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The Cln3 - Cdc28 kinase complex of *S.cerevisiae* is regulated by proteolysis and phosphorylation

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In *Saccharomyces cerevisiae*, several of the proteins involved in the Start decision have been identified; these include the Cdc28 protein kinase and three cyclin-like proteins, Cln1, Cln2 and Cln3. We find that Cln3 is a very unstable, low abundance protein. In contrast, the truncated Cln3-1 protein is stable, suggesting that the PEST-rich C-terminal third of Cln3 is necessary for rapid turnover. Cln3 associates with Cdc28 to form an active kinase complex that phosphorylates Cln3 itself and a co-precipitated substrate of 45 kDa. The *cdc34-2* allele, which encodes a defective ubiquitin conjugating enzyme, dramatically increases the kinase activity associated with Cln3, but does not affect the half-life of Cln3. The Cln - Cdc28 complex is inactivated by treatment with non-specific phosphatases; prolonged incubation with ATP restores kinase activity to the dephosphorylated kinase complex. It is thus possible that phosphate residues essential for Cln - Cdc28 kinase activity are added autocatalytically. The multiple post-translational controls on Cln3 activity may help Cln3 tether division to growth.
Key words: cell cycle/*CDC34*/G_1 cyclin/PEST hypothesis/Start

Introduction

In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* the Cdc28/Cdc2 protein kinase controls two critical cell cycle steps: the G_1 phase commitment to division ('Start') and mitosis (Reed, 1980; Nurse and Bissett, 1981; Piggott *et al.*, 1982). Cdc28/Cdc2 function is very highly conserved amongst eukaryotic species since the human *cdc2* gene complements yeast *cdc28/cdc2*$^-$ mutations (Lee and Nurse, 1987; Wittenberg and Reed, 1989). There is abundant evidence that Cdc2 co-ordinates mitosis in higher eukaryotes (reviewed by Nurse, 1990). Because of the homology and analogy with the yeast system, there is a strong general expectation and some limited evidence that a Cdc2-like protein kinase will regulate the mammalian G_1 commitment step (e.g. D'Urso *et al.*, 1990; Furukawa *et al.*, 1990; Fang and Newport, 1991; Hunter and Pines, 1991; Matsushime *et al.*, 1991). Despite enthusiasm for this idea, *S.cerevisiae* remains the only organism where several of the molecules intimately involved in G_1 commitment have been conclusively identified and even in this organism very little is known about the molecular nature of the commitment event.

The Cdc2 monomer has little or no kinase activity; instead, the active kinase is a complex composed of Cdc2 and a regulatory subunit called cyclin (Draetta *et al.*, 1989). Activity of the complex is controlled in part by the availability of cyclin and in part by tyrosine and threonine phosphorylation of Cdc2 (Gould and Nurse, 1989; Murray and Kirschner 1989; Solomon *et al.*, 1990, 1992; Gould *et al.*, 1991: Meijer *et al.*, 1991; Parker *et al.*, 1991). Mitotic cyclin abundance oscillates throughout the cell cycle, building up to a maximum in metaphase, just prior to catastrophic destruction in anaphase (Evans *et al.*, 1983). Versions of mitotic cyclin that cannot be degraded maintain Cdc2 kinase activity and block the cell cycle in mitosis (Murray *et al.*, 1989; Ghiara *et al.*, 1991). Mitotic cyclins are degraded by the ubiquitin proteolytic pathway and they are targeted to this pathway by an N-terminal consensus sequence called the destruction box (Glotzer *et al.*, 1991).

Genetic screens have identified three *S.cerevisiae* genes called *CLN1*, *CLN2* and *CLN3* that co-operate with *CDC28* at Start. *CLN1* and *CLN2* were identified as high copy plasmid suppressors of *cdc28-4* (Hadwiger *et al.*, 1989). *CLN3* was discovered because of a dominant mutation called *WHI1-1* (now *CLN3-1*) that accelerates cells through Start, resulting in a small cell size (a 'Wee' phenotype) and shortened G_1 phase (Sudbery *et al.*, 1980). A second allele of *CLN3* was isolated as the *DAF1-1* mutation (now *CLN3-2*) because of its dominant resistance to mating pheromone-induced G_1 arrest (Cross, 1988), a phenotype shared by *CLN3-1* (Nash *et al.*, 1988). Since wild-type *CLN3* is a dose dependent activator of Start, it has been proposed that *CLN3-1* encodes a hyperactive product (Nash *et al.*, 1988). The *CLN3-1* and *CLN3-2* mutations create stop codons that remove the C-terminal third of the protein, which is extremely rich in proline (P), serine (S) and threonine (T) residues (Nash *et al.*, 1988). Since such 'PEST' regions may be signals for proteolysis (Rogers *et al.*, 1986), we have proposed that the C-terminal third of Cln3 targets the protein for rapid turnover and that Cln3-1 may be a stable version of Cln3 (Nash *et al.*, 1988). Cln1 and Cln2 also have PEST-rich C-terminal regions (Hadwiger *et al.*, 1989).

Strikingly, sequence alignments show that all three *CLN* genes are distantly related to the mitotic cyclins (Nash *et al.*, 1988; Hadwiger *et al.*, 1989). Cln-deficient cells arrest in G_1 phase at Start with the same phenotype as *cdc28* mutants (Richardson *et al.*, 1989; Cross, 1990). The genetic properties of the three genes, the G_1 lethality of the triple mutant and the weak cyclin homology support the idea that Cln1, Cln2 and Cln3 are 'G_1 cyclins' (Nash *et al.*, 1988; Hadwiger *et al.*, 1989; reviewed by Reed, 1991). That is, Cln1, 2 and 3 may activate Cdc28 in G_1 phase to regulate Start and this Cln - Cdc28 interaction may be similar at the molecular level to the interaction between mitotic cyclins and Cdc2/Cdc28 that regulates mitosis.

