

# The anaphase-promoting complex is required in G<sub>1</sub> arrested yeast cells to inhibit B-type cyclin accumulation and to prevent uncontrolled entry into S-phase

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## SUMMARY

Inactivation of B-type cyclin dependent kinases due to ubiquitin-mediated cyclin proteolysis is necessary for the exit from mitosis. In *Saccharomyces cerevisiae*, proteolysis is initiated at the onset of anaphase and remains active until Cln1 and Cln2 cyclins appear in late G<sub>1</sub> of the subsequent cell cycle. A large particle called the anaphase-promoting complex (APC) which is composed of the TPR proteins Cdc16p/Cdc23p/Cdc27p and other proteins is required for B-type cyclin ubiquitination in both anaphase and during G<sub>1</sub> phase. The APC has an essential role for the separation of sister chromatids and for the exit from mitosis, but until now it was unclear whether the persistence of APC activity throughout G<sub>1</sub> had any physiological role. We show here that the APC is needed in G<sub>1</sub> arrested cells to inhibit premature appearance of B-type cyclins and to prevent

unscheduled initiation of DNA replication. When pheromone arrested cells of *cdc16* and *cdc23* mutants were shifted to the restrictive temperature, they underwent DNA replication in the presence of pheromone. DNA replication also occurred in a G<sub>1</sub> arrest induced by G<sub>1</sub> cyclin (Cln) depletion, indicating that mutant cells with a defective APC initiate DNA replication without the Cln G<sub>1</sub> cyclins, which are normally needed for the onset of S-phase. Degradation of Clb2p, Clb3p and Clb5p depends on the APC. This suggests that accumulation of any one of the six B-type cyclin proteins could account for the precocious replication of *cdc16* and *cdc23* mutants.

Key words: B-type cyclin proteolysis, CLB protein, DNA replication, G<sub>1</sub>-S transition, TPR protein

## INTRODUCTION

Association of the cyclin-dependent kinase CDK1 (*cdc2/CDC28*) with B-type cyclins, the mitotic cyclins, is needed for entry into mitosis; subsequent cyclin degradation in late mitosis is required for CDK1 inactivation and exit from mitosis (Murray et al., 1989; King et al., 1994; Murray, 1995). Mitotic cyclins are degraded via ubiquitin-dependent proteolysis (Glotzer et al., 1991; Deshaies, 1995; Zachariae and Nasmyth, 1996). B-type cyclin ubiquitination depends on an N-terminal 9 amino acid sequence motif called the destruction-box. It is tightly cell cycle regulated: inactive during S and G<sub>2</sub> phase and activated during mitosis (Amon et al., 1994; King et al., 1995; Sudakin et al., 1995).

A genetic screen in the yeast *Saccharomyces cerevisiae* revealed three essential genes, *CDC16*, *CDC23* and *CSE1*, which all are needed for Clb2p ubiquitination in vitro (Zachariae and Nasmyth, 1996) and Clb2p degradation in vivo (Irniger et al., 1995). Cdc16p and Cdc23p are members of a protein family which is characterised by multiple tetratripeptide repeats (TPR), 34 amino acid repeats implicated in protein-protein interaction (Goebel and Yanagida, 1991). The TPR proteins Cdc16p, Cdc23p and Cdc27p are part of a large multi-subunit complex (Lamb et al., 1994) which appears to be

very conserved in eukaryotes (Tugendreich et al., 1995; King et al., 1995; Zachariae et al., 1996). This particle, called the anaphase-promoting complex (APC) or cyclosome, whose role is to catalyse the ligation of ubiquitin molecules to cyclin B (Sudakin et al., 1995; Zachariae and Nasmyth, 1996) is needed for the metaphase to anaphase transition and for the exit from mitosis (Lamb et al., 1994; Irniger et al., 1995).

In the yeast *Saccharomyces cerevisiae*, cyclin instability initiated at anaphase persists during the subsequent G<sub>1</sub>-phase (Amon et al., 1994). The transition from G<sub>1</sub> to S phase in yeast is accompanied by a switch in the rates of proteolysis of B-type cyclins and their inhibitory protein p40-Sic1 (Amon et al., 1994; Schwob et al., 1994). This switch is due to the appearance in late G<sub>1</sub> of Cln1 and Cln2/Cdc28 kinases (Wittenberg et al., 1990), which shut off proteolysis of B-type cyclins and activate destruction of p40-Sic1, a B-type cyclin/Cdc28 kinase inhibitor (Amon et al., 1994; Schwob et al., 1994; Dirick et al., 1995). Both events promote activation of the B-type cyclin/Cdc28 kinases needed for the initiation of DNA replication (reviewed by Nasmyth, 1996). Pheromones are thought to cause a G<sub>1</sub> arrest primarily by inactivating the Cln/Cdc28 kinases (Chang, 1993; Peter et al., 1993), a consequence of which is the maintenance of APC activity and B-type cyclin instability.

Until now, it was unclear whether APC activity persisting throughout G<sub>1</sub> phase had any physiological role. It has been proposed that B-type cyclin instability during G<sub>1</sub> phase might help to prevent the appearance of B-type cyclin (Clb) associated kinases before the Cln associated kinases are activated, therefore ensuring a proper order of the events in G<sub>1</sub> phase (Amon et al., 1994). However, repression of B-type cyclin gene transcription and the presence of the p40-Sic1 CDK inhibitor might be sufficient to prevent the premature appearance of B-type cyclin dependent kinases during G<sub>1</sub> phase (Fitch et al., 1992; Schwob et al., 1994), in which case there would be no need for an additional mechanism controlling the accumulation of B-type cyclins.

We found that APC activity during G<sub>1</sub> phase does indeed have a physiological relevance. Pheromone arrested cells of temperature sensitive *cdc16-123* and *cdc23-1* mutants, which both are impaired in APC function, accumulated Clb2p during this G<sub>1</sub> arrest and afterwards replicated their DNA in the presence of pheromone. A similar effect was observed in a G<sub>1</sub> arrest due to G<sub>1</sub> cyclin (Cln) depletion indicating that these mutant cells initiate DNA replication without the Cln G<sub>1</sub> cyclins. We show that the B-type cyclins Clb3p and Clb5p are like Clb2p degraded via the APC. The abnormal replication in these mutants might therefore be a direct consequence of the accumulation of any of the six Clb B-type cyclins.

## MATERIALS AND METHODS

### Yeast strains, genetic techniques and media

The genotypes of yeast strains used in this study are listed in Table 1. Standard genetic techniques (Mortimer and Hawthorne, 1969) were used for manipulating yeast strains. Strains were transformed using the spheroplast method and integrative transformations were verified by Southern blot. When yeast cells were grown in complete medium, YEP medium supplemented either with 2% glucose (YEPD) or 2% raffinose (YEPraf) was used. When yeast cells were grown in a medium lacking methionine, a synthetic medium containing 0.8% yeast nitrogen base, 50 µg/ml uracil and adenine, supplemented with amino acids and 2% glucose was used (Rose et al., 1990).

