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Fission yeast pheromone blocks S-phase by inhibiting the G_1 cyclin B–p34^{cdc2} kinase

Bodo Stern and Paul Nurse

Cell Cycle Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

Yeast pheromones block cell cycle progression in G_1 in order to prepare mating partners for conjugation. We have investigated the mechanism underlying pheromone-induced G_1 arrest in the fission yeast *Schizosaccharomyces pombe*. We find that the G_1-specific transcription factor p65^{cdc10}–p72$^{res1/sct1}$ which controls the expression of S-phase genes is fully activated in pheromone, unlike the analogous control in budding yeast. In contrast, the G_1 function of p34^{cdc2} acting after activation of the G_1-specific transcription is blocked. Pheromone inhibits the p34^{cdc2} kinase associated with both the G_1-specific B-type cyclin p45^{cig2} and the B-type cyclin p56^{cdc13} and overexpression of p45^{cig2} or p47$^{cdc13\Delta 90}$ overcomes the pheromone-induced G_1 arrest. G_1 arrest is compromised in enlarged cells. We suggest that onset of S-phase is controlled by pheromone inhibiting the B-cyclin-associated kinase in G_1, and that increasing cell size contributes to the mechanism for pheromone adaptation. Thus, pheromone in fission and budding yeast acts similarly in inhibiting the G_1 cyclin-dependent kinase (CDK), but differs in its effects on the G_1/S transcriptional control, suggesting that inhibition of CDKs may be a more general mechanism for the control of G_1 progression compared with G_1/S transcriptional control.

Keywords: cyclin B–cdc2 kinase/pheromone/S-phase/*Schizosaccharomyces pombe*/transcription control

Introduction

An important step in the eukaryotic cell cycle is the control which determines whether a cell continues progression through G_1- to S-phase or undergoes an alternative developmental programme, such as withdrawal from the cell cycle or cellular differentiation (reviewed in Sherr, 1994). A good model system for studying this control is provided by the yeasts which become arrested in G_1 before initiating sexual differentiation. The addition of sexual pheromones to cells of either budding or fission yeast causes them to block in G_1, after which two haploid mating partners undergo conjugation to form a diploid zygote (reviewed in Hirsch and Cross, 1992; Nielsen and Davey, 1995). In both yeasts, a conserved MAP kinase pathway transduces the pheromone signal from membrane-located pheromone receptors (reviewed in Herskowitz, 1995). In the budding yeast, the MAP kinase pathway phosphorylates and activates the cyclin-dependent kinase (CDK) inhibitor FAR1 (Chang and Herskowitz, 1990; Peter *et al.*, 1993; Peter and Herskowitz, 1994). This phosphorylation facilitates binding of FAR1 to the CDK CDC28 associated with the G_1-specific CLN cyclins (Peter *et al.*, 1993; Peter and Herskowitz, 1994), and the inhibition of this CDK blocks cell cycle progression. The CLN–CDC28 kinase activity promotes the onset of S-phase in two ways (Dirick *et al.*, 1995): it activates the G_1-specific transcription factors necessary to transcribe genes required for S-phase (Cross and Tinkelenberg, 1991; Nasmyth and Dirick, 1991) and it inactivates SIC1, an inhibitor of the cyclin B-associated CDC28 kinase which is required for the initiation of S-phase (Mendenhall, 1993; Schwob *et al.*, 1994). Given that SIC1 is not essential for the pheromone response (Nugroho and Mendenhall, 1994), it is the inhibition of the G_1-specific transcription which is the crucial target for the pheromone-induced G_1 arrest in budding yeast.

The mechanism of G_1 arrest in fission yeast is much less well understood. Normally the sexual pheromones P- and M-factor only have an effect on nitrogen-starved fission yeast cells, because components of the pheromone signal transduction cascade are only expressed when nutrients are limiting (reviewed in Nielsen and Davey, 1995). However, pheromone effects on exponentially growing cells can be studied in a mutant background that mimics aspects of nitrogen starvation and reduces pheromone proteolysis (Davey and Nielsen, 1994; Imai and Yamamoto, 1994). Elimination of the adenylate cyclase gene *cyr1* lowers the intracellular cAMP level and leads to constitutive expression of genes under nutritional control, thus mimicking nitrogen starvation (Maeda *et al.*, 1990; Kawamukai *et al.*, 1991; Sugimoto, 1991), whilst elimination of the P-factor-degrading protease gene *sxa2* enhances the effects of P-factor addition (Imai, 1992; Ladds *et al.*, 1996). Exponentially growing cells of a *cyr1*Δ*sxa2*Δ double mutant respond to the addition of P-factor by undergoing G_1 arrest, although cells do eventually enter S-phase after a delay of ~1–2 generations (Imai and Yamamoto, 1994).

Similar to other eukaryotes, the onset of S-phase in fission yeast requires the execution of two functions: activation of the G_1-specific transcription factor consisting of p65^{cdc10} (Aves *et al.*, 1985; Lowndes *et al.*, 1992) and p72$^{res1/sct1}$ (Tanaka *et al.*, 1992; Caligiuri and Beach, 1993) and activation of the CDK cdc2 (Nurse and Bissett, 1981). Activation of p65^{cdc10}–p72$^{res1/sct1}$ brings about the periodic expression of genes required for S-phase. The G_1 function of *cdc2* requires the association of p34^{cdc2} with B-type cyclins. p45^{cig2} is the major partner of p34^{cdc2} in G_1 (Martin *et al.*, 1996; Mondesert *et al.*, 1996) but can be replaced by the mitotic B-cyclin p56^{cdc13} and to a minor extent by p48^{cig1} (Fisher and Nurse, 1996; Mondesert *et al.*, 1996). The dependecies between the p65^{cdc10}–p72$^{res1/sct1}$ transcription factor and p34^{cdc2} remain unknown, but it has been suggested that activation of

p65^{cdc10}–p72$^{res1/sct1}$ requires the G_1 form of p34^{cdc2} (Reymond *et al.*, 1993).

Here we have characterized the mechanism of P-factor-induced G_1 arrest using the fission yeast *cyr1*Δ*sxa2*Δ double mutant. We show that pheromone acts by inhibiting the p34^{cdc2} associated with B-type cyclins, but does not block activation of the p65^{cdc10}–p72$^{res1/sct1}$ transcription factor. This is a major difference in the mechanism of G_1 arrest from that operating in budding yeast. It also suggests that p34^{cdc2} is required after the activation of the G_1-specific transcription factor. The pheromone-induced G_1 arrest is sensitive to cell size as enlarged cells do not G_1 arrest in response to pheromone because the cyclin B-associated p34^{cdc2} kinase activity becomes activated despite the presence of pheromone. We propose a model in which the cyclin B-associated p34^{cdc2} kinase activity is inhibited by pheromone and this inhibition is lost during adaptation partly because of increasing cell size.

Results

Activity of the G_1-specific transcription factor is not inhibited by pheromone

The G_1-specific transcription factor complex p65^{cdc10}–p72$^{res1/sct1}$ (Aves *et al.*, 1985; Lowndes *et al.*, 1992; Tanaka, *et al.*, 1992; Caligiuri and Beach, 1993) controls the periodic expression of genes required for S-phase onset. These genes include *cdc22* encoding ribonucleotide reductase (Gordon and Fantes, 1986), *cig2* encoding a G_1/S B-type cyclin (Obara-Ishihara and Okayama, 1994) and *cdc18* (Kelly *et al.*, 1993) and *cdt1* (Hofmann and Beach, 1994) encoding replication initiation proteins. To follow the activity of the G_1 transcription factor complex after pheromone addition, we have monitored the levels of *cdc18* transcripts. These fluctuate in level during the cell cycle, being minimal in G_2 and maximal in G_1 and S-phase cells (Kelly *et al.*, 1993). Certain key experiments were also repeated measuring the levels of the other transcripts and these gave similar results. Within 2 h of P-factor pheromone addition to a *cyr1*Δ*sxa2*Δ double mutant, 40% of the cells were blocked in G_1, and after one generation (which is 6 h in this strain) nearly 100% of the cells were blocked (Figure 1A) (Davey and Nielsen, 1994; Imai and Yamamoto, 1994). Over the same time period, *cdc18* transcript levels increased at least 3-fold (Figure 1B and D) as did the *cig2* 3.2 kb transcript (Figure 1B). These results indicate that the G_1-specific transcription factor complex is active in cells blocked in G_1 by pheromone addition. The level of *cdc18* transcripts observed in the pheromone block was similar to that seen in cells in mid S-phase using hydroxyurea (Figure 1D), when the activity of the G_1-specific transcription factor is reported to be maximal (Fernandez-Sarabia *et al.*, 1993).

