

Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast

Uttam Surana¹, Angelika Amon, Celia Dowzer, Jeffrey McGrew², Breck Byers² and Kim Nasmyth³

Research Institute of Molecular Pathology, Dr Bohr Gasse 7, Vienna, Austria and ²Department of Genetics, SK-50, University of Washington, Seattle, WA 98195, USA

¹Present address: Institute of Molecular and Cellular Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511

The first two authors made equal contributions to this paper

³Corresponding author

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It is widely assumed that degradation of mitotic cyclins causes a decrease in mitotic cdc2/CDC28 kinase activity and thereby triggers the metaphase to anaphase transition. Two observations made on the budding yeast *Saccharomyces cerevisiae* are inconsistent with this scenario: (i) anaphase occurs in the presence of high levels of kinase in *cdc15* mutants and (ii) overproduction of a B-type mitotic cyclin causes arrest not in metaphase as previously reported but in telophase. Kinase destruction is therefore implicated in the exit from mitosis rather than the entry into anaphase. The behaviour of *esp1* mutants shows in addition that kinase destruction can occur in the absence of anaphase completion. The execution of anaphase and the destruction of CDC28 kinase activity therefore appear to take place independently of one another.

Key words: cell cycle/cyclins/metaphase–anaphase transition/mitotic kinase/*Saccharomyces cerevisiae*

Introduction

Entry into mitosis requires a protein kinase composed of a catalytic subunit encoded by the *cdc2/CDC28* gene in association with a mitotic cyclin (reviewed by Nurse, 1990). Homologues of both types of these proteins have been found in many eukaryotes, suggesting that mitotic controls are highly conserved. Such proteins are components of the maturation promoting activity (MPF) which upon injection causes immature *Xenopus* eggs or invertebrate oocytes to enter meiotic metaphase (Lohka *et al.*, 1988). Moreover, deregulation of the kinase through mutation causes premature entry into mitosis in the fission yeast *Schizosaccharomyces pombe* (Nurse and Thuriaux, 1980). Activation of the *cdc2/CDC28* kinase has therefore been proposed to serve as the trigger for entry into mitosis. This would involve association of *cdc2/CDC28* with cyclins (Solomon *et al.*, 1990) followed by dephosphorylation of a tyrosine residue within the N-terminal part (Gould and Nurse, 1989). Recent studies have, however, raised doubts whether this event alone is sufficient for entry into M phase (Osmani *et al.*, 1991; Amon *et al.*, 1992; Sorger and Murray, 1992).

Mitotic cyclins were first identified in sea urchin eggs on the basis of their periodic abundance during early cleavage divisions (Evans *et al.*, 1983). They accumulate during interphase and undergo abrupt degradation at the time of each division. Cell cycle oscillations of this sort, though not universal, are observed in a wide variety of organisms and cell types (Westendorf *et al.*, 1989; Lehner and O'Farrell, 1990). In extracts from *Xenopus* eggs which oscillate between mitosis and interphase, *cdc2* kinase activity fluctuates with a pattern very similar to that of mitotic cyclins, reaching a maximum at metaphase and disappearing as cells enter anaphase (Murray and Kirschner, 1989).

Rapid degradation of cyclin is thought to occur via the ubiquitin pathway requiring a conserved sequence called the 'destruction box' within the N-terminal part of the protein (Glotzer *et al.*, 1991). Mutated versions lacking the destruction box cause cycling *Xenopus* egg extracts to become arrested in mitosis in a state characterized by high kinase activity (Murray and Kirschner, 1989). This condition is similar to that found in the meiosis II metaphase arrest of unfertilized frog eggs caused by cytostatic factor (Lohka and Masui, 1984). These findings have led to the notion that cyclin destruction (and thereby kinase inactivation) triggers the metaphase to anaphase transition (Murray and Kirschner, 1989; Glotzer *et al.*, 1991). However, apart from a single preliminary study (Ghiara *et al.*, 1991), there has been no rigorous test of this hypothesis in living cells. In this paper, we describe experiments on the budding yeast *Saccharomyces cerevisiae* that question the universality, if not the validity, of this model.

B-type cyclins are encoded by at least four different genes in *S.cerevisiae*: *CLB1*, 2, 3 and 4 (Ghiara *et al.*, 1991; Surana *et al.*, 1991) Of these, *CLB2* seems the most important for mitosis because its deletion uniquely delays exit from G₂ (Surana *et al.*, 1991) whereas triple mutants lacking *CLB1*, 3 and 4 undergo nuclear division with nearly normal kinetics (Fitch *et al.*, 1992). We show here that *CDC28* kinase activity associated with *CLB2* fluctuates during the cell cycle, reaching a maximum just prior to anaphase and disappearing soon after. These cycles correspond with the pattern expected. But we also show that *cdc15* mutants, when incubated at the restrictive temperature, undergo anaphase without any decrease in *CLB2/CDC28* kinase activity. Moreover, expression of a stable *CLB2* mutant protein or over-expression of wild type *CLB2* causes cells to arrest not in metaphase but in telophase. Both observations imply that destruction of the *CLB2/CDC28* kinase is necessary for the final exit from mitosis and not, as has been widely assumed, for the onset of anaphase (Murray and Kirschner, 1989; Ghiara *et al.*, 1991; Glotzer *et al.*, 1991). In contrast, kinase destruction occurs normally in a mutant that fails to execute anaphase. The onset of anaphase and the destruction of kinase appear therefore to be independent of one another.

Results

Cell cycle regulation of CLB2/CDC28 kinase

Of all known B-type cyclins in *S.cerevisiae*, CLB2 plays the most important part in mitosis (Surana *et al.*, 1991; Fitch *et al.*, 1992). Immunoprecipitates of CLB2 have a strong histone H1 kinase activity that is CDC28 dependent (Amon *et al.*, 1992). To determine the cell cycle regulation of this kinase, wild type cells were synchronized by release from pheromone-induced G₁ arrest (Figure 1A). CLB2/CDC28 kinase activity rises 60 min after the release, reaches a maximum at 75 min and declines thereafter, reaching a minimum shortly after the completion of mitosis. A comparison with nuclear cytology (Figure 1B) reveals that the proportion of cells in late anaphase reaches a maximum at a time (90 min) when substantial kinase activity is still present (60% of the maximum). Thus, anaphase may have been initiated before substantial kinase destruction in many cells.

cdc15 mutants undergo anaphase with high kinase activity

If anaphase was triggered by kinase destruction, then *cdc15* mutants, which arrest in telophase with equal amounts of DNA widely separated by an extended spindle (Pringle and Hartwell, 1981), should have low kinase activity. To test this, *cdc15* mutant cells arrested in G₁ by incubation with pheromone at 25°C were released into fresh medium at 37°C (the restrictive temperature). By 100 min, the DNA masses of most cells were separated by extended spindles; a state we refer to as telophase (Figure 2B and C). During this period, CLB2/CDC28 kinase activity rose to maximal levels but did not show any dramatic decline (cf. Figures 2A and 5A). Approximately 70% of the kinase activity is still present at 180 min, by which time all cells in the culture had entered telophase. The level of CLB2 protein behaved similarly, except that it declined gradually during the arrest, to 60% of the maximum level. The distribution of DNA and the spindle morphology of *cdc15* mutants suggest that these cells undergo anaphase at the same time as wild type. Yet they do so without any loss of kinase activity. We note that the SWI5 protein, whose entry into the nucleus is prevented by phosphorylation of its nuclear localization signal by the CDC28 kinase, is found predominantly in the cytoplasm during a *cdc15* mutant telophase arrest (Moll *et al.*, 1991). Thus, the high levels of kinase that we measured in *cdc15* mutants using an *in vitro* assay probably reflect accurately a high kinase activity within the cell.

