

Mutations in *PRG1*, a yeast proteasome-related gene, cause defects in nuclear division and are suppressed by deletion of a mitotic cyclin gene

HAYA FRIEDMAN AND MICHAEL SNYDER

Department of Biology, Yale University, New Haven, CT 06511

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ABSTRACT Proteasomes are ubiquitous complexes exhibiting proteolytic activity *in vitro*. The function(s) of these enzymes *in vivo* is not known. To investigate the *in vivo* role of proteasomes, four temperature-sensitive alleles of the *Saccharomyces cerevisiae* proteasome-related gene, *PRG1*, were constructed and analyzed. At both the permissive and restrictive temperatures, many *prg1* cells have a large bud, contain replicated DNA, and have their nucleus positioned at the neck with a short spindle. These different phenotypes indicate a defect in nuclear division. Consistent with a nuclear division defect, *prg1* mutant strains lose a dispensable chromosome at a higher frequency than wild-type cells. Importantly, deletion of *CLB2*, a gene encoding a mitotic cyclin, suppresses the temperature-sensitive growth phenotype of *prg1* mutant strains. Our results indicate that proteasomes are important for nuclear division and suggest that they participate in degradation of the Clb2 protein (Clb2p).

Precise and specific degradation of proteins is essential for progression through the cell cycle (1). An important class of proteins that are degraded at discrete points during the cell cycle is the cyclins, which regulate entry into S phase and mitosis (2–4). One type of cyclin, cyclin B, is thought to be degraded via the ubiquitin pathway (5). In this pathway, 76-amino acid ubiquitin moieties are covalently attached onto proteins that are to be degraded (6, 7). Enzymes involved in recognizing and adding ubiquitin onto proteins and thereby targeting them for degradation have been identified; some of these enzymes are essential for cell growth (6–8).

The proteases involved in regulating cell cycle progression are not well defined. However, likely candidates to participate in this process are proteasomes. Proteasomes are large complexes composed of 15–20 related polypeptides; they are found in Archeobacteria and all eukaryotes examined thus far (9, 10). *In vitro* studies indicate that the proteasome has multicatalytic activity and can degrade both ubiquitinated and nonubiquitinated proteins (6, 10, 11). The association of proteasomes have been found to be associated with the mitotic spindle apparatus and chromosomes during mitosis suggesting that they may play a role in the progression through mitosis (12, 13). However, direct experiments to explore the *in vivo* role of proteasomes in cell cycle progression and nuclear division have not been performed.

Saccharomyces cerevisiae is a useful organism for studying the function of proteasomes *in vivo* because it is readily amenable to genetic analysis. Thus far, eight *S. cerevisiae* proteasome-related genes have been identified; seven are essential for cell growth (14–20). We have been studying one of the proteasome-related genes *PRG1/PRE2*. *PRG1* is highly homologous to the human *RING10* gene (55% identity over most of the protein), indicating that it is highly con-

served. Gene disruption experiments indicate that it is essential for cell growth (14, 20). *prg1/pre2* mutants are defective in chymotrypsin-like activity, suggesting a role for Prg1p in protein degradation (17).

S. cerevisiae has four mitotic cyclins—Clb1p, Clb2p, Clb3p, and Clb4p (21, 22)—which are potential candidates for proteasome degradation. All four mitotic cyclins are expressed only during nuclear division. Clb2p is thought to play a major role in regulating mitosis because single mutant *clb* and triple mutant *clb1 clb3 clb4* strains are viable; however, deletion of *CLB2* and any two other *CLB* genes results in lethality (22). Depletion of Clb2p in a strain lacking the other mitotic cyclins causes cells to arrest prior to mitosis (22). The mechanism by which Clb2p and the other mitotic cyclins are degraded is not known.

In this report, we have constructed temperature-sensitive mutations in *PRG1*. Analysis of *prg1^{ts}* mutant phenotypes reveals that a proteasome component is important for chromosome segregation and nuclear division. Our results indicate that protein degradation by proteasomes is necessary for specific processes between late S phase and the middle of mitosis. We demonstrate that a deletion of *CLB2* suppresses the temperature-sensitive growth defect of *prg1^{ts}* strains, indicating that mitotic cyclins may be *in vivo* substrates of proteasomes.

