Mutations in Yeast Protein Phosphatase Type 1 that Affect Targeting Subunit Binding†

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ABSTRACT: Protein phosphatase type 1 (PP1) is a major Ser/Thr protein phosphatase that is involved in many cellular processes. The activity of PP1 is controlled by regulatory subunits, many of which are thought to bind to a hydrophobic groove in PP1 via a short consensus sequence termed the V/IXF motif. To test this hypothesis, 11 variants of yeast PP1 (Glc7) were constructed in which one or more of the residues comprising the groove were changed to alanine. These variants were tested for their biological activity in vivo, for their biochemical activity in vitro, and for their ability to associate with three PP1 binding proteins. Five variants are unable to complement the essential function of PP1 in vivo although they are catalytically active in vitro. Many of the mutants are deficient in binding two V/IXF-containing subunits, Gac1 and Reg1, which regulate glycogen accumulation and glucose repression, respectively, but all retain the ability to associate with Sds22, a regulatory subunit that lacks this motif. The subcellular locations at which PP1 normally accumulates (bud neck, nucleolus, spindle pole body) were not occupied by one PP1 variant. Additionally, we provide evidence that mutations in the hydrophobic groove of PP1 affect substrate specificity. Together, these results demonstrate the importance of the hydrophobic groove for the interaction with regulatory subunits, for the proper subcellular localization of PP1 and for the substrate specificity of PP1.

Protein phosphatase 1 (PP1),[1] a highly conserved serine/threonine specific phosphatase, is involved in a wide range of cellular processes. In the cytoplasm and at the plasma membrane, it has roles in glycogen metabolism, muscle contraction, and neurotransmission (*1*, *2*). It is also abundant in the nucleus and functions in mitosis (*3*−*10*) and meiosis (*11*, *12*). In yeast and *Caenorhabditis elegans*, it acts to dephosphorylate histone H3 in opposition to the Ipl1/aurora protein kinase (*13*). As predicted from its broad range of substrates, PP1 is distributed widely in the cell but undergoes dynamic changes in its location during the cell cycle. For example, one mammalian isoform of PP1, PP1γ, moves from the nucleolus during interphase to the mitotic spindle during mitosis. A second isoform, PP1α, moves from the nuclear matrix during interphase to the centrosome during mitosis (*14*). The single PP1 isoform in budding yeast, Glc7, exhibits similar dynamics, accumulating at spindle pole bodies at the start of anaphase, at the actomyosin ring during cytokinesis, and at the site of the newly formed bud prior to bud emergence (*15*). During meiosis, Glc7 accumulates on chromosomes at pachytene (*12*).

How are the specificity and dynamics of this phosphatase regulated? It is widely accepted that PP1 carries out its diverse functions in vivo by the association of the catalytic subunit (PP1c) with different regulatory/targeting subunits (*2*, *16*). The paradigms for this model are the subunits involved in glycogen metabolism, including the G_M/R_{Gl} subunit (*17*), the G_L subunit (*18*), and the PTG subunit (*19*). These three subunits have different tissue distributions, but they all target PP1c to glycogen particles. In addition, these targeting subunits can also form complexes with one or more PP1 substrate proteins that regulate glycogen metabolism, including glycogen synthase, glycogen phosphorylase, and phosphorylase kinase (*20*−*22*). Another example of a targeting/regulatory subunit is the M_{110}/MYPT1 subunit found in smooth muscle (*23*−*25*), which is not significantly similar in sequence to the glycogen subunits. The M_{110}/MYPT1 subunit has distinct binding domains for PP1c and its substrate myosin. When the M_{110}/MYPT1 subunit forms a complex with PP1c, it enhances the dephosphorylation of myosin and suppresses the dephosphorylation of other substrates. The targeting mechanism is even employed by herpes simplex virus 1 (HSV1) to overcome a cellular defense system (*26*). Cells react to virus infection by activating double strand RNA kinase (PKR), which then phosphorylates translation initiation factor eIF2α to shut off protein synthesis (*27*, *28*). HSV1 counteracts this pathway by expressing the viral protein

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[1] Abbreviations: PP1, protein phosphatase type 1; PP1c, catalytic subunit of PP1; HSV1, Herpes simplex virus 1; PKR, double strand RNA activated protein kinase; I-1, inhibitor 1; DARPP-32, dopamine- and cAMP-regulated phosphoprotein, M_r 32 000; 2-DG, 2-deoxyglucose; GAD, Gal4 activation domain; DBD, DNA binding domain; β-gal, β-galactosidase; GFP, green fluorescent protein; MBP, myelin basic protein; PHP, phosphorylase a; G_M, glycogen-binding subunit of PP1 from striated muscle; G_L, glycogen-binding subunit of PP1 from liver; PTG, PP1 subunit targeting to glycogen; M110, myofibrillar-binding subunits of PP1; eIF2α, eucaryotic translation initiation factor 2α; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; X-gal, 5-broma-4-chloro-3-indolyl-β-D-galactoside.

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Table 1: Plasmids Used in This Study

plasmids	description	ref
pRS316	low copy *CEN URA3* shuttle vector	(*43*)
YEp352	high copy 2μ *URA3* shuttle vector	(*44*)
pNC160-GLC7	*GLC7* in low copy *CEN TRP1* shuttle vector	(*38*)
YEp-myc-SDS22	myc-SDS22 on high copy *URA3* plasmid	(*45*)
pG1-myc-Gac1$^{1-169}$	myc-Gac1$^{1-169}$ in high copy TRP1 plasmid	Wu et al.
pGAD-C2/pACT	2μ *LEU2* GAL4$_{768-881}$ (activation domain) shuttle vector for two-hybrid analysis	(*39*, *40*)
pAS1	2μ TRP1 GAL4$_{1-147}$ (DNA binding domain) shuttle vector for two-hybrid analysis	(*39*)
YCp50-HA-GLC7	*CEN4 URA3* shuttle plasmid with HA-*GLC7*	(*52*)
pAS1-GLC7	*GLC7* in pAS1	Wu et al.
pGAD-GLC7	*GLC7* in pGAD-C2	this study
pGAD-SDS22	*SDS22* in pACT	this study
pKD123	*REG1* in 2μ *URA3* LexA two hybrid vector	(*41*)

$\gamma_1$34.5, which forms a complex with PP1c and dephosphorylates eIF2α to prevent the shut-off of protein synthesis by PKR (*26*, *29*). The $\gamma_1$34.5 protein is not significantly similar in sequence to either G or M targeting subunits. Other binding proteins have been identified; these also show little similarity to one another or to the aforementioned subunits.

