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The Glc7 Type 1 Protein Phosphatase of *Saccharomyces cerevisiae* Is Required for Cell Cycle Progression in G_2/M

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We isolated a mutant carrying a conditional mutation in the *GLC7* gene, encoding the catalytic subunit of a type 1 protein phosphatase, by selection of suppressors that restored the growth defect of *cdc24* mutants at high temperature and simultaneously conferred cold-sensitive growth. This cold sensitivity for growth is caused by a single mutation ($glc7^{Y\text{-}170}$) at position 170 of the Glc7 protein, resulting in replacement of cysteine with tyrosine. Genetic analysis suggested that the $glc7^{Y\text{-}170}$ allele is associated with a recessive negative phenotype, reducing the activity of Glc7 in the cell. The $glc7^{Y\text{-}170}$ mutant missegregated chromosome III at the permissive temperature, arrested growth as large-budded cells at the restrictive temperature, exhibited a significant increase in the number of nuclei at or in the neck, and had a short spindle. Furthermore, the $glc7^{Y\text{-}170}$ mutant exhibited a high level of *CDC28*-dependent protein kinase activity when incubated at the restrictive temperature. These findings suggest that the $glc7^{Y\text{-}170}$ mutation is defective in the G_2/M phase of the cell cycle. Thus, type 1 protein phosphatase in *Saccharomyces cerevisiae* is essential for the G_2/M transition.

The phosphorylation and dephosphorylation of proteins play a pivotal role in transduction of extracellular signals and regulation of a number of pathways of cellular metabolism (2, 8). Recently, attention has focused on the role of reversible phosphorylation in the regulation of the cell division cycle, especially in mitotic control. Many proteins, including histones, lamins, and cytoskeletal proteins, undergo marked changes in phosphorylation during M phase (25, 32). Concentrated phosphorylation of these substrates is thought to be required to initiate and coordinate the radical reorganization of the chromosome, nuclear envelope, and cytoskeleton that occurs during this transition. A serine/threonine protein kinase crucial for this process is Cdc2 kinase, conserved in organisms from yeasts to humans (24, 26). This kinase is active only after forming a complex with a cyclin partner (13). Activation of Cdc2 kinase apparently plays a central role in the entry of cells into mitosis (26, 31).

The phosphorylation state of a particular protein is determined by the relative activities of the protein kinases and protein phosphatases which recognize it as a substrate (8, 14). Since protein phosphorylation is important for the G_2/M transition, it can be expected that protein phosphatases play an important role in this process. Indeed, type 1 protein phosphatases (PP1s) have been implicated as being required for progression through mitosis. Mutants of *Schizosaccharomyces pombe* and *Aspergillus nidulans* carrying cold-sensitive mutations in the $dis2^+$/$bws1^+$ and *bimG* genes, respectively, encoding PP1s, arrest growth in mitosis at the restrictive temperature (4, 12, 35). In *Drosophila melanogaster*, mutations in one of the four genes which encode PP1 isozymes result in abnormal mitosis, chromosome segregation, and chromosome condensation (1). In mammalian cells, microinjection of anti-PP1 antibody causes cells to arrest growth at metaphase (16). These results indicate that PP1 acts at the M phase in the cell cycle.

The budding yeast *Saccharomyces cerevisiae* contains one gene, *GLC7*/*DIS2S1*, which encodes PP1. The *GLC7* gene was originally identified in mutants that fail to accumulate glycogen (37). Glycogen metabolism in mammalian systems is regulated by protein phosphorylation, with the glycogenolytic enzymes being activated and glycogen synthase being inhibited by phosphorylation (9). It has been suggested that Glc7 is a glycogen synthase phosphatase because glycogen synthase activity from the *glc7-1* mutant remains in an inactive, phosphorylated form (17, 37). Recently, it was shown that Glc7 acts in opposition to the kinase activity of Gcn2 in modulating the level of eIF-2α phosphorylation (46). Thus, PP1 in *S. cerevisiae* is involved in translational control in addition to having a role in glycogen metabolism. Furthermore, as neither translational control nor glycogen synthesis is essential for cell viability, it appears that Glc7 performs additional functions, because a deletion of *GLC7* is unconditionally lethal (15). Therefore, it can be predicted that PP1 in *S. cerevisiae* is also involved in the control of mitosis, as described for other organisms. However, the role of Glc7 on cell cycle control is not well characterized. This might be because appropriate conditional mutations in *GLC7* have not been obtained.

We have now identified a cold-sensitive mutant of *GLC7* as a suppressor of the growth defect of the temperature-sensitive *cdc24* mutant at the restrictive temperature. The *CDC24* gene is required for the establishment of cell polarity. *cdc24* mutants fail to bud and are unable to grow at 35°C (42). We report here the evidence that PP1 in *S. cerevisiae* is involved in the G_2/M transition.

MATERIALS AND METHODS

Strains, media, and general methods. The yeast strains used in this study are listed in Table 1. The *glc7*Δ::*LEU2* mutation has been previously described (29). *Escherichia coli* DH5α was used for DNA manipulation, and DL23 was used for expression of the suc1 protein (6, 44). Standard yeast manipulations were carried out (38).

