34^{cdc2}$ in a pre-Start G_1 form, inhibiting its activity until the critical mass required to pass Start is achieved (Moreno and Nurse, 1994). Recent work has shown that deletion of the *rum1* gene increases $p34^{cdc2}$ G_1 activity *in vivo*, consistent with this model (Labib *et al.*, 1995).

Until now, fission yeast $p34^{cdc2}/G_1$ cyclin complexes have not been described, preventing an *in vitro* analysis of the inhibitory role of *rum1*. The mitotic $p34^{cdc2}$ kinase functions in a complex with the $p56^{cdc13}$ B-type cyclin (Booher *et al.*, 1989; Moreno *et al.*, 1989). Other fission yeast cyclins have been identified, such as puc1 and cig1, but these have not yet been shown to have a role in the

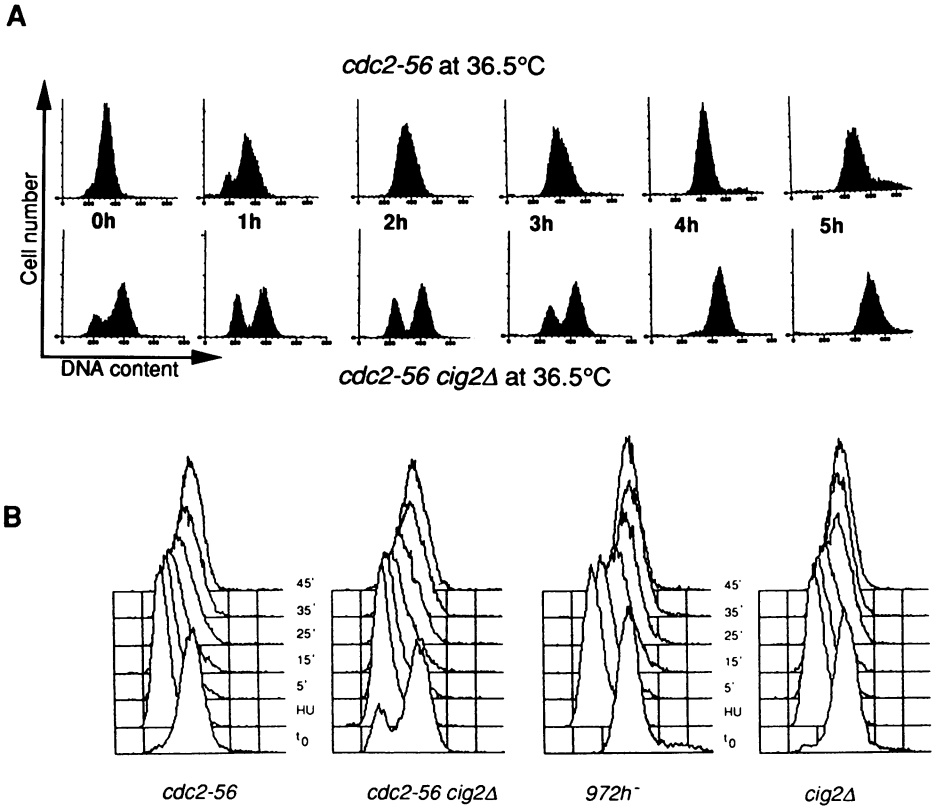

Fig. 1. *cig2* encodes a G_1 cyclin that regulates entry into S phase. (**A**) *cdc2-56* and *cdc2-56 cig2Δ* cells were grown to mid-exponential phase in minimal medium at 25°C, and then shifted to 36.5°C. Samples were taken every hour until 5 h, fixed in 70% ethanol, and processed for flow cytometry as described in Materials and methods. Flow cytometry of wild-type cells normally reveals a single peak of 2 C cells, since the majority are in G_2, whilst G_1 is completed rapidly after mitosis and S phase begins before the daughter cells have separated, so that a newly born cell has a 2 C DNA content. The *cdc2-56* mutant is slightly smaller than wild-type at 25°C, producing small G_1 cells that must delay G_1 progression transiently, and so a small population of 1 C cells is seen. Cells arrest in G_1 or G_2 at the restrictive temperature, depending on their position in the cell cycle, and so an increased peak of 1 C cells appears, and remains until cells leak past the G_1 block and undergo Start and S phase, before arresting in G_2. Deletion of *cig2* increases the proportion of 1 C cells at 25°C, whilst more cells arrest in G_1 at 36.5°C, and remain arrested for longer. (**B**) *972h⁻*, *cig2Δ*, *cdc2-56* and *cdc2-56 cig2Δ* strains were grown at 25°C, and then arrested in early S phase by addition of 10 mM hydroxyurea to the medium for 4 h. Cells were then washed three times in water and resuspended at 25°C in fresh medium lacking hydroxyurea. Samples were taken after 5, 15, 25, 35 and 45 min, and processed for flow cytometry. Deletion of *cig2* does not affect progression through S phase.

cell cycle at Start (Bueno *et al.*, 1991; Forsburg and Nurse, 1991, 1994; Connolly and Beach, 1994). The *cig2/cyc17* gene encodes a B-type cyclin that was originally thought to have a role in mitosis, but has since been shown to act as a negative regulator of the sexual cycle (Bueno and Russell, 1993; Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994). Cells lacking *cig2* have an enhanced ability to conjugate, and we thought that this may reflect a defect in passing Start, suggesting a role for cig2 as a G_1 cyclin. The abundance of *cig2* mRNA is cell cycle regulated and peaks at G_1/S phase (Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994). Here we show that the *cig2* gene product is indeed an important regulator of G_1 progression in the fission yeast cell cycle, functioning as a G_1 cyclin that positively regulates $p34^{cdc2}$ activity at Start. $p25^{rum1}$ inhibits cig2/cdc2 kinase activity *in vitro*, and *in vivo* may transiently inhibit $p34^{cdc2}$ kinase activity associated with cig2 until the critical size required for Start is reached. In cells that lack cig2, either cig1 or cdc13 are capable of promoting $p34^{cdc2}$ G_1 activity. Our data provide the basis for a model for the regulation of $p34^{cdc2}$ kinase activity in the G_1 phase of the fission yeast cell cycle.

Results

cig2 encodes a G_1 cyclin

If *cig2* encodes a G_1 cyclin that positively regulates $p34^{cdc2}$, deletion of *cig2* should reduce $p34^{cdc2}$ G_1 activity. Since total $p34^{cdc2}$ kinase activity detectable *in vitro* using histone H1 as a substrate only increases once cells have entered G_2 (Moreno *et al.*, 1989; Creanor and Mitchison, 1994), we initially used an *in vivo* approach to test this idea. When a *cdc2* temperature-sensitive mutant is placed at the restrictive temperature, cells become arrested in G_1 and G_2, and then either remain arrested on continued incubation, or leak past one or both of the block points. This depends on the particular allele chosen, and probably reflects the amount of residual $p34^{cdc2}$ activity at the restrictive temperature. If cells lacking *cig2* have reduced $p34^{cdc2}$ G_1 activity, shifting a $cdc2^{t.s.}$ *cig2Δ* strain to the restrictive temperature should result in more cells becoming arrested in G_1, and cells should remain arrested for a longer period. We previously used an analogous approach to show that cells lacking the *rum1* gene have increased $p34^{cdc2}$ G_1 activity, since $cdc2^{t.s.}$ *rum1Δ* cells are less able to arrest in G_1 at the restrictive temperature (Labib *et al.*, 1995).

