Genetic Interactions Between *GLC7*, *PPZ1* and *PPZ2* in *Saccharomyces cerevisiae*

Guglielmo M. Venturi, Andrew Bloecher, Tara Williams-Hart and Kelly Tatchell

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Shreveport, Louisiana 71130

Manuscript received December 2, 1999
Accepted for publication January 24, 2000

ABSTRACT

GLC7 encodes an essential serine/threonine protein type I phosphatase in *Saccharomyces cerevisiae.* Three other phosphatases (Ppz1p, Ppz2p, and Sal6p) share >59% identity in their catalytic region with Glc7p. *ppz1 ppz2* null mutants have no apparent growth defect on rich media. However, null alleles of *PPZ1* and *PPZ2*, in combination with mutant alleles of *GLC7*, confer a range of growth defects varying from slow growth to lethality. These results indicate that Glc7p, Ppz1p, and Ppz2p may have overlapping functions. To determine if this overlap extends to interaction with targeting subunits, Glc7p-binding proteins were tested for interaction in the two-hybrid system with the functional catalytic domain of Ppz1p. Ppz1p interacts strongly with a number of Glc7p regulatory subunits, including Glc8p, a protein that shares homology with mammalian PP1 inhibitor I2. Genetic data suggest that Glc8p positively affects both Glc7p and Ppz1p functions. Together our data suggest that Ppz1p and Ppz2p may have overlapping functions with Glc7p and that all three phosphatases may act through common regulatory proteins.

THE PPP family of serine/threonine protein phosphatases includes the well-studied enzymes PP1, PP2A, and calcineurin/PP2B (Barford 1996). The holoenzymes of these consist of a conserved catalytic subunit and one or more regulatory subunits. For PP1 (Egloff *et al.* 1997) and PP2A (Cohen 1989; Ferrigno *et al.* 1993; Depaoli-Roach *et al.* 1994), multiple unique regulatory subunits bind the catalytic subunit and either target the enzyme to its site of activity or otherwise regulate its activity. These regulatory subunits are thought to be necessary for the enzymes to carry out their wide range of physiological activities. In addition to PP1, PP2A, and PP2B, novel and generally less-well-studied phosphatases round out the gene family (Cohen 1997). Many of these novel phosphatases are very similar to either PP1 or PP2A within their catalytic domains but the extent to which targeting subunits contribute to their regulation is not well understood.

Saccharomyces cerevisiae contains 12 members of the PPP family of phosphatases, including 2 isoforms of PP2A, 2 isoforms of PP2B, 1 isoform of PP1, and 7 additional enzymes. These latter include three enzymes most similar to PP2A (Sit4p, Pph3p, and Ppg1p); three similar to PP1 (Ppq1p/Sal6p, Ppz1p, and Ppz2p); and Ppt1, a more distantly related member of the family (Stark 1996). The catalytic domains of the PP1 family members are >59% identical but Ppz1p, Ppz2p, and Sal6p have sequences upstream of the catalytic domain that are not found in Glc7p. The overall structures of these enzymes are diagrammed in Figure 1. Glc7p is the most extensively characterized member of this group. It is >70% identical to mammalian PP1 isoforms, for which crystal structures are known (Egloff *et al.* 1995, 1997; Goldberg *et al.* 1995), and participates in such diverse processes as cell cycle regulation (Francisco *et al.* 1994; Hisamoto *et al.* 1994; Black *et al.* 1995; MacKelvie *et al.* 1995; Bloecher and Tatchell 1999; Sassoon *et al.* 1999), glycogen metabolism (Feng *et al.* 1991; François *et al.* 1992; Cannon *et al.* 1994; Stuart *et al.* 1994), sporulation (Cannon *et al.* 1994; Tu *et al.* 1996), and glucose repression (Tu and Carlson 1994, 1995).

In contrast to the extensive investigations of Glc7p, much less is known about the substrates and physiological roles of Ppz1p, Ppz2p, and Sal6p/Ppq1p. *PPZ1* was cloned by virtue of its sequence similarity to other phosphatases (Da Cruz e Silva *et al.* 1991; Posas *et al.* 1992). *PPZ2* was cloned both in the same manner as *PPZ1* (Da Cruz e Silva *et al.* 1991) and as a dosage suppressor of recessive mutants in *MPK1*, a component of a MAP kinase pathway (Lee *et al.* 1993). Yeast strains lacking Ppz1p and Ppz2p are viable but exhibit an osmotic-remedial cell lysis defect in some genetic backgrounds (Hughes *et al.* 1993; Lee *et al.* 1993; Posas *et al.* 1993) and are resistant to high salt (Posas *et al.* 1995b). *PPQ1* was cloned on the basis of its sequence similarity to related phosphatases (Chen *et al.* 1993) and was identified independently by mutations that enhance translational suppressors (*SAL6*; Vincent *et al.* 1994). Like *PPZ1* and *PPZ2*, *PPQ1*/*SAL6* is not essential, but *ppq1* mutants have defects in protein synthesis (Chen *et al.* 1993) and are sensitive to protein synthesis inhibitors

Corresponding author: Kelly Tatchell, Department of Biochemistry and Molecular Biology, Louisiana State University Medical Ctr., 1501 Kings Hwy., Shreveport, LA 71130.
E-mail: ktatch@mail.sh.lsumc.edu

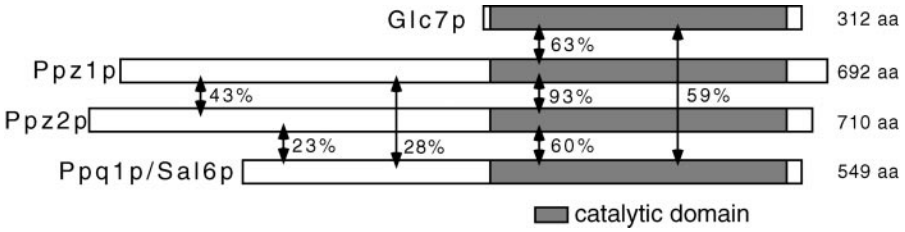


Figure 1.—Alignment of protein phosphatase sequences. Glc7p, Ppz1p, Ppz2p, and Sal6p share >60% identity at the amino acid sequence level in their catalytic domains. Sequences NH_2-terminal to the catalytic domains are less conserved.

(Chen *et al.* 1993; Vincent *et al.* 1994), suggesting that this enzyme functions in translational control.

The high sequence conservation within the catalytic domains of these four enzymes (Figure 1) and our finding that relatively few *GLC7* alleles isolated by alanine-scanning mutagenesis conferred conditional growth phenotypes (Baker *et al.* 1997) led us to ask if these enzymes share overlapping functions. The hypothesis that we have tested is that *PPZ1*, *PPZ2*, and *SAL6* are at least partially redundant with *GLC7* and that this redundancy is responsible for the relatively robust growth phenotype of many *GLC7* mutants.

MATERIALS AND METHODS

Strains, media, and growth conditions: The strains used in this study are listed in Table 1 and are all congenic to KT1112 with the exception of PJ69-4A. The *sal6::HIS3* disruption in strain KT1618 was constructed by digesting pAV194 (Vincent *et al.* 1994) with *Bam*HI and *Sal*I and transforming into yeast. The *sla1::URA3* disruption strain was kindly provided by D. Drubin. The *gip1::HIS3* disruption in strain AB104 was constructed using plasmid pJT26 as described in Tu *et al.* (1996). The *yol091w::kan*^{R} and *yal014c::kan*^{R} disruption strains were obtained from EUROSCARF (Frankfurt, Germany) and Research Genetics (Huntsville, AL), respectively. The *ppz1::TRP1*, *ppz1::URA3*, and *ppz2::LEU2* mutations in strains DL920, DL786, and DL789, respectively, were introduced into our genetic background by seven serial backcrosses. Yeast cells were grown either on YPD (1% yeast extract, 2% Bacto-peptone, 2% glucose) or on synthetic medium (0.67% yeast nitrogen base, 2% glucose) supplemented with the appropriate amino acids. Yeast genetic manipulations were carried out as described previously (Sherman and Hicks 1991). Sporulation was induced on medium containing 1% yeast extract, 2% peptone, and 2% potassium acetate. Glucose-repression defects were assessed by testing for growth on medium containing 1% yeast extract, 2% peptone, 200 μg/ml 2-DG (2-deoxyglucose; Sigma, St. Louis), and 2% sucrose under anaerobic conditions (GasPak; Difco, Detroit). *Escherichia coli* strains DH5αF′ and XL1-Blue were used for cloning and propagation of plasmids. Yeast transformations were carried out using the lithium acetate method (Gietz *et al.* 1992).

GLC8 was disrupted by a modified method described by Erdeniz *et al.* (1997). The *Kluyveromyces lactis URA3* gene was amplified by PCR using primers annealing to the ends of the *URA3* gene and containing adaptamer sequences. Both 5′ and 3′ flanking sequences of *GLC8* (~300 bp each) were amplified in separate PCR reactions. The primers for these reactions were designed such that two primers (BV-29 and BV-30) contained sequences complementary to the *GLC8* gene followed by sequences complementary to adaptamer sequences of the primers used to amplify the *K. lactis URA3* gene. The primers used to amplify the 5′ end of *GLC8* were BV-28 5′-CCCGAACA CTACCAAATCAATAGCAG-3′ and BV-29 5′-GATCCCCGGG AATTGCCATGCATTATTCTGCTGATGTGC-3′. The primers used to amplify the 3′ end region of *GLC8* were BV-30 5′-AA TTCCAGCTGACCACCATGGAAGCATTACGACGTAA GG-3′ and BV-31 5′-CGTTTCGCGTGATAACAAACAGTGC-3′. The PCR products from the three independent reactions were combined and the full-length product was amplified using primers BV-28 and BV-31. The resulting PCR product was transformed into KT1358, and Ura$^+$ colonies containing the disrupted gene were selected.

Plasmid construction: Plasmids used in this study are listed in Table 2. pUC19-*PPZ1* (kindly provided by D. Levin; Posas *et al.* 1992) contains the entire *PPZ1* coding sequence. pUC19-*PPZ1* was digested with *Pst*I and religated to change the orientation of the insert relative to the pUC19 *Hin*dIII site, creating pBV207. The change in orientation allowed the entire *PPZ1* gene to be released by digestion with *Hin*dIII. This fragment was ligated into the *Hin*dIII site of pNC160, yielding pBV216. A truncated *PPZ1* gene lacking the first 360 codons was generated in several steps. The *Cla*I-*Bgl*II fragment of *GLC7* in pHH1 [*Hin*dIII-*Sal*I clone of *HA-GLC7* from YCp50-*HA-GLC7* (Stuart *et al.* 1994) inserted into *Hin*dIII-*Xho*I in pBS-SK$^+$] was replaced with a 423-bp *Cla*I-*Bgl*II fragment of *PPZ1*, corresponding to codons 361–501, yielding pBV210. A 2114-bp *Bgl*II fragment of pUC19-*PPZ1*, encoding the remainder of the C-terminal domain of *PPZ1*, codons 502–692, was inserted in the correct orientation into pBV210 at the *Bgl*II site, yielding pBV211. pBV211 therefore encodes the catalytic domain of Ppz1p (codons 361–692) fused in frame with a hemagglutinin (HA) tag (Wilson *et al.* 1984) and the first 11 amino acids (aa) of Glc7p, under the control of the native *GLC7* promoter. The *GLC7:PPZ1* fusion was released from pBV211 with *Hin*dIII and inserted into the *Hin*dIII site of pNC160 (pBV212).