The elevated level of *cdc18* transcript was shown to be dependent upon continued *cdc10* function using a *cdc10^{ts}* (*cdc10-129*) mutant strain. P-factor was added to these cells at the permissive temperature of 25°C, which led to arrest in G_1 with elevated *cdc18* transcript levels (Figure 1C). The cells were then shifted to 36°C inactivating p65^{cdc10}, in the continued presence of pheromone. Within 1 h, the level of *cdc18* transcripts dropped dramatically, indicating that in the presence of pheromone the elevated levels of *cdc18* transcript are maintained by continued p65^{cdc10} activity. This decline of *cdc18* transcripts at 36°C was not due to the temperature shift as control cells subjected to the same temperature regime expressed high levels of *cdc18* (Figure 2B).

We conclude that pheromone arrests cells in G_1 by a mechanism which is independent of the G_1-specific transcription factor complex.

Pheromone blocks activation of G_1 p34^{cdc2}–p45^{cig2} kinase

Another important function required for G_1 progression in fission yeast is the CDK p34^{cdc2} protein kinase (Nurse and Bissett, 1981). To investigate the role of this protein kinase in the pheromone-induced G_1 block, we tested whether p34^{cdc2} is still required for cells to enter S-phase after they have been released from P-factor-induced G_1 arrest. For this experiment, we used a *cdc2^{ts}* (*cdc2.M26*) mutant strain which blocks G_1 progression at 36.5°C. Wild-type and the *cdc2^{ts}* strains were arrested in G_1 by addition of pheromone for 6 h at the permissive temperature of 25°C (Figure 2A). Both strains were shifted to 36.5°C for 1 h in the continued presence of pheromone, and then the pheromone was removed. The wild-type cells entered S-phase within a further 1 h (Figure 2A), whilst the *cdc2^{ts}* strain remained blocked in G_1 for the 3 h duration of the experiment (Figure 2A). In the wild-type strain, *cdc18* transcript levels were elevated in pheromone and remained elevated for 1 h after pheromone removal at 36.5°C, only falling as cells completed S-phase (Figure 2B). In the *cdc2^{ts}* strain, the levels of *cdc18* transcript continued to remain elevated at 36.5°C after pheromone removal. We conclude that p34^{cdc2} performs an essential function after the pheromone block point, and that this acts downstream of activation of the G_1-specific transcription factor complex.

Given that P-factor blocks prior to completion of some G_1 function of p34^{cdc2}, an obvious hypothesis is that the P-factor response pathway directly blocks the p34^{cdc2} activity required for G_1 progression. Recently it has been shown in fission yeast that a major p34^{cdc2} activity in G_1 is brought about by a complex between p34^{cdc2} and G_1/S B-type cyclin encoded by *cig2* (Fisher and Nurse, 1996; Martin *et al.*, 1996; Mondesert *et al.*, 1996). Therefore, we monitored p45^{cig2}-associated p34^{cdc2} protein kinase activity in a synchronous culture, both in the presence and absence of P-factor. A synchronous culture was prepared by elutriation and split in two when cells were in the early G_2-phase of the cell cycle. P-factor was added to one culture and the cells proceeded through mitosis and cell division (Figure 3A) and then arrested in G_1 in the next cell cycle (Figure 3B). Notably, septation in the pheromone-treated culture peaked nearly 40 min earlier than in the control culture. This observation suggests that pheromone not only affects G_1/S progression but also advances entry into mitosis by 0.1 of a cell cycle (see below). In the culture lacking pheromone, the p45^{cig2}-associated p34^{cdc2} kinase activity rose to a peak during G_1 and S-phase (Figure 3C and D), confirming earlier data (Martin *et al.*, 1996; Mondesert *et al.*, 1996). In contrast, in the culture containing pheromone, the peak in protein kinase activity was not observed (Figure 3C and D). This result was confirmed using cells synchronized in G_1 by nitrogen starvation and then released into rich

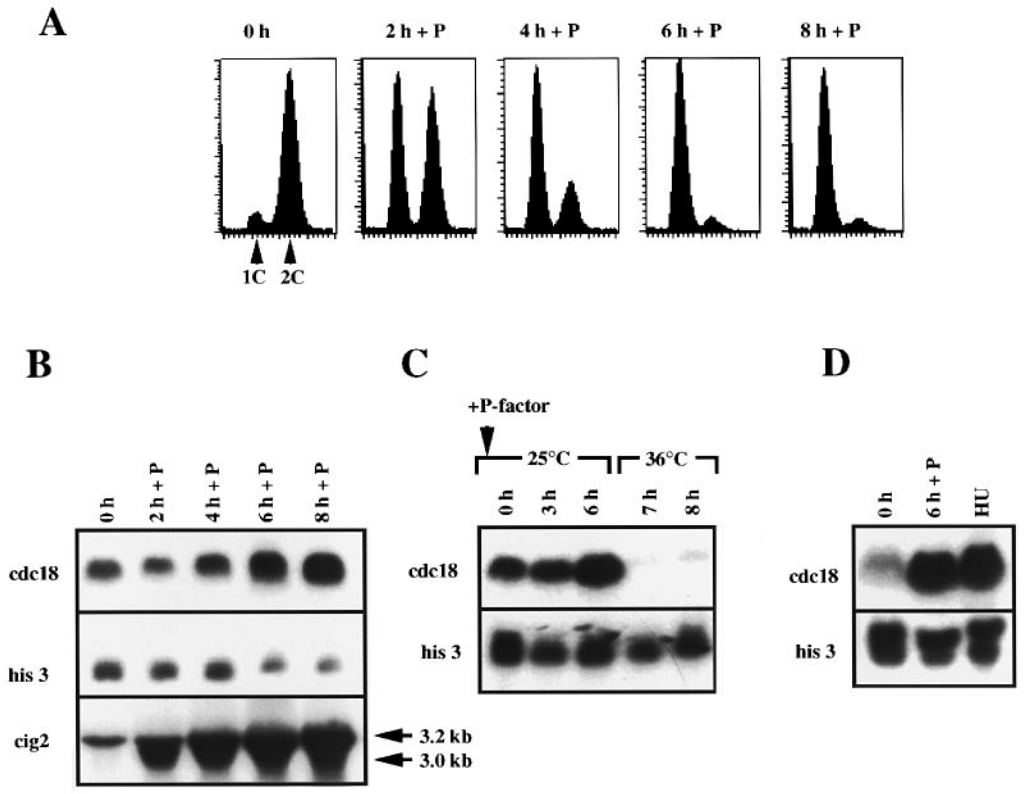

Fig. 1. Effects of P-factor on the activity of the G_1-specific $p65^{cdc10}$–$p72^{res1/sct1}$ transcription factor. (**A**) Flow cytometric analysis of a *cyr1*Δ*sxa2*Δ double mutant exposed to pheromone at 29°C for 8 h. The 1C and 2C DNA content are indicated by arrowheads. (**B**) RNA samples of the same experiment examined by Northern blotting for the expression of *cdc18*, *his3* and *cig2* transcripts. The upper 3.2 kb *cig2* transcript depends on the $p65^{cdc10}$–$p72^{res1/sct1}$ transcription factor (Obara-Ishihara and Okayama, 1994; data not shown). The lower 3 kb transcript is induced by pheromone. (**C**) A *cdc10.129cyr1*Δ*sxa2*Δ mutant strain blocked for 6 h in P-factor at the permissive temperature of 25°C was shifted to the restrictive temperature of 36°C. RNA samples were prepared at the indicated times and examined for *cdc18* and *his3* expression by Northern blotting. (**D**) A *cyr1*Δ*sxa2*Δ strain exposed to P-factor for 6 h at 29°C was washed and resuspended in pheromone-free medium containing 11 mM hydroxyurea. Samples for RNA preparation were taken at 0 and 6 h in P-factor and after 2.5 h in hydroxyurea.

medium. When the medium contained P-factor, cells remained in G_1 and had a low $p45^{cig2}$-associated protein kinase activity (Figure 4A). In contrast, control cells lacking P-factor activated the $p34^{cdc2}$–$p45^{cig2}$ kinase and entered S-phase 5 h after release from the G_1 block (Figure 4A). We conclude that P-factor blocks cells in G_1 by inhibition of the $p45^{cig2}$-associated $p34^{cdc2}$ kinase activity.