CLB2/CDC28 kinase activity and CLB2 protein disappear within 20 min of *cdc15* mutants being returned to the permissive temperature (Figure 3A) and the long spindles disappear 10 min later (Figure 3B). The mutant cells remain fully viable, promptly re-entering the next cell cycle (Price *et al.*, 1991) and re-acquiring kinase activity (Figure 3A). These data raise the possibility that it is the final exit from mitosis rather than the onset of anaphase that requires kinase destruction. Constitutive CLB2 protein instability could account for its disappearance during the recovery of *cdc15* mutants because *CLB2* transcription is repressed as cells enter G₁ (Surana *et al.*, 1991). Nevertheless, this is probably accompanied by a drop in the protein's stability because the amount of CLB2 protein fluctuates during the cell cycle, falling as cells enter G₁ and rising again as cells

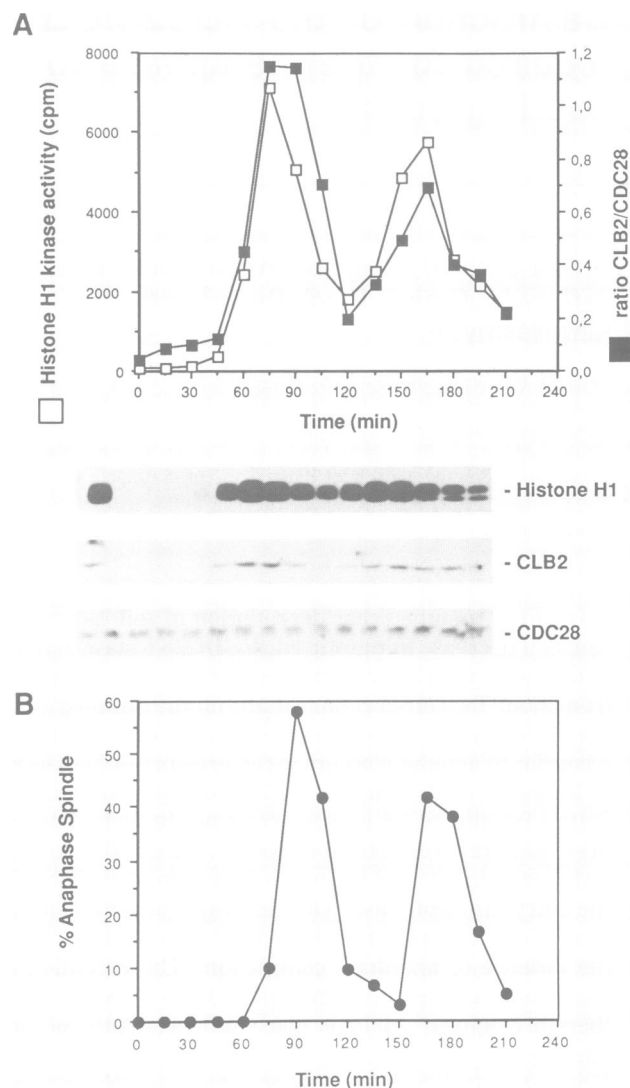


Fig. 1. CLB2/CDC28 kinase activity and CLB2 protein levels during the cell cycle. **A** *MATa bar1*⁻ derivative (K1534) of the wild type strain (K699/W303) synchronized in G₁ by α factor treatment for 100 min was released into fresh YEPD medium at 25°C. CLB2 was immunoprecipitated using a CLB2-specific antiserum and histone H1 kinase activity was measured (see Materials and methods). The CLB2 protein was detected by Western blot analysis and quantified with a Molecular Dynamics Phosphorimager. Cell samples were also fixed with formaldehyde and stained with anti-tubulin antibodies and diamidino-phenyl indole (DAPI) to visualize mitotic spindles and nuclei. The first lane in this and subsequent experiments shows the amount of CLB2-associated histone H1 kinase and CLB2 protein levels in exponentially growing cells prior to arrest and is not indicated in the graph. **(A)** Histone H1 kinase activity (open squares) measured by quantifying the radioactivity in the histone H1 band. The amount of CLB2 protein is plotted as the ratio of CLB2 to CDC28 (closed squares). The CDC28 protein was used as an internal control in the Western blots since its level does not fluctuate during the cell cycle. **(B)** The percentage of cells with anaphase spindles during the cell cycle. For each time point a total of at least 200 cells were counted.

enter G₂, even when *CLB2* is transcribed constitutively from the *GAL* promoter (Figure 3C).

Persistent CLB2 causes telophase arrest

The occurrence of anaphase in the absence of kinase destruction in *cdc15* mutants might only occur because *CDC15* is required to delay anaphase until kinase had been