MATERIALS AND METHODS

Yeast Strains, Media, and Genetic Manipulations. Yeast media and manipulations are presented by Sherman *et al.* (23). All yeast strains used in this study are congenic and derived from Y429 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 leu2-Δ98*) carrying chromosome fragment CFVII RAD2d (24) or Y432 (*MATa ura3-52 lys2-801 ade2-101 his3-Δ200*) carrying the same chromosome fragment. A yeast strain (YH1002; *MATa prg1-Δ1 ura3-52 lys2-801 ade2-101 trp1-1 leu2-Δ98 + pPRG1/URA3/CEN*) (14) that is disrupted at the *PRG1* locus and carries the *PRG1* gene on a *CEN/URA3* plasmid was constructed by one-step transformation (25). The *prg1-Δ1* allele [which contains a mTn3::*LEU2* transposon in the *PRG1* open reading frame (14)] was integrated into the genome of a wild-type diploid strain carrying the *PRG1* gene on a *CEN/URA3* plasmid. The diploid strain was sporulated and haploid *LEU2 URA3* prototrophs were selected. These haploids could not grow on plates containing 5-fluoroorotic acid, which selects for cells that have lost the *URA3* gene (26), but could grow if an additional *PRG1* plasmid was present in the strain.

Cell division at restrictive and permissive temperatures was monitored by counting the total number of cells at different times during incubation in rich medium. Buds that were $\geq 2/3$ rd the size of the mother were scored as a cell. Doubling times of *prg1* strains at the permissive temperatures were 3.2, 2.6, 2.5, and 2.2 hr for *prg1-1*, *prg1-2*, *prg1-3*, and *prg1-4* strains, respectively (wild-type doubling time, 1.75 hr). Cell viability was determined by plating aliquots of cell

cultures taken at different times after incubation at the restrictive temperature and counting the number of colonies that formed.

Construction of Temperature-Sensitive Mutant Strains. Temperature-sensitive mutant strains were created as described by Sikorski and Boeke (27). Briefly, a *TRP1/CEN* plasmid carrying the *PRG1* sequence was mutagenized *in vitro* by hydroxylamine; $\approx 3\%$ of the mutagenized plasmids failed to allow growth in medium lacking tryptophan. The mutagenized DNA was amplified in *Escherichia coli*, and DNA was prepared from 10,000 pooled colonies. This randomly mutated *PRG1* sequence library was introduced into YH1002; 13,200 Trp⁺ colonies were transferred to plates containing 5-fluoroorotic acid to select cells that had lost the *URA3* plasmid; and these colonies were subsequently screened for inability to grow at 38°C. Four temperature-sensitive alleles of *PRG1* were identified. Plasmids containing the different alleles were isolated, retested for the ability to mediate temperature-sensitive growth, and then sequenced by the dideoxynucleotide chain-termination method (28). Each *prg1* mutation contains a single G to A change, a transition consistent with that expected for hydroxylamine mutagenesis (27).

Immunofluorescence Analysis. Indirect immunofluorescence of yeast cells with anti-tubulin antibodies was performed as described (29). Rabbit anti-yeast β -tubulin was kindly provided by Frank Solomon (30). The rabbit antibodies were detected by using goat anti-rabbit antibodies conjugated to Texas Red (Cappel). Yeast nuclei were visualized with the DNA binding dye Hoechst 33258 (31).

Flow Cytometry Analysis. Cells were fixed in 70% ethanol, treated with RNase A (Sigma), and stained with propidium iodide as described by Hutter and Eipel (32). The distribution of the DNA content in 10,000 cells was determined by a Becton Dickinson flow-activated cell sorting machine.

Quantitation of Chromosome Derivative Loss Rate. Chromosome fragment CF VII RAD2d from strain YH1002 was crossed into the *prg1* temperature-sensitive strains. Quantitation of chromosome loss rates was determined by counting half-sectored colonies (33). Seventeen thousand cell divisions were evaluated for wild type, 6000 cell divisions were evaluated for *prg1-4* and *prg1-3*, and 2000 cell divisions were evaluated for *prg1-1* and *prg1-2*.