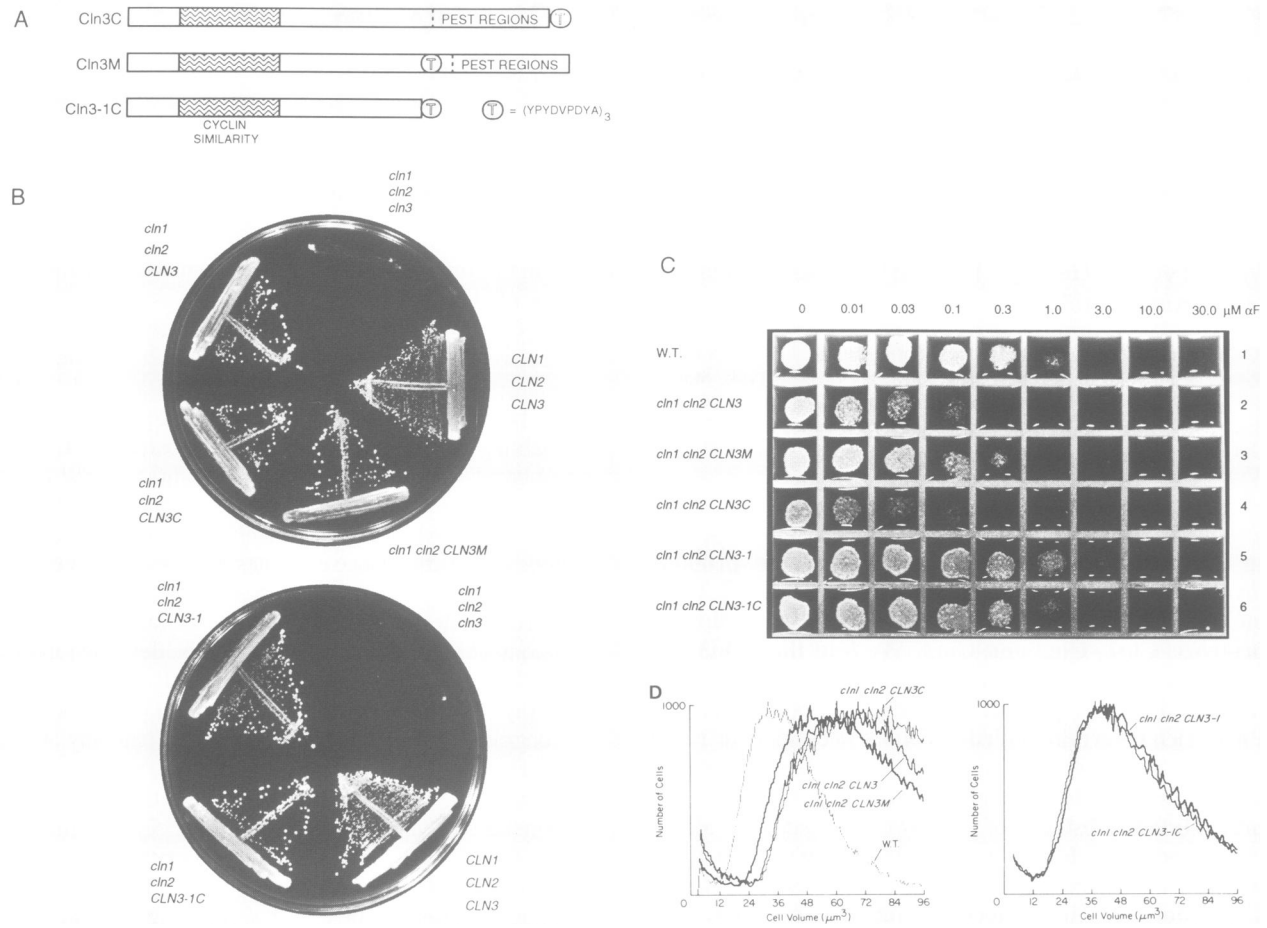

Fig. 1. Phenotypic effects of epitope tagged Cln3. (**A**) Structure of the tagged Cln3 proteins. Three tandem copies of the HA1 epitope were inserted at the indicated positions of the *CLN3* open reading frame (see Materials and methods for details). Nomenclature used for the tagged alleles is: *CLN3M*, middle tag position placed in frame at the *CLN3-1* mutation site; *CLN3C*, C-terminal tag position; *CLN3-1C*, tag inserted at the end of the *CLN3-1* reading frame immediately prior to the stop codon. PEST regions shown in the C-terminus of Cln3 and the region of cyclin similarity are defined in Nash *et al.* (1988). (**B**) Complementation of triple *cln* deletion by epitope tagged alleles of *CLN3*. Isogenic constructions were made in a *cln1 cln2* parental strain (see Materials and methods). The function of *CLN3* alleles was tested by examining growth on glucose plates. (**C**) Quantitative α-factor sensitivity of *cln1 cln2* strains bearing different *CLN3* alleles. The same strains as used in (B) were spotted on YEPD grids with the indicated α-factor concentrations and incubated for 3 days at room temperature. The α-factor resistance of *CLN3-1* strains depends in part on *CLN1* and *CLN2* (Dirick and Nasmyth, 1991; Nasmyth and Dirick, 1991); for this reason the *CLN3-1* strains shown here (rows 5 and 6) are somewhat sensitive to α-factor. None of the tag positions had any effect on the α-factor resistance of wild-type strains. (**D**) Size analysis of strains disrupted for *CLN1* and *CLN2* and bearing different *CLN3* alleles. Cultures of the strains used in (B) were sonicated and sized on a Coulter channelizer. The modal volume for each strain was: 32 μm^3, *CLN1 CLN2 CLN3* non-tagged parental strain (W.T.); 67 μm^3, *cln1 cln2 CLN3*; 60 μm^3, *cln1 cln2 CLN3M*; 68 μm^3, *cln1 cln2 CLN3C*; 37 μm^3, *cln1 cln2 CLN3-1*; 36 μm^3, *cln1 cln2 CLN3-1C*. Left panel shows the effect of the two tag positions in *CLN3*; right panel shows the effect of *CLN3-1C*. A typical *CLN1 CLN2 CLN3-1* strain has a modal volume of 23 μm^3. None of the tags had any effect on size in a wild-type background.

This idea, presently based mainly on genetic and sequence analysis, requires that Cln1, 2 and 3 have cyclin-like biochemical properties. Cln2 largely fulfils this expectation: it is physically associated with Cdc28 in a complex which can phosphorylate histone H1 *in vitro* and its abundance oscillates in the cell cycle with a peak in G_1 phase (Wittenberg *et al.*, 1990). Similar studies have not yet been done with Cln1 or Cln3. The amino acid sequence of Cln1 is ~75% identical to that of Cln2 and so it seems likely that their biochemical properties will be similar (Hadwiger *et al.*, 1989). On the other hand, Cln3 is only 20 to 25% identical to Cln1 and Cln2. In addition, *CLN3* transcription is regulated differently from *CLN1* and *CLN2*—it is not cell cycle regulated and it is not repressed by mating pheromone (Nash *et al.*, 1988; Wittenberg *et al.*, 1990). Since *CLN3* is quite different from *CLN1* and *CLN2*, we have examined the Cln3 protein to see whether it has properties expected of a cyclin.

We were particularly interested in Cln3 stability, since the popular idea that the cyclins are destabilized by their PEST-rich regions has not been tested. In addition, since mitotic cyclins are degraded by the ubiquitin pathway (Glotzer *et al.*, 1991), we asked if Cln3 was affected by a mutation in the ubiquitin conjugating enzyme encoded by *CDC34* (Goebl *et al.*, 1988). Finally, since the activity of mitotic Cdc2 is controlled by its phosphorylation state, we also examined the effects of phosphorylation on the Cln3−Cdc28 kinase complex.

Results

Detection of Cln3

Cln3 has proven to be a low abundance protein and antisera raised against recombinant protein or peptides could not detect Cln3 in extracts from wild-type cells. We used the epitope addition method (Field *et al.*, 1988) and over-

expression from the *GAL1* promoter (Johnston and Davis, 1984) to overcome this problem.

The wild-type chromosomal *CLN3* locus was replaced by a *GAL1 – CLN3* construct (Xiong *et al.*, 1991). The gene was strongly induced by galactose and rapidly repressed by glucose. Cells over-expressing Cln3 had short G_1 phases and became very small after growth for 4 h on galactose (see Table I). The *GAL1* promoter provided a convenient method for turning *CLN3* on and off, but still did not allow reliable detection of Cln3 with most antisera. To overcome this, the *CLN3* and *CLN3-1* open reading frames were tagged with three tandem copies of the hemagglutinin HA1 epitope (Figure 1A). Two alleles of *CLN3* were used: one was tagged at the end of the *CLN3* reading frame (*CLN3C* for C-terminus) and the other was tagged internally at a point corresponding to the *CLN3-1* mutation site (*CLN3M* for Middle). *CLN3-1* was tagged at only the end of its reading frame (*CLN3-1C* for C-terminus). For the strain concerned the full genotype will be described as for example *CLN3*, *CLN3M* or *CLN3C*, *CLN3-1* or *CLN3-1C*, but the encoded protein will be described only as either Cln3 or Cln3-1, omitting mention of the tag. The tagged proteins expressed from all three of these alleles could be detected with the 12CA5 anti-epitope monoclonal antibody (see below).

None of the tag positions had any phenotypic effect in a *CLN1 CLN2* background. As a stringent test of the function of tagged proteins, we constructed isogenic strains in which *CLN1* and *CLN2* were disrupted and viability was dependent on function of a gene encoding tagged Cln3 or Cln3-1. These strains (*cln1 cln2 CLN3M*, *cln1 cln2 CLN3C* and *cln1 cln2 CLN3-1C*) were compared to isogenic untagged strains (*cln1 cln2 CLN3* or *cln1 cln2 CLN3-1*) with respect to growth rate (Figure 1B), α-factor resistance (Figure 1C) and cell volume

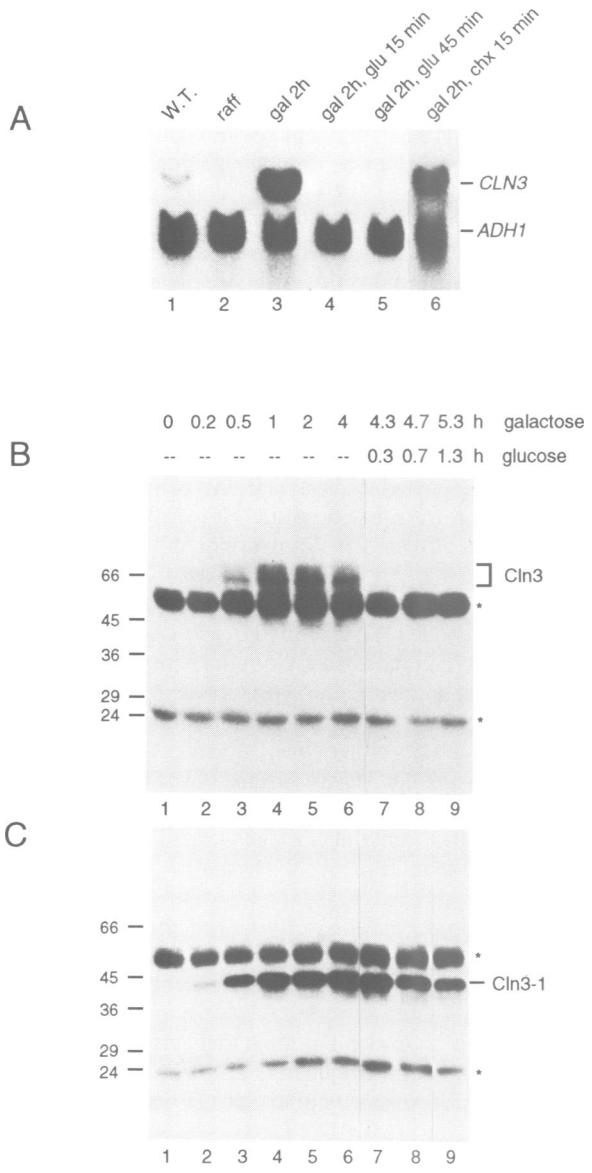

Fig. 2. Characterization of *CLN3* mRNA and protein stability. **(A)** *CLN3* mRNA is unstable. A Northern blot of total RNA was hybridized first with a *CLN3* probe and then with an *ADH1* probe without stripping the filter. The wild-type parental strain (BF338-3b) is shown in lane 1; lanes 2 through 6 show an isogenic *GAL1 – CLN3-1C* transformant (BF338-3b # 23) under different growth conditions. Cells were grown on 2% raffinose for 24 h (raff) and induced with 2% galactose for 2 h (gal). Galactose induced cultures were repressed with 2% glucose for either 15 or 45 min. Finally, to show that *CLN3* mRNA was not under the control of a labile repressor, a galactose induced culture was treated for 15 min with 50 μg/ml cycloheximide. **(B)** Induction and stability of Cln3. Cell lysates from a *GAL1 – CLN3M* culture (strain BF338-4b # 43) were immunoprecipitated with 12CA5 monoclonal antibody and then immunoblotted with the same antibody. Cultures were grown as follows: lane 1, raffinose; lanes 2–6, galactose for 0.2, 0.5, 1, 2 and 4 h respectively; lanes 7–9, galactose for 4 h then glucose for 0.3, 0.7 and 1.3 h respectively. Immunoreactivity of the secondary antibody with IgG heavy and light chains from the immunoprecipitation are shown by the asterisks; M_r markers are shown in kDa. **(C)** Induction and stability of Cln3-1. The experiment was carried out identically to that in (B) except that a *GAL1 – CLN3-1C* strain (BF338-3b # 23) was used.