For the two-hybrid screen, the catalytic domain of Ppz1p (aa 361–692) was fused to the DNA-binding domain of Gal4. In the first step of the construction, an *Eco*RI-*Sac*I fragment of *PPZ1* encoding the central catalytic portion of Ppz1p was inserted into pBluescript. Oligonucleotide primers were used to introduce a *Nco*I site at position +1081 in *PPZ1*. Primers used were BV-5 (5′-CAAAAAGCCCATGGATATTGATGAAAC TATCC-3′) and the complementary primer BV-6 (5′-GGATAG TTTCATCAATATCCATGGGCTTTTTG-3′), where the *Nco*I site is underlined. The two halves of the *Eco*RI-*Sac*I fragment of *PPZ1* were amplified independently by PCR using BV-5, BV-6, and primers complementary to the T7 and T3 promoter sequences in pBluescript, respectively. The two independent PCR products were then combined in a PCR reaction using the T7 and the T3 primers to recover the full-length *Eco*RI-*Sac*I *PPZ1* fragment containing the engineered *Nco*I site. The PCR product was digested with *Eco*RI-*Cla*I and inserted into pBV213 yielding pBV214. pBV213 is a plasmid containing a 2114-bp *Bgl*II fragment of *PPZ1* inserted into the *Bam*HI site of pUC18. The orientation of the *PPZ1 Bgl*II fragment in pBV213 is such that the *Eco*RI site of pUC18 is at the 5′ end of *PPZ1*. pBV214 was cut with *Nco*I and *Sal*I and the *Nco*I-*Sal*I

TABLE 1

***Saccharomyces cerevisiae* strains**

Strain	Genotype	Source
AB104	*MAT***a** *gip1::his3 ura3 leu2 his3*	This study
CC14	*MAT*α *pig1::URA3 ura3 leu2 his3*	Cheng *et al.* (1997)
DL786	*MAT***a** *ppz1::URA3 ura3 leu2 his4 trp1*	Lee *et al.* (1993)
DL789	*MAT***a** *ppz2::LEU2 ura3 leu2 his4 trp1*	Lee *et al.* (1993)
DL920	*MAT***a** *ppz2::TRP1 ura3 leu2 his4 trp1*	Lee *et al.* (1993)
KT1112	*MAT***a** *ura3 leu2 his3*	Stuart *et al.* (1994)
KT1113	*MAT*α *ura3 leu2 his3*	Frederick and Tatchell (1996)
KT1150	*MAT***a** *gac1::URA3 ura3 leu2*	This study
KT1357	*MAT***a** *ura3 leu2 his3 trp1*	Frederick and Tatchell (1996)
KT1358	*MAT*α *ura3 leu2 his3 trp1*	This study
KT1492	*MAT***a** *ppz1::URA3 ppz2::LEU2 ura3 leu2 his3*	This study
KT1511	*MAT***a** *reg2::URA3 ura3 leu2 his3 trp1*	This study
KT1596	*MAT*α *glc7-109 ura3 leu2 his3*	This study
KT1599	*MAT*α *glc7-132 ura3 leu2 his3*	This study
KT1601	*MAT***a** *ppz1::TRP1 ppz2::LEU2 ura3 leu2 his3 trp1*	This study
KT1621	*MAT*α *glc7-129 ura3 leu2 his3*	This study
KT1626	*MAT***a** *ppz1::URA3 ppz2::LEU2 ura3 leu2 his3*	This study
KT1628	*MAT***a** *ppz1::URA3 ppz2::LEU2 glc7-109 ura3 leu2 his3*	This study
KT1630	*MAT***a** *ppz1::URA3 ppz2::LEU2 glc7-132 ura3 leu2 his3*	This study
KT1638	*MAT***a** *glc7-109 ura3 leu2 his3*	This study
KT1639	*MAT***a** *glc7-132 ura3 leu2 his3*	This study
KT1714	*MAT*α *ppz1::TRP1 ppz2::LEU2 ura3 leu2 his3 trp1*	This study
EG716-6B	*MAT*α *ppz2::LEU2 reg1::URA3 leu2 ura3 his3 trp1*	This study
BV352	*MAT***a** *ppz1::TRP1 leu2 ura3 his3 trp1*	This study
BV427	*MAT***a** *glc7-127 ppz1::URA3 ppz2::LEU2 ura3 leu2 his3 trp1* w/pBV215	This study
BV428	*MAT***a** *glc7-127 ppz2::LEU2 ura3 leu2 his3 trp1*	This study
BV448	*MAT***a** *glc7-133 ura3 leu2 his3 trp1*	This study
BV449	*MAT***a** *glc7-109 ura3 leu2 his3 trp1*	This study
BV450	*MAT***a** *glc7-132 ura3 leu2 his3 trp1*	This study
BV451	*MAT***a** *glc7-127 ura3 leu2 his3 trp1*	This study
BV452	*MAT*α *ppz2::LEU2 ura3 leu2 trp1*	This study
BV459	*MAT*α *glc7-133 ura3 leu2 his3 trp1*	This study
BV460	*MAT*α *ppz1::URA3 ppz2::LEU2 ura3 leu2 his3 trp1*	This study
BV461	*MAT*α *ppz1::URA3 ppz2::LEU2 glc7-133 ura3 leu2 his3*	This study
BV470	*MAT*α *glc8::URA3 ura3 leu2 his3 trp1*	This study
BV471	*MAT***a** *glc8::URA3 ura3 leu2 his3 trp1*	This study
BV472	*MAT***a** *glc8::URA3 ura3 leu2 his3*	This study
BV473	*MAT*α *glc8::URA3 ura3 leu2 his3*	This study
BV475	*MAT***a** *ppz1::URA3 ura3 leu2 his3 trp1*	This study
BV477	*MAT***a** *glc7-127 glc8::URA3 ura3 leu2 his3 trp1*	This study
BV479	*MAT***a** *glc7-109 glc8::URA3 ura3 leu2 his3 trp1*	This study
BV481	*MAT***a** *glc7-133 glc8::URA3 ura3 leu2 his3 trp1*	This study
BV483	*MAT***a** *glc7-132 glc8::URA3 ura3 leu2 his3 trp1*	This study
BV519	*MAT*α *ppz1::TRP1 ura3 leu2 his3 trp1*	This study
BV533	*MAT***a** *ppz1::URA3 ppz2::LEU2 ura3 leu2 trp1*	This study
BV534	*MAT*α *ppz1::URA3 ppz2::LEU2 ura3 leu2 his3*	This study
BV543	*MAT*α *sla1::URA3 ura3 leu2 his3 trp1*	This study
BV546	*MAT*α *yol091w::kan*^{R} *ura3 leu2 his3 trp1*	This study
BV548	*MAT*α *yal014c::kan*^{R} *ura3 leu2 his3 trp1*	This study
PJ69-4A	*MAT***a** *ura3 leu2 his3 trp1 gal4*Δ *gal80*Δ *GAL2:ADE2 lys2::GAL1:HIS3 met2::GAL7:lacZ*	James *et al.* (1996)

fragment containing the catalytic domain of *PPZ1*, corresponding to amino acids 361–692, was inserted into the two-hybrid vector pAS-CYHII (Durfee *et al.* 1993), creating pBV222. The full-length Ppz1p was also fused to the Gal4-DNA-binding domain of the two-hybrid vector pAS-CYHII for use in our analysis. The N terminus of Ppz1p (aa 1–360) was amplified by PCR from pUC-19-*PPZ1* using primers BV-46 (5′-CCTTCCTTTTCACCATGGCTAATTCAAGTTC-3′), which introduced the underlined *Nco*I site at the start codon and changed the Gly at the second amino acid position to Ala to abolish N myristoylation, and BV-47 (5′-TGTTCTCTTAGCAG CGTAGCCCGCATCCAG-3′), which annealed downstream of

TABLE 2

Plasmids

Name	Vector	Description	Source
p1814	YCp50	*GLC8*	Cannon *et al.* (1994)
pAV194	Ylp5	*sal6::HIS3*	Vincent *et al.* (1994)
pUC19-*PPZ1*	pUC19	*Pst*I-*Pst*I *PPZ1* clone	Posas *et al.* (1992)
pNC160		*CEN TRP1*	Rhodes *et al.* (1990)
pBV212	pNC160	*HA:GLC7:PPZ1*$_{(aa\ 361–692)}$	This study
pBV215	pNC160	*GFP:PPZ1*	This study
pBV216	pNC160	*PPZ1*	This study
pSB17	pNC160	*HA:GLC7*	Baker *et al.* (1997)
pBV222	pAS-CYHII	*PPZ1*$_{(aa\ 361–692)}$	This study
pBV224	pAS-CYHII	*PPZ1*$_{(aa\ 1–692)}$	This study
YEp351		2 μm *LEU2*	Hill *et al.* (1986)
pBV223	YEp351	*GFP:PPZ1*	This study
pDF85	pAS1	*GAL4*$_{1–147}$-*GLC7*	This study
pCV6	pGAD2F	*GAL4*$_{768–881}$-*GAC1*	Stuart *et al.* (1994)
pSE1111	pACT	*SNF4*	S. Elledge (personal communication)
pDF112	pACT	*GLC8*	D. Frederick (personal communication)
pDF116	pACT	*REG2*	Frederick and Tatchell (1996)

the *Cla*I site at position +1081. The PCR product was digested with *Nco*I and *Cla*I and cloned into pBV222. During this cloning step the 441-bp *Nco*I-*Cla*I fragment of pBV222 was replaced with the native 441-bp *Cla*I fragment of pBV216 in the correct orientation, creating pBV224.

To construct a *GFP:PPZ1* gene fusion, the green fluorescent protein (GFP) variant GFP^{F64L,S65T} (Cormack *et al.* 1996) was amplified from the pRSETB:GFP template by PCR using primers BV-9 (5′-GGGCGCTCGAGTCCCCCCGCTGAATTCATGAG-3′) and BV-11 (5′-CGCGGCTCGAGTCTTTGTATAGTTCATCC-3′) that introduced the underlined *Xho*I sites flanking the GFP coding region. The PCR product was cut with *Xho*I and cloned into a unique *Xho*I site present in pUC19-*PPZ1* at nucleotide +54, creating pBV209. pBV209 was digested with *Hin*dIII to release the entire GFP:Ppz1p coding sequence, which was inserted into pNC160 and YEp351, yielding pBV215 and pBV223, respectively.

Genomic integration of *glc7-109, glc7-127, glc7-129, glc7-132,* and *glc7-133*: The previously described alanine-scanning alleles of *GLC7* were tested for function on the centromere vector pNC160 (Baker *et al.* 1997). To facilitate genetic analysis of these mutants, we integrated *glc7-109*, *glc7-127*, *glc7-129*, *glc7-132*, and *glc7-133* at the *GLC7* locus using a two-step gene replacement technique (Scherer and Davis 1979). The *Bgl*II-*Kpn*I restriction fragments from plasmids that contain these alleles were cloned into the *Bam*HI-*Kpn*I sites of the integrating vector pRS306 (Sikorski and Hieter 1989). These plasmids, lacking the 5′ end of the *GLC7* gene, were linearized at the unique *Sal*I site in *GLC7* and transformed into haploid yeast strains KT1112 and KT1113. Ura$^+$ transformants showed phenotypes previously reported for the plasmid-borne *glc7* mutants. These transformants were grown on medium containing 5-fluoroorotic acid to select for recombination events that looped out the integrated plasmids. 5-FOA-resistant Ura$^-$ segregants were screened for the phenotype of the *glc7* mutant. Southern analysis and genomic PCR were used to confirm that only a single *GLC7* gene was present in the *GLC7* locus and that the wild-type *GLC7* allele had looped out.