The media used include rich medium (YEPD), synthetic complete medium (SC), minimal medium (SD), and sporulation medium (38). SC lacking amino acids or other nutrients (e.g., SC-ura, which lacks uracil) was used to score auxotrophies and to select transformants. YEPG was identical to

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TABLE 1. Strains used in this study

Strain	Genotype
15Dau	*MAT***a** *ura3 leu2 his2 trp1 ade1*
KMY2-3A	*MAT***a** *leu2 his3*
Y147	*MAT***a** *cdc24-4 ura3 leu2 his3*
Y147-R19	*MAT***a** *glc7*$^{Y\text{-}170}$ *cdc24-4 ura3 leu2 his3*
NH102-2C	*MAT***a** *glc7*$^{Y\text{-}170}$ *ura3 leu2 trp1*
NH102-4D	*MAT*α *glc7*$^{Y\text{-}170}$ *ura3 leu2 his2 trp1*
NH103-4A	*MAT***a** *ura3 trp1 ade2*
NH103-4C	*MAT***a** *glc7*$^{Y\text{-}170}$ *ura3 trp1 ade2*
NH105	*MAT***a** *ura3 trp1 ade2*
	*MAT*α *ura3 leu2 trp1 lys2*
NH106	*MAT***a** *glc7*$^{Y\text{-}170}$ *ura3 trp1 ade2*
	*MAT*α *glc7*$^{Y\text{-}170}$ *ura3 leu2 trp1 lys2*
NH107	*MAT***a** *glc7Δ::LEU2 ura3 leu2 his3 ade8 met3*
	*MAT*α *ura3 leu2 his2 trp1 ade1*
NH107-1C	*MAT*α *glc7Δ::LEU2 ura3 leu2 trp1 ade8*

YEPD except that it contained 2% galactose instead of glucose.

Yeast cells were transformed by the lithium acetate method with single-stranded DNA as the carrier (19). Other recombinant DNA procedures were carried out as described by Sambrook et al. (39).

Cloning of the *STF1/GLC7* gene. Strain NH102-2C was transformed with a YCp50 yeast genomic library at 30°C and then replica plated to YEPD medium at 14°C. After 5 days of incubation, two transformant clones gave confluent growth. Two plasmids (pI1 and pI2) that rescue the cold-sensitive (Cs^-) phenotype of NH102-2C were recovered from *S. cerevisiae* by transforming *E. coli*. They have identical DNA regions, as determined by restriction site mapping (see Fig. 2). The nucleotide sequence of the *Hin*dIII-*Xho*I fragment was determined by the dideoxy chain termination technique (40).

Plasmid constructions. Plasmid pKAIGLC7 was constructed by ligating the *Bam*HI fragment of pI2 into the *Bam*HI-*Bgl*II sites of pNH10 (*URA3* as a selection marker in a YEp-based plasmid). A 7-kb *Bam*HI fragment from pI2 was inserted into a *Sal*I site of YCplac33, producing YCplacGLC7. pGAGLC7 has *GLC7* under the control of the *GAL1* promoter carried on a YCp-based plasmid with *URA3* as a selection marker (30). pGAglc7^{Y170} is the same as pGAGLC7 except for the *glc7*$^{Y\text{-}170}$ mutation.

Gap repair. The *glc7*$^{Y\text{-}170}$ allele was cloned by the gap repair method (38). The 4-kb *Xho*I-*Eco*RI fragment was excised from pKAIGLC7. Strain NH102-2C was transformed with this linear gapped plasmid by selection for uracil prototrophy at 30°C and then replica plated at 14°C. The gap-repaired plasmid, pK2, was recovered from Ura^+ and Cs^- transformants and tested by restriction analysis for restoration of the missing *Xho*I-*Eco*RI fragment. A 3.3-kb *Xho*I fragment from pK2 was inserted into a *Sal*I site of YCplac33, producing YCplac glc7^{Y170}.

Measurement of glycogen accumulation. Glycogen accumulation was qualitatively assessed by inverting agar plates containing colonies of the strains of interest over I_2 crystals contained in a glass dish. Colonies develop a brown color with an intensity that is proportional to the glycogen content (7).

Cell cycle analysis. For the cell cycle analysis of the *glc7*$^{Y\text{-}170}$ mutant, cells were grown to a density of 5×10^6 cells per ml at 30°C and then the culture was shifted to 14°C. Samples were removed at 3-h intervals and prepared for flow cytometry as described previously (33).

Immunofluorescence microscopy. Cells were processed for fluorescence and indirect immunofluorescence microscopy by using protocols described by Hagan and Hyams (20). Cells were fixed and stained for DNA with 25 μg of 4,6-diamidino-2-phenylindole (DAPI) per ml. Microtubule structures were observed following formaldehyde fixation with the antitubulin monoclonal antibody TAT-1 (48) and a fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Organon Teknika Corp.).

A Zeiss selective UV filter was used to view DAPI images, and a Zeiss selective fluorescein isothiocyanate filter was used to view the tubulin images. Photographs were taken on Kodak Tri-X-Pan film rated at 400 ASA.

Protein kinase assays. A method described by Wittenberg and Reed (47) was followed to make whole-cell extracts. All procedures were performed at 4°C or on ice. Cells were harvested by centrifugation and washed once in ice-cold water. They were then resuspended in 1 to 2 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM sodium PP_i) containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride and 1 μg each of aprotinin, leupeptin, and pepstatin A per ml) and disrupted by vortexing with 0.5-mm glass beads. The cell debris was removed by centrifugation at 15,000 × *g* for 10 min. Protein concentrations were determined by using the Bio-Rad assay kit with bovine serum albumin as the standard. All samples were adjusted to equal protein concentrations with lysis buffer and used for determination of kinase activity.

The Cdc28 kinase assays were performed essentially as described by Wittenberg and Reed (47) except that the suc1-Sepharose beads to which the crude extracts had been adsorbed were used for the assays. The suc1 beads were prepared as described by Brizuela and Beach (6). The cell extract (100 μl) was added to 60 μl of suc1 beads (equilibrated with lysis buffer), and the suspension was incubated on ice for 1 h. The beads were then washed three times in radioimmunoprecipitation assay (RIPA) buffer (lysis buffer containing 150 mM NaCl), once in lysis buffer without SDS or sodium PP_i, and finally twice with reaction buffer (20 mM Tris-HCl [pH 7.5], 7.5 mM $MgCl_2$, 1 mM $ZnSO_4$). For the kinase reaction, suc1 beads prepared in this way were suspended in reaction buffer (a final volume of 40 μl) and preincubated at 37°C for 20 min. One microliter of 0.5 mg of a solution of histone H1 (Boehringer Mannheim Biochemicals) per ml was added, along with 1 μl of [γ-^{32}P]ATP (1 mCi, 6,000 Ci/mmol; Amersham) and 3 μl of 2× reaction buffer, and the reaction mix was incubated at 37°C for 1 h. Reactions were terminated by the addition of 30 μl of 3× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, and products were analyzed by electrophoresis on an SDS–12% polyacrylamide gel. The gel was then dried and exposed to Fuji New RX film. Phosphorylated histone H1 was visualized by autoradiography.

