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The *EGP1* Gene May Be a Positive Regulator of Protein Phosphatase Type 1 in the Growth Control of *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae GLC7* gene encodes the catalytic subunit of type 1 protein phosphatase (PP1) and is required for cell growth. A cold-sensitive *glc7* mutant (*glc7*^{Y170}) arrests in G_2/M but remains viable at the restrictive temperature. In an effort to identify additional gene products that function in concert with PP1 to regulate growth, we isolated a mutation (*gpp1*) that exacerbated the growth phenotype of the *glc7*^{Y170} mutation, resulting in rapid death of the double mutant at the nonpermissive temperature. We identified an additional gene, *EGP1*, as an extra-copy suppressor of the *glc7*^{Y170} *gpp1-1* double mutant. The nucleotide sequence of *EGP1* predicts a leucine-rich repeat protein that is similar to Sds22, a protein from the fission yeast *Schizosaccharomyces pombe* that positively modulates PP1. *EGP1* is essential for cell growth but becomes dispensable upon overexpression of the *GLC7* gene. Egp1 and PP1 directly interact, as assayed by coimmunoprecipitation. These results suggest that Egp1 functions as a positive modulator of PP1 in the growth control of *S. cerevisiae*.

Phosphorylation and dephosphorylation of proteins play a pivotal role in the control of diverse cellular processes, including cell cycle regulation. The phosphorylation state of a particular protein is determined by the relative activities of the protein kinases and phosphatases that recognize it as a substrate (8, 9). Since protein phosphorylation is important for the G_2/M transition, it is expected that protein phosphatases will be shown to play an important role in this process. Indeed, studies indicate that type 1 protein phosphatase (PP1) is required for progression through mitosis. Mutations in PP1 in *Schizosaccharomyces pombe* (3, 36), *Aspergillus nidulans* (14), and *Drosophila melanogaster* (1) cause arrest of the cell cycle in midmitosis. In mammalian cells, microinjection of an anti-PP1 antibody causes cells to arrest at metaphase (4, 19).

PP1 in the budding yeast *Saccharomyces cerevisiae* is encoded by a single essential gene, *GLC7*/*DIS2S1* (18). Previous work suggests that in *S. cerevisiae*, PP1 is required for the control of mitosis as described for other organisms. We have shown that the cold-sensitive mutation *glc7*^{Y170}, which results in a cysteine-to-tyrosine substitution at position 170, causes arrest as large budded cells with undivided nuclei at the restrictive temperature (25). In mutant cells arrested at the restrictive temperature, the nucleus is positioned at or near the bud neck with a short intranuclear spindle. Furthermore, arrested cells exhibit elevated Cdc2/Cdc28 protein kinase activity. These results indicate that the *glc7*^{Y170} mutation is defective in the G_2/M phase of the cell cycle and that PP1 in *S. cerevisiae* is required for completion of mitosis.

The function of PP1 in cell cycle regulation adds to its previously demonstrated roles in the regulation of glycogen and cellular metabolism, modulation of protein synthesis, and relaxation of smooth muscle (7, 11, 51). To explain how a single enzyme can regulate such a diverse array of processes, Cohen and Cohen (10) introduced the concept of targeting subunits. They hypothesized that specific targeting or regulatory subunits direct PP1 to specific locations within the cell and selectively enhance phosphatase activity toward specific substrates. This model suggests that the functional specificity of PP1 is derived from such regulatory subunits. The best evidence for this model comes from studies of the phosphatase that regulates glycogen synthesis in skeletal muscle. This phosphatase consists of PP1 and a regulatory subunit that targets PP1 to the glycogen particle (26, 46). The *S. cerevisiae GAC1* gene is similar to the glycogen-binding subunit from skeletal muscle and has been shown to have a role in glycogen metabolism (21).

Similar mechanisms may be used to regulate and target PP1 activity to appropriate substrates during the cell cycle, particularly in mitosis. *sds22*$^+$ in *S. pombe*, isolated as a multicopy suppressor of a cold-sensitive PP1 mutant (*dis2-11*), is a candidate for such a cell cycle regulatory subunit (37). *sds22*$^+$ encodes a nuclear protein that physically interacts with PP1 and alters its substrate specificity. The *sds22*$^+$ gene is essential for the metaphase-anaphase transition during mitosis but is dispensable if *sds21*$^+$, one of two *S. pombe* PP1 structural genes, is overexpressed. On the basis of these results, Stone et al. proposed that Sds22 is a nuclear targeting subunit that enhances the activity of PP1 toward substrates required for mitosis (45).

To identify subunits of PP1 that participate in regulating the cell cycle, we have isolated mutations that enhanced the mutant phenotype of *glc7*^{Y170}. We report here the characterization of one such mutation, *gpp1*, and an extra-copy suppressor gene of *gpp1*, *EGP1*. *EGP1* encodes a leucine-rich repeat protein that is homologous to the fission yeast Sds22 protein and

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TABLE 1. Strains used in this study

Strain	Genotype
15D	*MAT***a**/*MAT*α *ura3*/*ura3 leu2*/*leu2 trp1*/*trp1 his2*/*his2 ade1*/*ade1*
15Dau	*MAT***a** *ura3 leu2 trp1 his2 ade1*
DF87	*MAT***a** *glc7*Δ::*LEU2 ura3 leu2 trp1* [pNC160:Ha-GLC7]
DF108	*MAT***a** *glc7*Δ::*LEU2 bar1-1 ura3 leu2* [pNC160:Ha-GLC7]
DF112	*MAT*α *glc7*Δ::*HIS3 gac1*::*URA3 ura3 leu2 his3 pep4*Δ*1* [YCp50:myc-GLC7]
KSC62	*MAT*α *ura3 leu2 trp1 his2 ade2*
NH102-2C	*MAT***a** *glc7*^{Y170} *ura3 leu2 trp1*
NH102-2C-M5	*MAT***a** *glc7*^{Y170} *gpp1-1 ura3 leu2 trp1*
NH109-3C	*MAT***a** *glc7*^{Y170} *gpp1-1 ura3 leu2 trp1 his2 ade1*
NH110	*MAT***a**/*MAT*α *egp1*Δ*1*::*LEU2*/*EGP1 ura3*/*ura3 leu2*/*leu2 trp1*/*trp1 his2*/*his2 ade1*/*ade1*
NH110-2B	*MAT***a** *egp1*Δ*1*::*LEU2 ura3 leu2 trp1 his2 ade1*
NH111	*MAT*α *EGP1*::*LEU2 ura3 leu2 trp1 his2 ade2*

which is essential for cell growth. Results from molecular and genetic analyses suggest that Egp1 is a positive modulator of PP1 in *S. cerevisiae*.

MATERIALS AND METHODS

Strains, media, and general methods. The yeast strains used in this study are listed in Table 1. *Escherichia coli* DH5α was used for DNA manipulations. Standard yeast manipulations were carried out (41).

The media used include rich medium (YEPD), synthetic complete medium (SC), nitrogen starvation medium, and sporulation medium (41). SC lacking amino acids or other nutrients (e.g., SC-Ura lacks uracil) was used to score auxotrophies and to select transformants. YEPG and SG were identical to YEPD and SC, respectively, except that glucose was replaced 2% galactose.

Yeast cells were transformed by the lithium acetate method with salmon sperm DNA as the carrier (22). Other recombinant DNA procedures were carried out as described by Sambrook et al. (42).

