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Regulation of B-type cyclin proteolysis by Cdc28-associated kinases in budding yeast

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In budding yeast, stability of the mitotic B-type cyclin Clb2 is tightly cell cycle-regulated. B-type cyclin proteolysis is initiated during anaphase and persists throughout the G_1 phase. Cln-Cdc28 kinase activity at START is required to repress B-type cyclin-specific proteolysis. Here, we show that Clb-dependent kinases, when expressed during G_1, are also capable of repressing the B-type cyclin proteolysis machinery. Furthermore, we find that inactivation of Cln- and Clb-Cdc28 kinases is sufficient to trigger Clb2 proteolysis and sister-chromatid separation in G_2/M phase-arrested cells, where the B-type cyclin-specific proteolysis machinery is normally inactive. Our results suggest that Cln- and Clb-dependent kinases are both capable of repressing B-type cyclin-specific proteolysis and that they are required to maintain the proteolysis machinery in an inactive state in S and G_2/M phase-arrested cells. We propose that in yeast, as cells pass through START, Cln-Cdc28-dependent kinases inactivate B-type cyclin proteolysis. As Cln-Cdc28-dependent kinases decline during G_2, Clb-Cdc28-dependent kinases take over this role, ensuring that B-type cyclin proteolysis is not activated during S phase and early mitosis.

Keywords: Cdc28-dependent kinases/Clb2/cyclin B proteolysis/*Saccharomyces cerevisiae*/Sic1

Introduction

Protein kinases whose activity is regulated by cyclins (cyclin-dependent kinases; Cdks) govern cell cycle progression. In the budding yeast *Saccharomyces cerevisiae*, a single cyclin-dependent kinase, Cdc28, regulates passage through the cell cycle. Its specificity is regulated by various cyclin proteins (reviewed by Nasmyth, 1993). Commitment to the cell cycle at START, spindle pole body duplication and bud formation are triggered by active kinase complexes composed of Cdc28 and the G_1 cyclins Cln1, 2 and 3. Initiation of S phase requires Cdc28 complexed with the S phase cyclins Clb5 and Clb6. Formation of a mitotic spindle, spindle elongation and nuclear division all rely on kinase complexes containing the B-type cyclins Clb1 to Clb5 (Nasmyth, 1993).

Activity of the different Cdc28-dependent kinase complexes is confined to appropriate stages of the cell cycle. Transcriptional control of cyclins and post-translational mechanisms restrict specific cyclin-dependent kinases to certain stages of the cell cycle (reviewed by Koch and Nasmyth, 1994; Nurse, 1994). B-type cyclin-specific proteolysis is a key post-translational control. B-type cyclins are stable during S phase and mitosis, but are abruptly degraded at the end of mitosis (Evans *et al.*, 1983). A sequence at the N-terminus of cyclin B, the destruction box, is required for this rapid degradation, and deletion of this nine amino acid motif stabilizes the protein (Glotzer *et al.*, 1991). Overexpression of such a stabilized cyclin B causes arrest of the cell cycle in late anaphase in a wide variety of organisms, including budding yeast (Murray *et al.*, 1989; Ghiara *et al.*, 1991; Luca *et al.*, 1991; Gallant and Nigg, 1992; Surana *et al.*, 1993; Sigrist *et al.*, 1995; Yamano *et al.*, 1996).

Degradation of cyclins is mediated by ubiquitin-dependent proteolysis. Recently the components of this ubiquitin-conjugating machinery have been identified (reviewed by Deshaies, 1995; Glotzer, 1995). Attachment of ubiquitin to cyclin B is mediated by the ubiquitin-conjugating enzymes UBC4 and UBCx (King *et al.*, 1995; Aristarkhov *et al.*, 1996; Yu *et al.*, 1996) and a 20S ubiquitin ligase complex known as the cyclosome or anaphase-promoting complex (APC; King *et al.*, 1995; Sudakin *et al.*, 1995; Zachariae *et al.*, 1996). This complex is comprised of eight subunits, four of which encode the previously identified proteins Cdc16, Cdc23, Cdc27 and Apc1/BimE which are highly conserved among eukaryotes (Irniger *et al.*, 1995; King *et al.*, 1995; Tugendreich *et al.*, 1995; Peters *et al.*, 1996; Zachariae *et al.*, 1996). In yeast, temperature-sensitive *cdc16*, *cdc27* and *cdc23* mutants arrest in metaphase when shifted to the restrictive temperature. This phenotype is consistent with the finding that proteins other than cyclin must be proteolysed in order for sister chromatids to segregate at the metaphase–anaphase transition (Holloway *et al.*, 1993).

B-type cyclin proteolysis is itself cell cycle-regulated. Work in embryonic extracts has shown that the cyclosome or APC is the target for this cell cycle regulation (King *et al.*, 1995; Sudakin *et al.*, 1995). How cyclosome/APC activity is regulated during the cell cycle is poorly understood. In clams, the Cdc2/cyclin B-dependent kinases are required for activation of B-type cyclin proteolysis (Lahav-Baratz *et al.*, 1995; Sudakin *et al.*, 1995). However, it is uncertain whether this effect is direct, because there is a lag phase between activation of Cdc2/cyclin B kinase and the activation of cyclin B degradation. In budding yeast, B-type cyclin proteolysis is initiated during anaphase (Irniger *et al.*, 1995) and continues to be active throughout G_1 (Amon *et al.*, 1994). The Cln-dependent kinases are required to repress Clb2 proteolysis as cells enter the cell cycle at START (Amon *et al.*, 1994; Dirick *et al.*, 1995). The mechanism(s) by which Cln-dependent kinases repress Clb2 proteolysis, how repression is maintained as the Cln-associated kinases decline during late S phase/G_2, and how Clb2 proteolysis is activated during mitosis is not understood.

Here, we investigate the role of Cln- and Clb-dependent

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kinases in repression of B-type cyclin-specific proteolysis during late G_1, S phase and early mitosis. We find that Clb-dependent kinases, like Cln-dependent kinases, can repress Clb2 proteolysis during G_1. Furthermore, we show that inactivation of Cln- and Clb-associated kinases is sufficient to induce proteolysis of Clb2 and to trigger sister-chromatid segregation in S and G_2/M phase-arrested cells where B-type cyclin proteolysis is normally inactive. These results suggest that Cln- and Clb-dependent kinases are required to keep the B-type cyclin-specific proteolysis machinery in an inactive state in S and G_2/M phase-arrested cells. We propose that, as Cln-dependent kinase levels decrease during G_2, Clb-dependent kinases assume the role of keeping B-type cyclin proteolysis inactive.

Results

Clb cyclins can inactivate Clb2-specific proteolysis during G_1

Inactivation of Clb2 proteolysis at START requires Cln-dependent kinase activity (Amon *et al.*, 1994; Dirick *et al.*, 1995), indicating that these kinases directly or indirectly repress B-type cyclin proteolysis. To further our understanding of how repression of B-type cyclin proteolysis is regulated, we determined whether inactivation of B-type cyclin proteolysis at START can be brought about solely by Cln-dependent kinases or whether Clb-dependent kinases, when expressed during G_1, are also capable of triggering inactivation of Clb2 proteolysis.