Growth Phenotype Analysis of *clb-Δ prg1^{ts}* Double Mutants. *prg1^{ts} clb2-Δ* double-mutant strains were created by mating a *clb2-Δ* strain (*MATα clb2-Δ::LEU2 PRG1*) (34) with either *prg1-2* (*MATα prg1-Δ::HIS3 CLB2 + pCEN URA3 PRG1 + pCEN TRP1 prg1-2*), *prg1-3* (as above except with *pCEN TRP1 prg1-3*), or *prg1-4* (as above except with *pCEN TRP1 prg1-4*) temperature-sensitive strains. After sporulation and dissection, segregants were scored for their relevant markers. Approximately one-half of the *prg1-Δ::HIS3* strains carrying the *prg1^{ts}* plasmid contained the *clb2-Δ* mutation, as expected (16, 18, and 14 tetrads were analyzed for *prg1-2*, *prg1-3*, and *prg1-4* strains, respectively). The growth of 8 *clb2-Δ prg1^{ts}* mutants and 12 *prg1^{ts}* segregants was checked on yeast extract/peptone/dextrose plates at 30°C and 38°C. All *clb2-Δ prg1^{ts}* double mutants grew at 38°C, whereas strains containing only the *prg1^{ts}* mutation did not. We also found that 16 of 33 *clb2-Δ prg1-Δ* segregants obtained from these crosses lacked both the *PRG1* and *prg1* plasmids (48% of total). This is close to the frequency expected given that approximately one-half of the segregants contain *PRG1* or *prg1* plasmids. In contrast, from these experiments and other crosses of *prg1-Δ* strains, we have found that of 111 deduced *prg1-Δ CLB* strains, only three lacked the *PRG1* plasmids (2.7% of total). These rare segregants are not temperature sensitive for growth and are assumed to have acquired additional suppressing mutations and/or chromosomes.

Similar studies were performed by creating four *clb4-Δ prg1^{ts}* double mutants from *MATα/MATα prg1-Δ1/PRG1 clb4-Δ::HIS3/CLB4* strains containing *pCEN3 URA3 PRG1* and the different *prg1^{ts}* plasmids. *prg1-Δ1 clb4-Δ* strains that lacked either plasmid were not recovered, but *prg1-Δ1 clb4-Δ* cells that contained the *prg1^{ts}* plasmids were found. Analysis of three to five different *prg1-Δ1 clb4-Δ prg1^{ts}* segregants for each *prg1* allele revealed that each strain was still temperature sensitive for growth, indicating that *clb4-Δ* does not suppress the *prg1^{ts}* mutations.

RESULTS

Construction of *PRG1* Temperature-Sensitive Mutants. To study the role of *PRG1* *in vivo*, temperature-sensitive mutants were generated by hydroxylamine mutagenesis and the plasmid shuffling procedure described in *Materials and Methods*. Four mutations, *prg1-1* to *-4*, were identified. Strains containing these mutations do not form colonies at 38°C and grow 30–80% slower at the permissive temperature than does an isogenic *PRG1* wild-type strain.

The nature of the mutation for each temperature-sensitive allele was determined by DNA sequence analysis. In each case, the mutant allele contained an amino acid substitution at a residue conserved in both the Prg1 and RING10 proteins (ref. 14; Fig. 1), indicating that the four conserved residues are important for *PRG1* function. The predicted amino acid changes in Prg1-2p, Prg1-3p, and Prg1-4p lie within a short stretch of 10 amino acids located 118 residues from the N terminus. The *prg1-1* mutation, which causes the most severe defects of the four mutations (see below), resides at codon 204. Heinemeyer *et al.* (20) identified two other *prg1/pre2* mutations: an A to V change at position 124 and a G to S change at position 259. These mutations did not cause a temperature-sensitive growth defect but did result in a loss of chymotrypsin-like activity and an accumulation of ubiquitinated proteins (17).

Each of the *prg1* mutant strains did not arrest with a uniform morphology at the restrictive temperature (38°C) in rich medium. After 4 hr at 38°C, *prg1-2*, *prg1-3*, and *prg1-4* cells, continue to divide at a rate 4.3, 2.9, and 2.6 times slower, respectively, than the rate of the corresponding strains at the permissive temperature. *prg1-1* and *prg1-2* cells lose viability after 8 hr at this temperature (90–50% loss of viability; data not shown). We assume that the combination of slow cell divisions and high inviability causes the temperature-sensitive growth defect observed on plates.