To test this idea, we utilized *cdc2-56*, a leaky $cdc2^{ts}$ mutant that causes a weak G_2 block and only arrests very transiently before Start. Duplication of the *cdc2-56* locus is sufficient to reverse the temperature-sensitive phenotype of this strain, indicating that *cdc2* function is not completely inactivated at the restrictive temperature (Carr *et al.*, 1989). We found that deletion of *cig2* dramatically affects the onset of S phase in this strain. When *cdc2-56* is placed at the restrictive temperature, 18% of cells transiently arrest in G_1, then leak past the block point and have undergone S phase by 2 h (Figure 1A). For the *cdc2-56 cig2Δ* double mutant, 20% of cells have a 1 C DNA content even at the permissive temperature (Figure 1A). This represents a defect in G_1 progression, rather than an advancement of mitosis, since *cdc2-56* and *cdc2-56 cig2Δ* enter mitosis at the same size (10.5 μm). Incubation of *cdc2-56 cig2Δ* cells at 36.5°C causes 40% to become arrested in G_1, and this arrest is maintained for 3 h, a generation time longer than for the *cdc2-56* single mutant (Figure 1A). These results indicate that deletion of *cig2* reduces $p34^{cdc2}$ G_1 activity *in vivo*, suggesting that cig2 has an important role as a G_1 cyclin in regulating the passage of Start.

Since B-type cyclins have been reported to have a role in progression through S phase in budding yeast (Epstein and Cross, 1992; Kuhne and Linder, 1993; Schwob and Nasmyth, 1993), an alternative interpretation of our data could be that cig2 has a similiar role in fission yeast, rather than regulating Start and entry into S phase. However, if *cdc2-56 cig2Δ* cells are grown at the permissive temperature and then arrested in early S phase with hydroxyurea, before shifting to the restrictive temperature in pre-warmed medium lacking hydroxyurea, all cells have completed S phase by 1 h (not shown), indicating that the data in Figure 1A represent a defect in G_1 progression rather than progression through S phase. A more detailed analysis clearly shows that deletion of *cig2* has no effect on S phase progression, and that the accumulation of 1 C cells for *cdc2-56 cig2Δ* at the permissive temperature represents a defect in G_1 progression (Figure 1B).

G_1 progression in small cells

Deletion of *cig2* in a wild-type strain has not been reported to affect G_1 progression (Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994), although we note that a very small 1 C population can be seen in flow cytometry (Figure 1B, see also Figure 4 of Obara-Ishihara and Okayama, 1994). It seems clear that cig2 cannot be the only fission yeast G_1 cyclin, and that at least one other apart from puc1 or cig1 remains to be found, since triple deletion of *puc1*, *cig1* and *cig2* is not lethal (Obara-Ishihara and Okayama, 1994). The identity of this remaining G_1 cyclin is considered below.

Why should deletion of *cig2* in *cdc2-56* affect G_1 progression so dramatically? Part of the explanation could be that $p34^{cdc2\text{-}56}$ has a defective interaction with other G_1 cyclins, so that *cig2* is particularly important for this strain. A second possibility is suggested by the fact that *cdc2-56* at the permissive temperature is 'semi-wee', meaning that cells enter mitosis at a reduced size with respect to wild-type (Nurse and Thuriaux, 1980). This produces small G_1 cells that have to delay the onset of S phase transiently until they achieve the critical mass required for Start (note the small population of 1 C *cdc2-56* cells in Figure 1). *cig2* may be particularly important, or its importance may be easier to observe, in small G_1 cells that must regulate progression past Start with respect to cell size.

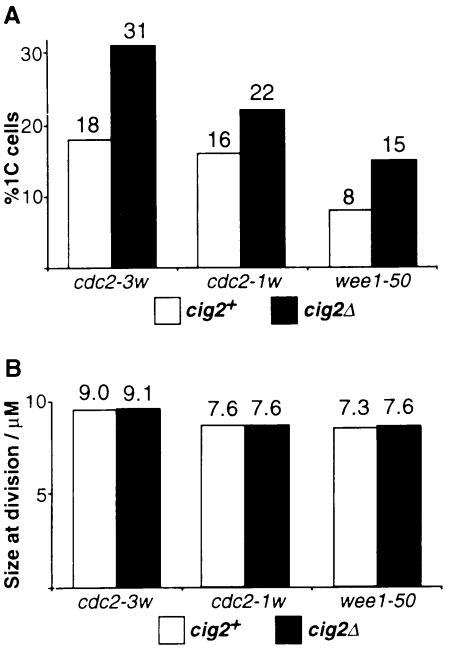

Fig. 2. *cig2* is important for G_1 progression in small cells. (**A**) The effect of deleting the *cig2* gene in *cdc2-3w*, *cdc2-1w* and *wee1-50* was investigated by flow cytometry. Cells were grown in minimal medium at 32°C, except for *wee1-50* and *wee1-50 cig2Δ* cells, which were initially grown at 25°C, before shifting to the restrictive temperature of 35°C for 4 h. Samples were fixed in 70% ethanol and processed for flow cytometry as described in Materials and methods, and the proportion of 1 C cells quantified. Deletion of *cig2* increases the proportion of 1 C cells in all cases. (**B**) The cell size at mitosis was determined for the same strains as in (A), as described in Materials and methods.

We therefore examined the effects of deleting *cig2* in a variety of cell cycle mutants that enter mitosis at a reduced cell size. As shown in Figure 2, an increase in the proportion of 1 C cells was observed in all cases (Figure 2A), whilst the cell size at mitosis was not reduced (Figure 2B), showing that *cig2* is important for G_1 progression in these small cells. The *wee1-50 cig2Δ* double mutant is of particular interest, since this strain has wild-type $p34^{cdc2}$, so the observed effect is likely to reflect the importance of *cig2* for G_1 progression in small cells.

It has been shown previously that the cell size at which a wild-type strain initiates mitosis increases with temperature, so that shifting cells from 25 to 35°C causes a transient inhibition of mitotic entry, until G_2 cells achieve the new size required (Nurse, 1975). We found that shifting cells lacking the *cig2* gene from 25 to 36°C caused a transient accumulation of 1 C cells, suggesting that Start is also inhibited until a slightly greater cell mass is achieved, and that *cig2* is important for G_1 progression in these conditions (Figure 3). A similar effect is not seen for wild-type cells, indicating that the level of inhibition is too small to be significant in cells that express *cig2* (Figure 3).

G_1 progression in rapidly growing wild-type size cells

Is *cig2* also important for G_1 progression in rapidly growing cells that initiate S phase almost immediately after mitosis, since the critical mass required to pass Start is already achieved as they enter G_1? We looked at the effect of deleting the *cig2* gene in *cdc2-33*, a typical $cdc2^{t.s.}$ mutant with wild-type size at the permissive temperature, that causes a tight G_2 arrest and a transient G_1 arrest at 36.5°C. When *cdc2-33* cells are placed at the restrictive temperature, ~15% arrest in G_1 and remain arrested for up to 4 h, before leaking past the block point and undergoing S phase (Figure 4). In contrast, a small population of 1 C cells is observed even at the permissive temperature for the *cdc2-33 cig2Δ* double mutant, whilst 28% of cells become arrested in G_1 at 36.5°C, and these cells remain arrested for up to 6 h (Figure 4). A similiar result was obtained with *cdc2-M26*, where 16% of cells arrest in G_1 after 1 h at 36.5°C, compared with 27% for the *cdc2-M26 cig2Δ* double mutant. These results again indicate that deletion of *cig2* reduces $p34^{cdc2}$ G_1 activity *in vivo*, suggesting that the *cig2* gene product acts as a G_1 cyclin in wild-type size fission yeast cells.