Analysis of chromosome III missegregation. Quantitative measurement of chromosome III loss and recombination was performed essentially as described previously (18). Strains to be tested were plated as single cells on YEPD plates and allowed to grow at 30°C to colonies consisting of ~50,000 cells. For each experiment, four colonies were plugged from the agar with a sterile stick and resuspended in 100 μl of YEPD. Of these cells, 0.05% were plated on YEPD to determine viable cell numbers. Mating was initiated by adding the remaining cells to 500,000 *MAT*α haploid testers (KMY2-3A) in a final volume of 145 μl. After 6 h at 30°C, the mating mixture was plated on medium selecting for prototrophic triploids. The colonies that formed were counted and scored for a Leu^+ phenotype by replica plating to SC-leu. Wild-type *MAT*α

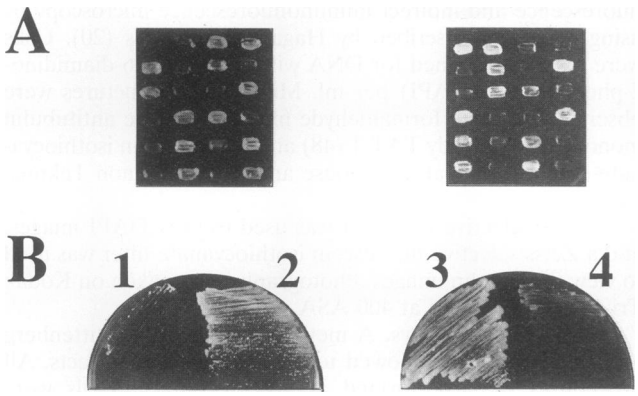

FIG. 1. Growth property of *cdc24-4 stf1-1* double mutants. (A) Y147-R19 (*cdc24-4 stf1-1*) was crossed with NH101-7C (*cdc24-4 STF1*), and the resulting diploid was sporulated and tetrads were dissected. The spores were allowed to germinate at 30°C and transferred to YEPD at 14°C (left panel) or 35°C (right panel). Each row of cells was derived from one tetrad. (B) Y147-R19 was transformed with YCp50 (segments 1 and 3) or pI1 (YCp50-STF1/GLC7) (segments 2 and 4). Transformants were patched onto SC-ura and incubated at 14°C (left panel) or 35°C (right panel).

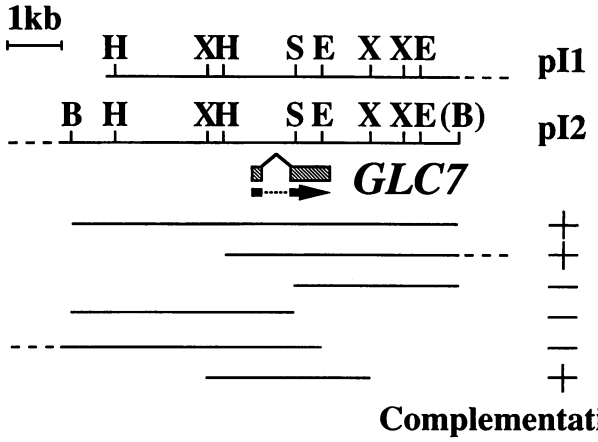

FIG. 2. Restriction map of the suppressor DNA fragment. The restriction maps of inserts from plasmids pI1 and pI2 are aligned. The site in parentheses is located in the multiple cloning region of the vector. The hatched boxes below the restriction map represent the exon regions of the *GLC7* open reading frame determined from the DNA sequence, and the line between the two boxes represents an intron. The ability of each plasmid to complement the growth defect conferred by *stf1-1* at 14°C is indicated on the right: +, complementation; −, no complementation. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I.

haploids were used as a control to determine mating efficiency. The number of maters obtained in each experiment was corrected on the basis of this efficiency.

RESULTS

Isolation of suppressors of *cdc24*. Cell polarity of *S. cerevisiae* is controlled by *CDC42* and *CDC24*, which encode a small G protein and its guanine-nucleotide-releasing protein, respectively (42, 49). The *cdc24* and *cdc42* mutants lose cell polarity and are unable to grow at 35°C. To identify factors involved in regulation of the Cdc24/Cdc42 pathway, we decided to isolate mutants that would suppress the growth defect of *cdc24* at 35°C and simultaneously could not grow at 14°C. Cells of the *cdc24-4* strain (Y147) were plated on YEPD plates and incubated at 35°C. Next, colonies that grew at 35°C were screened for cold sensitivity (Cs^-) by replica plating to YEPD at 14°C and incubating for 4 days. We isolated a total of 64 *cdc24* suppressor mutants that did not grow at 14°C. They were crossed with a *cdc24-4* strain, and the resulting diploids were sporulated and dissected. Of the 17 candidates tested, 8 showed that the ability to suppress *cdc24* and the Cs^- phenotype cosegregated; all Cs^- segregants grew at 35°C (Fig. 1A). This demonstrates that for each suppressor mutant, both phenotypes are caused by a mutation in a single gene. Genetic analysis indicated that these suppressor mutations are recessive with regard to both phenotypes and define six complementation groups. The suppressor loci were designated *stf* (for suppressor of *cdc* twenty-four). When suppressor mutants (*stf cdc24-4*) were crossed with a wild-type strain, tetrads resulted in a 2+:2− segregation at 14°C on YEPD, whereas they showed 4+:0−, 3+:1−, and 2+:2− segregations at 35°C. Thus, each single *stf* mutation caused cold-sensitive growth independently of *cdc24*. We further characterized the *stf1-1* mutation in this study. The *stf1-1* mutation also suppressed the growth defect of *cdc42-1* mutants at 35°C (data not shown).