Mutant isolation. A *glc7*^{Y170} strain (NH102-2C) was mutagenized with ethyl methanesulfonate to a viability of 20%. Mutagenized cells were grown as single colonies on YEPD plates at 30°C, the permissive temperature for *glc7*^{Y170}. Colonies were replica plated onto YEPD and potassium acetate plates, both of which were incubated at 14°C for 6 days. Replicas were made of these plates onto YEPD. After a subsequent 3-day incubation at 30°C, growth of each colony was compared with that of the *glc7*^{Y170} strain. We isolated 13 colonies that died rapidly on YEPD but not on potassium acetate plates at 14°C. These candidates were then transformed with YCplacGLC7 carrying *GLC7* (25) or the YCplac33 empty vector (22) and tested for dependence of the lethal phenotype on the *glc7*^{Y170} mutation. We further characterized five mutants whose lethal phenotype was suppressed by wild-type *GLC7*.

Cloning of the *EGP1* gene. Strain NH102-2C-M5 (*glc7*^{Y170} *gpp1-1*) was transformed with a YCp50 yeast genomic library at 30°C and replica plated to YEPD medium at 14°C. After 8 days of incubation, the plates were replica plated to YEPD medium again and incubated at 30°C. Five colonies grew; two which did not accumulate glycogen were selected. Plasmids (p2-7 and p2-9) were recovered from these yeast cells by transforming *E. coli*. They exhibited the same restriction site pattern and could rescue the lethality of NH102-2C-M5 at 14°C.

The cloned insert could represent either the actual *gpp1* locus or a suppressor locus. To distinguish between these possibilities, we integrated the clone into the yeast genome by homologous recombination and tested for linkage to *gpp1-1*. A 3.5-kb *Bam*HI fragment from pBT2-9B (see below) was cloned into the *Bam*HI site of pUC18, producing pUC2-9B. A 2.2-kb *Sal*I-*Xho*I fragment carrying *LEU2* from YEp13 was inserted into the *Xho*I site (see Fig. 5) of pUC2-9B. This construct was cut with *Bam*HI and transformed into KSC62 (Table 1). Southern blot analysis of genomic DNA from the resulting strain, NH111, confirmed that the integration occurred at the *EGP1* locus. NH111 was mated to the *glc7*^{Y170} *gpp1-1* strain, NH109-3C. When sporulated, the Leu$^+$ phenotype showed no linkage with the *gpp1-1* mutation. Therefore, this cloned DNA does not contain the *GPP1* locus but instead encodes an unlinked suppressor locus. This locus was designated *EGP1* (extra-copy suppressor of *glc7 gpp1*). Furthermore, NH111 was crossed with the *egp1*Δ*1*::*LEU2* strain (NH110-2B) carrying plasmid YCp2-9B (*EGP1*$^+$). After sporulation and tetrad dissection of the diploid, all segregants were Leu$^+$. The nucleotide sequence of *EGP1* gene was determined by the dideoxy-chain termination technique (43).

Plasmid construction. Plasmids YCp2-9B, YEp2-9B, and pBT2-9B were constructed by inserting the 3.5-kb *Bam*HI fragment containing the *EGP1* open reading frame (ORF) from plasmid p2-9 into the *Bam*HI sites of YCplac33, YEplac112 (23), and pBluescript(SK+), respectively. To create YCpGEGP1, which contains the *EGP1* gene under the control of the *GAL1* promoter, *EGP1* was amplified by PCR from plasmid pBT2-9B, using primers designed to introduce a *Bam*HI site at the 5′ (5′-CCACATATAGGGATCCTTATGGAT-3′) end of the *EGP1* ORF. The PCR product was digested with *Bam*HI, cloned into the *Bam*HI site of pBluescript (SK+), and then subcloned into YCpG33 (13). Plasmids YEpGLC7 and YEpglc7^{Y170} were constructed by inserting the 3.3-kb *Xho*I fragments from pI1 and pK2 (25), respectively, into YEplac195 (23). Plasmid YCpGLC7 was constructed by inserting the 3.3-kb *Xho*I fragment from YEplacGLC7 (25) into YCplac33. Plasmid pNC160:Ha-GLC7 was constructed by ligating a *Hin*dIII-*Xho*I fragment encoding *Ha* (hemagglutinin gene)-*GLC7* at the *Hin*dIII-*Sal*I sites of pNC160 (40).

Disruption of *EGP1*. Plasmid pUC2-9BE was constructed by inserting a 1.4-kb *Eco*RI-*Bam*HI fragment from pBT2-9B into pUC18. A *Sac*I-*Xho*I fragment carrying *LEU2* from YEp13 was substituted for a *Sac*I-*Xho*I fragment of *EGP1* in pUC2-9BE to make the *egp1*Δ*1*::*LEU2* plasmid pDE1 (see Fig. 5). To make the *egp1*Δ*2*::*LEU2* plasmid, pBT2-9B was cut with *Eco*RI and self-ligated to delete a 1.4-kb *Eco*RI-*Bam*HI insert. Then, a 2.4-kb *Sal*I fragment from pDE1, which contains the *LEU2* gene and 3′ noncoding region of *EGP1*, was inserted into the plasmid to make pDE2 (see Fig. 5). This effectively deletes all but the first four amino acids of Egp1. The *egp1*Δ*1*::*LEU2* and *egp1*Δ*2*::*LEU2* mutations were introduced into the wild-type yeast diploid strain 15D (13) by one-step gene replacement (41). Although these constructions delete 134 bp of the noncoding region beyond the end of the *EGP1* ORF, this region is not required for the function of the neighboring gene, *MST1* (39). Furthermore, the phenotypes caused by the *egp1*Δ*1*::*LEU2* mutation were fully suppressed by YCpEGP1myc, which contains only the *EGP1* ORF and does not contain the 3′ noncoding region (data not shown).

Immunofluorescence microscopy. Cells were processed for fluorescence and indirect immunofluorescence microscopy as described previously (25). 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, using the antitubulin monoclonal antibody TAT-1 (52) and a fluorescein isothiocyanate-conjugated goat anti-mouse antibody as described previously (25).

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 (ASA 400).

Epitope-tagged *EGP1* gene. The DNA sequences encoding the epitope recognized by the anti-myc monoclonal antibody 9E10 (17), LEQKLISEEDLN, were inserted in *EGP1* at the position corresponding to the putative COOH terminus of Egp1. For this construction, two oligonucleotides, 5′-TCTAGAGCAAAAG CTCATTTCTGAAGAGGACTTGAATTGA-3′ and 5′-AAGCTTCAATTCA AGTCCTCTTCAGAAATGAGCTTTTGCT-3′, respectively, were made. They were annealed and inserted into the *Xba*I-*Hin*dIII site of YCplac33 to produce YCp33myc. Sequence analysis was used to confirm that the inserted sequence was correct. To create a fusion gene that encodes myc-tagged Egp1 at the COOH terminus, *EGP1* was amplified by PCR from plasmid pBT2-9B, using primers designed to introduce a *Bam*HI site at the 5′ (5′-CTAAGGATCCGTCCCCG CTCT-3′) and 3′ (5′-CAAGGATCCCCTCTTATATATGTCGCATCAAT-3′) ends of the gene. The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of YCp33myc to give YCpEGP1myc. Sequence analysis was used to confirm that the myc-*EGP1* fusion was in the correct frame. Since *EGP1* is an essential gene (see Results), we transformed YCpEGP1myc or YCp33myc into an *egp1*Δ*1*::*LEU2* strain (NH110-2B) carrying YEp2-9B, which contains the wild-type *EGP1* gene with the *TRP1* marker. By plasmid shuffling, only cells containing YCpEGP1 myc could become Trp$^-$, indicating that the plasmid could rescue the lethality of an *egp1*Δ deletion. Plasmid YEpEGP1myc was constructed by inserting a *Kpn*I-*Hin*dIII fragment from YCpEGP1myc into the *Kpn*I-*Hin*dIII site of YEplac195. YEpEGP1myc was introduced into a *glc7*Δ::*LEU2* strain carrying pNC160:Ha-GLC7 (DF87) and used for the immunoprecipitation assay.