To this end we generated a strain which was deleted for *cln1*, *cln2* and *cln3* but kept alive by expression of *CLN2* from the methionine-repressible *MET3* promoter (Amon *et al.*, 1994). In the presence of methionine, cells arrest in G_1 due to lack of G_1 (Cln) cyclins. In addition, this strain carried a single copy of either the wild-type *CLB2* gene (*GAL–CLB2*) or a version of *CLB2* which lacks the destruction box (*GAL–CLB2-db*Δ) under the control of the galactose-inducible *GAL1-10* promoter. During G_1, Clb2 protein expressed from the *GAL1-10* promoter fails to accumulate due to continuous B-type cyclin-specific proteolysis activity, whereas Clb2-dbΔ protein accumulates and forms active Clb2-associated kinase during G_1 (Amon *et al.*, 1994). To analyze the consequences of ectopic expression of mitotic kinase on Clb2 stability during G_1, these cells also carried a *CLB2–lacZ* reporter fusion under the control of the constitutive, weak *Schizosaccharomyces pombe ADH* promoter (*ADH–CLB2–lacZ*). During G_1, Clb2–lacZ is normally unstable (Irniger *et al.*, 1995).

Cells were arrested in G_1 by cultivating them in the presence of methionine. After 5 h, when 90% of the cells were arrested in G_1 (Figure 1A), expression of *CLB2* or *CLB2-db*Δ was induced by addition of galactose (Figure 1B). This led to the accumulation of Clb2-dbΔ protein and associated kinase, but not to accumulation of wild-type Clb2 protein (Figure 1C and D). Expression of the Clb2-dbΔ protein, but not of wild-type Clb2, also led to accumulation of the Clb2–lacZ fusion protein (Figure 1C). This accumulation was due to formation of Clb2-dependent kinase because Clb2–lacZ failed to accumulate at 37°C in the strain described above carrying a temperature-sensitive *cdc28-4* allele (data not shown). Expression of Clb2-dbΔ also triggered entry into S phase (Figure 1A; Amon *et al.*, 1994). We therefore cannot exclude the possibility that cyclins other than Clb2, i.e. Clb5 and Clb6, contribute to the inactivation of B-type cyclin proteolysis. These results, however, show that inactivation of Clb2 proteolysis is not specific to Cln-dependent kinases, but that Clb-dependent kinases are also capable of inactivating B-type cyclin proteolysis.

Inactivation of Clb- and Cln-dependent kinases induces Clb2 decay in nocodazole-arrested cells

The requirement of Cdc28 kinase activity to switch off B-type cyclin proteolysis during G_1 could be transient; they could act only to initiate repression. Alternatively, Cln- and Clb-dependent kinases could be continuously required to maintain inhibition of B-type cyclin proteolysis throughout S phase, G_2 and early mitosis. To address this question we analyzed the consequences of inactivating Clb- and Cln-dependent kinases on Clb2 stability in G_2/M phase-arrested cells. The most direct way to address this question is to use a temperature-sensitive *CDC28* allele. However, we have found that in the temperature-sensitive alleles available, kinase complexes formed at the permissive temperature cannot be readily inactivated upon shift to the restrictive temperature (A.Amon, unpublished observations). To inactivate Cln- and Clb-dependent kinases efficiently, we took advantage of the fact that the cyclin-dependent kinase inhibitor (CDK inhibitor) Sic1 specifically inactivates Clb-dependent kinases (Mendenhall, 1993; Schwob *et al.*, 1994) and that α-factor pheromone induces production of the CDK inhibitor Far1, which specifically inhibits the Cln-dependent kinases (reviewed by Cross, 1995). To inhibit Clb-dependent kinases efficiently a strain was constructed that contains five copies of the *SIC1* gene under the control of the galactose-inducible *GAL1-10* promoter (*GAL–SIC1* strain). In the presence of galactose, cells arrest with a phenotype similar to that exhibited by cells lacking all B-type cyclin-associated kinases (data not shown; Schwob *et al.*, 1994), indicating that Sic1 levels sufficient to inhibit all Clb-dependent kinases are generated.

To analyze the consequences of inactivating Clb- and Cln-dependent kinases on Clb2 proteolysis, *GAL–SIC1* cells were arrested with the microtubule-depolymerizing drug nocodazole in G_2/M phase, where Clb-dependent kinase activity is high, Cln-dependent kinase activity is low and where Clb2 is stable (half-life >120 min; Amon *et al.*, 1994). When arrest was complete, galactose was added to inhibit Clb-dependent kinases. Inhibition of Clb-associated kinases results in the activation of Cln-associated kinases (Dahmann *et al.*, 1995), which inactivate Clb2 proteolysis (Amon *et al.*, 1994). To prevent accumulation of Cln-dependent kinases, α-factor pheromone was added to the cultures to induce production of the Cln kinase-specific inhibitor Far1. Pheromone treatment efficiently inhibited Cln-dependent kinases. Rebudding, which is indicative of active Cln-dependent kinases and is induced by inactivation of Clb-associated kinases (Dahmann *et al.*, 1995), was inhibited; instead, cells formed a mating projection (data not shown). Within 60 min of galactose addition, Clb2-associated kinase activity (and presumably that of other B-type cyclin-dependent kinases) dropped to basal levels (Figure 2B). Clb2 protein levels started to decline soon after kinase activity dropped