***STF1* is identical to *GLC7*.** The *STF1* gene was cloned from a YCp50 yeast genomic library by complementation of the cold-sensitive phenotype. After approximately 2,000 transformants had been screened, two complementing plasmids (pI1 and pI2) were isolated. They were distinct but overlapping as determined by restriction mapping. The complementing region was localized to a 2.7-kb *Hin*dIII-*Xho*I fragment, which was included in both plasmids (Fig. 2). Determination of the nucleotide sequence within this region showed that it contains a previously described gene called *GLC7*, encoding a type 1 protein phosphatase (15, 35). The assignment of its phosphatase function was based on the deduced amino acid sequence of Glc7, which is 81% identical to the catalytic subunit of rabbit skeletal muscle type 1 protein phosphatase (3). Henceforth, we refer to the *STF1* gene as *GLC7* and to the *stf1-1* mutation as $glc7^{Y\text{-}170}$ (see below).

To demonstrate that *GLC7* is responsible for both the cold sensitivity and the *cdc24-4* suppression activity, plasmids YCp50 and pI1 (YCp50 containing *GLC7*) were introduced into the *cdc24-4* $glc7^{Y\text{-}170}$ strain. The transformants containing the *GLC7* plasmid grew at 14°C but not at 35°C, while those containing the control vector grew at 35°C but not at 14°C (Fig. 1B). These results indicate that the $glc7^{Y\text{-}170}$ mutation confers both suppression of *cdc24-4* and cold-sensitive growth.

GLC7 is involved in the regulation of glycogen metabolism. The *glc7-1* mutation was identified as conferring a defect in the steady-state levels of glycogen (37). This reduction in glycogen accumulation appears to result from diminished activation of glycogen synthase, which requires the PP1 activity of Glc7. Therefore, the measurement of glycogen accumulation is the conventional way to assess PP1 activity in the $glc7^{Y\text{-}170}$ mutant. To determine whether the $glc7^{Y\text{-}170}$ allele affects glycogen synthesis, the $glc7^{Y\text{-}170}$ mutant strain was transformed with low-copy-number plasmids containing wild-type *GLC7* or the vector alone and examined for glycogen accumulation. The $glc7^{Y\text{-}170}$ mutant cells containing *GLC7* accumulated high levels of glycogen compared with those containing the vector alone (Fig. 3), indicating that the $glc7^{Y\text{-}170}$ mutation is defective for the function of Glc7 involved in glycogen accumulation. Therefore, these results suggest that the activity of Glc7 is decreased in the $glc7^{Y\text{-}170}$ mutant even at the permissive temperature.

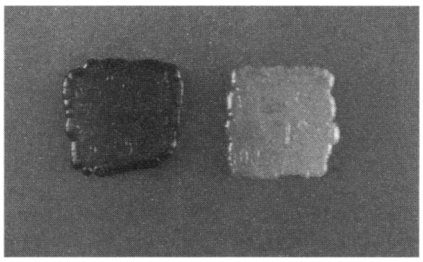

FIG. 3. Glycogen accumulation. NH102-4D ($glc7^{Y\text{-}170}$) was transformed with pI2 (YCp50-GLC7) (left) or YCp50 (right). Transformants were grown on SC-ura medium at 30°C and tested for glycogen accumulation by iodine vapor staining. Dark staining indicates glycogen accumulation.

Identification of the mutation site. To identify the site of the mutation in *GLC7* responsible for creating the Cs$^-$ phenotype, the $glc7^{Y\text{-}170}$ gene was isolated by the gap repair method as described in Materials and Methods. Subcloning analysis revealed that the mutation site resides within the *Sal*I-*Eco*RI segment of the *GLC7* gene (Fig. 4). The nucleotide sequence of this region from the mutant gene indicated a single nucleotide change (from G to A) that causes a single alteration of the amino acid sequence (from cysteine to tyrosine at position 170) (Fig. 5A). Cysteine at position 170 is a highly conserved residue among PP1s of different species, but *S. pombe* Dis2 and *A. nidulans* BimG have threonine residues at the corresponding positions (Fig. 5B). Interestingly, this cysteine residue is also conserved in PP2A and PP2B.

In order to confirm that the single $glc7^{Y\text{-}170}$ mutation is sufficient to cause cold-sensitive growth, we constructed a *glc7* deletion (*glc7*Δ::*LEU2*) strain carrying the $glc7^{Y\text{-}170}$ allele on a plasmid. The *glc7*Δ::*LEU2* allele contains the *LEU2* gene inserted in place of 803 bp of *GLC7* coding sequences (29). This allele was used to replace one copy of the wild-type *GLC7* gene in a diploid strain, producing NH107 (*glc7*Δ::*LEU2*/*GLC7*). After sporulation of this strain, all dissected tetrads contained only two viable spores and all spores were phenotypically Leu$^-$. This confirms the previous result (15) that *GLC7* is essential for growth. Next, the $glc7^{Y\text{-}170}$ allele was introduced into NH107 on the YCplac33 (*URA3*) plasmid and the resulting diploid transformants were sporulated. In tetrads dissected for each transformant at 30°C, all viable Leu$^+$ (*glc7*Δ::*LEU2*) spores were also Ura$^+$, indicating that they contained the *glc7*Δ::*LEU2* allele on the chromosome and the $glc7^{Y\text{-}170}$ allele on the plasmid. All of them exhibited the Cs$^-$ phenotype (data not shown). We conclude that the single $glc7^{Y\text{-}170}$ mutation is the cause of the cold-sensitive phenotype.