Although the lack of similarity between targeting subunits suggests that the protein−protein interactions between PP1c and a given targeting subunit may be unique, a PP1c-binding motif (R/K-V/I-X-F), hereafter referred to as the V/IXF motif, has been identified in many targeting subunits of PP1c. This motif was identified independently from the cocrystal structure of PP1c and a small peptide derived from the G_M subunit (*30*), from PP1c-binding peptides in a peptide display library (*31*), and from structure/function analysis of the PP1 inhibitors I-1 (32) and DARPP-32 (*33*, *34*). Although the V/IXF motif is very common among predicted peptide sequences, existing in more than 10% of known proteins (*30*), its importance in binding PP1c has been confirmed for many targeting subunits (*26*, *30*, *33*−*37*). The G_M-PP1c crystal structure revealed that a hydrophobic channel on the surface of PP1c interacts primarily with the $V_{66}S_{67}F_{68}$ sequence of the G_M peptide. This hydrophobic channel is formed at the interface of two β-sheets that are remote from the catalytic center of PP1c.

The presence of the V/IXF motif in many regulatory/targeting subunits leads to the prediction that this hydrophobic groove on PP1c will play a key role regulating the specificity of the phosphatase. We have tested this prediction by constructing eleven variants of yeast PP1c (Glc7) in which one or more amino acid residue(s) in the hydrophobic groove has been changed to alanine. Each variant was expressed in yeast and tested for expression, phosphatase activity, ability to associate with PP1c-binding proteins, and biological function. Together, these results allow us to conclude that the hydrophobic groove is necessary for binding V/IXF-containing regulatory/targeting subunits, for biological activity, and for proper subcellular localization. Moreover, changes in phosphatase activity and trypsin sensitivity of some variants also suggests that some residues that constitute the hydrophobic groove may play a more direct role in protein structure and in regulating phosphatase activity rather than simply in the binding of regulatory/targeting subunits.

MATERIALS AND METHODS

Strains Media and Yeast Methods. Heterozygous diploid yeast strain SB78 (*MAT***a**/*MAT*α *ura3-52/ura3-52 trp1-1/trp1-1 leu2/leu2 GLC7/glc7::LEU2*) (*38*) was used to study *GLC7* mutants. Strains Y190 (*MAT***a** *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3, 112 URA3::GAL-lacZ, LYS2::GAL-HIS3 cyh*^{r}), PJ69-4A (*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) and KDY109 (*MAT***a** *ade2-101 gal4 gal80 his3-200 leu2-1 trp1-901 ura3::HIS3::(lexA$_{op}$)$_8$-lacZ*) were used for two hybrid assays (*39*−*41*). Yeast cells were grown in YEPD (1% yeast extract, 2% bacto peptone, 2% glucose) or synthetic dropout (SD) media (0.67% yeast nitrogen base, 2% glucose supplemented with amino acids). Yeast transformation was performed using the lithium acetate method (*42*). Sporulation efficiency was assayed on plates or in liquid culture using YPA media (1% yeast extract, 2% peptone, 2% potassium acetate).

Site-Directed Mutagenesis and Plasmid Construction. *Escherichia coli* strains DH5α and XL1-Blue were used to propagate plasmids. Plasmids used in this study are listed in Table 1. Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, USA) following the manufacturer's protocol. Mutations were screened by DNA sequencing using Sequenase kit version 2.0 (Amersham Life Science Inc, USA). The entire sequence of each *GLC7* mutant allele was determined at Iowa State University DNA facility and no additional mutation was found. Pairs of oligonucleotide primers used to generate mutations are listed in Table 2, and point mutations generated and used in this study are listed in Table 3. The plasmid pBluescript containing the *Hin*d III/*Xho* I fragment of HA-*GLC7* was used as the template to generate the *glc7-I168A*, *glc7-F256A*, *glc7-L288A*, *glc7-C290A*, *glc7-F292A*, *glc7-E241 L242*, and *glc7-E288 F292* alleles. To generate *glc7-F256 F292*, a pBluescript plasmid containing *glc7-F292A* was used as template and primers F256A+/F256A− were used to introduce the second mutation. To generate *glc7-143*, which contains three point mutations, a pBluescript plasmid containing *glc7-E241 L242* was used as the template and primers I168A+/I168A− were used to introduce the third mutation. To generate *glc7*−*144*, which also contains three point mutations, a pBluescript plasmid containing *glc7-E288 F292* was used as the template and primers F256A+/F256A− were used to introduce the third point mutation. To construct *glc7-145*, which contains six point mutations, the 888 bp *Apa* I fragment from pBluescript-*glc7-144* was swapped into the *Apa* I site of pBluescript-*glc7-143*. After these mutations were made and sequenced in pBluescript, the 2.7 kb *Xho* I/*Hin*d III fragment containing the *GLC7* gene, and its natural promoter was inserted into the *Xho* I/*Hin*d III sites of the low copy plasmid pRS316 (*URA3*) (*43*) for expression in

Table 2: Oligonucleotides Used for Site-Directed Mutagenesis in This Study

name	sequence (5′ to 3′)
I168A+	GCAATTATTGATGAGAAAGCCTTCTGTATGCATGG
I168A-	CCATGCATACAGAAGGCTTTCTCATCAATAATTGC
F256A+	GATGGTTATGAAGCCTTTAGTAAAAGAC
F256A-	GTCTTTTACTAAAGGCTTCATAACCATC
L288A+	GTTGATGAAAGTGCATTATGTTCTTTTC
L288A-	GAAAAGAACATAATGCACTTTCATCAAC
C290A+	GAAAGTTTATTAGCTTCTTTTCAAATT
C290A-	AATTTGAAAAGAAGCTAATAAACTTTC
F292A+	GTTTATTATGTTCTGCTCAAATTTTAAAGC
F292A-	GCTTTAAAATTTGAGCAGAACATAATAAAC
E241L242+	CAGAAACAAGATATGGCGGCGATTTGCAGGGCC
E241L242-	GGCCCTGCAAATCGCCGCCATATCTTGTTTCTG
L288F292+	GTGTTGATGAAAGTGCATTATGTTCTGCTCAAATTTTAAAGCC
L288F292-	GGCTTTAAAATTTGAGCAGAACATAATGCACTTTCATCAACAC

Table 3: List of *GLC7* Mutants Created and Used in This Study

name of the allele	residues changed to Ala	phenotype[a]
glc7-I168A	Ile168	glc±, spo-
glc7-F256A	Phe256	ts, glc±, 2-DG^r, CsCls
glc7-L288A	Leu288	glc±
glc7-C290A	Cys290	wild type[b]
glc7-F292A	Phe292	ts, glc±, spo- 2-DG^r, CsCls
glc7-E241 L242	Glu241, Leu242	ts, 2-DG^r, CsCls
glc7-E288 F292	Leu288, Phe292	lethal
glc7−143	Ile168, Glu241, Leu242	lethal
glc7−144	Phe256, Leu288, Phe292	lethal
glc7−145	Ile168, Glu241, Leu242, Phe256, Leu288, Phe292	lethal
glc7-F256 F292	Phe256, Phe292	lethal