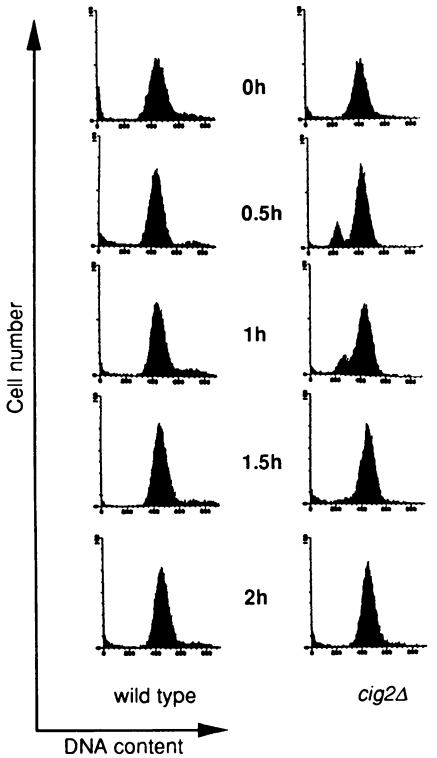

Fig. 3. A transient G_1 delay is seen when *cig2Δ* cells are shifted from 25 to 35°C. *972h*⁻ and *cig2Δ* cells were grown to mid-exponential phase in minimal medium at 25°C, and then shifted to 35°C for the indicated times. Flow cytometry shows that a transient 1 C population is observed for cells lacking *cig2*, but not for wild-type cells, indicating that G_1 progression is delayed in these circumstances.

cig2-associated $p34^{cdc2}$ kinase activity peaks in G_1

Our genetic data demonstrate the importance of cig2 for G_1 progression in the fission yeast cell cycle. We examined cig2 protein and associated $p34^{cdc2}$ kinase activity, and find that both are periodic in the cell cycle, peaking in G_1. Since the G_1 phase of wild-type cells is very short, we used a synchronous culture of *wee1-50* cells, which have an extended G_1 phase at the restrictive temperature (see above). $p34^{cdc2}$ kinase activity associated with cig2 accumulates in G_1 cells, when cdc13-associated activity is at its lowest level, and peaks as cells enter S phase (Figure 5A and B: the data is particularly clear in the first cycle, between 60 and 200 min, when the synchrony of the culture is at its best). In contrast, cig2-associated kinase activity is low in dividing cells, when cdc2/cdc13 kinase activity is at its peak. The levels of cig2 protein show a similiar periodicity, and can therefore explain the G_1 specificity of the associated $p34^{cdc2}$ kinase activity (Figure 5C).

Our results identify the *cig2* gene product as a G_1 cyclin that regulates the onset of S phase in the fission yeast cell cycle. In order to understand how G_1 progression is controlled, two key issues have still to be addressed. Other G_1 cyclins remain to be identified, that are presumably able to promote progession past Start in the absence of cig2. In addition, the role of $p25^{rum1}$ at Start must be elucidated.

cig2 and rum1 have opposing roles in G_1 regulation

The *rum1* gene encodes a negative regulator of G_1 progression, proposed to maintain $p34^{cdc2}$ in an inactive form. Cells lacking *rum1* have increased $p34^{cdc2}$ G_1 activity and are unable to arrest cell cycle progression before Start,

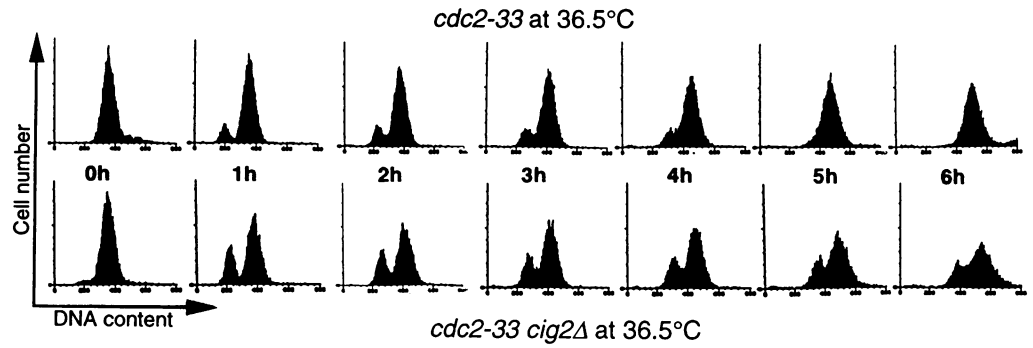

Fig. 4. Deletion of *cig2* reduces $p34^{cdc2}$ G_1 activity *in vivo*. *cdc2-33* and *cdc2-33 cig2Δ* cells were grown to mid-exponential phase in minimal medium at 25°C, and then shifted to 36.5°C. Samples were taken every hour until 6 h, fixed in 70% ethanol and processed for flow cytometry as described in Materials and methods. A small population of 1 C cells is seen for *cdc2-33 cig2Δ* at the permissive temperature, whilst at 36.5°C more cells become arrested in G_1 than for the *cdc2-33* single mutant, and these cells remain arrested to the end of the experiment.