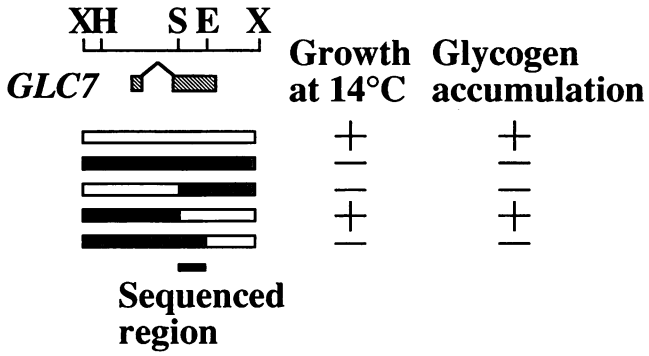

FIG. 4. Mapping of the region containing the $glc7^{Y\text{-}170}$ mutation. The hatched boxes below the restriction map represent the exon regions of the *GLC7* open reading frames, and the line between the two boxes represents an intron. Chimeric plasmids were constructed from YCplacGLC7 (the wild-type gene) (unshaded box) and YCplac glc7^{Y170} (the mutant gene) (shaded box). These chimeric plasmids were introduced into strain NH102-2C ($glc7^{Y\text{-}170}$) and examined for the ability to complement the $glc7^{Y\text{-}170}$ mutation. The ability of each plasmid to complement the growth defect at 14°C and the glycogen accumulation defect conferred by $glc7^{Y\text{-}170}$ is indicated on the right: +, complementation; −, no complementation. Glycogen accumulation was tested by iodine vapor staining as described in the legend to Fig. 3. Abbreviations: E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I.

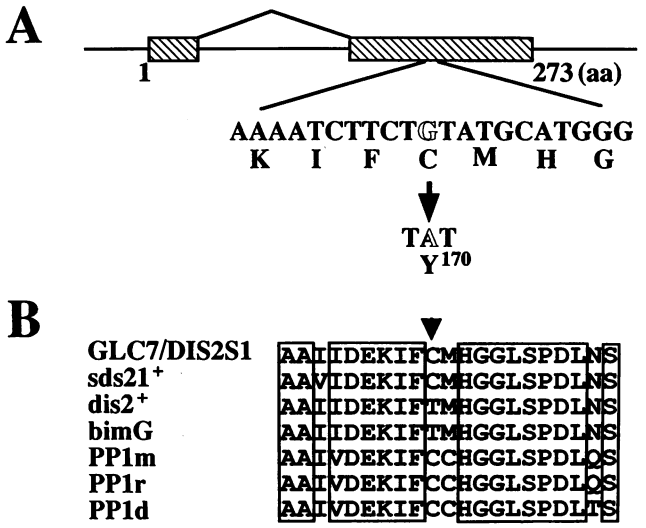

FIG. 5. A single amino acid substitution conferred by the $glc7^{Y\text{-}170}$ mutation. (A) The nucleotide sequence of the *Sal*I-*Eco*RI segment of the $glc7^{Y\text{-}170}$ gene (Fig. 4) was determined, and a substitution (from G to A) in the 170th codon was found. This causes a change of the corresponding amino acid from cysteine (C) to tyrosine (Y). The hatched boxes indicate the *GLC7* open reading frame, and the line between the boxes indicates an intron. aa, amino acids. (B) The cysteine residue (indicated by the arrow) in the mutation site is conserved between PP1s. The residues identical in all PP1s are boxed. sds21$^+$ and dis2$^+$, *S. pombe* PP1 (35); bimG, *A. nidulans* PP1 (12); PP1m, mouse PP1 (35); PP1r, rabbit PP1 (3); PP1d, *D. melanogaster* PP1 (11).

The results presented thus far suggest that the $glc7^{Y\text{-}170}$ allele is defective for Glc7 function and is associated with a recessive phenotype. Additional support for this idea is provided by the fact that overexpression of $glc7^{Y\text{-}170}$ does not cause any effect on cellular growth in *GLC7*$^+$ cells. The expectation is that if the $glc7^{Y\text{-}170}$ mutation gained a new activity for growth control, as in the case of the *S. pombe* *dis2-11* mutation (35), overexpression of $glc7^{Y\text{-}170}$ should show an effect dominant to that of *GLC7*$^+$ with regard to growth. When *GLC7*$^+$ controlled by the galactose-inducible *GAL1* promoter was introduced into wild-type *GLC7*$^+$ strain 15Dau on plasmid pGAGLC7, cells stopped growing in the medium containing galactose (Fig. 6). This result indicates that overexpression of *GLC7*$^+$ has a toxic effect on cellular growth. On the other hand, overexpression of the $glc7^{Y\text{-}170}$ allele from the *GAL1* promoter (pGAglc7^{Y170}) did not confer any detectable effect on cellular growth in the 15Dau strain. As expected, the *glc7*Δ::*LEU2* strain NH107-1C carrying pGAglc7^{Y170} exhibited galactose-dependent growth (Fig. 6) and the Cs$^-$ phenotype (data not shown). These results support the conclusion that

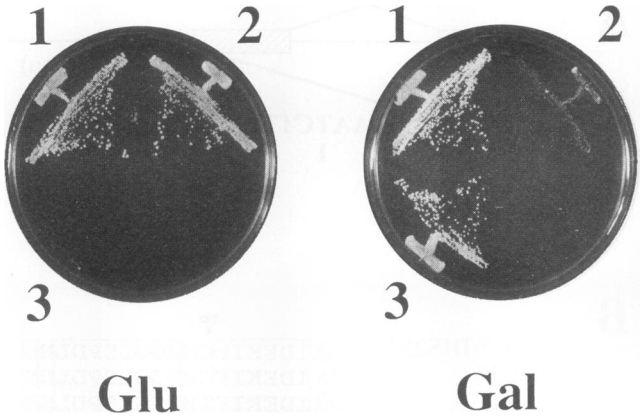

FIG. 6. Effect of overexpression of *GLC7* on cellular growth. Cells carrying pGAGLC7 (*GAL1 GLC7*$^+$) or pGAglc7^{Y170} (*GAL1 glc7*$^{Y-170}$) were grown for 2 days on YEPD (Glu) (left panel) or YEPG containing 2% galactose (Gal) (right panel). Segments 1, 15Dau (*GLC7*$^+$) carrying pGAglc7^{Y170}; segments 2, 15Dau carrying pGAGLC7; segments 3, NH107-1C (*glc7*Δ::*LEU2*) carrying pGAglc7^{Y170}.