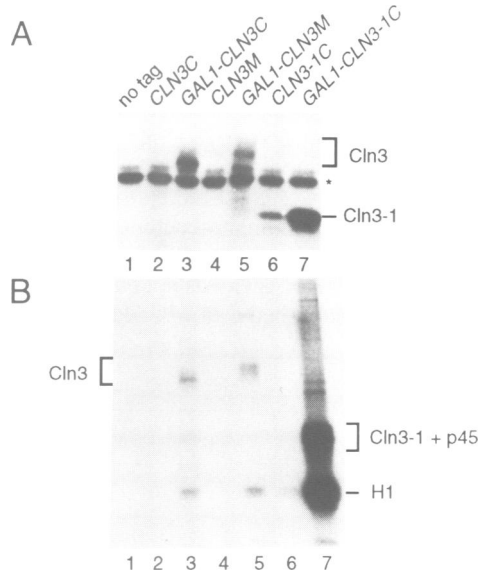

Fig. 3. Cln3 has an associated kinase activity. **(A)** Immunoblot analysis of Cln3 abundance in strains expressing various Cln3 constructs. Cell lysates were immunoprecipitated with 12CA5 monoclonal antibody and three-quarters of the immunoprecipitates were immunoblotted with the same antibody. Strains bearing the following alleles of *CLN3* were used: untagged *CLN3* (RN202-2b, lane 1); *CLN3C* (MT222, lane 2); *GAL1 – CLN3C* (BF410-2d, lane 3); *CLN3M* (BF338-4b # 43p, lane 4); *GAL1 – CLN3M* (BF338-4b # 43, lane 5); *CLN3-1C* (BF338-3b # 23p, lane 6); *GAL1 – CLN3-1C* (BF338-3b # 23, lane 7). The position of the IgG heavy chain is shown by the asterisk. Note that Cln3C migrates slightly faster than Cln3M. **(B)** *In vitro* kinase assays of Cln3 immunoprecipitates. The remaining quarter of the immunoprecipitates used in (A) were assayed for histone H1 kinase activity. Phosphorylated Cln3-1 and p45 were not well resolved in this gel system.

(Figure 1D). The latter in particular is a very sensitive indicator of the activity of *CLN3* in a cell (Nash *et al.*, 1988). Since the tags had very little phenotypic effect, we believe that they did not significantly affect Cln3 function. If anything, *CLN3C* was slightly hypoactive for cell size and α-factor resistance whereas *CLN3M* was slightly hyperactive. Despite these subtle effects (which were seen only in a *cln1 cln2* background), identical biochemical results were obtained with both *CLN3C* and *CLN3M*.

Cln3 is extremely unstable but the truncated, PEST-less Cln3-1 is stable

In galactose medium, the *GAL1–CLN3* construct expressed about 60-fold more mRNA than the wild-type *CLN3* gene (Figure 2A, lane 1 versus lane 3). Repression of the *GAL1* promoter by addition of glucose showed that this *CLN3* transcript was extremely unstable (Figure 2A, lane 4). This being so, the determination of Cln3 protein levels after glucose repression allowed an estimate of Cln3 half-life; the same technique has been used to determine the stability of Cln2 (Wittenberg *et al.*, 1990). Cln3 was very unstable, with a half-life of ~10 min (Figure 2B, lanes 7–9). In striking contrast, Cln3-1 was very stable, with a half-life of ~2 h (Figure 2C, lanes 7–9). Therefore, the PEST-rich C-terminal third of Cln3 is necessary for rapid turnover.

It was possible that over-expression of Cln3 induced Cln3 turnover by some homeostatic mechanism. However, when synthesis of the Cln3 protein expressed from its own promoter was inhibited by cycloheximide, the Cln3 half-life was also ~10 min (unpublished data). The addition of glucose in shutting off the *GAL1* promoter was not the cause of Cln3 instability, since when the *GAL1–CLN3* gene construct was shut off by washing out galactose medium and resuspending the cells in raffinose medium, the Cln3 signal again decayed rapidly (unpublished data).

In all cases, detection of Cln3 required prior immunoprecipitation from large amounts of cell lysate. No subpopulation of Cln3 was obscured by the IgG band since a similar pattern of Cln3 immunoreactivity was observed if the 12CA5 monoclonal antibody was first cross-linked to protein A beads so that the IgG was not eluted upon boiling in sample buffer (unpublished data). In addition, when Cln3 was tagged with nine copies of the HA1 epitope it was electrophoretically retarded, but this did not cause the emergence of any hidden species from behind the IgG bands (unpublished data). We have found that Cln3 is a much less abundant protein than either Cln1 or Cln2 and that detection of wild-type levels of Cln3 requires an enhanced chemiluminescent system (Tyers,M., Tokiwa,G. and Futcher, B., in preparation). Cln3-1 was at least 10-fold more abundant than Cln3 and could be detected even when expressed from its own promoter (Figure 3A; Table I).

Cln3 has an associated protein kinase activity

The amount of Cln3 in various immunoprecipitates was determined by immunoblot analysis (Figure 3A). Fractions of the same immunoprecipitates were incubated with [γ^{32}P]ATP and tested for kinase activity towards the exogenous substrate histone H1. A small amount of background kinase activity was non-specifically adsorbed onto protein A beads (e.g. Figure 3B, lane 1). Strains expressing wild-type levels of Cln3 had very low levels of immunoprecipitated kinase activity which could not be distinguished from background. However, specific kinase activity was detected in immunoprecipitates from strains over-expressing Cln3 (Figure 3B, lanes 3 and 5; see also Figure 4C). Of several exogenous substrates tested, including histones H2A, H2B, H3 and casein, the best was histone H1. The kinase activity phosphorylated Cln3 itself and an as yet unidentified, co-precipitated protein of 45 kDa which we refer to as p45. Cln3-1 immunoprecipitates had a kinase activity that was about equal to that of immunoprecipitates from strains over-expressing Cln3 (Figure 3B, lane 6). Over-expression of Cln3-1 greatly increased the amount of associated kinase activity (Figure 3B, lane 7).

Cln3-associated kinase activity and the abundance of phosphorylated Cln3 are dramatically elevated in strains defective for the ubiquitin conjugating enzyme Cdc34

Since Cln3 is an unstable protein that acts at Start and since the Cdc34 ubiquitin conjugating enzyme is required for cell cycle progression shortly after Start (Pringle and Hartwell,

Table I. Influence of various *CLN3* alleles on Cln3 abundance, associated kinase activity, sensitivity to mating pheromone and cell size

Allele[a]	Protein abundance[b]	Kinase activity[c]	α-factor sensitivity[d]	Cell size[e]
CLN3	1	1	S	39
CLN3 cdc34	2	10	S	48
GAL1–CLN3	20	10	S	17
GAL1–CLN3 cdc34	20	200	R	19
CLN3-1	10	10	R	18
CLN3-1 cdc34	20	200	R	34
GAL1–CLN3-1	50	2000	R	20
GAL1–CLN3-1 cdc34	50	4000	R	22

[a]Cells bearing *GAL1* constructs were grown on galactose for 4 h; *cdc34-2* strains were grown at permissive temperature.
[b]Protein abundance was estimated by β-imaging of ^{125}I signals on the immunoblots shown in Figure 3A and Figure 4A; the signal detected for Cln3 was not corrected for background and was arbitrarily assigned a value of 1.
[c]Kinase activities were estimated by Cerenkov counting of excised histone H1 bands. The value for *CLN3* was not corrected for background, non-specific activity and was arbitrarily assigned a value of 1. Values are the mean of at least three independent experiments. For genotypes where the activity is indicated as different (e.g. *CLN3* versus *CLN3-1*), the ranges did not overlap. Under the same assay conditions, p13^{suc1} beads precipitated a relative kinase activity of 20 000 c.p.m.
[d]A strain unable to form colonies in the presence of 3×10^{-6} M α-factor was scored as sensitive (S), while a strain able to form colonies in the presence of 3×10^{-5} M α-factor was scored as resistant (R).
[e]Values are the mean of modal volume (in μm^3) of log phase cells for at least three independent experiments.