To test this possibility further, we added P-factor to a culture expressing *cig2* to high level from an integrated plasmid containing the *cig2* gene, and to a second control culture harbouring an identical empty vector plasmid. Addition of P-factor to the cells with the vector plasmid resulted in G_1 arrest (Figure 5A) and little further cell division after 6 h (Figure 5B). In contrast, the cells containing the *cig2* plasmid failed to arrest in G_1 (Figure 5A) and continued to divide (Figure 5B). There was an initial transient delay in G_1, but most cells had undergone S-phase by 10 h when the control cells were still fully arrested. This demonstrates that the G_1 arrest due to P-factor addition can be overcome by elevated $p45^{cig2}$ levels and strongly supports the idea that P-factor induces G_1 arrest by inhibiting the $p45^{cig2}$-associated $p34^{cdc2}$ kinase activity.

The initial G_1 delay in *cig2*-overexpressing cells suggests that high levels of *cig2* cannot completely override the pheromone effect. A *cig2*-independent mechanism might, therefore, contribute to the pheromone-induced G_1 arrest. In particular, the *cdc2* kinase associated with other B-type cyclins might also be inhibited by pheromone, given the redundancy of B-type cyclins at the G_1–S transition (Fisher and Nurse, 1996; Martin *et al.*, 1996; Mondesert *et al.*, 1996). The major G_1 cyclin that compensates for the loss of *cig2* is the mitotic B-cyclin *cdc13* which becomes activated later in the cell cycle (Fisher and Nurse, 1996; Mondesert *et al.*, 1996). We found that the activity of the $p34^{cdc2}$–$p56^{cdc13}$ kinase was inhibited in the P-factor-induced G_1 arrest (Figure 6A). The kinase activity associated with a third B-type cyclin, $p48^{cig1}$ (Bueno *et al.*, 1991; Bueno and Russell, 1993), was not investigated as suitable anti-$p48^{cig1}$ antibodies were not available. However, the $p48^{cig1}$-associated kinase activity has been shown to follow closely the $p56^{cdc13}$-associated kinase activity (Basi and Draetta, 1995), and the contribution of *cig1* to the promotion of S-phase is minor compared with *cig2* and *cdc13* (Fisher and Nurse, 1996).

We overexpressed *cdc13* lacking the N-terminal cyclin destruction box (*cdc13*Δ*90*) to investigate whether high levels of $p56^{cdc13}$ can also override the pheromone arrest. We chose a non-degradable version because levels of $p56^{cdc13}$ are known to be low in G_1, most likely due to an activated cyclin proteolysis machinery (Hayles *et al.*, 1994). The terminal phenotype of cells overexpressing *cdc13*Δ*90* is mitotic arrest (J.Hayles, personal communication). However, in the current experiment P-factor was

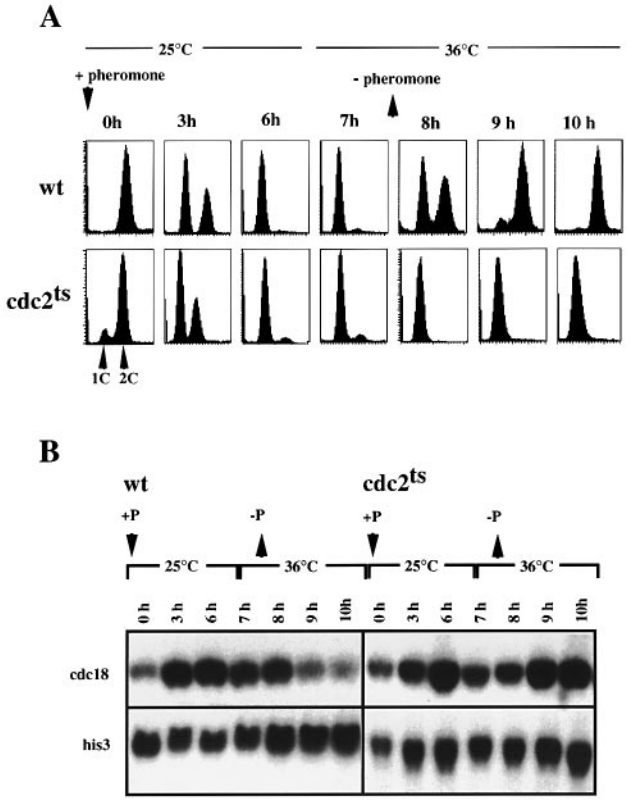

Fig. 2. The *cdc2* function in G_1 is required after activation of *cdc10* and is blocked by pheromone. *Cyr1*Δ*sxa2*Δ and *cdc2-M26 cyr1*Δ*sxa2*Δ strains were blocked in pheromone at 25°C for 6 h, shifted to 36.5°C and P-factor washed out 1 h later. Samples were taken at the indicated times and examined for DNA content by flow cytometric analysis (**A**) and for expression of *cdc18* and *his3* by Northern blotting (**B**).

added 20 h after induction of the *nmt* promoter when p47$^{cdc13\Delta90}$ levels were not yet maximal (data not shown) and cells were still proliferating with an almost normal generation time (Figure 5B). Ten hours after addition of P-factor, the control culture was still arrested in G_1 while nearly all cells with high levels of p47$^{cdc13\Delta90}$ were in G_2, indicating that the pheromone-induced G_1 arrest was severely compromised (Figure 5A).

Thus, both the p45^{cig2}- and the p56^{cdc13}-associated kinase activities are down-regulated in pheromone, and high levels of either p45^{cig2} or p47$^{cdc13\Delta90}$ can overcome the pheromone-induced G_1 arrest. We conclude that P-factor induces G_1 arrest by inhibiting the activity of the p34^{cdc2} kinase associated with B-type cyclins.

Pheromone does not block activation of p45^{cig2}-associated kinase in enlarged cells

It has been described that the pheromone-induced G_1 arrest is transient and cells eventually adapt to P-factor by entering S-phase (Davey and Nielsen, 1994; Imai and Yamamoto, 1994). The beginning of this adaptation is shown in Figure 6A, where 10 h after P-factor addition cells can be observed to begin entry into S-phase. At a later stage (15–23 h, Figure 6B), cells in P-factor proliferate with a generation time indistinguishable from control cells. However, unlike the control cells, the adapting P-factor-treated cells spend an extended period in the G_1-phase. In pheromone-free medium, cells spend 10% of their cell cycle in G_1 (Nasmyth *et al.*, 1979) whereas P-factor-adapting cells spend 60% of their cell cycle in G_1.

The adaptation starts with an increase of p45^{cig2}-associated p34^{cdc2} kinase activity (Figure 6A), consistent with the idea that adaptation to P-factor involves up-regulation of the p45^{cig2}-associated p34^{cdc2} kinase activity. The notion that p45^{cig2}-associated kinase plays a role in adapting cells is also supported by the observation that adaptation is slightly delayed in a *cig2*Δ mutant (Figure 6C). However, *cig2* is not essential for the adaptation process as *cig2* mutant cells eventually adapt (Figure 6C). The p56^{cdc13}-associated kinase activity might compensate for *cig2* in a *cig2* mutant because this kinase activity, which drops to very low levels in G_1 arrest, reappears at 10 h concomitant with the onset of S-phase.