Immunoprecipitation. Preparation of ^{35}S-labeled extracts and immunoprecipitation assays were performed as previously described (47). Immunoprecipitates were resuspended in 30 μl of protein gel loading dye (0.6 M Tris [pH 6.8], 10% glycerol, 2% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 0.05% bromophenol blue), boiled for 3 min, and electrophoresed on SDS–10% polyacrylamide gels as described previously (30). After electrophoresis, the gels were fixed in 20% methanol–10% acetic acid and soaked in Amplify fluorography solution (Amersham). The dried gels were fluorographed with X-Omat R film (Kodak) at −70°C.

For cell cycle arrest experiments, strain DF108 transformed with YEpEGP1myc was pregrown for 2 days in selective minimal media at 30°C. These cells were used to inoculate four YEPD cultures at a density of 3×10^6. Following a 3-h incubation at 30°C, cultures were treated with either 20 μg of benomyl per ml, 15 mg of hydroxyurea per ml, 3×10^{-7} M α-factor, or 1% dimethyl sulfoxide. Cultures were grown for an additional 3 h at 30°C (25°C for benomyl-treated cells) and then harvested for preparation of extracts and immunoprecipitation. Phosphatase inhibitors (1 mM $NaPP_i$, 1 mM sodium vanadate, 10 mM NaF, and 10 mM β-glycerylphosphate) were included in the standard breaking buffer.

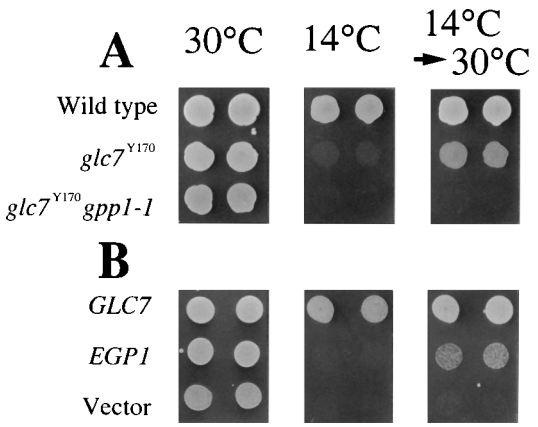

FIG. 1. Growth properties of *glc7*^{Y170} *gpp1-1* cells. (A) A total of 4×10^5 cells from each strain grown in YEPD liquid medium at 30°C were spotted onto YEPD plates and incubated at 30°C for 2 days (left panel) or 14°C for 5 days (middle panel). After 5 days of incubation at 14°C, plates were shifted to 30°C and incubated for 1 day (right panel). Strains used: row 1, wild-type strain 15Dau; row 2, *glc7*^{Y170} strain NH102-2C; row 3, *glc7*^{Y170} *gpp1-1* strain NH102-2C-M5. (B) A total of 4×10^5 cells from each strain grown in SC-Ura liquid medium at 30°C were spotted on YEPD and incubated at 30°C for 3 days (left panel) or 14°C for 8 days (middle panel). After 8 days at 14°C, plates were shifted to 30°C and incubated for 2 days (right panel). The strains used are NH102-2C-M5 carrying YCpGLC7 (*GLC7*; row 1), YCp2-9B (*EGP1*; row 2), and YCplac33 (Vector; row 3).

RESULTS

Isolation of the *gpp1* mutation. *glc7*^{Y170} cells remain viable at the G_2/M phase when shifted to the nonpermissive temperature of 14°C (25). In an effort to identify new components that function with PP1 in the regulation of the G_2/M transition, we isolated mutations that led to a loss of cell viability in *glc7*^{Y170} cells during an extended incubation at 14°C. We reasoned that genes whose mutations exacerbated the mutant phenotype in *glc7*^{Y170} cells might participate with *GLC7* in regulating the G_2/M transition. A conceptually similar scheme was used to search for downstream effectors of the *Drosophila sevenless* protein kinase (44). Cells of the *glc7*^{Y170} strain (NH102-2C) were mutagenized with ethyl methanesulfonate and spread on YEPD plates at a density to give individual colonies at the permissive temperature (30°C). Colonies were then replica plated to YEPD at 14°C. After 6 days, cells were replica transferred back to YEPD at 30°C. Potential mutants were those that recovered poorly from this regimen relative to recovery on a potassium acetate plate on which yeast cells arrest in G_1 (see Materials and Methods). This control provided some specificity for mutants sensitive to arrest at G_2/M. We isolated five colonies unable to form colonies on the final YEPD replica plate following 3 days of incubation at 30°C (Fig. 1). Genetic analysis indicated that these mutations are recessive and define at least two complementation groups. The loci were designated *gpp* (*glc7*^{Y170} phenotype potentiator). We further characterize the *gpp1-1* mutation in this study.

When a *glc7*^{Y170} *gpp1-1* mutant strain was crossed to a wild-type strain, tetrads resulted in a 2+:2− segregation for growth at 14°C on YEPD, whereas they showed 4+:0−, 3+:1−, and 2+:2− segregation for survival at 14°C. These results indicated that the potentiating mutation (*gpp1-1*) was unlinked to *GLC7* and that the *gpp1-1* mutation was not lethal in the wild-type *GLC7* background. To test the possible effect of the *gpp1-1* mutation on cell cycle progression in *glc7*^{Y170} cells, growth-arrested populations were analyzed microscopically (Fig. 2). Exponentially growing cultures of the *glc7*^{Y170} or *glc7*^{Y170} *gpp1-1* cells under permissive conditions (30°C) were shifted to the restrictive temperature (14°C). As described previously (25), approximately 90% of the *glc7*^{Y170} cells arrested growth as large-budded cells with a single nucleus at the neck of the bud. On the other hand, the *glc7*^{Y170} *gpp1-1* cells arrested as a morphologically heterogeneous population with greatly elongated buds, multiple buds, and/or amorphous shapes. DAPI staining showed that the double-mutant cells contained separated nuclei and were often multinucleate. The latter phenotype could be due either to fragmented nuclear DNA or to an