1981; Goebl *et al.*, 1988), we asked if the *cdc34-2* mutation influenced Cln3. Two dramatic effects were seen at both the permissive and the restrictive temperature. First, the abundance of an electrophoretically retarded, phosphorylated form of Cln3 was greatly increased in immunoprecipitates from *cdc34-2* strains (Figure 4A). This form was rare in *CDC34* cells and usually not visible except by *in vivo* ^{32}P-labeling (unpublished data). Second, the amount of Cln3-associated kinase activity was elevated 10- to 20-fold in *cdc34-2* strains when compared with isogenic *CDC34* strains (Figure 4B, lane 3 versus lane 4). Over-expression of Cln3 was not required for the effect, since *CLN3 cdc34-2* strains had more kinase activity than isogenic *CLN3 CDC34* strains (Figure 4B). Despite the large increase in kinase activity, total Cln3 abundance increased no more than 2-fold in the *cdc34-2* background (Figure 4A). Thus, *cdc34-2* seemed to increase the amount of kinase activity

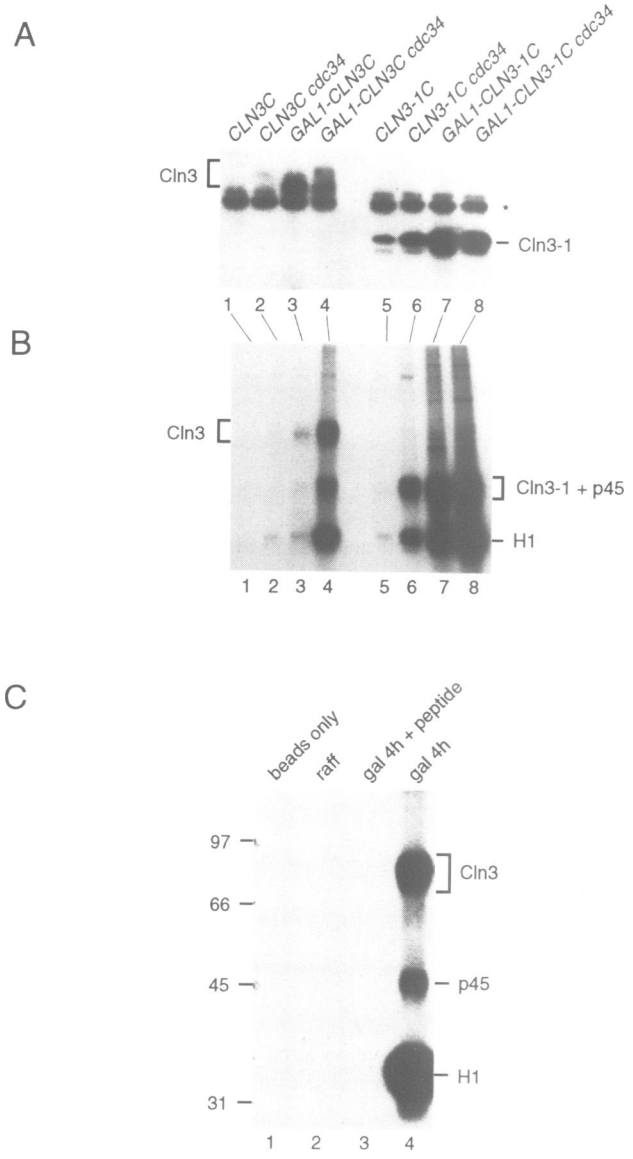

Fig. 4. Cln3-associated kinase activity is dramatically increased by the *cdc34-2* mutation. (**A**) Immunoblot analysis of Cln3 and Cln3-1 in wild-type and *cdc34-2* strains. Cell lysates from the indicated strains were immunoprecipitated and three-quarters immunoblotted with 12CA5 monoclonal antibody. Relevant genotypes of the strains used were: *CLN3C* (MT222, lane 1); *CLN3C cdc34-2* (MT212, lane 2); *GAL1–CLN3C* (BF410-2d, lane 3); *GAL1–CLN3C cdc34-2* (BF410-7c, lane 4); *CLN3-1C* (BF338-3b#23p, lane 5); *CLN3-1C cdc34-2* (MT217, lane 6); *GAL1–CLN3-1C* (BF338-3b#23, lane 7); *GAL1–CLN3-1C cdc34-2* (RN201-1c, lane 8). The position of the IgG heavy chain is shown by the asterisk. (**B**) Comparison of kinase activity in wild-type and *cdc34-2* strains. Kinase assays were performed on a quarter of the immunoprecipitates used in (A). (**C**) Specific association of kinase activity with Cln3. Kinase assays were performed on immunoprecipitates from a *GAL1–CLN3M cdc34-2* strain (RN204-3c). Lysates were prepared from cultures grown on raffinose for 24 h (lane 2) and induced for 4 h with galactose (lanes 1, 3 and 4). Immunoprecipitations were carried out with 12CA5 monoclonal antibody, except for lane 1 in which antibody was omitted. The sample shown in lane 3 was immunoprecipitated in the presence of excess (50 μg) HA1 peptide. M_r markers (in kDa) are indicated.

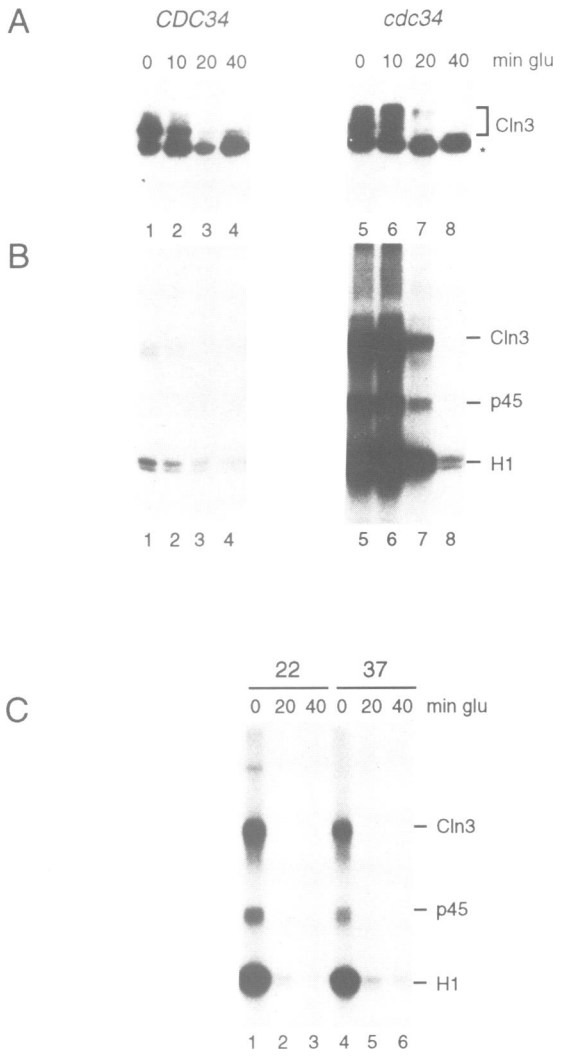

Fig. 5. The *cdc34-2* mutation does not alter the half-life of Cln3. (**A**) Immunoblot analysis of Cln3 stability in wild-type and *cdc34-2* strains. Cultures were induced with galactose for 4 h and then repressed with glucose for the indicated periods of time. Cell lysates were immunoprecipitated and three-quarters were immunoblotted with 12CA5 monoclonal antibody. Strains used were: *GAL1–CLN3C* CDC34 (BF410-2d, lanes 1–4) and *GAL1–CLN3C cdc34-2* (BF410-7c, lanes 5–8). The IgG heavy chain is shown by the asterisk. (**B**) Stability of Cln3-associated kinase activity in wild-type and *cdc34-2* strains. Kinase assays were performed on a quarter of the immunoprecipitates used in (A). (**C**) The half-life of Cln3 is not temperature sensitive in a *cdc34-2* background. *GAL1–CLN3M cdc34-2* cultures (RN204-3c) were grown in galactose at either the permissive (22°C) or restrictive temperature (37°C) for 4 h then treated with glucose for the indicated periods of time. Arrest at the restrictive temperature was confirmed by microscopy. Cell lysates were immunoprecipitated and assayed for kinase activity.

