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Type 1 Protein Phosphatase Acts in Opposition to Ipl1 Protein Kinase in Regulating Yeast Chromosome Segregation

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The *IPL1* gene is required for high-fidelity chromosome segregation in the budding yeast *Saccharomyces cerevisiae*. Conditional *ipl1*^{ts} mutants missegregate chromosomes severely at 37°C. Here, we report that *IPL1* encodes an essential putative protein kinase whose function is required during the later part of each cell cycle. At 26°C, the permissive growth temperature, *ipl1* mutant cells are defective in the recovery from a transient G_2/M-phase arrest caused by the antimicrotubule drug nocodazole. In an effort to identify additional gene products that participate with the Ipl1 protein kinase in regulating chromosome segregation in yeast, a truncated version of the previously identified *DIS2S1/GLC7* gene was isolated as a dosage-dependent suppressor of *ipl1*^{ts} mutations. *DIS2S1/GLC7* is predicted to encode a catalytic subunit ($PP1_C$) of type 1 protein phosphatase. Overexpression of the full-length *DIS2S1/GLC7* gene results in chromosome missegregation in wild-type cells and exacerbates the mutant phenotype in *ipl1* cells. In addition, the *glc7-1* mutation can partially suppress the *ipl1-1* mutation. These results suggest that type 1 protein phosphatase acts in opposition to the Ipl1 protein kinase in vivo to ensure the high fidelity of chromosome segregation.

As a eukaryotic cell progresses through the cell cycle and divides, a complete set of chromosomes is segregated faithfully to each of the two progeny cells. The fidelity of this chromosome segregation process is remarkably high in normal cells. Errors in chromosome segregation result in aneuploidy, which often causes cell death and is implicated in oncogenesis and birth defects in humans. While little is known about the mechanisms that govern proper chromosome segregation, it is clear that the functions of many proteins must be transiently modified, such that the different cellular processes associated with chromosome segregation—including DNA replication, chromosome condensation, attachment of microtubules to kinetochores, formation of the mitotic spindle apparatus, and ultimately chromosome separation—can be precisely executed in a strict temporal order.

The reversible phosphorylation of proteins is a major mechanism for the control of protein functions in eukaryotes. Phosphorylation and dephosphorylation of serine and threonine residues of proteins have been shown to be involved in the control of diverse cellular processes, including chromosome segregation (12, 16). The phosphorylation state of a given protein is determined by the relative activities of the protein kinase(s) and phosphatase(s) that recognize it as a substrate. A number of protein kinases (2, 5, 19, 34, 43, 59) and phosphatases (1, 15, 18, 26, 27, 36, 37, 40) are known to be involved in the control of chromosome segregation in different organisms. However, in most cases, the functional relationships between these protein kinases and phosphatases are not known.

The budding yeast *Saccharomyces cerevisiae* is an excellent system for studying the control of chromosome segregation. The major structural and mechanistic aspects of chromosome segregation appear to be largely conserved between yeast and higher eukaryotes. Errors in chromosome segregation can be detected by scoring chromosome-specific markers in highly sensitive genetic assays. We have previously described one such genetic assay, which detects chromosome gain in yeast cells that missegregate chromosomes at increased frequencies (9). By this assay, a number of temperature-sensitive (Ts^-) conditional *S. cerevisiae ipl* mutants that missegregate chromosomes at 37°C were isolated. Results from flow cytometry analysis of one such mutant, the *ipl1* mutant, suggested that this mutant suffers from severe chromosomal nondisjunction at 37°C, giving rise to progeny cells that have too many or too few chromosomes.

We report here that the *IPL1* gene encodes a putative protein kinase that is essential for cell viability. To identify other gene products that may interact functionally with the Ipl1 protein kinase, we isolated dosage-dependent suppressors of *ipl1*^{ts} mutants. One such suppressor isolated is a truncated form of a previously identified gene named *DIS2S1* (40) or *GLC7* (6, 17), which encodes the catalytic subunit ($PP1_C$) of type 1 protein phosphatase (PP1). Results from the genetic analysis of *ipl1* and *glc7* mutants suggest that PP1 acts in opposition to the Ipl1 protein kinase in regulating chromosome segregation in yeast cells.

MATERIALS AND METHODS

Strains, media, and genetic techniques. The yeast strains used in this study are listed in Table 1. The *ipl1-4* strain CCY98-3D-1-1 was constructed by a recombination-mediated two-step gene replacement procedure (51), replacing the *IPL1* gene of CCY98-3D-1 with the *ipl1-4* mutant allele present on pCC321. The diploid strain CBY1830-20 was constructed by a one-step gene disruption procedure (49), replacing one of the two *IPL1* genes in DBY1830 with the *ipl1-Δ3::HIS3* allele present on pCC305. This disruption was confirmed by DNA hybridization. The *Escherichia coli* strain DB1142 (*leu pro thr hsdR hsdM recA*) was used as a host for plasmids.

Rich medium (YEPD), synthetic minimal medium (SD), and SD with necessary supplements were prepared as described elsewhere (53).

Cells were routinely grown at 26°C unless otherwise specified. Glycogen accumulation was determined qualitatively by staining patches of yeast cells with iodine vapor (10). Chromosome gain assay was carried out as described previously (9), by

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TABLE 1. Yeast strains used in this study[a]

Strain	Genotype
DBY1826	**a** *ade2 his3-Δ200 ura3-52 leu2-3,112*
DBY1828	**a** *ade2 his3-Δ200 ura3-52 leu2-3,112 trp1-1*
DBY1830	**a**/α *ade2/+ lys2-801/+ his3-Δ200/his3-Δ200 ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-1/trp1-1*
CBY1830-20	**a**/α *ade2/+ lys2-801/+ his3-Δ200/his3-Δ200 ura3-52/ura3-52 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ipl1-Δ3::HIS3/+*
CCY69-4C-1	α *lys2-801 his3-Δ200 ura3-52 ipl1-1*
CCY72-3D-1	α *lys2-801 his3-Δ200 ura3-52 ipl1-2*
CCY98-3D-1	α *hom3 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102*
CCY98-3D-1-1	α *hom3 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 ipl1-4*
CCY107-8D-1	α *ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 ipl1-1*
CCY108-15C-1	**a** *ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 ipl1-2*
CCY220-2A	**a** *lys2-801 his3-Δ200 ura3-52 ipl1-2*
CCY220-12D	**a** *lys2-801 his3-Δ200 ura3-52*
CCY357-7C	**a** *ade2 his3-Δ200 ura3-52 leu2-3,112 ipl1-1*
CCY358-2D	**a** *ade2 his3-Δ200 ura3-52 leu2-3,112 ipl1-2*
CCY435-15C	α *ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 glc7-1*
CCY464-1D	α *ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 ipl1-1*
CCY464-4C	α *ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 ipl1-1 glc7-1*
CCY464-5D	α *ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102*
CCY464-6C	α *ade2 his3-Δ200 ura3-52 lys2-Δ101::HIS3::lys2-Δ102 glc7-1*
CCY482-13D	**a** *ade2 his3-Δ200 ura3-52 leu2-3,112 lys2-Δ101::HIS3::lys2-Δ102*