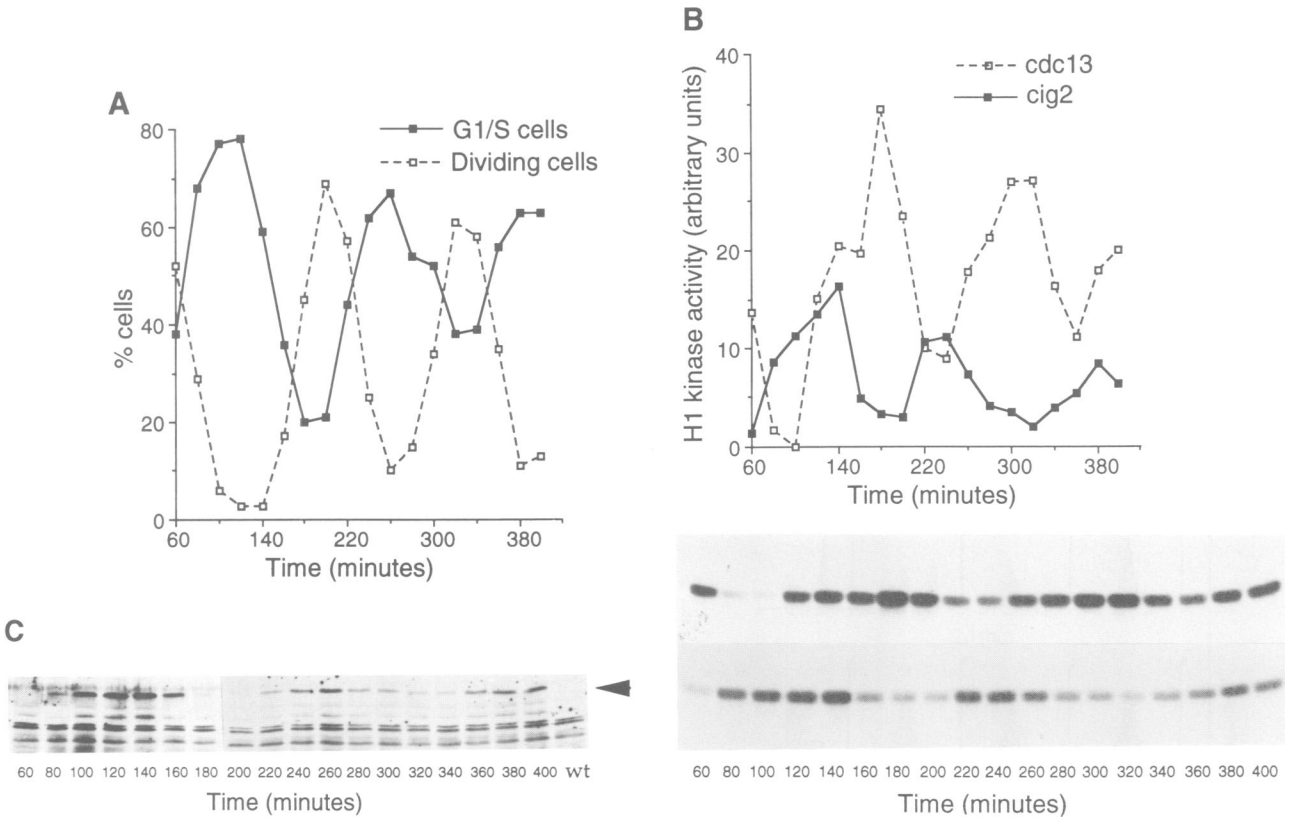

Fig. 5. cig2-associated p34^{cdc2} kinase activity peaks in G_1. In order to examine cdc2 kinase activity associated with cig2, we used a strain in which the *cig2* gene was replaced with a version containing three copies of the influenza virus HA epitope at the C-terminus (see Materials and methods). Anti-HA antibodies specifically immunoprecipitate H1 kinase activity from *cig2HA* strains, and this activity dissappears in a *cdc2-33* mutant, showing that it is dependent upon p34^{cdc2} (data not shown). A synchronous population of *wee1-50 cig2HA* cells was prepared at 25°C by elutriation, and the culture shifted to 36°C, resulting in entry into mitosis at a reduced cell size. The culture was left for 1 h to stabilize at 36°C, and then samples taken every 20 min. (**A**) Ethanol-fixed samples were stained with DAPI and Calcofluor and the proportion of binucleate cells determined by microscopic examination (open squares). DNA content of the same samples was measured by flow cytometry, and the fraction of G_1/S cells calculated as described in Materials and methods (filled squares). (**B**) Protein extracts were made and H1 kinase activity assayed in immunoprecipitates of cdc13 and cig2HA. The upper panel shows a quantification of the data presented in the lower panel. In the lower panel the top set of reactions correspond to cdc13, and the bottom set to cig2. Whilst cdc13/cdc2 kinase activity peaks in mitosis, cig2/cdc2 activity reaches its maximum in G_1. (**C**) Western blotting with anti-HA antibodies of samples from the same protein extracts demonstrated that cig2 protein levels show a similiar periodicity to cig2/cdc2 H1 kinase activity, peaking in G_1. The arrowhead marks the position of cig2HA. A wild-type control lacking the *cig2HA* gene is included, showing that most of the other bands seen in the blot are unrelated to cig2.

resulting in sterility and a failure to enter the sexual cycle (Moreno and Nurse, 1994; Labib *et al.*, 1995). In contrast, cells lacking *cig2* have the opposite phenotype—decreased p34^{cdc2} G_1 activity and an enhanced ability to arrest before Start, resulting in hyperfertility (this study; Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994).

We found that deletion of *cig2* restores fertility to a strain lacking the *rum1* gene, indicating that cells are once more able to arrest before Start (Figure 6A). Whilst *rum1* is also required to prevent entry into mitosis from the pre-Start G_1 interval (Moreno and Nurse, 1994), deletion of *cig2* does not alter this requirement, since a *cdc10-129 rum1Δ cig2Δ* triple mutant still enters mitosis at the restrictive temperature, showing that deletion of *cig2* only suppresses the failure of cells lacking the *rum1* gene to delay progression past Start. A *cig2Δ rum1Δ* double mutant has reduced fertility compared with wild-type however, and the ability to arrest in G_1 upon nitrogen starvation is also less than for wild-type (Figure 6B). Whilst it is clear that *cig2* has an important role at Start, *rum1* cannot exclusively be an inhibitor of cig2/cdc2, since in this case the *cig2Δ rum1Δ* double mutant would behave like the *cig2Δ* single mutant. Instead our data suggests that, in the absence of *rum1*, some other p34^{cdc2}/cyclin complex has increased G_1 activity, so that even when *cig2* is deleted, the cell is less able to arrest at Start than a wild-type strain.

Regulation of G_1 progression by cig2, cig1 and p25^{rum1}

Overexpression of p25^{rum1} in a G_2 cell strongly inhibits cdc2/cdc13 kinase activity, and produces multiple rounds of S phase in the absence of mitosis, equivalent to deletion of the *cdc13* gene (Hayles *et al.*, 1994; Moreno and Nurse, 1994). It has therefore been proposed that the cdc2/cdc13 complex defines a cell as being in G_2, and recent work has shown that p25^{rum1} potently inhibits cdc2/cdc13 kinase activity *in vitro* (Hayles *et al.*, 1994; Correa-Bordes and Nurse, 1995; Figure 7C).

Before cells overexpressing rum1 begin to re-replicate, a transient accumulation of 1 C cells is seen (Moreno and Nurse, 1994; Figure 7B). This suggests that rum1 is also capable of inhibiting cdc2/G_1 cyclin complexes, though such inhibition must be transient or weak, since cells

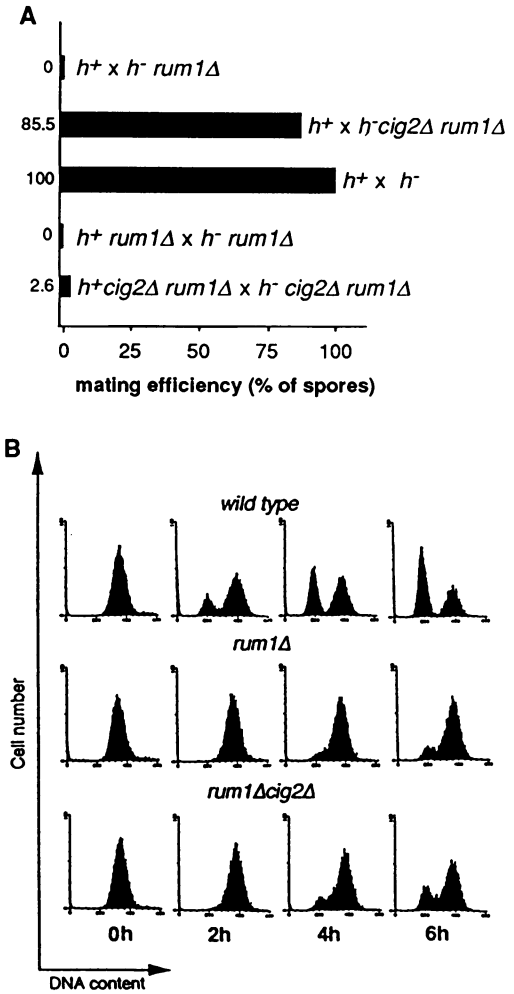

Fig. 6. Deletion of *cig2* suppresses the sterility of cells lacking the *rum1* gene. (**A**) *rum1Δ* h^-, *rum1Δ cig2Δ* h^- and wild-type h^- cells were grown in yeast extract medium at 32°C and the cultures adjusted to a cell density of 2.5×10^6 cells/ml, before mixing with equivalent cultures of *rum1Δ* h^+, *rum1Δ cig2Δ* h^+ or wild-type h^+ cells at the same density. Aliquots were spotted on malt extract plates and incubated for 48 h at 25°C. Cells from the cross were then resuspended in water at a concentration of 2.5×10^6 cells/ml, treated with helicase for 24 h to kill vegetative cells and the number of spores was determined microscopically using a haemocytometer. Microscopic examination of crosses prior to helicase treatment confirmed that low percentage spore formation corresponded to a failure in zygote formation. Deletion of *cig2* restores fertility to cells lacking *rum1*, though the *cig2Δ rum1Δ* double mutant remains less fertile than wild-type cells (h^- *rum1Δ cig2Δ* conjugates very efficiently with a h^+ wild-type strain, but less efficiently with h^+*rum1Δ cig2Δ*). (**B**) The ability of *rum1Δ*, *rum1Δ cig2Δ* and wild-type cells to arrest in G_1 in medium lacking nitrogen was examined, by growing cells to mid-exponential phase in minimal medium at 28°C, then washing three times in water and resuspending at a density of 2×10^6 cells/ml in fresh medium lacking a nitrogen source. Incubation was continued at 28°C and samples taken and processed for flow cytometry as described in Materials and methods. The ability of *rum1Δ cig2Δ* cells to arrest with a 1 C DNA content is greater than for the *rum1Δ* single mutant but less than for wild-type, whilst the ability of *rum1Δ cig2Δ* cells to conjugate shows that these cells can arrest cell cycle progression before Start, unlike the *rum1Δ* single mutant.

subsequently undergo multiple rounds of S phase. Each of these rounds is dependent upon $p34^{cdc2}$ kinase activity, again implying that rum1 inhibition of total cdc2/G_1 cyclin activity can only be transient or weak (Moreno and Nurse, 1994).