[a] glc±, reduced glycogen accumulation; 2-DG^r, resistance to 2-deoxyglucose; CsCl^s, sensitivity to YPD medium containing 100 mM CsCl; ts, reduced growth rate above 37 °C; spo-, sporulation less than 5% on YPA plates and in liquid YPA media. [b] This mutant behaved like a wild-type *GLC7* strain with regard to glycogen accumulation, glucose repression, CsCl sensitivity and ability to grow at temperatures ranging from 11 to 40 °C.

yeast. The *Xho* I/*Hin*d III fragments containing *glc7-E288 F292* and *glc7-F256 F292* were also inserted into the *Sal* I/*Hin*d III sites of the high copy plasmid YEp352 (*URA3*) (*44*) for overexpression studies. For yeast two-hybrid assays, two sets of plasmids were constructed. For the first set, mutant alleles were cloned into the *GAL4* DNA binding domain (GBD) fusion plasmid pAS1 (*39*). This was done by swapping the *Cla* I/*Xho* I fragments containing mutant *GLC7* alleles from plasmid pRS316 into the *Cla* I/*Sal* I sites of pAS1 containing wild-type *GLC7*. This set of plasmids was used to study two-hybrid interactions with Sds22. For the second set, mutant alleles were cloned into the *GAL4* transcription activation domain (GAD) fusion plasmid pGAD-C2 (*40*). This was done in two steps. First, the *Bam*H I fragment containing wild-type *GLC7* from plasmid pAS1-*GLC7* was inserted into the *Bam*H I site of plasmid pGAD-C2 to generate pGAD-*GLC7*. Then, the *Cla* I/*Xho* I fragments containing mutant *GLC7* alleles from pRS316 plasmids were swapped into the *Cla* I/*Sal* I sites of pGAD-*GLC7*. To construct pGAD-*SDS22*, the *Eco*R I/*Xho* I fragment containing *SDS22* from Ycp2-9b (*45*) was inserted into pBluescript and then the *SDS22* gene was removed from the resulting plasmid by digesting with *Bam*H I/*Xho* I and inserted into pACT (*39*) to produce the in-frame fusion with the *GAL4* activation domain.

Biochemical and Analytical Procedures. Immunoprecipitation analysis and immunoblot analysis were performed as previously described (*46*). To assay Gac1/Glc7 interaction, pG1-myc-Gac1$^{1-169}$ and pRS316-HA-Glc7 were coexpressed in yeast cells. To assay Sds22/Glc7 interaction, YEp-myc-Sds22 and pNC160-HA-Glc7 were used. For qualitative glycogen assays, yeast cells were grown on SD plates at 30 or 37 °C and stained with iodine vapor (*47*). For quantitative glycogen assays, yeast cells were grown in liquid synthetic media and cells were harvested by centrifugation. Glycogen was converted to glucose as described (*48*), and then the glucose concentration was determined by the glucose oxidase method (Sigma procedure 510) (*49*). Glycogen levels were normalized to the dry weight of yeast cells. To assay invertase activity, strains were grown in 10 mL of selective synthetic medium containing 5% glucose to a cell density of 1 $\times$ 10^7 cells/mL. A total of 1 mL of cell culture was harvested for each invertase assay. Invertase activity was assayed in whole cells as described previously (*50*).

Yeast Two-Hybrid Assays. To assay the interaction between *Glc7* mutants and regulatory subunits, yeast two-hybrid assays were used. Strain KDY109 (*41*) was used to study the interaction between Glc7 and Reg1. Strain Y190 (*39*) was used to study the interaction between Glc7 and Sds22. The two different yeast two-hybrid strains differ in their genetic backgrounds but both carry a *lacZ* reporter. In all cases, we quantitatively assayed the activation of reporter gene by measuring β-galactosidase (β-gal) activity using the chloroform method as described (*51*). The qualitative filter assay was performed using cells grown on a nitrocellulose membrane (*51*).

Phosphatase Activity Assays. Protein phosphatase activities of wild-type and mutant Glc7 proteins were assayed using immunoprecipitated Glc7. A YCp50 plasmid containing the wild-type HA-*GLC7* (*52*) or pRS316 plasmids containing

HA-tagged mutant alleles were transformed into strain SB78. Transformants were grown to 1×10^8 cells/mL in 50 mL of synthetic -ura medium. Cell extracts were made in breaking buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, 5% glycerol, pH 7.0 with 1 mM phenylmethylsulfonyl fluoride and a 1/300 dilution of protease inhibitor cocktail of 5 mg [each] of chymostatin, leueptin, antipain, and pepstatin in 20 mL of 50% ethanol) and immunoprecipitated with 12CA5 anti-HA antibody basically as described (*46*). Briefly, 100 μL of cell extract containing 1 mg of total protein and 1 μL of 12CA5 antisera were incubated for 1 h at 4 °C. After centrifugation at 12 000 rpm for 10 min in a microcentrifuge, the supernatant was transferred to a new tube containing 30 μL of Protein G agarose (Gibco BRL) equilibrated in radioimmunoprecipitation assay buffer without sodium dodecyl sulfate (SDS) (50 mM Tris, 1% Triton X-100, 0.5% sodium deoxycholate, 200 mM NaCl, pH 7.0). The suspension was rocked at 4 °C for 1 h and centrifuged at 1000 rpm for 10 s. The agarose beads were washed three times with 0.5 mL of wash buffer (25% radioimmunoprecipitation assay buffer without SDS: 75% breaking buffer) before phosphatase assay. Phosphatase assay was performed using either ^{32}P-labeled rabbit phosphorylase a or ^{32}P-labeled myelin basic protein (MBP) as substrate at a concentration of 10 μM. Phosphorylase a phosphatase and MBP phosphatase assay kits were purchased from Gibco BRL and New England Biolabs, respectively, and used according to the manufacturer's instructions. The protein G agarose bound to HA-Glc7 was washed once in 500 μL of phosphorylase phosphatase assay buffer (20 mM imidizole-HCl, pH 7.6, 0.1% β-mercaptoethanol, 0.1 mM EDTA, 1 mg/mL BSA) or MBP phosphatase assay buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij 35) and resuspended in 150 μL of phosphorylase or MBP phosphatase assay buffer. Forty microliter aliquots were transferred to new tubes and 20 μL of labeled substrate was added to each tube and then incubated at 30 °C for 10 min. A total of 180 μL of 20% TCA was added to terminate the reaction and protein was precipitated on ice for 10 min. After centrifugation for 3 min at 12 000 rpm, 200 μL samples of supernatant were counted in a scintillation counter. The acid-soluble ^{32}P activity reflects the phosphatase activity. The phosphatase assays were repeated 3 times, and the average activity is shown. In all phosphatase activity assays, reactions were terminated before 20% of total substrate was consumed. The phosphatase reaction was linear within this range.

