G₁ cyclin turnover and nutrient uptake are controlled by a common pathway in yeast

Yves Barral,^{1,2} Stefan Jentsch,^{2,3} and Carl Mann^{1,4}

¹Service de Biochimie et Génétique Moléculaire, Département de Biologie Cellulaire et Moléculaire, Commissariat à l'Energie Atomique, Centre d'Etudes de Saclay, F-91191 Gif-sur-Yvette, France; ²Friedrich Miescher Laboratorium der Max Planck Gesellschaft, D-72026 Tübingen, Germany

Entry into a new cell cycle is triggered by environmental signals at a point called Start in G₁ phase. A key regulator of this transition step in yeast is the CDC28 kinase together with its short-lived regulatory subunits called G₁-cyclins or CLN proteins. To identify genes involved in G₁-cyclin degradation, we employed a genetic screen by selecting for stable CLN1- β -galactosidase fusion proteins. Surprisingly, one group of mutants was found to be allelic to *GRR1*, a gene previously described to be involved in glucose uptake, glucose repression, and divalent cation transport. In *grr1* mutants, both CLN1 and CLN2 cyclins are significantly stabilized. A suppressor analysis indicated that G₁-cyclin stabilization in *grr1* was not a consequence of the nutrient uptake defect. This suggests that the *GRR1* gene product is part of a common regulatory pathway linking two functions important for cell growth, nutrient uptake, and G₁ cyclin-controlled cell division.

[Key Words: G₁ cyclins; proteolysis; nutrient uptake]

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Cell cycle progression in eukaryotes is controlled predominantly at the G₁/S and G₂/M transition steps. In the G₁ phase of yeast, at a point called Start, the cell integrates environmental signals (the presence or absence of sex pheromones and needed nutrients) in deciding whether to begin a new mitotic cycle or whether to switch to a distinct differentiation pathway, such as mating (for haploid cells), meiosis and sporulation (for diploid cells), or entry into a quiescent, stationary phase state (Pringle and Hartwell 1981). Transition at Start occurs upon activation of the CDC28 kinase through its association with the G₁ cyclins, including CLN1, CLN2, and CLN3 (Reed 1992). The three cyclins are not fully equivalent. CLN3 is present at a low level throughout the cell cycle, whereas CLN1 and CLN2 accumulate abruptly near Start in G₁ phase (Wittenberg et al. 1990; Tyers et al. 1993). CLN3 may act upstream of the other G₁ cyclins by contributing to the transcriptional activation of their genes in a positive feedback loop (Wittenberg et al. 1990; Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991; Nasmyth and Dirick 1991; Ogas et al. 1991; Johnston 1992; Tyers et al. 1993; Cross et al. 1994; Stuart and Wittenberg 1994). This transcriptional

activation is responsible for the sharp increase of the G₁-cyclin concentration in cells at Start.

The G₁ cyclins are unstable proteins with half-lives on the order of 10 minutes (Wittenberg et al. 1990; Tyers et al. 1992, 1993). The three CLN proteins contain PEST sequences in their carboxy-terminal domains (Nash et al. 1988; Hadwiger et al. 1989). These sequences, rich in proline, aspartate, glutamate, serine, and threonine residues and often flanked by basic residues, have been found in several unstable proteins (Rogers et al. 1986). A truncated version of CLN3 lacking PEST sequences is stable (Tyers et al. 1992; Cross and Blake 1993). Little is known, however, about the mechanisms and potential regulation of G₁-cyclin turnover.

The degradation of the mitotic A and B cyclins is better understood. This degradation is ubiquitin-dependent, and a short amino-terminal sequence, the destruction box, is involved in directing the turnover of these cyclins (Glotzer et al. 1991). Overexpression of destruction box mutants of mitotic B-type cyclins in yeast arrests cell division with cells unable to exit from mitosis (Surana et al. 1993). In contrast, no evidence has yet been found for a stringent necessity to degrade the G₁ cyclins. Cells expressing a truncated, stabilized CLN3 enter prematurely into new cell cycles with a consequently reduced G₁ phase and cell size (Sudbery et al. 1980; Cross 1988; Nash et al. 1988; Tyers et al. 1992; Cross and Blake 1993). Similar phenotypes have been reported for cells

³Present address: ZMBH, Universität Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany.

⁴Corresponding author.

expressing a truncated *CLN2* gene that is presumed to encode a stabilized cyclin (Hadwiger et al. 1989). Thus, one role of G_1 -cyclin turnover is to couple the timing of the initiation of new cell cycles to cell growth.

To better understand the role of CLN protein degradation, we developed a screen for mutants in which CLN1 is stabilized using an inducible CLN1- β -galactosidase (β -gal) fusion protein. CLN1's degradation signal made the entire fusion protein unstable. This feature allowed us to monitor CLN1 stability through the levels of β -gal activity within cells expressing the fusion protein. One complementation group of *trans*-acting mutants that stabilizes CLN1 is described here. Surprisingly, the corresponding wild-type gene turned out to be the previously identified gene *GRR1* (*CAT80/COT2*) implicated in the regulation of glucose uptake, catabolite repression, and divalent cation transport (Bailey and Woodward 1984; Flick and Johnston 1991; Conklin et al. 1993; Özcan et al. 1994). A suppressor of *grr1* has been characterized previously. This suppressor, *rgt1*, reverses the transport and catabolite repression defects of the *grr1* mutant (Erickson and Johnston 1994; Vallier et al. 1994). We show that this suppressor does not reverse the cyclin turnover defect of *grr1*, indicating that the stabilization of the G_1 cyclin is not the consequence of the known transport defects. This result suggests that *GRR1* is part of a common pathway that regulates both nutrient uptake and G_1 -cyclin proteolysis.