***prg1* Mutants Are Defective in Nuclear Division.** Analysis of the nuclear morphology and the tubulin distribution in *prg1* mutant cells revealed a defect in nuclear division. *prg1* mutant strains grown at the permissive and restrictive temperatures were stained with anti-tubulin antibodies and Hoechst 33258, a DNA binding dye. The number of cells at each stage of the cell cycle was quantified for each mutant grown at the per-

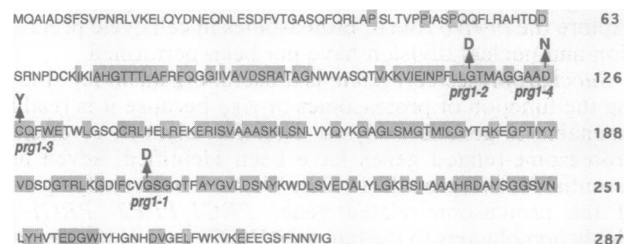


FIG. 1. Sequence analysis of *prg1* temperature-sensitive alleles. Shaded areas are conserved residues found in both the RING10 and Prg1p putative protein sequences (14). Numbers refer to amino acid residue positions in the predicted PRG1 protein sequence. Mutated residues in Prg1^{ts} proteins are indicated.

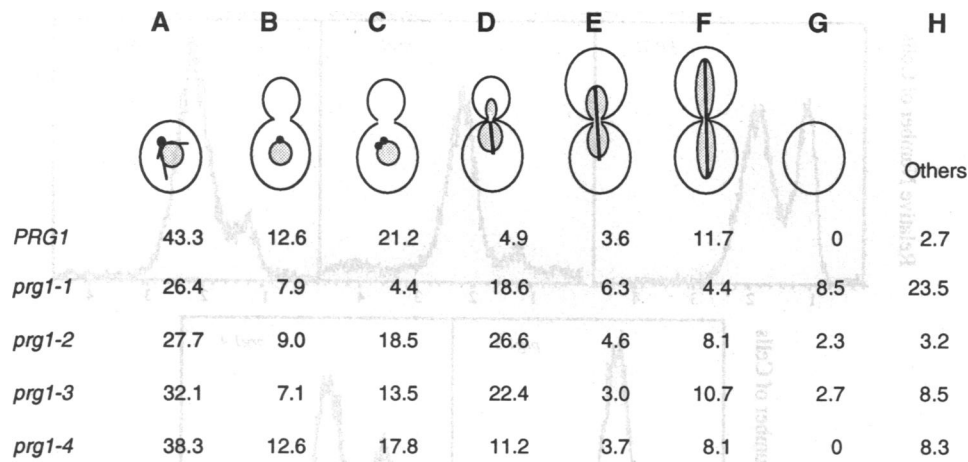


FIG. 2. Percentage of *prg1^{ts}* cells at different stages of the cell cycle. *prg1* cells were grown at the permissive temperature and stained with Hoechst 33258 and anti-tubulin antibodies. For each strain 450–600 cells were scored in the indicated categories according to cell morphology, nuclear position, and spindle apparatus morphology. Numbers indicate percentage of cells in each category.

missive temperature and was compared to the number of wild-type cells (Fig. 2). For each mutant, a large fraction of cells contained a single nucleus and short spindle (see Fig. 3 for *prg1-2* cells; see Fig. 2, lane D, for results of all mutants). The nucleus was usually positioned near the mother/bud neck or stretched between the two cells. The fraction of *prg1-1*, *prg1-2*, *prg1-3*, and *prg1-4* cells with this phenotype was 3.8-, 5.4-, 4.6-, and 2.3-fold higher, respectively, than for wild-type cells (Fig. 2). *prg1-1*, *prg1-2*, and *prg1-3* cultures also accumulate a significant fraction of cells without a nucleus (Fig. 2, lane G).

prg1-1 cells, which have the slowest growth rate of the four mutants at the permissive temperature (see *Materials and*

Methods), display the largest fraction of anucleated cells and the highest number of cells in the category “others” (Fig. 2, lane H). This category includes cells with an elongated/deformed bud (30% for *prg1-1*), unbudded cells with a short spindle (36% for *prg1-1*), cells with two nuclei (26%), and a fraction (14%) of cells without tubulin staining.

prg1-1 and *prg1-2* were also analyzed after incubation at the restrictive temperature for 3 hr. The distribution of cell types was similar to that of cell populations grown at the permissive temperature (data not shown). Therefore, the nuclear division defect is not enhanced at the restrictive temperature (see below). Nevertheless, although *prg1* cells did not exhibit a terminal cell division cycle (*cdc*) arrest phenotype, the accumulation of cells with a short spindle and anucleated cells indicates that *prg1* cells have a nuclear division defect.