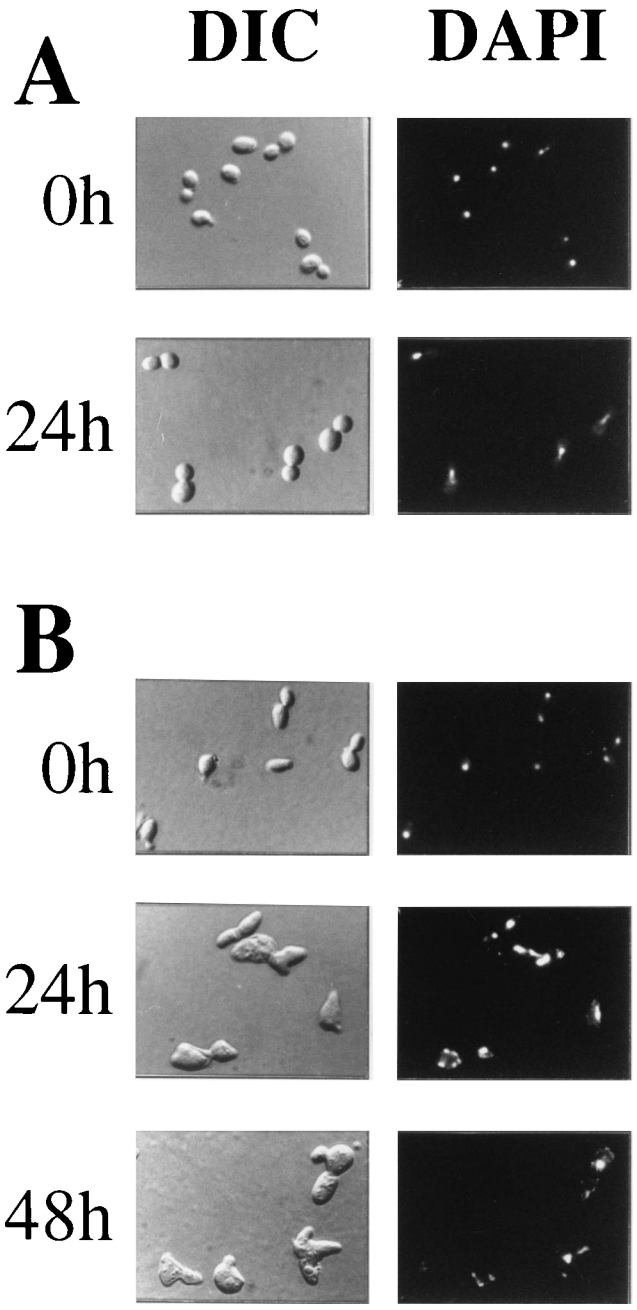

FIG. 2. Morphology of cells. Logarithmic cultures of *glc7*^{Y170} (NH102-2C; A) and *glc7*^{Y170} *gpp1-1* (NH102-2C-M5; B) strains grown in YEPD at 30°C were shifted to 14°C. Aliquots of cultures were fixed at 0, 24, and 48 h after the shift. Fixed cells were stained with DAPI to visualize DNA and were also examined with Nomarski optics (differential interference contrast [DIC]).

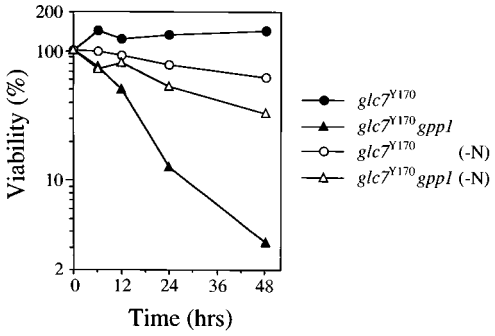

FIG. 3. Viability of cells. Logarithmic cultures of *glc7*^{Y170} (NH102-2C) (circles) and *glc7*^{Y170} *gpp1-1* (NH102-2C-M5) (triangles) strains grown in YEPD at 30°C were divided into two portions. Cells in one part (closed symbols) were shifted to 14°C. Cells in the other part (open symbols) were harvested, washed, and suspended in nitrogen starvation medium. After incubation at 30°C for 24 h, cultures were shifted to 14°C. Aliquots were removed at the indicated time points, and the number of viable CFU on YEPD agar at 30°C was determined.

excess of mitochondria in these cells. After prolonged incubation at 14°C (48 h), the abnormal morphologies were enhanced.

The *gpp1-1* mutation leads to cell death in *glc7*^{Y170} cells, which arrest at the G_2/M transition. We anticipated that if cell death correlated with the G_2/M arrest, blocking cell cycle progression at a point before G_2/M might rescue *glc7*^{Y170} cells from the lethality caused by the *gpp1-1* mutation. To test this possibility, we examined if blocking cells in G_0 phase by nitrogen starvation could prevent lethality of the *glc7*^{Y170} *gpp1-1* double mutation. Cultures of *glc7*^{Y170} or *glc7*^{Y170} *gpp1-1* cells were first grown at 30°C and then starved for nitrogen to arrest cells in G_0. Following this treatment, the cultures were transferred to 14°C. At various time points, aliquots were removed and tested for viability by plating at 30°C (Fig. 3). Transfer of *glc7*^{Y170} *gpp1-1* cells from 30 to 14°C in YEPD led to a loss of cell viability, whereas *glc7*^{Y170} *GPP1*$^{+}$ cells retained cell viability. However, nitrogen starvation blocked the loss of cell viability. Furthermore, arrest of cells in S phase by hydroxyurea treatment partially prevented the lethality of the double mutation (data not shown). Thus, blockage or delay of cell cycle progression at a point before G_2/M phase largely rescued *glc7*^{Y170} cells from the lethality caused by the *gpp1-1* mutation. This finding suggests that the cell death in *glc7*^{Y170} *gpp1-1* requires progression into G_2/M.

Identification of *EGP1*, a suppressor of *gpp1-1*. In an attempt to clone the *GPP1* gene, yeast strain NH102-2C-M5 (*glc7*^{Y170} *gpp1-1*) was transformed with a yeast genomic library constructed in YCp50, and approximately 2,000 independent transformants were screened for complementation of the lethal phenotype of *glc7*^{Y170} *gpp1-1* cells at 14°C. Five colonies were isolated, three of which reverted two traits associated with *glc7*^{Y170}, cold sensitivity and failure to accumulate glycogen, suggesting that they contained a plasmid bearing the wild-type *GLC7* gene. Plasmids were recovered from the remaining two transformants. The two plasmids were identical; they conferred viability to NH102-2C-M5 at 14°C but failed to restore glycogen accumulation (data not shown) or colony formation at 14°C (Fig. 1B). Plasmid integration and genetic linkage experiments (see Materials and Methods) revealed that the cloned chromosomal locus was unlinked to *gpp1-1*. Therefore, this new locus represents an extra-copy suppressor of *gpp1-1*. We have named the gene at this locus *EGP1*, for extra-copy suppressor of *glc7 gpp1*.

Subcloning was used to identify the size of the smallest DNA fragment carrying the *EGP1* gene (see Fig. 5). The nucleotide sequence revealed a single long ORF that potentially encodes a protein of 338 amino acids. DNA sequence comparison showed that the *EGP1* gene corresponds to *YKL193c* of chromosome XI (6, 15). A search of the GenBank database revealed that the *EGP1* product is 47% identical to the fission yeast *sds22*$^{+}$ gene product (Fig. 4). It has been proposed that Sds22 is a regulatory subunit of the Dis2/Sds21 PP1 catalytic subunits and that Sds22-bound phosphatase carries a key phosphatase activity essential for progression from metaphase to anaphase (37, 45). Sds22 consists of leucine-rich 22-amino-acid repeat motifs that are characterized by regularly spaced leucine residues and a strictly conserved asparagine residue at position 17 within each repeat (Fig. 4). The Egp1 protein contains 11 internal repeats of this 22-residue motif. The first 60 residues in the NH_2-terminal region and the last 33 residues in the COOH-terminal region do not contain the repeat element, but these regions are also conserved between Egp1 and Sds22. The NH_2-terminal region is highly acidic, containing 19 aspartic acid or glutamic acid residues.

***EGP1* is an essential gene.** To determine the functional importance of Egp1 in vivo, a diploid yeast strain with a portion of one of its two *EGP1* genes replaced by the *LEU2* gene was constructed (Fig. 5A). Southern blot analysis confirmed that one of the two *EGP1* genes in the original diploid was replaced with the *LEU2* marker (data not shown). Sporulation and tetrad analysis of this heterozygous (*EGP1*/*egp1*Δ*1*::*LEU2*) diploid strain (NH110) showed that only two of the four spores per tetrad were viable at 30°C on rich YEPD medium (Fig. 5B). All viable spores were Leu^{-}, indicating that the inviable spores contain the *egp1*Δ*1*::*LEU2* replacement. Microscopic examination revealed that mutant spores from NH110 germinated and completed one to two rounds of cell division, and then one-half of them arrested with a uniform large bud morphology. This inviability does not result from a defect acquired during spore germination. When the *EGP1*/*egp1*Δ*1*::*LEU2* diploid containing the wild-type *EGP1* gene on a *URA3-CEN* plasmid (YCp2-9B) was sporulated and the tetrad was dissected, haploid *egp1*Δ*1*::*LEU2* progeny containing the wild-type *EGP1* gene on YCp2-9B were obtained. This strain cannot segregate viable cells that have lost the plasmid. These results show that *EGP1* is essential. The *egp1*Δ*1*::*LEU2* cells carrying YCp2-9B showed normal phenotypes with regard to growth rate and cell cycle progression.