We investigated the effects of overexpressing $p25^{rum1}$ in wild-type cells, or in strains lacking either or both of the *cig1* and *cig2* genes. In all cases, rum1 overexpression strongly inhibited $p34^{cdc2}$ kinase activity associated with immunoprecipitates of cdc13 (Figure 7A). In contrast, we only observed partial inhibition of the total kinase activity associated with immunoprecipitates of $p34^{cdc2}$. This again suggests that only a fraction of cdc2/cyclin complexes are sensitive to rum1 inhibition. Maximum inhibition was observed after 16 h, as rum1 overexpression was beginning to peak, and this corresponded to accumulation of a small 1 C population of cells (Figure 7B). rum1 overexpression in cells lacking the *cig2* gene initially caused more cells to arrest transiently in G_1, consistent with cig2's role as a G_1 cyclin (Figure 7B). Subsequent re-replication occurred with similar kinetics to wild-type. Deletion of *cig1* also caused more cells to arrest in G_1 initially, but in this case subsequent re-replication was delayed with respect to wild-type (the reasons for this are considered below). This suggests that cig1 also has a role in G_1 progression, in addition to cig2.

Deletion of both *cig1* and *cig2* completely abolishes re-replication when rum1 is overexpressed, and instead cells become arrested in G_1 and G_2 in approximately equal proportions (Figure 7B). This demonstrates that cig1 and cig2 promote entry into S phase in re-replicating cells overexpressing rum1. It is therefore unlikely that rum1 can efficiently inhibit $p34^{cdc2}$ kinase activity associated with these two cyclins *in vivo*. Consistent with this conclusion, rum1 overexpression in cells lacking both cig1 and cig2 inhibits total $p34^{cdc2}$ kinase activity to the same low levels as cdc13-associated activity (Figure 7A).

We examined the ability of purified bacterially produced $p25^{rum1}$ to inhibit $p34^{cdc2}$ kinase activity in immunoprecipitates of cig1, cig2 and cdc13 from wild-type cells. Whilst rum1 potently inhibits cdc13-associated kinase activity, $p34^{cdc2}$ activity associated with cig1 is not inhibited at all (Figure 7C). Interestingly, we find that $p25^{rum1}$ is capable *in vitro* of inhibiting cig2-associated $p34^{cdc2}$ kinase activity (Figure 7C). We think, therefore, that rum1 may transiently inhibit cdc2/cig2 activity *in vivo*, until cig2 protein levels accumulate to sufficiently high levels to overcome the inhibitory effects of rum1.

We conclude that both cig1 and cig2 can act as G_1 cyclins. It has recently been shown that triple deletion of *cig1*, *cig2* and *cdc13* results in G_1 arrest, demonstrating that cdc13 regulates the onset of S phase in the absence of cig1 and cig2 (Fisher and Nurse, 1996). $p25^{rum1}$ overexpression in cells lacking both cig1 and cig2 inhibits cdc2/cdc13 kinase activity, and this therefore explains why cell cycle progression is blocked both in G_1 and G_2. Our data suggest that cig2 is likely to be the principal fission yeast cyclin regulating G_1 progression. Whilst cdc13 levels and associated kinase activity are very low in G_1 cells and peak in mitosis, cig2/cdc2 kinase activity peaks in G_1 and is low in dividing cells. $p34^{cdc2}$ kinase activity associated with cig1 has been reported to peak in mitosis with similiar kinetics to cdc13 (Basi and Draetta, 1995). In contrast to deletion of *cig2*, deletion of *cig1* does not restore the ability of a *rum1Δ* strain to arrest before Start and enter the sexual cycle (not shown). In addition, deletion of *cig1* does not affect G_1 progression in *wee1-50* cells at 36°C, nor in wild-type cells shifted