[a] Most of the strains were constructed specifically for this study, the exceptions being DBY1826, DBY1828, and DBY1830, which are from D. Botstein's laboratory collection. The origin of some of the markers used is indicated in the text.

using yeast strains that carried the chromosome II marker *lys2-Δ101::HIS3::lys2-Δ102*, which allowed chromosome II numbers to be monitored.

Cell cycle synchronization and temperature shift experiments. Cultures of cells (~5 × 10^6 cells per ml) growing exponentially at 26°C in YEPD, pH 4.0, were treated with synthetic α-factor (4 to 8 μg/ml; Sigma Chemical Co., St. Louis, Mo.) for 3 to 4 h (until over 95% of the cells became arrested as unbudded cells). The cells were then washed twice with water and resuspended in YEPD, pH 4.0, that was kept at room temperature or prewarmed at 37°C. α-Factor, hydroxyurea (Sigma Chemical Co.), or nocodazole (Sigma Chemical Co.) was added where appropriate, and cells were incubated at 26 or 37°C thereafter. Cell viability was measured by plating briefly sonicated and appropriately diluted samples on YEPD at 26°C. Cell morphology was monitored microscopically following a brief sonication of cells that had been fixed in 5% formaldehyde. Fluorescent staining of microtubules and DNA was carried out as described previously (46).

Cloning of *ipl1-1* and *ipl1-2* mutant alleles. The *ipl1-1* and *ipl1-2* mutant alleles were cloned by transforming CCY69-4C-1 (*ipl1-1*) and CCY72-3D-1 (*ipl1-2*), respectively, with linearized pCC234 that lacked the *Bcl*I-*Mlu*I DNA fragment (containing the *IPL1* gene). Gap-repaired and circularized plasmid (42) was recovered from Ura^+ yeast transformants, and the mutant alleles on these plasmids were sequenced (50) with the Sequenase system (U.S. Biochemical, Cleveland, Ohio).

Cloning of dosage-dependent suppressors of *ipl1-1*. Plasmids containing dosage-dependent suppressors of *ipl1-1* were isolated by transforming the *ipl1-1* strain CCY107-8D-1 with a yeast genomic library constructed in the high-copy-number plasmid YEp24 (8). Ura^+ transformants were selected by plating cells on supplemented SD lacking uracil. After 24 h at 26°C, plates containing Ura^+ transformants were shifted to 35°C. After 3 more days, Ts^+ Ura^+ transformants were identified and plasmids were recovered from such transformants into *E. coli*. The ability of these plasmids to complement the Ts^- phenotype of *ipl1-1* (CCY107-8D-1), *ipl1-2* (CCY108-15C-1), and *ipl1-4* (CCY98-3D-1-1) mutants at 35 or 37°C was retested.

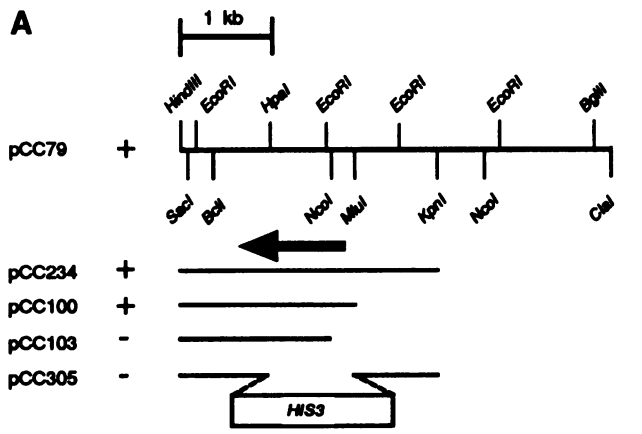
DNA manipulation. Functional localization of *IPL1* was done by subcloning DNA fragments into the low-copy-number *URA3*-CEN plasmid YCp50 (48) or pRS316 (55). Functional localization of the dosage-dependent suppressor was done by subcloning DNA fragments into the high-copy-number *URA3*-2μm plasmid pSM217 (55) or YEp24 (4). pCC305, used for disruption of *IPL1*, was constructed by replacing the ~1-kb *Mlu*I-*Hpa*I fragment (containing most of *IPL1*) of pCC234 with the ~1.8-kb *Bss*HII-*Nru*I fragment (containing *HIS3*) of YEp6 (35).

The *URA3*-CEN plasmid (pCC299) containing the *ipl1-4* allele was constructed by site-directed mutagenesis (29) of pCC234 with DNA primer 85A (5′-TGTCCCACAGACA GCTTTCCTTCT-3′). The *URA3*-integrating plasmid pCC321 was constructed by cloning the ~2.9-kb *Hin*dIII-*Kpn*I fragment (containing the *ipl1-4* allele) of pCC299 into the *Hin*dIII-*Kpn*I sites of pRS306 (55).

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number for the *IPL1* region is U07163.

RESULTS

***IPL1* encodes a putative protein kinase that is essential for cell viability.** To elucidate the molecular nature of the chromosome segregation defect observed in *ipl1* mutants, we carried out a molecular analysis of the *IPL1* gene (9). *IPL1* was localized to one end of a 2-kb DNA fragment (Fig. 1A). Sequencing of this region revealed a single long open reading frame (ORF) (Fig. 1B) that potentially encodes a protein of 367 amino acids, with a pI of 9.74 and a predicted molecular mass of 43 kDa, which is in good agreement with the apparent molecular mass (~45 kDa) of the Ipl1 protein (Ipl1p) as determined by immunoblotting (data not shown). Among the carboxyl-terminal 270 amino acids of Ipl1p are all of the conserved residues characteristic of a serine/threonine-specific protein kinase (22). A search of the GenBank data base revealed that the three yeast cyclic AMP-dependent protein kinase (PKA) catalytic subunits, encoded by the *TPK1*, *TPK2*,