DNA sequencing: The sequence of the *Nco*I-*Cla*I PCR fragment used in the construction of pBV222 was determined. Specific oligonucleotides were designed to allow sequence analysis of both strands using Sequenase (United States Biochemical, Cleveland) and the dideoxy-chain termination method. Primer BV-7 (5′-TCCGGAAACAAAAACGCTCC-3′) was used at the 5′ end and primer BV-8 (5′-GCTACGATAGCAGCTAATGG-3′) was used at the 3′ end. Comparison of the nucleotide sequence to wild type was performed using a BLAST search at the Stanford Genomic Resources Center. The sequence of the *Nco*I-*Cla*I fragment used in the construction of pBV224 was also determined. The primers used were GV-3 (5′-CCATTTGGATCATTGAAGGTG-3′), GV-4 (5′-TCAAACGTCCCTGATCCCTC-3′), and GV-5 (5′-AGACAACGACATCTCGCAC-3′). Primers and templates were sent to the DNA Sequencing Facility at Iowa State University and comparison of the nucleotide sequence to wild type was performed using a BLAST search at the Stanford Genomic Resources Center.

Immunoblot analysis: Immunoblot analysis was performed on total cell extracts. Cell extracts were prepared by growing cells to mid-log phase, breaking the cells with glass beads in the presence of 5% trichloroacetic acid, and precipitating total protein (Davis *et al.* 1993). The pelleted protein samples were resuspended in a 6-m urea buffer, electrophoresed through an SDS-polyacrylamide gel, and immunoblotted with monoclonal HA antisera (12CA5; Wilson *et al.* 1984) at a dilution of 1:1000 and horseradish-peroxidase-conjugated secondary antibody at a dilution of 1:1000. The ECL detection system (Amersham, Arlington Heights, IL) was used to detect antibody binding.

Two-hybrid assay: Yeast strain PJ69-4A (James *et al.* 1996) was used for the two-hybrid assay. *GLC7*, *PPZ1*$_{(aa\ 361–692)}$, and *PPZ1*$_{(aa\ 1–692)}$ were fused in frame to the Gal4DBD in pAS-CYHII. Glc7p-interacting proteins were fused in frame to the Gal4-AD in either pACTII or pGAD. Protein-protein interactions were measured by assaying β-galactosidase enzyme activity (Kaiser *et al.* 1994). Assays were performed in triplicates and the mean and simple standard deviation were calculated.

RESULTS

To test the hypothesis that Glc7p, Ppz1p, Ppz2p, and Sal6p/Ppq1p have overlapping functions, we examined

TABLE 3

Summary of *glc7 ppz1 ppz2* triple-mutant phenotypes

glc7 allele	*glc7* mutation	*glc7*[a]	*glc7 ppz1 ppz2*[a,b]
glc7-109	K259A, R260A	Salt sensitive, high glycogen, caffeine^S	High glycogen, slg, YPGE$^-$, caffeine^S, ts, cs
glc7-127	K110A, K112A	slg, low glycogen, weakly salt sensitive, 2-DG^r, spo$^-$	Inviable
glc7-129	D137A, E138A	slg, cold-sensitive mitotic defect, low glycogen, spo$^-$	Inviable
glc7-132	D165A, E166A, K167A	slg, low glycogen, weakly salt sensitive	Low glycogen, slg, cs, caffeine^S
glc7-133	R186A, R187A, R190A, Q298A	2-DG^r	Very slow growth, cell lysis defect

[a] The defects are reduced sporulation (spo$^-$), slow growth at 24–30° (slg), temperature sensitivity (ts), cold sensitivity (cs), 2-deoxyglucose resistance (2-DG^r), and failure to utilize glycerol and ethanol (YPGE$^-$).
[b] With the exception of salt sensitivity, *glc7* traits were not affected in *PPZ1 PPZ2*, *ppz1::URA3 PPZ2*, or *PPZ1 ppz2::LEU2* backgrounds.

the phenotypes of yeast strains containing mutations in more than one of these phosphatase genes. A set of congenic strains was generated, each containing a gene deletion in *PPZ1*, *PPZ2*, and/or *SAL6*. These strains were crossed to *GLC7* mutants. In our genetic background *ppz1::URA3* and *ppz2::LEU2* mutants, hereafter referred to as *ppz1* and *ppz2*, respectively, have relatively mild growth phenotypes, growing at near wild-type rates at temperatures ranging from 24° to 37°. *ppz1 ppz2* double mutants grow slowly at 11° and 15°, indicating that *PPZ1* and *PPZ2* likely have overlapping functions necessary for growth at low temperature. As reported, *ppz1* mutants were resistant to high NaCl concentrations (Posas *et al.* 1995b) and were hypersensitive to caffeine (Hughes *et al.* 1993; Posas *et al.* 1993). *ppz1 ppz2* strains also showed the salt resistance phenotype of *ppz1 PPZ2* strains. *sal6::HIS3* mutants, hereafter referred to as *sal6*, were also robust in our genetic background, exhibiting no obvious growth defect at temperatures ranging from 11° to 37° on rich and synthetic media. As reported, *ppq1*/*sal6* mutants were mildly paromomycin sensitive (Vincent *et al.* 1994), consistent with the role of *PPQ1* in translational proofreading (Chen *et al.* 1993; Vincent *et al.* 1994).

To determine the effects of combining mutations in the phosphatase genes, tetrad analysis was carried out on diploid strains heterozygous for *sal6*, *ppz1*, *ppz2*, and *glc7* mutations. The *sal6*, *ppz1*, and *ppz2* mutations were scored by the associated auxotrophic markers and each *glc7* mutation was scored by its associated phenotype. The *glc7* alleles used in this analysis are listed in Table 3 with the accompanying phenotype of each mutant. The *ppz1 ppz2 glc7* mutants were tested for their ability to grow using different carbon sources, at different temperatures, and in the presence of different salts and other growth inhibitors (*e.g.*, 0.9 m NaCl and caffeine). The results of tetrad analysis of meiotic progeny of diploid strains heterozygous for *glc7* and *ppz1*, *ppz2*, or *sal6* are summarized in Table 4 and Figure 2. Whereas *sal6* showed no genetic interaction with *glc7* or *ppz1 ppz2* mutations, multiple genetic interactions were observed between *glc7* and *ppz1 ppz2* mutations.

***glc7* mutants display complex genetic interactions with *ppz1* and *ppz2*:** In crosses between each *glc7* mutant and the *ppz1 ppz2* strain, all spore clones of the triple mutant genotype (*glc7 ppz1 ppz2*) grew significantly more slowly than spore clones of the other genotypes. The *glc7-127* and *glc7-129* triple mutants germinated but never grew into macrocolonies. For *glc7-109*, *glc7-132*, and *glc7-133*, the triple mutants formed reproducibly smaller colonies than any single mutant. Representative tetrads are illustrated in Figure 2A. To illustrate in more detail the complex phenotypes displayed by these mutant combinations, representative strains from these crosses were grown on YPD medium and tested for growth in a variety of conditions. As shown in the second and third rows of Figure 2B, the triple mutants grow more slowly than strains of other genotypes on YPD at 37° and 11°, respectively. Microscopic examination and flow cytometry analysis of these cells grown at low temperature revealed no obvious cell cycle arrest (data not shown). The fourth row of Figure 2B illustrates a growth defect of the triple mutants on rich media containing nonfermentable carbon sources ethanol and glycerol (YPGE); the *glc7-132 ppz1 ppz2* strain grows slowly while the *glc7-109 ppz1 ppz2* strain fails to grow on YPGE.

ppz1 mutants are hypersensitive to caffeine (Hughes *et al.* 1993; Posas *et al.* 1993). In our strain background *glc7-109* and *glc7-132* strains grow more slowly than wild type on medium containing 10 mm caffeine but are less sensitive than *ppz1* mutants (data not shown). As shown in the sixth row of Figure 2B, the *ppz1 ppz2*, *ppz1 ppz2 glc7-109*, and *ppz1 ppz2 glc7-132* strains fail to grow in the presence of 5 mm caffeine. The triple mutants are

TABLE 4

Summary of genetic interactions between *glc7*, *ppz1*, *ppz2*, and *sal6*

Relevant genotype	Tetrads analyzed	No. of spores of relevant genotype[a]	Growth rate of relevant genotype[b]	Viability of other spore clones (%)
glc7-109 ppz1 ppz2	11	4	Slow growth (4/4)	37/40 (92.5)
glc7-127 ppz1 ppz2	9	6	Inviable (6/6)	29/30 (96.7)
glc7-129 ppz1 ppz2	10	6	Inviable (6/6)	34/34 (100)
glc7-132 ppz1 ppz2	10	4	Slow growth (4/4)	34/36 (94.4)
glc7-133 ppz1 ppz2	10	5	Very slow growth (5/5)	35/35 (100)
ppz1 ppz2 sal6	10	8	Wild type (8/8)	31/32 (96.9)
glc7-109 sal6	12	11	Wild type (11/11)	37/37 (100)
glc7-127 sal6	11	9	*glc7-127*[c] (9/9)	35/35 (100)
glc7-129 sal6	11	8	*glc7-129*[c] (8/8)	32/36 (88.9)
glc7-132 sal6	11	10	*glc7-132*[c] (10/10)	32/34 (94.1)
glc7-133 sal6	11	10	Wild type (10/10)	34/34 (100)

[a] Where the spore clones did not grow into macrocolonies, the genotype of the mutants was inferred from the genotypes of viable spore clones in the tetrad, assuming no gene conversion.
[b] Growth rate was assessed by colony size of spore clones.
[c] Growth rate was comparable to the *glc7* mutant alone.

consistently more sensitive to caffeine than *ppz1 ppz2* mutants alone (Figure 2C).

ppz1 ppz2 double mutants exhibit an osmotic-remedial cell lysis defect at high temperatures (*e.g.*, 37°) in some genetic backgrounds (Lee *et al.* 1993), suggesting a role for these phosphatases in some aspect of cell wall integrity. *ppz1 ppz2 glc7-133* mutants grow slowly at all temperatures and are inviable at 37°. Microscopic examination of *ppz1 ppz2 glc7-133* mutants revealed massive cell lysis (approaching 100%) at 37°. We found that 1 m sorbitol only partially suppressed the growth defect of the triple mutant at 37°. As shown in Figure 2D, growth at 37° was not restored to wild-type levels by sorbitol, and sorbitol actually repressed growth of the *ppz1 ppz2 glc7-133* mutant at 30°. These results suggest that Glc7p, Ppz1p, and Ppz2p may all function in cell wall maintenance but further studies will be necessary to sort out possible roles.

***PPZ1* and *PPZ2* act redundantly in their genetic interactions with *GLC7*:** Several growth defects have been noted for strains containing disruptions of *PPZ1*. *ppz1* mutants show increased salt tolerance (Posas *et al.* 1995b) and caffeine sensitivity (Hughes *et al.* 1993; Posas *et al.* 1993). Disruption of *ppz2* has little influence on these defects. However, most of the genetic interactions observed between different *glc7* alleles and *ppz1* and *ppz2* require that both *ppz1* and *ppz2* be disrupted. For example, *glc7-127* and *glc7-129* strains bearing disruptions of either *ppz1* or *ppz2* are viable, whereas the triple mutants are inviable (Table 4). Likewise, the slow-growth phenotype of *ppz1 ppz2* strains containing *glc7-133*, *glc7-109*, or *glc7-132* is not observed if either *PPZ1* or *PPZ2* is functional. One exception to this rule is *glc7-109*. The salt sensitivity conferred by *glc7-109* is suppressed by *ppz1* alone (data not shown).