What signal leads to the reactivation of the p34^{cdc2} kinase activity associated with B-cyclins during adaptation to pheromone? Cell size measurement revealed that the p45^{cig2}–p34^{cdc2} kinase activity was correlated with cell size (Figure 6A). An initial cell size decrease is the result of the pheromone-induced advancement of mitosis (Figure 3A). Cell size starts to increase 6 h after addition of pheromone as a result of the formation of conjugation tubes (‘shmooing’). A similar cell size profile has been described for a *cyr1* mutant and a *pat1-114* mutant treated with M-factor (Davey and Nielsen, 1994). Cell elongation coincides with an up-regulation of the p45^{cig2}–p34^{cdc2} kinase activity (Figure 6A). This result suggests that the pheromone-dependent G_1 arrest might be weakened in enlarged cells because the pheromone-mediated inhibition of the p45^{cig2}–p34^{cdc2} kinase is counterbalanced by the increasing cell size. To test this hypothesis, we created long cells using a *cdc25^{ts}* allele which blocks cell cycle progression in G_2 at the restrictive temperature of 36°C. A *cdc25-22 cyr1*Δ*sxa2*Δ triple mutant was incubated at 36°C for 6 h before release to the permissive temperature. Pheromone was added after 5 h at the restrictive temperature to half of the culture. Upon release, both pheromone-treated and untreated cells underwent a synchronous mitosis as seen from the peak of septation. The control cells continued proliferation as indicated by a synchronous S-phase after 60 min (4C peak in the FACS analysis, Figure 7C), an additional cell number doubling (Figure 7A) and a second septation peak at ~200 min (Figure 7B). Similarly, the majority of cells in pheromone entered S-phase, as indicated by the 4C peak at 60 min (Figure 7C). In addition, the septation index peaked a second time at 180 min (Figure 7B) and the cell number nearly quadrupled during the time course (Figure 7A). All these observations indicate that the cells went through two cell divisions despite the presence of pheromone. After the completion of two cell divisions during which the cell size is considerably reduced compared with the G_2-arrested starting culture, the cells finally arrested in G_1 (Figure 7C). Figure 7D shows that the p45^{cig2}–p34^{cdc2} kinase activity and the p45^{cig2} protein level reach a maximum during S-phase very similar to the untreated control. From these results, we conclude that pheromone does not inhibit the p45^{cig2}–p34^{cdc2} kinase activity in highly enlarged cells.

The failure of enlarged *cdc25-22* cells to arrest in the first G_1 is not due to an increased pheromone requirement because a 5-fold higher pheromone concentration gives

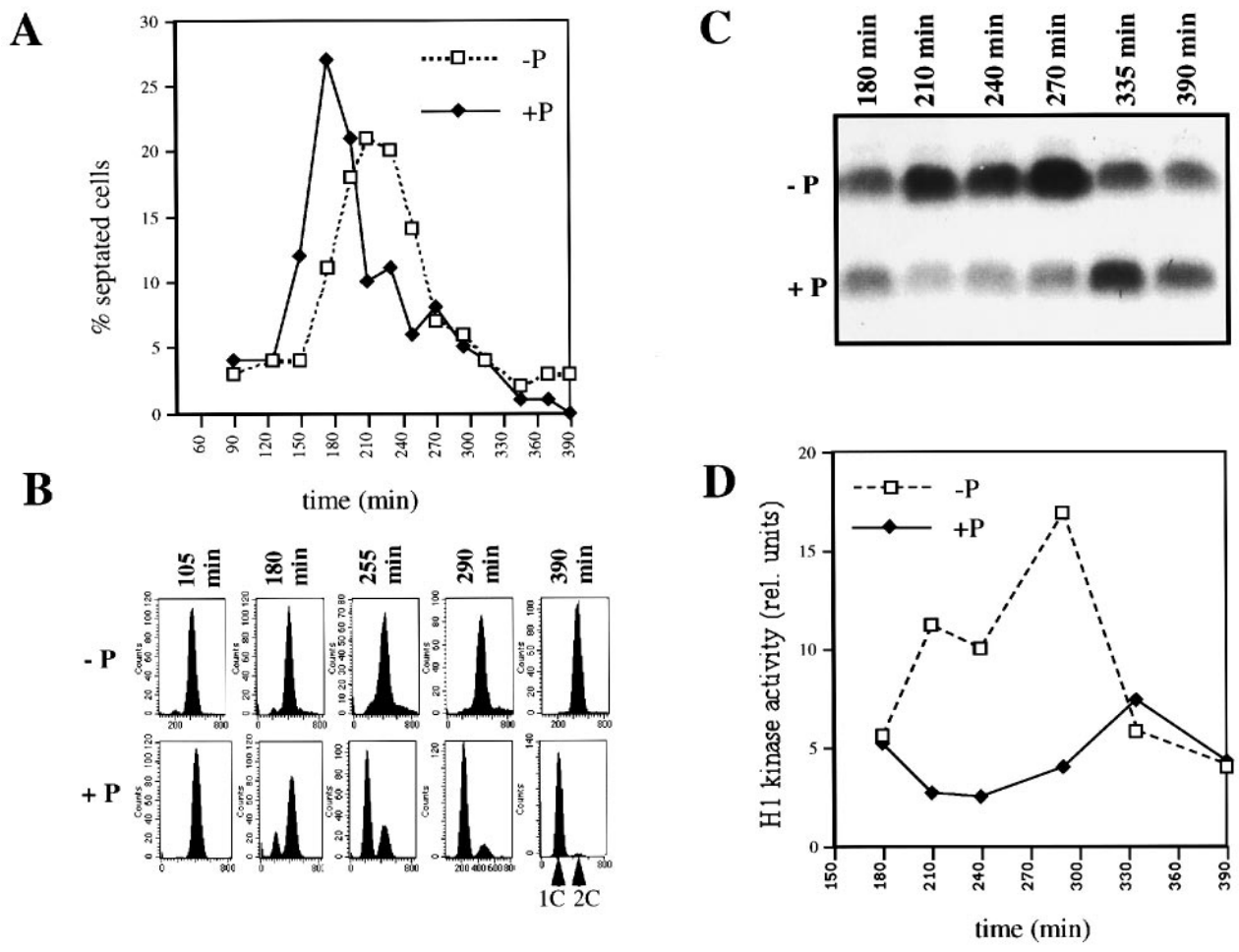

Fig. 3. p45^{cig2}-associated kinase activity is not activated in pheromone-treated cells synchronized by elutriation. A *cyr1*Δ*sxa2*Δ culture was synchronized by elutriation, and pheromone added to half of the culture 45 min after elutriation when cells were in G_2. The percentage of septated cells is shown in (**A**) and flow cytometric analysis of the DNA content in (**B**), with 1C and 2C DNA content indicated by arrowheads. Autoradiograph of the p45^{cig2}-associated kinase activity in the absence (–P) or presence (+P) of P-factor at the indicated times after elutriation is shown in (**C**), and (**D**) shows a quantification of the kinase data.

similar results (data not shown). In addition, the bypass of the pheromone response in the first cycle is not due to an insufficient exposure time to P-factor. *Cdc25-22* cells that were incubated for only 4 h at 36°C, including 1 h in P-factor, arrested mostly in the first cycle after release to 25°C (Figure 7E). This indicates that a 1 h pre-incubation in P-factor is sufficient to activate the pheromone response. Figure 7E also shows that the longer the incubation at 36°C, the more cells fail to arrest in the first cell cycle after the release. Because the incubation time at 36°C reflects cell size, this result supports the notion that a cell size-dependent signal counteracts the inhibitory effect of pheromone on the cell cycle.

Pheromone has distinct effects in G_1 and G_2

We observed that pheromone stimulation of G_2 cells has a significant affect on growth rate. When pheromone-treated *cdc25-22* cells were grown for 90 min at 36°C, followed by a 3.5 h incubation in P-factor at 36°C, the mean cell size was reduced by 30% compared with control cells incubated at 36°C without P-factor (Figure 8A). These G_2 cells had a reduced growth rate. Consistent with the results presented in the previous paragraph, most of these 'smaller' *cdc25-22* cells arrest in the first cell cycle after relase to 25°C (data not shown).

The data in Figure 3A show that P-factor advances septation in an elutriated culture. Thus, P-factor affects G_2 cells in two ways: it reduces growth rate (Figure 8A) and it accelerates entry into mitosis (Figure 3A). A nutritional reduction of growth rate is known to cause mitosis at a smaller cell size (Fantes and Nurse, 1977), and so pheromone could have a similar effect.

We have shown that in G_1 cells P-factor blocks the activation of cyclinB p56^{cdc13}-associated p34^{cdc2} but in G_2 cells mitosis is advanced which requires the activation of the p56^{cdc13}-associated p34^{cdc2} kinase. Figure 8B shows that the p56^{cdc13}–p34^{cdc2} kinase is, indeed, activated during mitosis in P-factor-treated cells, suggesting that pheromone does not block the activation of the mitotic kinase activity in G_2 cells. We conclude that P-factor signalling is active in both G_1 and G_2, but has different effects on p34^{cdc2} kinase activity.