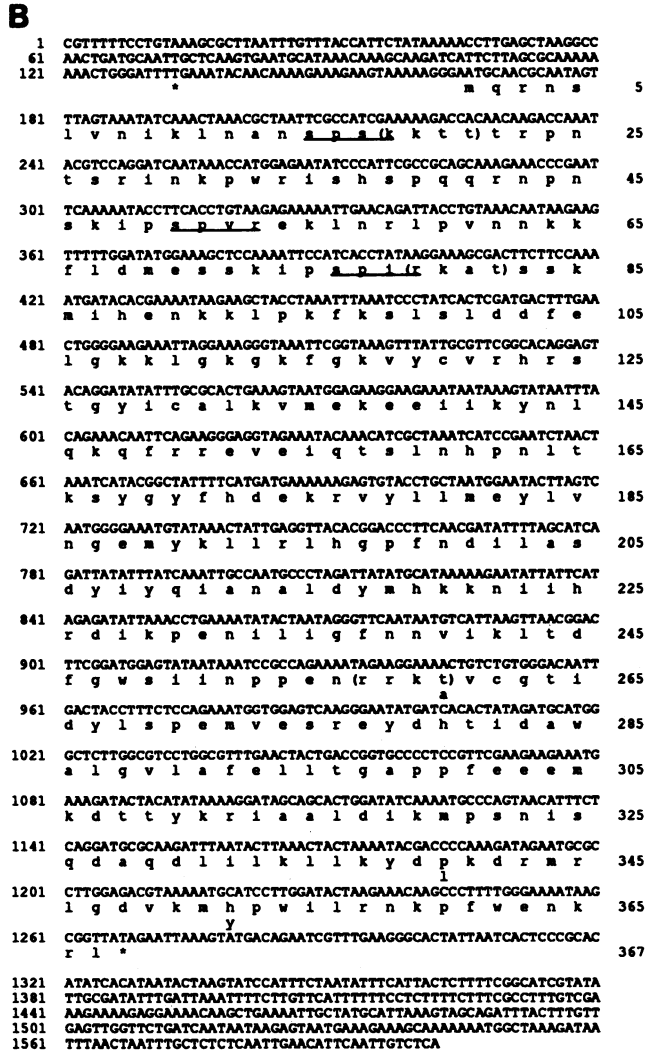


FIG. 1. (A) Functional localization of the *IPL1* gene. The ability (+) or inability (−) of the different *URA3*-CEN plasmids (containing the DNA fragments shown) to complement the temperature-sensitive growth phenotype of an *ipl1-1* mutant (CCY69-4C-1) at 37°C is indicated. The location and orientation of the predicted *IPL1* ORF are represented by the arrow. (B) Nucleotide sequence of the *IPL1* region and predicted amino acid sequence of the Ipl1 protein kinase. The upstream and downstream in-frame stop codons are shown as asterisks. Sequences that match the consensus for sites phosphorylated by the $p34^{cdc2/CDC28}$ protein kinase are underlined. Sequences that match the consensus for sites phosphorylated by PKA are enclosed in parentheses. The extra residues listed under positions 260, 340, and 352 represent the alternative residues predicted to be encoded by the *ipl1-4*, *ipl1-1*, and *ipl1-2* mutant alleles, respectively.

and *TPK3* genes (7, 32, 60), are among the closest relatives, being 35 to 37% identical in primary sequence to Ipl1p.

To confirm that the predicted ORF indeed encodes Ipl1p, we also cloned and sequenced two temperature-sensitive *ipl1* mutant alleles (9). As expected, each *ipl1* allele has a single base change within the predicted *IPL1* ORF. The *ipl1-1* mutation (CCC to CTC) causes a change of Pro-340 to Leu-340, while the *ipl1-2* mutation (CAT to TAT) causes a change of His-352 to Tyr-352 (Fig. 1B). The former change is located within the conserved subdomain XI found in eukaryotic protein kinases (22).

To determine the functional importance of Ipl1p in vivo, a diploid yeast strain with one of its two *IPL1* genes replaced by the *HIS3* gene was constructed (Fig. 1A). Sporulation and tetrad analysis of this heterozygous (*IPL1/ipl1-Δ3::HIS3 his3-Δ200/his3-Δ200*) diploid strain (CCY1830-20) showed that only two of the four spores per tetrad are viable at 26°C on YEPD. All viable spores are His^-, indicating that the inviable spores contain the *IPL1* gene replacement. *ipl1-Δ3::HIS3* haploid cells carrying a functional *IPL1* gene on a *URA3*-CEN plasmid (pCC100) are viable but cannot segregate viable cells that have lost the plasmid. These results showed that the *IPL1* gene is essential for the viability of yeast cells.

Protein kinases are often regulated by phosphorylation. For example, phosphorylation of Thr-167 in $p34^{cdc2}$ of *Schizosaccharomyces pombe* is required for its function in vivo (21). Present in Ipl1p are three sequences that match the consensus for sites phosphorylated by PKA (R/K-R/K-X-S/T) (44) and three other sequences that match the consensus for sites phosphorylated by the $p34^{cdc2/CDC28}$ protein kinase (S/T-P-X-R/K) (39) (Fig. 1B). Two of these latter sequences form part of a longer repeat (S-K-I-P-S-P-I/V-R) of unknown function. We used site-directed mutagenesis to create the *ipl1-4* mutant allele, which alters one of the putative PKA phosphorylation sites by replacing Thr-260 (equivalent to Thr-167 of $p34^{cdc2}$) with alanine (Fig. 1B). The resulting mutant is alive at 26°C, but is temperature sensitive for growth at 35°C.