Since *S. cerevisiae* cells establish a short spindle during S phase (35), it is possible that the nuclear division defect in *prg1* mutant cells lies either before, during, or after DNA synthesis. To further determine where the defect resides, the DNA content of the different mutant cells grown at the permissive temperature and those shifted to the restrictive temperature for 3 hr was examined by fluorescence-activated cell sorting analysis (Fig. 4). When haploid wild-type cells were analyzed at either 25°C or 38°C, two peaks were observed; at each temperature, ≈40% of the cells have a 1n content of DNA and ≈60% have a 2n DNA content. In contrast, when *prg1* mutant strains were incubated at either 25°C or 38°C, the fraction of 1n cells was severely reduced, and the vast majority of cells had a 2n or greater content of DNA. (Fig. 4 shows the results for cells grown at 25°C; analysis of cells at 38°C yields similar results.) The fraction of cells with 1n content of DNA inversely correlates with the severity of the mutation: *prg1-1* cells have the fewest number of cells with 1n content of DNA and *prg1-4* cells have the most. Furthermore, *prg1-1* cultures, which have a substantial fraction of anucleate and multinucleate cells detected by cytological analysis, have a small population with <1n DNA and a second population with >2n DNA. In summary, these observations indicate that *prg1* mutants replicate their DNA but are defective in subsequent nuclear division events.

The Frequency of Chromosome Loss Is Increased in *prg1* Mutants. *PRG1* was originally identified as a suppressor of *crcl* (calcium-regulated component), a mutation that exhibited a high frequency of chromosome loss (unpublished results; ref. 14). This suppression effect, in conjunction with the role of Prg1p in nuclear division, suggested that *prg1* cells might lose chromosomes. This hypothesis was tested by

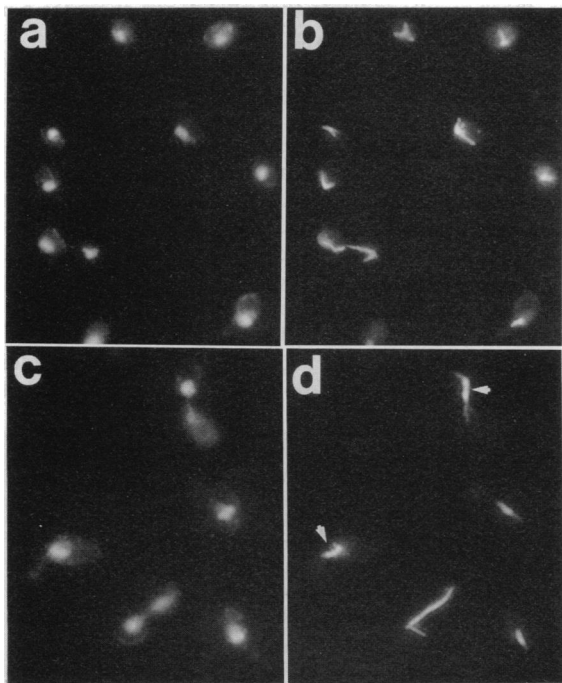


FIG. 3. *prg1* temperature-sensitive mutants often have a single nucleus at the neck between the mother and daughter cell and a short spindle. *prg1-2* (c and d) and the isogenic wild-type *PRG1* strain (a and b) were stained with Hoechst 33258 (a and c) and with rabbit anti- β -tubulin antibody (b and d) at the permissive temperature. Arrows in d indicate cells with short spindles and a nucleus at the neck. A similar pattern was seen at the restrictive temperature (data not shown).