RESULTS

Generation of Mutations in the Hydrophobic Groove of GLC7. The G_M-PP1c crystal structure reveals that a hydrophobic groove on the surface of PP1c interacts primarily with the $V_{66}S_{67}F_{68}$ sequence of the G_M peptide (*30*). Figure 1 shows the amino acid residues involved in the interaction with the V/IXF motif of the G_M subunit. Residues F257, C291, and F293 of PP1c interact with the residue F68′ of the G_M peptide while residues I169, L243, D242, L289, and C291 of PP1c interact with the residue V66′ of the G_M peptide. With the exception of residue D242, which is substituted by a conserved E residue in yeast PP1c, all other residues in this hydrophobic groove are identical in the yeast and mammalian proteins. To study the structure−function relationship of this hydrophobic groove, amino acid residues

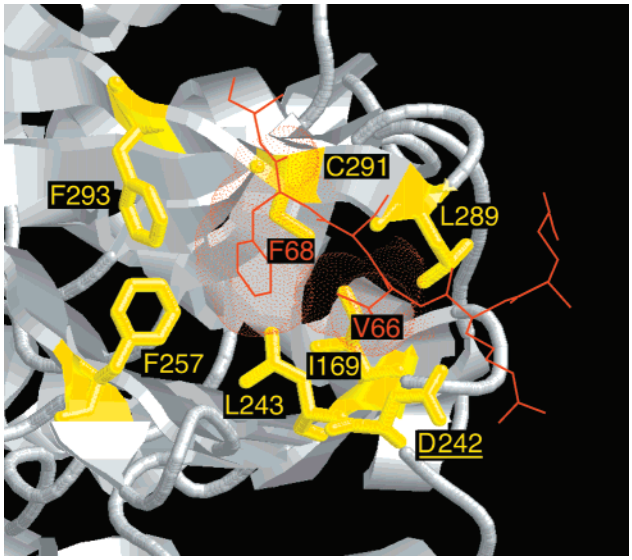

FIGURE 1: Structure of the PP1-$G_{M[63-75]}$ complex. The residues of PP1c that make contact with $G_{M[63-75]}$ are shown in yellow. The structure of $G_{M[63-75]}$ is shown in red. van der Waals radii of key residues that contact PP1 (V66 and F68) are designated with red dots. The coordinates of human PP1γ1 (*30*) were used to create this model using Rasmol 2.6 software (Roger Sayle, Glaxo Wellcome Medicines Research Centre). All residues of PP1c that are marked in this figure are identical in to those in Glc7 with the exception of D242 which is E241 in Glc7. Note that yeast Glc7 lacks one amino acid residue at its NH_2-terminus that is present in PP1. Therefore, the related Glc7 and PP1 amino acid residues are offset by one residue (i.e., F293 in PP1 is F292 in Glc7).

I168, E241, L242, F256, L288, C290, F292 of yeast PP1c (Glc7) were altered by site-directed mutagenesis. For the convenience of subsequent protein detection, mutagenesis was performed on a *GLC7* gene with sequence encoding the HA-epitope (YPYDVPYDYAT) inserted near the 5′ end of the gene (*52*). It was previously shown that HA-*GLC7* is fully functional in vivo (*46*, *52*). Table 3 lists the *GLC7* mutants used in this study.

Expression and Biological Activity of Glc7 Variant Proteins. To assay the biological activity of the PP1 variants, each was expressed from the low copy yeast shuttle vector pRS316 and tested for its ability to provide the essential function in a *glc7*Δ strain. For this test, pRS316 plasmids carrying the *GLC7* alleles were transformed into the heterozygous diploid strain SB78 (*GLC7*/*glc7::LEU2*, *ura3*/*ura3*, *leu2*/*leu2*, *his3*/*his3*). The transformants were sporulated and meiotic haploid spore clones were isolated and scored for the presence of the disrupted *glc7* gene (marked with the *LEU2*) and for the plasmid borne *GLC7* variant gene (marked with *TRP1*). Because *GLC7* is an essential gene, the *glc7::LEU2* mutation is only recovered in haploid progeny that retain a pRS316 plasmid with a functional *GLC7* gene. For all alleles containing a single point mutation and for *glc7-E241 L242*, which contains two missense mutations, spore clones were identified that contained the *glc7::LEU2* null allele and the pRS316-*GLC7* plasmid, indicating that these alleles can complement a *glc7* disruption for cell growth and viability. These alleles are referred to hereafter as viable alleles (Table 3). In contrast, we recovered no Leu$^+$ spore clones from transformants with pRS316 plasmids containing other *GLC7* alleles, indicating that these

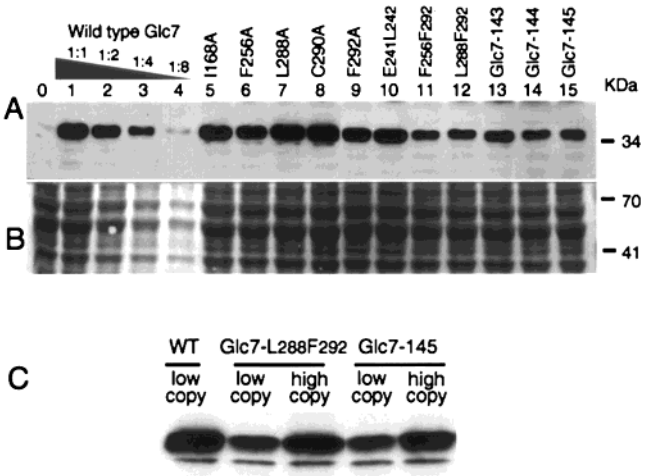

FIGURE 2: Steady-state levels of mutant Glc7 proteins. Cell extracts were prepared from yeast strain SB78 transformed with wild-type or mutant HA-*GLC7* plasmids and subjected to SDS−polyacrylamide gel electrophoresis followed by immunoblot analysis, using anti-HA monoclonal antibody 12CA5. (A) Immunoblot; (B) Loading control as shown by Ponceau S staining of the membrane used for immunoblot analysis. Approximately equal amounts of total protein (~50 μg) were loaded in all lanes except in lane 2−4, which contain 2-fold serial dilutions of lane 1. Lane 0, cell extract from a strain containing the vector pNC160. (C) Immunoblot as in panel A with cell extracts prepared from strains expressing wild-type Glc7 from a low copy plasmid and Glc7-E288 F292 and Glc7-145 from low and high copy plasmids, respectively.

alleles cannot complement the lethality of a *glc7::LEU2* disruption. These alleles are referred to hereafter as lethal alleles (Table 3).