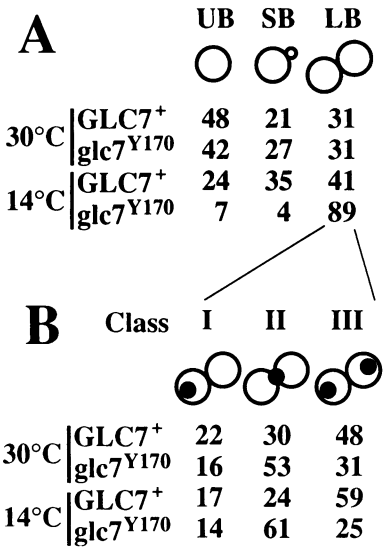

FIG. 8. Cell cycle arrest morphology in the *glc7*$^{Y-170}$ mutant. Log-phase cultures of NH102-4D (*glc7*$^{Y-170}$) carrying pI2 (YCp50-GLC7) (*GLC7*$^+$) or YCp50 (*glc7*$^{Y-170}$) at 30°C were shifted to 14°C for 14 h. Pre- and postshift cell samples were fixed, stained with DAPI, and observed by light microscopy and fluorescence microscopy. At least 500 cells were scored. (A) Percentage of cells with the indicated morphology: UB, unbudded; SB, small budded; LB, large budded. (B) Percentage of large-budded cells with the nuclear DNA staining region indicated characteristic of class I to class III cells.

phenotypes associated with the *glc7*$^{Y-170}$ allele are caused by loss of function.

The *glc7*$^{Y-170}$ mutation confers cell cycle arrest in the G_2/M phase. When *glc7*$^{Y-170}$ cells growing at 30°C were shifted to 14°C, cell proliferation ceased after 12 h. This arrest is reversible; cells transferred to 14°C for 30 h did not lose viability when returned to the permissive temperature (30°C) (data not shown). The arresting population was stained for DNA content with propidium iodide and analyzed by flow cytometry (Fig. 7). The proportion of cells with replicated DNA increased while that of cells with unreplicated DNA decreased after arrest at the restrictive temperature. This result indicates that the *glc7*$^{Y-170}$ mutation causes cells to arrest growth at the G_2/M phase of the cell cycle.

To assess where in the cell cycle the *glc7*$^{Y-170}$ cells accumulate, growth-arrested populations were analyzed microscopically (Fig. 8). When exponentially growing cultures of the *glc7*$^{Y-170}$ mutant carrying pI2 (YCp50-GLC7) (*GLC7*$^+$) or YCp50 (*glc7*$^{Y-170}$) at 30°C were shifted to 14°C for 14 h, approximately 90% of the *glc7*$^{Y-170}$ cells had arrested growth at the large-budded stage, compared with only 40% of the *GLC7*$^+$ cells. The numbers and positions of nuclear chromosomal DNA masses in the large-budded cells were determined by staining with the DNA-specific fluorescence dye DAPI. After incubation at 14°C for 14 h, about 60% of the large-budded *glc7*$^{Y-170}$ cells had a single nucleus at or through the neck between the mother and daughter cells (class II), while only 24% of the *GLC7*$^+$ cells had this morphology. Following a shift to 14°C, samples of wild-type and *glc7*$^{Y-170}$ cells were prepared for antitubulin immunofluorescence microscopy (Fig. 9). Figure 9B shows typical large-budded *glc7*$^{Y-170}$ cells. The class II *glc7*$^{Y-170}$ cells had a short mitotic spindle, consisting of a brightly stained bar of nuclear microtubules, with more faintly staining cytoplasmic microtubules connected to each end. These data demonstrate that the *glc7*$^{Y-170}$ mutation causes growth arrest in cells with a morphology indicative of the G_2/M preanaphase portion of the cell cycle.

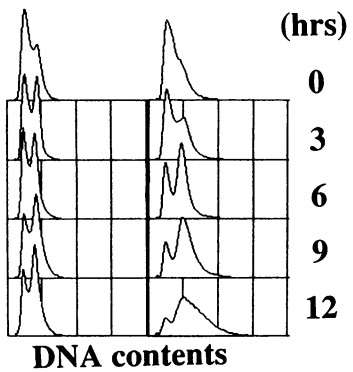

FIG. 7. Cell cycle analysis of the *glc7*$^{Y-170}$ mutant. Cells of NH103-4A (*GLC7*$^+$) (left) and NH103-4C (*glc7*$^{Y-170}$) (right) grown to exponential phase at 30°C were shifted to 14°C. Cellular DNA contents were determined by flow cytometric analysis at the indicated times. Peak broadening at late times is a result of autofluorescence due to large increases in cell size.

The G_2/M transition is controlled by the activation and inactivation of the Cdc2/Cdc28 protein kinase. In order to characterize the *glc7*$^{Y-170}$ G_2/M arrest phenotype further, we assayed the *CDC28*-dependent histone H1 kinase activity in the *glc7*$^{Y-170}$ mutant. The Cdc28 kinase was purified from the extracts by using suc1-Sepharose beads, and the level of this kinase activity was assayed by using bovine histone H1 as a substrate (Fig. 10). Extracts from *cdc28-4* mutant cells had little or no kinase activity (data not shown). This confirms that the kinase activity associated with suc1 beads was *CDC28* dependent. In asynchronous wild-type cells incubated at 14 or 30°C and *glc7*$^{Y-170}$ mutant cells incubated at 30°C, the *CDC28*-dependent kinase activities were detected. When the *GLC7*$^+$ cells were blocked with the microtubule-destabilizing agent nocodazole, the kinase activity was increased. Similarly, when *glc7*$^{Y-170}$ mutant cells were shifted to 14°C, the kinase activity was found to increase as cells arrested their growth. Thus, the