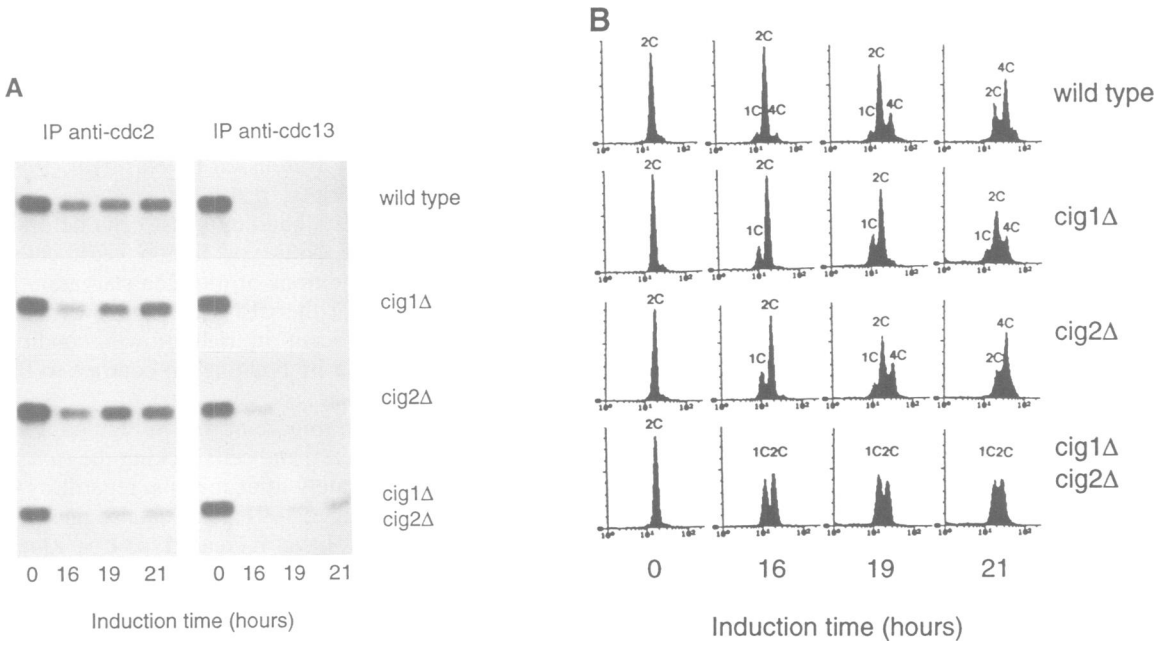

Fig. 7. Regulation of G_1 progression by cig2, cig1 and $p25^{rum1}$. (**A**) The *rum1* gene was overexpressed from the thiamine-repressible *nmt1* promoter (Maundrell, 1989), in wild-type, *cig1*Δ, *cig2*Δ or *cig1*Δ *cig2*Δ cells containing an integrated copy of pREP3X(*sup3-5*) $rum1^+$. Cells were grown to mid-exponential phase at 25°C in minimal medium containing 5 μg/ml thiamine, then washed three times in water and resuspended in fresh medium lacking thiamine, at a density calculated to produce 4×10^6 cells/ml after 16 h, when expression from the *nmt1* promoter peaks. Samples were taken at the indicated times, protein extracts made and H1 kinase activity associated with immunoprecipitates of cdc2 and cdc13 assayed as described in Materials and methods. Whilst cdc13/cdc2 kinase activity is constitutively inhibited, total cdc2 activity is only partially inhibited. The rum1-resistant activity disappears when *cig1* and *cig2* are both deleted, implying that cig1 and cig2 are not inhibited by rum1 to the same degree as cdc13. Overexpression of rum1 in a cell that lacks cig1 results in transient inhibition of cig2-dependent kinase activity. (**B**) Samples from the same time course were processed for flow cytometry as above. See text for details. (**C**) cig1-, cig2- and cdc13-associated cdc2 kinase activity was immunoprecipitated from wild-type cells, and purified bacterially produced $p25^{rum1}$ added to the concentrations shown. Whilst rum1 potently inhibits cdc13/cdc2, and can also inhibit cig2/cdc2, cig1/cdc2 is not affected.

from 25 to 36°C (data not shown; see above for the effects of deleting *cig2* in these cases). We think, therefore, that in a wild-type cell cig2 has a major role in promoting G_1 progression at Start. In cells that lack cig2, either cig1 or cdc13 can compensate, but entry into S phase is delayed with respect to wild-type. This becomes particularly clear in small G_1 cells that must regulate progression past Start with respect to cell size.

$p25^{rum1}$ strongly inhibits cdc2/cdc13 activity, and may have a role in preventing accumulation of this complex in pre-Start G_1 cells (Hayles and Nurse, 1995). Small G_1 cells lacking the *rum1* gene are advanced into S phase, and this may be due, in part, to increased levels of cdc2/cdc13 kinase activity. We find that $p25^{rum1}$ is capable of inhibiting cig2/cdc2 activity *in vitro*, and may act *in vivo* as a transient inhibitor of cig2-associated $p34^{cdc2}$ kinase activity, until the cell reaches the minimum size required to pass Start. In contrast, cig1/cdc2 kinase activity is not inhibited by $p25^{rum1}$, and must therefore be regulated in some other manner.

No evidence that puc1 acts as a G_1 cyclin in the mitotic cell cycle

The fission yeast *puc1* gene product shares sequence homology with budding yeast G_1 cyclins, but has not been shown to have a role in G_1 progression in fission yeast, and the available data suggest a role in negative regulation of the meiotic cell cycle (Forsburg and Nurse, 1991, 1994). We looked at the effects of deleting *puc1* in circumstances where deletion of *cig2* was found to affect the onset of S phase. We observed no effect on G_1 progression when *puc1* was deleted in *cdc2-56*, *cdc2-3w*, *cdc2-1w*, *cdc2-33* or *rum1*Δ (data not shown). Overexpression of *rum1* in *puc1*Δ or wild-type cells transiently inhibits G_1 progression to the same degree (not shown).

Our data do not suggest, therefore, that puc1 functions as a G_1 cyclin in a similiar fashion to cig2 and cig1.

Discussion

The $p34^{cdc2}$ kinase is essential for G_1 progression past Start in fission yeast (Nurse and Bissett, 1981). Here we show that $p34^{cdc2}$ kinase activity is periodically associated with the cig2 B-type cyclin in the G_1 phase of the cell cycle. cig2 protein levels also peak in G_1/S and are low in dividing cells, and regulated expression is therefore likely to explain the observed periodicity of association with $p34^{cdc2}$. It has been shown previously that *cig2* mRNA levels show a similiar pattern of expression, and this periodicity depends upon the *res1* gene product, which acts together with $p85^{cdc10}$ in a transcriptional complex in G_1 (Lowndes *et al.*, 1992; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994).

Cells lacking *cig2* have an increased ability to arrest cell cycle progression before Start and are therefore hyperfertile (Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994). No other fission yeast cyclin has been found to be periodically expressed in G_1, and deletion of other cyclin genes such as *cig1* or *puc1* does not produce hyperfertility (Forsburg and Nurse, 1994; Obara-Ishihara and Okayama, 1994). We found that deleting *cig2* delays the onset of S phase in small G_1 cells that regulate Start with respect to cell size, and specifically reduces $p34^{cdc2}$ G_1 activity *in vivo*. Such effects are not observed when either *cig1* or *puc1* are deleted. $p34^{cdc2}$ kinase activity associated with cig1 shows similiar periodicity to the mitotic cyclin cdc13 throughout the cell cycle, and peaks in mitosis (Booher *et al.*, 1989; Moreno *et al.*, 1989; Basi and Draetta, 1995). We found that whilst cdc2/cdc13 kinase activity is very low in G_1 cells, $p34^{cdc2}$ kinase activity associated with cig2 peaks around G_1/S. We think, therefore, that cig2 is likely to be the principal G_1 cyclin regulating $p34^{cdc2}$ at Start in fission yeast. It has been shown recently that triple deletion of *cig1*, *cig2* and *cdc13* blocks cell cycle progression in G_1, showing that cig1 or cdc13 are capable of promoting G_1 progression in the absence of cig2 (Fisher and Nurse, accompanying manuscript). We find that overexpression of $p25^{rum1}$ in a cell that lacks both cig1 and cig2 inhibits cdc13 activity, and blocks cell cycle progression in G_1 and G_2, consistent with this idea. These three cyclins are therefore sufficient to explain $p34^{cdc2}$ G_1 activity in fission yeast, and there is still no evidence that puc1 has any role in the mitotic cell cycle. Our data suggest that, in wild-type cells, cig2 is most important for cell cycle progression in G_1 cells, when cig1- and cdc13-associated $p34^{cdc2}$ kinase activity are at their minimum. cdc13 is essential for mitosis and cig1 may also normally function late in the cell cycle in wild-type cells. The fact that either of these cyclins can compensate for the absence of cig2 suggests a quantitative model for cell cycle regulation, where the total kinase activity associated with $p34^{cdc2}$ may be of central importance. Since cig2/cdc2 kinase activity peaks early in the cell cycle, this complex may ordinarily control G_1 progression. Regulation of cig2 is likely to have a role in linking G_1 progression to achievement of the critical cell size required for Start.

Fission yeast therefore use B-type cyclins to regulate Start and the onset of S phase, together with entry into mitosis. In budding yeast, CLN1, 2 and 3 represent distinct G_1 cyclins regulating Start and bud formation, whilst B-type cyclins subsequently promote the onset of S phase and mitotic entry (discussed by Nasmyth, 1993; Schwob *et al.*, 1994). Whilst mating pheromone inhibits CLN activity and arrests budding yeast cells before Start even in rich medium, wild-type fission yeast cells can only conjugate in conditions of nitrogen starvation. It is therefore possible that the CLNs may have evolved to allow conjugation to occur in rich growth conditions, or to facilitate division by budding, in contrast to the situation for fission yeast.

$p25^{rum1}$ has an important role in regulating G_1 progression in fission yeast, and cells lacking the *rum1* gene enter S phase immediately after mitosis, regardless of cell size (Moreno and Nurse, 1994). Our data suggest that G_1 cells lacking $p25^{rum1}$ have increased $p34^{cdc2}$ kinase activity associated with cig2 and cdc13. Since both of these complexes can promote G_1 progression in fission yeast, this explains why cells are advanced into S phase. In conditions where a wild-type cell must delay G_1 progression, $p25^{rum1}$ prevents accumulation of an active cdc2/cdc13 complex (Correa-Bordes and Nurse, 1995), and may transiently inhibit cdc2/cig2 kinase activity until this complex accumulates to high enough levels to overcome rum1 inhibition. cdc2/cig2 kinase activity may in turn negatively regulate $p25^{rum1}$, facilitating G_1 progression past Start. Indeed, consensus phosphorylation sites for the $p34^{cdc2}$ kinase are found in the sequence of $p25^{rum1}$ (Moreno and Nurse, 1994). A model for the regulation of fission yeast G_1 progression by cig2 and rum1 is shown in Figure 8.