Results

Construction of unstable CLN1-fusion proteins

To study the degradation of G_1 cyclins, we made *CLN1-lacZ* fusion genes and placed them under the control of the *GAL10* promoter. Fusions were made with either the entire CLN1 protein (CLN1- β -gal) or a form deleted for the PEST region (CLN1 Δ PEST- β -gal) (Fig. 1A). The PEST

sequences are thought to be determinants of CLN protein turnover (Tyers et al. 1992). These constructs were first used to determine whether the degradation signals of CLN1 would be recognized in the context of a CLN1- β -gal fusion protein.

The stability of the fusion proteins was estimated by the levels of β -gal activity within colonies as revealed by an agar overlay assay using the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). Expression of the CLN1- β -gal fusion protein in wild-type cells gave a low β -gal activity as indicated by a weakly colored colony (Fig. 1B). In contrast, the colonies expressing CLN1 Δ PEST- β -gal gave a strong coloration. On Western blots, the intact CLN1- β -gal fusion protein was not detectable under conditions in which CLN1 Δ PEST- β -gal was observed easily (data not shown).

A pulse-chase analysis showed that the half-life of CLN1- β -gal in yeast cells was ~ 10 min, whereas the Δ PEST version was largely stable over the 30 min of the experiment (Fig. 2). The instability of the CLN1- β -gal fusion protein thus depends on the presence of the carboxy-terminal PEST sequences. Similarly, CLN1 Δ PEST, a truncated CLN1 not fused to β -gal, was also stabilized relative to the intact CLN1 (data not shown). We conclude that the carboxy-terminal PEST sequences of CLN1 are necessary for its rapid turnover and that the degradation of the CLN1- β -gal fusion protein is likely to be mediated by the same degradation mechanism as for CLN1 itself.

We then examined whether the fusion proteins are functional as G_1 cyclins *in vivo*. We used a strain mutated in all three *CLN* genes and kept alive by a *CLN3* gene carried on a plasmid with *URA3* as a marker. 5-Fluoro-orotic acid (FOA) is toxic to *Ura*⁺ yeast cells (Boeke et al. 1984); hence, this strain cannot grow on FOA-containing media because the plasmid is required for its vi-

Figure 1. [A] Plasmids expressing CLN1- β -gal and CLN1 Δ PEST- β -gal. pDR1 contains the entire *CLN1* gene fused to the *lacZ*-coding sequence of the Fusionator (pSJ101) plasmid (Johnson 1991), whereas pDR5 contains a carboxy-terminal deletion of *CLN1* in phase with *lacZ*. [B] β -Gal colony-color assay of wild-type or *toc* mutant yeast cells expressing *CLN1-lacZ* or *CLN1 Δ PEST-lacZ*. Cells (10^5) of the FM135 *reg1-501* background were spotted on an SDGal-LEU plate and incubated overnight. Cells were then stained for β -gal activity with an X-gal-agar overlay as described in Materials and methods.

Figure 2. Comparison of the stability of CLN1-β-gal and CLN1ΔPEST-β-gal by pulse-chase analysis. Wild-type (YW02) cells expressing *CLN1-lacZ* or *CLN1ΔPEST-lacZ* from the *GAL10* promoter were labeled for 5 min with [³⁵S]methionine/cysteine followed by a chase with an excess of cold methionine and cysteine. Samples were taken at 0, 10, and 30 min of chase and analyzed by immunoprecipitation with a monoclonal anti-β-gal antibody (Promega Biotech). The arrows indicate the position of the unstable CLN1-β-gal fusion protein and the stable CLN1ΔPEST-β-gal fusion protein. These proteins were identified by the fact that they were only found in cells transformed with pDR1 or pDR5, they were of the predicted size, and they were also precipitated with anti-CLN1 antibodies. A quantification of the fusion protein bands is shown next to the fluorographs.

ability. The expression of *CLN1-lacZ*, but not *CLN1ΔPEST-lacZ*, in this strain allowed these cells to lose the *CLN3*-bearing plasmid and grow on an FOA-containing medium. Thus, by this complementation test, the CLN1-β-gal fusion protein can provide CLN function to yeast cells but the CLN1ΔPEST protein is nonfunctional.

We also observed that the overexpression in wild-type cells of the CLN1-β-gal fusion protein, but not CLN1ΔPEST-β-gal, induced an aberrant morphology in 10–30% of cells in the population (Fig. 3, cf. A and B). These cells had elongated buds and were sometimes multibudded. The multibudded cells generally contained

a nucleus within each bud, suggesting that DNA replication and nuclear division occurred at each budding event. Overexpression of wild-type *CLN1* gave rise to similar morphological defects at the same frequency as for overexpression of *CLN1-lacZ*, indicating that this phenotype is the result of high CLN1 activity rather than an aberrant function of the fusion protein. The *CLN1-lacZ* gene is expressed from a 2μ multicopy plasmid (Fig. 1A) whose copy number, and thus the expected level of expression from the *CLN1-lacZ* gene, is variable within the cell population (Rose and Broach 1990). Thus, the multibudded phenotype may be restricted to those cells with the highest copy number of the *CLN1-lacZ*-bearing