In *S. pombe*, overexpression of *sds21*$^{+}$, encoding one of two PP1 isozymes, rescues the lethality of the *sds22* deletion mutant (37). Therefore, we examined whether inviable *egp1*Δ*1*::*LEU2* cells can be rescued by overexpression of *GLC7*. A multicopy *URA3*-containing plasmid carrying the *GLC7* gene (YEpGLC7) or the *URA3*-containing control plasmid (YEp195) was introduced into the *egp1*Δ*1*::*LEU2* strain (NH110-2B) containing the *EGP1* gene on the *TRP1*-containing plasmid (YEpEGP1). We then tested the ability of the transformants to survive loss of plasmid YEpEGP1. With the transformants carrying the control plasmid (*URA3*) and the *EGP1* plasmid (*TRP1*), we found no Trp^{-} segregants (Table 2). When the *GLC7* plasmid (*URA3*) was present in addition to the *EGP1* plasmid (*TRP1*), Trp^{-} segregants were observed (Table 2), demonstrating that *egp1*Δ*1*::*LEU2* is rescued by overexpression of *GLC7*. In contrast, overexpression of *glc7*^{Y170} did not rescue the lethality of *egp1*Δ*1*::*LEU2* (Table 2). A similar experiment showed that overexpression of *EGP1* on the YEp plasmid (YEp2-9B) failed to suppress the lethality of *glc7*Δ::*LEU2* cells (data not shown). These results are consistent with the premise that *EGP1* encodes a positive regulatory subunit of PP1.

To characterize the phenotype of cells defective in *EGP1*, we

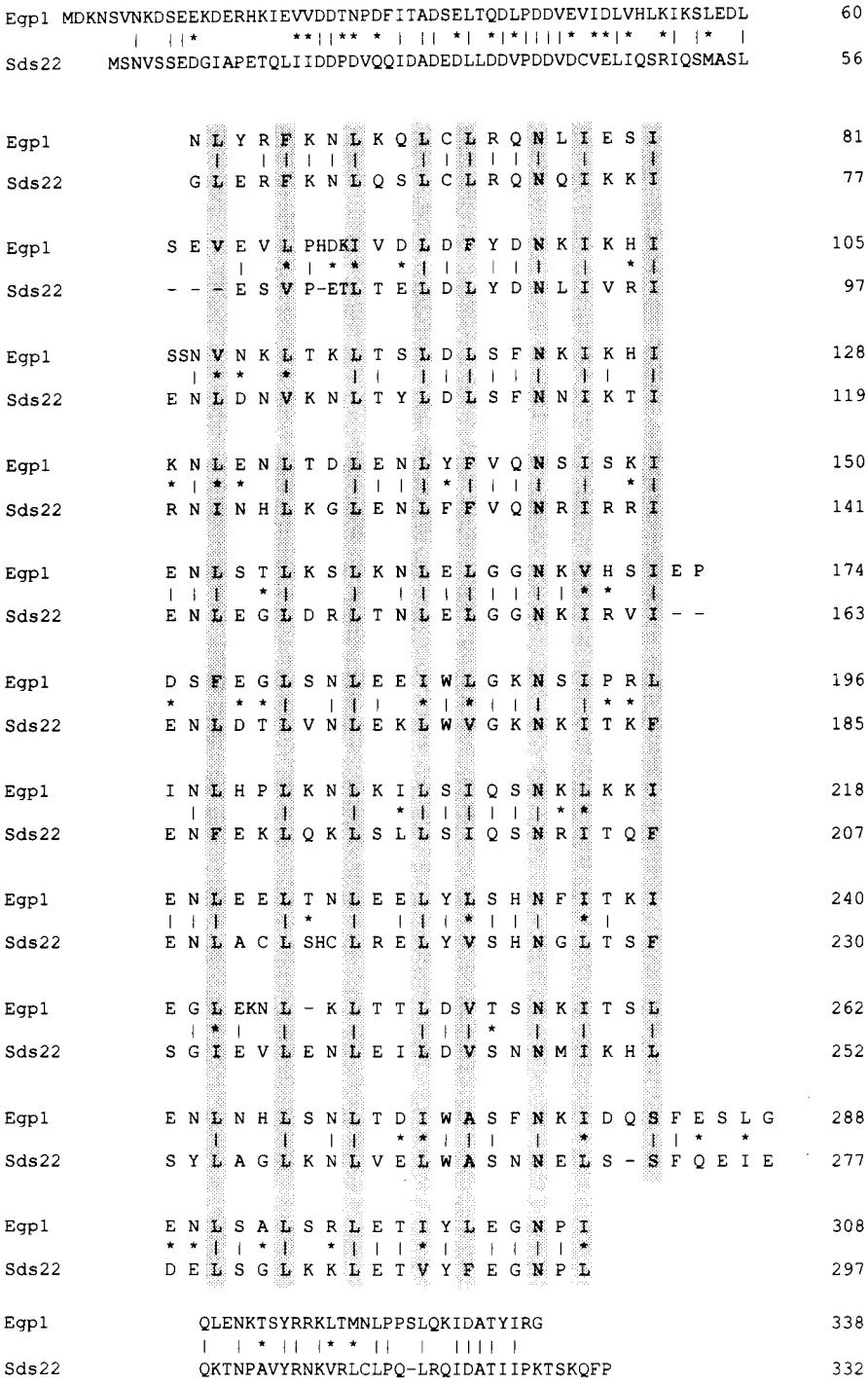

FIG. 4. Comparison of amino acid sequences of Egp1 and *S. pombe* Sds22. Amino acids identical between Egp1 and *S. pombe* Sds22 are indicated by vertical lines, and conservative replacements are indicated by asterisks. The 11 tandem leucine-rich repeats are aligned. The leucine-rich repeat motifs are indicated by shadowing.

constructed a plasmid, YCpGEGP1, in which *EGP1* was placed under the control of the *GAL1* promoter, allowing *EGP1* to be induced and repressed on galactose- and glucose-containing media, respectively. The plasmid was used to study the consequences of *EGP1* depletion in vegetatively growing cultures. Strain NH110-2B (*egp1Δ1*::*LEU2*) harboring YCpGEGP1 grew normally in galactose medium (YEPG). When *EGP1* gene expression was repressed by shifting the cells to glucose (YEPD), cell division began to slow after 24 h and finally stopped by 40 h with a decrease in viability (Fig. 6). The long lag time needed to completely shut down cell division in the repressed cultures is probably due to overexpression of *EGP1* in galactose, thus leading to a high residual level of the protein, which requires several generations to be depleted. After 40 h in glucose, approximately 60% of the *EGP1*-depleted cells were arrested in the large-bud stage of the cell cycle, compared with

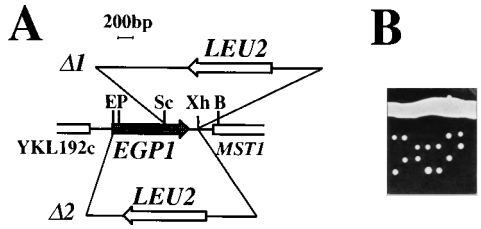

FIG. 5. Disruption of *EGP1*. (A) Structure of the *EGP1* deletion. The adjacent gene *YKL192c* is similar to the *Neurospora crassa* mitochondrial acyl carrier protein gene (6), and *MST1* encodes a mitochondrial threonyl-tRNA synthetase (39). The region extending leftward from *Xho*I is not required for *MST1* function (39). Restriction sites: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; Sc, *Sac*I; Xh, *Xho*I. (B) An *EGP1*/*egp1*Δ*1*::*LEU2* heterozygous diploid was sporulated, dissected, and grown for 4 days at 30°C. The four spores from individual asci are aligned vertically. Only two Leu$^-$ cells out of four spores grew on the YEPD plate after germination at 30°C.