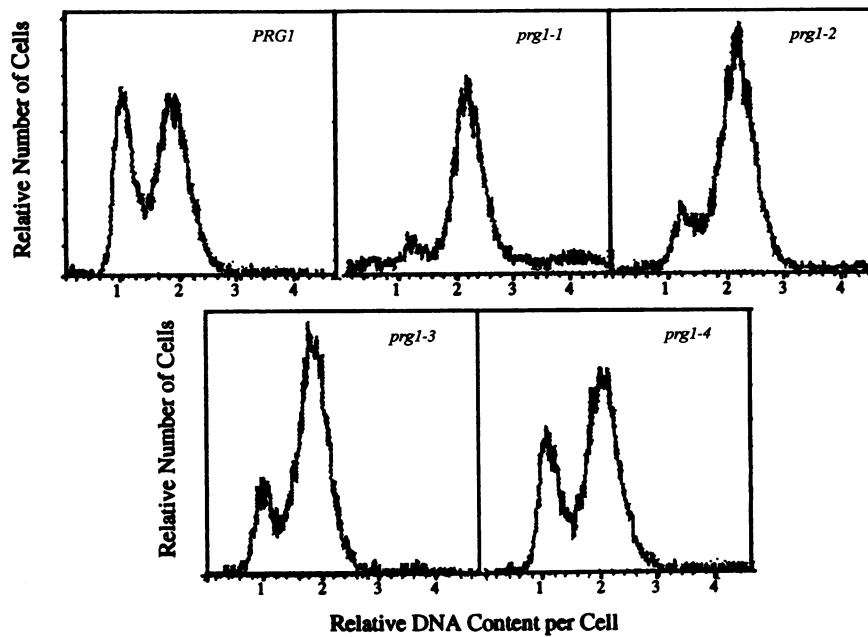


FIG. 4. Proportion of cells that contain duplicated DNA is enriched in *prg1^{ts}* populations. Cells grown at the permissive temperature were stained with propidium iodide and analyzed by fluorescence-activated cell sorting. Peak at position 1 corresponds to unreplicated DNA ($1n$) and that at position 2 corresponds to replicated DNA ($2n$). Similar results were obtained at the restrictive temperature (data not shown).

measuring the loss frequency of a nonessential chromosome VII derivative in *prg1* strains. The results presented in Table 1 indicate that each of the different mutant strains exhibits a substantial increase in chromosome loss at the permissive temperature compared to that of wild-type cells: chromosome loss ranged from an 86-fold increase for *prg1-1* to a 12-fold increase for *prg1-4*. Consistent with the results described above, the frequency of chromosome loss correlated with the severity of the growth defects and other phenotypes for the different *prg1* mutant strains. Thus, *PRG1* is important for chromosome maintenance and nuclear division in yeast.

Deletion of *CLB2* Suppresses the Temperature-Sensitive Growth Phenotype of *prg1^{ts}* Strains. Cyclins are known to regulate cell cycle progression, including nuclear division, and the degradation of mitotic cyclins is important for progression through mitosis (2, 36). It was therefore possible that *prg1* defects are due, at least in part, to an inability to degrade cyclin(s).

This possibility was examined by genetic tests in which a mitotic cyclin gene, *CLB2* or *CLB4*, was deleted in each of *prg1-2*, *prg1-3*, and *prg1-4* strains. As shown in Fig. 5, wild-type and *CLB2* deletion strains grow at 38°C, while *prg1-4* mutant cells do not. The *prg1-4 clb2-Δ* strains formed normal-sized colonies, indicating the *clb2-Δ* suppresses the *prg1-4* temperature-sensitive growth defect. Identical results were obtained for the *prg1-2* and *prg1-3* strains. Interestingly, the *clb4-Δ* mutation did not suppress the growth defects of

prg1-1, *prg1-2*, *prg1-3*, or *prg1-4* cells (data not shown; see Discussion).

We also found that a *clb2-Δ* mutation would suppress a complete deletion of the *PRG1* gene. As described in *Materials and Methods*, *prg1-Δ clb2-Δ* segregants from *prg1-Δ/PRG1 clb2-Δ/CLB2* strains were viable. *prg1* null cells containing the *clb4-Δ* mutation were not viable, as expected. Thus, these results indicate that *clb2-Δ* can suppress a *prg1* null strain, whereas *clb4-Δ* cannot.

DISCUSSION

In this study, we report the generation of strains containing four different temperature-sensitive mutations in the *PRG1* proteasome-related gene. Characterization of each of these strains indicates that *PRG1* is important for nuclear division in yeast. At both the restrictive and permissive temperatures the majority of cells contain replicated DNA. Furthermore, a significant fraction of cells contain a single nucleus at the neck and a short spindle. These results suggest that the nuclear division defect in *prg1* strains probably resides at early to midmitosis. Immunofluorescence studies of other

Table 1. Quantitation of chromosome VII fragment loss in various *prg1^{ts}* strains

Strain	Loss of CF per division, fold increase
<i>PRG1</i>	1 (5×10^{-4})
<i>prg1-1</i>	86
<i>prg1-2</i>	70
<i>prg1-3</i>	28
<i>prg1-4</i>	12

CF, chromosome fragment.