Some phenotypic traits of *glc7* mutants are independent of *ppz1* and *ppz2*: In contrast to growth rate, caffeine sensitivity, carbon source utilization, and salt sensitivity, other traits of *glc7* mutants appear to be independent of the *PPZ* genotype. *glc7-109* mutants hyperaccumulate glycogen, as shown in Figure 2B by the dark brown staining with iodine. This trait is not affected by deletion of either *ppz1* or *ppz2*. In a similar manner both *glc7-132* and *glc7-132 ppz1 ppz2* strains accumulate low levels of glycogen (Figure 2B). Likewise, the sporulation competency and deficiency of *glc7-109* and *glc7-132* strains, respectively, are not altered by the *PPZ* genotype (data not shown). *glc7* mutants that are defective in glucose repression, including *glc7-133* and *glc7-127*, are resistant to 2-DG (Baker *et al.* 1997; Tu and Carlson 1994). *ppz1 ppz2* mutants are not 2-DG resistant, nor are any *ppz1 ppz2* mutants in combination with any of our *glc7* alleles, other than *glc7-133* (*glc7-127 ppz1 ppz2* strains are inviable). In a similar manner, the conditional mitotic arrest observed in other *glc7* mutants (Hisamoto *et al.* 1995; MacKelvie *et al.* 1995), including *glc7-129* (Bloecher and Tatchell 1999), is not observed in any *ppz1 ppz2 glc7* mutants. These results indicate that glucose repression and mitotic regulation are two additional pathways regulated solely by *GLC7*.

***reg1* and *sla1* mutants display genetic interactions with *ppz1* and *ppz2*:** The hypothesis that PP1/Glc7p is regulated by targeting subunits leads to the prediction that mutations in at least some of the genes encoding Glc7p regulatory subunits will result in synthetic phenotypes with *ppz1* and *ppz2* mutations. We tested this hypothesis by crossing *reg1*, *reg2*, *sla1*, *gip1*, *gac1*, *pig1*, *yol091w*, and *yal014c* null mutants, which encode bona fide and putative Glc7p-binding proteins, with *ppz1* and *ppz2* null mutants. As predicted from our earlier genetic analysis,

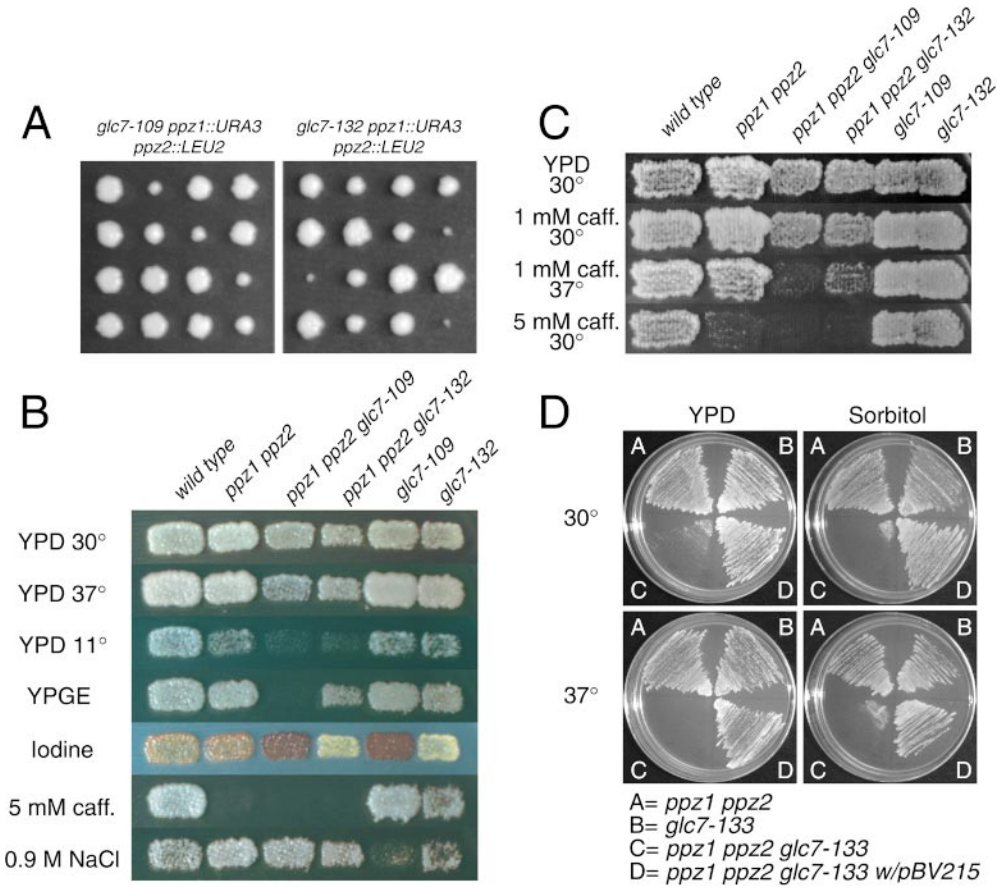


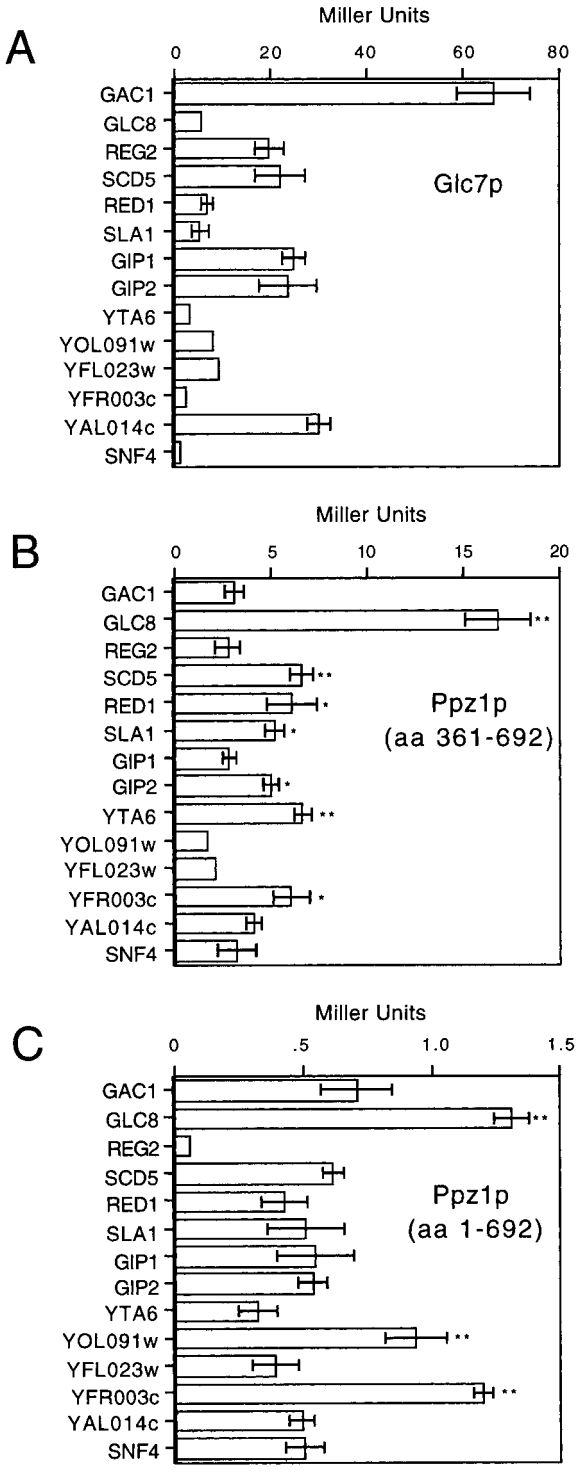
Figure 2.—Genetic interactions between *GLC7*, *PPZ1*, and *PPZ2*. (A) Ascosporal clones from crosses between a *ppz1::URA3 ppz2::LEU2* (KT1492) strain and *glc7-109* (KT1596) or *glc7-132* (KT1599) strains. The four colonies in each row represent a tetrad. The smaller colonies are always those that contain *ppz1::URA3*, *ppz2::LEU2*, and either *glc7-109* or *glc7-132*. Colonies of all other genotypes grow at wild-type rates. (B) Clones from the tetrads represented in A were patched onto YPD medium, allowed to grow overnight at 30°, and replica plated onto the media indicated on the left. The plates used for scoring growth at 30° and glycogen accumulation were incubated for 1 day. The plates containing 5 mm caffeine and 0.9 m NaCl were incubated for 2 days and the other plates were incubated for 3 days at their respective temperatures. Unless otherwise stated the plates were incubated at 30°. (C) The strains of indicated genotype were grown on media containing the indicated concentrations of caffeine at indicated temperatures for 1 day. (D) Strains containing either *ppz1 ppz2* (BV460), *glc7-133* (BV459), *ppz1 ppz2 glc7-133* (BV461), and *ppz1 ppz2 glc7-133* (BV461) with pBV215 (*GFP:PPZ1* in pNC160) were streaked onto either YPD media or YPD media containing 0.5 m sorbitol. The plates were incubated for 1 day at their respective temperatures.

ppz1 ppz2 null mutants displayed no obvious growth defects in combinations with mutants affecting either glycogen accumulation [*e.g.*, *gac1* (François *et al.* 1992; Stuart *et al.* 1994) and *pig1* (Cheng *et al.* 1997)] or sporulation [*e.g.*, *gip1* (Tu *et al.* 1996) and *yol091w* (Tu *et al.* 1996; Pearson *et al.* 1998)]. *ppz1 ppz2* null mutants also did not show any obvious growth defects in combination with either *reg2* or *yal014c* null mutants. Reg2p is a Glc7p-binding protein involved in growth control (Frederick and Tatchell 1996) and Yal014c is a putative Glc7p-binding protein with unknown function.

On the other hand, synthetic growth defects were observed in crosses between *ppz1 ppz2* mutants and either *reg1* or *sla1* null mutants. Reg1p is required for glucose repression (Tu and Carlson 1995) and its activity requires the ability to bind Glc7p (Alms *et al.* 1999; Dombek *et al.* 1999). *reg1* mutants also have a slow-growth phenotype (Matsumoto *et al.* 1983; Tung *et al.* 1992). As judged by the colony size of *reg1::URA3 ppz1::TRP1* and *reg1::URA3 ppz2* double mutants, *ppz1* and *ppz2* alone have no adverse effect on the growth rate of *reg1::URA3* mutants. However, *reg1::URA3* mutants are inviable in combination with both *ppz1* and *ppz2*. From 11 tetrads analyzed in a cross between EG716-6B (*MAT*α *reg1::URA3 ppz2::LEU2*) and BV352 (*MAT***a** *ppz1::TRP1*), the five spore clones judged to be *reg1::URA3 ppz1 ppz2* failed to grow into macrocolonies. Four of the triple mutants arrested as microcolonies and one arrested as a single cell. All the remaining spore clones germinated and grew into macrocolonies. The fact that *ppz1 ppz2* mutants are not defective in glucose repression yet are lethal in combination with *reg1* suggests that the activities of Ppz1p and Reg1p that are responsible for the synthetic lethality of the triple mutant are not related to glucose repression.

Sla1p was originally identified in a synthetic lethal screen with Abp1p (actin-binding protein). Sla1p has been shown to be required for the proper formation of the cortical actin cytoskeleton (Holtzman *et al.* 1993) and is required for the proper localization of Rho1p and Sla2p (Ayscough *et al.* 1999). As determined by the colony size of *sla1::URA3 ppz1* and *sla1::URA3 ppz2* double mutants, *ppz1* and *ppz2* alone have no adverse effect on the growth rate of *sla1::URA3* mutants. However, *sla1::URA3* mutants are inviable in combination with both *ppz1* and *ppz2*. From 10 tetrads analyzed from a cross between BV533 (*MAT***a** *ppz1::URA3 ppz2::LEU2*) and BV543 (*MAT*α *sla1::URA3*), the four spore clones

judged to be the triple mutants arrested as microcolonies and failed to grow into macrocolonies. Microscopic examination of the triple mutants revealed a cell lysis phenotype reminiscent of the cell lysis phenotype observed with the *glc7-133 ppz1 ppz2* triple mutant. All but one of the remaining spore clones germinated and grew into macrocolonies. The synthetic phenotype of the *reg1 ppz1 ppz2* and the *sla1 ppz1 ppz2* mutants provides further evidence for possible overlapping roles for Glc7p and Ppz1/2p.