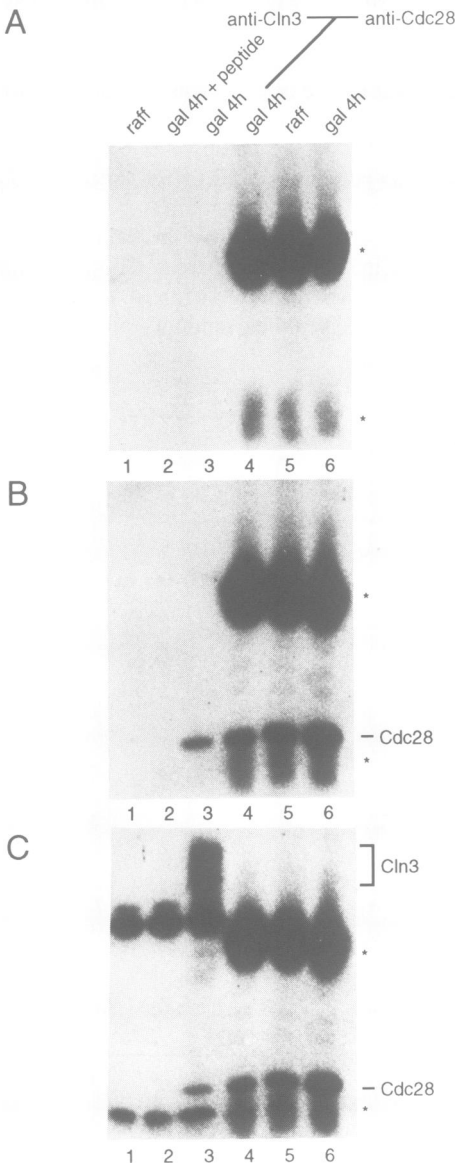

Fig. 6. Cdc28 co-precipitates with Cln3. (**A**) Immunoblot probed with pre-immune serum. Immunoprecipitations were carried out with 12CA5 mouse monoclonal antibody (lanes 1–3) and anti-Cdc28 serum from rabbit (lanes 4–6). Lysates were made from either a *GAL1–CLN3M cdc34-2* strain (RN204-3c, lanes 1, 2, 3, 5 and 6) or a *GAL1–CLN3M CDC34* strain (BF338-4b#43, lane 4). Cultures were grown on raffinose for 24 h (lanes 1 and 5) and subsequently induced with galactose for 4 h (lanes 2, 3, 4 and 6). The sample in lane 2 was immunoprecipitated in the presence of excess competing HA peptide. Reactivity of rabbit IgG heavy and light chains with the anti-rabbit secondary antibody (in lanes 4–6) is shown by the asterisks. This secondary antibody did not cross-react with mouse IgG (lanes 1–3). (**B**) Parallel immunoblot to that shown in (A) probed with anti-Cdc28 serum from rabbit. Lane order was the same as in (A). The positions of Cdc28 and rabbit IgG heavy and light chains (*) are indicated. (**C**) Immunoblot from (B) reprobed with 12CA5 monoclonal antibody to show the presence of Cln3 in immunoprecipitates. The original signal from the anti-rabbit secondary antibody was not stripped off prior to reprobing. The positions of IgG heavy and light chains from both mouse and rabbit are shown by asterisks.

immunoprecipitated with Cln3, rather than Cln3 abundance. These observations are summarized in Table I.

The kinase activity associated with Cln3-1 was also increased ~10-fold by the *cdc34-2* mutation (Figure 4B).

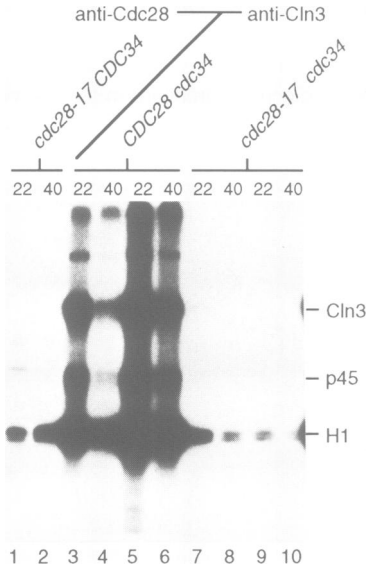

Fig. 7. Cln3-associated kinase is *CDC28*-dependent and inherently temperature sensitive. Immunoprecipitates from the indicated strains were split in two and assayed for kinase activity *in vitro* at either 22°C or 40°C. The first two lanes are control anti-Cdc28 immunoprecipitations from a *GAL1–CLN3M cdc28-17* strain (BF399-1a). The remaining immunoprecipitates were carried out with 12CA5 monoclonal antibody on lysates from strains with the following genotypes: *GAL1–CLN3M cdc34-2* (BF399-2a, lanes 3 and 4); *GAL1–CLN3M cdc34-2* (BF399-3a, lanes 5 and 6); *GAL1–CLN3M cdc34-2 cdc28-17* (BF399-6d, lanes 7 and 8); *GAL1–CLN3M cdc34-2 cdc28-17* (BF399-10b, lanes 9 and 10).

This shows that the effect of *cdc34-2* is independent of the PEST-rich regions. At best, the *cdc34-2* mutation caused only a slight increase in the half-life of Cln3 and its associated kinase activity (Figure 5A and B). Half-life and abundance were the same in *cdc34-2* cells cycling at the permissive temperature as in cells arrested at the restrictive temperature (Figure 5C).

Tetrad dissection showed that the elevated kinase activity and the phosphorylated Cln3 phenotype co-segregated with each other and with the *cdc34-2* mutation. Rescue of the *cdc34-2* defect with a plasmid carrying the wild-type *CDC34* gene caused the Cln3-associated kinase activity and the proportion of phosphorylated Cln3 to revert to wild-type levels (unpublished data). These results demonstrated that the defect in Cdc34 was responsible for the effects on Cln3. The effect of the *cdc34-2* mutation on Cln3 *in vitro* was paralleled by a pronounced biological effect: while *GAL1–CLN3* strains were sensitive to mating pheromone, *GAL1–CLN3 cdc34-2* strains were completely resistant to mating pheromone (see Table I). This suggested that *cdc34-2* increased the Cln3-associated kinase activity *in vivo* as well as *in vitro*. Despite the increased Cln3-associated kinase activity *in vitro*, *cdc34-2* mutant cells were not Wee, but instead were slightly larger than wild-type (see Table I). However, the mutation may cause a slight cell cycle pause at the G_1–S transition, which when coupled with continued growth, could mask any slight Wee phenotype.

The increased Cln3-associated kinase activity in *cdc34-2* strains allowed a conclusive demonstration of its specificity. Immunoprecipitations performed without 12CA5 monoclonal antibody (i.e. with protein A beads alone) or from cells grown without galactose induction had negligible kinase activity compared to galactose-induced *GAL1–CLN3M*