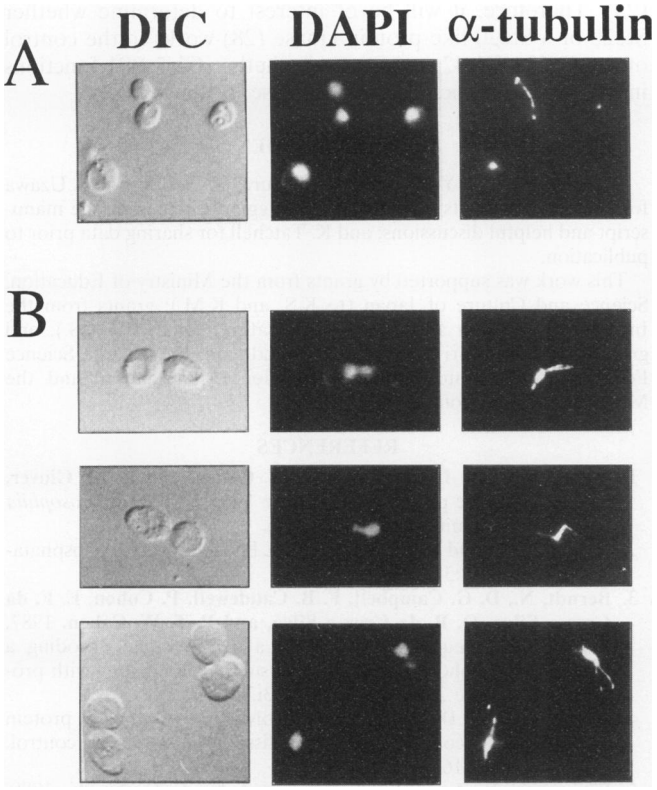

FIG. 9. Cell cycle arrest phenotype conferred by the *glc7*$^{Y\text{-}170}$ mutation. Cells of NH102-4D (*glc7*$^{Y\text{-}170}$) carrying pI2 (YCp50-GLC7) (A) or YCp50 (B) were incubated at 14°C for 14 h. Cells were fixed in formaldehyde and stained with antitubulin antibodies to visualize spindles. A differential interference contrast (DIC) micrograph of the cell (left panel), corresponding to nuclei stained with DAPI (middle panel), and spindles (α-tubulin) (right panel) are shown.

high level of Cdc28 kinase activity observed for the *glc7*$^{Y\text{-}170}$ mutant at the restrictive temperature is consistent with the preanaphase arrest phenotype.

The *glc7*$^{Y\text{-}170}$ mutation increases the rate of chromosome loss. Many mutants that arrest growth at the G_2/M phase have a defect in chromosome segregation (10, 18, 21, 45). Hartwell and Smith (21) have previously proposed that chromosome missegregation mutations fall into two groups: those affecting the pathways of DNA metabolism and those affecting the mitotic segregation machinery. On the basis of their proposal, a mutation conferring a defect in DNA metabolism was expected to cause increased rates of both chromosome loss and mitotic recombination, while a mitotic segregation mutation

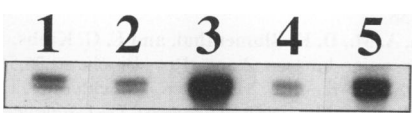

FIG. 10. *CDC28*-dependent kinase activity in the *glc7*$^{Y\text{-}170}$ mutant. Exponentially growing cells of NH102-4D (*glc7*$^{Y\text{-}170}$) carrying pI2 (YCp50-GLC7) at 30°C (lane 1) were shifted to 14°C and incubated in the absence (lane 2) or presence (lane 3) of nocodazole (15 μg/ml) for 14 h. Exponentially growing cells of NH102-4D carrying YCp50 at 30°C (lane 4) were shifted to 14°C and incubated for 14 h (lane 5). Cell extracts were prepared and histone H1 kinase activity was measured by the suc1-Sepharose bead assay (see Materials and Methods).

TABLE 2. Mitotic chromosome III missegregation and recombination frequencies in the *glc7*$^{Y\text{-}170}$ mutant[a]

Strain	Relevant genotype	Chromosome missegregation frequency (10^{-6})	Recombination frequency (10^{-5})
NH105	*GLC7/GLC7*	9 (1)	1.8 (1)
NH106	*glc7*$^{Y\text{-}170}$/*glc7*$^{Y\text{-}170}$	760 (85)	1.9 (1)

[a] The frequencies with which mating-competent diploids arose by mitotic recombination versus chromosome III missegregation in *GLC7/GLC7* (NH105) and *glc7*$^{Y\text{-}170}$/*glc7*$^{Y\text{-}170}$ (NH106) diploids were analyzed as described in Materials and Methods. Mating-competent diploids were scored as prototrophic triploids after incubation with a *leu2* mating tester (KMY2-3A). The fractions of these diploids that had mated by mitotic recombination because they were homozygous for *MAT*α were distinguished from those that had missegregated chromosome III by replica plating to plates lacking leucine. Frequencies were calculated from these numbers by the method described by Gerring et al. (18). Numbers in parentheses are normalized to wild-type (*GLC7/GLC7*) backgrounds.

was expected to cause only an increase in chromosome loss events. The effect of the *glc7*$^{Y\text{-}170}$ mutation on chromosome stability was examined by measuring the rates of mitotic missegregation and recombination of two markers, *MAT*α and *LEU2*, located on opposite arms of chromosome III (see Materials and Methods). The mitotic missegregation rate of chromosome III was elevated about 90-fold in the *glc7*$^{Y\text{-}170}$ homozygous diploid, while the mitotic recombination rate was not increased (Table 2). Thus, the *glc7*$^{Y\text{-}170}$ mutation dramatically increases chromosome III missegregation rates above wild-type levels and has no effect on mitotic recombination.