Ppz1p interacts with some Glc7p-binding proteins in the two-hybrid system: The synthetic growth defects of *ppz1 ppz2 glc7* mutants could be explained if a substrate(s) shared by all three phosphatases must be dephosphorylated to maintain normal cell viability or growth. If the substrate specificity of these phosphatases is regulated by targeting subunits, as is Glc7p, then one might predict that the three phosphatases would share at least a subset of Glc7p regulatory or targeting subunits. We tested two Ppz1p-containing fusion proteins in the two-hybrid assay with a panel of Glc7p-binding protein fusions. pAS-PPZ1$_{(aa\ 1–692)}$ contains the entire *PPZ1* open reading frame while pAS-PPZ1$_{(aa\ 361–692)}$ contains only the catalytic domain of Ppz1p. In pAS-PPZ1$_{(aa\ 1–692)}$ the codon encoding the N-myristoylated glycine residue was changed to alanine to avoid possible complications due to N myristoylation, a known modification of Ppz1p (Clotet *et al.* 1996). For example, Andrulis *et al.* (1998) noted that membrane association of a GAL4-DNA-binding domain fusion protein to the nuclear periphery can alter gene expression. The Glc7p-binding proteins tested for Ppz1p interaction included Gac1p, a glycogen-specific regulatory protein (François *et al.* 1992; Stuart *et al.* 1994); Gip1p, which is required for meiosis and sporulation (Tu *et al.* 1996); and Reg2p, which is involved in growth control (Frederick and Tatchell 1996). Each of these proteins has been shown to interact with Glc7p both *in vivo* and *in vitro*. We also tested proteins that were identified in the two-hybrid system as Glc7p-interacting proteins (Tu *et al.* 1996) but which have not yet been confirmed by other methods to interact with Glc7p. These included Red1p, which is involved in synaptonemal complex formation (Bailis and Roeder 1998); Scd5p, which is involved in vesicular trafficking (Nelson *et al.* 1996); Sla1p, an actin-binding protein (Holtzman *et al.* 1993); and the products of several uncharacterized open reading frames: *YTA6*, *GIP2*, *YFR003c*, *YFL023w*, *YOL091w*, and *YAL014c*. Glc8p, a PP1-inhibitor-2-related protein (Cannon *et al.* 1994; Tung *et al.* 1995) that weakly interacts with Glc7p

Figure 3.—Two-hybrid interactions between Ppz1p$_{(aa\ 1–692)}$, Ppz1p$_{(aa\ 361–692)}$, and Glc7p-binding proteins. Yeast strain PJ69-4A, which contains the *GAL7-LacZ* reporter gene, was cotransformed with either pAS-*GLC7* (A), pAS-*PPZ1*$_{(aa\ 361–692)}$ (B), or pAS-*PPZ1*$_{(aa\ 1–692)}$ (C) along with pACT plasmids containing one of the following clones: pGAD-*GIP1*, pGAD-*GIP2*, pGAD-*RED1*, pGAD-*SCD5*, pGAD-*SLA1*, pGAD-*YTA6*, pGAD-*YFL023W*, pGAD-*YOL091W*, pGAD-*YAL014*, or pGAD-*YFR003C* (Tu *et al.* 1996); pSE1111 (pACT-*SNF4*), pDF112 (pACT-*GLC8*), or pDF116 (pACT-*REG2*). The pACT-*SNF4* clone was used as a negative control. Protein-protein interactions were assayed by measuring β-galactosidase activity. A single asterisk denotes statistically significant change *vs.* the negative control with $P < 0.05$ and a double asterisk denotes a statistically significant change *vs.* the negative control with $P < 0.01$. The levels of all the protein-protein interactions between Glc7p and the various subunits were significantly different ($P < 0.01$) than the negative control.

in the two-hybrid assay (Ramaswamy *et al.* 1998), was also tested in our analysis.

pAS-PPZ1$_{(aa\ 1–692)}$, pAS-PPZ1$_{(aa\ 361–692)}$, and pAS-GLC7 were transformed with one of the Glc7p-binding pACT fusions into strain PJ69-4A and transformants were tested for protein-protein interactions by measuring β-galactosidase (β-gal) activity. As shown in Figure 3A, Glc7p interacted most strongly with Gac1p (β-gal levels 60-fold above Snf4p), whereas Glc8p, Red1p, Sla1p, Yta6p, or Yfr003c with Glc7p resulted in 2- to 6-fold higher β-gal activity than with Snf4p, which we used as the negative control (Figure 3A). All other Glc7p-binding proteins showed an intermediate level of interaction with Glc7p, exhibiting β-gal levels 18- to 27-fold higher than Snf4p (Figure 3A).

In contrast to Glc7p, which interacted with all Glc7p-binding proteins, full-length Ppz1p interacted with only a subset of Glc7p-binding proteins (Figure 3C). Glc8p and Yfr003c showed the strongest interaction, consistently giving β-gal levels 2.5-fold higher than the negative control while Yol091w exhibited β-gal levels 2-fold higher than the negative control. Eliminating the NH_2-terminal extension of Ppz1p resulted in a systematic increase in β-galactosidase expression with all the Glc7p-binding proteins as well as the negative control. Ppz1p$_{(aa\ 361–692)}$ displayed a significant interaction with Glc8p, at least 5-fold above the negative control (Figure 3B). Scd5p, Red1p, Sla1p, Gip2p, Yta6p, and Yfr003c also significantly interacted with the truncated Ppz1p (1.5- to 2-fold above the negative control; Figure 3B). de Nadal *et al.* (1998) also noted that Hal3p, a negative regulator of Ppz1p, shows enhanced binding to the Ppz1p catalytic domain in the absence of the NH_2-terminal domain. The observed increase in β-galactosidase activity between the truncated Ppz1p and the full-length Ppz1p is not simply due to increased protein expression, since immunoblot analysis demonstrated that the full-length Ppz1p is actually more abundant than the truncated version (data not shown). Together, these results support the possibility that Glc7p and Ppz1p share a subset of Glc7p regulatory subunits.

The catalytic domain of Ppz1p complements a *ppz1* null mutant: Our *PPZ1* clone with the NH_2-terminal deletion interacted strongly with a number of Glc7p-binding proteins. However, Clotet *et al.* (1996) reported that a similar deletion expressed from the *PPZ1* promoter did not complement the caffeine sensitivity or LiCl resistance of a *ppz1* null mutant, although overexpression of the catalytic domain did partially complement the *ppz1* mutant. This raises the possibility that the NH_2-terminal domain may be critical for specificity and brings into question the significance of the two-hybrid interactions between the catalytic domain of Ppz1p$_{(aa\ 361–692)}$ and the Glc7p regulatory proteins. As suggested by Clotet *et al.* (1996), the catalytic domain of Ppz1p may lack activity towards specific substrates but retain some nonspecific ser/thr phosphatase activity. Overexpression of this fragment could partially meet the demand for Ppz1p activity. Alternatively, the catalytic domain could contain the essential determinants of Ppz1p specificity, with the amino-terminal domain serving to control subcellular location, regulate stability, or modulate the binding of regulatory proteins. To address this question we constructed a *GLC7:PPZ1* fusion clone comprised of the *GLC7* promoter, a sequence encoding an HA epitope, and the first 10 codons of *GLC7* fused in frame to the coding regions for the catalytic domain and the C terminus of Ppz1p$_{(aa\ 361–692)}$. We transformed CEN plasmids containing either the *GLC7-PPZ1*$_{(aa\ 361–692)}$ fusion or the full-length *PPZ1* into yeast to test expression and biological activity. Immunoblot analysis (Figure 4B) indicates that the Glc7-Ppz1p$_{(aa\ 361–692)}$ fusion protein is stably expressed in yeast at steady-state levels only slightly lower than Glc7p. This is probably a higher level of expression than that of native Ppz1p. A direct comparison of GFP:Glc7p (A. Bloecher and K. Tatchell, unpublished results) and full-length GFP:Ppz1p (see below) protein levels expressed from CEN vectors under the control of their respective natural promoters revealed that GFP:Glc7p is at least 10-fold more abundant than GFP:Ppz1p (data not shown). As shown in Figure 4A, the Glc7-Ppz1p$_{(aa\ 361–692)}$ protein retains the activity of full-length Ppz1p, as shown by its ability to complement not only the caffeine sensitivity of a *ppz1* strain (Figure 4A), but also the lethality of a

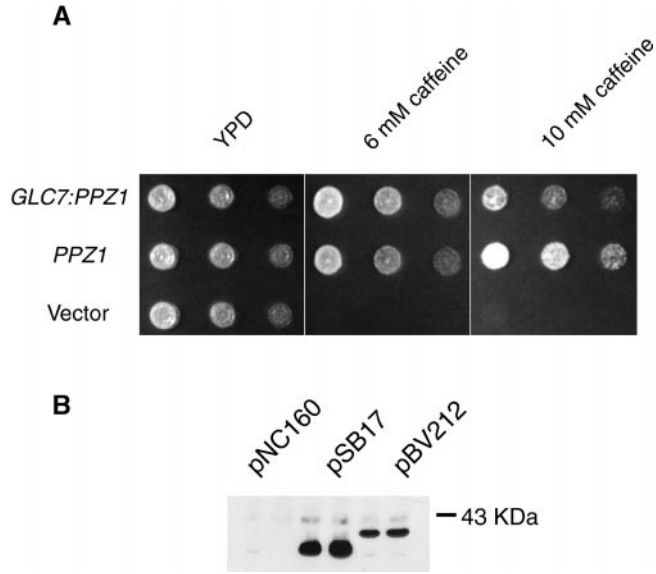


Figure 4.—Complementation of *ppz1::URA3* by an NH_2-truncated form of Ppz1p. (A) A *ppz1::URA3* (BV475) strain was transformed with pBV212 (HA-tagged catalytic domain of Ppz1p), pBV216 (wild-type *PPZ1*), and pNC160 (empty vector). Transformants were spotted on YPD medium, on YPD medium containing 6 mm caffeine, and on YPD medium containing 10 mm caffeine for 1, 3, and 5 days, respectively, at 30°. (B) Immunoblot analysis was performed on protein extracted from strain BV475 containing either pBV212 (HA-tagged NH_2-truncated Ppz1p), pSB17 (HA-tagged Glc7p), or pNC160. The blot was probed with the 12CA5 anti-HA monoclonal antibody.

ppz1 ppz2 glc7-127 triple mutant and the slow-growth phenotype of *ppz1 ppz2 glc7-109* and *ppz1 ppz2 glc7-133* triple mutants (data not shown). In contrast, the Glc7-Ppz1p$_{(aa\ 361–692)}$ fusion failed to complement any of the defects associated strictly with the loss of *GLC7*, including the lethality of a *glc7* disruption, the 2-DG resistance of *glc7-127* and *glc7-133*, and the LiCl sensitivity of *glc7-109* (data not shown). In summary, the ability of the Glc7-Ppz1p$_{(aa\ 361–692)}$ fusion to complement a *ppz1* null mutation suggests that the catalytic domain of Ppz1p retains key determinants of Ppz1p function while its failure to complement any *glc7*-associated defects suggests that it has not gained a nonspecific ser/thr phosphatase activity. Likewise, it has also been noted that a *SAL6* clone containing a partial deletion (130 amino acids) of the NH_2 terminus still complemented *sal6* phenotypes (Vincent *et al.* 1994). Together, these results indicate that the essential determinants of Ppz1p reside within the catalytic domain and strengthen the significance of the two-hybrid interactions. The discrepancy between these results and those of Clotet *et al.* (1996), where a truncated *PPZ1* clone failed to complement a *ppz1* null mutant, is likely due to differences in the levels of expression between the two clones. Ppz1p is expressed at a low level, relative to Glc7p, but is localized strictly to membranes. When an NH_2-terminal truncated Ppz1p is expressed from its native promoter, the local concentration of Ppz1p in the membrane is probably insufficient for function. Increased expression of the truncated Ppz1p from the more active *GLC7* promoter may result in an increase in the local concentration of Ppz1p at membranes to the point where it is able to act on specific substrates. This model is compatible with the existing data.