### Plasmid constructions

The standard protocols have been used for DNA manipulation (Maniatis et al., 1982). Construction of the plasmid containing the *pGAL-CLB5-ha3* and the *pGAL-CLB5ΔDB-ha3* constructs were described by Schwob et al. (1994). To generate the *pGAL-CLB3-ha3* fusion, the *CLB3* gene containing a triple HA epitope at its C terminus (a gift from B. Futcher) was PCR-amplified in order to introduce a *EcoRI* site immediately upstream of its AUG translation initiation codon and a *SalI* site 104 bp downstream of the stop codon. The resulting 1.7 kb *EcoRI-SalI* fragment and a 0.7 kb *BamHI-EcoRI GAL1-10* promoter fragment were then cloned in a triple ligation into YIplac211 (Gietz and Sugino, 1988).

### Cell cycle arrests

Prior to each cell cycle arrest, cultures were grown to log phase, usually to an *A*<sub>600</sub> of 0.3 to 0.6. In all cases when a gene was expressed from the *GAL1-10* promoter, cells were pre-grown in medium containing raffinose as the only carbon source. The *GAL1-10* promoter was induced by the addition of 2% galactose.

Cells were arrested in G<sub>1</sub> phase either by α-factor pheromone or by cyclin deprivation. To arrest cells in G<sub>1</sub> with α-factor pheromone, 0.5 µg/ml α-factor was added to *bar1* strains. After every 120 minutes

additional α-factor (0.25 µg/ml) was added to prevent a drop in the α-factor concentration. To arrest cells in G<sub>1</sub> by Cln cyclin deprivation, strains deleted for G<sub>1</sub> cyclins (*cln1*, *cln2*, *cln3*), but kept alive by a *pMET-CLN2* fusion, were pre-grown in minimal medium lacking methionine. Cells were arrested in G<sub>1</sub> by the addition of methionine to a 2 mM end-concentration. Every 120 minutes additional methionine (1 mM) was added to prevent a decrease in the methionine concentration.

To arrest cells with the microtubule depolymerizing drug nocodazole, 15 µg/ml nocodazole was added.

### FACScan analysis

To determine the DNA content, cells were fixed in 70% ethanol and stained with propidium-iodide as described by Epstein and Cross (1992). The stained cells were analysed on a Becton-Dickinson FACScan.

### Western blot analysis

Western blotting was performed as described by Surana et al. (1993) using the enhanced chemiluminescence detection system (ECL, Amersham). Clb2p- and Cdc28p- antibodies were used in 1:2,000 and 1:500 dilutions, respectively. To detect HA tagged cyclins, the HA antibody (12CA5) was used in a 1:100 dilution.

### Pulse chase experiments and immunoprecipitations

In vivo labellings of yeast cells with [<sup>35</sup>S]methionine for pulse chase experiments and immunoprecipitations were performed as described previously (Amon et al., 1994; Irmiger et al., 1995). The HA antibody (12CA5) was used in a 1:100 dilution to immunoprecipitate the tagged cyclins. After SDS-polyacrylamide gel-electrophoresis, the bands were visualized on X-ray films (Kodak). For each experiment, control cultures to determine the budding index and the DNA content were grown under identical conditions except that the radiolabelled methionine was omitted.

**Table 1. Yeast strains used in this study**

Strains	Relevant genotypes
K1534	<i>MATa bar1::hisG</i>
K2944	<i>MATa cdc15-2 bar1::hisG</i>
K3413	<i>MATa cln1::hisG cln2Δ cln3::LEU2</i> <i>MET3-CLN2/TRP1 SSD1</i>
K4386	<i>MATa cdc23-1 bar1::hisG</i>
K4438	<i>MATa cdc16-123 bar1::hisG</i>
K4554	<i>MATa cdc28-4 bar1::hisG GAL-CLB5ha3/URA3</i>
K4555	<i>MATa cdc28-4 bar1::hisG GAL-CLB5ha3 ΔDB/URA3</i>
K4620	<i>MATa cdc28-4 cdc23-1 bar1::hisG</i> <i>GAL-CLB5ha3/URA3</i>
K4623	<i>MATa cdc28-4 cdc16-123 bar1::hisG</i> <i>GAL-CLB5ha3/URA3</i>
K5040	<i>MATa cse1-22 bar1::hisG</i>
K5310	<i>MATa cdc23-1 bar1::hisG clb5::URA3 clb6::LEU2</i>
K5445	<i>MATa cdc23-1 bar1::hisG clb3::TRP1 clb4::HIS3</i>
K5446	<i>MATa cdc23-1 cdc4-1 bar1::hisG</i>
K5505	<i>MATa bar1::hisG GAL-CLB3ha3/URA3</i>
K5506	<i>MATa cdc16-123 bar1::hisG GAL-CLB3ha3/URA3</i>
K5507	<i>MATa cdc23-1 bar1::hisG GAL-CLB3ha3/URA3</i>
K5575	<i>MATa cdc20-1 bar1::hisG</i>
K5675	<i>MATa cdc16-123 bar1::hisG clb1::hisG clb2-ts</i> <i>clb3::TRP1 clb4::HIS3</i>
K6219	<i>MATa cdc23-304 cln1::hisG cln2Δ cln3::LEU2</i> <i>MET3-CLN2/TRP1 SSD1</i>
YS1193	<i>MATa cdc23-1 bar1::hisG GAL-SIC1</i>
YS1194	<i>MATa cdc23-1 bar1::hisG SIC1ha4</i>
YS1195	<i>MATa cdc23-1 cdc4-1 bar1::hisG SIC1ha4</i>

All strains are isogenic derivatives of W303 (K699): *MATa, ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL*.