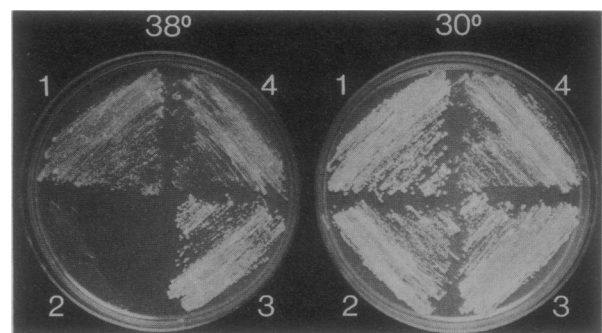


FIG. 5. A *CLB2* deletion suppresses the *prg1* temperature-sensitive growth phenotype. The growth of wild type (1), *prg1-4* (2), *clb2-Δ* (3), and *clb2-Δ prg1-4* (4) was examined at the permissive (30°C) and the restrictive (38°C) temperatures on plates containing rich medium.

eukaryotic cells indicate that proteasomes are localized to the spindle apparatus and/or chromosomes during metaphase but not during anaphase (12, 13). This localization is consistent with an early or midmitotic defect for proteasome function.

The *prg1* mutant strains do not have uniform arrest (i.e., *cdc*) phenotype at the restrictive temperature, and the various phenotypes of the *prg1* mutants are not exacerbated at the restrictive temperature. It is plausible to conclude that the temperature-sensitive growth defect of these strains is not a result of increased proteasome dysfunction but rather is a consequence of increased chromosome missegregation or some other defect at the elevated temperature. Consistent with the former possibility, *prg1-4* cells show a dramatic increase in chromosome missegregation at 35°C (10-fold over cells incubated at 24°C; data not shown). Other mutants of *S. cerevisiae* that do not have a *cdc* arrest, but are important for nuclear division and chromosome maintenance, have been identified; these include *ndc1* (nuclear division cycle) (37), *mpsi1* (monopolar spindle) (38), *top2* (topoisomerase II) (39), and *cmd1* (calmodulin) (40). The nonuniform arrest phenotype in these cases might be due, at least in part, to the consequences of chromosome imbalance in these strains.

The nuclear division defect of proteasomes is not specific to mutations in the Prg1p subunit. Recently, mutations in two probable components of the 26S proteasome complex, *cim5* and *cim3* of *S. cerevisiae*, have been shown to cause a nuclear division defect that is very similar to *prg1* cells (C. Mann, personal communication).

We hypothesize that the Prg1 protein, as part of the proteasome, functions in degradation of regulatory proteins such as cyclins and that the inability to degrade these proteins leads to a nuclear division defect, resulting in chromosome loss. Consistent with this hypothesis, a deletion of *CLB2*, a gene encoding a regulatory protein necessary for mitotic entry, suppresses the *prg1* temperature-sensitive growth phenotype. Although overproduction of Clb2p normally results in a block in late anaphase (36), it is possible that Clb2p also functions early in mitosis when it is still accumulating along with Clb1p, Clb3p, and Clb4p. Thus, removal of Clb2p will help pass an earlier mitotic block caused by overproduction of cyclin B proteins.

A deletion of *CLB4* did not suppress the temperature-sensitive growth defect of *prg1^{ts}* strains. Either Clb4p is not a substrate for the Prg1 proteasome or, more likely, Clb4p plays a smaller role in regulating nuclear division than Clb2p. Consistent with this latter hypothesis, deletion of *CLB2* causes a more severe growth defect when combined with mutations in other *CLB* genes than deletion of *CLB4* does (22).

These studies indicate that proteasomes are necessary for proper progression through nuclear division and that Clb2p is a likely candidate for degradation by proteasomes. One likely pathway for the removal of cyclins is that they are first ubiquitinated as described for cyclin B in vertebrate cells (5) and then targeted for degradation by proteasomes. It is also possible that additional regulatory or structural proteins are degraded by the proteasomes.

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