***GLC8* regulates *PPZ* and *GLC7* functions *in vivo*:** Our two-hybrid data suggest that previously identified Glc7p-interacting proteins can also associate with the catalytic domain of Ppz1p$_{(aa\ 361–692)}$. To test the significance of these data, we chose to investigate the interaction between Glc8p and Ppz1p in more detail because Glc8p has a well-documented role in Glc7p regulation and because Glc8p showed the strongest interaction with Ppz1p. Glc8p is a heat-stable protein most similar to inhibitor-2, a well-characterized PP1 regulatory protein (Bollen *et al.* 1994; Kakinoki *et al.* 1997). The *glc8* null mutation reduces glycogen levels and suppresses the chromosome-loss phenotype of *ipl1-1* (Cannon *et al.* 1994; Tung *et al.* 1995), two traits associated with reduced Glc7p activity. Overexpression of Glc8p also suppresses *ipl1-1* (Tung *et al.* 1995), suggesting that Glc8p can exert a negative role on Glc7p when overexpressed. Glc7p phosphatase activity is reduced in *glc8* null mutants (Tung *et al.* 1995), implying that Glc8p can directly modulate Glc7p activity. Surprisingly, Glc8p only weakly

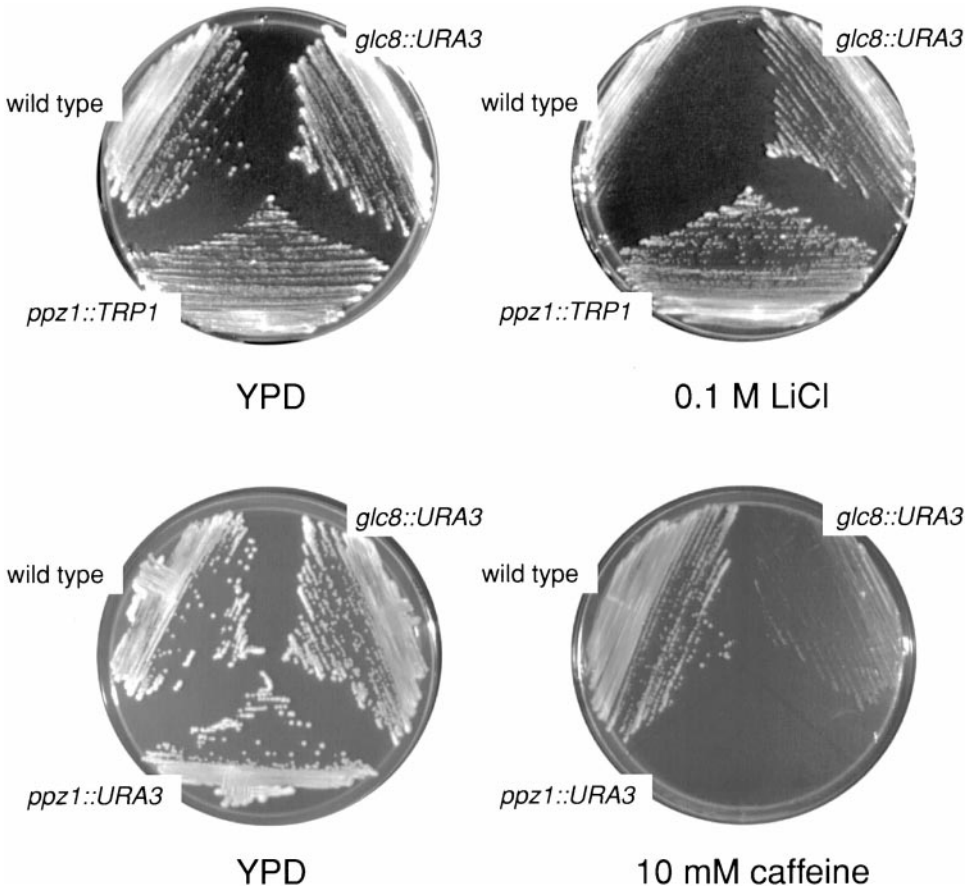


Figure 5.—Phenotype of *glc8::URA3*. (Top) Wild-type (KT-1357), *ppz1::TRP1* (BV519), and *glc8::URA3* (BV470) yeast strains were plated onto YPD medium or YPD medium containing 0.1 m LiCl and grown at 37° for 2 days. (Bottom) Wild-type (KT1112), *ppz1::URA3* (BV475), and *glc8::URA3* (BV472) yeast strains were plated onto YPD medium or YPD medium containing 10 mm caffeine and were grown at 30° for 2 days.

TABLE 5

Summary of genetic interactions between *glc8*, *glc7*, *ppz1*, and *ppz2*

Relevant genotype	Tetrads analyzed	No. of spores of relevant genotype[a]	Growth rate of relevant genotype[b]	Viability of other spore clones (%)
ppz1 ppz2 glc8	11	5	Wild type (5/5)	39/39 (100)
glc7-109 glc8	12	11	Slow growth (11/11)	36/37 (97.3)
glc7-127 glc8	11	7	Slow growth (7/7)	32/37 (86.5)
glc7-129 glc8	11	9	Inviable (9/9)	33/35 (94.3)
glc7-132 glc8	11	12	*glc7-132*[c] (12/12)	32/32 (100)
glc7-133 glc8	12	13	Very slow growth (13/13)	32/35 (91.4)

[a] Where the spore clones did not grow into macrocolonies, the genotype of the mutants was inferred from the genotypes of viable spore clones in the tetrad, assuming no gene conversion.
[b] Growth rate was assessed by colony size of spore clones.
[c] Growth rate was comparable to the *glc7* mutant alone.

interacts with Glc7p in the two-hybrid assay (Figure 3A; Ramaswamy *et al.* 1998) and has not been reported to associate with Glc7p in a more direct binding assay.

To test for genetic interactions between *GLC8*, *PPZ1*, and *PPZ2* we first constructed a *glc8::URA3* null mutant and examined it for defects reported for *ppz1* mutants. As reported previously (Cannon *et al.* 1994), *glc8::URA* null mutants, hereafter referred to as *glc8*, have a mild glycogen accumulation defect. As shown in Figure 5, *glc8* mutants are also tolerant to 0.1 m LiCl and sensitive to 10 mm caffeine. The LiCl tolerance and caffeine sensitivity of *glc8* are intermediate between that of *ppz1* and of wild type and are more pronounced at 37°. These results are consistent with Glc8p acting as a positive modulator of both Ppz1p and Glc7p activity.

If Glc8p acts as a positive regulator of Ppz1p we would predict that *glc8* would also suppress the salt sensitivity of *glc7* mutants. To test this, we mated *glc8* mutants to strains containing five different *glc7* alleles and characterized the meiotic progeny (Table 5). As shown in Figure 6, *glc8* rescues the NaCl salt sensitivity of *glc7-109*, *glc7-132*, and *glc7-127*. The temperature-sensitive growth defect of the *glc7-133 glc8* strain (Figure 6, row 2) is partially rescued by the addition of 1 m sorbitol (Figure 6, row 3), reminiscent of the partially osmotic-remedial growth defect of *glc7-133 ppz1 ppz2* (Figure 2D). We also characterized the meiotic progeny of a cross between a *glc8* strain and a *ppz1::TRP1 ppz2::LEU2* strain (Table 5). The triple mutants were viable and relatively robust in growth, but were slightly more sensitive to caffeine than the *ppz1::TRP1 ppz2::LEU2* double mutants (data not shown). Since *glc7* and *ppz1* both cause caffeine sensitivity, the enhanced caffeine sensitivity of *glc8 ppz1 ppz2* mutants could be due to an effect of the *glc8* null mutation on Glc7p function. Together, these results lend support to the hypothesis that Glc8p positively regulates both Ppzp and Glc7p.

Overexpression of Ppz1p has been shown to reduce the growth rate of wild-type cells (Clotet *et al.* 1996). If Glc8p is indeed a positive modulator of Ppz1p function, we reasoned that mutations in *GLC8* might alleviate this slow-growth defect. To test this hypothesis, we constructed a chimeric *GFP:PPZ1* gene fusion (Figure 7A) that contained the *GFP* open reading frame between

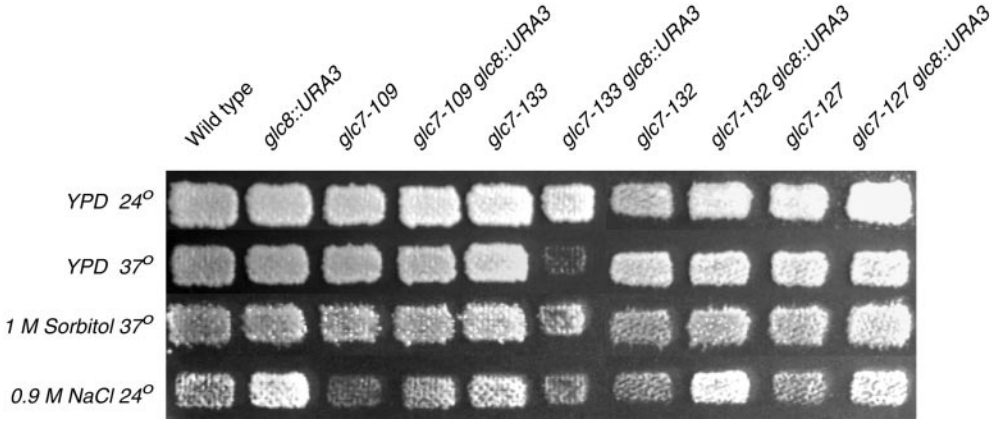


Figure 6.—Genetic interactions between *glc7* and *glc8* mutations. Yeast strains with the relevant genotype designated were grown on YPD medium at 24° for 2 days and then replica plated onto the media listed on the left. The 0.9 m NaCl plates were incubated at 24° for 3 days. The 1 m sorbitol plates were incubated at 37° for 2 days and the YPD plates were incubated either at 24° for 3 days or at 37° for 1 day. Strains used were as follows: wild-type (KT1358), *glc8::URA3* (BV470), *glc7-109* (BV449), *glc7-109 glc8::URA3* (BV479), *glc7-133* (BV448), *glc7-133 glc8::URA3* (BV481), *glc7-132* (BV450), *glc7-132 glc8::URA3* (BV483), *glc7-127* (BV451), and *glc7-127 glc8::URA3* (BV477).