It has been suggested that the cdc2/cdc13 complex defines a cell as being in G_2, and that $p25^{rum1}$ defines a cell as being in pre-Start G_1 (Hayles *et al.*, 1994; Moreno and Nurse, 1994). Overexpression of $p25^{rum1}$ produces multiple rounds of S phase in the absence of mitosis, equivalent to deletion of the *cdc13* gene, and this can be explained by rum1 inhibition of cdc13/cdc2 kinase activity. We show that such re-replication from G_2 is principally dependent upon cig1, and to a lesser extent upon cig2. Our data explain this difference, since $p25^{rum1}$ does not inhibit cig1/cdc2, but can inhibit cig2-associated $p34^{cdc2}$ kinase activity. Whilst cig2 levels peak in G_1 and are low late in the cell cycle, $p34^{cdc2}$ kinase activity associated with cig1 peaks in mitosis (this work; Basi and Draetta, 1995). It is likely, therefore, that whilst $p25^{rum1}$ overexpression in a G_2 cell inhibits cdc2/cdc13 kinase activity, cdc2/cig1 activity remains and so promotes re-replication (Figure 8C). Such re-replication from G_2 does not reflect the normal situation of a G_1 cell, where cig2 is of principal importance for determining the onset of S phase, since cig1- and cdc13-associated $p34^{cdc2}$ kinase activity is very low (Figure 8A and B).

In addition to regulating G_1 progression, it was proposed originally that $p25^{rum1}$ is necessary to prevent entry into mitosis from pre-Start G_1 (Moreno and Nurse, 1994). Deletion of *rum1* in a $cdc10^{ts}$ mutant that ordinarily blocks G_1 progression at Start, preventing the onset of S phase, results in the onset of mitosis from G_1. In contrast, deletion of *rum1* in wild-type G_1 cells abolishes the pre-Start G_1

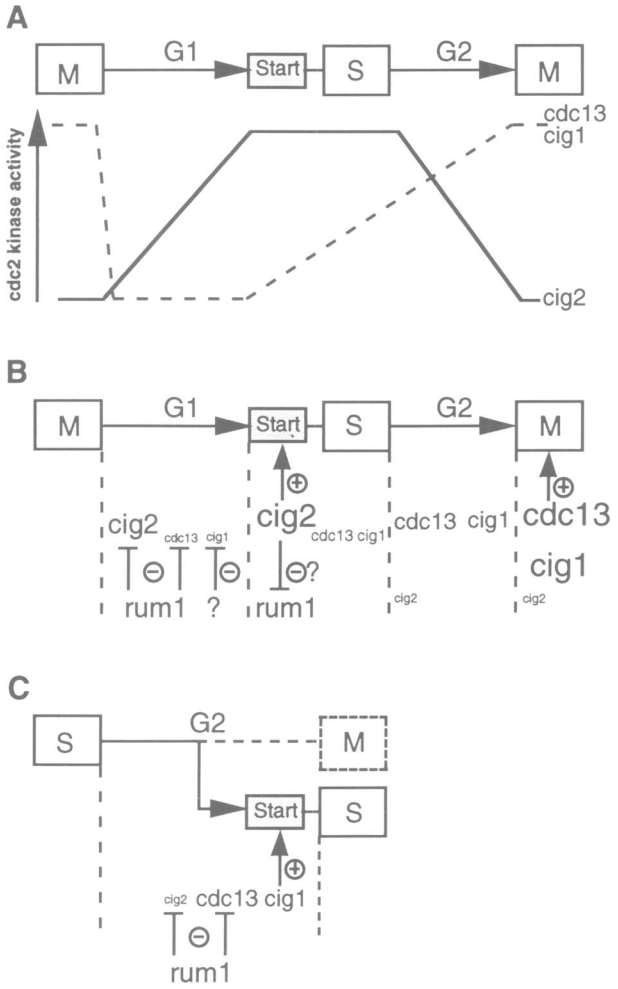

Fig. 8. A model for the regulation of G_1 progression in fission yeast. cig1, cig2 and cdc13 are B-type cyclins that activate $p34^{cdc2}$ kinase activity. For the sake of simplicity, only the cyclin partners of cdc2 are shown in the diagram, and in each case the letter size indicates the level of cdc2 kinase activity associated with the particular cyclin at that point in the cell cycle. (**A**) Whilst levels of cdc13 and cig1 peak in mitosis, cig2 accumulates in G_1 cells and promotes Start and the onset of S phase. (**B**) $p25^{rum1}$ transiently inhibits cig2/cdc2 until the cell reaches the minimum size required to pass Start, at which point cig2 expression may be induced, and $p25^{rum1}$ inhibited by phosphorylation. cig1 and cdc13 are only present at very low levels in G_1 cells (indicated by small type). $p25^{rum1}$ may have a role in preventing accumulation of cdc13 before Start, but cig1 is regulated independently. Whilst cdc13/cdc2 and cig1/cdc2 peak in mitosis (large type), cig2 levels are low. The presence of the cdc13/cdc2 complex in G_2 cells inhibits re-entry into S phase. (**C**) Overexpression of $p25^{rum1}$ in a G_2 cell inhibits cdc13/cdc2 kinase activity, allowing cig1/cdc2 to promote Start and re-replication in the absence of mitosis.

interval and causes premature S phase, rather than entry into the subsequent mitosis (Moreno and Nurse, 1994). In these circumstances, increased $p34^{cdc2}$ kinase activity associated with cig2 and cdc13 advances cells into S phase, thereby activating the post-Start checkpoint that inhibits mitosis until S phase is completed (Moreno and Nurse, 1994; Hayles and Nurse, 1995). In cells that lack both *rum1* and *cdc10*, the post-Start checkpoint cannot be activated since S phase is not initiated. These cells, therefore, can continue to accumulate cdc2/cdc13 kinase activity, resulting in entry into mitosis from G_1 (Correa-Bordes and Nurse, 1995).

We believe, therefore, that the principal function of $p25^{rum1}$ is to regulate G_1 progression. Transient inhibition of cig2/cdc2 may regulate the timing of Start with regard to cell size, whilst inhibition of cdc13/cdc2 can both ensure that this complex does not promote progression past Start, and also prevent premature entry into mitosis from G_1. Since $p25^{rum1}$ inhibits cdc2/B-type cyclin complexes in G_1, it thus resembles the budding yeast $p40^{SIC1}$ cyclin-dependent kinase (cdk) inhibitor (Mendenhall, 1993; Donovan *et al.*, 1994; Nugroho and Mendenhall, 1994; Schwob *et al.*, 1994). Whilst Start is regulated by the CLNs in *Saccharomyces cerevisiae*, $p40^{SIC1}$ regulates B-type cyclins that promote the onset of S phase (Schwob *et al.*, 1994). In fission yeast, B-type cyclins regulate both Start and the onset of S phase, and inhibition by $p25^{rum1}$ therefore links cell cycle progression to the G_1 size control. It has been noted recently that $p25^{rum1}$ and $p40^{SIC1}$ share limited sequence homology (M.Mendenhall, personal communication), whilst expression of *SIC1* complements deletion of the *rum1* gene in fission yeast (I.Gonzalez and S.Moreno, unpublished data). cdk inhibitors described in animal cells link cell cycle progression in G_1/S to external signals such as growth factors or contact inhibition, or to DNA damage (for a recent review, see Sherr and Roberts, 1995). In contrast, $p25^{rum1}$ and $p40^{SIC1}$ seem to act in the midst of a normal cell cycle. $p40^{SIC1}$ is known to be degraded at the G_1/S boundary, and this depends upon the CDC34 ubiquitin conjugating enzyme (Schwob *et al.*, 1994). A human homologue of *CDC34* has been described recently (Plon *et al.*, 1993), raising the possibility that vertebrate cells may also have a cdk inhibitor that acts in an analogous fashion to $p40^{SIC1}$ and $p25^{rum1}$.