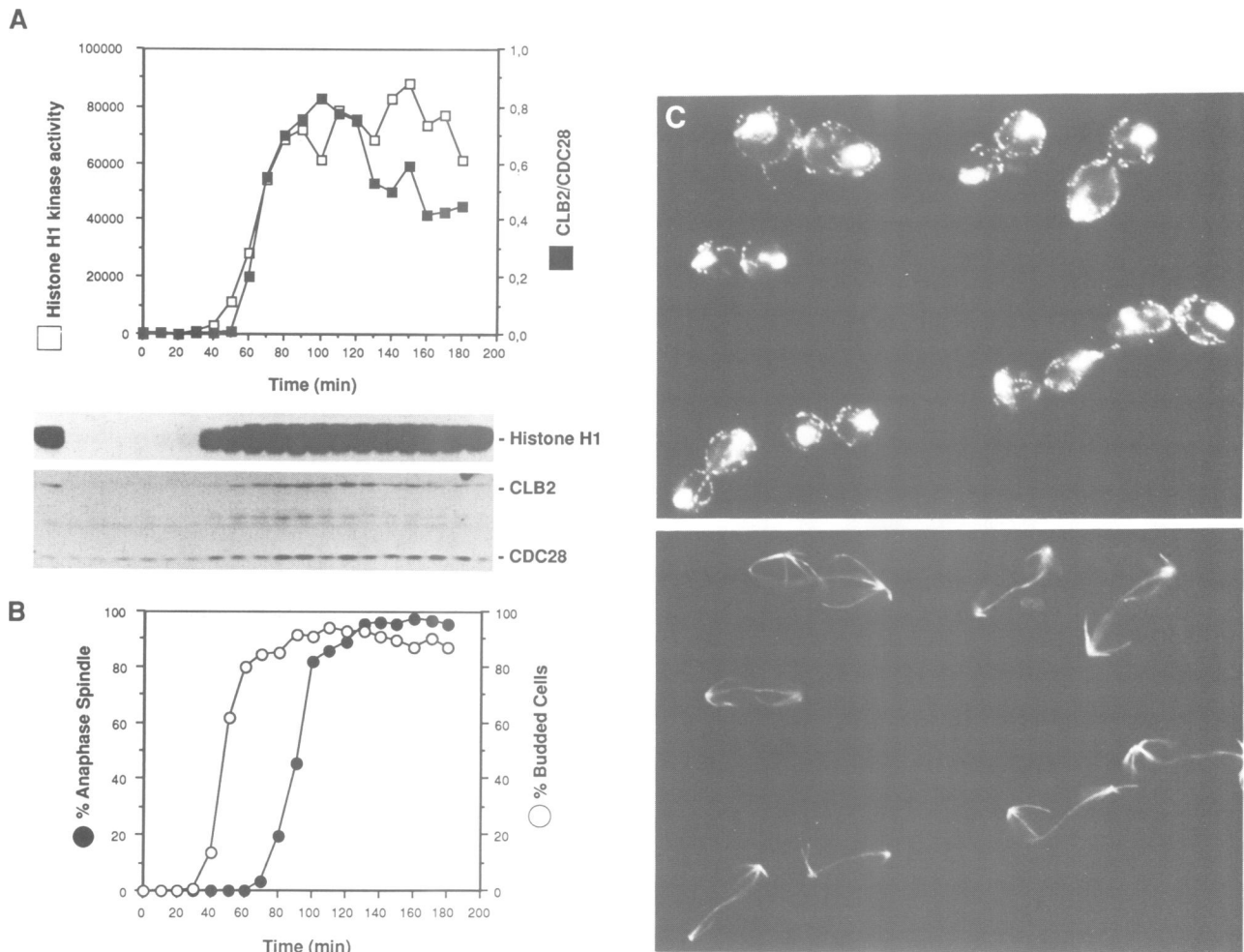


Fig. 2. CLB2/CDC28 kinase activity as *cdc15* mutant cells undergo anaphase. *MATa bar1⁻ cdc15-2* mutant cells (K2798) growing at 25°C were synchronized in G₁ by α factor treatment (150 min) and then released into fresh medium at 37°C. CLB2/CDC28 kinase activity, the amount of CLB2 protein relative to CDC28 and the proportion of cells containing anaphase spindle were measured as in Figure 1. (A) CLB2/CDC28 kinase activity (open squares) and the ratio of CLB2 to CDC28 protein (filled squares) in *cdc15* mutant cells as they progress through the cell cycle at 37°C. CDC28 protein levels do not usually vary during the cell cycle (see Figure 1). It is not clear why there is a slight increase as cells enter G₂ in this experiment. (B) Percentage of budded cells (open circles) and of cells containing anaphase spindles (closed circles). (C) Cell cycle arrest phenotype of *cdc15* mutant cells 180 min after the release from G₁ block. DNA stained with DAPI above and microtubules stained by *in situ* immunofluorescence using anti-tubulin antibodies below.

destroyed. If this were the case, stabilization of the kinase in wild type (i.e. *CDC15*⁺) might cause metaphase arrest, as described for *Xenopus* extracts. Expression from the *GAL1* promoter of a mutant CLB2 protein lacking a destruction box is lethal when the cells are grown on galactose (data not shown). To analyse the cause of this lethality, G₁ cells obtained by growth to stationary phase in raffinose medium (in which *GAL1* is inactive) were inoculated into fresh medium containing galactose. Expression of the mutant CLB2 protein had little or no effect on DNA segregation and spindle elongation but prevented cells from exiting mitosis (Figure 4A). Greater than 90% of the cells arrest in telophase within the first cell cycle on galactose, with chromosomes separated by a long mitotic spindle, as in *cdc15* mutants. Whereas a single copy of the wild type *CLB2* gene expressed from *GAL1* is tolerated by cells, four copies cause a telophase cell cycle arrest similar to that caused by a single copy of the destruction box mutant (Figure 4B). These cells initiate chromosome separation with the same kinetics as wild type cells (Figure 4C). Our data therefore suggest that kinase

destruction is not required for the onset of anaphase, even in *CDC15*⁺ cells, but may instead be necessary for the final exit from mitosis and entry into G₁. This conclusion is inconsistent with a report that a nondegradable version of CLB1 causes yeast cells to arrest in a metaphase-like state (Ghiara *et al.*, 1991). We have re-analysed the behaviour of this strain and find that it too arrests in telophase (data not shown). It is noteworthy that *CLB2* overexpression hinders not only exit from mitosis but also bud formation. Thus, many cells arrest with extended spindles and well separated DNA masses within an unbudded cell (Figure 4A and B; see also Ghiara *et al.*, 1991).

Kinase destruction and exit from mitosis without completing anaphase

The delay in kinase destruction until the time of recovery from *cdc15* arrest raises the possibility that kinase destruction is dependent on the completion of chromosome segregation rather than vice versa. To test this, we measured the kinase activity of *esp1* mutants, which accumulate extra spindle pole

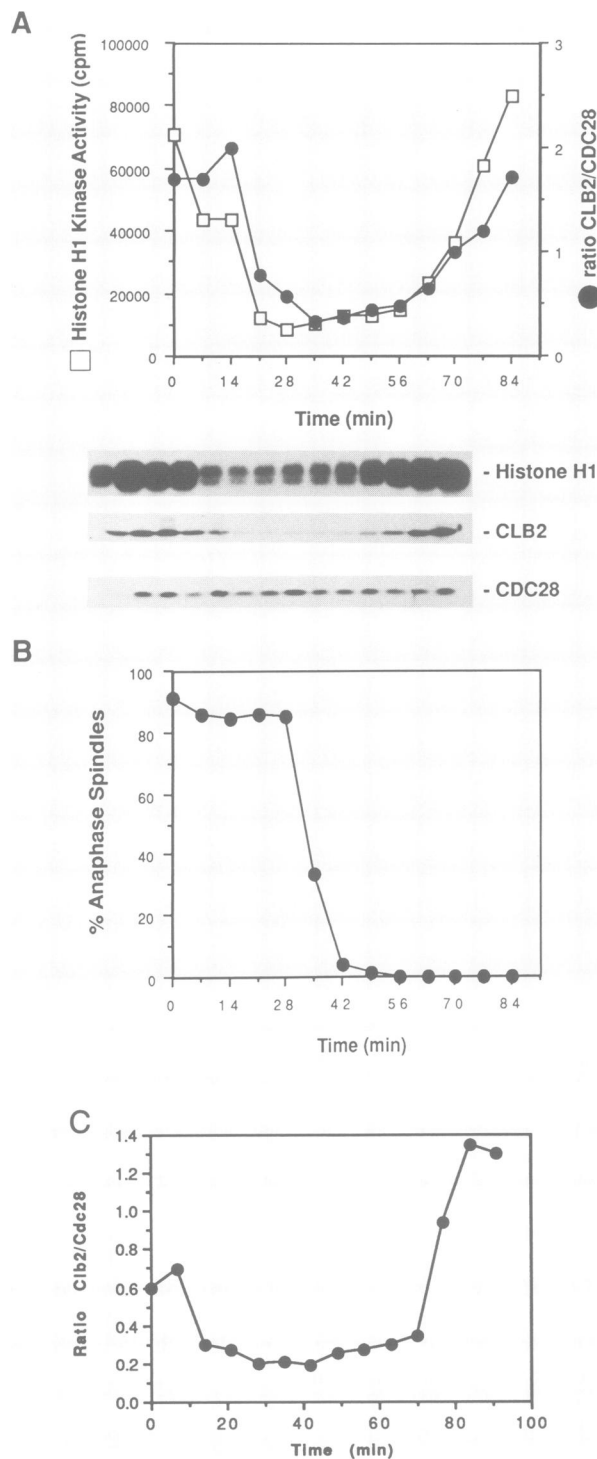


Fig. 3. Loss of CLB2/CDC28 kinase activity as *cdc15* mutant cells recover from arrest. (A) CLB2/CDC28 kinase activity (open squares) and the ratio of CLB2 to CDC28 protein (closed circles). The corresponding blots are shown below the graph. (B) Percentage of cells containing anaphase spindles. A log phase culture of *MATa bar1::URA3 cdc15-2* cells (K2944) growing at 25°C was transferred to 37°C for 150 min. The arrested cells were filtered and resuspended in YEPD medium at 25°C. (C) The ratio of CLB2/CDC28 protein in *cdc15* mutant cells expressing a single copy of *CLB2* from the *GAL1-10* promoter returned to 25°C after arrest at 37°C. A log phase culture of K2742 (*MATa BAR1 cdc15-2 clb2::LEU2 GAL-CLB2::URA3*) growing at 25°C in YEP medium containing raffinose plus galactose was transferred to 37°C for 150 min. The arrested cells were filtered and resuspended in fresh medium at 25°C.