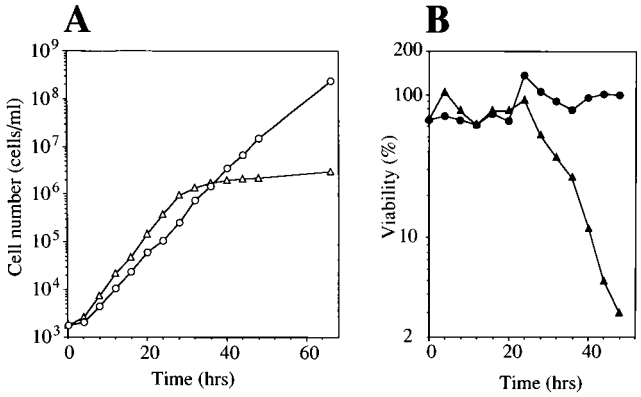

FIG. 6. Loss of *EGP1* function. A logarithmic culture of strain NH110-2B (*egp1*Δ*1*::*LEU2*) carrying YCpGEGP1 (*GAL1p-EGP1*) grown in SG-Ura containing 2% galactose at 25°C was diluted directly into YEPG (circles) and YEPD (triangles). At the times indicated, cell number (A) and viability (B) were assayed. Cell number was determined by phase-contrast light microscopy with a hemacytometer. Percent viability was determined by comparing the colony number obtained after incubation for 48 h in YEPG with that expected from the number of cells observed in the plated sample.

only 23% of cells grown in the presence of galactose. The majority of the remaining *EGP1*-depleted cells were unbudded (29% + 7% + 4% = 40%) (Fig. 7).

The numbers and positions of nuclear chromosomal DNA masses were determined by staining cells with the DNA-specific fluorescence dye DAPI (Fig. 7 and 8). Most notably, many *EGP1*- depleted large-budded cells contained two DAPI-staining masses in one cell body (15%) (Fig. 8A, cells 1 to 3). Cultures of the *egp1*Δ*1*::*LEU2* cells in the glucose-containing medium also contained significant numbers of other aberrant morphological types. Approximately 7% of the total *EGP1*-depleted cells were multinucleate (unbudded cells containing two or more nuclei) (Fig. 8A, cell 4) and aploid (unbudded cells with no nuclear DNA staining) (Fig. 8A, cell 5). These two classes presumably arose from cytokinesis of large-budded cells containing two nuclei in one cell body. The *egp1*Δ*1*::*LEU2* cells overexpressing *GLC7* did not contain abnormal nuclear morphologies (Fig. 7 and 8).

In *S. cerevisiae*, microtubules are required for separation of chromosomes during mitosis. In addition, they are required for the migration of the dividing nucleus to the neck between mother and daughter cell bodies (27). To determine if microtubule structure or spindle orientation was affected in the *EGP1*-depleted cells containing two or more nuclei in one cell body, we observed the organization of microtubules by indirect immunofluorescence using an antitubulin antibody. A spindle structure in the *EGP1*-depleted large-budded cells displaying a binucleated phenotype was seen to connect the two DAPI-staining regions, but it was misaligned with respect to the mother/bud axis (Fig. 8A, cells 1 and 2). These results suggest that the binucleate phenotype in large-budded cells arose from the misorientation of spindles. In addition, some *EGP1*-depleted large-budded cells had abnormal spindle structures (Fig. 8A, cell 3). Thus, Egp1 and PP1 appear to be involved in regulation of microtubule-related functions.

Another type of *EGP1* deletion mutation (*egp1*Δ*2*::*LEU2*) was also constructed (Fig. 5; see Materials and Methods). This mutation effectively deletes all but the first four amino acids of Egp1. The *egp1*Δ*2*::*LEU2* mutation conferred the same phenotype as the *egp1*Δ*1*::*LEU2* allele (data not shown).

Coimmunoprecipitation of Egp1 and Glc7. To demonstrate a direct interaction between PP1 and Egp1, we developed an immunoprecipitation assay that used epitope-tagged derivatives of the two proteins. A low-copy plasmid containing an *Ha-GLC7* gene, previously shown to encode a fully functional protein (47), was introduced into a strain carrying a gene disruption of *GLC7*. Either a low-copy-number plasmid (YCpEGP1myc) or a high-copy-number plasmid (YEpEGP1myc) containing a myc-tagged *EGP1* gene was also introduced. The *myc-EGP1* gene, in which the myc epitope was introduced near the COOH terminus of Egp1, suppressed the lethality of an *egp1*Δ*2*::*LEU2* deletion. Cultures of the *Ha-GLC7* strain containing either of the *myc-EGP1* plasmids or a vector control were labeled in vivo with ^{35}S protein labeling mix, cell extracts were prepared, and Ha-PP1 or myc-Egp1 was precipitated with

TABLE 2. Suppression of *egp1*Δ*1*::*LEU2* by overexpression of *GLC7*

Plasmid[a]	Gene	No. of Trp$^-$ colonies/ total no. of colonies[b]
YCp2-9B	*EGP1*	310/315
YEpGLC7	*GLC7*	461/490
YEpglc7^{Y170}	*glc7*^{Y170}	0/499
YEplac195	Vector	0/236

[a] Each plasmid with the *URA3* marker was transformed into NH110-2B (*egp1*Δ*1*::*LEU2*) carrying YEpEGP1 with the *TRP1* marker.

[b] Each strain was inoculated in SC-Ura containing Trp and cultured for full growth. The cultures were diluted and plated out on SC-Ura plates and then replica plated onto SC-Trp. Stability of the Trp$^+$ marker is indicated as the ratio of the number of Trp$^-$ colonies to that of colonies on an SC-Ura plate. The ratio of Trp$^-$ cells to total cells reflects the proportion of cells in the population that lost plasmid YEpEGP1.

	Normal nuclei					Abnormal nuclei		
Gal	58	18	2	10	11	0	<0.5	<0.5
Glu	29	1	9	17	18	7	4	15
GLC7	39	21	8	18	13	0	0	<1

FIG. 7. Quantitation of arrested morphologies. Samples were prepared from cultures as described in the legend to Fig. 6. Cells were fixed, stained with DAPI, and observed by light microscopy and fluorescence microscopy. At least 300 cells were scored. The data represent the average results of three experiments. Gal, log-phase cells of NH110-2B (*egp1*Δ*1*::*LEU2*) carrying YCpGEGP1 (*GAL1p-EGP1*) from YEPG; Glu, arrested cells of NH110-2B carrying YCpGEGP1 from YEPD at 40 h; GLC7, log-phase cells of NH110-2B carrying YEpGLC7 from YEPD.