***IPL1* function is required during the later part of the cell cycle.** To determine if Ipl1p activity is required at any particular time during the yeast cell cycle, we used synchronized cultures to test the effect of Ipl1p inactivation on cell viability. Amongst the three recessive temperature-sensitive *ipl1* mutant alleles available, *ipl1-2* confers the lowest restrictive growth temperature; *ipl1-2* mutants fail to form colonies at ≥33°C. Wild-type and *ipl1-2* mutant cells were arrested in G_1 phase by α-factor treatment at 26°C, the permissive growth temperature. After the removal of α-factor, these synchronized cells were then allowed to progress through the cell cycle at 37°C, the restrictive growth temperature for *ipl1-2* mutant cells. Under these conditions, wild-type cells remained fully viable and progressed through many cell cycles (data not shown). *ipl1-2* mutant cells also remained fully viable for the first 54 min after release from α-factor arrest (Fig. 2). During this period, a large fraction of cells had become small budded, indicative of progression into S phase (45). However, by 80 min after release from α-factor arrest, cell viability was greatly reduced, and this correlated with the appearance of large-budded cells. This observation indicates that cell death occurs

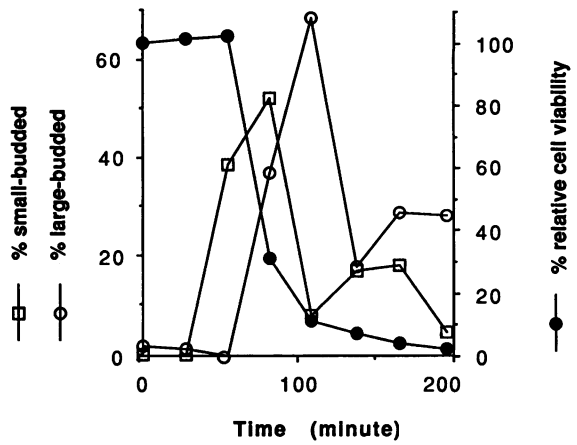


FIG. 2. Viability and cell morphology of *ipl1-2* mutant cells after transfer to 37°C. CCY220-2A mutant cells were arrested in G_1 by α-factor treatment for 4 h at 26°C, followed (at 0 min) by transfer to fresh medium without α-factor at 37°C. At the indicated times, aliquots were plated to assess viable cell count and fixed and examined microscopically to determine the distribution of cell morphology. Relative viability was calculated with the viable cell count at 0 min as a reference.

TABLE 2. Effect of drug treatment on *ipl1-2* cell viability[a]

Strain genotype	Drug(s)	Relative (3.25 h/0 h) no. of viable cells at:	
		26°C	37°C
IPL1	None	3.01 (3.01)	2.88
	α-Factor	0.80 (ND)	0.83
	HU	0.75 (0.82)	0.66
	Nocodazole	0.50 (0.56)	0.59
	Nocodazole and HU	ND (0.68)	ND
ipl1-2	None	2.05 (2.55)	0.02
	α-Factor	0.71 (ND)	0.71
	HU	0.72 (0.67)	0.50
	Nocodazole	0.02 (0.06)	0.03
	Nocodazole and HU	ND (0.59)	ND

[a] Wild-type (CCY220-12D) or *ipl1-2* (CCY220-2A) mutant cells in YEPD, pH 4.0, were arrested in G_1 (>95% unbudded) by α-factor treatment for 3.5 h. Cells were washed twice with water to remove α-factor and then resuspended in YEPD, pH 4.0, that had been prewarmed at 26 or 37°C, in either the presence or absence of drugs. These cultures were then incubated for 3.25 h at the temperatures indicated. The drug concentrations used were: 8 μg/ml for α-factor, 0.1 M for hydroxyurea (HU), and 15 μg/ml for nocodazole. Appropriately diluted and briefly sonicated samples were plated on YEPD, and colonies were scored after 3 days at 26°C to determine the numbers of viable cells at 0 and 3.25 h. The results from a separate experiment that did not involve a temperature shift to 37°C are shown in parentheses. ND, not determined.

during progression through G_2 phase and/or mitosis (45). Further incubation resulted in increased cell inviability and also disappearance of large-budded cells, indicative of exit from mitosis. These results suggest that Ipl1 protein kinase activity is not required during the early part of the yeast cell cycle but is absolutely required during G_2 phase and/or mitosis. The absence of Ipl1p function presumably leads to chromosome missegregation and cell death without arrest at G_2/M phase.

If the above interpretation is correct, we might expect blockage of cell cycle progression at points before G_2/mitosis to rescue *ipl1-2* mutant cells from the lethality caused by Ipl1p inactivation. We thus monitored the cell cycle progression of G_1-synchronized wild-type and *ipl1-2* mutant cells in the presence or absence of α-factor, hydroxyurea, or nocodazole. In the presence of α-factor, G_1-synchronized cells should remain in G_1 phase and not enter the cell cycle. In the presence of hydroxyurea or nocodazole, G_1-synchronized cells should progress to S phase or G_2/M phase, respectively, and then become arrested. As expected, our results showed that in the absence of any drugs, wild-type cells progressed through the cell cycle and the number of viable cells increased during a 3.25-h incubation at 26 or 37°C (Table 2). Similarly, the number of viable *ipl1-2* mutant cells also increased during the same incubation period at 26°C. Also, as expected, only ~2% of *ipl1-2* mutant cells remained viable after 3.25 h at 37°C. In the presence of α-factor or hydroxyurea, wild-type as well as *ipl1-2* mutant cells did not give rise to increased numbers of viable cells; this is to be expected. Instead, there was a relatively small drop in viability at both 26 and 37°C. A comparison of the levels of viability of *ipl1-2* mutant cells incubated at 37°C in the presence or absence of α-factor or hydroxyurea showed that blockage of cell cycle progression at points before G_2/M phase largely rescued yeast cells from the lethality caused by Ipl1p inactivation. This observation is consistent with the proposed requirement of *IPL1* function in mitosis. Results from nocodazole-treated cells will be described below.

Microtubule structures appear normal, but DNA staining appears abnormal, in *ipl1* mutants. We have previously shown by flow cytometry that *ipl1-2* mutant cells missegregate chromosomes severely at 37°C. To determine if this defect is caused by structural alterations of the mitotic spindle, we examined yeast microtubules and DNA by fluorescence microscopy. Our results showed that the chromosome segregation defect seen in *ipl1* mutants is not caused by gross defects in microtubule organization. At both 26 and 37°C, the microtubule structures of *ipl1-2* mutant cells are indistinguishable from those of wild-type cells (Fig. 3). For example, antitubulin staining revealed normal microtubule structures in *ipl1-2* mutant cells that had been incubated at 37°C for 105 min (from Fig. 2). However, DNA staining appeared abnormal (Fig. 3). The intensities of DNA staining located near the opposite ends of many elongated spindles were clearly uneven, suggesting that unequal numbers of chromosomes segregated to the two poles. These experiments were repeated with asynchronous cultures of *ipl1-2* mutant cells, and similar staining patterns were observed (data not shown). Thus, *ipl1-2* cells missegregate chromosomes at 37°C, even though they contain microtubule structures of normal appearance at this temperature.