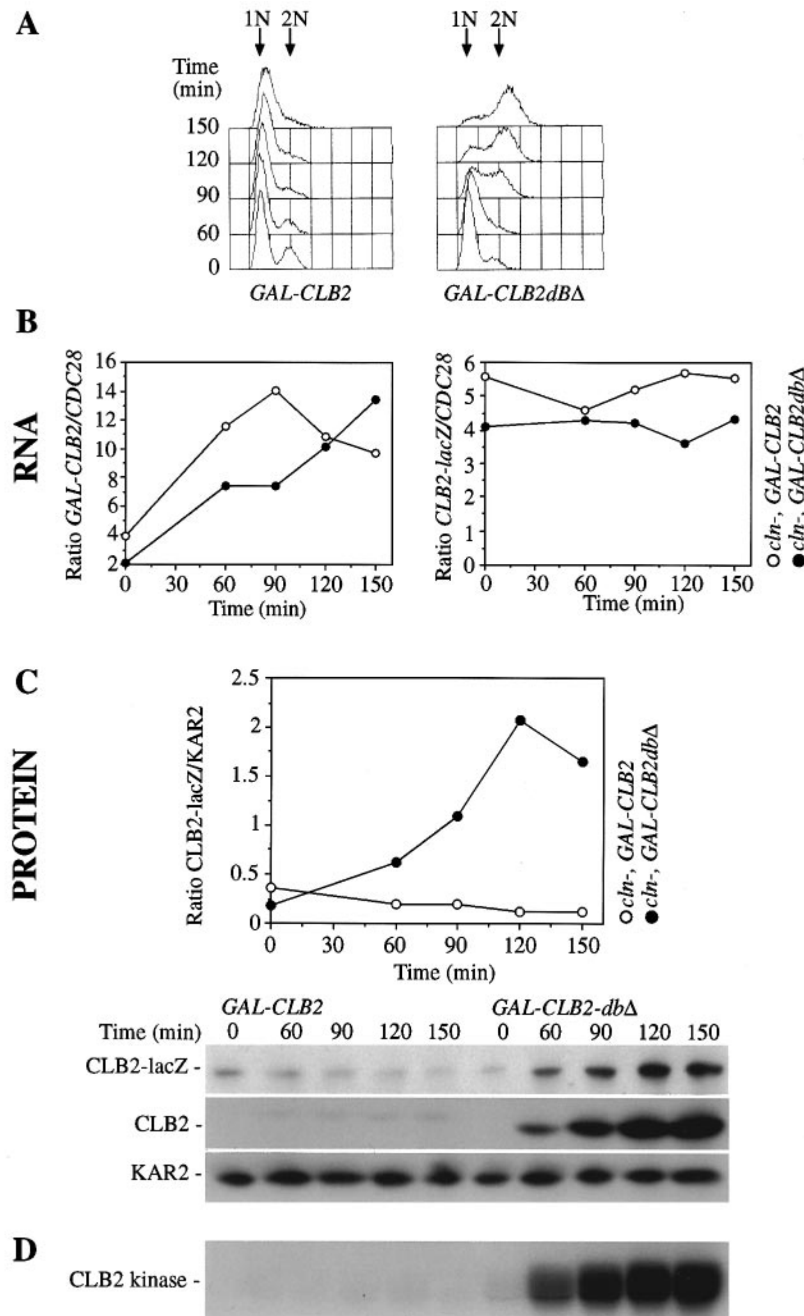

Fig. 1. Expression of stable Clb2 during G_1 leads to inactivation of Clb2-specific proteolysis. Cells of genotype *MAT***a**, *cln1Δ*, *cln2Δ*, *cln3::LEU2*, *MET–CLN2::TRP1*, *ADH–CLB2–lacZ::HIS3* either carrying a *GAL–CLB2::URA3* fusion (A801) or a *GAL–CLB2dbΔ::URA3* fusion (A908) were arrested in G_1 by incubating cells in the presence of methionine. After 5 h, galactose was added and samples were withdrawn at the indicated time to analyze: (**A**) DNA content; (**B**) *GAL–CLB2* and *CLB2–lacZ* RNA; (**C**) Clb2 and Clb2–lacZ protein; and (**D**) Clb2-associated Histone H1 kinase activity. The graph shown in (C) compares the levels of Clb2–lacZ accumulating in cells expressing *GAL–CLB2* with *GAL–CLB2dbΔ*. In (C) Kar2 was used as an internal loading control.

(Figure 2C). In contrast, pheromone treatment and galactose addition did not induce Clb2 decay in control cultures lacking the *GAL–SIC1* construct (Figure 2C). Expression of Sic1 alone (no α-factor addition) led to some decline in Clb2 protein levels (data not shown). However, the decrease in Clb2 protein levels was only 5-fold in the absence of pheromone (data not shown), whereas the decline was 50-fold in presence of pheromone (Figure 2C). We conclude that ectopic expression of *SIC1* and simultaneous pheromone treatment induces Clb2 decay in nocodazole-arrested cells, in which Clb2 is normally stable. The simplest explanation for this observation is that inactivation of Cln- and Clb-associated kinases induces Clb2 decline.

Because *CLB2* transcription depends on active Clb-dependent kinase (Amon *et al.*, 1993), inhibition of the Clb-dependent kinases by *GAL–SIC1* also leads to the loss of cyclin transcription (Figure 2D). Although the half-life of Clb2 is >120 min in nocodazole-arrested cells, it is possible that loss of transcription could partly account for the loss of Clb2 protein caused by inactivation of Clb-Cdc28 kinases. To eliminate transcriptional effects, we

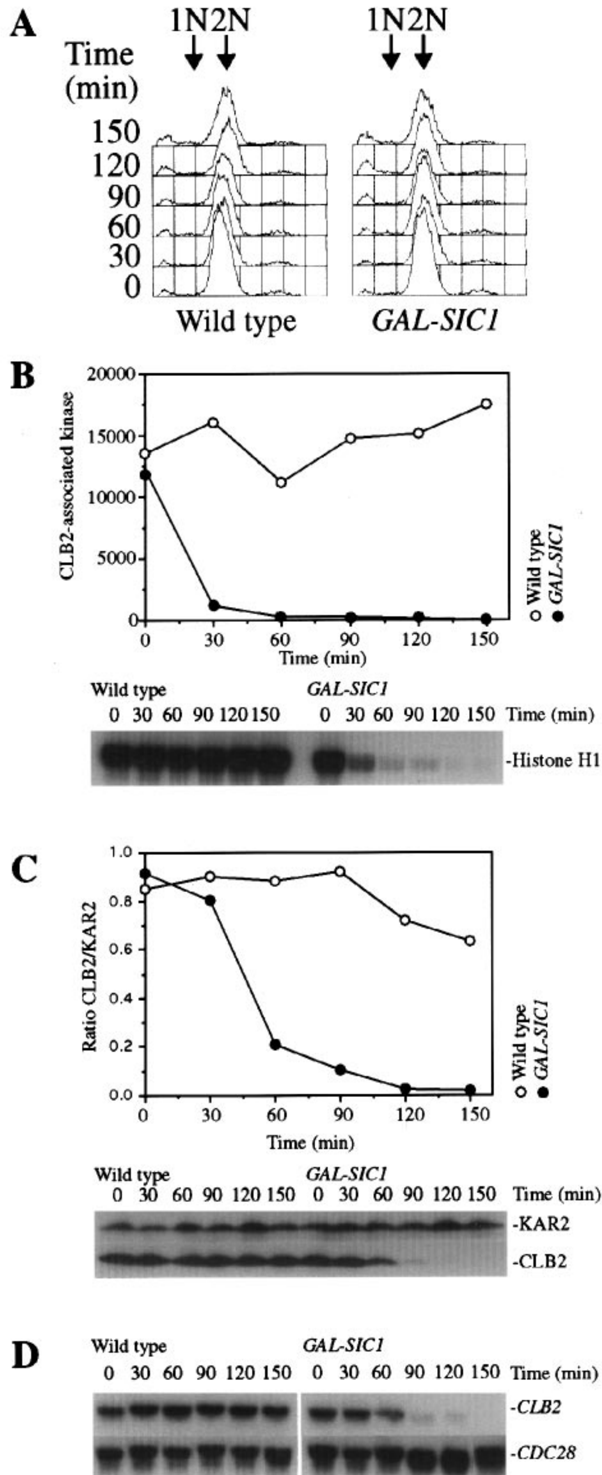

Fig. 2. Ectopic expression of *SIC1* and α-factor treatment induces Clb2 decay in nocodazole-arrested cells. Wild-type cells (K1534; *MAT***a**, *bar1::HisG*; ○) or cells carrying five copies of *GAL–SIC1* (A701; *MAT***a**, *bar1::HisG*, *5x GAL–SIC1::TRP1*; ●) were arrested in YEPraf medium with 15 μg/ml nocodazole at 25°C. After 165 min, 2% galactose and 7 μg/ml α-factor were added. Samples were withdrawn at the indicated times to analyze DNA content (**A**), Clb2-associated kinase activity (**B**), Clb2 protein (**C**) and *CLB2* RNA (**D**). In (D), *CDC28* RNA was used as an internal loading control.

repeated our experiment with a strain carrying the *CLB2* gene under the control of the constitutive *S.pombe ADH* promoter. Clb2-associated kinase activity was repressed to basal levels within 60 min (Figure 3B) and the protein declined soon thereafter (Figure 3C). Under these conditions, transcription of *CLB2* was not affected by induction of *SIC1* (Figure 3D), demonstrating that a post-transcriptional mechanism is responsible for the disappearance of Clb2 upon inactivation of Cln- and Clb-associated kinases.