bodies because of their failure to complete anaphase (Baum *et al.*, 1988; McGrew *et al.*, 1992). *esp1* mutants form a short mitotic spindle of normal appearance, but aberrant behaviour at the normal time of spindle elongation results in the failure of nuclear division. The bulk of the nuclear material segregates to one of the two daughter cells, which thereby become diploid or hyperploid, while the other receives little or none of the genome (P.Baum, L.Goetsch and B.Byers, manuscript submitted). To explore the kinetics of kinase activity in *esp1* mutants, wild type and congenic *esp1* mutant cells arrested in G₁ with pheromone were released into fresh medium at 37°C. The mutant cells budded normally and formed short mitotic spindles with the same kinetics as wild type but neither spindle elongation nor chromosome segregation occurred (data not shown). Remarkably, CLB2/CDC28 kinase activity oscillated in the mutant cells as in wild type, decreasing just prior to spindle dissolution and then reappearing (Figure 5A and B). *CLN1* transcripts, which normally arise late in G₁, rose in abundance soon after mitosis/cytokinesis (Figure 5C), indicating that the now hyperploid *esp1* cells were capable of undergoing Start-related functions. Subsequently, in parallel with the second rise in kinase activity, short mitotic spindles were again formed. Thus, kinase destruction is not dependent on the successful completion of anaphase since *esp1* mutants remain capable of destroying kinase, exiting from mitosis and re-entering the cell cycle.

***CDC15* is required for kinase destruction in *esp1* mutants**

One explanation for our results is that kinase destruction and anaphase are independent consequences of metaphase. Anaphase execution requires *ESP1* but not *CDC15*, whereas kinase destruction needs *CDC15* but not *ESP1* (model A; Figure 6). An alternative explanation is that *ESP1* is needed not only for anaphase/nuclear division but also to ensure that kinase destruction only occurs once anaphase is complete. Thus, *ESP1* could be a negative regulator of kinase destruction that must be antagonized by *CDC15* (model B; Figure 6). These two possibilities can be distinguished by analysing the phenotype of *cdc15 esp1* double mutants. The first model predicts that the double mutant should arrest with high kinase activity and should not undergo nuclear division, whereas the second model predicts that the double mutant should resemble the *esp1* single mutant, losing kinase activity. We find that when *cdc15 esp1* double mutant cells previously synchronized in G₁ are incubated at 37°C, the cells arrest with an undivided nucleus (Figure 7A) and only gradually lose kinase activity (Figure 7B). Short mitotic spindles form with similar kinetics in wild type and *esp1* mutants. However, unlike the *cdc15* mutant or the wild type strain, spindles in the *esp1 cdc15* double mutant continue to resemble the G₂ or metaphase spindles (seen in *cdc13* or *cdc20* mutants at the restrictive temperature; Weinert and Hartwell, 1988; Sethi *et al.*, 1992). Neither nuclear division nor spindle elongation occur in the double mutant. It is difficult to assess the integrity of spindles after extensive incubation due to their distortion and to an accumulation of cytoplasmic microtubules. The spindles disintegrate more quickly than the extended spindles that accumulate in *cdc15* single mutants. Unlike *esp1* single mutants, *esp1 cdc15* double mutant cells neither undergo cytokinesis nor form

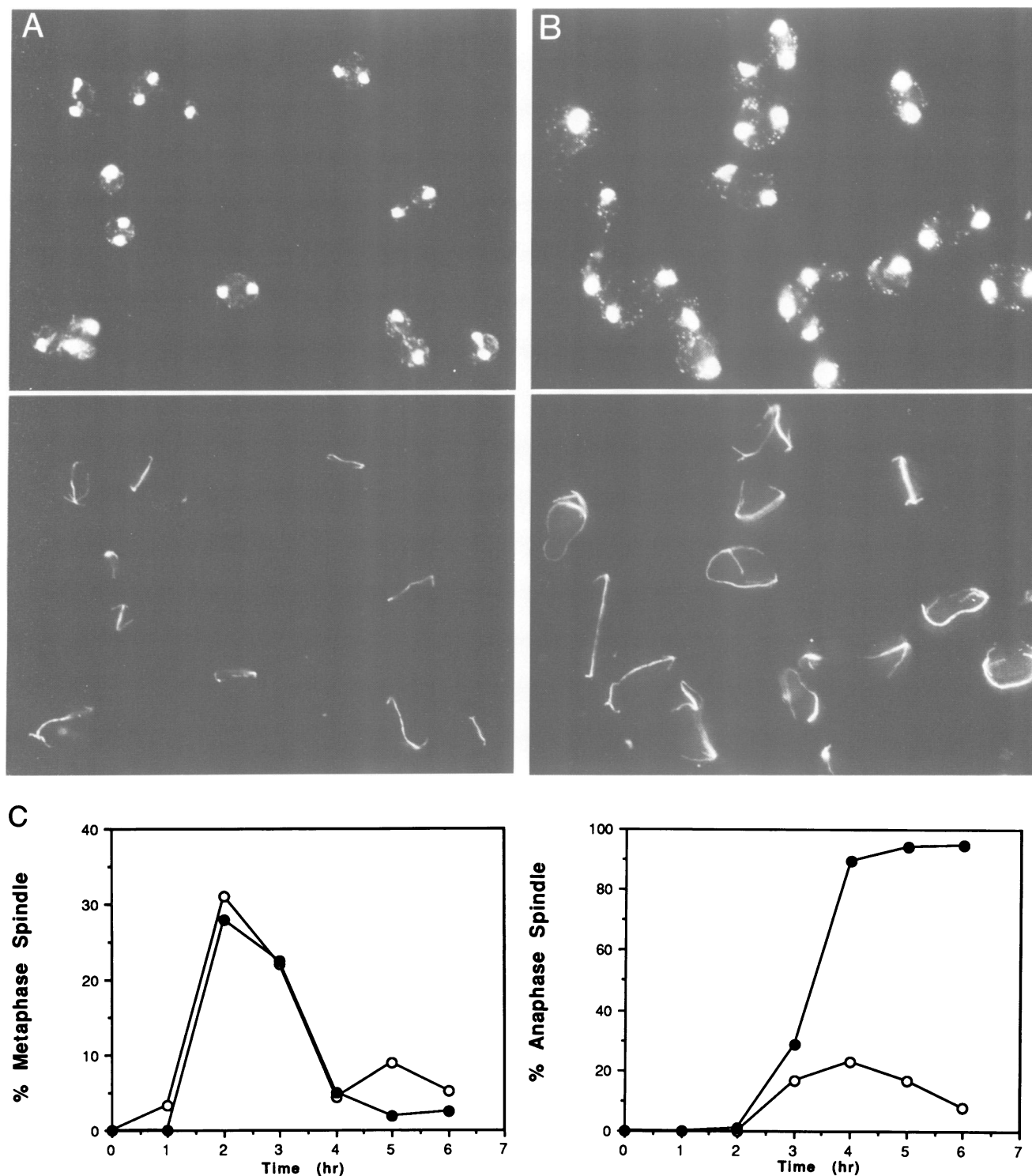


Fig. 4. Cell cycle arrest due to persistent CLB2. Cells were synchronized in G₁ by growth to stationary phase on YEP plates containing raffinose for 48 h at 25°C and then inoculated into fresh medium containing raffinose and galactose. Fixed cell samples were analysed by *in situ* immunofluorescence. (A) DNA (above) and tubulin (below) staining of haploid cells (A552; *MATa clb2::LEU2 1XGAL-CLB2-dbΔ::TRP1*) containing a single copy of a *clb2* destruction box mutant expressed from the *GAL* promoter, 5 h after inoculation. (B) DNA (above) and tubulin (below) staining of diploid cells (A469; *MATa/MATα 8XGAL-CLB2::TRP1*) containing eight or more copies of the wild type *CLB2* gene expressed from the *GAL* promoter, 5 h after inoculation. (C) Percentage of cells containing G₂/metaphase (left) or anaphase (right) spindles following inoculation into galactose medium. Open circles, wild type; filled circles, multiple *GAL-CLB2*.

new buds (Figure 7A). Thus, both kinase destruction and cytokinesis in *esp1* mutants are dependent on *CDC15*. The phenotype of the double mutant favours model A over model B.