***ipl1-2* mutant cells are defective in recovery from a transient G_2/M phase arrest caused by exposure to nocodazole.** In the cell viability experiment described above (Table 2), the microtubule-destabilizing drug nocodazole caused only a moderate decrease in the viability of wild-type cells incubated at 26 or 37°C, indicating that most wild-type cells can resume growth upon removal of nocodazole. In contrast, this drug caused a very large decrease in viability for *ipl1-2* mutant cells, even at the permissive growth temperature of 26°C. Failure of *ipl1-2* mutant cells to resume growth upon removal of nocodazole may be interpreted in at least two ways. First, since nocodazole causes disassembly of almost all microtubule structures in yeast cells (data not shown), these mutant cells may be defective in the feedback mechanism that detects microtubule depolymerization and thus fail to arrest at G_2/M phase, resulting in cell death (23, 31). However, this possibility can be ruled out because over 80% of the wild-type as well as *ipl1-2* mutant cells remained arrested as uninucleate, large-budded cells after

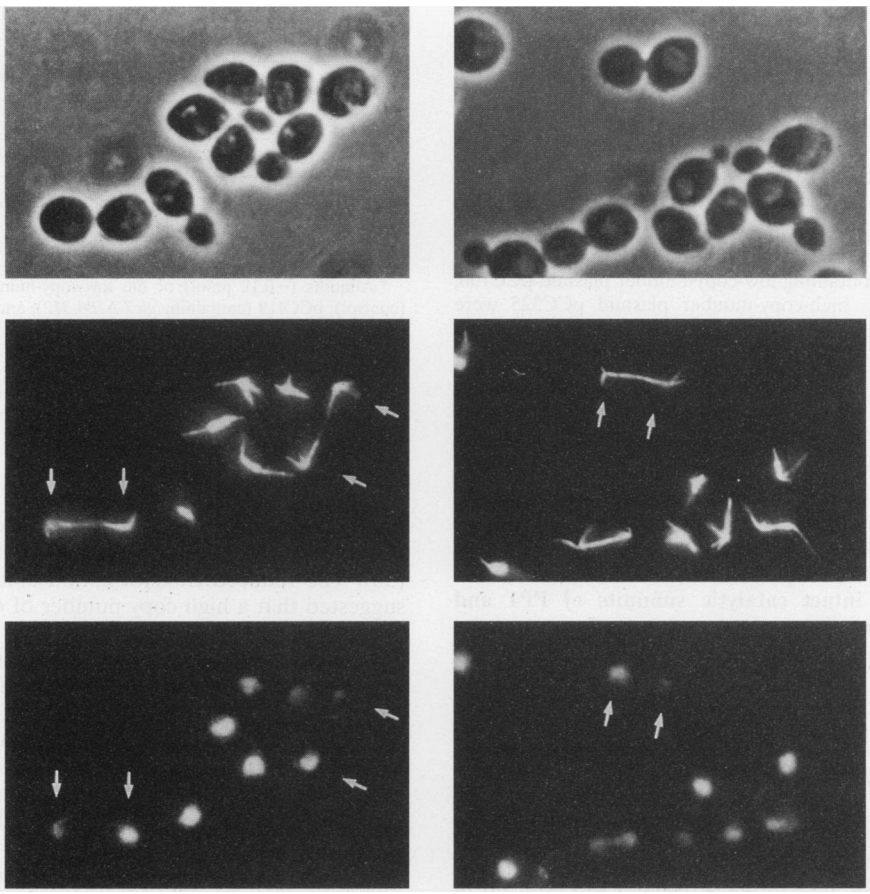


FIG. 3. Fluorescence microscopy of *ipl1-2* mutant cells incubated at 37°C for 105 min (from Fig. 2). Phase-contrast images (top panels), antitubulin staining (middle panels), and 4′,6-diamidino-2-phenylindole (DAPI) staining (bottom panels) are shown. The arrows identify cells with uneven intensities of DAPI staining found at the opposite ends of elongated spindles.

exposure to nocodazole for 3.25 h (data not shown). Alternatively, the *ipl1* cells may be defective in the proper reassembly of the mitotic spindle after the removal of nocodazole, or the reassembled microtubules may be defective in their ability to interact with other cellular components such as spindle pole bodies and kinetochores. So far, immunofluorescence microscopic studies have failed to reveal any major differences between wild-type and *ipl1-2* cells in their ability to reassemble microtubules after a transient exposure to nocodazole (data not shown). However, chromosome missegregation is apparent in many *ipl1-2* cells. For example, 45 min after the removal of nocodazole, about 40% of *ipl1-2*, but only about 6% of wild-type, cells that have elongated spindles appear to have uneven amounts of DNA staining located at the opposite ends of the spindles. Thus, *ipl1-2* cells missegregate chromosomes when incubated at 37°C or after a transient G_2/M phase arrest caused by exposure to nocodazole at 26°C.

The lethal effect of nocodazole on *ipl1-2* cells could be caused directly by its microtubule depolymerization function or indirectly by the G_2/M phase arrest brought about by microtubule depolymerization. To distinguish between these two possibilities, G_1-synchronized *ipl1-2* cells were allowed to progress through the cell cycle at 26°C in the presence of both hydroxyurea and nocodazole. These cells should become arrested in S phase with depolymerized microtubules. Our results showed that under this condition, most *ipl1-2* cells remain viable and can recover after the removal of hydroxyurea and nocodazole (Table 2). Thus, transient G_2/M phase arrest, and not microtubule depolymerization, appears to be the cause for the loss of viability observed in *ipl1-2* cells.

Overexpression of a truncated catalytic subunit of PP1 can compensate for a partial loss of Ipl1 protein kinase function. To identify other gene products that may function with Ipl1p in the regulation of chromosome segregation, we isolated genes that in high copy number can suppress the temperature-sensitive (Ts^-) growth defect of *ipl1-1* mutants. We obtained five suppressors, one of which will be described here. This suppressor can suppress the Ts^- phenotypes of both *ipl1-1* and *ipl1-4* mutants at 35°C but not the Ts^- phenotype of the *ipl1-2* mutant at 35°C or the inviability of the *ipl1-Δ3::HIS3* mutant at 26°C, suggesting that suppression requires residual Ipl1 protein kinase activity presumed to be present in *ipl1-1* and *ipl1-4* mutants at 35°C.

ipl1-1 mutants carrying the high-copy-number plasmid pCC325 can grow well at 35 but not at 37°C (Fig. 4). The suppressor gene present on this plasmid was localized to one end of the DNA insert (Fig. 5). Sequence analysis of this region showed that it contains a portion of a previously described gene called *DIS2S1* (40), predicted to encode a catalytic subunit ($PP1_C$) of PP1. More recent work showed that *DIS2S1* is identical to *GLC7*, which is an essential gene that is required for glycogen accumulation (6, 11, 17, 20, 58). Consistent with the predicted enzymatic activity of the Glc7 protein, assayable PP1 activity is reduced by about 60% in extracts prepared from