## RESULTS

**Pheromone arrested *cdc16-123* and *cdc23-1* mutants accumulate Clb2p and enter into S-phase**

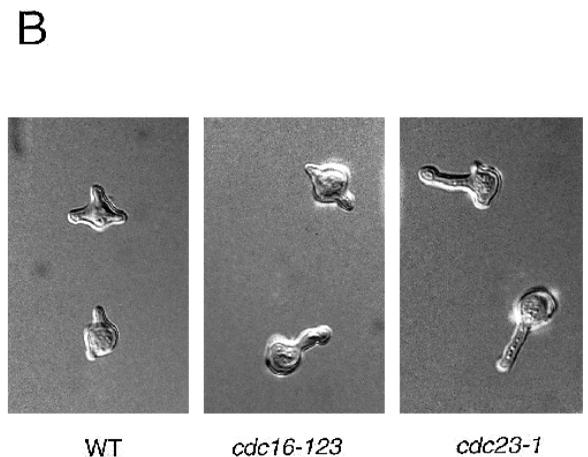
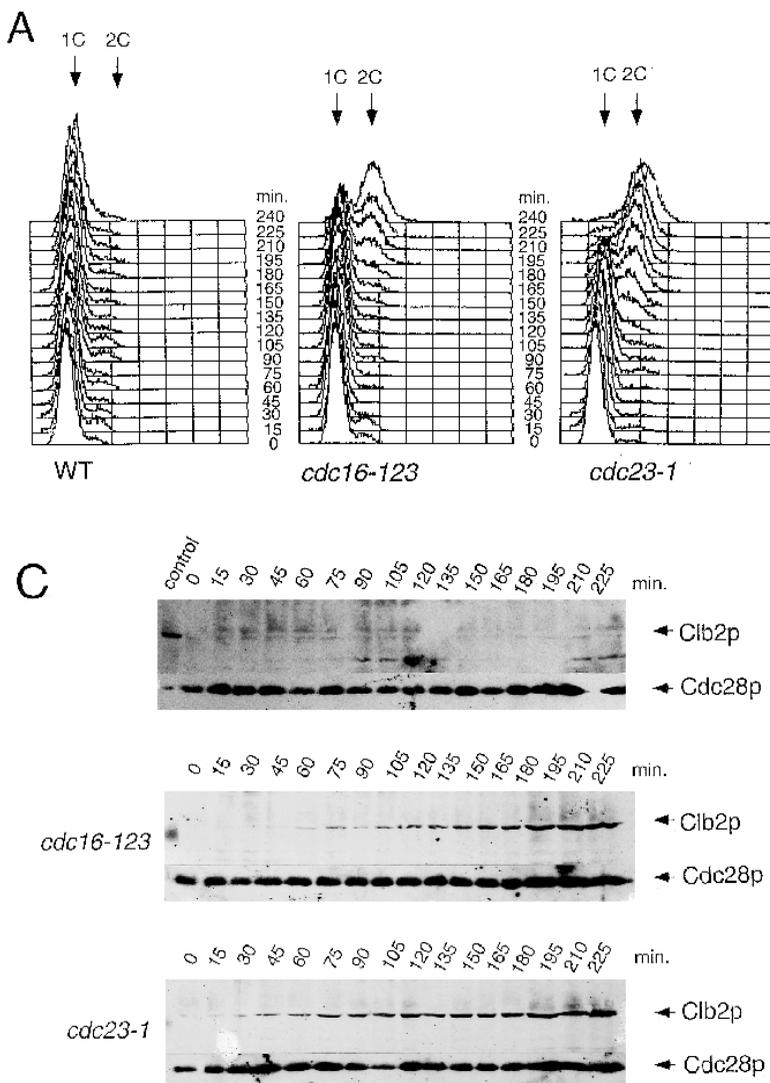
We investigated the role of the anaphase-promoting complex (APC) during G<sub>1</sub> by using temperature-sensitive *cdc16-123* and *cdc23-1* mutants. In both of these mutants APC function is impaired at the restrictive temperature and as a consequence the half-life of Clb2p during G<sub>1</sub>, which is less than 1 minute in wild-type cells, is increased to more than 20 minutes (Irniger et al., 1995).

To analyse whether the APC is important for pheromone induced G<sub>1</sub> arrest, *cdc16-123* and *cdc23-1* mutants growing at their permissive temperature (25°C) were first arrested in G<sub>1</sub> by the addition of  $\alpha$ -factor pheromone. The cultures were then shifted to the restrictive temperature (37°C) and incubated further in the presence of pheromone. Virtually all *cdc16-123* and *cdc23-1* mutant cells underwent DNA replication between 2 and 4 hours after the temperature shift despite the continued presence of pheromone (Fig. 1A), whereas wild-type cells remained permanently arrested in G<sub>1</sub>. Most of the mutant cells (>70%) did not bud, but instead formed abnormally elongated

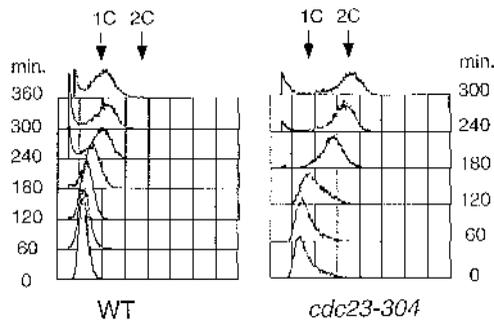
shmoo (Fig. 1B). The absence of budding indicates that the entry into S phase of *cdc16-123* and *cdc23-1* mutants in the presence of  $\alpha$ -factor is not simply due to their becoming refractory to pheromone.

To test this hypothesis we analysed whether mutants defective in APC function replicate their DNA in a different G<sub>1</sub> arrest, induced by G<sub>1</sub> cyclin (Cln-cyclins) depletion. Since we had a *cdc23-304* mutant in the triple *cln* deletion background (*cln1, cln2, cln3*), this mutant, which was identified in the screen for mutants defective in cyclin proteolysis (Irniger et al., 1995), was used for this experiment. A *cdc23-304* strain lacking all the Cln G<sub>1</sub> cyclins, kept alive by a methionine-repressible *MET3-CLN2* fusion, was arrested at the permissive temperature in G<sub>1</sub> phase by the addition of methionine. Upon a shift to the restrictive temperature, these mutant cells efficiently replicated their DNA in the presence of methionine, that is in the absence of the Cln G<sub>1</sub> cyclins (Fig. 2). We conclude from these results that G<sub>1</sub> cells defective in APC function enter into S-phase without the Cln cyclins, which are normally needed for the G<sub>1</sub>/S-transition.

Since DNA replication is normally triggered by the Cdc28 kinase associated with any of the six yeast Clb B-type cyclins,



**Fig. 1.** *cdc16-123* and *cdc23-1* mutants enter S-phase in the presence of  $\alpha$ -factor pheromone. Wild-type (K1534), *cdc16-123* (K4438) and *cdc23-1* (K4386) strains (all *bar1::hisG*) were arrested at the permissive temperature (25°C) in G<sub>1</sub> by  $\alpha$ -factor pheromone for 3 hours, shifted to the restrictive temperature (37°C) and incubated further in the presence of pheromone. (A) DNA content determined by FACS analysis. The timepoints after the temperature shift are indicated. (B) Photographs of cells 225 minutes after the temperature shift. (C) Clb2p levels detected by western blotting. Cdc28p was used for standardisation. The control for Clb2p in the WT-western blot is the 180 minute sample from *cdc16-123*.



**Fig. 2.** *cdc23-304* mutants enter S-phase in the absence of G<sub>1</sub> cyclins. Wild-type (K3413) and *cdc23-304* (K6219) strains, which lack the G<sub>1</sub> cyclins *CLN1*, *CLN2* and *CLN3* and are kept alive by *CLN2* expressed from the methionine repressible *MET3* promoter, were arrested in G<sub>1</sub> phase by the addition of methionine at 25°C for 3 hours. The cultures were shifted to 37°C and further incubated in the presence of methionine. The DNA content was analysed by FACScan analysis. The timepoints after the temperature shift are indicated.

the DNA replication of mutant cells could be a consequence of the accumulation of B-type cyclins. Indeed Clb2p accumulated prior to DNA replication in pheromone arrested *cdc16-123* and *cdc23-1* mutants (Fig. 1C). In the *cdc23-1* mutant Clb2p was detectable 45 minutes after the temperature shift and the cells initiated DNA replication after 135 minutes. In *cdc16-123* mutant cells Clb2p accumulated after 75 minutes and DNA replication was initiated after 180 minutes. Therefore *cdc23-1* cells both accumulated Clb2p and entered S phase earlier than *cdc16-123* cells. This timing of the initiation of DNA replication was fully reproducible in several independent experiments.