The role of kinase destruction in the exit from mitosis of *esp1* mutants

The failure of *cdc15 esp1* double mutants to exit from mitosis might result either from persistent kinase activity or from

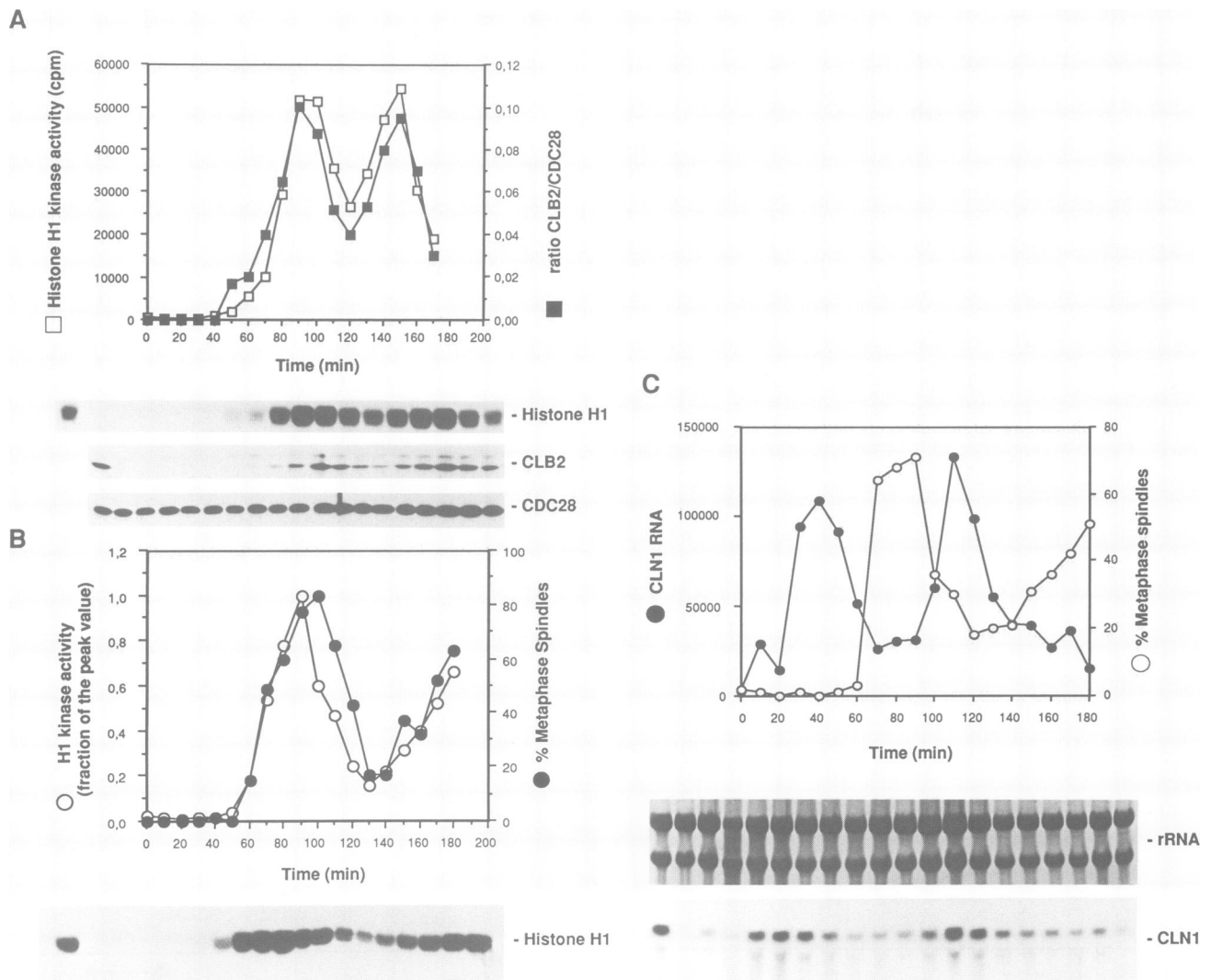


Fig. 5. A comparison of CLB2/CDC28 kinase in synchronous cultures of wild type (K1534) and *esp1* mutant (K2975; *MATa esp1-1 bar1::URA3*) cells at 37°C. Cells grown at 25°C were synchronized in G₁ by α factor treatment for 150 min and then released into fresh YEPD medium at 37°C. (A) CLB2/CDC28 kinase activity (open squares) and the ratio of CLB2 to CDC28 protein (closed squares) in the wild type cells. (B) CLB2/CDC28 kinase activity (open circles) and the percentage of cells with G₂/metaphase spindles (closed circles) in *esp1* mutant cells. No anaphase spindles are seen in these cells since these mutants fail to execute anaphase. (C) *CLN1* transcripts measured by Northern blotting as *esp1* mutant cells progress through the cell cycle at 37°C. RNA levels were quantified using a Molecular Dynamics Phosphorimager.

some more direct consequence of the *cdc15* defect. To assess the role of kinase destruction in the exit of *esp1* mutants from mitosis without destruction of nuclear DNA, we have analysed the effect of overproducing CLB2. *esp1* mutant cells carrying several copies of the CLB2 destruction box mutant gene, each expressed from a *GAL1* promoter, were synchronized in G₁ by growth to stationary phase on raffinose medium. These cells form short mitotic spindles upon inoculation into galactose medium at 37°C but never undergo nuclear division or cytokinesis (data not shown). Furthermore, they never bud a second time or re-initiate DNA replication. Whereas, *esp1* mutants divide to produce cells without any nuclear DNA and hyperploid cells, the over-expression of CLB2 causes cells to arrest with a 2N DNA content (Figure 8). CLB2 overproduction also causes mitotic spindles to persist longer than would otherwise be the case for *esp1* mutants, although spindle breakdown continues to occur in a sizeable fraction of the cells (data not shown). Kinase destruction thus appears to play an important role in the exit of *esp1* mutant cells from mitosis.