Figure 3. Morphology of haploid wild-type and *grr1* cells. (A) Wild-type (CMY826) containing the Fusionator vector. (B) Wild-type (CMY826)/pDR1 cells overexpressing *CLN1-lacZ* from the *GAL10* promoter of the Fusionator vector. (C) *grr1* (Ely186) containing the Fusionator vector. (D) *grr1* (Ely186)/pDR6 cells overexpressing *CLN1* from the *GAL10* promoter of the Fusionator vector. Cells were grown in SGal without leucine. Nuclear DNA was stained with DAPI, and the cells were photographed with a combination of epifluorescence and phase-contrast microscopy. All cells are shown at the same magnification; bar in D, 10 μm.

plasmid. A phenotype of elongated buds was also seen for cells expressing *CLN2* from the *GAL1* promoter (Lew and Reed 1993).

Isolation of mutants affecting *CLN1* degradation

The *CLN1-lacZ* construct was used for the isolation of mutants in which the fusion protein was stabilized. Because the overexpression of a stabilized CLN1 protein might be lethal, we expressed the *CLN1-lacZ* fusion gene from the galactose-inducible *GAL10* promoter in a *reg1* strain that allowed induction from the *GAL10* promoter in the presence of glucose (Hovland et al. 1989). This strain was mutagenized with ethylmethane sulfonate (EMS), and cells were plated on a selective medium for the reporter plasmid. The colonies were then replica-plated to an induction medium for the *GAL10* promoter (2% glucose + 2% galactose), and the β -gal activity of the fusion protein was visualized with the X-gal colony assay.

Of 50,000 colonies screened, 25 exhibited an increased blue coloration indicative of a potentially stabilized fusion protein (e.g., see Fig. 1B). The expression of CLN1- β -gal after galactose induction led to much greater morphological defects in the mutants than in the wild-type strain for all but one of the mutants. Some of the mutants exhibited morphological defects (elongated cells, multiple buds) even after the loss of the *CLN1-lacZ* plasmid. This phenotype was taken as a sign that the endogenous CLN proteins might be stabilized in these mutants.

Of 25 mutants, 12 were found to belong to the same complementation group. This complementation group was called *toc1* (turn over of cyclin), and further studies were concentrated on these mutants. The *toc1* mutants exhibited a strong coloration (Fig. 1B), and *toc1* cells were elongated even in the absence of the *CLN1-lacZ* construct (Fig. 3C). Overexpression in *toc1* cells of CLN1 from the *GAL10* promoter greatly enhanced the morphological defects of these cells. Most of these cells had multiple, highly elongated buds (Fig. 3D), and some showed nuclear segregation defects.

The *TOC1* gene is identical to *GRR1*

We noticed that the *toc1* phenotype was more pronounced in a *trp1* background. Such cells grew exponentially at 37°C, but most of them were unable to exit stationary phase at 37°C. Few *toc1 trp1* cells were able to form a colony when stationary phase cells were plated on fresh medium at 37°C, but when transferred after a few days from 37 to 23°C, these cells resumed growth. At 23°C, stationary-phase *toc1 trp1* cells were fully capable of resuming growth in fresh medium.

These observations were exploited to isolate a plasmid from a genomic library that complements the *toc1* mutation. The *toc1 trp1* cells were transformed with the library and then plated on selective medium at 37°C. The plates were incubated for several days after appearance of the colonies to ensure that the cells were in stationary

phase before replica-plating to fresh medium at 37°C. One of five transformed colonies that grew on fresh medium at 37°C had lost the *toc1* morphological phenotype in a plasmid-dependent fashion. This plasmid was recovered in *Escherichia coli* and shown to be the *TOC1* gene as detailed in Materials and methods. The DNA sequence of *TOC1* was compared with data bank sequences and was found to be identical to the gene *GRR1* sequenced previously (Flick and Johnston 1991). We verified that *toc1* and *grr1* mutants do not complement each other and that *toc1* is meiotically linked to *grr1*. Furthermore, *grr1* mutants have a morphological phenotype similar to *toc1* mutants, and *grr1 trp1* cells grow slower than *grr1 TRP1* cells (Bailey and Woodward 1984; Flick and Johnston 1991; Conklin et al. 1993). We conclude that *toc1* and *grr1* are allelic, and we will thus henceforth refer to our mutants as *grr1*. *GRR1* is implicated in the regulation of glucose uptake, glucose repression, and divalent cation transport (Bailey and Woodward 1984; Flick and Johnston 1991; Conklin et al. 1993; Özcan et al. 1994).

*CLN1- β -gal fusion protein is stabilized in *grr1* mutants*

The *grr1* (glucose repression resistance) mutant was first isolated as a mutation abolishing glucose repression (Bailey and Woodward 1984; Flick and Johnston 1991; Gancedo 1992). Glucose repression is a regulatory mechanism of yeast that reduces the expression of many genes that are dispensable when cells are growing with glucose as the carbon source (Gancedo 1992). As our mutant was isolated using a reporter gene under *GAL* promoter control, we tested whether the increased β -gal activity in our *grr1* (*toc1-1*) mutant was attributable to a transcriptional activation of the *GAL* promoter rather than a stabilization of the CLN1- β -gal fusion protein. To this end, the *lacZ* gene under the control of the *GAL10* promoter was introduced into *reg1* and *reg1 toc1* strains. After induction of the *GAL10* promoter, the β -gal activity was four times higher in the *reg1 toc1* strain compared with the *reg1* strain. In contrast, the expression of *CLN1-lacZ* gave 40–50 times more β -gal activity in the *reg1 toc1* double mutant compared to *reg1* alone. Thus, the observed increase of the β -gal activity of *grr1* mutants expressing the *GAL*-controlled *CLN1-lacZ* construct was largely caused by a stabilization of CLN proteins and not merely a consequence of a transcriptional activation of the reporter gene. Pulse-chase experiments (not shown) confirmed that the CLN1- β -gal fusion protein is indeed stabilized in *grr1* mutants.