DISCUSSION

In this study, we identified and characterized a cold-sensitive mutation in the *GLC7* gene encoding the *S. cerevisiae* PP1. A single mutation (*glc7*$^{Y\text{-}170}$) in *GLC7* resulting in the change of cysteine to tyrosine at position 170 causes cold-sensitive growth. The *glc7*$^{Y\text{-}170}$ mutants arrest growth as large-budded cells with undivided nuclei at the restrictive temperature. Flow cytometric analysis showed that these cells had replicated their DNA. In the mutant cells, the nucleus is positioned at or near the neck between the mother and daughter cells and the spindle is short and intranuclear at the restrictive temperature. These phenotypes are characteristic of cells with growth arrested at preanaphase. Cell morphology and DNA content do not critically distinguish G_2 and M phases in *S. cerevisiae*. However, when the mutant cells arrest growth at the restrictive temperature, they exhibit an elevated Cdc2/Cdc28 protein kinase activity. Similar elevation of the kinase activity is observed when wild-type cells are treated with the microtubule-destabilizing agent nocodazole, resulting in mitotic arrest (22). Furthermore, mutations of PP1 in *S. pombe* (34), *A. nidulans* (12), and *Drosophila melanogaster* (1) cause arrest of the cell cycle in mitosis. It seems likely, therefore, that the *glc7*$^{Y\text{-}170}$ mutation causes the cells to accumulate in M phase. Taken together, these results suggest that PP1 in *S. cerevisiae* is involved in the mitotic transition.

S. pombe has two genes, *dis2*$^+$ and *sds21*$^+$, encoding PP1, and while a single disruption has no effect on cell growth, disruptions in both genes cause cell lethality (35). Accordingly, the cold-sensitive mutation in *dis2* is semidominant. Similarly, conditional mutations in *bimG* of *A. nidulans* have lethal effects in mitosis, but it seems that deletion of this gene is not lethal because of the presence of functionally equivalent homologs. Thus, phenotypes associated with the mutation of PP1 in *S. pombe* and *A. nidulans* are not strictly due to a loss of function. On the other hand, *S. cerevisiae* has only one gene,

GLC7, encoding PP1 and deletion of this gene is lethal. Therefore, phenotypes associated with the recessive *glc7*$^{Y\text{-}170}$ mutation are caused by a loss of function. This raises an interesting possibility that the G_2/M arrest caused by the *GLC7* mutation is specific for *glc7*$^{Y\text{-}170}$. In fact, a mutant carrying a temperature-sensitive mutation of *GLC7*, resulting in replacement of glycine with serine at position 62, did not show the G_2/M arrest (29). The specific cell cycle regulation phenotype associated with the *glc7*$^{Y\text{-}170}$ mutation might reflect a defective interaction with a regulatory subunit or substrate. Functional specificity of PP1 appears to be derived from regulatory subunits that target the catalytic subunit to the appropriate site in the cell or alter its substrate specificity. The best example of this kind of specificity comes from studies of a protein phosphatase that regulates glycogen synthesis in skeletal muscle. This phosphatase consists of PP1 and a regulatory subunit that targets PP1 to the glycogen particle. The *S. cerevisiae GAC1* gene may encode such a glycogen-specific regulatory subunit of Glc7 (17). Mutants with the *glc7-1* mutation are defective in glycogen accumulation but do not exhibit the cell cycle defect (15). The glycogen accumulation defect of *glc7-1* appears to be due to the inability of the mutant Glc7 phosphatase to interact with Gac1 (41). Similarly, an analogous regulatory subunit(s) could determine the function of PP1 in the control of cell cycle. The *sds22*$^+$ gene in the fission yeast *S. pombe* encodes a nuclear protein which interacts with PP1 and is essential for the metaphase-anaphase transition during mitosis (36, 43). This suggests that Sds22 is a targeting subunit which enhances the activity of PP1 towards nuclear substrates required for mitosis. It is possible that the *glc7*$^{Y\text{-}170}$ mutation might impair an interaction with such a cell cycle regulatory subunit. However, the *glc7*$^{Y\text{-}170}$ mutation also impairs glycogen accumulation. This observation could be explained if the mutant form of PP1, encoded by *glc7*$^{Y\text{-}170}$, was also defective for Gac1 interaction. Thus, the *glc7*$^{Y\text{-}170}$ mutation will be useful for identification of the regulatory subunit of PP1 critical for its role in mitosis.

Identification of the Glc7 target protein(s) is important for our understanding of the role of Glc7 in the control of cell cycle progression. A number of roles of PP1 in mitosis have been suggested. These include involvement in the dephosphorylation of mitotic substrates (5, 27), spindle dynamics (1), and sister chromatid disjoining (34). These putative roles suggest that the substrates of Glc7 could be regulators of mitosis, or of the spindle apparatus or other nuclear structural components. Indeed, the molecular motors in the kinetocores, responsible for the direction of chromosome movement, are controlled by phosphorylation and dephosphorylation. One might speculate that their dephosphorylation by PP1 might be part of this process.

Finally, we should discuss the relationship of *GLC7* to *CDC24*. Cdc24 acts as a GDP/GTP exchange factor for a small GTP-binding protein, Cdc42. The pathway mediated by Cdc24/Cdc42 regulates cell polarity. The *glc7*$^{Y\text{-}170}$ mutation can suppress not only the *cdc24-4* mutation but also the *cdc42-1* mutation, indicating that suppression by *glc7*$^{Y\text{-}170}$ is not allele specific. This implies that the Cdc24/Cdc42 pathway is negatively regulated by Glc7 PP1. Conversely, a protein kinase could play a positive role in the regulation of the Cdc24/Cdc42 pathway. Consistent with this possibility, it has recently been reported that in mammalian cells, the GTP-bound form of Cdc42Hs specifically complexes with a serine/threonine kinase (PAK), leading to kinase activation (28). This suggests that PAK participates in pathways regulated by Cdc42Hs. This kinase sequence is related to that of the *S. cerevisiae* Ste20 protein kinase, involved in the pheromone response pathway (23). Therefore, it will be of interest to determine whether Ste20 or a Ste20-like protein kinase (28) works in the control of the Cdc24/Cdc42 pathway and whether Glc7 PP1 functions in opposition to such a protein kinase in the pathway.

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