These results demonstrate that there is a correlation between the appearance of Clb2p and the initiation of DNA replication.

*CLB* genes are not transcribed at high levels during pheromone induced G<sub>1</sub> arrest (Surana et al., 1991; Richardson et al., 1992) and therefore the accumulation of Clb2p in the mutants might be due to abnormally high expression of this gene. However, in both *cdc16-123* and *cdc23-1* mutants *CLB2* transcript levels did not increase compared to wild-type before the cells initiated DNA replication (data not shown).

### DNA replication in the presence of pheromone occurs predominantly in cyclin proteolysis mutants

To test whether the DNA replication in the presence of pheromone is specific for mutants defective in B-type cyclin proteolysis or rather a general phenomenon, we incubated different temperature-sensitive cell cycle mutants in the

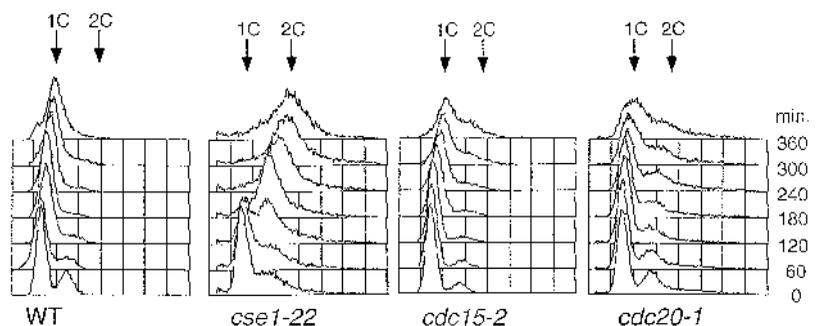
presence of pheromone. The *cse1-22* mutant, which is also defective for Clb2p ubiquitination and proteolysis in G<sub>1</sub> phase (Irniger et al., 1995; Zachariae and Nasmyth, 1996), behaved similarly to *cdc16-123* and *cdc23-1* mutants and cells efficiently replicated their DNA (Fig. 3). *CDC15* and *CDC20* are additional genes which have an essential role during mitosis, but in contrast to *cse1-22*, both *cdc15-2* and *cdc20-1* mutants are not impaired in Clb2p ubiquitination in G<sub>1</sub> phase (Zachariae and Nasmyth, 1996). When arrested with pheromone, these mutants behaved like wild-type and did not significantly replicate their DNA, indicating that the entry into S phase in the presence of pheromone appears to be a phenomenon occurring predominantly in mutants defective in cyclin proteolysis.

### B-type cyclins Clb3p and Clb5p are degraded via the APC

There are six different B-type cyclins in budding yeast and each of them can trigger DNA replication (Schwob et al., 1994). Therefore any one could contribute to the unscheduled replication in cyclin proteolysis mutants. However, so far only Clb2p has been shown to be rapidly degraded via Cdc16 and Cdc23 proteins (Irniger et al., 1995). We therefore tested whether degradation of other B-type cyclins is similar to Clb2p proteolysis.

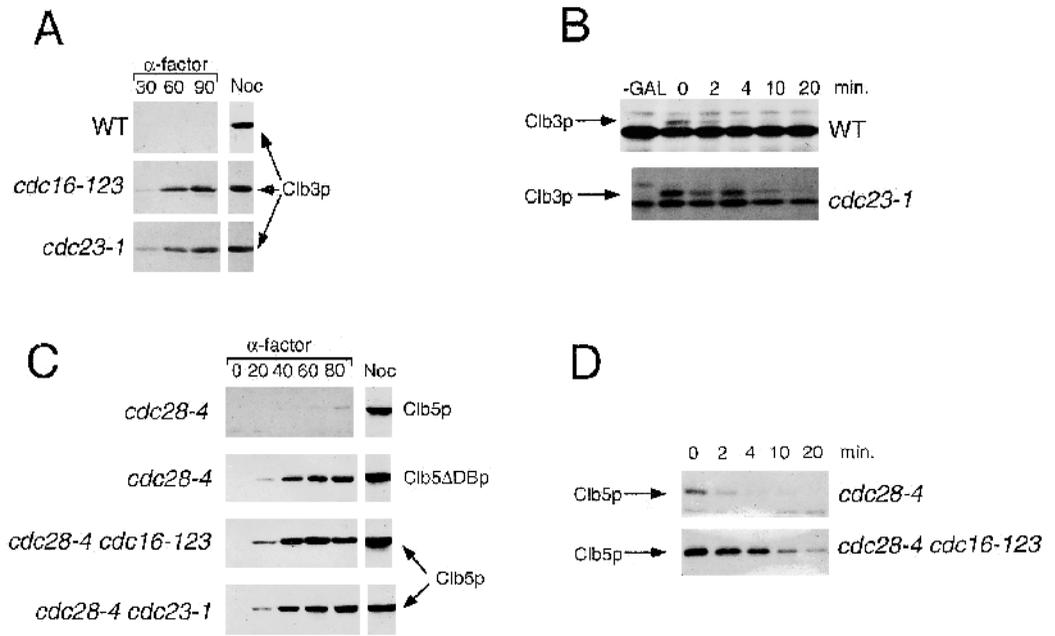
Clb3p, which is like Clb2p a mitotic cyclin, is rapidly degraded in a Cdc16/23 dependent manner during G<sub>1</sub>. The expression of a C-terminally HA-tagged Clb3 protein from the *GAL1* promoter does not result in the accumulation of any Clb3p in pheromone arrested wild-type cells, whereas in *cdc16-123* and *cdc23-1* mutants, Clb3p levels gradually accumulated (Fig. 4A). In a nocodazole induced metaphase arrest, where B-type cyclin proteolysis is inactive (Amon et al., 1994), Clb3p accumulated to equal levels in wild-type and mutant cells. Pulse chase experiments revealed that the half-life of Clb3p during G<sub>1</sub> is increased from 2 minutes in wild-type cells to 10 minutes in the *cdc23-1* mutant (Fig. 4B).