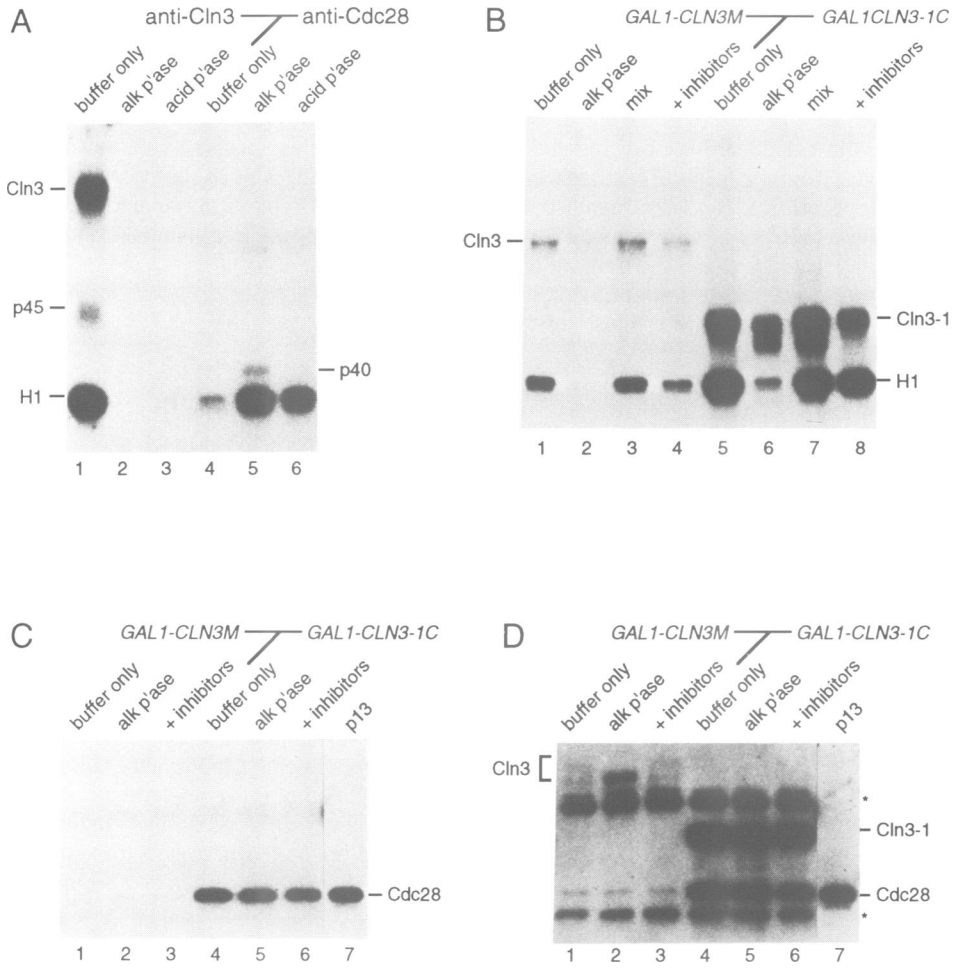

Fig. 8. Phosphorylation of the Cln3–Cdc28 complex is essential for kinase activity. (**A**) Phosphatase treatment inactivates the Cln3–Cdc28 kinase complex but activates mitotic Cdc28 kinase complexes. Lysates from a *GAL1–CLN3M cdc34-2* strain (RN204-3c) induced with galactose for 4 h were immunoprecipitated with either 12CA5 monoclonal antibody (lanes 1–3) or anti-Cdc28 serum (lanes 4–6). Washed immunoprecipitates were incubated with buffer only (lanes 1 and 4), 1 U alkaline phosphatase (lanes 2 and 5) or 0.06 U potato acid phosphatase (lanes 3 and 6), washed twice in kinase buffer to remove the phosphatase from the immunoprecipitates and then assayed for kinase activity. (**B**) Inactivation of Cln3 and Cln3-1 kinase activity is prevented by phosphatase inhibitors and is not due to residual phosphatase activity. Lysates from a *GAL1–CLN3M cdc34-2* strain (RN204-3c, lanes 1–4) and a *GAL1–CLN3-1C cdc34-2* strain (RN201-1c, lanes 5–8) were immunoprecipitated and split into five aliquots at the last wash step. After incubation under the following conditions a quarter of each aliquot was assayed for kinase activity: buffer alone (lanes 1 and 5); 1 U alkaline phosphatase (lanes 2 and 6); alkaline phosphatase-treated aliquot mixed with buffer-treated aliquot just prior to kinase assay (lanes 3 and 7); 1 U alkaline phosphatase in the presence of 50 mM NaF and 25 mM sodium pyrophosphate (lanes 4 and 8). (**C**) Phosphatase treatment does not cause the dissociation of Cln3 and Cdc28. The remaining three-quarters of the indicated immunoprecipitates used in (B) were immunoblotted and probed with anti-Cdc28 serum. The immunoprecipitates used were treated with buffer (lanes 1 and 4), with alkaline phosphatase (lanes 2 and 5) or with alkaline phosphatase in the presence of phosphatase inhibitors (lanes 3 and 6). A control Cdc28 signal was obtained by precipitating one-fifth the amount of RN204-3c lysate used for 12CA5 immunoprecipitations with $p13^{suc1}$ beads (lane 7). (**D**) Phosphatase treatment does not cause degradation of Cln3. The same immunoblot as in (C) was reprobed with 12CA5 monoclonal antibody. The original signal was not stripped prior to reprobing. Note that phosphatase treatment shifts all of the Cln3 immunoreactivity to the fastest migrating form. The positions of IgG heavy and light chains are shown by asterisks.

cdc34-2 cells (Figure 4C). Excess epitope peptide blocked immunoprecipitation of Cln3 and the associated kinase activity (Figure 4C).

Cln3 forms an active kinase complex with Cdc28

Physical interaction between Cln3 and Cdc28 was demonstrated by immunoblotting anti-Cln3 immunoprecipitates with anti-Cdc28 antibody (directed against the N-terminal 11 residues of Cdc28). As an example, anti-Cln3 immunoprecipitates from a *GAL1–CLN3M cdc34-2* strain contained substantial amounts of Cdc28 polypeptide (Figure 6B, lane 3). Control lanes probed with pre-immune serum did not show any immunoreactivity other than IgG from the immunoprecipitations (Figure 6A). Control immunoprecipitations also had no Cdc28 immunoreactivity (Figure 6B, lanes 1 and 2). Thus, Cln3 forms a specific complex with Cdc28.

If the Cdc28 catalytic subunit accounts for the kinase activity associated with Cln3 then Cln3 immunoprecipitates from temperature sensitive *cdc28* strains might have a reduced or temperature sensitive kinase activity *in vitro*. However, we note that the Cdc2 or Cdc28 kinase activity immunoprecipitated from $cdc2^{ts}$ and *cdc28* strains is often not significantly temperature sensitive *in vitro* (Reed *et al.*, 1985; Booher *et al.*, 1989; our unpublished results). The Cln3-associated kinase activity from extracts from *cdc28-17*, *cdc28-4* and *cdc28-13* strains was severely reduced compared to related *CDC28* strains (Figure 7, lanes 7–10; and

unpublished data). The small amount of remaining activity was moderately temperature sensitive *in vitro*, but interpretation of this was complicated by the fact that Cln3-associated kinase activity from *CDC28* strains was also somewhat temperature sensitive *in vitro* (e.g. Figure 7, lanes 3–6). For comparison, the Cdc28 activity precipitated by the anti-Cdc28 antibody from *cdc28-17* extracts was moderately temperature sensitive *in vitro* against co-precipitated substrates, but its activity against histone H1 was not (e.g. Figure 7, lanes 1 and 2). Since the total amount of Cln3-associated activity was *CDC28*-dependent, we conclude that Cdc28 is responsible for most of the Cln3-associated kinase activity.

Phosphorylation of the Cln3–Cdc28 complex is necessary for kinase activity

Dephosphorylation of Cln3 immunoprecipitates with either alkaline or potato acid phosphatase eliminated at least 95% of the associated kinase activity (Figure 8A, lanes 1–3). In contrast, the kinase activity of anti-Cdc28 immunoprecipitates was increased several-fold by phosphatase treatment (Figure 8A, lanes 4–6). Most of the activity in the Cdc28 immunoprecipitates was presumably due to the mitotic Clb–Cdc28 form of the kinase since this is far more abundant than the Cln form (Surana *et al.*, 1991) and since the antibody used does not appear to precipitate Cln–Cdc28 complexes (e.g. Figure 6C, lanes 4–6; and unpublished data). Similar activation by non-specific phosphatases has been obtained for starfish M phase Cdc2 complexes (Pondaven *et al.*, 1990). Any phosphorylated residues on these mitotic Cdc28 complexes that are essential for kinase activity must be inaccessible to non-specific phosphatases.

Inclusion of phosphatase inhibitors in the phosphatase reaction prevented inactivation of the Cln3-associated kinase (Figure 8B, lane 4) and dephosphorylated Cln3 immunoprecipitates did not inhibit the kinase activity of mock-treated immunoprecipitates (Figure 8B, lane 3). Phosphatase treatment did not cause dissociation of Cdc28 from Cln3 and neither Cdc28 nor Cln3 were degraded during the phosphatase reaction (Figure 8C and D). All of the Cln3 signal was converted to the highest mobility form by the phosphatase; this confirmed that the lower mobility species were phosphorylated forms of Cln3 (Figure 8D, lane 2). The gel system used could not distinguish between various phosphorylated forms of Cdc28 and so we could not determine its phosphorylation state in the Cln3 complex.

Similar results were obtained with Cln3-1 immunoprecipitates (Figure 8B, C and D) and also with Cln1 and Cln2 immunoprecipitates (unpublished data). The phosphatase-treated immunoprecipitates always retained some residual kinase activity (e.g. Figure 8B, lanes 2 and 6). This may have been for one or more of the following reasons: incomplete dephosphorylation; partial activity even in the absence of the activating phosphate(s); or the presence of other kinases not susceptible to inactivation by phosphatase.

Since phosphorylation of the Cln3–Cdc28 complex was necessary for activity, we wondered if the activating phosphate(s) could be added autocatalytically. Prolonged incubation of dephosphorylated Cln3 immunoprecipitates in the presence, but not in the absence, of ATP partially restored the kinase activity of the complexes (Figure 9, lanes 1–3). Reactivation was particularly effective with Cln3-1 immunoprecipitates, for which at least 50% of the initial kinase activity could be recovered (Figure 9, lanes 4–6). Interestingly, a 38 kDa substrate (p38) became apparent in kinase reactions of dephosphorylated Cln3-1 immunoprecipitates (Figure 8B, lane 6; Figure 9, lane 6). The identity of this species is unknown. Although reactivation of the Cln3–Cdc28 complex may have occurred via autophosphorylation, we cannot rule out the possibility that some non-specifically precipitated kinase was responsible for reactivation.

Discussion

Cln3 instability

Cln3 is an extremely unstable, low abundance protein. Since the half-life of Cln3 is much shorter than half the cell cycle, Cln3 must be unstable at many or all points in the cycle. This is qualitatively very different from the once per cycle destruction of some mitotic cyclins. Continuous instability in the absence of transcriptional periodicity would make Cln3 abundance responsive to the metabolic state of the cell but would not lead to oscillation through the cell cycle. In fact, neither Cln3 abundance nor its associated kinase activity appear to vary through the cell cycle (Tyers,M., Tokiwa,G. and Futcher.B., in preparation). A reduced rate of synthesis would very rapidly reduce steady state levels of Cln3 protein, perhaps allowing Cln3 to tether division to growth. The idea that Cln3 helps to co-ordinate division with growth is consistent with gene dosage studies which show that Start is accelerated by increased amounts of *CLN3* (Nash *et al.*, 1988). Unstable molecules ('trigger proteins') which link division to growth have long been postulated in mammalian cells (reviewed in Pardee, 1989) and there is some indirect evidence for such molecules acting at Start in *S.cerevisiae* (Moore, 1988; Ko and Moore, 1990).

What is the signal for Cln3 turnover? The C-terminal third of Cln3 is extremely rich in proline (P), serine (S) and

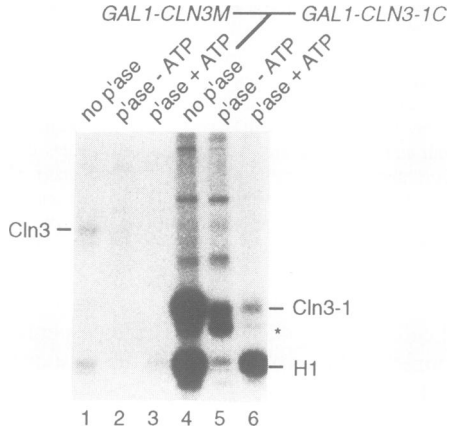

Fig. 9. Reactivation of phosphatase-treated Cln3 immunoprecipitates. Control immunoprecipitates that were not phosphatased (no p'ase) indicate the input amount of kinase activity. Immunoprecipitates treated with 1 U alkaline phosphatase were incubated in the absence (p'ase–ATP) or presence (p'ase + ATP) of 100 μM ATP for 1 h prior to assay for kinase activity. Cln3 immunoprecipitates were from strain RN204-3c (lanes 1–3); Cln3-1 immunoprecipitates were from strain RN201-1c (lanes 4–6). The asterisk indicates a 38 kDa substrate that was prominent in kinase assays of phosphatase-treated Cln3-1 immunoprecipitates.

threonine (T), and it has been proposed that 'PEST' regions rich in these amino acids might be signals for proteolysis (Rogers *et al.*, 1986). We have now shown that the hyperactive, truncated Cln3-1 protein, which lacks these PEST-rich regions, does not turn over rapidly. Furthermore, both Cln1 and Cln2 have similar PEST-rich regions (Hadwiger *et al.*, 1989) and both of these proteins are unstable (for Cln1, our unpublished data; for Cln2, Wittenberg *et al.*, 1990). The *CLN2-1* allele also lacks PEST regions and has genetic phenotypes consistent with the idea that it encodes a stabilized protein (Hadwiger *et al.*, 1989). All of these observations suggest that the signal for continuous, rapid turnover lies in the C-terminal third of the Cln proteins. Whether the PEST regions themselves constitute the signal has not yet been determined.