Cyclin decay induced by inactivation of Cln- and Clb-associated kinases is mediated by B-type cyclin-specific proteolysis

The decline in Clb2 protein levels brought about by inactivation of Cln- and Clb-dependent kinases was due to B-type cyclin-specific proteolysis because it was absent in a strain specifically defective for the B-type cyclin-specific proteolysis machinery, in *cdc23-1* mutants (Figure 4). Cells containing *GAL–SIC1* that were either wild-type for *CDC23* (a component of the ubiquitin ligase) or carrying a mutant *cdc23-1* allele were arrested with nocodazole. When the arrest was complete, galactose and α-factor were added. Clb2-associated kinase was efficiently inhibited within 60 min after galactose induction in both cultures (Figure 4C) and Clb2 protein levels declined soon thereafter in cells wild-type for *CDC23* (Figure 4C). In contrast, Clb2 protein levels remained high in *cdc23-1* mutants (Figure 4B). Other cell cycle parameters were similar in the two cultures: cells remained arrested with a 2N DNA content (Figure 4A), formed mating projections with similar kinetics (data not shown) and *CLB2* RNA levels declined with similar kinetics (Figure 4D). Similar results were obtained with cells arrested in early S phase by hydroxyurea (Figure 5). Inactivation of Cln- and Clb-dependent kinases by *GAL–SIC1* induction and pheromone treatment led to a decrease in Clb2 protein levels in cells wild-type for *CDC23*, but not in cells carrying a *cdc23-1* allele (Figure 5).

The most rigorous way of determining whether inactivation of Cln- and Clb-dependent kinases induces Clb2 proteolysis is to determine the half-life of Clb2 soon after kinase inactivation is complete. However, in spite of repeated efforts, we were unable to measure the half-life of Clb2 under conditions where ectopic expression of *GAL–SIC1* and pheromone treatment induce Clb2 degradation. We failed to detect labeled Clb2 protein even after very short pulses with [^{35}S]methionine. High-level expression of Clb2 to detect the protein was ineffective because the resulting increase in Clb2-associated kinase activity leads to incomplete inactivation of Clb-dependent kinases by *SIC1* and therefore to poor induction of Clb2 proteolysis (data not shown). However, the fact that Clb2 protein levels do not decline in *cdc23-1* mutants shows that B-type cyclin-specific proteolysis is responsible for the decay of Clb2 induced by inactivation of Cln- and Clb-dependent kinases. These results also suggest that Cln- and Clb-dependent kinases are required to keep Clb2-specific proteolysis in an inactive state in S and G_2/M phase-arrested cells.

Clb2 proteolysis is a consequence of Cln- and Clb-dependent kinase inactivation

To determine whether Clb2 proteolysis induced by ectopic expression of *SIC1* and simultaneous pheromone treatment

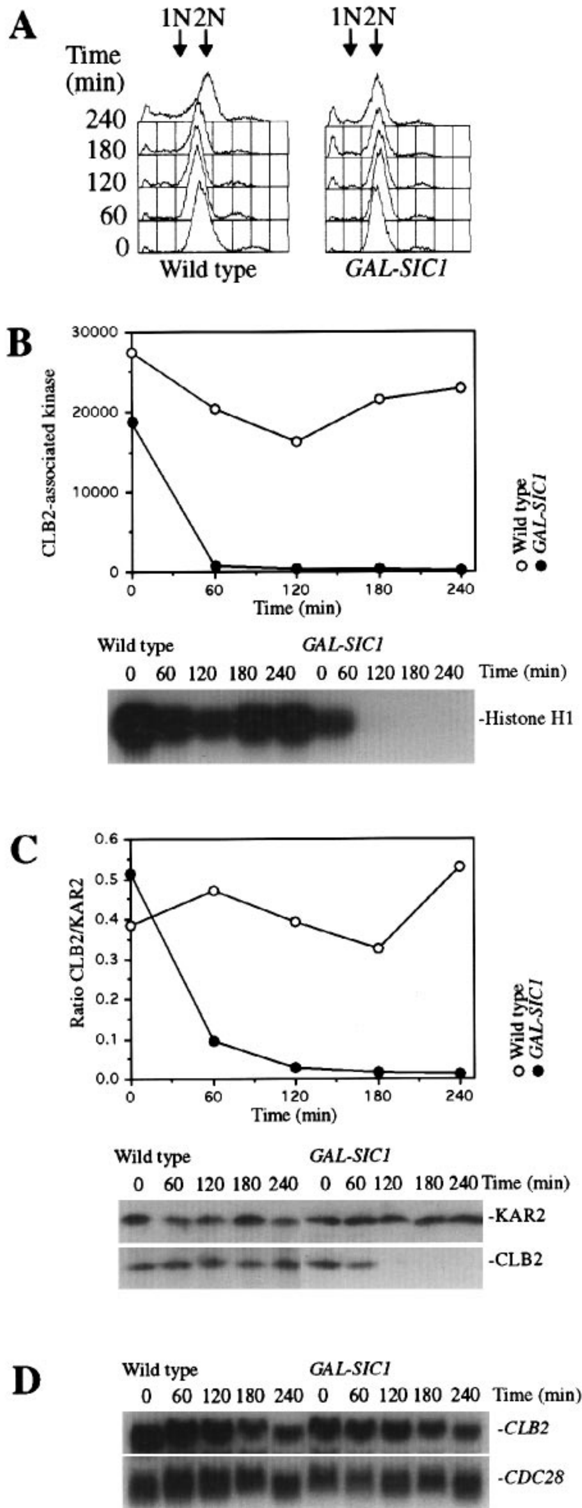

Fig. 3. Post-transcriptional mechanisms are responsible for Clb2 decay induced by inactivation of Cln- and Clb-dependent kinases. Cells deleted for *CLB2* carrying *CLB2* under the control of the constitutive *S.pombe ADH* promoter (A724; *MAT***a**, *clb2::LEU2*, *ADH–CLB2::URA3*; ○) or an *ADH–CLB2* strain carrying five copies of *GAL–SIC1* (A725; *MAT***a**, *clb2::LEU2*, *ADH–CLB2::URA3*, *5x GAL–SIC1::TRP1*; ●) were grown and arrested as described in the legend to Figure 2. After galactose and α-factor addition, cell samples from indicated times points were analyzed for DNA content (**A**), Clb2-associated kinase activity (**B**), Clb2 protein (**C**) and *CLB2* RNA (**D**).