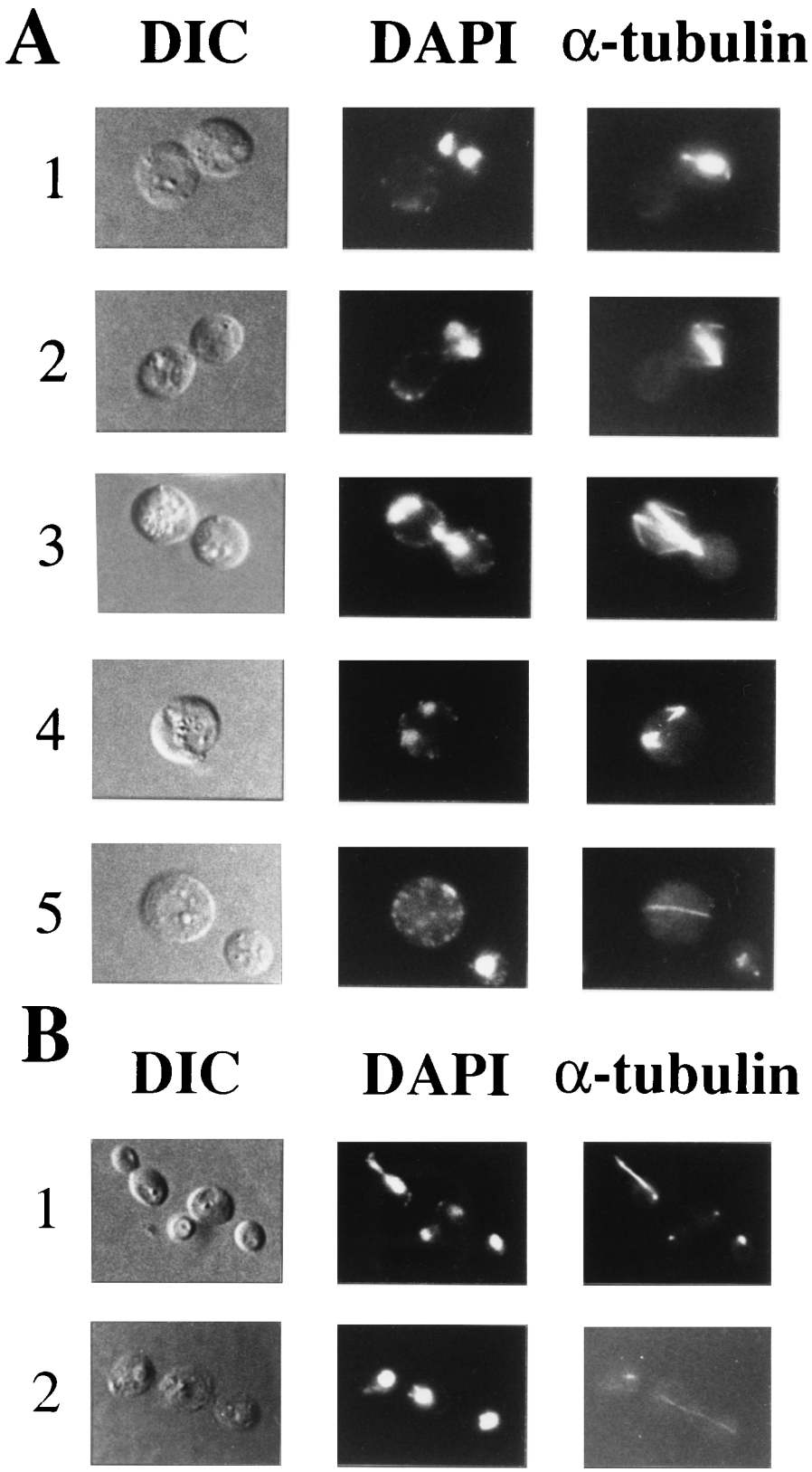

FIG. 8. Abnormal nuclear distribution and aberrant microtubular structures in *egp1Δ1*::*LEU2* cells. Cells of NH110-2B (*egp1Δ1*::*LEU2*) carrying YCpGEGP1 (*GAL1p-EGP1*) or YEpGLC7 were prepared as described in the legends to Fig. 6 and 7. Cells were fixed in formaldehyde and stained with antitubulin antibodies to visualize spindles. Cells are shown in differential interference contrast (DIC), stained with DAPI for nuclei, and stained with α-tubulin for spindles. (A) Cells of NH110-2B carrying YCpGEGP1 arrested in YEPD at 40 h. Cells 1 to 3 are large-budded cells with mislocalized nuclei; cells 4 and 5 are examples of unbudded cells with two nuclei and without a nucleus, respectively. (B) Cells 1 and 2 are log-phase cells of NH110-2B carrying YCpGEGP1 in YEPG and NH110-2B carrying YEpGLC7 in YEPD, respectively.

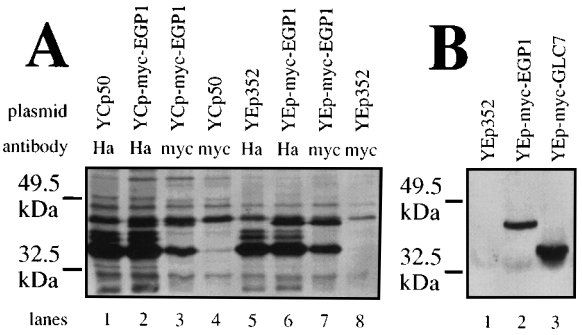

FIG. 9. Coimmunoprecipitation of Egp1 and Glc7. (A) Yeast strains were labeled with ^{35}S protein labeling mix; extracts were prepared, immunoprecipitated, and electrophoresed on an SDS–10% polyacrylamide gel as described in Materials and Methods. The autoradiograph of the dried gel is presented. The relevant genotype of each strain and the antibody used in the immunoprecipitation are listed above each lane. For lanes 1 to 4, 180 μl of extract was incubated with 20 μl of antibody solution. For lanes 5 to 8, 90 μl of extract was incubated with 10 μl of antibody solution. Lanes 1 and 4, DF87 (*glc7*Δ::*LEU2* [pNC160: Ha-GLC7]) carrying YCp50; lanes 2 and 3, DF87 carrying YCpEGP1myc; lanes 5 and 8, DF87 carrying YEp352; lanes 6 and 7, DF87 carrying YEpEGP1myc. Upper and lower hash marks indicate the positions of myc-Egp1 and Ha-Glc7, respectively. (B) Western blotting analysis of yeast strains expressing myc epitope-tagged Egp1 and PP1. Cell extracts were made from strain DF87 carrying the empty YEp352 vector (lane 1), DF87 carrying YEpEGP1myc (lane 2), or DF12 (*glc7*Δ::*HIS3* [YCp50:myc-GLC7]).

antibody 12CA5 or 9E10, respectively. The precipitates were electrophoresed on SDS-polyacrylamide gels, and dried gels were autoradiographed. As shown in Fig. 9A, lanes 1 and 5, monoclonal antibody 12CA5 precipitates a 36-kDa protein with the predicted molecular mass of Ha-Glc7. The anti-myc monoclonal antibody 9E10 specifically precipitates a protein of approximately 44 kDa (Fig. 9A, lanes 3 and 7) with the same mobility as the myc-Egp1 observed by immunoblot analysis (Fig. 9B, lane 2). In strains containing both Ha-Glc7 and myc-Egp1, Ha-Glc7 is coprecipitated with the myc-specific antibody (Fig. 9A, lanes 3 and 7). In the same strains, the Ha-specific antibody coprecipitates a band identical in molecular weight to myc-Egp1 (Fig. 9A, lanes 2 and 6). Coimmunoprecipitation of myc-Egp1 and Ha-Glc7 was also observed when immunoprecipitates from unlabeled extracts were detected by Western blotting (immunoblotting) (data not shown). Antibody 12CA5 did not immunoprecipitate myc-Egp1 from strains lacking Ha-Glc7 (data not shown). These results demonstrate that Ha-Glc7 and myc-Egp1 associate in our cell extracts and reciprocally coprecipitate in our assay. The observation that the amounts of Egp1 present in lanes 2 and 3 of Fig. 9A are nearly equal suggests that all of the Egp1 is associated with PP1 in cell extracts.