Discussion

We have shown that activation of the p34^{cdc2} kinase associated with the major G_1 cyclin p45^{cig2} is blocked

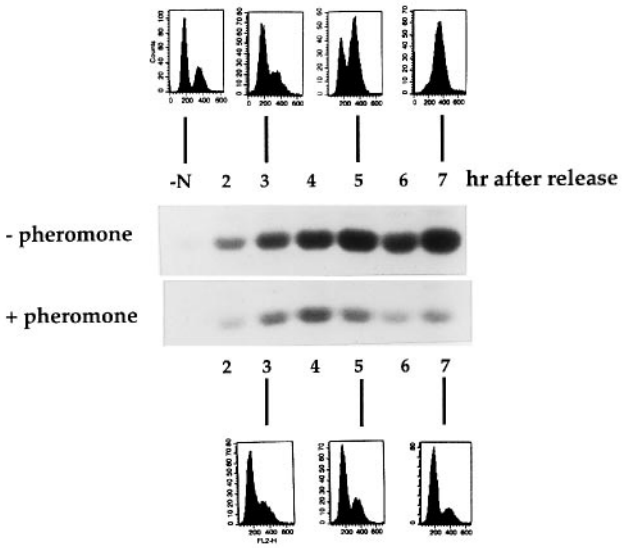

Fig. 4. Pheromone blocks the activation of p45^{cig2}-associated kinase activity in cells synchronized by nitrogen starvation. A *cyr1*Δ*sxa2*Δ mutant was arrested in G_1 by nitrogen starvation for 20 h. Immediately after resupplying a nitrogen source, pheromone was added to half the culture. Samples for DNA content analysis and p45^{cig2} kinase assay were taken at the indicated times.

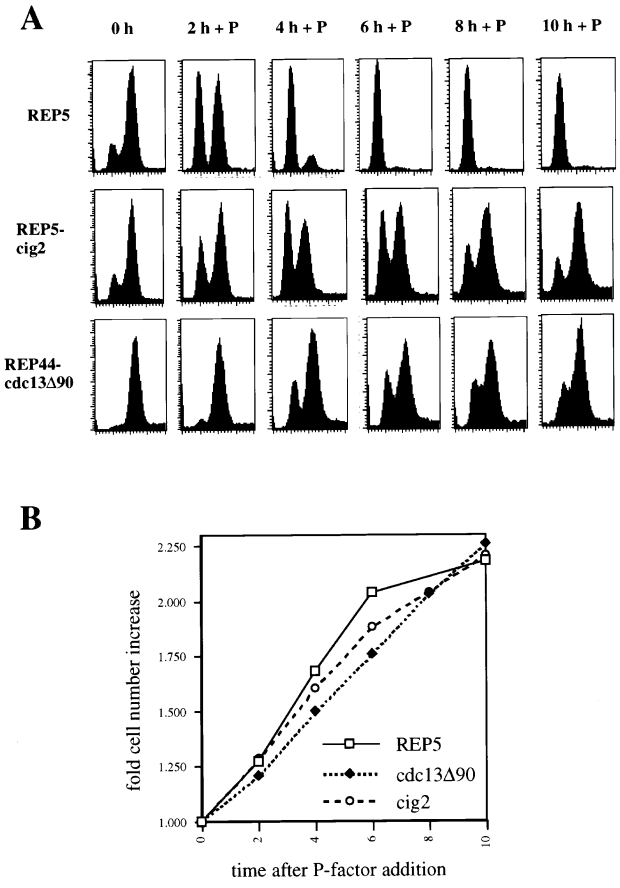

Fig. 5. Overexpression of p45^{cig2} or p47$^{cdc13\Delta90}$ compromises the pheromone-induced G_1 arrest. *cyr1*Δ*sxa2*Δ mutants with the indicated integrated plasmids were exposed to pheromone at 20 (REP5, REP44-sup3-5 cdc13Δ90) or 24 h (REP5 cig2) after induction of the *nmt* promotor. Samples were taken to analyse DNA content (**A**) and cell number (**B**).

during pheromone-induced G_1 arrest in fission yeast (Figures 3 and 4). The functional redundancy of p45^{cig2} and p56^{cdc13} in G_1 requires the inhibition of the p34^{cdc2} kinase activity associated with both B-type cyclins and, indeed, pheromone also blocks the p56^{cdc13}-associated kinase activity in G_1 (Figure 6A). The inhibition of both the p45^{cig2}- and the p56^{cdc13}-associated kinase activities is essential as ectopic expression of either p45^{cig2} or p47$^{cdc13\Delta90}$ compromises pheromone-induced G_1 arrest (Figure 5A). This indicates that the cyclin B-associated p34^{cdc2} kinase activity is a crucial target for pheromone to bring about a G_1 arrest. The observation that *cig2* mRNA levels increase in pheromone (Figure 1A) and *cdc13* mRNA levels remain unchanged (data not shown) suggests that regulation of B-type cyclins is post-transcriptional.

In contrast to the effects on the cyclin B-associated p34^{cdc2} kinase, there were no pheromone effects on the G_1-specific p65^{cdc10}–p72$^{res1/sct1}$ transcription factor complex. This indicates that *cdc2* functions downstream or parallel to the activation of the p65^{cdc10}–p72$^{res1/sct1}$ transcription factor. Given that pheromone-dependent regulation of G_1 progression occurs with a fully activated transcription factor, it seems unlikely that the transcriptional regulation of S-phase genes constitutes a general regulatory step at the G_1–S transition in fission yeast. This suggests that p34^{cdc2}–p45^{cig2} is not required for the expression of replication proteins such as p65^{cdc18} but acts closer to the replication initiation event, possibly by activating the origin recognition complex via phosphorylation (Leatherwood *et al.*, 1996). It is possible that p34^{cdc2} in association with different cyclins fulfils a second function earlier in G_1 which might be required for the activation of G_1-specific transcription, but such a function cannot be subject to inhibition by the pheromone pathway.

Pheromone-induced G_1 arrest is compromised in enlarged cells (Figures 7 and 8). This is consistent with the analysis of the 'adaptation' process starting after ~1–2 generations in pheromone (Figure 6A). Since pheromone-arrested G_1 cells continue growth, cell size increases from 6 h onwards (Figure 6A). Entry into S-phase starts at 10 h and is not due to a declining pheromone activity in the medium, as the same medium can arrest fresh cells in G_1 (Davey and Nielsen, 1994, and data not shown). We suggest that adapting cells can enter S-phase more readily after they have aquired a critical cell size. Because pheromone activity maintains the increased cell size requirement for the G_1–S transition, the G_1 portion of the cell cycle increases to >60% without affecting the generation time (Figure 6B). This interpretation is consistent with changes in cell size monitored in a previous study of the response to M-factor (Davey and Nielsen, 1994). Therefore, fission yeast pheromone appears not to be acting simply as an 'antimitotic' factor like budding yeast pheromone, but rather it delays the onset of S-phase as a consequence of the increasing cell size requirement for entry into S-phase.

Re-entry into the cell cycle may be promoted by the increasing cell size during pheromone-induced cell cycle arrest. However, this is not the sole adaptation mechanism in wild-type *Schizosaccharomyces pombe* cells where the P-factor-degrading protease *sxa2* is involved in the