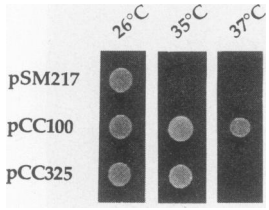


FIG. 4. Growth of an *ipl1-1* mutant (CCY107-8D-1) carrying various plasmids. Suspensions of cells carrying high-copy-number control plasmid pSM217, *IPL1*-containing low-copy-number plasmid pCC100, or suppressor-containing high-copy-number plasmid pCC325 were spotted on YEPD plates and allowed to grow at the indicated temperatures for 42 h.

glc7-1 mutant cells (17). The *DIS2S1* gene in pCC325 is truncated, missing codons 186 to 312 from the 3′ end. The truncated *DIS2S1* gene is fused in frame with vector sequence to generate a chimera, predicted to encode a protein of 195 residues. We will refer to the full-length wild-type gene as *GLC7* and to our cloned suppressor as *glc7-Δ186-312*.

Overexpression of intact catalytic subunits of PP1 and overexpression of truncated catalytic subunits have opposite effects in *ipl1* mutants. Since $PP1_C$s isolated from diverse sources have highly conserved primary sequences (>75% identity over their entire lengths) (13), the severely truncated protein encoded by the *glc7-Δ186-312* allele is unlikely to be active enzymatically. Thus, we wished to determine whether suppression of the *ipl1* mutations required truncation of the *GLC7* gene. For this purpose, we transformed wild-type and *ipl1* mutant cells with low- or high-copy-number *URA3* plasmids containing *GLC7* or *glc7-Δ186-312*. With the exception of the *GLC7*-bearing high-copy-number plasmid pCC414, all of these plasmids could transform wild-type and *ipl1-1* cells efficiently to Ura^+ at 26°C (Table 3). The transformation efficiency with pCC414 was somewhat reduced in wild-type and *ipl1-1* cells and greatly reduced in *ipl1-2* cells, which are more temperature sensitive than *ipl1-1* cells (data not shown) and display a chromosome segregation defect even at 26°C (9). *ipl1* mutants transformed by pCC414 produced colonies that were very heterogeneous in size, and their Ts^- phenotypes could not be reliably assessed because such cells might have unstable genomes (see the discussion of chromosome gain below). It is known that extreme overexpression of *GLC7* through the action of a strong promoter is lethal to even wild-type cells (33). The reduced transformation efficiency by pCC414 thus suggested that a high copy number of *GLC7* may be deleterious to wild-type and *ipl1-1* mutants and may not be tolerated in the more defective *ipl1-2* mutant. Consistent with this idea is the observation that a heterozygous *ipl1-2/IPL1* diploid strain containing a high-copy-number plasmid carrying *GLC7* produced, upon sporulation, no viable plasmid-bearing spores that were of the *ipl1-2* genotype. Thus, overexpression of the intact *GLC7* gene has an effect in *ipl1* mutants opposite that of the overexpression of the *glc7-Δ186-312* allele.

TABLE 3. Transformation efficiency of plasmids containing *GLC7* or *glc7-Δ186-312*[a]

Strain genotype	Plasmid efficiency (no. of transformants)					
	Low copy no.			High copy no.		
	Control	*glc7-Δ186-312*	*GLC7*	Control	*glc7-Δ186-312*	*GLC7*
IPL1	2,154	6,330	4,332	8,694	2,658	654
ipl1-1	4,344	7,092	4,674	10,974	5,208	414
ipl1-2	1,590	9,636	468	7,656	4,290	18

[a] Aliquots (~0.12 pmol) of the low-copy-number *URA3* plasmids pRS316 (control), pCC419 (containing *glc7-Δ186-312*), and pCC413 (containing *GLC7*) and the high-copy-number *URA3* plasmids pSM217 (control), pCC418 (containing *glc7-Δ186-312*), and pCC414 (containing *GLC7*) were used to transform the yeast strains DBY1826 (*IPL1*), CCY357-7C (*ipl1-1*), and CCY358-2D (*ipl1-2*) (24). Ura^+ transformants were selected at 26°C on supplemented SD lacking uracil. The number of transformants obtained from a typical experiment is shown here.

The *glc7-1* mutation suppresses the Ts^- phenotype caused by the *ipl1-1* mutation. The fact that overexpression of *glc7-Δ186-312* and *GLC7* had opposite effects in *ipl1* mutants suggested that the *glc7-Δ186-312* allele functions as a dominant negative mutation, resulting in reduced *GLC7* function (see Discussion for an explanation). If this is true, we may expect chromosomal loss-of-function mutations in *GLC7* to also suppress the *ipl1-1* mutation. The recessive *glc7-1* mutation (6) represents a likely loss-of-function mutation, because PP1 activity is reduced by about 60% in extracts prepared from *glc7-1* mutant cells (17). To determine whether the *glc7-1* mutation can suppress the *ipl1-1* mutation, we analyzed tetrads from a cross between *ipl1-1 GLC7* (CCY357-7C) and *IPL1 glc7-1* (CCY435-15C) strains. All 19 *ipl1-1 glc7-1* spores we examined were Ts^+ for growth at 35°C, indicating that *glc7-1* suppresses the Ts^- phenotype caused by the *ipl1-1* mutation (Fig. 6). The failure of *glc7-1* to suppress the *ipl1-1* mutant phenotype at 37°C suggested that suppression requires residual

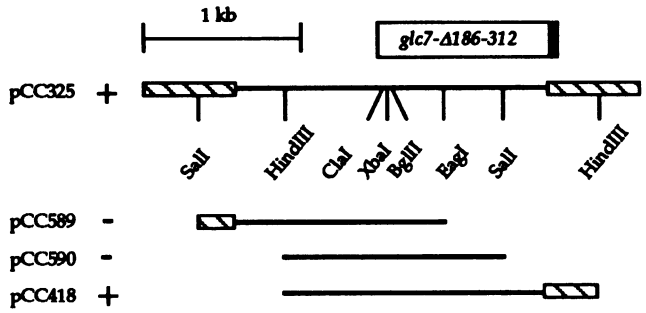


FIG. 5. Functional localization of the dosage-dependent suppressor of *ipl1-1* on plasmid pCC325. The vector sequence (hatched box), the 5′ two-thirds of the *GLC7* gene (open box), including a 525-bp intron, and the ORF from YEp24 (shaded box) that is fused in frame with the truncated *GLC7* gene are indicated. The ability (+) or inability (−) of the different high-copy-number plasmids (containing the DNA fragments shown) to suppress the Ts^- phenotype of an *ipl1-1* mutant (CCY107-8D-1) at 35°C is indicated.

