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

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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-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. 1992, 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
expressing a truncated CLN2 gene that is presumed to encode a stabilized cyclin [Hadwiger et al. 1989]. Thus, one role of G1 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 G1 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 G1 cyclin proteolysis.

Results

Construction of unstable CLN1-fusion proteins

To study the degradation of G1 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 (CLN1APEST-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 CLN1APEST-β-gal gave a strong coloration. On Western blots, the intact CLN1-β-gal fusion protein was not detectable under conditions in which CLN1APEST-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 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, CLN1APEST, 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 G1 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 viability. We show that the fusion proteins restore viability in this strain, indicating that they are functional as G1 cyclins in vivo.

Figure 1. [A] Plasmids expressing CLN1-β-gal and CLN1APEST-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 CLN1APEST-lacZ. Cells (10⁵) 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 CLN1APEST-β-gal by pulse-chase analysis. Wild-type (YW02) cells expressing CLN1-lacZ or CLN1APEST-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 CLN1APEST-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.

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 (Howland et al. 1989). This strain was mutagenized with ethylmethane sulphonate (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 trpl background. Such cells grew exponentially at 37°C, but most of them were unable to exit stationary phase at 37°C. Few toc1 trpl 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 trpl 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 trpl 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 trpl 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 G1 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 $G_1$ 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 $G_1$ 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 $[^{35}S]$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 $[^{35}S]$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.
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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 GRRI + 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.

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 2μ 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.

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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

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[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
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 un budded G1 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 defects 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 G1-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 G1-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 G1 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.
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Figure 8. Model for GRR1 function. See Discussion for details.

Figure 8

GRR1

?  

GRT1

G1 cyclin stability

Nutrient transport

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 G1-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 G1-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 G2 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 G2 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 substrates, 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 BamHI-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 Xhol–BamHI fragment into the SalI–BamHI sites of the Fusionator plasmid [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 CLN1APEST–lacZ plasmid was made by digesting Fusionator–CLN1–lacZ with Ncol, filling in the extremities, cutting with SmaI, and religating the plasmid.

Cloning of TOC1 and its identification as GRR1

A plasmid from a pSEY8 yeast genomic DNA library that com-
implemented the inability of a tocl 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 BglII 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 BglII 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 TOCl/tocl 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. Tetrads with the toc1 mutation in all of 30 tetrads examined.

For the immunoprecipitation, the cells were quickly chilled on ice, centrifuged, and then resuspended in 0.5 ml of the same medium at 30°C. [35S]Methionine/cysteine (Amersham) 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 3 ml of unlabeled medium at 30°C. 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 3 ml of unlabeled medium 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 3 ml of unlabeled medium 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 3 ml of unlabeled medium at 30°C.

**Table 1. S. cerevisiae strains**

| Strain | Relevant genotype | Source |
|--------|-----------------|--------|
| CMY374 | MATa ura3       | this study |
| CMY394 | MATa ura3-52 leu2Δ1 his3Δ200 lys2-801 ade2-101 trp1Δ1 | this study |
| CMY604 | MATa cll1::TRP1 cln2::HIS3 cln3Δ1 ura3 trpl his3 leu2 ade1/YCp50-CLN3 | this study |
| CMY826 | MATa ura3-52 leu2Δ1 his3Δ200 lys2-801 ade2-101 trp1Δ163 bar1::LEU2 | this study |
| FM135  | MATa ura3-52 leu2 gal1 pep4 prb1 reg1-501 | M. Johnston, pers. comm. |
| Ely2   | MATa ura3-52 leu2 gal1 pep4 prb1 reg1-501 toc1-3 | this study |
| Ely6   | MATa ura3-52 leu2 gal1 pep4 prb1 reg1-501 toc1-1 | this study |
| Ely9   | MATa ura3-52 leu2 gal1 pep4 prb1 reg1-501 toc1-2 | this study |
| Ely161 | MATa ura3-52 leu2 trpl-1 lys2-801 gal1 toc1-1 | this study |
| Ely186 | MATa ura3-52 leu2-3-112 lys2-801 ade2-101 his3-200 grr1::URA3 | this study |
| Ely227 | MATa ura3-52 leu2Δ1 his3Δ200 lys2-801 ade2-101 trp1Δ1 cdc24-8 | this study |
| Ely245 | MATa ura3-1 leu2-3-112 his3-11-15 TRP1 ade2-1 cln3::CLN3C | this study |
| Ely246 | MATa ura3-1 leu2-3-115 his3-11-15 TRP1 ade2-1 cln2::CLN2C-LEU2 | this study |
| Ely247 | MATa ura3-1 leu2-3-115 his3-11-15 TRP1 ade2-1 cln3::CLN3C grr1::URA3 | this study |
| Ely248 | MATa ura3-1 leu2-3-115 his3-11-15 TRP1 ade2-1 cln2::CLN2C-LEU2 grr1::URA3 | this study |
| Ely254 | MATa ura3-52 leu2Δ1 his3Δ200 lys2-801 ade2-101 cdc28-4 grl::URA3 | this study |
| MT263  | MATa ura3-1 leu2-3-112 his3-11-15 trp-1 ade2-1 cln3::CLN3C | B. Futcher, pers. comm. |
| GT108  | MATa ura3-1 leu2-3-112 his3-11-15 trp-1 ade2-1 cln3::CLN3C | B. Futcher, pers. comm. |
| YW01   | MATa ura3-52 leu2-3-112 lys2-801 trp1-101 his3Δ200 | W. Seufert, pers. comm. |
| YW02   | MATa ura3-52 leu2-3-112 lys2-801 trp1-101 his3Δ200 | W. Seufert, pers. comm. |
| YM2953 | MATa ura3-52 leu2-3-112 lys2-801 his3Δ200 grr1::LEU2 | M. Johnston, pers. comm. |
| YM2957 | MATa ura3-52 his3Δ200 ade2-101 lys2-801 gal80Δ grr1::18-1829 LEU2::pRY181(GAL1-lacZ) | M. Johnston, pers. comm. |
| YM3378 | MATa ura3-52 his3Δ200 ade2-101 lys2-801 gal80Δ grr1::18-1829 grr1::18-1829 LEU2::pRY181(GAL1-lacZ) | M. Johnston, pers. comm. |

Pathway for cyclin turnover and nutrient uptake

Northern blot analysis

Congenic CMY826 GRR1Δ 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 (Stratagene). 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 (Stratagen). Hybridization was performed as described (Church and Gilbert 1984), and radioactive signals were revealed and quantified with a PhosphorImager.
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 μl 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 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 OD_{600} 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× 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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