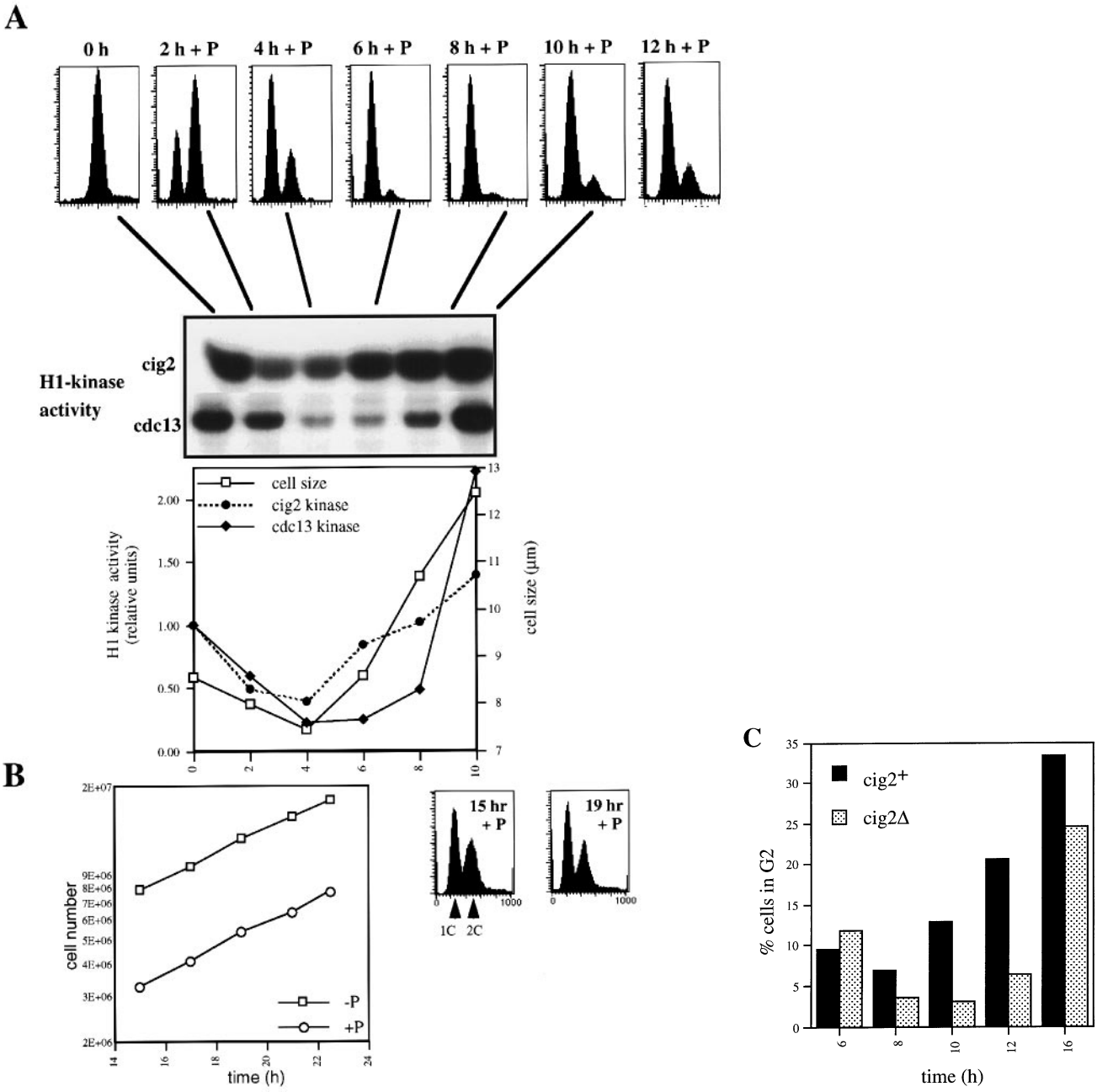

Fig. 6. An increase in cell size and p45^{cig2}-associated kinase activity precedes the adaptation to pheromone. (**A**) A *cyr1*Δ*sxa2*Δ strain was exposed to P-factor for 12 h at 25°C. Samples for DNA content analysis (upper panel) and p45^{cig2}- and p56^{cdc13}-associated kinase activities (middle panel) were taken every 2 h. The lower panel shows a quantification of the two kinase activities and the mean cell size of ethanol-fixed cells. (**B**) Growth curve of a *cyr1*Δ*sxa2*Δ strain in the presence or absence of P-factor. Cell number was followed between 15 and 23 h (left panel). The right panel shows a DNA profile of the *cyr1*Δ*sxa2*Δ culture 15 and 19 h after addition of P-factor. (**C**) Adaptation to pheromone is delayed in a *cig2* mutant. A *cyr1*Δ*sxa2*Δ mutant and a *cig2*Δ*cyr1*Δ*sxa2*Δ mutant were treated for 16 h with P-factor. The increasing percentage of G2 cells reflects the adaptation to pheromone.

adaptation process (Imai, 1992; Imai and Yamamoto, 1994).

The cell size signal may overcome the effect of pheromone by activation of the cyclin B–p34^{cdc2} kinase in G_1, given that the p45^{cig2}-associated kinase is activated normally in enlarged *cdc25* mutant cells in the presence of P-factor (Figure 7D) and that the p45^{cig2}- and the p56^{cdc13}-associated p34^{cdc2} kinase activities increase when cells enlarge after pheromone addition (Figure 6A). We conclude that two opposing signals—an inhibitory pheromone signal and an activating cell size signal—converge on the same effector, the cyclin B–p34^{cdc2} G_1 kinase activities, and that these kinase activities can promote S-phase once a certain threshold level is obtained.

The addition of P-factor to G_2 cells advances onset of mitosis (Figure 3A) and reduces growth rate (Figure 8A) resulting in a reduced cell size (Davey and Nielsen, 1994; Figure 6A). Given that an increase in cell size contributes to the recovery from pheromone-induced cell cycle arrest, it is plausible that the cell size reduction initially facilitates