Each viable allele was tested for its function in biological pathways known to require Glc7, including glycogen accumulation, glucose repression, cell cycle control, sporulation, and ion homeostasis (for review see ref *53*). The phenotype of each mutant is summarized in Table 3. *glc7-F256A*, *glc7-F292A*, and *glc7-E241 L242* all conferred multiple defects including abnormal glycogen accumulation, CsCl sensitivity, 2-deoxyglucose (2-DG) resistance, and temperature sensitivity for growth. *GLC7* mutants with such pleiotropic phenotypes have not been previously reported. The *glc7-I168A* mutant was also sporulation deficient. *glc7-L288A* and *glc7-C290A* conferred no obvious defects in these assays. *glc7-F256A*, *glc7-F292A* and *glc7-E241 L242* caused temperature-sensitive growth. *glc7-F256A* strains failed to grow at temperatures above 37 °C, whereas *glc7-F292A* and *glc7-E241 L242* strains failed to grow above 40 °C. Like several previously characterized conditional alleles of *GLC7* (*5*, *7*, *9*, *10*, *38*), *glc7-F256A* caused cell cycle arrest with large budded cells at restrictive temperature.

The pleiotropic phenotypes observed for many of the mutants are consistent with the hypothesis that the hydrophobic groove is critical for phosphatase function. Alternatively, these mutations could destabilize the structure of Glc7, resulting in reduced steady-state expression. To test the latter possibility, immunoblot analysis was performed on cell extracts from strain SB78 containing the mutant *GLC7* genes. Our results, presented in Figure 2, show that all variant proteins accumulate in yeast. The products of the viable alleles accumulate at levels comparable to that of wild-type Glc7, while those of the lethal alleles are reduced 2−4-fold relative to that of wild type. To test whether the lethality

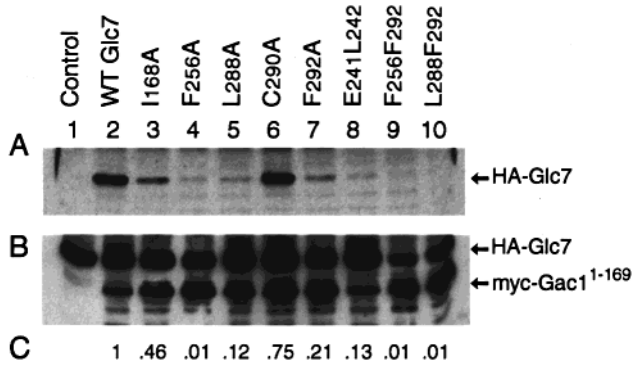

FIGURE 3: Association of HA-Glc7 with myc-Gac1$^{1-169}$ in immune complexes. Cell extracts were prepared from yeast strains expressing HA-Glc7 variants and myc- Gac1$^{1-169}$. (A) Immunoblot of HA-Glc7 variants that coprecipitated with myc- Gac1$^{1-169}$. Cell extracts were immunoprecipitated with anti-myc antibody and probed with anti-HA antibody. (B) Immunoblot of total cell extracts probed with both anti-HA and anti-myc antibodies, showing the levels of HA-Glc7 and myc-Gac1$^{1-169}$ that were present in each extract. (C) Fraction of Glc7 in the myc-Gac1 immunoprecipitates as determined by densitometric scanning of the Glc7 signal and normalizing to the wild-type Glc7.

could be caused by reduced protein levels, two lethal alleles (*glc7-E288 F292* and *glc7-145*) were cloned into the high copy 2μ plasmid YEp352 and these plasmids were introduced into strain SB78. Immunoblot analysis of the transformants revealed that the protein levels of Glc7-E288 F292 and Glc7-145 were close to the level of the wild-type Glc7 (Figure 2C). However, these high copy plasmids could not complement a *glc7* disruption, indicating that the lethality is not caused by reduced expression of the Glc7 proteins.

Interaction of Glc7 Variants with Known Glc7-Interacting Proteins. Since the hydrophobic groove is a potential docking site for many PP1c binding proteins, we characterized the interaction between mutant proteins and several known Glc7 interacting proteins using co-immunoprecipitation assays and quantitative yeast two hybrid assays. For these assays, we selected two regulatory subunits (Gac1 and Reg1), which contain bona fide V/IXF motifs and a third regulatory subunit (Sds22) that has no such motif.

Gac1 is a glycogen-specific regulatory subunit for Glc7 (*54*). We have shown that the NH_2-terminal fragment of 93 amino acid of Gac1 is necessary and sufficient to interact with Glc7 (*69*). The $K_{69}N_{70}V_{71}R_{72}F_{73}$ sequence in Gac1 matches the consensus V/IXF motif and the single missense mutation F73A in Gac1 totally abolishes binding to Glc7, suggesting that the V/IXF in Gac1 is crucial for the Gac1/Glc7 binding (*69*). To test the hypothesis that the hydrophobic groove on Glc7 is indeed the docking site for the V/IXF motif of Gac1, we assayed the physical interaction between the mutant Glc7 proteins and Gac1 by co-immunoprecipitation (Figure 3). HA-Glc7 protein was coexpressed with myc-tagged Gac1$^{1-169}$ in yeast. Total cell extracts were made and immunoprecipitated with anti-myc antibody. The immunoprecipitates were electrophoresed on an SDS−polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-HA antibody. As shown in Figure 3, wild type HA-Glc7 was associated with Gac1 in the immune complex. The interactions between most Glc7 variants and Gac1 were weaker except Glc7-C290A, which interacted with Gac1 as strongly as wild type. The results suggest that the

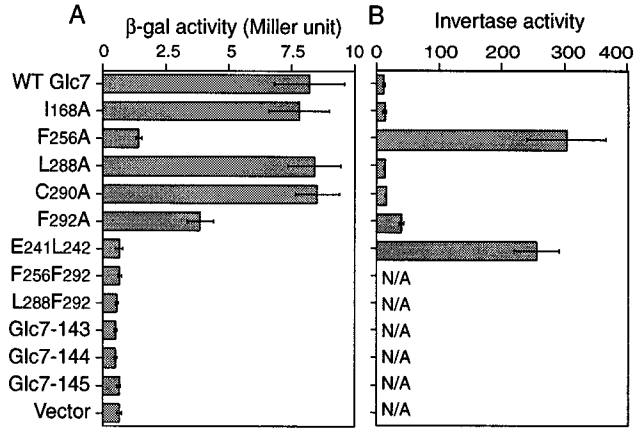

FIGURE 4: Two-hybrid analysis of Reg1/Glc7 interactions and invertase activities in mutant strains. (A) β-gal activity was assayed in KDY109 strains expressing both the LexA DBD-Reg1 fusion and the indicated GAD-Glc7 fusion protein. (B) Invertase activities in wild-type and the corresponding *GLC7* mutant strains. The activity is expressed as μmol of glucose released/min/100 mg of cells (dry weight). All activities shown are the mean of activities from at least three independent transformants. Error bars represent standard errors. N/A, not applicable because these mutants cannot support biological activity.