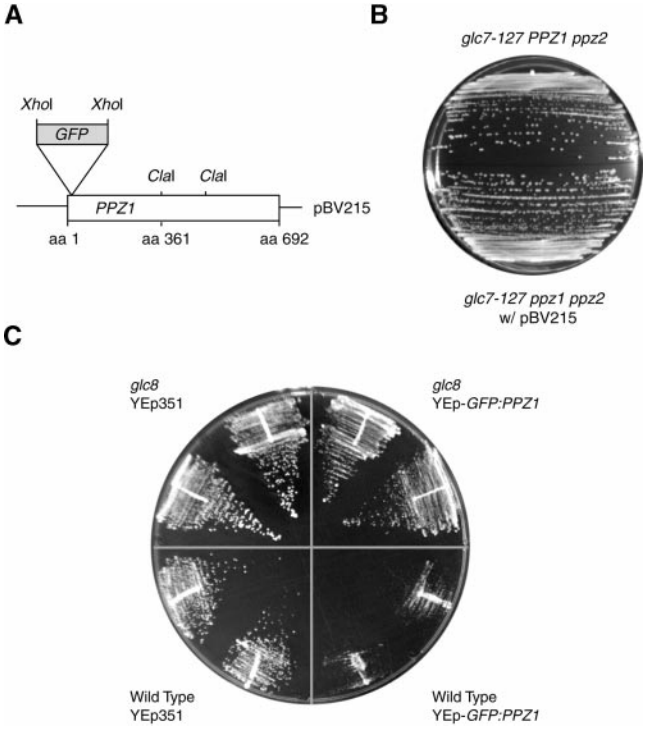


Figure 7.—Mutations in *glc8* suppress the growth defect conferred by GFP:Ppz1p overexpression. (A) Schematic diagram of *GFP:PPZ1* construction. (B) Complementation of the *glc7-127 ppz1::URA3 ppz2::LEU2* lethal phenotype by *GFP:PPZ1* (pBV215) on a low-copy vector. Strains *glc7-127 ppz1::URA3 ppz2::LEU2* kept alive with pBV215 (BV427) and *glc7-127 ppz2::LEU2* (BV428) were grown on YPD medium for 3 days at 24°. (C) Strains KT1357 and BV470 were transformed with either YEp351 or pBV223 (YEp351-*GFP:PPZ1*). Transformants were grown on synthetic medium lacking leucine for 2 days at 30°.

the 18th and 19th codon of *PPZ1* and assayed it for biological activity. The GFP:Ppz1p on a low-copy vector was able to rescue the lethality of a *ppz1 ppz2 glc7-127* triple-mutant strain (Figure 7B) as well as the caffeine sensitivity of a *ppz1* strain (data not shown). Fluorescence microscopy revealed that GFP:Ppz1p localized primarily with internal and plasma membranes of yeast cells (data not shown). Expression of the *GFP:PPZ1* fusion from a high-copy plasmid reduced the growth rate of wild-type but not of *glc8* mutant strains (Figure 7C), providing further evidence that Glc8p positively affects Ppz1p function. To test whether Glc8p might affect the stability of GFP:Ppz1p, we performed immunoblot analysis for GFP on strains containing the *GFP:PPZ1* fusion integrated at the *URA3* locus either in a *ppz1* background or a *ppz1 glc8* double-mutant background. The levels of GFP:Ppz1p are equivalent in either background (data not shown), strongly suggesting that Glc8p affects GFP:Ppz1p activity. Together, these results suggest that Glc8p acts as a modulator of both Glc7p and Ppz1/Ppz2p.

DISCUSSION

Our tests for genetic interactions between *GLC7*, *PPZ1*, *PPZ2*, and *SAL6* were prompted after our limited success at isolating conditional alleles of *glc7* by alanine-scanning mutagenesis (Baker *et al.* 1997). Our model for this approach was the isolation and characterization of alanine-scanning alleles of the actin gene *ACT1* (Wertman *et al.* 1992). We felt that actin would be a good model for our effort because actin and protein phosphatase type 1 are both products of single essential genes, both exhibit a high degree of evolutionary conservation, and both have a large number of binding partners. However, only 2 out of 22 *glc7* mutants had conditional growth phenotypes, in contrast to the 16 out of 36 conditional mutants identified for *act1* (Wertman *et al.* 1992). Although many of our mutants contained missense mutations in highly conserved amino acid residues, few of these displayed strong or conditional growth phenotypes. One explanation for these results is that other phosphatases can partially compensate for the lack of Glc7p function. The most likely candidates for phosphatases with overlapping function are Ppz1p, Ppz2p, and Sal6p. These are most similar in sequence to Glc7p in their catalytic domains but none is essential for viability (Vincent *et al.* 1994; Posas *et al.* 1992, 1993; Chen *et al.* 1993). The results of genetic analysis presented here are largely consistent with this hypothesis. All *glc7* alanine-scanning alleles tested conferred either a more severe growth defect or lethality in a *ppz1 ppz2* null background. Some of the changes observed were qualitatively similar but quantitatively more severe. For example, *ppz1 ppz2* mutants and some *glc7* mutants are sensitive to caffeine but the triple mutants are more sensitive. In other cases qualitatively new traits are observed in the triple mutant. For example, *glc7-109 ppz1 ppz2* mutants are unable to utilize ethanol efficiently as a carbon source but this defect is not observed for any *glc7* mutant nor for *ppz1 ppz2* mutants.

The activity of type 1 protein phosphatase is regulated by targeting and/or regulatory subunits (Hubbard and Cohen 1993). We predicted that if Glc7p and Ppzp have overlapping activities then some Glc7p-binding proteins would also interact with Ppzp. Precedence for this is provided by the association of Tap42p with both the PP2A and the Sit4p phosphatases (Di Como and Arndt 1996). The results of our two-hybrid assay confirmed this prediction for Ppz1p and Glc7p. Most of the Glc7-binding proteins that also associate with Ppz1p have not been characterized, but Glc8p, which has been shown to regulate Glc7p activity (Tung *et al.* 1995), interacts more strongly with Ppz1p in the two-hybrid system than with Glc7p. The results of our genetic analysis indicate that Glc8p also regulates the activity of Ppz1p, as shown by the caffeine sensitivity and partial salt resistance of *glc8* mutants and the ability of *glc8* to suppress the slow growth associated with Ppz1p overexpression.

The complex genetic interactions observed between *GLC7*, *PPZ1*, and *PPZ2* could be explained by a "nested overlap" hypothesis. In this model each phosphatase would have substrates that it is uniquely capable of dephosphorylating, whereas other substrates are shared by all three. Thus, each phosphatase would have unique as well as overlapping cellular functions. For *GLC7*, unique roles include glycogen synthesis, glucose repression, regulation of mitosis, and sporulation. For *PPZ1*, possible unique roles are regulation of the response to salt stress by the regulation of *ENA1* (Posas *et al.* 1995b) and the control of the G1/S phase transition of the cell cycle (Clotet *et al.* 1999). We do not yet know the physiological pathway(s) in which Glc7p, Ppz1p, and Ppz2p act redundantly. The cell lysis defect of *glc7-133 ppz1 ppz2* mutants suggests that cell wall synthesis or maintainance may be such a pathway. The failure of *glc7-109 ppz1 ppz2* mutants to utilize ethanol or glycerol as a carbon source suggests that mitochondrial function may require either activity. This type of functional redundancy has been observed for other protein phosphatases. *PPH21* and *PPH22*, which encode isoforms of the catalytic subunit of PP2A, exhibit genetic interactions with *PPH3*, another PP2A-like phosphatase gene (Ronne *et al.* 1991).

Not all data are consistent with the nested overlap hypothesis. If Glc7p and Ppzp have overlapping activities we would predict that they would exhibit similar enzymatic activities. However, Posas *et al.* (1995a) found that the *in vitro* specificity of Ppz1p is qualitatively different from that of Glc7p. Recombinant Ppz1p was found to effectively dephosphorylate histone H1, myelin basic protein, and casein but was ineffective toward rabbit glycogen phosphorylase, a common substrate for PP1. The caveat for this experiment is that regulatory subunits may influence enzyme specificity, thus differential binding could result in differential specificity. It has also been noted that recombinant PP1 has enzymatic properties different from PP1 synthesized *in vivo* (Alessi *et al.* 1993; MacKintosh *et al.* 1996).

The apparent functional overlap between Glc7p and Ppzp proteins could also reside at the level of the substrate, such that the function of a pathway would require the dephosphorylation of only one of two or more functionally redundant substrate proteins. One of these proteins could be a substrate of Glc7p while the other could be a substrate of Ppz1p/Ppz2p. The two phosphatase substrates could even be part of the same protein. One site(s) on such a protein would be a substrate of Glc7p while another site(s) would only be a substrate for Ppz1p. As long as one of the two sites is dephosphorylated the pathway can function. One appealing feature of this model is that the two phosphatases need not have similar substrate specificities. Distinguishing among these possibilities will require better understanding of the pathways regulated redundantly by Glc7p and the Ppzp enzymes.

We thank David Levin, Marian Carlson, Kelly Tindall, Philip James, Sue Liebman, Debra Frederick, Steve Elledge, Donald Pappas, and John Cannon for strains and plasmids used in this study. We also thank Lucy Robinson and Eric Aamodt for critically reading this manuscript. This work was supported by National Institutes of Health grant GM-477899.

LITERATURE CITED

Alessi, C. R., A. J. Street, P. Cohen and P. T. W. Cohen, 1993 Inhibitor-2 functions like a chaperone to fold three expressed isoforms of mammalian protein phosphatase-1 into a conformation with the specificity and regulatory properties of the native enzyme. Eur. J. Biochem. **213:** 1055–1066.

Alms, G. R., P. Sanz, M. Carlson and T. A. J. Haystead, 1999 Reglp targets protein phosphatase 1 to dephosphorylate hexokinase II in *Saccharomyces cerevisiae*: characterizing the effect of a phosphatase subunit on the yeast proteome. EMBO J. **18:** 4157–4168.

Andrulis, E. D., A. M. Neiman, D. C. Zappulla and R. Sternglanz, 1998 Perinuclear localization of chromatin facilitates transcriptional silencing. Nature **394:** 592–595. (erratum: Nature **395:** 525).

Ayscough, K. R., J. J. Eby, T. Lila, H. Dewar, K. G. Kozminski *et al.*, 1999 Sla1p is a functionally modular component of the yeast cortical actin cytoskeleton required for correct localization of both Rho1p-GTPase and Sla2p, a protein with talin homology. Mol. Biol. Cell **10:** 1061–1075.

Bailis, J. M., and G. S. Roeder, 1998 Synaptonemal complex morphogenesis and sister-chromatid cohesion require Mek1-dependent phosphorylation of a meiotic chromosomal protein. Genes Dev. **12:** 3551–3563.

Baker, S. H., D. L. Frederick, A. Bloecher and K. Tatchell, 1997 Alanine scanning mutagenesis of protein phosphatase type 1 in the yeast *Saccharomyces cerevisiae*. Genetics **145:** 615–626.

Barford, D., 1996 Molecular mechanisms of the protein serine/threonine phosphatases. Trends Biochem. Sci. **21:** 407–412.

Black, S., P. D. Andrews, A. A. Sneddon and M. J. R. Stark, 1995 A regulated *MET3-GLC7* gene fusion provides evidence of a mitotic role for *Saccharomyces cerevisiae*. Yeast **11:** 747–759.

Bloecher, A., and K. Tatchell, 1999 Defects in *Saccharomyces cerevisiae* protein phosphatase type I activate the spindle/kinetochore checkpoint. Genes Dev. **13:** 517–522.

Bollen, M., A. A. DePaoli-Roach and W. Stalmans, 1994 Native cytosolic protein phosphatase-1 (PP-1S) containing modulator (inhibitor-2) is an active enzyme. FEBS Lett. **344:** 196–200.

Cannon, J. F., J. R. Pringle, A. Fiechter and M. Khalil, 1994 Characterization of glycogen-deficient *glc* mutants of *Saccharomyces cerevisiae*. Genetics **136:** 485–503.

Chen, M. X., Y. H. Chen and P. T. W. Cohen, 1993 PPQ, a novel protein phosphatase containing a Ser+Asn-rich amino-terminal domain, is involved in the regulation of protein synthesis. Eur. J. Biochem. **218:** 689–699.

Cheng, C., D. Huang and P. J. Roach, 1997 Yeast *PIG* genes: *PIG1* encodes a putative type 1 phosphatase subunit that interacts with the yeast glycogen synthase Gsy2p. Yeast **13:** 1–8.

Clotet, J., F. Posas, E. de Nadal and J. Ariño, 1996 The NH_2-terminal extension of protein phoshatase *PPZ1* has an essential functional role. J. Biol. Chem. **271:** 26349–26355.

Clotet, J., E. Gari, M. Aldea and J. Ariño, 1999 The yeast ser/thr phosphatases sit4 and ppz1 play opposite roles in regulation of the cell cycle. Mol. Cell. Biol. **19:** 2408–2415.

Cohen, P., 1989 The structure and regulation of protein phosphatases. Annu. Rev. Biochem. **58:** 453–508.