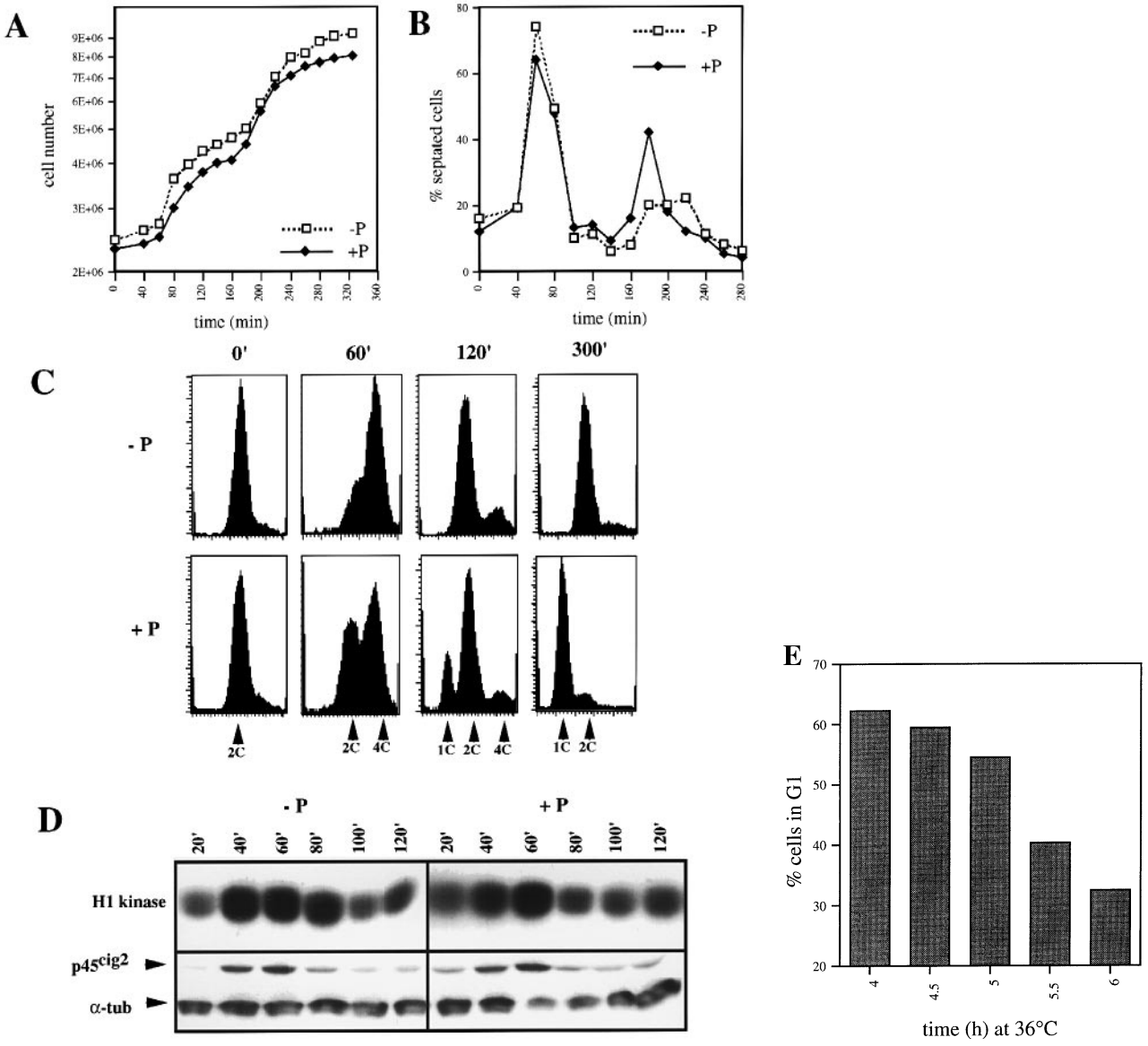

Fig. 7. Pheromone does not block cell cycle progression and activation of the p45^{cig2}-associated kinase activity in enlarged cells. A *cdc25-22 cyr1*Δ *sxa2*Δ mutant was blocked in G_2 by a 6 h incubation at 36°C with P-factor being present during the last hour. After release to 25°C, cell number (**A**), septation index (**B**), DNA content (**C**) and p45^{cig2} protein level and associated kinase activity (**D**) was analysed. (**E**) Correlation of cell size and failure to arrest in G_1 in response to pheromone. A *cdc25-22 cyr1*Δ *sxa2*Δ mutant was incubated at the restrictive temperature for various times between 4 and 6 h and P-factor added 1 h before release to 25°C. The incubation time at 36°C reflects increasing cell size and the figure shows the percentage of G_1 cells after completion of the first cell division (160 min after release from the *cdc25* block).

G_1 arrest. The effects of pheromone on the onset of mitosis and on growth rate demonstrate that G_2 cells are not refractory to the pheromone signal. However, P-factor still allows activation of the mitotic p56^{cdc13}-associated p34^{cdc2} kinase in G_2 (Figure 8B) while it inhibits the same kinase in G_1 (Figures 3 and 4). One possibility is that P-factor mediates the different cell cycle responses in G_1 and G_2 through distinct signalling molecules. A candidate for a G_1-specific signalling molecule is p25^{rum1}, which is a potent inhibitor of the cyclin B-associated p34^{cdc2} kinase (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995). p25^{rum1} is only expressed in G_1 (Correa-Bordes and Nurse, 1995) and thus could mediate the pheromone-induced inhibition of the cyclin B kinase in G_1 without affecting progression through mitosis.

The cell cycle effects of P-factor are reminiscent of the response to nitrogen starvation which also induces G_1 arrest preceded by an advanced mitosis and a slower growth rate in G_2 (Egel and Egel-Mitani, 1974). Given that both pheromone addition and nitrogen starvation have similar effects on G_1 and G_2 cells, they might operate through the same signalling pathway in bringing about G_1 arrest.

Comparison with S.cerevisiae

The pheromone-induced G_1 arrest which we have described here shows similarities and differences with budding yeast. In both yeasts, inhibition of a CDK step is crucial to block cell cycle progression in G_1. In *Saccharomyces cerevisiae*, the G_1-specific CDK complex comprising the CDC28 protein kinase and the G_1 CLN cyclins is inhibited by FAR1 which is phosphorylated by

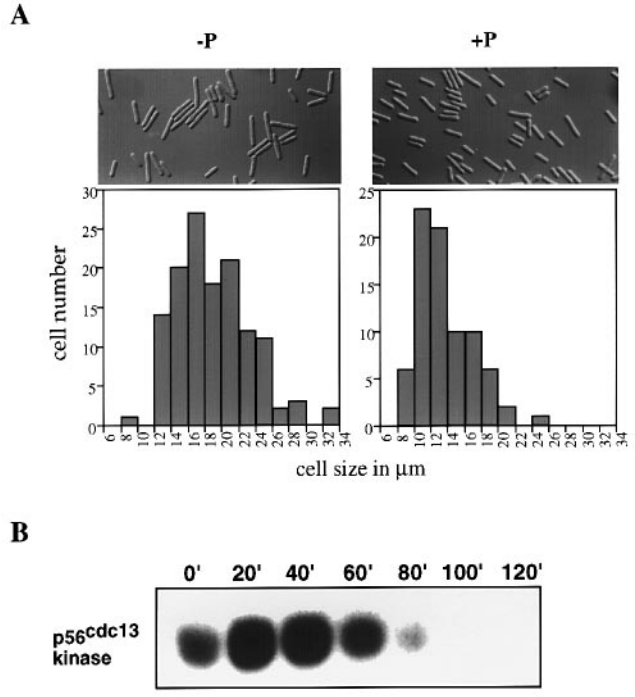

Fig. 8 An exponential growing *cdc25-22 cyr1*Δ *sxa2*Δ mutant was shifted to 36°C and P-factor added after 90 min. After a further 3.5 h incubation at 36°C, the culture was released to the permissive temperature. The cell size distribution in the *cdc25-22* block was compared with control cells that had not been exposed to P-factor (**A**). The mean cell size is 21.7 ± 5.3 μm without P-factor and 13.8 ± 3.1 μm in the presence of P-factor. After the release we followed the $p56^{cdc13}$-associated kinase activity (**B**).

the pheromone-activated MAP kinase FUS3 (Peter *et al.*, 1993; Peter and Herskowitz, 1994). Loss of FAR1 (Chang and Herskowitz, 1990) and mutations that stabilize CLN cyclins confer resistance to pheromone (Cross, 1988; Nash *et al.*, 1988; Hadwiger *et al.*, 1989; Lanker *et al.*, 1996). The CLN–CDC28 kinase promotes progression through G_1 by activating the SWI4–SWI6 and MBP1–SWI6 transcription factors which are required for the cell cycle-specific expression of genes in late G_1 and S-phase (reviewed in Koch and Nasmyth, 1994; Koch *et al.*, 1996). Full activation of the CLN-associated CDC28 kinase activity itself requires activation of SWI4–SWI6 as both *CLN1* and *CLN*2 are under the transcriptional control of SWI4–SWI6 (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). Thus, during pheromone arrest, activation of the G_1-specific transcription factors is viewed as the rate-limiting step for the onset of S-phase (Tyers *et al.*, 1993; Dirick *et al.*, 1995). Our observation in *S.pombe* that the $p65^{cdc10}$–$p72^{res1/sct1}$ transcription factor is fully activated in the pheromone-induced G_1 arrest contrasts with this view and suggests that, at least in fission yeast, transcriptional control is not the major regulatory step at the G_1–S transition during pheromone arrest. We note that an artificial promotor containing the MBP1–SWI6-responsive element confers periodic expression of a reporter gene in *S.cerevisiae* but is not repressed in α-factor (Lowndes *et al.*, 1991). One possible interpretation of this result is that expression of S-phase genes in α-factor is not blocked via inhibition of the MBP1–SWI6 transcription factor but via an MBP1–SWI6-independent mechanism. Such a pheromone-specific mechanism might not exist in fission yeast so that the G_1-specific transcription factor is fully activated in pheromone.

We conclude that in both budding and fission yeast G_1–CDKs are inhibited by pheromone. In budding yeast, the CLN-associated CDC28 kinase activity is inhibited by pheromone. Fission yeast pheromone, however, inhibits the activities of cyclin B–$p34^{cdc2}$ in G_1 which occur later in the cell cycle. As a result, the CLN–CDC28-dependent G_1-specific transcription is down-regulated in budding yeast whereas the corresponding transcription factor in fission yeast, which does not depend on the cyclin B–$p34^{cdc2}$ kinase, is fully activated. We conclude that inhibition of CDKs in G_1 is a more general mechanism for the control of S-phase onset compared with G_1/S transcriptional control.

Materials and methods

Fission yeast strains and methods

The following new strains were constructed: *h⁻cyr1Δ::LEU2⁺sxa2Δ::ura4⁺leu1-32 ura4-D18*; *h⁻cdc10-129cyr1Δ::LEU2⁺sxa2Δ::ura4⁺leu1-32ura4-D18*; *h⁻cdc2-M26cyr1Δ::LEU2⁺sxa2Δ::ura4⁺leu1-32ura4-D18⁺*; *h⁻cdc25-22cyr1Δ::LEU2⁺sxa2Δ::ura4⁺leu1-32ura4-D18*; *h⁻cig2Δ::ura4⁺cyr1Δ::LEU2⁺sxa2Δ::ura4⁺leu1-32ura4-D18*; *h⁻cyr1Δ::ura4⁺sxa2Δ::ura4⁺leu1-32ura4-D18ade6-704 REP5* integrant; *h⁻cyr1Δ::ura4⁺sxa2Δ::ura4⁺leu1-32ura4-D18ade6-704REP5-cig2* integrant; *h⁻cyr1Δ::ura4⁺sxa2Δ::ura4⁺leu1-32ura4-D18ade6-704REP44-sup3-5-cdc13Δ90*. The media and growth conditions were as described by Moreno *et al.* (1991).