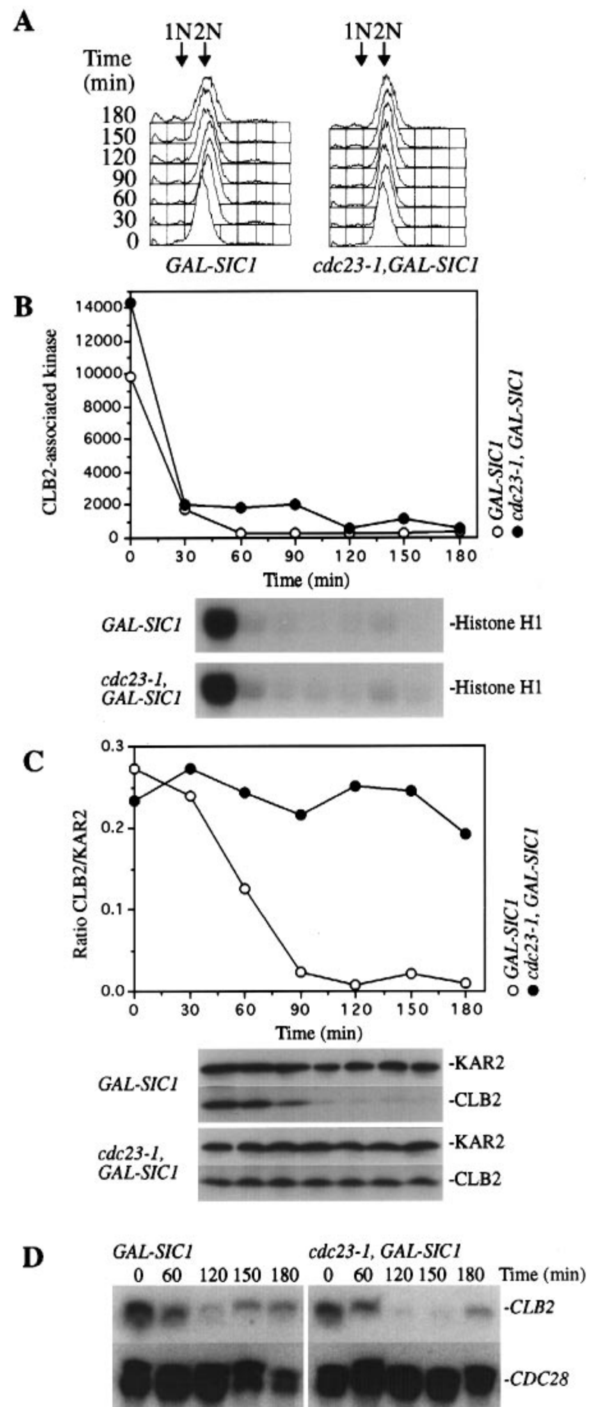

Fig. 4. Cyclin B-specific proteolysis is responsible for the decline in Clb2 levels induced by inactivation of Cln- and Clb-associated kinases. *GAL–SIC1* cells (A701; ○) and *GAL–SIC1* cells carrying a *cdc23-1* mutation (A731; *MAT***a**, *bar1::HisG*, *cdc23-1*, *5x GAL–SIC1::TRP1*; ●) were grown and arrested at 25°C as described in the legend to Figure 2 and induced with galactose and 10 μg/ml α-factor at 25°C. DNA content (**A**), and the total amount of Clb2-associated kinase activity (**B**), Clb2 protein (**C**) and *CLB2* RNA (**D**) were determined at the indicated times. The experiment was carried out at 25°C because inhibition of B-type associated kinases by ectopic expression of *SIC1* from the *GAL1-10* promoter is not as complete at higher temperatures, i.e. at 37°C, the restrictive temperature for the *cdc23-1* mutation. However, *cdc23-1* mutants are partly defective in Clb2 degradation even at 25°C (Irniger *et al.*, 1995).

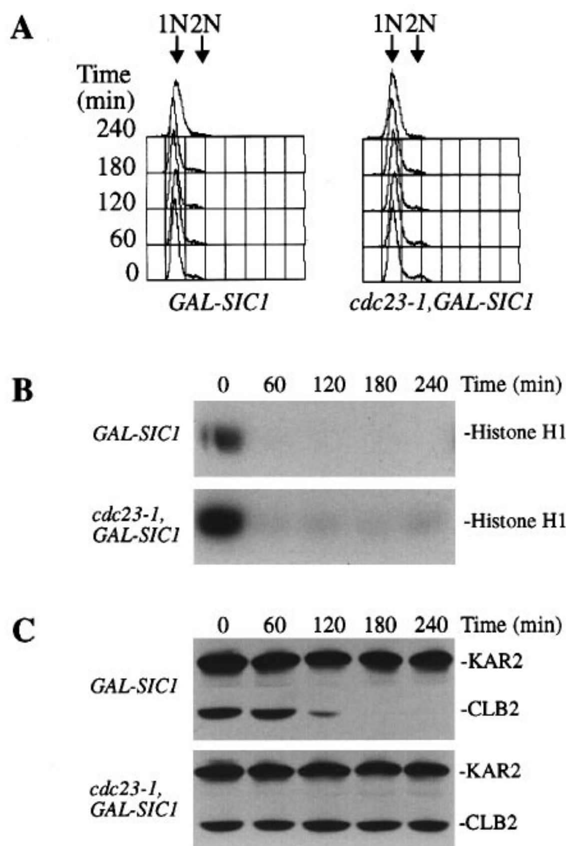

Fig. 5. Inactivation of Cdc28-associated kinases induces Clb2 proteolysis in hydroxyurea-arrested cells. *GAL–SIC1* cells (A701) and *GAL–SIC1* cells carrying a *cdc23-1* mutation (A731) were arrested at 25°C with 20 mg/ml hydroxyurea. After 180 min, galactose and 7 μg/ml α-factor were added. DNA content (**A**), the total amount of Clb2-associated kinase activity (**B**) and Clb2 protein (**C**) were determined at the indicated times. The experiment was performed at 25°C due to reasons pointed out in legend to Figure 4.

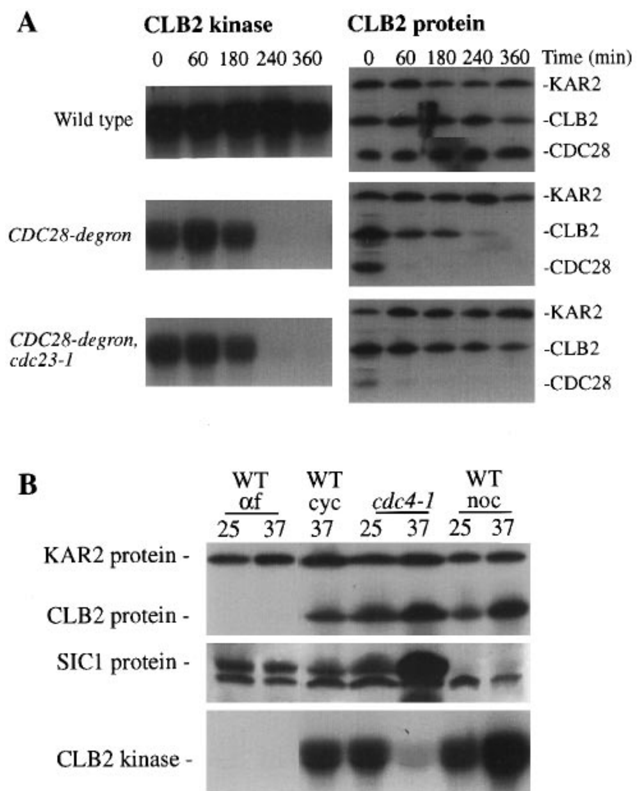

Fig. 6. Activation of Clb2 proteolysis occurs by inhibition of Cdc28-associated kinases, not by Sic1 binding. (**A**) Wild-type cells (K699) or cells carrying a *GAL–CDC28-degron* fusion as their sole source of *CDC28* either wild-type for *CDC23* (A735; *MAT***a**, *GAL–CDC28-degron–URA3::cdc28*) or carrying a *cdc23-1* mutation (A739; *MAT***a**, *cdc23-1*, *GAL–CDC28-degron–URA3::cdc28*) were arrested in YEPraf+gal medium containing 15 μg/ml nocodazole at 25°C. After 180 min cells were filtered and transferred into YEPraf medium containing glucose and 15 μg/ml nocodazole prewarmed to 37°C. Samples were withdrawn at the indicated times to analyze Clb2-associated kinase activity and the levels of Clb2 and Cdc28 protein. (**B**) Cells deleted for *CLB2* carrying *CLB2* under the control of the constitutive *S.pombe ADH* promoter (K3241; *MAT***a**, *bar1::HisGURA3HisG*, *clb2::LEU2*, *ADH–CLB2::TRP1*) were grown in YEPD to exponential phase (cyc) or arrested with either α-factor (1 μg/ml; αf) or 15 μg/ml nocodazole (noc) for 180 min at 25°C or 37°C. *cdc4-1* mutants carrying an *ADH–CLB2* fusion (K4016; *MAT***a**, *cdc4-1*, *bar1::HisGURA3HisG*, *clb2::LEU2*, *ADH–CLB2::TRP1*) were grown either at 25°C or arrested at 37°C for 180 min.