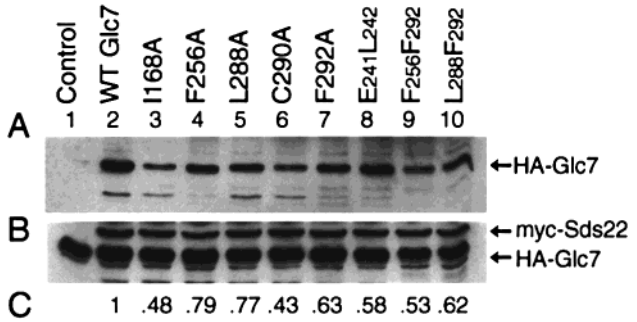

FIGURE 5: Association of Glc7 with Sds22. (A) Immunoblot with anti-HA antibody showing HA-Glc7 variants that coprecipitate with myc-Sds22. Cell extracts prepared from strains coexpressing HA-Glc7 proteins and myc-Sds22 were immunoprecipitated with anti-myc antibody and probed with anti-HA-antibody. (B) Immunoblot of total cell extracts probed with anti-HA and anti-myc antibodies, showing the levels of HA-Glc7 and myc-Sds22that were present in each extract. (C) Fraction of Glc7 in the myc-Sds22 immunoprecipitates as determined by densitometric scanning of the Glc7 signal and normalizing to the wild-type Glc7.

hydrophobic channel is indeed the cognate docking site for theV/IXF motif on Gac1.

Reg1 is a Glc7 subunit that regulates the phosphorylation state of several proteins acting in the glucose repression pathway, including Snf1, Mig1, and Hxk2 (*55*−*57*). Glucose repression refers to the phenomenon in which the expression of genes encoding enzymes required for the utilization of carbon sources other than glucose is highly repressed by the presence of glucose in the culture media (reviewed in ref *58*). For example, the expression of invertase, which is necessary for sucrose utilization, is repressed at high glucose concentrations and is used frequently to assay for a defect in glucose repression. Defects in the glucose repression pathway can also be assessed by growth on medium containing 2-deoxyglucose (2-DG), which is a nonmetabolizable glucose analogue that inhibits the utilization of sucrose by normal cells but not by glucose derepressed mutants such as *reg1* (*59*). Reg1 has a conserved V/IXF motif (IHF in this case) that is essential for its interaction with Glc7 (*41*, *57*). This interaction is required for glucose repression (*41*, *55*, *57*). Several of our mutants were resistant to 2-DG (Table 3), suggesting that they may be defective for association with Reg1. To investigate the interaction between Reg1 and Glc7, we used a two-hybrid assay developed by Dombek et al. (*41*), which was shown to correlate with the association of Reg1 and Glc7 in a direct binding assay (*41*, *60*). In this assay, Reg1 is fused to the DNA binding domain (BD) of LexA and Glc7 is fused to the activation domain (AD) of Gal4. The interaction between LexA-BD-Reg1 and GAD-Glc7 was assayed by measuring the activation of the *lacZ* reporter gene in strain KDY109 (Figure 4A). Reg1 failed to interact with the products of the five lethal alleles. Glc7 proteins encoded by *glc7-F256A*, *glc7-F292A* and *glc7-E241 L242* also showed reduced interactions with Reg1. To assess the significance of these interactions, we assayed the expression of invertase in *GLC7* mutant strains grown at high glucose concentrations, as a measure of glucose repression. Yeast strains lacking Reg1 or containing mutant forms of Glc7 that are unable to bind Reg1 express invertase constitutively. As shown in Figure 4B, strains carrying *glc7-F256A*, *glc7-F292A* and *glc7-E241 L242* express higher than normal levels of invertase, indicating a defect in glucose repression. Moreover, the level of invertase expressed in each strain was inversely correlated with the level of interaction between Reg1 and Glc7, suggesting that the two hybrid assay reflects the level of interaction between Reg1 and Glc7 in vivo.

The third Glc7 binding protein we investigated was Sds22, a non-V/IXF motif protein. *sds22*$^+$ from *Schizosaccharomyces pombe* was originally isolated as a multicopy suppressor of *dis2-11* (*61*), a cold-sensitive mutant in a *S. pombe* PP1 gene. *sds22*$^+$ is essential and a conditional allele of *sds22* causes arrest in mitosis at the restrictive temperature (*61*). The *Saccharomyces cerevisiae* homolog, SDS22, whose product has a similar repeating structure made up of eleven 22-aa leucine-rich repeats, is also essential (*9*, *45*). Proteins closely related to Sds22 have been identified in human cells (*62*) and in rat liver nuclei, and both proteins were found to bind PP1 and inhibit its activity (*63*). Although Sds22 has no V/IXF motif in its sequence, it has been shown to interact with Glc7 in both co-immunoprecipitation and two hybrid assays (*9*, *45*). We used both assays to measure the interaction between Sds22 and Glc7 mutant proteins. The results of a coprecipitation assay are shown in Figure 5A, in which the level of Glc7 bound to Sds22 from whole cell extracts was assayed by immunoblot analysis. All variants of Glc7 tested bound Sds22, including the lethal alleles that failed to interact with Gac1 and Reg1. The Glc7/Sds22 interaction was also tested with the two hybrid system. Glc7 was fused to the BD domain of *GAL4* and Sds22 was fused to the AD domain of *GAL4*. The activation of the *lacZ* reporter gene was measured by a filter assay using X-gal as a substrate. Strains containing wild-type Glc7 and all mutant proteins developed blue color indicative of β-gal activity, while the strain containing empty vector remained white (data not shown). Together, these results support the hypothesis that the hydrophobic groove identified in the G_M-PP1c cocrystal structure is a key binding site for the V/IXF-containing proteins but is not important for the binding of Sds22, a Glc7-binding protein that lacks this motif.