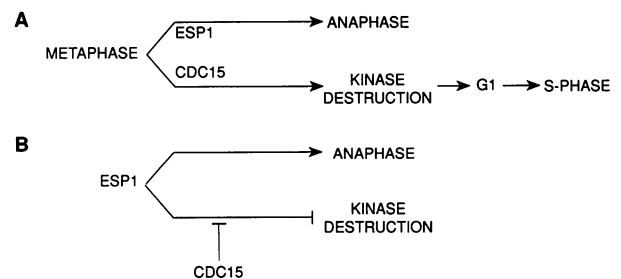


Fig. 6. Two alternative schemes to explain why *esp1* mutants destroy mitotic kinase in the absence of anaphase. (A) Anaphase and kinase destruction, although both dependent on the successful completion of metaphase, are independent events. While *CDC15* is necessary for the destruction of CLB2/CDC28 kinase, *ESP1* is required for anaphase execution. Thus *esp1* mutant cells can destroy the kinase, exit mitosis, enter the new cycle and initiate another round of DNA replication without having executed anaphase. (B) *ESP1* is required not only for anaphase but also for preventing kinase destruction until anaphase is completed. The *CDC15* gene product mediates CLB2/CDC28 kinase destruction by negatively regulating *ESP1*.

Discussion

The role of cyclin degradation in mitosis

Initiating the segregation of sister chromatids at anaphase is possibly the most irrevocable event during the cell cycle. The mitotic arrest caused by expressing non-degradable cyclins in *Xenopus* egg extracts or in yeast cells suggested that anaphase onset requires destruction of the cdc2/CDC28 protein kinase due to degradation of its B-type cyclin subunit (Murray and Kirschner, 1989; Ghiara *et al.*, 1991; Glotzer *et al.*, 1991). This paper describes two experiments on the budding yeast *S.cerevisiae* that are inconsistent with cyclin destruction initiating anaphase: (i) *cdc15* cell cycle mutants seem to undergo anaphase without any noticeable drop in the CDC28 kinase activity associated with the CLB2 B-type cyclin and (ii) the expression of a stable derivative of the CLB2 cyclin or the over-expression of wild type protein seems to inhibit exit from rather than entry into anaphase. Our data indicate that cyclin destruction is required for exit from mitosis but not for the initiation of anaphase, as was previously assumed. Our measurement of kinase activity in *esp1* mutants, which enter and exit mitosis without completing anaphase, suggests that kinase destruction and anaphase execution are independent of each other.

One concern is whether *cdc15* mutants and cells overproducing cyclins really undergo anaphase. Their mitotic spindles clearly elongate and DNA segregates in equal amounts to the opposite poles of the cell (Figures 2C and 4B). It is very hard to imagine how these changes could occur without the concomitant separation of sister chromatid centromeres. Our data therefore suggest that at least anaphase B occurs in the absence of kinase destruction. It is not yet possible to establish whether anaphase A also takes place. A second concern is the inconsistency between our observation of a telophase arrest due to persistent cyclin B expression and the metaphase-like arrest reported by Ghiara *et al.* (1991). We re-analysed the strain used by the above authors and found that it also arrests in telophase. The discrepancy between our work and that on *Xenopus* extracts is harder to explain. It may be relevant that wild type cyclins are unstable in extracts arrested by non-degradable cyclins, suggesting that the arrested state is distinct from metaphase (Glotzer *et al.*, 1991). Might this arrest actually represent a subsequent stage that is more similar to early anaphase? In this regard it is worth noting that expression of a stable version of chicken cyclin B in HeLa cells does not seem to prevent cells attempting some form of chromosome separation (Gallant and Nigg, 1992). Kinase activity clearly decreases before anaphase is completed in *Xenopus* extracts (Shamu and Murray, 1992), but this does not imply that the decrease is necessary for triggering anaphase.

Our work does not exclude the possibility that destruction of cyclins other than CLB1 and CLB2 is responsible for triggering anaphase in yeast. CLB3 and CLB4 are unlikely candidates because they are dispensable for mitosis (Fitch *et al.*, 1992). One would therefore have to invoke the destruction of a novel cyclin in anaphase initiation. A cyclin of this sort could, however, make only a minor contribution to the total histone H1 kinase activity of the cell, because total suc1-binding kinase activity also fails to drop during the onset of anaphase in *cdc15* mutants (data not shown).

The conclusion that kinase destruction is needed for exit from mitosis rather than entry into anaphase raises the possibility that the CDC28 kinase has a role during anaphase

itself. Loss of *CLB1*, *2*, *3* and *4* functions prevents cells from separating spindle pole bodies and from forming mitotic spindles (Fitch *et al.*, 1992). Cyclin B is presumably necessary for the function of these mitotic spindles at least up to metaphase but possibly also during anaphase. It is arguable that the forces exerted by mitotic spindles during metaphase and anaphase are similar and that the crucial difference lies in the attachment of sister chromatids at the kinetochore. Merely breaking this attachment could initiate anaphase. Thus, cyclins might have similar functions in the behaviour of metaphase and anaphase spindles. However, the large decrease in histone H1 kinase activity that occurs before anaphase has been completed in *Xenopus* extracts implies that low levels of kinase, if any, are sufficient for the later stages of anaphase, at least in amphibians (Shamu and Murray, 1992).

CDC15 and the mechanism of kinase destruction

The failure of *cdc15* mutants to degrade the CLB2/CDC28 kinase is not simply due to the state in which they arrest. *CDC15* is also necessary for kinase destruction in *esp1* mutants that cannot execute anaphase. *CDC15*, which itself encodes a kinase (Schweitzer and Philipsen, 1991), could therefore have a direct role in mediating CLB2/CDC28 kinase destruction. It is nevertheless possible that the failure to inactivate CDC28 kinase in *cdc15* mutants is due to a regulatory system or checkpoint control (Weinert and Hartwell, 1989) that detects a defect in some aspect of the mitotic apparatus in *cdc15* mutants and sends an inhibitory signal to the process responsible for cyclin destruction; that is, *CDC15* may not be directly involved in kinase destruction. In this regard, it is interesting that yeast cells spend an appreciable fraction (10%) of their cell cycle in late anaphase/telophase. This may be a stage during which cells delay the final exit from mitosis (due to retention of CDC28 kinase activity) while they check the fidelity of spindle behaviour.

The actual mechanism of kinase destruction in yeast is far from clear. The *CLB2* gene continues to be transcribed during a *cdc15* arrest but is rapidly repressed upon the restoration of *CDC15* function (Surana *et al.*, 1991). Thus, constitutive cyclin protein instability could in principle explain the disappearance of the kinase. A change in CLB2 protein stability also contributes because CLB2 protein levels fluctuate following a *cdc15* release, declining as cells enter G₁ and rising as they enter G₂, even when *CLB2* is continuously transcribed from the *GAL1* promoter.

ESP1 and the independence of anaphase and kinase destruction

Previous studies have demonstrated that *esp1* mutants accumulate extra spindle pole bodies when transferred to nonpermissive conditions, suggesting a role for *ESP1* in regulation of the spindle pole body duplication cycle (Baum *et al.*, 1988). More recent analysis indicated, however, that the accumulation of extra spindle pole bodies is a secondary consequence of a defect in nuclear division (J.McGrew, L.Goetsch, B.Byers and P.Baum, manuscript submitted). When mutant cells are synchronized in G₁ and then permitted to enter the cell cycle in the absence of *ESP1* function, spindle pole body duplication and spindle formation proceed normally, but anaphase is defective and neither spindle elongation nor chromosome segregation occur.