Cohen, P. T., 1997 Novel protein serine/threonine phosphatases: variety is the spice of life. Trends Biochem. Sci. **22:** 245–251.

Cormack, B. P., R. H. Valdivia and S. Falkow, 1996 FACS-optimized mutants of the green fluorescent protein (GFP). Gene **173:** 33–38.

Da Cruz e Silva, E. F., V. Hughes, P. McDonald, M. J. Stark and P. T. Cohen, 1991 Protein phosphatase 2Bw and protein phosphatase Z are *Saccharomyces cerevisiae* enzymes. Biochim. Biophys. Acta **1089:** 269–272.

Davis, N. G., J. L. Horecka and G. F. Sprague, Jr., 1993 Cis- and

trans-acting functions required for endocytosis of the yeast pheromone receptors. J. Cell Biol. **122:** 53–65.

de Nadal, E., J. Clotet, F. Posas, R. Serrano, N. Gomez *et al.*, 1998 The yeast halotolerance determinant Hal3p is an inhibitory subunit of the Ppz1p Ser/Thr protein phosphatase. Proc. Natl. Acad. Sci. USA **95:** 7357–7362.

Depaoli-Roach, A. A., I. K. Park, V. Cerovsky, C. Csortos, S. D. Durbin *et al.*, 1994 Serine/threonine protein phosphatases in the control of cell function. Adv. Enzyme Regul. **34:** 199–224.

Di Como, C. J., and K. T. Arndt, 1996 Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. Genes Dev. **10:** 1904–1916.

Dombek, K. M., V. Voronkova, A. Raney and E. T. Young, 1999 Functional analysis of the yeast Glc7-binding protein Reg1 identifies a protein phosphatase type 1 binding motif as essential for repression of ADH2 expression. Mol. Cell. Biol. **19:** 6029–6040.

Durfee, T., K. Becherer, P. L. Chen, S. H. Yeh, Y. Yang *et al.*, 1993 The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev. **7:** 555–569.

Egloff, M. P., P. T. Cohen, P. Reinemer and D. Barford, 1995 Crystal structure of the catalytic subunit of human protein phosphatase 1 and its complex with tungstate. J. Mol. Biol. **254:** 942–959.

Egloff, M. P., D. F. Johnson, G. Moorhead, P. T. Cohen, P. Cohen *et al.*, 1997 Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. EMBO J. **16:** 1876–1887.

Erdeniz, N., U. H. Mortensen and R. Rothstein, 1997 Cloning-free PCR-based allele replacement methods. Genome Res. **7:** 1174–1183.

Feng, Z., S. E. Wilson, Z.-Y. Peng, K. K. Schlender, E. M. Reimann *et al.*, 1991 The yeast *GLC7* gene required for glycogen accumulation encodes a type 1 protein phosphatase. J. Biol. Chem. **266:** 23796–23801.

Ferrigno, P., T. A. Langan and P. Cohen, 1993 Protein phosphatase 2A1 is the major enzyme in vertebrate cell extracts that dephosphorylates several physiological substrates for cyclin-dependent protein kinases. Mol. Biol. Cell **4:** 669–677.

Francisco, L., W. Wang and C. S. M. Chan, 1994 Type 1 protein phosphatase acts in opposition to Ipl1 protein kinase in regulating yeast chromosome segregation. Mol. Cell. Biol. **14:** 4731–4740.

François, J. M., S. Thompson-Jaeger, J. Skroch, U. Zellenka, W. Spevak *et al.*, 1992 *GAC1* may encode a regulatory subunit for protein phosphatase type 1 in *Saccharomyces cerevisiae*. EMBO J. **11:** 87–96.

Frederick, D. L., and K. Tatchell, 1996 The *REG2* gene of *Saccharomyces cerevisiae* encodes a type1 protein phosphatase-binding protein that functions with Reg1p and the Snf1p protein kinase to regulate growth. Mol. Cell. Biol. **16:** 2922–2931.

Gietz, D., A. St. Jean, R. A. Woods and R. H. Schiestl, 1992 Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:** 1425.

Goldberg, J., H. B. Huang, Y. G. Kwon, P. Greengard, A. C. Nairn *et al.*, 1995 Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature **376:** 745–753.

Hill, J. E., A. M. Myers, T. J. Koerner and A. Tzagoloff, 1986 Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2:** 163–167.

Hisamoto, N., K. Sugimoto and K. Matsumoto, 1994 The Glc7 type 1 protein phosphatase of *Saccharomyces cerevisiae* is required for cell cycle progression in G2/M. Mol. Cell. Biol. **14:** 3158–3165.

Hisamoto, N., D. L. Frederick, K. Sugimoto, K. Tatchell and K. Matsumoto, 1995 The *EGP1* gene may be a positive regulator of protein phosphatase type 1 in the growth control of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:** 3767–3776.

Holtzman, D. A., S. Yang and D. G. Drubin, 1993 Synthetic-lethal interactions identify two novel genes, *SLA1* and *SLA2*, that control membrane cytoskeleton assembly in *Saccharomyces cerevisiae*. J. Cell Biol. **122:** 635–644.

Hubbard, M. J., and P. Cohen, 1993 On target with a new mechanism for the regulation of protein phosphorylation. Trends Biochem. Sci. **18:** 172–177.

Hughes, V., A. Muller, M. J. R. Stark and P. T. W. Cohen, 1993 Both isoforms of protein phosphatase Z are essential for the maintenance of cell size and integrity in *Saccharomyces cerevisiae* in response to osmotic stress. Eur. J. Biochem. **216:** 269–279.

James, P., J. Halladay and E. A. Craig, 1996 Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics **144:** 1425–1436.

Kaiser, C., S. Michaelis and A. Mitchell, 1994 Assay of β-galactosidase in yeast, pp. 171–173 in *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kakinoki, Y., J. Somers and D. L. Brautigan, 1997 Multisite phosphorylation and the nuclear localization of phosphatase inhibitor 2-green fluorescent protein fusion protein during S phase of the cell growth cycle. J. Biol. Chem. **272:** 32308–32314.

Lee, K. S., L. K. Hines and D. E. Levin, 1993 A pair of functionally redundant yeast genes (*PPZ1* and *PPZ2*) encoding type 1-related protein phosphatase function within the PKC-mediated pathway. Mol. Cell. Biol. **13:** 5843–5853.

MacKelvie, S. H., P. D. Andrews and M. J. R. Stark, 1995 The *Saccharomyces cerevisiae* gene *SDS22* encodes a potential regulator of the mitotic function of yeast type 1 protein phosphatase. Mol. Cell. Biol. **15:** 3777–3785.

MacKintosh, C., A. J. Garton, A. McDonnell, D. Barford, P. T. Cohen *et al.*, 1996 Further evidence that inhibitor-2 acts like a chaperone to fold PP1 into its native conformation. FEBS Lett. **397:** 235–238.

Matsumoto, K., T. Yoshimatsu and Y. Oshima, 1983 Recessive mutations conferring resistance to carbon catabolite repression of galactokinase synthesis in *Saccharomyces cerevisiae*. J. Bacteriol. **153:** 1405–1414.

Nelson, K. K., M. Holmer and S. K. Lemmon, 1996 *SCD5*, a suppressor of clathrin deficiency, encodes a novel protein with a late secretory function in yeast. Mol. Biol. Cell **7:** 245–260.

Pearson, B. M., Y. Hernando and M. Schweizer, 1998 Construction of PCR-ligated long flanking homology cassettes for use in the functional analysis of six unknown open reading frames from the left and right arms of *Saccharomyces cerevisiae* chromosome XV. Yeast **14:** 391–399.

Posas, F., A. Casamayor, N. Morral and J. Arino, 1992 Molecular cloning and analysis of a yeast protein phosphatase with an unusual amino-terminal region. J. Biol. Chem. **267:** 11734–11740.

Posas, F., A. Casamayor and J. Arino, 1993 The PPZ protein phosphatases are involved in the maintenance of osmotic stability of yeast cells. FEBS Lett. **318:** 282–286.

Posas, F., M. Bollen, W. Stalmans and J. Arino, 1995a Biochemical characterization of recombinant yeast PPZ1, a protein phosphatase involved in salt tolerance. FEBS Lett. **368:** 39–44.

Posas, F., M. Camps and J. Arino, 1995b The PPZ protein phosphatases are important determinants of salt tolerance in yeast cells. J. Biol. Chem. **270:** 13036–13041.

Ramaswamy, N. T., L. Li, M. Khalil and J. F. Cannon, 1998 Regulation of yeast glycogen metabolism and sporulation by Glc7p protein phosphatase. Genetics **149:** 57–72.

Rhodes, N., M. Company and B. Errede, 1990 A yeast-*Escherichia coli* shuttle vector containing the M13 origin of replication. Plasmid **23:** 159–162.

Ronne, H., M. Carlberg, G. Z. Hu and J. O. Nehlin, 1991 Protein phosphatase 2A in *Saccharomyces cerevisiae*: effects on cell growth and bud morphogenesis. Mol. Cell. Biol. **11:** 4876–4884.

Sassoon, I., F. F. Severin, P. D. Andrews, M. R. Taba, K. B. Kaplan *et al.*, 1999 Regulation of *Saccharomyces cerevisiae* kinetochores by the type 1 phosphatase Glc7p. Genes Dev. **13:** 545–555.

Scherer, S., and R. W. Davis, 1979 Replacement of chromosome segments with altered DNA sequences constructed *in vitro*. Proc. Natl. Acad. Sci. USA **76:** 4951–4955.

Sherman, F., and J. Hicks, 1991 Micromanipulation and dissection of asci. Methods Enzymol. **194:** 21–37.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Stark, M. J. R., 1996 Yeast protein serine/threonine phosphatases: multiple roles and diverse regulation. Yeast **12:** 1647–1675.

Stuart, J. S., D. L. Frederick, C. M. Varner and K. Tatchell, 1994 The mutant type 1 protein phosphatase encoded by *glc7-1* from *Saccharomyces cerevisiae* fails to interact productively with the *GAC1*-encoded regulatory subunit. Mol. Cell. Biol. **14:** 896–905.

Tu, J., and M. Carlson, 1994 The *GLC7* type 1 protein phosphatase is required for glucose repression in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **14:** 6789–6796.

Tu, J., and M. Carlson, 1995 REG1 binds to protein phosphatase

type 1 and regulates glucose repression in Saccharomyces cerevisiae. EMBO J. **14:** 5939–5946.

Tu, J., W. Song and M. Carlson, 1996 Protein phosphatase type 1 interacts with proteins required for meiosis and other cellular processes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:** 4199–4206.

Tung, K.-S., L. L. Norbeck, S. L. Nolan, N. S. Atkinson and A. K. Hopper, 1992 *SRN1*, a yeast gene involved in RNA processing, is identical to *HEX2*/*REG1*, a negative regulator in glucose repression. Mol. Cell. Biol. **12:** 2673–2680.

Tung, H. Y., W. Wang and C. S. Chan, 1995 Regulation of chromosome segregation by Glc8p, a structural homolog of mammalian inhibitor-2 that functions as both an activator and an inhibitor of yeast protein phosphatase 1. Mol. Cell. Biol. **15:** 6064–6074.

Vincent, A., G. Newman and S. W. Liebman, 1994 The yeast translational allosuppressor, *SAL6*: a new member of the PP1-like phosphatase family with a long serine-rich N-terminal extension. Genetics **138:** 597–607.

Wertman, K. F., D. G. Drubin and D. Botstein, 1992 Systematic mutational analysis of the yeast *ACT1* gene. Genetics **132:** 337–350.

Wilson, I. A., H. L. Niman, R. A. Houghten, A. R. Cherenson, M. L. Connolly *et al.*, 1984 The structure of an antigenic determinant in a protein. Cell **37:** 767–778.

Communicating editor: M. Carlson