Clb1p and Clb4p are highly related to Clb2p and Clb3p, respectively (Fitch et al., 1992; Richardson et al., 1992), and their proteolysis is likely to be similarly regulated. Clb5 and 6 represent a third class of B-type cyclins, which are important in triggering S phase in wild-type cells (Epstein and Cross, 1992; Schwob and Nasmyth, 1993). Previous data indicated that proteolysis of Clb5p and Clb2p appear to be different. Whereas Clb2p proteolysis is switched off in S and G<sub>2</sub> phase (Amon et al., 1994), Clb5p is also degraded during an S phase arrest induced by hydroxyurea or during a nocodazole arrest, with a half-life of 10-15 minutes (Seufert et al., 1995).



**Fig. 3.** DNA replication in the presence of pheromone is specific for cyclin proteolysis mutants. Wild-type cells (K1534), *cse1-22* (K5040), *cdc15-2* (K2944) and *cdc20-1* (K5575) mutants (all *bar1::hisG*) were arrested at the permissive temperature (25°C) in G<sub>1</sub> by pheromone for 3 hours. The temperature was shifted to 37°C and the cultures were further incubated in the presence of pheromone. The DNA content was determined by FACScan analysis. The timepoints after the temperature shift are indicated.

**Fig. 4.** Clb3 and Clb5 protein degradation during G<sub>1</sub> is dependent on Cdc16 and Cdc23 proteins. (A) Clb3p expressed from the *GALI*-promoter does not accumulate in G<sub>1</sub> arrested wild-type cells, but in *cdc16-123* and *cdc23-1* G<sub>1</sub> cells. Wild type (K5505), a *cdc16-123* (K5506) and a *cdc23-1* mutant (K5507), all *bar1::hisG* and containing a *GAL-CLB3* construct (*CLB3* C-terminally tagged with the HA-epitope) were arrested in G<sub>1</sub> by pheromone at 25°C. Galactose was added to express the fusion protein and the temperature was shifted to 37°C. Clb3 protein was visualised in western blots using the HA antibody. As a control, the *GAL-CLB3-ha3* construct was also expressed in cells arrested in M-phase by nocodazole, a period in which B-type cyclin proteolysis is inactive (Surana et al., 1993).



(B) The half-life of Clb3p during G<sub>1</sub> is fivefold increased in *cdc23-1* mutants. Wild-type (K5505) and *cdc23-1* (K5507) cells were arrested in G<sub>1</sub> by pheromone at 25°C. Galactose was added to express the *GAL-CLB3* fusion and the cultures were shifted to 37°C for 20 minutes. The cells were labelled with [<sup>35</sup>S]methionine for 5 minutes and subsequently chased with cold methionine. The cells remained arrested during the course of this experiment, as judged by FACScan analysis (not shown). Epitope-tagged Clb3p was immunoprecipitated using the HA antibody. 0 minutes represents the timepoint when cold methionine was added. (C) Clb5p expressed from the *GALI*-promoter does not accumulate in G<sub>1</sub> arrested *cdc28-4* cells, but in *cdc28-4 cdc16-123* and *cdc28-4 cdc23-1* double mutants. A *cdc28-4* mutant (K4554), *cdc28-4 cdc16-123* (K4623) and *cdc28-4 cdc23-1* (K4620) double mutants, all *bar1::hisG* and containing a *GAL-CLB5* construct (*CLB5* C-terminally tagged with the HA-epitope) as well as a *cdc28-4* mutant, *bar1::hisG* and containing a *GAL-CLB5 $\Delta$ DB* construct (destruction box of *CLB5* deleted; K4555), were arrested in G<sub>1</sub> by pheromone at 25°C. Galactose was added to express the fusion proteins and the temperature was shifted to 37°C. Clb5 protein was visualised in western blots using the HA antibody. As a control, the *GAL-CLB5-ha3* construct was also expressed in cells arrested in M-phase by nocodazole. The *cdc28-4* mutation was required to prevent an otherwise rapid entry into S-phase upon expression of the *CLB5* cyclin. (D) The half-life of Clb5p during G<sub>1</sub> is approximately fivefold increased in *cdc16-123* mutants. Both a *cdc28-4* mutant (K4554) and a *cdc28-4 cdc16-123* double mutant (K4623) were arrested in G<sub>1</sub> by pheromone at 25°C and pulse-chased as described in B. Epitope-tagged Clb5p was immunoprecipitated using the HA antibody. 0 minutes represents the timepoint when cold methionine was added. The cells remained arrested during the course of this experiment, as judged by FACScan analysis (not shown).

However, Clb5p is even more unstable during G<sub>1</sub> phase than in S or G<sub>2</sub> phase (Seufert et al., 1995).

To elucidate whether Clb5p proteolysis during G<sub>1</sub> is mediated by APC activity, we tested the stability of Clb5p in a pheromone arrested *cdc28-4* mutant. This mutant was used in this experiment to prevent cells from entering S-phase upon the expression of the S-phase cyclin Clb5p. In this G<sub>1</sub> arrest, Clb5p was detectable only in very low amounts when expressed from the *GALI*-promoter, whereas a version of Clb5p lacking its nine amino acid destruction box (*Clb5 $\Delta$ DBp*) accumulated to high levels. Similarly, Clb5p accumulated in *cdc28-4* mutants which in addition contain *cdc16-123* or *cdc23-1* mutations (Fig. 4C). The half-life of Clb5p in the *cdc28-4* mutant is not more than 1-2 minutes, but is increased to 10 minutes when *CDC16* function is eliminated (Fig. 4D), indicating that efficient proteolysis of Clb5p also requires the activity of the APC.

Taken together, our results suggest that most or each of the Clb B-type cyclins are substrates of the APC during G<sub>1</sub> phase.

To test whether the entry into S phase of the cyclin proteolysis mutants depends on the accumulation of a specific Clb protein, *cdc16-123* and *cdc23-1* mutants strains were constructed which lack different *CLB* genes. The unscheduled

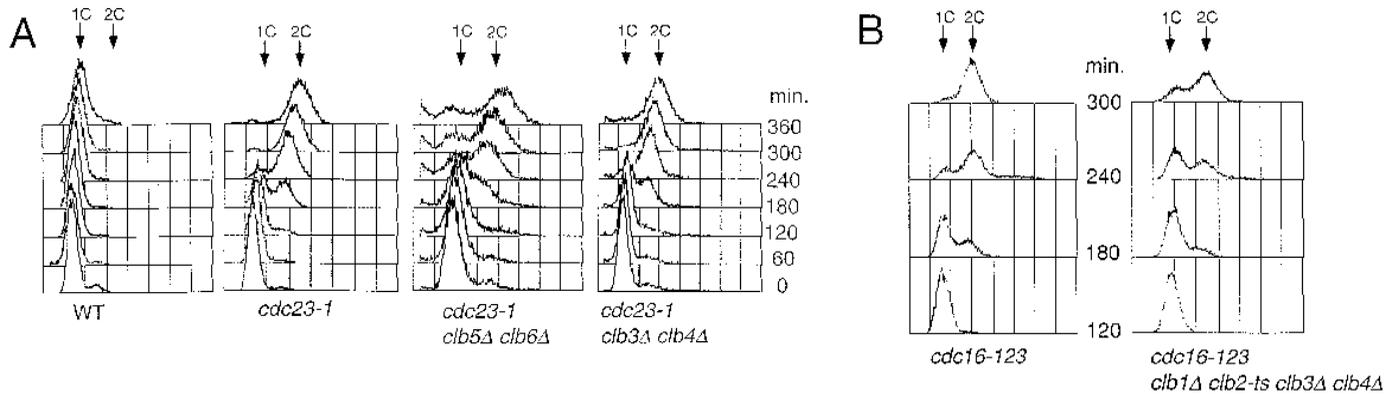
DNA replication of *cdc23-1* mutants was neither dependent on the Clb5p/Clb6p nor on the Clb3p/Clb4p B-type cyclin pairs (Fig. 5A). *cdc23-1* mutants containing deletions of either of these cyclin pairs efficiently entered S-phase without any obvious delay.