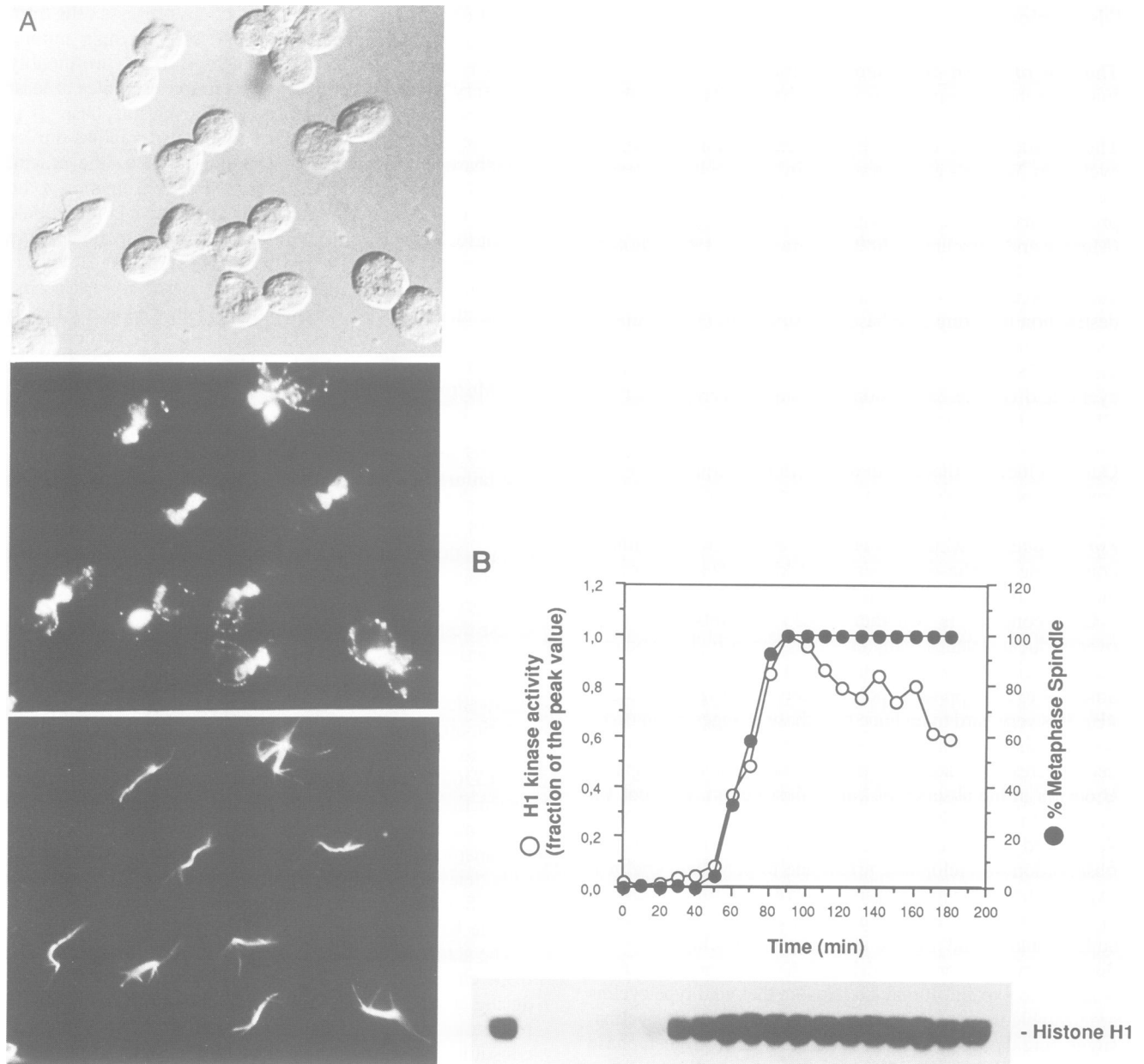


Fig. 7. The phenotype of *cdc15 esp1* double mutants. *cdc15 esp1* double mutant cells (K2952; *MATa esp1-1 cdc15-2*) were synchronized in G_1 by α factor treatment at 25°C and cells were released in YEPD medium at 37°C. (A) Cell morphology as viewed under Nomarski optics (top), DNA staining (middle), and tubulin staining (bottom) 150 min after growth at 37°C. (B) CLB2/CDC28 kinase activity and percentage of cells showing metaphase-like spindles following release from the G_1 arrest. No anaphase spindles were detected in these mutants.

Nevertheless, the mitotic spindles disappear at the same time as in wild type and the cells proceed into cytokinesis, with the result that one of the two progeny cells inherits both chromosome sets as well as both spindle poles while its sister is apolar and largely aploid. The diploid daughter then enters a new cell cycle, thus leading to polyploidization and spindle multipolarity because of a primary defect in spindle behaviour rather than any intrinsic defect in regulation of spindle pole duplication. Assays reported in the present work reveal that CLB2/CDC28 kinase activity falls at about the time of spindle dissolution, just as is the case when anaphase occurs normally in wild type. We therefore conclude that kinase destruction does not depend upon anaphase chromosome segregation, but must instead be under independent control. Whether kinase destruction is sufficient

for exit of *esp1* cells from one cycle and entry into the next remains to be resolved. We note in this regard that the polyploidization resulting from defects in certain *S.pombe cdc2* alleles upon heat shock and nitrogen starvation (Broek *et al.*, 1991) suggests that kinase destruction may be sufficient for premature transit from one cycle into the next in some organisms.

We do not know what aspect of anaphase is defective in *esp1* mutants. The short mitotic spindles in *esp1 cdc15* double mutants appear to be less stable than those in *cdc15* single mutants. In contrast, mitotic spindles are stable when *esp1* mutants are arrested in G_2 by the *cdc13* mutation (data not shown), so it may well be the case that an *esp1* deficiency leads to a defect in spindle stability only during anaphase.

ESP1 is partially homologous to the *cut1* gene from

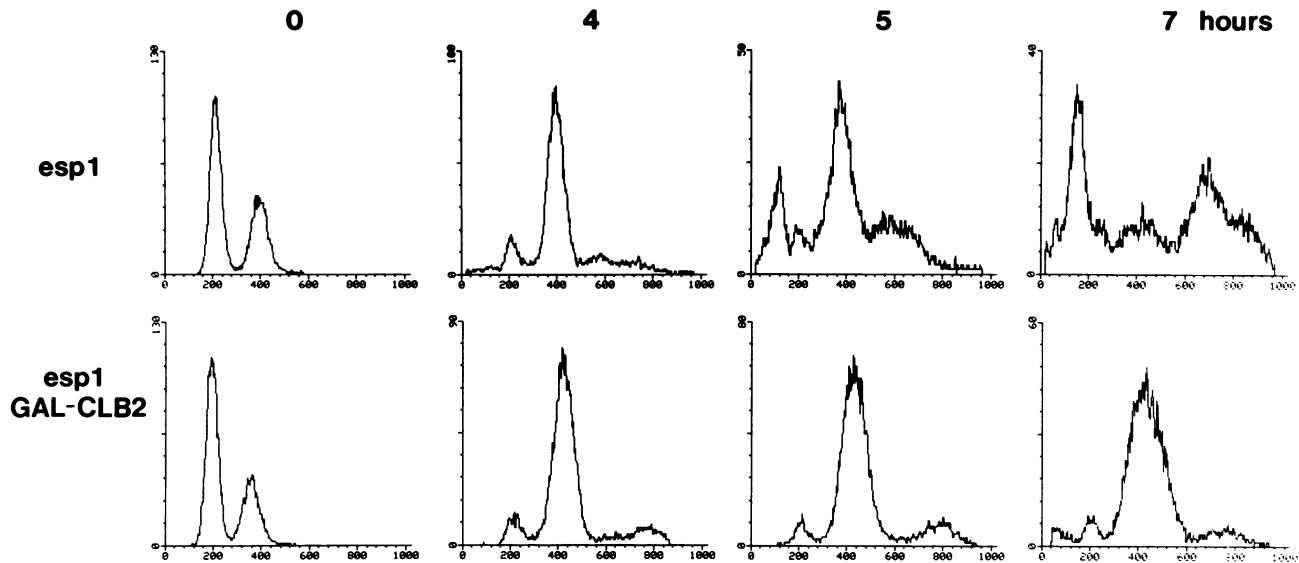


Fig. 8. CLB2 overproduction prevents the exit from mitosis of *esp1* mutants. A *MATa esp1-1* strain (K2788) and a congenic derivative containing multiple copies of a *GAL-CLB2-dbΔ* fusion integrated at the *TRP1* locus (K2978) grown on YEP raffinose plates until stationary phase (3 days at 25°C) were inoculated into YEP medium containing raffinose and galactose medium at 37°C. Cellular DNA contents were determined by FACS analysis at the indicated times.