is due to inactivation of Cln- and Clb-associated kinases rather than other mechanisms, we wished to inactivate Cln- and Clb-associated kinases by other means. To this end, we generated a strain whose altered Cdc28 protein can be degraded by shifting cells to 37°C ('*CDC28-degron*'; Dohmen *et al.*, 1994). Cells carrying such a *CDC28-degron* fusion under the control of the *GAL1-10* promoter were arrested with nocodazole. Upon temperature shift to 37°C, *CDC28* transcription was repressed by glucose addition. Although the bulk of the Cdc28 protein was degraded within 60 min, Cdc28 associated with Clb2 was more stable, as judged by the continuous presence of Clb2-associated kinase activity (Figure 6A). By 3 h, Clb2-associated kinase disappeared and soon thereafter Clb2 protein levels dropped. In contrast, Clb2 protein levels remained elevated in *cdc23-1* mutants (Figure 6A), suggesting that B-type cyclin-specific proteolysis is responsible for Clb2 decay. These data suggest that inactivation of all Cdc28-associated kinases by targeted degradation of the Cdc28 protein induces Clb2 proteolysis, supporting our hypothesis that inactivation of Cln- and Clb-associated kinases leads to proteolysis of Clb2 in a *CDC23*-dependent manner.

Sic1 directly binds Clb kinase complexes (Mendenhall, 1993; Schwob *et al.*, 1994). To investigate the possibility that binding of Sic1 to the Clb kinase complex targets Clb2 for degradation, we analyzed whether Clb2 protein can accumulate in *cdc4* mutants. In *cdc4* mutants arrested at the restrictive temperature, Sic1 protein fails to be degraded and accumulates to high levels, resulting in the inhibition of all Clb-associated kinases (Schwob *et al.*, 1994). In contrast, the levels of Cln-associated kinases remain high in *cdc4* mutants since this class of kinases is not inhibited by Sic1 (Tyers *et al.*, 1993; Schwob *et al.*, 1994). Thus, in *cdc4* mutants, both high levels of Sic1 and high levels of Cln-associated kinases are present, enabling us to distinguish between the following possibilities. If binding *per se* of Sic1 is required to target Clb2 for proteolysis, Clb2 protein should be unstable in *cdc4* mutants. Alternatively, if inactivation of Cln- and Clb-dependent kinases is required to induce Clb2-specific proteolysis, Clb2 should be stable. We found that in *cdc4* mutants Clb2 protein accumulated to levels as high as in

stages of the cell cycle where B-type cyclin-specific proteolysis is inactive (in nocodazole-arrested cells, Figure 6B), despite the presence of high levels of functional Sic1 protein (Figure 6B). This result suggests that, although Sic1 binds and inhibits all Clb2-associated kinases, Clb2 remains stable presumably due to high levels of Cln-associated kinases (Amon *et al.*, 1994; Schwob *et al.*, 1994). Indeed, removal of Cln-dependent kinases in *cdc4* mutants leads to Clb2 degradation (Amon *et al.*, 1994). We conclude that binding of Sic1 to the Clb/Cdc28 kinase complex does not target Clb2 for proteolysis.

Cln- and Clb-associated kinases are required to inhibit sister-chromatid separation in G_2/M phase-arrested cells

Cln- and Clb-associated kinases are required to inhibit Clb2 proteolysis in S and G_2/M phase-arrested cells. To determine whether Cln- and Clb-dependent kinases are also required to inhibit proteolysis of other substrates of the B-type cyclin-specific proteolysis machinery, we analyzed the consequences of inactivating Cln- and Clb-dependent kinases on sister-chromatid cohesion. Release of sister-chromatid cohesion at the metaphase–anaphase transition is controlled by the B-type cyclin-specific proteolysis machinery (Holloway *et al.*, 1993; Irniger *et al.*, 1995). Although the target of the proteolysis machinery remains to be identified, the status of sister-chromatid cohesion can be monitored by *in situ* hybridization to chromosomes (Guacci *et al.*, 1994). When sister chromatids are attached, one hybridization signal is observed in nuclei, whereas when cohesion is lost, two signals are visible. *GAL–SIC1* cells were arrested with nocodazole, followed by galactose and α-factor addition. In cultures lacking the *GAL–SIC1* construct, 85% of the cells showed one hybridization signal per nucleus throughout the course of the experiment, regardless of whether the probe used maps near the centromere (centromere probe) or more distally (arm probe, Figure 7). In contrast, inactivation of Cln- and Clb-dependent kinases led to loss of sister-chromatid cohesion, as judged by the appearance of two hybridization signals per nucleus. By 3 h, cohesion was lost in ~80% of the cells (Figure 7). Loss of cohesion was delayed in *cdc23-1* mutants (Figure 7), demonstrating that sister-chromatid separation depends on *CDC23* and thus on APC activity. Our results suggest that Cln- and Clb-associated kinases are not only required to repress Clb2 proteolysis in G_2/M phase-arrested cells but also proteolysis of other substrates such as proteins required for sister-chromatid cohesion.

Discussion

Cell cycle-regulated B-type cyclin proteolysis is a key regulator of Clb-associated kinase activity. It plays an important role in inactivation of Clb-dependent kinases as cells exit mitosis and, at least in yeast and mammalian cells, ensures that the mitotic kinase does not accumulate during G_1 (Amon *et al.*, 1994; Brandeis and Hunt, 1996). Cln-dependent kinases induce inactivation of Clb2-specific proteolysis as cells pass through START (Amon *et al.*, 1994; Dirick *et al.*, 1995). Our results suggest that Cln- and Clb-dependent kinases are required to keep the B-type cyclin-specific proteolysis machinery inactive in S and G_2/M phase-arrested cells.

Repression of B-type cyclin-specific proteolysis during late G_1

Inactivation of B-type cyclin proteolysis during G_1 is not specific to Cln-dependent kinases. Ectopic expression of Clb2-dependent kinase during G_1 allowed an otherwise unstable Clb2–lacZ fusion to accumulate. We can exclude the possibility that inactivation of Clb2 proteolysis is due to Cln activity brought about by incomplete repression of the *MET3* promoter because, firstly, Clb2–lacZ failed to accumulate in Cln-depleted cells expressing the wild-type *CLB2* gene. Secondly, although cells expressing the Clb2-dbΔ protein entered S phase, budding (which is indicative of Cln kinase activity) was completely inhibited (A.Amon, unpublished observations). It is also unlikely that G_1 cyclins other than Clns such as Hcs26 inactivate Clb2-specific proteolysis since Clb-dependent kinases repress transcription of *CLN1*, *CLN2* and *HCS26* (Amon *et al.*, 1993). On the other hand, since ectopic expression of Clb2 also triggered entry into S phase, Clb cyclins other than Clb2 (i.e. Clb5 and Clb6) could contribute to the inactivation of Clb2-specific proteolysis.