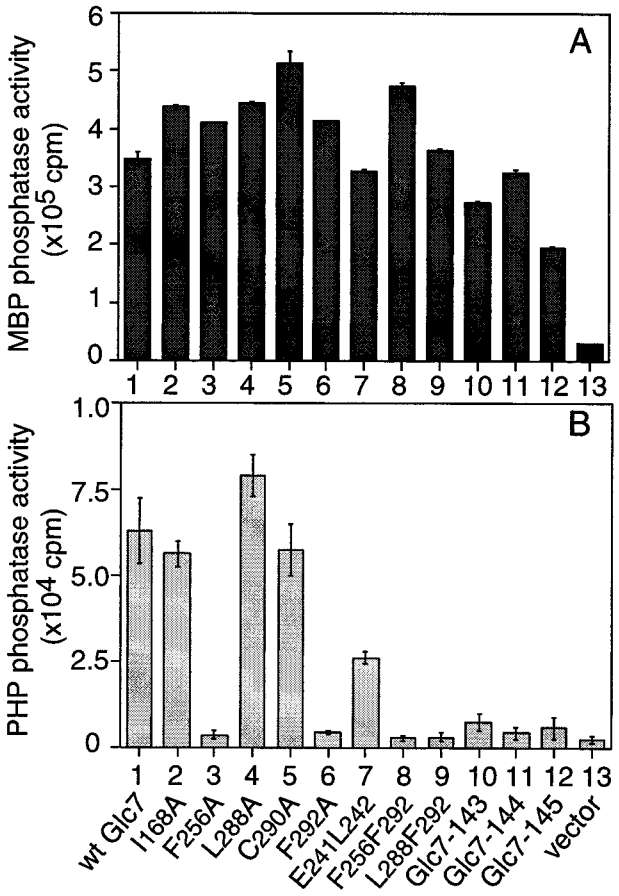

FIGURE 6: In vitro phosphatase activity of wild-type and mutant Glc7 proteins. Immunoprecipitates of cell extracts prepared from strain SB78 transformed with low copy HA-*GLC7* plasmids were assayed for MBP phosphatase activity (A) or Phosphorylase a (PHP) phosphatase activity (B) (See Experimental Procedures). Lanes: WT Glc7, lane 1; Glc7-I168Ap, lane 2; Glc7-F256Ap, lane 3; Glc7-L288Ap, lane 4; Glc7-C290Ap, lane 5; Glc7-F292Ap, lane 6; Glc7-E241 L242p, lane 7; Glc7-F256 F292p, lane 8; Glc7-E288 F292p, lane 9; Glc7-143p, lane 10; Glc7-144p, lane 11; Glc7-145p, lane 12; and vector control, lane 13. All activities are the mean of at least three independent measurements and error bars represent the standard errors.

All Glc7 Variants Are Catalytically Active but Some Exhibit Altered Substrate Specificity. The data presented so far are consistent with the hypothesis that the hydrophobic channel is necessary for binding at least two and possibly all V/IXF-containing Glc7 targeting subunits, but we have not ruled out the possibility that the lethality of some alleles is caused by lack of catalytic activity of the PP1 variants. To test this possibility, phosphatase assays were conducted on the wild-type Glc7 and all 11 mutant proteins. We immunoprecipitated Glc7 from crude extracts prepared from strains containing each of the 11 mutants and assayed phosphatase activity directly in the immune complexes. The levels of Glc7 in the immunoprecipitates were assayed by immunoblot analysis and found to be similar to those in the total extracts (data not shown). We used both myelin basic protein (MBP) labeled by PKA and rabbit phosphorylase a labeled by phosphorylase kinase as substrates in our phosphatase assays. Immunoprecipitates of cell extracts from a strain lacking the HA epitope-tagged Glc7 had low MBP and phosphorylase phosphatase activity (Figure 6, column 13), indicating that our assay was specific for HA-tagged

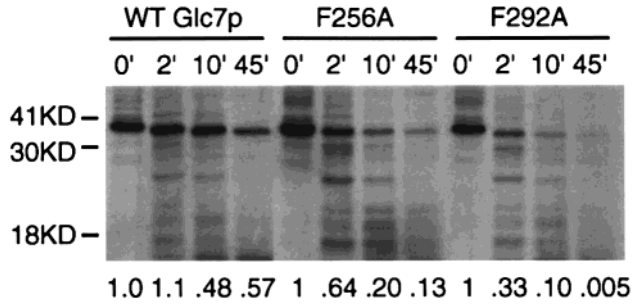

FIGURE 7: Trypsin treatment of immunoprecipitated Glc7 protein. ^{35}S-Met-labeled cell extracts were prepared from SB78 strains transformed with HA-*GLC7* plasmids. One milligram protein samples of crude extract were immunoprecipitated with anti-HA antibody followed by trypsin digestion at a concentration of 2 μg/mL. The reactions were terminated at the indicated times by adding 2× SDS loading buffer and samples were boiled for 5 min followed by electrophoresis on 10% SDS−polyacrylamide gels. The dried gels were exposed to X-ray film for 2 weeks. Numbers below each lane refer to the fraction of Glc7 remaining as determined by densitometric scans of the autoradiogram.

proteins. The immunoprecipitates containing wild-type and HA-Glc7 variants all showed strong phosphatase activity against MBP (Figure 6A). However, the immunoprecipitates containing Glc7 encoded by *glc7-F256A*, *glc7-F292A*, and the lethal alleles showed very low levels of phosphorylase a phosphatase activity (Figure 6B).

Since our phosphatase assays were performed on relatively crude preparations, it is possible that the altered phosphatase activity reflects changes in subunit composition of the PP1 holoenzymes present in the immune complexes. Glc7-F256A, Glc7-F292A, and the variant proteins encoded by the lethal alleles could fail to bind to certain subunits that activate phosphorylase a activity or could bind with higher affinity to inhibitory subunits. Limited proteolysis with trypsin has been used with PP1 to unmask cryptic activity (*64*). Therefore, we treated the immunoprecipitates containing HA-Glc7 with 2 μg/mL of trypsin for 10 min before the phosphatase assay. Under these conditions wild-type Glc7 migrated with slightly increased electrophoretic mobility (Figure 7), indicating the presence of a trypsin-resistant core. This form is due to proteolysis at the COOH terminus of the protein, because immunoblot analysis of the trypsin treated samples reveals that the HA epitope is retained in the more rapidly migrating form (data not shown). The phosphorylase phosphatase activity of this material was 2.3-fold higher than before trypsin treatment. Surprisingly, Glc7-F256A and Glc7-F292A were more sensitive to trypsin. After digestion with 2 μg/mL trypsin, little of the resistant core remained after 10 min of digestion and additional species were observed that are not observed for the wild-type protein. At a higher concentration of trypsin (20 μg/mL) the two variants were almost completely degraded after 45 min, whereas the wild type was largely resistant (data not shown). These results indicate that the alteration of key residues in the V/IXF-binding groove can alter the structure of Glc7. It is possible that this conformational change could account for the altered substrate specificity of Glc7-F256A and Glc7-F292A since these two mutants had lost most of their phosphorylase a phosphatase activity.