One consequence of Cln3-1 stability is that the protein accumulates to relatively high levels. Because *CLN3* is a dose-dependent activator of Start, this provides a basis for the small cell size phenotype of *CLN3-1* strains (Sudbery *et al.*, 1980; Nash *et al.*, 1988). *GAL – CLN3* cells and *CLN3-1* cells both have more Cln3 or Cln3-1 protein than wild-type and both have more Cln3 – Cdc28 kinase activity, so we presume that the additional Cdc28 kinase activity is the reason for the Wee phenotype and the acceleration of Start. Notably, the non-degradable Cln3-1 does not cause a G_1 block analogous to the mitotic block caused by indestructible mitotic cyclins (Ghiara *et al.*, 1991; Glotzer *et al.*, 1991); this may be because Start and mitosis are quite different kinds of events. The mechanism whereby Cln3-1 confers α-factor resistance is less clear, but this probably depends at least in part on *CLN1* and *CLN2* and is not merely a consequence of increased *CLN3* dosage (Dirick and Nasmyth, 1991; Nasmyth and Dirick, 1991; Tyers,M., Tokiwa,G. and Futcher,B., in preparation).

Regulation of Cln3 by CDC34

The *CDC34* gene encodes a ubiquitin conjugating enzyme (Goebl *et al.*, 1988; Haas *et al.*, 1991) which may target proteins for destruction *in vivo* (reviewed by Jentsch *et al.*, 1990). We tested the effect of the *cdc34-2* mutation on Cln3 stability with the idea that Cdc34 might be responsible for rapid Cln3 turnover. This did not seem to be the case, because steady state levels and half-life of Cln3 were only slightly increased by the *cdc34-2* mutation, either at the permissive or the restrictive temperature. However, the *in vitro* kinase activity associated with Cln3 and the abundance of phosphorylated Cln3 were both dramatically increased in *cdc34-2* strains. The causal relationship between these two effects is not known: increased kinase activity might cause the hyperphosphorylation of Cln3 or the high levels of phosphorylated Cln3 might elevate the associated kinase activity. The *cdc34-2* mutation also increased the kinase activity associated with the stable Cln3-1 protein. Thus, Cdc34 is not required for the normal rapid turnover of Cln3, and the Cdc34-dependent inhibition of Cln3-associated kinase activity does not require the PEST-rich C-terminal third of the protein. These *in vitro* effects probably reflect an *in vivo* change in the activity of Cln3, because there is a strong genetic interaction between *cdc34-2* and *GAL1 – CLN3* for the phenotype of α-factor resistance (Tyers,M., Tokiwa,G. and Futcher,B., in preparation). The effects of *cdc34-2* seem to be specific for Cln3 since we could not detect any similar effects on Cln1 and Cln2 (unpublished data). We note that *cdc34-2* is a temperature sensitive lethal mutation even in a *cln3* deletion background and over-expression of *CLN3* does not exacerbate the *cdc34-2* phenotype (unpublished data). Thus, while Cln3 may be a target of Cdc34, it is not the essential target.

The mechanism by which *cdc34-2* affects Cln3 is unclear.

Table II Strains used in this study

Strain	Relevant genotype
BF305-15d	*MATa his3 leu2 trp1 ura3*
BF305-15d#21	*MATa his3 leu2 trp1 ura3 cln1::HIS3 cln2::TRP1 cln3::URA3 – GAL1 – CLN3*
BF#21-*CLN3M*	*MATa his3 leu2 trp1 ura3 cln1::HIS3 cln2::TRP1 cln3::URA3 – CLN3M*
BF#21-*CLN3C*	*MATa his3 leu2 trp1 ura3 cln1::HIS3 cln2::TRP1 cln3::URA3 – CLN3C*
BF#21-*CLN3*	*MATa his3 leu2 trp1 ura3 cln1::HIS3 cln2::TRP1 cln3::URA3 – CLN3*
BF#21-*CLN3-1C*	*MATa his3 leu2 trp1 ura3 cln1::HIS3 cln2::TRP1 cln3::URA3 – CLN3-1C*
BF#21-*CLN3*-1	*MATa his3 leu2 trp1 ura3 cln1::HIS3 cln2::TRP1 cln3::URA3 – CLN3-1*
BF338-3b#23	*MATa ura3 cln3::URA3 – GAL1 – CLN3-1C*
BF338-3b#23p	*MATa ura3 CLN3-1C*
BF338-4b#43	*MATa ura3 cln3::URA3 – GAL1 – CLN3M*
BF338-4b#43p	*MATa ura3 CLN3M*
BF399-1a	*MATa ura3 cln3::URA3 – GAL1 – CLN3M cdc28-17*
BF399-2a	*MATa ura3 cln3::URA3 – GAL1 – CLN3M cdc34-2*
BF399-3a	*MATa ura3 cln3::URA3 – GAL1 – CLN3M cdc34-2*
BF399-6d	*MATa ura3 cln3::URA3 – GAL1 – CLN3M cdc28-17 cdc34-2*
BF399-10b	*MATa ura3 cln3::URA3 – GAL1 – CLN3M cdc28-17 cdc34-2*
BF410-2d	*MATa ura3 cln3::URA3 – GAL1 – CLN3C*
BF410-7c	*MATa ura3 cln3::URA3 – GAL1 – CLN3C cdc34-2*
RN201-1c	*MATa ura3 cln3::URA3 – GAL1 – CLN3-1C cdc34-2*
RN202-2b	*MATa ura3*
RN204-3c	*MATα ura3 cln3::URA3 – GAL1 – CLN3M cdc34-2*
MT 212	*MATa his3 leu2 ura3 cln3::URA3 – CLN3C cdc34-2*
MT 217	*MATa his3 leu2 ura3 cln3::URA3 – CLN3-1C cdc34-2*
MT 222	*MATa his3 leu2 ura3 cln3::URA3 – CLN3C*

Since the *cdc34* effects are independent of cell cycle position (Tyers,M., Tokiwa,G. and Futcher,B., in preparation), the *cdc34-2* mutation does not act on Cln3 by merely delaying the cells at some stage where Cln3 is phosphorylated and most active. Cdc34 may influence Cln3 indirectly, for instance, by turning over a kinase that phosphorylates and activates the Cln3−Cdc28 complex. Alternatively, Cdc34 might act directly to cause degradation of the highly phosphorylated forms of Cln3 that correlate with elevated Cln3−Cdc28 kinase activity, effectively targeting the active Cln3−Cdc28 kinase complex for turnover. Although the precise mechanism remains to be defined, it is clear that the *cdc34-2* mutation uncovers a second post-translational control on Cln3 activity.

Control of Cln activity by phosphorylation

Dephosphorylation of the Cln3−Cdc28 complex dramatically reduced its kinase activity, implying that the complex carries one or more phosphates important for its kinase activity *in vitro*. The same is true of Cln1− and Cln2−Cdc28 complexes (unpublished data). It is a completely open question whether the critical phosphate(s) in the Cln complex is on Cdc28, the Cln or some other component. Reactivation of Cln−Cdc28 kinase activity by incubation with ATP *in vitro* is consistent with autocatalytic addition of the activating phosphate. Since Cln3, p38 and p45 are substrates for the kinase *in vitro*, they are candidates for the proteins carrying the critical phosphate(s). If the critical activating phosphate is on Cln3 and if Cdc34 targets phosphorylated Cln3 for turnover, then the increase in Cln3-associated kinase activity in *cdc34-2* cells as well as the inactivation−reactivation effects may be explained by the same mechanism. Recent evidence suggests that for *Xenopus* and *S.pombe* Cdc2, phosphorylation of threonine 161 (167 for *S.pombe*) is essential for activity (Solomon *et al.*, 1990; Gould *et al.*, 1991; Solomon *et al.*, 1992) and that this phosphate can be removed by type 2A protein phosphatases (Gould *et al.*, 1991; Lee *et al.*, 1991). It is possible that the critical phosphate in Cln−Cdc28 complexes is on the corresponding threonine (169) of Cdc28, although the fact that we have not observed phosphorylation of Cdc28 during reactivation *in vitro* tends to argue against this. Also, addition of the activating phosphate on mitotic Cdc2 in *Xenopus* does not appear to occur in an autocatalytic manner (Solomon *et al.*, 1992).