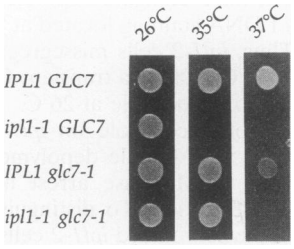


FIG. 6. Suppression of the Ts^- phenotype of *ipl1-1* mutants by the *glc7-1* mutation. Suspensions of the following yeast strains were spotted on YEPD plates and allowed to grow at the indicated temperatures for 42 h: CCY464-5D (*IPL1 GLC7*), CCY464-1D (*ipl1-1 GLC7*), CCY464-6C (*IPL1 glc7-1*), and CCY464-4C (*ipl1-1 glc7-1*).

TABLE 4. Frequencies of recombination and chromosome gain for wild-type haploid strains with genetically marked chromosome II[a]

Plasmid gene	Frequency	
	Recombination (Lys^+)	Chromosome gain × recombination (His^+ Lys^+)
Control	$(7.2 \pm 2.8) \times 10^{-4}$	$(10.4 \pm 2.3) \times 10^{-7}$
IPL1	$(5.6 \pm 1.1) \times 10^{-4}$	$(8.5 \pm 1.7) \times 10^{-7}$
GLC7	$(5.8 \pm 1.1) \times 10^{-4}$	$(4.9 \pm 2.1) \times 10^{-5}$
glc7-Δ186-312	$(5.8 \pm 1.1) \times 10^{-4}$	$(8.0 \pm 2.3) \times 10^{-7}$

[a] The wild-type haploid yeast strain CCY482-13D was transformed to Ura^+ with the high-copy-number *URA3* plasmids containing the genes shown: pSM217 (control), pCC146 (*IPL1*), pCC510 (*GLC7*), and pCC418 (*glc7-Δ186-312*). Cells from individual Ura^+ transformants were grown to stationary phase at 26°C in 5 ml of SD supplemented with Casamino Acids and adenine (i.e., lacking uracil). Appropriately diluted aliquots were plated on selective medium lacking uracil (total cell count), on selective medium lacking uracil and lysine (Lys^+ cell count), and on selective medium lacking uracil, lysine, and histidine (His^+ Lys^+ cell count). Colonies were scored after 3 days at 26°C. The mean frequencies and standard deviations obtained from eight independent cultures of each strain are shown here.

Ipl1 protein kinase activity presumed to be missing at 37°C. In this same cross, we also examined whether the *ipl1-1* mutation can suppress the glycogen accumulation defect caused by *glc7-1*. Our results indicated that *ipl1-1* cannot rescue this defect, even at the elevated temperature of 35°C.

Overexpression of $PP1_C$ causes chromosome gain. So far, our results show that the lethality caused by a (presumed) partial reduction in Ipl1 protein kinase activity can be compensated by a reduction in PP1 activity, thus suggesting that Ipl1 protein kinase activity is counteracted by PP1 activity in vivo. If this idea is correct, we might expect cells overproducing $PP1_C$ to have a phenotype similar to that of *ipl1* mutants. Since *ipl1* mutants are known to have increased frequencies of chromosome gain (9), we examined the effect of increased dosage of *IPL1*, *GLC7*, or *glc7-Δ186-312* on chromosome stability in wild-type cells, using an assay that measured the copy number of chromosome II (9). In this assay, intrachromosomal recombination frequency at the *lys2-Δ101*::*HIS3*::*lys2-Δ102* locus on chromosome II was measured as the Lys^+ frequency, and the product of this frequency with the frequency of the gain of chromosome II was measured as the Lys^+ His^+ frequency.

As shown in Table 4, an increased dosage of *IPL1* or *glc7-Δ186-312* had no significant effect on the frequency of recombination or chromosome gain. Similar results were obtained with the *glc7-1* mutant (data not shown). In contrast, increased dosage of *GLC7* caused a large (~47-fold) increase in the frequency of chromosome gain while having little effect on the recombination frequency. This result suggests that overexpression of $PP1_C$ causes chromosome gain, a phenotype also associated with mutants defective in the Ipl1 protein kinase, thus supporting the idea that Ipl1 protein kinase activity is counteracted by PP1 activity in vivo.

DISCUSSION

We have previously shown by flow cytometry and a chromosome gain genetic assay that conditional $ipl1^{ts}$ mutants suffer from severe chromosome missegregation at the restrictive growth temperature of 37°C (9). In this report, we showed that the missegregation of chromosomes can be detected cytologically and that this defect is not associated with readily detectable alterations in the appearance of the mitotic spindle (Fig. 3). This observation suggests that *IPL1* function is not required for spindle pole body duplication and separation or spindle assembly and disassembly. Furthermore, at the otherwise permissive growth temperature of 26°C, most *ipl1-2* mutant cells die after a transient G_2/M phase arrest caused by the microtubule-depolymerizing drug nocodazole (Table 2). While the underlying cause for this lethality is unknown, it appears also to be associated with chromosome missegregation.

DNA sequence analysis revealed that the *IPL1* gene encodes a protein homologous to protein-serine/threonine kinases. The primary sequence of the Ipl1 protein kinase is not particularly homologous to that of the Mck1 protein kinase, which is also important for chromosome segregation in yeast (14, 38, 54). These two protein kinases differ in one important aspect: the Ipl1 protein is essential for cell viability, whereas the Mck1 protein is not, suggesting that these two enzymes may have different endogenous substrates that must be phosphorylated for chromosome segregation to occur normally. The Cdc5 (28), Cdc15 (52), Dbf2 (25), and Dbf20 (61) protein kinases are required for late nuclear division and thus also may be involved in mitosis and chromosome segregation in yeast cells. Functionally, the Ipl1 protein kinase differs from these four protein kinases in not being required for nuclear division, again suggesting that these protein kinases may have different physiological substrates.