*CLN1 and CLN2, but not CLN3, are stabilized in the *grr1* mutant*

We determined directly the half-life of the endogenous G_1 cyclins as expressed from their normal chromosomal promoters by pulse-chase analysis and immunoprecipitation in *grr1* and wild-type cells. A polyclonal antibody that recognized both CLN1 and CLN2 was used for half-life determinations of the combined pool of these two

related cyclins (Fig. 4A). In addition, the half-lives of CLN2 and CLN3 were individually monitored using a monoclonal antibody that recognized epitope-tagged versions of these two proteins (Tyers et al. 1992, 1993). Consistent with previous estimates (Wittenberg et al. 1990; Tyers et al. 1992, 1993), CLN1, CLN2, and CLN3 all had half-lives of 5–10 min in wild type (Fig. 4, WT). In *grr1* mutants, however, the CLN1 and CLN2 cyclins were significantly stabilized (Fig. 4). Whereas the combined pool of CLN1 and CLN2 had a half-life of >30 min (Fig. 4A), CLN2 analyzed with the monoclonal antibody exhibited a half-life of ~20 min (Fig. 4B). In contrast to these two G₁ cyclins, we found no significant stabilization of CLN3 in *grr1* mutants (Fig. 4C). We conclude that CLN1 and CLN2 turnover involves the GRR1 protein but CLN3 degradation may be controlled by a distinct pathway.

The pulse-chase data indicated that the stabilization of CLN1 and CLN2 in *grr1* cells resulted in an accumulation of higher molecular-weight forms of these two

proteins. As G₁ cyclins are known to be phosphorylated in vivo (Wittenberg et al. 1990; Tyers et al. 1992), we tested whether CLN1 and CLN2 are hyperphosphorylated in *grr1* cells. These higher molecular-weight forms of CLN1 and CLN2 disappeared when the immunoprecipitated material was subjected to alkaline phosphatase treatment before electrophoresis (data not shown). Importantly, we observed that CLN1 and CLN2 phosphorylation is greatly reduced in a *cdc28-4 grr1* double mutant (Fig. 5A). Thus, CLN1 and CLN2 hyperphosphorylation in the *grr1* mutant must be induced, directly or indirectly, by the CDC28 kinase with which the cyclins are associated.

As judged by the amount of immunoprecipitated material (Fig. 4A), CLN1 and CLN2 were synthesized at significantly higher levels in our *grr1* strain compared with wild-type. *CLN1* and *CLN2* transcription are known to be activated by the CDC28 kinase associated with any one of the three cyclins, whereas *CLN3* transcription is insensitive to this positive feedback loop

Figure 4. Stability of CLN1, CLN2, and CLN3 in wild-type and *grr1* cells. (A) Congenic WT (CMY374) and *grr1::URA3* (Ely186) cells growing in SD medium were labeled for 5 min with [³⁵S]methionine/cysteine and then chased for 10 and 30 min in the presence of an excess of unlabeled methionine and cysteine. CLN1 and CLN2 were immunoprecipitated with a polyclonal anti-CLN1 antibody that cross-reacts with CLN2. The immunoprecipitates were then analyzed by SDS-PAGE and fluorography. CLN1 and CLN2 were identified by their expected size, and the competition of their immunoprecipitation by the presence of an excess of unlabeled CLN1 purified from *E. coli* (data not shown). HA1-tagged forms of CLN2 (B) and CLN3 (C) were immunoprecipitated from WT (Ely245, Ely246) and *grr1::URA3* cells (Ely247, Ely248) labeled for 5 min with [³⁵S]methionine/cysteine and then chased for 10 and 30 min as detailed in Materials and Methods. The protein indicated with an asterisk (*) is a stable yeast protein that is recognized by the 12CA5 mouse monoclonal anti-HA1 antibody. A quantification of the levels of the CLN protein bands is shown at right of the fluorographs. Note that the films were exposed for the same time period in A but not B and C. Thus, the quantity of CLN synthesized in wild-type vs. *grr1* cells is directly comparable only in A.

Figure 5. (A) Absence of modified CLN1 and CLN2 in a *grr1 cdc28-4* double mutant. CLN1 and CLN2 were immunoprecipitated from a *grr1* mutant (YM3378) and a *grr1 cdc28-4* double mutant (Ely254). Log-phase cells in SD medium at 24°C were shifted to 33°C for 2 hr and then labeled for 10 min with [³⁵S]methionine/cysteine. The *cdc28-4* mutation inactivates the CDC28 kinase activity at 33°C. The arrow indicates the position of unmodified CLN1. (B) The transcription of *CLN1* and *CLN2* is enhanced in the *grr1* mutant. Total RNA was extracted from congenic CMY826 *GRR1*⁺ and YM2957 *grr1* cells as described in Materials and methods. The RNAs were electrophoresed on agarose gels, transferred to membranes, and probed separately with radiolabeled fragments of the *CLN1*, *CLN2*, *CLN3*, and *ACT1* coding sequences. Bands were detected and quantified with a PhosphorImager, and the *CLN1*, *CLN2*, and *CLN3* signals were normalized to the levels of the *ACT1* mRNA.

(Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). The steady-state levels of *CLN1* and *CLN2* mRNAs is ~20- and 10-fold higher, respectively, in the *grr1* mutant compared with wild-type cells, whereas *CLN3* mRNA levels are equivalent in the two cell types (Fig. 5). The higher synthesis rates of *CLN1* and *CLN2* in *grr1* mutants are likely to be a consequence of *CLN1* and *CLN2* stabilization leading to increased *CLN1* and *CLN2* transcription, but stabilization of *CLN1* and *CLN2* mRNA in the mutant cannot be ruled out. Clearly, the increased synthesis of *CLN1* and *CLN2* does not overload the CLN-degradation system, because expression of *CLN1* to very high levels from the *GAL10* promoter on a multicopy plasmid had no influence on the short half-life of the protein (Fig. 6A).

G₁-cyclin stabilization in *grr1* is not a consequence of nutrient uptake defects

The *rgt1* (restores glucose transport) mutation was isolated as a suppressor of the glucose transport defects of a *snf3* mutant (Marshall-Carlson et al. 1991) and as a suppressor of the glucose transport, catabolite repression, and cobalt transport defects of *grr1* mutants (Erickson and Johnston 1994). Moreover, the *rgt1* suppressor also

restores a wild-type growth rate to *grr1* cells on glucose-based media. Thus, we wondered whether the *G₁*-cyclin stabilization in *grr1* cells was a consequence of the transport defects of the mutant or whether the stabilization was a direct consequence of the *grr1* mutation. Pulse-chase analysis showed that *CLN1* and *CLN2* were indeed both stabilized and hyperphosphorylated in the *grr1 rgt1* double mutant (Fig. 6A). Hence, we conclude that the stabilization of *G₁* cyclins in the *grr1* mutant is not a secondary effect of its transport defects and that *G₁*-cyclin turnover and nutrient uptake are two separate functions controlled by a common pathway that requires the *GRR1* protein.

Cell cycle defects of *grr1* mutants

The results shown above demonstrate that in wild-type

Figure 6. (A) A vast overexpression of *CLN1* in a wild-type strain does not saturate the *CLN1* proteolysis system. *CLN1* was immunoprecipitated from an equal quantity of congenic wild-type CMY826/pDR6 cells (expressing *CLN1* from the *GAL10* promoter on a 2 μ multicopy plasmid) or *grr1* (YM2957) cells growing in SGAL medium after labeling at 30°C for 5 min with [³⁵S]methionine/cysteine ($t=0$) or after further incubation for 10 and 30 min in the presence of excess nonradioactive methionine and cysteine. A graph of the percent *CLN1* radioactivity relative to the amount obtained from a pulse labeling of the wild-type strain overexpressing *CLN1* is shown below the fluorographs; (○) wild type with pGAL10::*CLN1*; (●) *grr1*. No saturation of the *CLN1* degradation system is observed even after overexpressing *CLN1* in the wild type to overall higher levels than exist in the *grr1* mutant. (B) *CLN1* and *CLN2* proteolysis is not restored by the *rgt1* suppressor mutation in *grr1* cells. The stability of *CLN1* and *CLN2* was examined by pulse-chase analysis in a *grr1 rgt1* double mutant (YM3378) using an anti-*CLN1* polyclonal antibody as for the experiment of Fig. 4A.

cells CLN1 and CLN2 cyclins are turned over rapidly by a GRR1-mediated proteolysis pathway. Because the abnormally elongated cell shape of *grr1* cells is also observed in a *grr1 rgt1* double mutant (Erickson and Johnston 1994; Vallier et al. 1994), we reasoned that the morphology of these cells is caused, at least in part, by the stabilization of the CLN1 and CLN2 cyclins but not by the transport defects of *grr1* cells. Further evidence for this interpretation comes from the observation that overexpression of *CLN1* or *CLN2* in wild-type cells leads to similar defects with elongated cells showing polarized cell growth (Fig. 3A; Lew and Reed 1993). To directly analyze the cell cycle defects caused by a GRR1 deficiency, we examined the DNA content of *grr1*, *grr1 rgt1* double mutants and wild-type cells by flow cytometry and classified the morphology of the different cell types. Figure 7A shows that the fraction of cells with a 1N DNA content was greatly reduced in the *grr1* (8%) and *grr1 rgt1* (18%) mutants compared with wild type (45%). Microscopic examination of these cells (Fig. 7B) indicated that this effect was accompanied by a decrease in the fraction of unbudded G_1 cells of both mutants compared with wild type. Apparently, *grr1* and *grr1 rgt1* cells entered S phase prematurely and, owing to segregation defects, accumulated as budded cells with one or two nuclei. Also characteristic for *grr1* cells were cells with multiple buds and more than two nuclei. However, this phenotype and the slow growth phenotype of *grr1* mutants are presumably the results of its nutrient transport defect because the *rgt1* mutation largely suppresses both the formation of these abnormal multibudded cells (Fig.