Experiments with the *cyr1Δ::LEU2* strain were carried out in minimal medium supplemented with leucine since *LEU2* at the *cyr1* locus does not fully complement the *leu1-32* allele.

P-factor was synthesized by a solid phase method using an automated synthesizer. The peptide was stored in methanol at a concentration of 5 mg/ml and was used at a concentration of 1.5 μg/ml in liquid culture and at a concentration of 3 μg/ml on minimal agar plates.

For the construction of the integrants, a *h⁻ cyr1Δ::ura4⁺ sxa2Δ::ura4⁺ leu1-32 ura4-D18 ade6-704* strain was transformed with the plasmids pREP5, pREP5-cig2 and REP44-sup3-5 cdc13Δ90 (see Construction of plasmids below). REP5 contains the full-strength thiamine-repressible *nmt1* promoter and the *sup3-5* marker (Maundrell, 1993), whereas the REP44-sup3-5 plasmid contains a mutated, medium strength *nmt1* promoter (Basi *et al.*, 1993) with both *LEU2* and a *sup3-5* markers. Transformants that formed white colonies, indicating stable integration of the plasmid, were selected. To induce expression of *cig2* and *cdc13Δ90* from the thiamine-repressible *nmt* promoter, cells were grown in minimal medium containing 5 μg/ml thiamine to mid-exponential phase, spun down, washed three times in minimal medium and resuspended in fresh medium lacking thiamine at a concentration of 1×10^6 cells/ml. Pheromone was added 20 h (*cdc13Δ90*) and 24 h (*cig2*) after induction of the *nmt* promoter. Expression of $p45^{cig2}$ and $p47^{cdc13\Delta90}$ was verified by Western blotting (data not shown).

Flow cytometric analysis

A total of 2×10^6 cells were fixed in 70% ethanol, washed in 3 ml of 50 mM Na_3 citrate and resuspended in 1 ml of 50 mM Na_3 citrate, 0.1 mg of RNase A, 2 μg/ml of propidium iodide. Analysis was carried out as described (Sazer and Sherwood, 1990) using a Becton-Dickinson FACScan.

Cell number

For cell number determination, cells were fixed in 3.7% formaldehyde and 1% saline and counted on a Sysmex Microcellcounter F-800 on the White Blood Cell channel.

Cell length measurement

Ethanol-fixed cells were rehydrated in saline and photographed. The negatives were scanned with a Polaroid Sprint Scan 35 into Adobe Photoshop™ 3.0. Cell length was measured using the NIH image 1.59 programme. The presented data represent the average size of all cells in culture. The size of ethanol-fixed cells is slighly reduced compared with living cells because of fixation.

Synchronized cultures

Elutriation of a *cyr1*Δ *sxa2*Δ strain was carried out using a Beckman J6 centrifuge and elutriator rotor. P-factor was added to half of the culture 45 min after the elutriation.

For starvation-induced synchronization in G_1, a *cyr1*Δ*sxa2*Δ strain was deprived of nitrogen for 20 h, then NH_4Cl was added to a concentration of 5 g/l and P-factor was added to half the culture.

RNA preparation and Northern blot

RNA was prepared by glass bead lysis in the presence of phenol and SDS and subsequently was separated on a formaldehyde gel. 10 μg as measured by OD_{260} was loaded in each track. Probes for blotting were prepared by random oligo priming with [^{32}P]dATP using a Prime-It kit (Stratagene). The template DNA for the probes were a *Nde*I–*Bam*HI *cdc18*$^{+}$ fragment from a REP1-cdc18cDNA plasmid, a *Nde*I–*Eco*RV *cig2* fragment from a genomic *cig2* clone in pAL-SK and a *Sal*I–*Kpn*I *his3* fragment from a pKS*his3* plasmid.

Construction of plasmids

A 2.2kb *Nde*I–*Xma*I fragment from a 4.7 kb genomic *cig2* clone in pAL-SK was cloned into pREP5 allowing expression from the *nmt* promoter.

The non-degradable cdc13 was constructed by first introducing an *Nde*I site at the start codon and then removing the first 89 amino acids using site-directed mutagenesis (Bio-Rad) (J.Hayles, unpublished result). A 1.45 kb *Nde*I fragment containing the open reading frame of *cdc13*Δ*90* was cloned into REP44. Subsequently, a 0.5 kb *Pst*I fragment containing the *sup3-5* marker was cloned into REP44 cdc13Δ90 creating REP44sup3-5 cdc13Δ90.

p45^{cig2} antibodies

Bacterially produced full-length p45^{cig2} (from inclusion bodies) was used to raise four polyclonal antibodies in rabbits (M.O'Connell and J.Correa-Bordes, unpublished results). The crude sera from all rabbits immunoprecipitated H1 kinase activity from a *cig2* strain but not a *cig2*Δ strain. The H1 kinase activity was also dependent on *cdc2* (J.Correa-Bordes, personal communication). For kinase assays, the crude anti-p45^{cig2} serum MOC8 was used. p45^{cig2} was detected on Western blots with affinity-purified anti-p45^{cig2} MOC6 antibodies. For affinity purification, 5 mg of bacterially produced p45^{cig2} was run on a 10% SDS–polyacrylamide gel and Western blotted to a nitrocellulose membrane. A 1 cm wide strip with the main p45^{cig2} band was cut out, washed in phosphate-buffered saline (PBS), blocked with 5% milk and incubated overnight in 2 ml of the polyclonal anti-cig2 serum MOC6. The strip was washed three times in PBS, eluted in 1 ml of 1 mM EDTA, 0.2 M glycine pH 2.5 for 10 min and immediately equilibrated to pH 7.0–7.5 with an equal volume of 0.1 M Tris base. The affinity-purified anti-cig2 antibody detected a 45 kDa protein in wild-type cell extracts but not in *cig2*Δ extracts.

Kinase assays

For kinase assays, soluble extracts from 2–6×10^8 cells were made using HB buffer (Moreno *et al*., 1991) with the following protease inhibitors: aprotinin 20 μg/ml; benzamidin 1 mM; bepstatin 10 μg/ml; leupeptin 20 μg/ml; TLCK 50 μg/ml; TPCK 50 μg/ml; pepstatin 1.8 μg/ml; and phenylmethylsulfonyl fluoride (PMSF) 1 mM. The p45^{cig2}-associated p34^{cdc2} kinase activity was immunoprecipitated from 700 μg (Figure 3) or 1.4 (Figure 7), 2 (Figure 4A) or 6 mg (Figure 6) of protein extract using 10–20 μl of crude anti-p45^{cig2} polyclonal serum. The kinase assay was linear for varying protein levels and for incubation times up to 40 min at 30°C. p56^{cdc13}-associated kinase activity was immunoprecipitated with anti-p56^{cdc13} serum SP4 from 800 (Figure 6A) and 400 μg of extract (Figure 8). The immunocomplexes were precipitated with protein A–Sepharose CL-4B (Pharmacia) and washed three times in HB buffer. For histone H1 kinase activity, immunoprecipitates were resuspended in 40 μl of HB buffer containing 200 μM ATP, 1 mg/ml histone H1 (Calbiochem) and 40 μCi/ml of [γ-^{32}P]ATP and were incubated at 30°C for 20 min. The reactions were stopped with 10 μl of 5× SDS sample buffer, denatured at 100°C for 4 min and samples were run on a 12% SDS–polyacrylamide gel. Phosphorylated histone H1 was detected by autoradiography and quantitated using a Phosphoimager.

Western blot

A total of 5× SDS sample buffer was added to soluble protein extracts and 40 μg of protein from each sample were electrophoresed using a 10% SDS–polyacrylamide gel (Laemmli, 1970). For Western blots, the protein was blotted to Immobilon™-P membrane (Millipore) and detected using ECL (Amersham). Dilutions of the antibodies were 1:1000 for the anti-p45^{cig2} affinity-purified polyclonal antibody and 1:50 000 for the anti-α-tubulin monoclonal antibody (Sigma T5168).

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