Since deletion of *CLB2* is lethal for *cdc23-1* mutants (Irniger et al., 1995), we assessed the role of the mitotic cyclins Clb1-4 in the *cdc16-123* mutant. A *cdc16-123 clb1 $\Delta$  clb2-ts clb3 $\Delta$  clb4 $\Delta$*  mutant was constructed by genetic crosses and incubated in the presence of pheromone at 37°C, i.e. in the absence of all the mitotic cyclins. This mutant strain entered S-phase, but less efficiently than the *cdc16-123* mutant, with a delay of approximately one hour (Fig. 5B). These data suggest that accumulation of Clbs 1-4 contributes to the replication of *cdc16-123* mutants in the presence of pheromone.

We conclude that the abnormal replication of *cdc16-123* and *cdc23-1* mutants during a pheromone arrest could be due to the appearance of any one of the Clb B-type cyclins and is not dependent on one specific Clb protein.

### Precocious DNA replication in *cdc23-1* mutants is dependent on p40-Sic1 degradation

We then asked whether the abnormal replication of mutants



**Fig. 5.** (A) DNA replication in the *cdc23-1* mutant is independent of either *CLB5/CLB6* deletions or *CLB3/CLB4* deletions. A *cdc23-1* (K4386) mutant, a *cdc23-1 clb5::URA3 clb6::LEU2* (K5310) mutant and a *cdc23-1 clb3::TRP1 clb4::HIS3* (K5445) mutant, all *bar1::hisG*, were arrested at the permissive temperature in G<sub>1</sub> by pheromone for 3 hours. The temperature was shifted to 37°C and the cultures were further incubated in the presence of pheromone. The DNA content was determined by FACS analysis, the timepoints after the temperature shift are indicated. (B) DNA replication in the *cdc16-123* mutant is delayed in the absence of Clbs1-4. A *cdc16-123* mutant (K4438) and a *cdc16-123 clb1Δ clb2-ts clb3::TRP1 clb4::HIS3 bar1::hisG* mutant (K5675) were treated as described in A. The DNA content 120, 180, 240 and 300 minutes after the temperature shift to 37°C is shown.

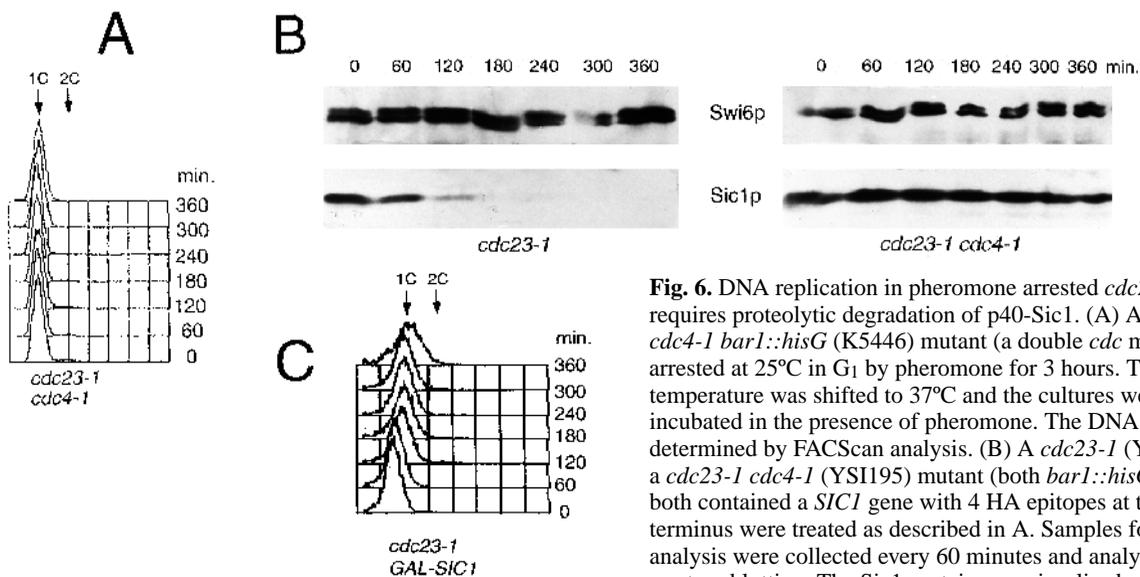
defective in APC function is dependent on Clb proteins at all. Although it is known that association of Cdc28p with at least one of the six Clb proteins is essential for DNA replication (Schwob et al., 1994), it might be possible that proteins other than Clbs accumulate abnormally in *cdc16* and *cdc23* mutants and bypass the need for the Clb proteins for DNA replication.

The most direct experiment to test whether DNA replication is dependent on B-type cyclins would be to create an APC mutant which lacks all *CLB* genes. Due to the technical difficulties of constructing a sextuple *clb* deletion strain in the *cdc16* and *cdc23* background we tested the dependence of DNA replication on B-type cyclin associated kinases by con-

structing a *cdc23-1 cdc4-1* double mutant. *cdc4-1* mutants fail to degrade the CDK inhibitor p40-Sic1. As a consequence all Clb/Cdc28 kinases are inhibited and the mutant cells are unable to enter S-phase (Schwob et al., 1994).

Indeed *cdc23-1 cdc4-1* double mutant cells did not replicate their DNA when treated with  $\alpha$ -factor (Fig. 6A). By using a *cdc23-1 cdc4-1* double mutant which contains an HA-tagged version of p40-Sic1, we found that p40-Sic1 remains stable in these cells, whereas it gets completely degraded in the *cdc23-1* single mutant (Fig. 6B).