To explore the possibility that Egp1 regulates PP1 in a cell cycle-dependent manner, extracts were prepared and immunoprecipitation was carried out as described above, using cultures arrested at various stages of the cell cycle. No change was detected in either the level of Egp1 expression or its ability to associate with PP1 in cells arrested in G_1 with α-factor, in S phase with hydroxyurea, or in mitosis with benomyl (data not shown).

DISCUSSION

PP1 is known to complex with regulatory or targeting subunits that influence the activity of PP1 toward various substrates. For example, a glycogen-binding subunit that associates with PP1 in a holoenzyme isolated from skeletal muscle has been identified (26, 46). This subunit targets PP1 to the glycogen particle. A related subunit activates glycogen synthase in *S. cerevisiae* (47). An analogous regulatory subunit(s) may function to direct PP1 in cell cycle control. The *sds22*$^+$ gene product in the fission yeast *S. pombe* has been proposed to function as a targeting subunit that enhances the activity of PP1 toward nuclear substrates required for mitosis (45). In this study, we identified the *EGP1* gene, which encodes a protein highly homologous to fission yeast Sds22, as an extra-copy suppressor of the *glc7*^{Y170} *gpp1-1* double mutation. The *glc7*^{Y170} mutant arrests at the G_2/M phase of the cell cycle at the restrictive temperature but does not lose viability (25). The *gpp1-1* mutation causes loss of viability in *glc7*^{Y170} cells arrested at the restrictive temperature. This lethality requires cell cycle progression beyond S phase, but the function and DNA sequence of *GPP1* are still unknown.

The *S. pombe sds22*$^+$ gene was isolated as a multicopy suppressor of the *dis2-11* mutation, a dominant negative allele of PP1 (37). *sds22*$^+$ encodes a nuclear protein and is essential for the mitotic metaphase/anaphase transition. Sds22 physically interacts with the Dis2/Sds21 PP1s, and Sds22-associated phosphatase activity has altered substrate specificity (45). On the basis of these results, Stone et al. have proposed that Sds22 targets the Dis2/Sds21 PP1s to substrates that must be dephosphorylated for cell cycle progression from metaphase to anaphase (45). Several lines of evidence presented here indicate that Egp1 may have a similar role in the budding yeast *S. cerevisiae*. First, *EGP1* and *sds22*$^+$ are both essential genes, mutations in which confer a cell cycle defect. Differences are observed in the terminal phenotypes of *egp1* and *sds22* mutants. Whereas an *sds22* temperature-sensitive mutant arrests in midmitosis with a short spindle, condensed chromosomes, and elevated H1 kinase activity, *EGP1*-depleted cells contain an abundance of large-budded cells with an elevated frequency of cells with a misorientated spindle. This difference in terminal phenotype could reflect physiological differences between the two yeast species or differences in the type of mutation used in the two experiments—a mutation conferring temperature sensitivity in *S. pombe* and depletion of the wild-type protein in *S. cerevisiae*. Irrespective of the differences, however, disruption mutations in both genes are rescued by increased expression of PP1, suggesting that both gene products are modulators of PP1 in their respective hosts.

A second line of evidence supporting a modulatory role of Egp1 in PP1 activity comes from the physical interaction observed by immunoprecipitation. From a given extract, the same quantity of Egp1 can be immunoprecipitated directly, with antibodies directed against the epitope on Egp1, or indirectly, with antibodies directed against PP1. The immunoprecipitation is reciprocal; PP1 also coprecipitates with Egp1. However, the level of PP1 that coprecipitates is significantly less than the total amount of PP1 in the extract. This observation suggests that Egp1 is not bound to all of the PP1 in the cell, and one would predict that only a fraction of PP1 associated with Egp1 is required for the mitotic role. The interaction between PP1 and Egp1 contrasts with the interaction between PP1 and its glycogen-specific subunit Gac1. In the latter case, under the same immunoprecipitation conditions as used for Egp1, only about half of the Gac1 coprecipitated with PP1, and PP1 could not be reproducibly coprecipitated with Gac1 (47). These differences could reflect differences in binding affinity or the locations of binding sites with respect to the sites of the antibody epitopes.

The 11 tandem repeats of a leucine-rich 22-amino-acid sequence, which make up the bulk of Egp1 and Sds22, also suggest that these proteins function in protein-protein interactions. Tandemly repeated motifs similar to that of Sds22/Egp1

have been found in various other proteins, including adenylyl cyclase in yeasts (28, 53), leucine-rich glycoprotein in human serum (49), platelet membrane receptor GP1bα (32), and *Drosophila* Toll (24). The role of this type of tandemly repeated motif is unclear, but the motif probably participates in protein-protein interactions. The repeats in adenylyl cyclase from *S. cerevisiae* appear to interact with Ras proteins and are necessary for adenylyl cyclase activation by Ras (48). Deletion analysis of the *sds22*$^+$ gene suggests that the central repeated region of Sds22 is important for its association with the Dis2/Sds21 PP1s (45).

Depletion of Egp1 by glucose-mediated repression of the *EGP1* gene under control of the *GAL1* promoter results in a gradual arrest of cell division at the G_2/M phase, although the block is incomplete (60% large-budded cells). A significant fraction of the large-budded cells contain two DAPI-staining regions, often connected by a misorientated spindle. Subsequent cytokinesis produces one cell with two nuclei and one without a nucleus. Similar phenotypes have been observed for several previously described mutants (2, 5, 12, 16, 27, 29, 31, 33–35, 38, 50), notably mutants with mutations in components of either the actin or microtubule cytoskeleton. Huffaker et al. (27) have shown that cytoplasmic microtubules are required for nuclear migration and spindle orientation prior to mitosis. Support for the role of Egp1 and/or PP1 in a microtubule-related function also comes from the fact that *egp1*Δ::*LEU2* cells overexpressing *GLC7* are viable but supersensitive to the microtubule-depolymerizing drug thiabendazole (data not shown). In addition, tubulin staining of *egp1*Δ::*LEU2* cells containing two nuclei in one cell body revealed that cells have deformed or abnormal spindle structures. These results raise the possibility that Egp1 itself exerts its effect as a regulator of a microtubule-related component. However, *egp1*Δ::*LEU2* cells overexpressing *GLC7* did not contain binucleate cells. Therefore, it seems likely that *S. cerevisiae* PP1 is involved in regulation of microtubule-related function.

In contrast to the rather nonspecific phenotype of cells depleted in Egp1, *glc7*^{Y170} mutants arrest uniformly at the nonpermissive temperature; more than 90% of the cells contain a single nucleus at the bud neck, a short mitotic spindle, and elevated H1 kinase activity. Binucleate cells are not observed. How do we reconcile the different phenotypes of mutants with mutations in PP1 and those with mutations in its putative positive regulator Egp1? Although a number of explanations are possible, we favor the hypothesis that PP1 is required for a number of steps in mitosis. Because of the nature of the *glc7*^{Y170} mutation, strains containing this *glc7* allele exhibit a tight arrest at an early stage of mitosis. However, PP1 is likely to be required later in the mitosis as well. Francisco et al. (20) have implicated *GLC7* in the control of mitotic chromosome segregation, and PP1 mutation in *S. pombe* causes mitotic defects in chromosome disjunction and the separation of nuclei during anaphase (3, 37). Egp1 could be a PP1 cofactor that is required for a number of these steps. As a result, Egp1 depletion results in nonuniform arrest. Are there additional subunits or components of PP1 that control its mitotic functions? Recently, we isolated two different genes (tentatively designated *EGP2* and *EGP3*) that suppressed the lethal phenotype of *glc7*^{Y170} *gpp1-1* cells but did not suppress the *glc7*^{Y170} single defect, using a yeast genomic library constructed on a high-copy-number plasmid (data not shown). These gene products are candidates for components involved in regulating PP1. They should prove valuable for addressing the regulation of PP1 and its role in the control of progression through the cell cycle.

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