Repression of B-type cyclin proteolysis in S and G_2/M phase-arrested cells

Ectopic expression of *SIC1* and pheromone treatment led to the activation of B-type cyclin proteolysis in nocodazole- and hydroxyurea-arrested cells. We believe that this is due to inactivation of Cln- and Clb-associated kinases for two reasons. First, we could exclude the possibility that binding of Sic1 to the Clb2-Cdc28 kinase complex targets Clb2 to be proteolysed; Clb2 protein is stable in *cdc4* mutants although Sic1 accumulates to high levels and binds to all Clb2-dependent kinases. Cln-dependent kinases repress Clb2-proteolysis in *cdc4* mutants. When Cln-dependent kinases are removed in *cdc4* mutants Clb2 becomes unstable (Amon *et al.*, 1994). Secondly, targeted degradation of the Cdc28 kinase subunit also induces Clb2 proteolysis in nocodazole-arrested cells and, as proteolysis induced by ectopic expression of *SIC1* and pheromone treatment, depends on a functional anaphase promoting complex. Thus, we suggest that Cln- and Clb-dependent kinase activity is not only necessary to inactivate B-type cyclin-specific proteolysis during G_1 but both Clb- and Cln-dependent kinases are also required to keep Clb2 proteolysis in an inactive state in S and G_2/M phase-arrested cells. In nocodazole-arrested cells, Clb-dependent kinases are probably responsible mainly for repression of B-type cyclin proteolysis because these cells contain high levels of Clb-associated kinases but low levels of Cln-dependent kinases. In hydroxyurea-arrested cells perhaps both Cln- and Clb-dependent kinases contribute to the repression of B-type cyclin-specific proteolysis since these cells contain intermediate levels of Clb-dependent kinase activity.

Clb cyclins are not the only proteins whose stability is regulated by the B-type cyclin-specific proteolysis machinery. Degradation of a non-cyclin protein required for sister-chromatid cohesion is thought to be under the control of the B-type cyclin-specific proteolysis machinery. Degradation of this protein is thought to trigger sister-chromatid segregation at the metaphase–anaphase trans-

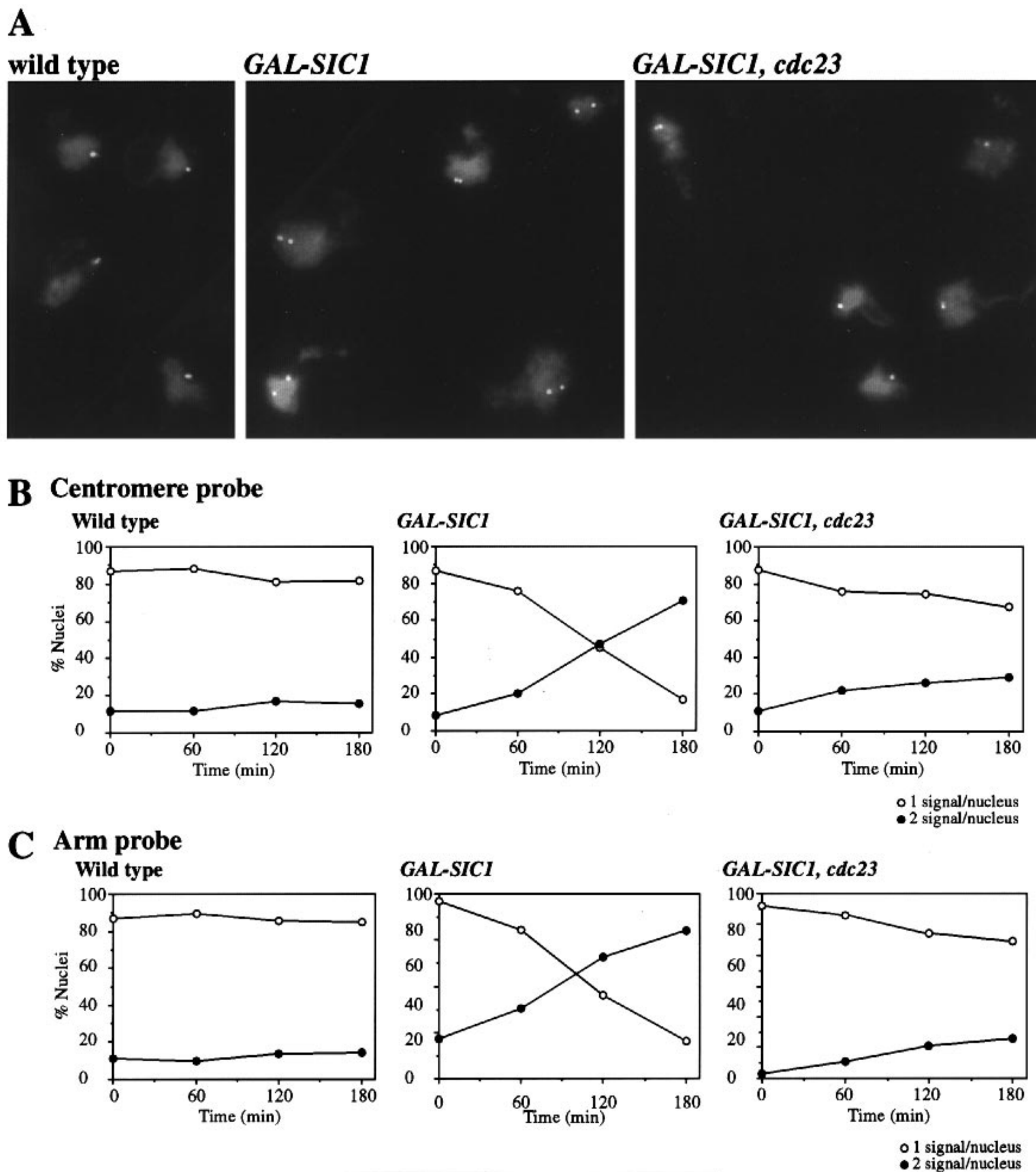

Fig. 7. Inactivation of Cln- and Clb-associated kinases induces sister-chromatid separation in a *CDC23*-dependent manner. Wild-type cells (K1534), *GAL–SIC1* cells (A701) and *GAL–SIC1* cells carrying a *cdc23-1* mutation (A731) were grown and arrested at 25°C as described in the legend to Figure 2. Cells were then induced with galactose and 10 μg/ml α-factor at 25°C. Samples were withdrawn at the indicated times to analyze the status of sister-chromatid cohesion by scoring the number of hybridization signals per nucleus (see Materials and methods). One signal per nucleus (○) indicates that sister chromatids are attached, two signals within one nucleus (●) indicate that sister-chromatid cohesion is lost. Status of cohesion was determined at the centromere (**B**) and along chromosomal arms (**C**). The photograph in (**A**) shows status of sister-chromatid cohesion at chromosomal arms 180 min after galactose induction and α-factor addition. The experiment was performed at 25°C due to reasons pointed out in legend to Figure 4.

ition (Holloway *et al.*, 1993). Furthermore, B-type cyclin proteolysis plays an important role during S phase, restricting DNA replication to once per cell cycle (Heichman and Roberts, 1996). Are Cln- and Clb-dependent kinases required to repress degradation of these various substrates in S and G_2/M phase-arrested cells? Inactivation of Cln- and Clb-associated kinases leads to sister-chromatid separation in G_2/M phase-arrested cells in a *CDC23*-dependent manner, suggesting that Cln- and Clb-dependent kinases are also required to repress degradation of proteins required for sister-chromatid cohesion during G_2/M phase. Whether Cln- and Clb-dependent kinases are required to repress degradation of a potential DNA-synthesis initiator protein (Heichman and Roberts, 1996), whose degradation depends on B-type cyclin-specific proteolysis, we do not yet know. It is, however, tempting to speculate that, in S and G_2/M phase-arrested cells, Cln- and Clb-dependent kinases are required to repress degradation of many if not all substrates of the B-type cyclin proteolysis machinery.