To support the findings that p40-Sic1 degradation is required for the abnormal S phase entry of *cdc23-1* cells, we expressed



**Fig. 6.** DNA replication in pheromone arrested *cdc23-1* mutant requires proteolytic degradation of p40-Sic1. (A) A *cdc23-1 cdc4-1 bar1::hisG* (K5446) mutant (a double *cdc* mutant) was arrested at 25°C in G<sub>1</sub> by pheromone for 3 hours. The temperature was shifted to 37°C and the cultures were further incubated in the presence of pheromone. The DNA content was determined by FACS analysis. (B) A *cdc23-1* (YS1194) and a *cdc23-1 cdc4-1* (YS1195) mutant (both *bar1::hisG*) which both contained a *SIC1* gene with 4 HA epitopes at the C terminus were treated as described in A. Samples for protein analysis were collected every 60 minutes and analysed by western blotting. The Sic1 protein was visualised with the

12CA5 antibody. The Swi6 protein was used as a loading control. The *cdc23-1 cdc4-1* mutant did not replicate (similar to the strain used in A), whereas the *cdc23-1* strain replicated in a similar manner as shown in Figs. 1 and 5. (C) A *cdc23-1* mutant (YS1193), *bar1::hisG* and containing a single copy of a *GAL-SIC1ha4* construct, was cultivated in YEPrf and arrested at 25°C with pheromone for 3 hours. Galactose was added to express the *SIC1ha4* construct. After 1 hour the culture was shifted to 34°C and further incubated in the presence of pheromone and the DNA content was determined by FACS analysis. In this experiment 34°C was used because we found that induction of the *GAL1* promoter is often inefficient at temperatures higher than 35°C. The *cdc23-1* control strain underwent DNA replication under these conditions (not shown).

the *SIC1* gene to high levels in these cells (Fig. 6C). A *GAL-SIC1* construct completely blocked DNA replication in *cdc23-1* cells indicating that *cdc23-1* mutants are unable to replicate their DNA in the presence of p40-Sic1 which prevents the activation of Clb associated kinase.

These observations and the finding that replication is delayed in the absence of Clbs 1-4 suggest that the abnormal DNA replication of APC mutants in the presence of pheromone is caused by the appearance of B-type cyclins.

## DISCUSSION

### A role for the APC during G<sub>1</sub>

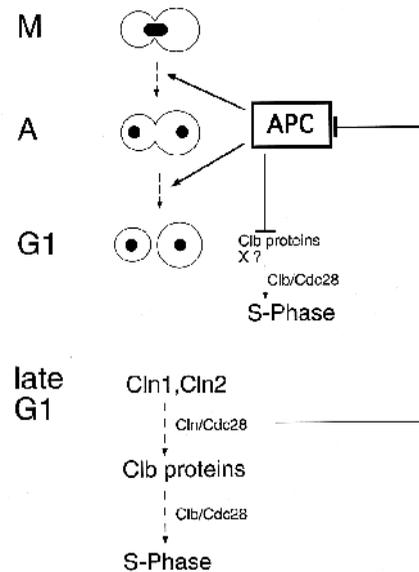
B-type cyclin degradation depends on ubiquitination which is mediated by the activity of the anaphase-promoting complex (King et al., 1995; Sudakin et al., 1995; Zachariae and Nasmyth, 1996). The APC plays a fundamental role during mitosis, because it is essential for both sister chromatid separation and spindle depolymerisation (Lamb et al., 1994; Irniger et al., 1995; Nasmyth, 1996). We have shown here that the APC not only has an important function in mitosis, but also during G<sub>1</sub> arrests, where it is needed to prevent precocious entry into S-phase (Fig. 7). Temperature-sensitive *cdc16-123* and *cdc23-1* mutants, pre-arrested in G<sub>1</sub> with  $\alpha$ -factor pheromone at their permissive temperature and subsequently shifted to their restrictive temperature entered S-phase in the presence of pheromone (Fig. 1). Remarkably, virtually all mutant cells completed DNA replication after 4 hours incubation at the restrictive temperature. Most mutant cells did not form buds, indicating that their entry into S-phase is not simply a consequence of becoming insensitive to alpha-factor pheromone. This is supported by the findings that the *cdc23* mutation also allowed DNA replication in cells deprived of G<sub>1</sub> cyclins.

What might induce DNA replication in G<sub>1</sub> cells with a defective APC? Good candidates are the B-type cyclins, because we have shown that at least three of them are degraded by the APC and because they are essential for DNA replication in yeast (Schwob et al., 1994). Replication is normally triggered by Cdc28p associated with Clb5 and Clb6 proteins, but in their absence Clbs 1-4, the mitotic cyclins, can substitute and each of the six B-type cyclins is capable of triggering S-phase in yeast.

Although it is known that transcription of the *CLB* genes is repressed during G<sub>1</sub> phase (Surana et al., 1991; Fitch et al., 1992; Richardson et al., 1992), the abnormal entry into S-phase of mutants impaired in APC function may be a direct consequence of the accumulation of B-type cyclins during G<sub>1</sub> phase. In pheromone arrested *cdc16-123* and *cdc23-1* mutants there is clearly a correlation between the appearance of the mitotic B-type cyclin Clb2p and the initiation of DNA replication (Fig. 1). Intriguingly, Clb2p accumulated prior to the initiation of DNA replication in both mutants. Clb2p accumulation and initiation of DNA replication occurred earlier in the *cdc23-1* mutant cells than in *cdc16-123* cells. The more severe defect of *cdc23-1* mutants at the permissive temperature may cause the more rapid appearance of this phenotype in *cdc23-1* cells.

### The B-type cyclin substrates of the APC

Our finding that members of each of the three classes of B-type cyclins (Clbs 1/2, Clbs 3/4 and Clbs 5/6) are degraded via



**Fig. 7.** Diverse roles of the anaphase-promoting complex (APC) in the yeast cell cycle. The APC has at least three important roles during the cell cycle. (i) At the metaphase to anaphase transition, the APC is needed for the separation of sister chromatids, probably for ubiquitin-mediated proteolysis of a protein which inhibits sister chromatid separation. (ii) At the end of anaphase, the APC is required for the proteolytic degradation of B-type cyclins. (iii) During G<sub>1</sub>, continued proteolysis of B-type cyclins mediated by the APC is important for preventing inappropriate entry into S-phase. The APC might have another yet unidentified substrate (X) whose appearance during G<sub>1</sub> contributes to the initiation of DNA replication. APC activity in G<sub>1</sub> is a prerequisite for the proper control of the G<sub>1</sub>/S transition, which is mediated by the G<sub>1</sub> (CLN) cyclins. The appearance of the CLB proteins and its associated kinases is normally dependent on the CLN associated kinases, which turn off the APC.

the Cdc16/Cdc23 proteins during G<sub>1</sub> phase (Fig. 4) indicate that all Clbs are probably substrates of the APC. The accumulation of any one of the Clb proteins might therefore induce precocious DNA replication in  $\alpha$ -factor treated cells. Replication was not dependent on the accumulation of one specific Clb, because any one of them can be inactivated and cells still undergo DNA replication. Nevertheless two lines of evidence show that DNA replication is dependent on Clb associated kinases. First, deletion of the mitotic cyclins Clbs 1-4 resulted in a delayed entry into S-phase showing that their accumulation is needed for efficient DNA replication. Second, DNA replication did not occur in the *cdc23-1 cdc4-1* double mutant which fails to degrade the Clb/Cdc28 kinase inhibitor p40-Sic1 and which therefore is unable to form active Clb/Cdc28 kinases.