Regardless of where the phosphate is, the fact that the active complex has an essential, accessible phosphate may be very important for regulation of Cln kinase activity. In particular, the Cln1,2−Cdc28 complexes could be inactivated after Start by a phosphatase. This would serve to interrupt the positive feedback loop of *CLN1* and *CLN2* transcription (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Nasmyth and Dirick, 1991) and lead to the observed drop in *CLN1* and *CLN2* mRNA levels after Start (Wittenberg *et al.*, 1990). A number of phosphatase genes have been characterized in *S.cerevisiae* and one of these, *SIT4*, is required for the completion of Start (Sutton *et al.*, 1991).

It seems very likely that the three Cln proteins play a role at Start which is broadly analogous to the role of the mitotic cyclins at the G_2−M transition. Cln3 has an associated kinase activity which is largely due to the Cdc28 catalytic subunit. This does not rule out the possibility that other catalytic subunits such as Kss1, Fus3 and Sgv1 (Courchesne *et al.*, 1989; Elion *et al.*, 1990; Irie *et al.*, 1991) may also associate with Cln3. *CLN3* is not highly regulated at the transcriptional level, but the Cln3 protein and its associated kinase activity are regulated by several post-translational mechanisms: PEST (or at least C-terminal) -dependent degradation, phosphorylation of some element of the kinase complex and inhibition by a Cdc34-dependent mechanism, possibly specific proteolysis of phosphorylated Cln3. Post-transcriptional regulation may be the most appropriate level of regulation for a protein whose function is to directly monitor cellular metabolism, as we suspect Cln3 does.

Materials and methods

Strains and cell culture

The genotypes of the strains used in this study are shown in Table II. All yeast media and transformation procedures used are detailed in Guthrie and Fink (1991) and in Rose *et al.* (1990). Complementation of the triple *cln* disruption strain [described in detail in Xiong *et al.*, (1991)] was carried out by rescue with a *CLN2* high copy plasmid, followed by transformation to Ura^+ with various *CLN3* alleles and loss of the *CLN2* plasmid. Transformations were confirmed by Southern analysis and PCR of the epitope region.

Induction of the *GAL1* promoter was achieved by adding galactose to a final concentration of 2% to a log phase culture of cells grown in YEP-raffinose for at least 24 h;. the promoter was repressed by addition of glucose to a final concentration of 2%. All cultures were subjected to size analysis with a Coulter channelizer and counted with a model ZM Coulter Counter.

Sensitivity of various strains to mating pheromone was quantified by plating a small number of cells (~1000) on a grid of either YEPD or YEP-galactose containing increasing amounts of α-factor (Nash *et al.*, 1988). The ability to form colonies was determined after growth at room temperature for 2−3 days.

Epitope addition

The *CLN3* gene was tagged by insertion of a triple tandem HA1 epitope (Field *et al.*, 1988). All molecular biology procedures for mutagenesis and plasmid construction were carried out essentially as described in standard manuals (e.g. Sambrook *et al.*, 1989). A single epitope sequence was inserted by oligonucleotide directed mutagenesis at either the stop codon of *CLN3* (mutagenic oligonucleotide sequence: 5′-CTCAAGAAAACTCGCTACC-CATACGATGTTCCAGATTACGCTTGAAACGACAAAA-3′) or at the *CLN3-1* mutation site (mutagenic oligonucleotide sequence: 5′-TACAAT-ACTATCTTTTACCCATACGATGTTCCAGATTACGCTT/CAGTCCTTTG-3′). In the latter case degeneracy in the oligonucleotide was used to create either a stop codon after the epitope sequence (*CLN3-1C*) or a read through into the remainder of the *CLN3* coding region (*CLN3M*). The single epitope tag did not provide enough sensitivity to detect Cln3; therefore a second round of mutagenesis was used to insert two more in-frame copies of the epitope into the first copy (mutagenic oligonucleotide sequence: 5′-TACCCATACGATGTACCTGACTATGCGGGCTATCCC-TATGACGTCCCGGACTATGCAGGATCCTATCCGTATGACGTTCC-AGATTACGC-3′). A *Bam*HI site placed in the oligonucleotide for diagnostic purposes is underlined. All mutations were confirmed by dideoxy sequencing (Sanger *et al.*, 1977). After mutagenesis, the *Eco*RI−*Hpa*I fragment encompassing the mutated region was subcloned into plasmid pBF30 which carries the *CLN3* chromosomal region and is marked with *URA3* (Nash *et al.*, 1988). Expression of various *CLN3* alleles from the *GAL1* promoter was achieved by either replacement of the chromosomal *CLN3* upstream region (−402 to −108) with a *GAL1* promoter fragment (Xiong *et al.*, 1991) or by replacement of the resident *CLN3* gene with the same promoter construct fused to various tagged alleles of *CLN3*.

Northern analysis

Total RNA (15 μg) was quantified by fluorometry (Nash *et al.*, 1988), electrophoresed on a 1% agarose−6% formaldehyde gel (Rose *et al.*, 1990) and transferred to Hybond-N membrane (Amersham). A *CLN3* fragment (nucleotides −108 to 1171) and an *ADH1* fragment were radioactively labeled and hybridized to the membrane as described by Allshire (1990). Relative amounts of mRNA species were quantified by β-imaging.

Immunoprecipitation and immunoblot analysis

The 12CA5 mouse monoclonal antibody to the influenza hemagglutinin peptide HA1 was prepared as ascites fluid as described (Field *et al.*, 1988). The synthetic peptide corresponding to the epitope recognized by the 12CA5

antibody (YPYDVPDYA) was synthesized by Dan Marshak (Cold Spring Harbor, NY). Antiserum directed against the N-terminal 11 residues of Cdc28 was prepared as described (Mendenhall *et al.*, 1987). This anti-Cdc28 serum (# 182) immunoprecipitates only a minor fraction of the total amount of Cdc28 (e.g. < 10% of the amount that is bound by $p13^{suc1}$ beads). The $p13^{suc1}$ bead reagent was prepared as described (Brizuela *et al.*, 1987).

Adequate detection of Cln3 required 100 ml of yeast culture at a density of 2×10^7 cells per ml. Cells were pelleted, washed once with ice cold water and resuspended in 1–2 vol of Buffer 3 (Draetta *et al.*, 1989). The following inhibitors were included during cell lysis: 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.6 mM dimethylaminopurine, 10 μg/ml soybean trypsin inhibitor and 1 μg/ml TPCK. Cells were broken by vortexing with glass beads (5×30 s bursts) after which the lysate was cleared by two spins for 10 min at 13 000 *g*. Typically this yielded 10 mg of protein in 0.5 ml lysate; this was used for immunoprecipitations. In any given experiment all samples were normalized to contain the same amount of total protein (Bradford, 1976). Cln3 was stable in these lysates even if incubated for 30°C for 30 min. Lysate was incubated with 0.25 μl 12CA5 ascites fluid (or 2.5 μl # 182 anti-Cdc28 serum from rabbit) for 1–3 h on ice and then rocked in the presence of protein A beads for 1–3 h at 4°C. Beads were collected by gentle centrifugation (< 1000 *g*) for 5 s and washed four times with Buffer 3. After resuspension in 1×kinase buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) samples were split 1:3; one quarter was transferred to a fresh tube for kinase assays. The remainder was aspirated dry and then boiled briefly in 2×loading buffer prior to SDS–PAGE on a 10% gel.

Protein gels were transferred to nitrocellulose with a semi-dry transfer apparatus (Millipore) and probed with a 1:2000 dilution of 12CA5 ascites fluid and 1:1000 dilution of [^{125}I]-goat anti-mouse IgG. Immunoblots probed for Cdc28 were incubated with 1:1000 dilution of serum # 182 followed by a 1:1000 dilution of [^{125}I]-goat anti-rabbit IgG. Quantitative immunoreactivity of particular bands was estimated with a Phosphor-Imager (Molecular Dynamics).

Protein kinase assays

Fractions of immunoprecipitates for kinase assays were washed once more with kinase reaction buffer, aspirated and pre-incubated at 37°C for 10 min. Kinase assays were initiated by adding 5 μl of kinase buffer containing 10 μCi [γ-^{32}P]ATP (final ATP concentration = 1 μM) and 1 μg bovine histone H1. Reactions were terminated after 15 min with 10 μl of 2× sample buffer and heated to 95°C for 2 min before electrophoresis. Incorporation of ^{32}P into substrates was quantified by counting Cerenkov emissions or by β-imaging using a Phosphor-Imager.

Phosphatase treatment of immunoprecipitations was carried out with 1 U of calf intestinal alkaline phosphatase (Boehringer Mannheim) in 20 μl of 50 mM Tris–HCl pH 8.0 at 37°C for 15 min. Alternatively, beads were treated with 0.06 U of potato acid phosphatase (Boehringer Mannheim) in 20 μl of reaction buffer (50 mM PIPES pH 6.0, 0.1% β-mercaptoethanol, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor and 1 mM PMSF) at 37°C for 10 min (Pondaven *et al.*, 1990). When used, phosphatase inhibitors (50 mM NaF and 25 mM sodium pyrophosphate or 1 mM Na_3VO_4) were added prior to addition of the enzyme. Reactions were stopped on ice and washed twice with 1 ml of kinase reaction buffer before proceeding with kinase assays.

Reactivation of phosphatase-inactivated Cln3 immunoprecipitates was carried out by incubating the washed, phosphatase treated beads in 20 μl kinase reaction buffer containing 100 μM ATP for 1 h at 22°C with rotation. Beads were then washed once with reaction buffer and then assayed for histone H1 kinase activity.

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