8B) and the growth defect of *grr1* cells (Erickson and Johnston 1994; Vallier et al 1994).

Discussion

To further our understanding of the mechanism and biological role of G_1 -cyclin proteolysis, we designed a screen for mutants affecting CLN1 degradation. Fusion of CLN1 to β -gal conferred instability to the otherwise stable β -gal protein. This instability depended on the same carboxy-terminal sequences that signal the degradation of CLN1. Mutants that stabilize the CLN1- β -gal fusion protein were isolated using a colony-color screen that monitors β -gal activity. One predominant group of mutants was represented by *grr1* alleles. These mutants also stabilized endogenous CLN1 and CLN2 cyclins.

GRR1 is thought to regulate glucose and divalent cation transport by inactivating RGT1, a putative transcriptional repressor of diverse genes encoding nutrient transporters (Erickson and Johnston 1994; Özcan et al. 1994; Vallier et al. 1994). We show here that GRR1 is also involved in G_1 -cyclin degradation and that the stabilization of CLN1 and CLN2 in *grr1* mutants occurs independently of known nutrient uptake defects. In contrast, proteolysis of CLN3, a G_1 cyclin acting upstream of CLN1 and CLN2 (Tyers et al. 1993), is largely unaffected by the *grr1* mutation.

Model for GRR1 action

GRR1 encodes a 135-kD, weakly expressed protein that

Figure 7. Cell cycle parameters of *grr1*, *grr1 rgt1* and wild-type cells by flow cytometry and morphology. (A) Wild-type (CMY826), *grr1* (YM2957), and *grr1 rgt1* (YM3378) log-phase cells in SD medium were analyzed for DNA content by fluorocytometry. (B) Morphological classification of the different cell types found in the wild-type and mutant cell populations prepared for flow cytometry. The percentage of cells with a 1N DNA content was obtained from the flow cytometric analysis.

Figure 8. Model for *GRR1* function. See Discussion for details.

is found in a particulate fraction (Flick and Johnston 1991). Aside from the observation of leucine-rich repeats that might mediate protein–protein interactions, the deduced amino acid sequence of *GRR1* provides no clue as to its biochemical function. The model in Figure 8 proposes that *GRR1* activates G_1 -cyclin turnover and inhibits the putative transcriptional repressor *RGT1*. *GRR1* could be a component of a proteolytic system that degrades *CLN1*, *CLN2*, and *RGT1*. Alternatively, *GRR1* might modulate G_1 -cyclin stability and *RGT1* function by affecting the phosphorylation state of the proteins. One role of *GRR1* may be to coordinate the timing of Start to the availability of nutrients by regulating cyclin proteolysis and transporter gene transcription. Nutrients are known to modulate the timing of Start in yeast: Cells growing slowly perform Start at smaller cell sizes than fast-growing cells (Pringle and Hartwell 1981). Activation of the *GRR1* pathway in the presence of glucose is thought to increase the expression of certain glucose transporters (Erickson and Johnston 1994; Özcan et al. 1994; Vallier et al. 1994). Our results indicate that this activation would also lead to an increased proteolysis of *CLN1* and *CLN2* with a consequent delay in the passage of Start. Conversely, in the absence of glucose, reduced activity of the *GRR1* pathway would be expected to down-regulate the expression of the glucose transporters and decrease *CLN1* and *CLN2* degradation. The higher levels of *CLN1* and *CLN2* in the absence of glucose would advance the time at which yeast cells execute Start relative to that of cells growing in the presence of glucose. We propose that the *GRR1* pathway acts along with the RAS/cAMP pathway (Baroni et al. 1994; Tokiwa et al. 1994) in coordinating cell growth with cell division.

GRR1, a regulator of pseudohyphal growth?

A particularly interesting observation of *grr1* mutants concerns their morphological phenotype. *grr1* cells produce very elongated buds that do not separate from the mother cell as easily as buds from wild-type cells. The

elongated-cell phenotype could be the result of the increased presence and activity of *CLN1* and *CLN2* in the S and G_2 phases in *grr1*, because it is known that constitutive overexpression of *CLN1* or *CLN2*, but not *CLN3*, can promote a similar hyperpolarized growth in the wild type (Lew and Reed 1993). The observed half-life of *CLN1* and *CLN2* of >30 min in the *grr1* mutant is consistent with the presence of these proteins during the S and G_2 phases. The morphology of the *grr1* cells resembles that of wild-type cells under conditions of pseudohyphal growth. *Saccharomyces cerevisiae* undergoes pseudohyphal differentiation upon limited nitrogen starvation (Gimeno et al. 1992). We propose that increased *CLN1* and *CLN2* stability could be an important factor in switching from normal budding to pseudohyphal growth. Hence, this switch to an alternative lifestyle may also be regulated by a signaling pathway that involves *GRR1*.

Materials and methods

Media, genetic methods, and strain construction

YPD (yeast extract, peptone, dextrose medium) and SD (synthetic dextrose minimal medium) were prepared as described (Sherman 1991). SGal contained 2% galactose instead of glucose, and SDGal contained 2% dextrose + 2% galactose.