Materials and methods

Fission yeast strains and methods

The following strains were used: *972h⁻*, *cig1Δ::ura4⁺ ura4-D18 h⁻*, *cig2Δ::ura4⁺ ura4-D18 h⁻*, *puc1Δ::ura4⁺ ura4-D18 h⁻*, *cig1Δ::ura4⁺ cig2Δ::ura4⁺ ura4-D18 h⁻*, *cdc2-56 h⁻*, *cdc2-56 cig2Δ::ura4⁺ ura4-D18 h⁻*, *cdc2-56 puc1Δ::ura4⁺ ura4-D18 h⁻*, *cdc2-3w h⁻*, *cdc2-3w cig2Δ::ura4⁺ ura4-D18 h⁻*, *cdc2-3w puc1Δ::ura4⁺ ura4-D18 h⁻*, *cdc2-1w h⁻*, *cdc2-1w cig2Δ::ura4⁺ ura4-D18 h⁻*, *cdc2-1w puc1Δ::ura4⁺ ura4-D18 h⁻*, *wee1-50 h⁻*, *wee1-50 cig1Δ::ura4⁺ ura4-D18 h⁻*, *wee1-50 cig2Δ::ura4⁺ ura4-D18 h⁻*, *wee1-50 puc1Δ::ura4⁺ ura4-D18 h⁻*, *cdc2-33 h⁻*, *cdc2-33 cig2Δ::ura4⁺ ura4-D18 h⁻*, *cdc2-33 puc1Δ::ura4⁺ ura4-D18 h⁻*, *ade6-704 ura4-D18 leu1-32 h⁻*, *cdc10-129 rum1Δ::ura4⁺ cig2Δ::ura4⁺ ura4-D18 h⁻*. The *cig1Δ::ura4⁺ ura4-D18 h⁻* strain was described by Bueno *et al.* (1991), and involves deletion of the entire open reading frame. The *cig2Δ::ura4⁺ ura4-D18 h⁻* strain was described by Obara-Ishihara and Okayama (1994), and involves deletion of the N-terminal 80% of the protein The *puc1Δ::ura4⁺ ura4-D18 h⁻* strain was described by Forsburg and Nurse (1994), and involves deletion of the C-terminal 80% of the protein. Basic fission yeast methods were as described by Moreno *et al.* (1991).

For the experiment described in Figure 7A and B, pREP3X(*sup3-5*) *rum1⁺* was integrated in the strain *ade6-704 ura4-D18 leu1-32 h⁻*, using the *ade6-704/sup3-5* system (Hofer *et al.*, 1979; Carr *et al.*, 1989) and the lithium acetate transformation method (Okazaki *et al.*, 1990).

Since deletion of *rum1* causes sterility, the *cig2Δ rum1Δ* double mutant was made by crossing *cig2Δ::ura4⁺ ura4-D18 leu1-32 h⁻* to *rum1Δ::ura4⁺ ura4D-18 leu1-32 ade6-M210 h⁺* transformed with pREP3X *rum1⁺*, so that *rum1* is expressed from the plasmid, and the double mutant was checked subsequently to ensure that the plasmid had been lost. Tetrad analysis was performed, and the identity of the *cig2Δ rum1Δ* double mutant confirmed by Southern blotting. *cig1Δ rum1Δ* and *puc1Δ rum1Δ* strains were made in the same way.

All experiments in liquid culture were carried out in minimal medium, starting with a cell density of $2–4\times10^6$ cells/ml, corresponding to mid-exponential phase growth. Temperature shift experiments were carried

out using a water bath at 36.5°C, and the temperature was checked carefully, since the G_1 arrest of *cdc2*^{ts} mutants is weaker at lower temperatures.

To induce expression from the *nmt1* promoter, cells were grown to mid-exponential phase in minimal medium containing 5 μg/ml thiamine, then spun down and washed three times with water, before resuspending in fresh medium lacking thiamine at a density calculated to produce 4×10^6 cells/ml at the time of peak expression from the *nmt1* promoter.

Epitope tagging of cig2

A restriction site for *Not*I was introduced at the 3′ end of the *cig2* open reading frame, and used to insert a fragment corresponding to three consecutive copies of the haemagglutin epitope tag (HA tag). A 4.8 kb *Pst*I–*Spe*I genomic fragment containing *cig2HA* was transformed into a *cig2Δ::ura4*$^+$ *ura4-D18* strain, and 5-fluoroorotic acid used to select ura$^-$ colonies (Boeke *et al.*, 1984). Southern blotting and PCR analysis of DNA isolated from such a colony confirmed that the *cig2Δ::ura4*$^+$ locus had been specifically replaced with *cig2HA* by homologous recombination. In order to demonstrate that cig2HA is functional, we compared the ability of wild-type and *cig2HA* strains to arrest in G_1 in medium lacking a nitrogen source. Whilst *cig2Δ* cells have an enhanced ability to arrest in G_1, *cig2HA* behaves in an identical fashion to wild-type.

H1 kinase assays and Western blots

For H1 kinase assays, extracts from 3×10^8 cells were made using HB15 buffer (Moreno *et al.*, 1989, 1991), spun at 4°C in a microfuge for 10 min, and the protein concentration determined by a Bradford assay. Samples of 500 μg of extract were immunoprecipitated at 0°C for 1 h, using 2 μl of SP4 anti-cdc13 polyclonal antibody, 2 μl of anti-HA monoclonal antibody, 2 μl of anti-cig1 antibody or 2 μl of affinity-purified C2 anti-cdc2 antibody. Protein A–Sepharose was then added for 30 min at 4°C and the immunoprecipitates washed four times with HB buffer. Immunoprecipitates were resuspended in 20 μl of HB containing 200 μM ATP, 1 mg/ml histone H1 (Boehringer Mannheim) and 40 μCi/ml [γ-^{32}P]ATP, and incubated at 30°C for 20 min. The reactions were stopped with 40 μl of 2× SDS sample buffer, denatured at 100°C for 5 min and samples run on a 14% SDS–PAGE gel. Phosphorylated histone H1 was detected by autoradiography.

For Western blots of cig2 and cdc2, 20 μg of protein extract from each sample was run on a 14% SDS–PAGE gel, which was then blotted to nitrocellulose and probed with anti-cdc2 or anti-HA antibodies.

Flow cytometry and microscopy

About 10^7 cells were spun down, washed once with water, then fixed in 70% ethanol and processed for flow cytometry or DAPI staining, as detailed previously (Sazer and Sherwood, 1990; Moreno, *et al.*, 1991). A Becton-Dickinson FACScan was used for flow cytometry. For the experiment described in Figure 5A, the proportion of G_1/S cells represents the percentage of cells with a DNA content less than a value midway between 1 C and 2 C.

Cell size measurements were made using ethanol-fixed cells that were subsequently rehydrated and DAPI stained, and then 20 mitotic cells in each case were measured using a Zeiss Axiophot microscope with a micrometer scale for calibration.

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