Our temperature shift experiments with synchronized $ipl1\text{-}2^{ts}$ mutant cells showed that Ipl1p function is required during the later part of the cell cycle (Fig. 2) and that the lethality caused by Ipl1p inactivation requires cell cycle progression beyond S phase (Table 2). These results are consistent with the requirement of *IPL1* function in chromosome segregation during mitosis. Furthermore, our results showed that yeast cells die within a single cell cycle upon inactivation of Ipl1p, indicating that the substrate(s) of the Ipl1 protein kinase has to be phosphorylated de novo during each cell cycle. This may be due to degradation or dephosphorylation of the substrate(s) in each cell cycle. The latter possibility is supported by our finding that the lethality caused by partial inactivation of Ipl1p can be ameliorated by in vivo perturbations that presumably result in reduced PP1 activity.

We isolated a truncated form of the *GLC7* gene (*glc7-Δ186-312*) on the basis of its ability in high copy numbers to suppress the *ipl1-1* mutation. The full-length *GLC7* gene encodes $PP1_C$, the catalytic subunit of PP1. The suppression of a protein kinase defect by overexpression of a truncated *GLC7* gene (*glc7-Δ209-312*) was also observed in *gcn2-507* mutants (62), which are defective in the protein kinase that phosphorylates the translation initiation factor eIF-2α. Several lines of evidence suggest that overexpression of the *glc7-Δ186-312* gene product suppresses the Ipl1 protein kinase defect by interfering with the chromosomally encoded wild-type Glc7 $PP1_C$. First, the recessive chromosomal *glc7-1* mutation, which causes a dramatic reduction in glycogen accumulation and PP1 activity in vivo (6, 17, 20, 58), also can suppress the *ipl1-1* mutation (Fig. 6). Second, overexpression of the full-length *GLC7* gene in wild-type cells results in a great increase in the frequency of chromosome gain (Table 4), a phenotype similar to that seen in *ipl1* mutants (9). Third, overexpression of the full-length *GLC7* gene in *ipl1-2* mutant cells appears to result in cell death rather than suppression of the Ts^- phenotype (Table 3). These results together suggest that overexpression of *glc7-Δ186-312* leads to reduced PP1 activity in vivo, and this in turn compensates for a reduction in Ipl1 protein kinase activity.

$PP1_C$ is known to be complexed with regulatory or targeting subunits in vivo in different organisms (3, 12), and the functional role of $PP1_C$ in its uncomplexed form is unknown. The regulatory or targeting subunits influence the activity of $PP1_C$

towards various substrates in vitro. Some of the *GLC7*-encoded yeast $PP1_C$ is probably also complexed with other proteins (17, 58). Thus, it is likely that deletion of the C-terminal 127 residues from Glc7p inactivates the phosphatase activity but still allows the *glc7-Δ186-312* gene product to compete with wild-type Glc7 $PP1_C$ for binding to regulatory and/or targeting subunits, resulting in a reduction in the level of functional $PP1_C$ complexes (towards certain substrates) when *glc7-Δ186-312* is overexpressed. This is an interpretation that was also proposed by Wek et al. (62). It is interesting that overexpression of the *glc7-Δ96-312* allele (carried on pCC590), unlike overexpression of the *glc7-Δ186-312* allele, fails to suppress the *ipl1-1* mutation (Fig. 5). If the truncated proteins encoded by the two mutant alleles are equally stable, this result would suggest that the domain spanning residues 96 through 185 of Glc7 $PP1_C$ is involved in the interaction of Glc7p with at least one regulatory or targeting subunit that is important for chromosome segregation.

The results described above strongly suggest that PP1 acts in opposition to the Ipl1 protein kinase in vivo. This functional interaction is likely to be specific, because the *pph22-δ1::HIS3* mutation, which inactivates one of at least two genes encoding the catalytic subunit of PP2A (47, 56), cannot suppress the *ipl1-1* mutation (our unpublished results). Assayable PP2A activity is known to be reduced by about 33% in crude extracts prepared from *pph22* deletion mutants (56). The functional relationship between Ipl1p and PP1 may be interpreted in several ways. First, Ipl1p and PP1 may have common endogenous substrates that must be phosphorylated during the later part of each cell cycle for chromosome segregation to occur normally; a partial reduction in the phosphorylating activity (due to the *ipl1-1* or *ipl1-4* mutation) may be compensated by a partial reduction in the dephosphorylating activity (due to perturbations that reduce PP1 activity), whereas absence of the phosphorylating activity cannot be compensated by absence of the dephosphorylating activity. Consistent with this idea is the observation that cells lacking both *IPL1* and *GLC7* are inviable (our unpublished results). Second, the Ipl1 protein kinase itself may require phosphorylation to be active, and PP1 may dephosphorylate and inactivate the Ipl1 protein kinase in vivo. Third, PP1 may need to be phosphorylated and (partially) inactivated by Ipl1p, and hyperactive PP1 causes chromosome missegregation. If this is true, a regulatory subunit of PP1 would be the likely target for Ipl1p, because yeast $PP1_C$ phosphorylation is not detectable in vivo (58). Finally, Ipl1p and PP1 may have different substrates that function in the same chromosome segregation pathway. Clearly, more complicated functional relationships are possible and they will be the subject of our study in the future.

The study of PP1 mutants in *Drosophila melanogaster* (1), *Aspergillus nidulans* (15), and *S. pombe* (26, 40, 41, 57), as well as microinjection experiments in rat fibroblasts (18), has clearly demonstrated the absolute requirement of PP1 in mitosis in these organisms. Recently, the analysis of conditional *glc7* mutant phenotypes also suggested that PP1 may be required for mitosis in *S. cerevisiae* (22a, 56a). The function of PP1 in mitosis in *S. cerevisiae* probably is not entirely limited to its action against the Ipl1 protein kinase. The lethality caused by the absence of *GLC7* cannot be suppressed by the simultaneous absence of *IPL1*. The chromosome gain phenotype caused by overexpression of *GLC7*, though similar to that seen in *ipl1* mutants, also cannot be reversed by simultaneous overexpression of *IPL1* (our unpublished results). It has been shown that increasing the level of PP1 by microinjection in an anaphase B rat fibroblast results in accelerated exit from mitosis (18). It will be interesting to find out whether the chromosome gain defect seen in yeast cells overexpressing *GLC7* may also be due to premature exit from mitosis.

Ultimately, our understanding of the functional roles of the Ipl1 protein kinase and PP1 requires identification of the physiological substrates for these two enzymes. Possible candidate substrates include the kinetochore protein complex CBF3, which requires phosphorylation for its in vitro centromere DNA-binding activity (30). Using mutant cells that have unusually high or low Ipl1 protein kinase or PP1 activity, we hope to identify physiological substrates for these two enzymes in the near future.

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