Standard genetic methods were used for crosses and tetrad analysis. EMS mutagenesis was performed as described (Lawrence 1991). Yeast transformations were done using a lithium acetate procedure (Gietz et al. 1992). The β -gal overlay assay was a modification of a protocol developed by S. Johnson (1991): 10 ml of X-gal agar [0.5 M potassium phosphate buffer (pH 7.0), 6% dimethyl formamide, 0.1% SDS, 0.1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), and 0.5% agarose] at 60°C were poured over the colonies on plates followed by an incubation at room temperature, 30°C, or 37°C until color development was evident. The DMF and SDS uniformly permeabilize the cells to the X-gal chromogenic substrate, and the phosphate buffer maintains the pH at the optimum for β -gal activity.

Strains *CLN2C grr1* and *CLN3C grr1* were obtained by converting the strains MT263 (*CLN2C*) and GT108 (*CLN3C*) to tryptophan prototrophy by transformation with a *TRP1* integrative plasmid and then disrupting the *GRR1* gene by transformation with *Bam*HI-digested pBM2101 [*grr1::hisG-URA3-hisG*] (Flick and Johnston 1991). Disruption of *GRR1* was signaled by the characteristic *grr1* morphological phenotype.

S. cerevisiae strains are listed in Table 1.

Plasmid construction

The *CLN1* gene was isolated from genomic DNA by PCR and cloned as a *Xho*I–*Bam*HI fragment into the *Sal*I–*Bam*HI sites of the Fusionator plasmid (pSJ101) (Johnson 1991) such that a *CLN1-lacZ* fusion gene was created and expressed from the *GAL10* promoter (Fig. 1A). The *CLN1* clone used in this study complemented in a galactose-dependent fashion a *cln1 cln2 cln3* triple mutant. The *CLN1 Δ PEST-lacZ* plasmid was made by digesting Fusionator–*CLN1-lacZ* with *Nco*I, filling in the extremities, cutting with *Sma*I, and religating the plasmid.

Cloning of TOC1 and its identification as *GRR1*

A plasmid from a pSEY8 yeast genomic DNA library that com-

Table 1. *S. cerevisiae* strains

Strain	Relevant genotype	Source
CMY374	<i>MATα ura3</i>	this study
CMY394	<i>MATα ura3-52 leu2Δ1 his3Δ200 lys2-801 ade2-101 trp1Δ1</i>	this study
CMY604	<i>MATα cln1::TRP1 cln2::HIS3 cln3Δ ura3 trp1 his3 leu2 ade1/YCp50-CLN3</i>	this study
CMY826	<i>MATα ura3-52 leu2Δ1 his3Δ200 lys2-801 ade2-101 trp1Δ63 bar1::LEU2</i>	this study
FM135	<i>MATα ura3-52 leu2 gal1 pep4 prb1 reg1-501</i>	M. Johnston, pers. comm.
Ely2	<i>MATα ura3-52 leu2 gal1 pep4 prb1 reg1-501 toc1-3</i>	this study
Ely6	<i>MATα ura3-52 leu2 gal1 pep4 prb1 reg1-501 toc1-1</i>	this study
Ely9	<i>MATα ura3-52 leu2 gal1 pep4 prb1 reg1-501 toc1-2</i>	this study
Ely161	<i>MATα ura3-52 leu2 trp1-1 lys2-801 gal1 toc1-1</i>	this study
Ely186	<i>MATα ura3-52 leu2-3,-112 lys2-801 ade2-101 his3-200 grr1::URA3</i>	this study
Ely227	<i>MATα ura3-52 leu2Δ1 his3Δ200 lys2-801 ade2-101 trp1Δ1 cdc28-4</i>	this study
Ely245	<i>MATα ura3-1 leu2-3,-112 his3-11,-15 TRP1 ade2-1 cln3::CLN3C</i>	this study
Ely246	<i>MATα ura3-1 leu2-3,-115 his3-11,-15 TRP1 ade2-1 cln2::CLN2C-LEU2</i>	this study
Ely247	<i>MATα ura3-1 leu2-3,-115 his3-11,-15 TRP1 ade2-1 cln3::CLN3C grr1::URA3</i>	this study
Ely248	<i>MATα ura3-1 leu2-3,-115 his3-11,-15 TRP1 ade2-1 cln2::CLN2C-LEU2 grr1::URA3</i>	this study
Ely254	<i>MATα ura3-52 leu2Δ1 his3Δ200 lys2-801 ade2-101 cdc28-4 grr1::URA3</i>	this study
MT263	<i>MATα ura3-1 leu2-3,-112 his3-11,-15 trp1-1 ade2-1 cln2::CLN2C-LEU2</i>	B. Futcher, pers. comm.
GT108	<i>MATα ura3-1 leu2-3,-112 his3-11,-15 trp1-1 ade2-1 cln3::CLN3C</i>	B. Futcher, pers. comm.
YWO1	<i>MATα ura3-52 leu2-3,-112 lys2-801 trp1-101 his3Δ200</i>	W. Seufert, pers. comm.
YWO2	<i>MATα ura3-52 leu2-3,-112 lys2-801 trp1-101 his3Δ200</i>	W. Seufert, pers. comm.
YM2953	<i>MATα ura3-52 leu2-3,-112 lys2-801 his3Δ200 grr1::LEU2</i>	M. Johnston, pers. comm.
YM2957	<i>MATα ura3-52 his3Δ200 ade2-101 lys2-801 gal80Δ grr1Δ-1829 LEU2::pRY181(GAL1-lacZ)</i>	M. Johnston, pers. comm.
YM3378	<i>MATα ura3-52 his3Δ200 ade2-101 lys2-801 gal80Δ grr1Δ-1829 rgt1-101 LEU2::pRY181(GAL1-lacZ)</i>	M. Johnston, pers. comm.