S.pombe and the *BIMB* gene of *Aspergillus nidulans* (Uzawa *et al.*, 1990; May *et al.*, 1992). *cut1* mutants undergo cytokinesis without completing nuclear division and *bimB* mutants can re-replicate DNA in the absence of completing mitosis. Thus, *cut1* and *bimB* may perform an equivalent mitotic function to *ESP1*. The ability to exit from the mitotic state without completing anaphase may be a property common to many if not most eukaryotic organisms. In *Drosophila* embryos homozygous for the *plutonium* and *pan gu* mutations (Shamanski and Orr-Weaver, 1991), DNA synthesis, centriole duplication and mitotic spindle cycles continue in the absence of nuclear division, resulting in giant polyploid nuclei. Natural polyploidization is widespread in plants and animals. For example, cells in the trophoblast of mouse embryos are polyploid (Ilgren, 1981), as are many cells in adult liver. Most larval tissues in *Drosophila*, other than neurons and imaginal discs, undergo polyteny. Conceivably, the inherent independence of anaphase and *cdc2/CDC28* kinase destruction could be a conserved feature of mitosis that is exploited for polyploidization.

Materials and methods

Strains and growth conditions

All strains used in this study were derivatives of the wild type strain W303. The *cdc15* and *esp1* mutants were made isogenic by backcrossing at least three times to the wild type strain. Cells were grown in YEP medium containing adenine (50 mg/l) supplemented either with glucose (2%) or both galactose (2%) and raffinose (2%).

Synchronization by treatment with α factor and release

For experiments involving synchronous cultures, cells were grown to exponential phase at 25°C in YEP medium containing glucose (YEPD) and α factor was added to a final concentration of 1 μ g/ml. After 150 min of α factor treatment cells were filtered, washed with 3 vol YEPD medium and resuspended in fresh YEPD medium. Samples were withdrawn every 10 min for measurement of *CDC28/CLB2* kinase activity and total *CLB2* protein and for visualization of spindles and nuclei. In some experiments where induction by galactose was required, cells were first synchronized in stationary phase by growth on YEP+raffinose plates for 48 h. They were then scraped and resuspended in YEP medium containing raffinose and galactose pre-equilibrated either at 25°C or at 37°C.

Genetic manipulations

All DNA manipulations were performed according to Maniatis *et al.* (1982). The *CLB2* gene was fused to the *GAL1-10* promoter by introducing an *EcoRI* site at its AUG codon by PCR and cloning a *BamHI-EcoRI GAL* fragment with an *EcoRI-BamHI CLB2* fragment into the *BamHI* site of the *TRP1*-based integrative vector YIPlac204 (Gietz and Sugino, 1988). To generate a nondegradable version of *CLB2*, the destruction box (²⁴RLALNNVTN³⁴) was deleted using M13 mutagenesis (Amersham). The mutant allele (*CLB2-dbΔ*) was cloned under the control of the *GAL10* promoter as described above.

Preparation of cell extracts and kinase assay

For the preparation of crude extracts, cells were harvested by centrifugation at 4°C and washed twice with ice-cold stop-mix (Surana *et al.*, 1991). The cell pellet was resuspended in ice-cold lysis buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl pH 7.2, 20 mg/l leupeptin, 40 mg/l aprotinin, 0.1 mM sodium orthovanadate and 15 mM *p*-nitrophenyl phosphate). An equal volume of glass beads was added to this suspension and cells were broken by two bursts of vigorous vortexing for 3 min at 4°C. The glass beads and cell debris were removed by centrifugation for 5 min followed by two more centrifugations for 15 min each in a microfuge. The supernatant was used for assaying the *CLB2*-associated kinase activity.

CLB2/CDC28 kinase was immunoprecipitated by mixing cell extracts containing 100–150 μ g total protein with affinity purified *CLB2*-specific antibodies (1:40 dilution; Amon *et al.*, 1992); the mixture was incubated on ice for 1 h. To each tube, 10 μ l of protein A-Sepharose beads pre-equilibrated with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl and 50 mM Tris-HCl pH 7.2) were added and the tubes were incubated at 4°C for 1 h with gentle mixing. The beads were then collected by centrifugation and washed five times with RIPA buffer and three times with 25 mM MOPS buffer pH 7.2. For the kinase reaction, immunoprecipitates prepared this way were resuspended in 6 μ l of HBI buffer (Surana *et al.*, 1991) and incubated at 25°C for 7 min. 10 μ l of KIN buffer (2 mg/ml histone H1, 200 μ M [γ -³²P]ATP, ~100 c.p.m./pmol in 25 mM MOPS pH 7.2) were then added and the reaction mix was incubated at 25°C for 12 min. The reaction was terminated by adding 5 μ l of 5 \times PAGE sample buffer, boiled for 3 min and loaded on a 12.5% SDS-PAGE gel (Laemmli, 1970). Phosphorylated histone H1 was visualized by autoradiography. For quantification, bands corresponding to phosphorylated histone H1 were cut out from dried gels and the amount of radioactivity was determined using a liquid scintillation counter. In some experiments, a Phosphorimage analyser (Molecular Dynamics) was used for quantification. Control experiments in which the amount of extract was varied showed that the kinase activity measured in this way was proportional to the amount of extract immunoprecipitated. No kinase activity can be detected in *cdc28-4* mutants using the above assay even when the cells are grown at 25°C.

Western blot analysis

For determination of total CLB2 protein, cells were harvested by centrifugation, washed once with 10 mM Tris-HCl buffer pH 7.5 and resuspended in 200 μ l of breakage buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 mM DTT, 1 mM PMSF, 500 μ M TPCK, 25 μ M TLCK and 2 μ g/ml pepstatin). An equal volume of glass beads was added and cells were broken by vigorous vortexing for 3 min. 10 μ l were withdrawn for protein determination. To the remainder, 100 μ l of 3 \times PAGE sample buffer were added and the mixture was immediately boiled for 3 min. The glass beads and cell debris were removed by centrifugation. The cell extract containing 50 μ g of total protein was loaded on a 12.5% SDS-PAGE gel. The gel was soaked in transfer buffer containing 10% methanol and proteins were transferred to cellulose nitrate filters using an electroblotter (LKB). The filters were pretreated with milk solution (3% non-fat dry milk in PBS) for 30 min and then treated with CLB2 antibodies (1:300 dilution) or CDC28-specific antibodies (1:500) for 2 h at room temperature. Finally, these filters were probed with ¹²⁵I-labelled protein A for 75 min and washed three times with milk solution followed by a wash with PBS. The CLB2 and CDC28 protein bands were visualized by autoradiography.

Other techniques

The methods described by Cross and Tinkelenberg (1991) and Price *et al.* (1991) were used for RNA isolation and the Northern blot analysis respectively. *In situ* immunofluorescence and photomicroscopy were performed according to Nasmyth *et al.* (1990). Flow cytometric DNA quantification was performed as described by Lew *et al.* (1992) and Epstein and Cross (1992).

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