Despite the finding, that both Clb2p and Clb5p are degraded via Cdc16/Cdc23 proteins, our results and previous reports have revealed differences in Clb2p and Clb5p proteolysis. (i) Unlike CLB2, whose proteolysis is switched off in S and G<sub>2</sub> phase (Amon et al., 1994), Clb5p is unstable throughout the cell cycle, with a half-life of 10-15 minutes during S and G<sub>2</sub> phase (Seufert et al., 1995). (ii) In contrast to Clb2p, whose stability drastically increases in *cdc16-123* and *cdc23-1* mutants (Irniger et al., 1995), Clb5p is only partially stabilised in these mutants (Fig. 4D). These data could be explained if

there were two different modes of Clb5p proteolysis: an APC-independent mechanism that does not vary much during the cell cycle and an APC-dependent mechanism that is cell cycle regulated; inactive in S and G<sub>2</sub> cells but active when APC is turned on in anaphase until late G<sub>1</sub>.

Indeed it has previously been shown that Clb5p is more unstable in G<sub>1</sub> arrested cells than in S or G<sub>2</sub> cells (Seufert et al., 1995). A difference between our results and Seufert's data is the stability of Clb5p during G<sub>1</sub>: 1-2 minutes in *cdc28* G<sub>1</sub> cells versus 5 minutes in wild-type G<sub>1</sub> cells. We have used a *cdc28-4* mutant for our experiments, because expression of *CLB5* from the *GALI* promoter rapidly induces the cells to enter S-phase. The *cdc28-4* mutation prevented the escape of cells from the G<sub>1</sub> arrest, even if Clb5 protein is expressed to high levels. There could be two possible explanations for why Clb5p appears to be more stable in wild-type than in *cdc28* G<sub>1</sub> cells. First, this might be an experimental artifact, because wild-type cells might escape from the G<sub>1</sub> arrest upon Clb5p expression and enter a cell cycle state where Clb5p is more stable. A second possibility could be that Cdc28p might partially stabilize Clb5p during G<sub>1</sub> phase. Interestingly, the half-life of Clb5p in telophase arrested cells (induced by a *cdc15* mutation) is similar to that in *cdc28* G<sub>1</sub> cells (S. Irniger and K. Nasmyth, unpublished observations).

The findings that Clb2p proteolysis is more dependent on the APC than Clb5p proteolysis could explain why DNA replication in APC mutants is more efficient when the mitotic cyclins are present (Fig. 5B). An accumulation of Clb2p to high levels is possible, because it is drastically stabilised in these mutants.

Although DNA replication of *cdc16-123* and *cdc23-1* mutants is dependent on the formation of Clb associated kinases, it is possible that additional, non-cyclin proteins appear together with the Clb proteins and contribute to precocious entry into S-phase (Fig. 7). It is likely that the APC has in addition to B-type cyclins other target proteins (Holloway et al., 1993; Irniger et al., 1995; Heichman and Roberts, 1996; Cohen-Fix et al., 1996). Recent observations suggested that *cdc16* and *cdc27* mutants overreplicate their DNA while arrested in metaphase and that the APC is involved in the degradation of a protein that acts as an 'S phase initiator' (Heichman and Roberts, 1996). However, the identity of such a protein is unknown.

The failure of APC mutants to degrade B-type cyclins could, however, be solely responsible for their entry into S-phase in pheromone treated cells, because previous experiments have shown that expression of a stable version of Clb2p from the *GALI* promoter also triggers S phase in pheromone arrested or Cln-depleted cells (Amon et al., 1994).

Another interesting question is, why the potent inhibitor of the Clb/Cdc28 kinase, p40-Sic1, is not capable of inhibiting DNA replication in pheromone arrested *cdc16* and *cdc23* mutants, but instead is degraded in these cells (Fig. 6B). One possible explanation might be that the accumulation of Clb proteins to high levels overcomes the inhibitory capacity of p40-Sic1. Indeed, it has been observed that Clb5p overexpression in G<sub>1</sub> cells triggers p40-Sic1 proteolysis and DNA replication (E. Schwob and K. Nasmyth, unpublished observations). We propose that accumulating Clb proteins in *cdc16* and *cdc23* G<sub>1</sub> cells exceed the level at which p40-Sic1 protein is able to inhibit all Clb associated kinases. The Clb/Cdc28 kinase

molecules that are now active trigger p40-Sic1 degradation, which in turn results in increased kinase levels and subsequent entry into S-phase.

Taken together, our data indicate that neither repression of *CLB* gene expression nor the CDK inhibitor p40-Sic1 is sufficient to prevent the appearance of active Clb kinases in G<sub>1</sub> cells defective in Clb proteolysis. Accumulation of B-type cyclins and maybe other APC target proteins is sufficient to induce DNA replication and to bypass the block normally imposed on DNA replication through the inhibition of Cln/Cdc28 kinases in pheromone arrested cells.

### Is APC activity during G<sub>1</sub> important in other eukaryotes?

The anaphase-promoting complex appears to be conserved from yeast to human (Lamb et al., 1994; King et al., 1995; Tugendreich et al., 1995). Given the highly conserved role of this particle in mitosis, it would not be surprising if the function of the APC during G<sub>1</sub> phase were important in other eukaryotic organisms.

It is interesting in this regard that mutations in *nuc2* cause sterility due to a failure in blocking DNA replication upon starvation (Kumada et al., 1995); *nuc2* is an *S. pombe* homologue to *CDC27*, previously thought to be needed exclusively for anaphase. Our data suggest that this may be caused by the deregulated accumulation of B-type cyclins during the G<sub>1</sub> arrest that precedes conjugation in *S. pombe*.

Recently, it was shown that, as in yeast cells, mitotic cyclin degradation persists from the end of mitosis until the onset of S phase in mouse fibroblasts and is also active in G<sub>0</sub> and differentiated myoblasts (Brandeis and Hunt, 1996). Although in these cells a physiological relevance for cyclin instability remains to be shown, it appears that APC activity persisting throughout G<sub>1</sub> phase is a conserved mechanism in eukaryotes to prevent the premature appearance of mitotic cyclins.

An example for the potential importance of B-type cyclin instability during G<sub>1</sub> might be the accumulation of mitotic cyclins prior to G<sub>1</sub> cyclins, which has been observed in certain human breast cancer cells (Keyomarsi et al., 1993). It will be an interesting task to elucidate whether the deranged appearance of cyclins in tumor cells is, at least partially, caused by a defective or misregulated APC. Considering the drastic effect in yeast (almost 100% G<sub>2</sub> cells in the presence of pheromone), one can imagine that in any eukaryotic G<sub>1</sub>, G<sub>0</sub> or differentiated cell which is impaired in proper APC function, B-type cyclins could accumulate after a certain period, circumvent the normal control of the G<sub>1</sub>/S transition and promote entry into S-phase.

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