plemented the inability of a *toc1* mutant to exit from stationary phase at 37°C was obtained as described in Results. This plasmid was recovered in *E. coli*, the restriction map of the plasmid was determined, and a *TRP1* marker was introduced in a unique *Bgl*II site located within the yeast DNA fragment. This insertion disrupted the ability of the plasmid to complement the *toc1* mutant. DNA sequence was then obtained from either side of the *Bgl*II site, and these sequences were found to be identical to a region of the *GRR1* DNA sequence. The *grr1::TRP1* insert was isolated and used to transform a *TOC1/toc1* heterozygous diploid. A minority of the transformants had a *toc1* phenotype and grew very slowly. One transformant with a *toc1* phenotype and one transformant with the wild-type phenotype were sporulated, and tetrad asci were dissected. Diploids with the *toc1* phenotype gave rise to few complete tetrads, but all of the growing spores exhibited the *toc1* morphological phenotype. For the diploid transformant with a wild-type phenotype, spore viability was much higher and the *grr1::TRP1* marker segregated in opposition with the *toc1* mutation in all of 30 tetrads examined. We conclude that the *TOC1* gene was cloned and that it corresponds to the *GRR1* gene isolated previously.

Northern blot analysis

Congenic CMY826 *GRR1*⁺ and YM2957 *grr1* cells were grown in log phase in YPD to an OD₆₀₀ of 0.5. Cells were spun down, and total RNA was extracted as described (Schmitt et al. 1990). RNAs were denatured with glyoxal, electrophoresed on a 1% agarose gel in sodium phosphate buffer, transferred to a Nytran membrane (Schleicher & Schuell) and fixed by UV irradiation (Stratalinker). Membranes were separately probed with [³²P]dATP-labeled DNA fragments containing the coding region of the *CLN1*, *CLN2*, *CLN3*, or *ACT1* gene. DNA fragments were labeled with a random-priming kit (Stratagene). Hybridization

was performed as described (Church and Gilbert 1984), and radioactive signals were revealed and quantified with a PhosphorImager.

Immunoprecipitation and pulse-chase analysis

For pulse-chase analysis, 30 ml of culture in SD medium at an OD₆₀₀ of 0.5 was centrifuged and resuspended in 0.9 ml of the same medium at 30°C. [³⁵S]Methionine/cysteine (Amersham) (500 μ Ci) was added, and the cells were incubated for 5 min at 30°C. Cells (0.3 ml) were then removed for the 0-min time point, and 1 ml of chase medium (containing 0.4 mg/ml of unlabeled methionine and cysteine) was added to the remaining culture for chase periods of 10 and 30 min.

For the immunoprecipitation, the cells were quickly chilled on ice, centrifuged, and then resuspended in 150 μ l of cold immunoprecipitation buffer (1% Triton X-100, 0.2% SDS, 0.3% deoxycholate, 50 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl at pH 8.0) containing the protease inhibitors PMSF at 1 mM and leupeptin, aprotinin, chymostatin, and pepstatin at 2 μ g/ml. An equal volume of glass beads was added, and cells were lysed with six pulses of vortexing for 30 sec separated by 30-sec incubations on ice. An additional 1 ml of immunoprecipitation buffer was added, and the extract was clarified by centrifugation for 15 min in an Eppendorf centrifuge at 4°C. The supernatant was then incubated for 1 hr with 2 μ g of antibody at 4°C. *CLN1* was immunoprecipitated with rabbit polyclonal antibodies prepared against denatured *CLN1* purified from inclusion bodies after overexpression in *E. coli* (P. Guegen and C. Mann, unpubl.), whereas the *CLN2C* and *CLN3C* proteins were immunoprecipitated with the 12CA5 mouse monoclonal antibody directed against the HA1 epitope (Field et al. 1988). Protein A-Sepharose beads (30 μ l) were then added, and the mixture was further incubated for 1 hr. The Sepharose beads were washed three

times with the immunoprecipitation buffer. The supernatant was entirely removed from the pellet of beads after each wash using a Hamilton syringe. The beads were then resuspended in 0.6 ml of IP buffer and transferred to a fresh tube and washed once more. In the case of very low signals, the beads were eluted twice with 15 μ l of 1% SDS for 5 min at room temperature, diluted in the immunoprecipitation buffer, and reprecipitated as described above. This double immunoprecipitation procedure greatly reduced the background radioactivity. The beads were then resuspended in sample buffer, boiled, and the released proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The radiolabeled proteins were detected either with a PhosphorImager (Molecular Dynamics) or by treating the gel for fluorography with Amplify (Amersham) and exposure of a photographic film.

Flow cytometry and light microscopy techniques

For flow cytometry, log-phase cells were harvested at an OD₆₀₀ of 0.5 and cells were fixed in 70% ethanol and stained with propidium iodide as described previously (Mann et al. 1992). Cells were photographed with a 100 $\times$ objective using a combination of fluorescence and phase-contrast microscopy with a Zeiss Axioplan microscope. 4',6'-Diamidino-2-phenylindole (DAPI) staining of cellular DNA was carried out as described (Pringle et al. 1991).

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G1 cyclin turnover and nutrient uptake are controlled by a common pathway in yeast.

Y Barral, S Jentsch and C Mann

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