The role of Cln- and Clb-dependent kinases in repression of mitotic cyclin proteolysis

Our results suggest that Cln- and Clb-dependent kinases are required to repress B-type cyclin-specific proteolysis in S and G_2/M phase-arrested cells. One interpretation of these results is that they reflect the requirement of Cln- and Clb-dependent kinases to repress B-type cyclin-specific proteolysis during S-phase and early mitosis in a normal cell cycle. These findings, however, are also consistent with the notion that inactivation of Cln- and Clb-dependent kinases leads to inactivation of the surveillance mechanisms that inhibit onset of B-type cyclin-specific proteolysis in nocodazole- or hydroxyurea-arrested cells. However, Cln- and Clb-dependent kinases are also required to inhibit mitotic cyclin proteolysis in a *cdc4* arrest (Amon *et al.*, 1994; this study), which is caused by a failure to degrade Sic1 and not by a surveillance mechanisms (Schwob *et al.*, 1994). We therefore favor the idea that the requirement of Cln- and Clb-dependent kinases to repress B-type cyclin proteolysis in hydroxyurea- and nocodazole-arrested cells reflects the requirement of these kinases to repress B-type cyclin proteolysis in S phase and early mitosis during a normal cell cycle.

How Cln- and Clb-dependent kinases repress B-type cyclin-specific proteolysis and whether this is a direct effect is not yet known. Some component(s) of the proteolytic machinery (perhaps APC) could be inhibited by Cln- and Clb-dependent kinases. Alternatively, substrate recognition could be regulated by Cln- and Clb-dependent phosphorylation. Given that inactivation of Cln- and Clb-dependent kinases leads to both degradation of Clb2 and of proteins required to hold sister chromatids together, we favor the idea that the proteolysis machinery itself, directly or indirectly, is inhibited by Cln- and Clb-dependent kinases.

Activation of B-type cyclin-specific proteolysis

A key question is how the B-type cyclin proteolysis machinery is activated during anaphase. *In vitro* reconstitution of cyclin B proteolysis using partially purified components obtained from clam oocytes suggests that cyclin B/Cdc2 kinase activates B-type cyclin-specific proteolysis (Lahav-Baratz *et al.*, 1995; Sudakin *et al.*, 1995). There are indications that the Clb-dependent kinases might also be required to activate B-type cyclin proteolysis in yeast. Deletion of *CLB2* in a *cdc23-1* mutant is lethal even at 25°C (Irniger *et al.*, 1995). This has been interpreted to suggest that Clb2-dependent kinase activity is involved in activation of B-type cyclin-specific proteolysis (Irniger *et al.*, 1995). Interestingly, our results suggest that Clb-dependent kinases are required to repress the B-type cyclin-specific proteolysis during S phase and G_2/M phase. Perhaps Clb-dependent kinases play a dual role in the regulation of B-type cyclin proteolysis: on one hand they ensure that the B-type cyclin proteolysis machinery is not activated prematurely; on the other hand they might sow the seeds of their own destruction by activating a pathway that leads to the activation of the B-type cyclin-specific proteolysis machinery. Precedent for the notion that Clb-dependent kinases may have two opposing roles in the same process is provided by the finding that Clb-dependent kinases play a dual role in regulation of DNA replication. They are required for initiation of DNA replication and at the same time prevent re-replication (Dahmann *et al.*, 1995).

A model for the regulation of B-type cyclin proteolysis in yeast

Based on our findings, we propose the following model for how B-type cyclin-specific proteolysis is regulated during the cell cycle in yeast. Although the G_1 cyclin Cln3 is present during G_1, its associated kinase is either inactive or insufficiently active to repress B-type cyclin-specific proteolysis (Dirick *et al.*, 1995). As cells progress through G_1, Cln-associated kinases, which are not subjected to B-type cyclin-specific proteolysis, rise and at the G_1/S phase transition are sufficiently active to repress B-type cyclin proteolysis. During S phase/G_2, the Cln kinases decline and the Clb kinases assume the role of inhibiting B-type cyclin proteolysis. How proteolysis is activated during anaphase is an important question which remains to be addressed.

Materials and methods

Plasmids and strains

All strains were derivatives of strain W303 (also called K699). Strains carrying *CLB2* constructs were described previously (Amon *et al.*, 1994). To generate a *GAL–CDC28-degron* fusion an *Eco*RI fragment carrying the *Arg-DHFRts–CDC28* fusion from pPW66R (Dohmen *et al.*, 1994) was cloned under the control of the *GAL1-10* promoter and placed into Yiplac211 (Gietz and Sugino, 1988). The construct was integrated at the *CDC28* locus as described by Dohmen *et al.* (1994). The *GAL–SIC1* construct was transplaced into yeast as described by Nugroho and Mendenhall (1994). To generate a *S.pombe ADH–CLB2–lacZ* fusion we replaced the *GAL1-10* promoter of a *GAL–CLB2–lacZ* fusion (Irniger *et al.*, 1995) by the *S.pombe ADH* promoter.

Cell cycle arrest and release conditions

For cell cycle arrest by depleting cells of G_1 cyclins (Figure 1) cells were grown to log phase in –met raf medium at 25°C, filtered and arrested in YEPraf medium containing 2 mM methionine for 5 h before galactose addition. In experiments where Cln- and Clb-associated kinases were inactivated by expressing *SIC1* from the *GAL1-10* promoter and simultaneous pheromone treatment, cells were grown to exponential phase in YEPraf at 25°C and 15 μg/ml nocodazole (Figures 2, 3, 4 and 7) or 20 mg/ml hydroxyurea (Figure 5) was added. When arrest was complete (after 165 min), galactose (2%) and α-factor (7 μg/ml or 10 μg/ml) were added. A further aliquot of nocodazole (7.5 μg/ml) or hydroxyurea (5 mg/ml) was added to prevent cells from escaping from the arrest.

Techniques

In situ hybridization to yeast chromosomes was performed as described by Guacci *et al.* (1994) using probes mapping to the *CEN4* locus (cosmid 70938) and to the arm of chromosome 16 (cosmid 70912). For quantification at least 150 nuclei were scored per time point. Western blot analysis of total amount of Clb2, Cdc28, Sic1 and Kar2 protein in extracts was determined as described by Surana *et al.* (1993) or by an enhanced chemiluminescence detection system (Irniger *et al.*, 1994; Schwob *et al.*, 1995). Anti-Sic1 antibodies were used at a 1:200 dilution. Equal loading of gel lanes was shown by probing blots with anti-Kar2 antiserum (1:3000 dilution; Rose *et al.*, 1989). Incorporation of ^{32}P into histone H1 and the total amount of protein in extracts was quantitated using a FujiX BAS2000 phosphorimager. All other techniques were performed as described (Amon *et al.*, 1993 and references therein).

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