Cellular Localization of Certain Variant Proteins Was Affected. Glc7 is targeted to the nucleus, bud neck, spindle pole bodies, and the actomyosin ring during vegetative

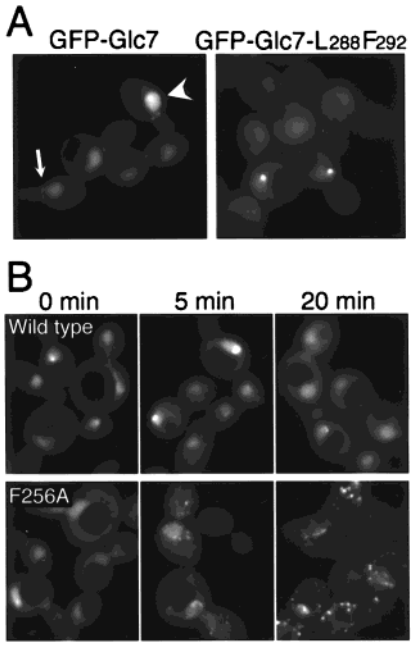

FIGURE 8: Subcellular localization of GFP-Glc7-E288 F292 and GFP-Glc7-F256A. Visualization of GFP fluorescence in yeast strains bearing low copy plasmids containing *GFP-GLC7, GFP-glc7-E288 F292* and *GFP-glc7-F256A*. (A) Fluorescence from GFP-Glc7 and GFP-Glc7-E288 F292. The higher levels of GFP-Glc7 seen at the bud neck (arrow) and in the nucleolus (arrowhead) are not observed for GFP-Glc7-E288 F292. (B) Fluorescence from strains containing GFP-Glc7 and GFP-Glc7-F256A before and after a temperature shift from 24 to 37 °C. GFP-Glc7F256A rapidly becomes punctate in appearance after a shift to 37 °C.

growth (*15*) and to pachytene chromosomes during meiosis (*12*). Some of these associations are dynamic. For example, Glc7 associates with spindle pole bodies, the yeast microtubule organizing centers, only from the start of anaphase of mitosis to cytokinesis, and a portion of the enzyme co-localizes with the actomyosin ring for 10−15 min during cytokinesis (*15*). Although the meiosis-specific Glc7p binding protein Red1p is necessary for the association of Glc7 with pachytene chromosomes (*12*), the binding proteins responsible for the localization of Glc7 during vegetative growth are unknown. To investigate the role of the V/IXF-binding hydrophobic groove in the localization of Glc7, we fused the gene encoding a bright variant of the green fluorescent protein (*65*) (GFP) to *glc7-F256A*, a viable but pleiotropic allele, and to *glc7-E288 F292*, a lethal allele. The gene fusions were expressed in a wild-type yeast strain from the pRS316 vector using the *GLC7* promoter. The wild-type GFP-Glc7 accumulates at the same locations as Glc7 and fully complements a *glc7* null mutation (*15*); therefore, the patterns of GFP-fluorescence should reflect the sites of the Glc7 accumulation. As shown in Figure 8A, wild-type GFP-Glc7 accumulates at the bud neck (arrows, left panel) and the nucleolus (arrowhead, left panel). In contrast, GFP-Glc7-E288 F292p is excluded from the vacuole but is otherwise distributed uniformly throughout the cell with a slight increase observed in the nucleus (Figure 8A, right panel). GFP alone has a similar distribution (data not shown). We did observe a bright spot in ~15% cells. From this result, we conclude that the Glc7 targeting subunits responsible for the subcellular localization of Glc7 contain functional V/IXF-motifs.

In contrast to the uniform distribution of GFP-Glc7-E288 F292, GFP-Glc7-F256A was retained at the bud neck and the nucleolus at 24 °C. Since cells containing Glc7-F256A as their sole source of Glc7 are temperature-sensitive for growth, we assayed the location of GFP-Glc7-F256A after a shift to the restrictive temperature, 37 °C. As shown in Figure 8B, after a 5 min shift to 37 °C the fluorescence pattern became punctate. Small spots were observed in both the cytoplasm and the nucleus. The intensity of the spots and their numbers increased with time at the restrictive temperature. This rapid change of localization may account for the temperature sensitivity of the allele.

DISCUSSION

In this paper, we report the results of systematically mutating residues in yeast PP1 that are proposed to associate with the V/IXF motif of many targeting subunits. The biological function of each variant protein was studied, and the interaction of each with known regulatory subunits was assayed. Overall, our results support the hypothesis that a single hydrophobic channel of PP1 interacts with V/IXF-containing subunits. Many of our variants have defects in binding to multiple targeting subunits and most variants with two or more amino acid substitutions fail to associate with the two V/IXF proteins that we tested. However, these variants retain the ability to bind Sds22, a known Glc7 binding protein that lacks a V/IXF motif. Phenotypic characteristics of these mutants corroborate the biochemical results. The products of those mutants that fail to complement the lethality of a *glc7::LEU2* null mutation fail to associate with either V/IXF-motif protein. In addition, a fusion of one lethal allele to the GFP gene results in a protein that fails to localize to the nucleolus, bud neck, actomyosin ring, and spindle pole bodies, sites normally associated with increased concentrations of Glc7 (*15*). Glc7 products of viable alleles that failed to associate with multiple targeting subunits conferred pleiotropic phenotypes. A good example is *glc7-F256A*, whose product fails to associate with Gac1 and Reg1 and which confers defects in glycogen accumulation and glucose repression. Although *GLC7* mutants have been previously identified with defects in either glucose repression (*38*, *66*) or glycogen synthesis (*11*, *38*, *67*, *68*), *glc7-F256A* is the first with defects in both processes and corresponding defects in binding Reg1 and Gac1.

The cocrystal structure of PP1c and $G_{M[63-57]}$ revealed that the major interactions between PP1c and the G_M peptide involve hydrophobic interactions betweenV66′ and F68′ of the canonical V/IXF motif in G_M and amino acid residues within the hydrophobic channel. V66′ interacts with I169, L243, L268, and C291 while F68′ interacts with F257, F293, and C291 (Figure 1). Among our mutant collection, those with altered residues predicted to interact with the phenylalanine of the V/IXF (F256 and F292), exhibited more deleterious phenotypes than those mutants with altered residues predicted to interact with the valine of the V/IXF (I168 L288). Glc7-F256A and Glc7-F292A interacted poorly with both Gac1 and Reg1 and the corresponding mutants had the most pleiotropic phenotypes. In contrast, Glc7-I168A and Glc7-L288A interacted normally with Reg1 and the phenotypes of the corresponding mutants were much less severe. This variation is consistent with results from studies on mutants in V/IXF-containing proteins in which the V/I

and F residues were mutated separately. In the case of $G_{M[63-75]}$ (*30*), DARPP-32 (*33*), and Gac1 (*69*), substitution of the F residue with A reduces binding to PP1c to a greater extent than substituting the V/I residue with A. Together, these results are consistent with the hypothesis that the predominant hydrophobic interactions between PP1c and the V/IXF motif are between the phenylalanine residue in the V/IXF motif and the two phenylalanine residues in the hydrophobic channel of PP1c. Egloff et al. (*30*) observed that C291 in PP1 made hydrophobic contacts with both V66′ and F68′ of $G_{M[63-57]}$. However, glc7-C290A has a wild-type phenotype and its product interacts productively with both Gac1 and Reg1. On the basis of these results, we propose that C290 may play only a minor role in binding to V/IXF-containing proteins.

It is likely that many if not all PP1 targeting subunits will have more than one site of interaction with PP1c. Biochemical studies reveal that the PP1 subunits DARPP-32 (*33*), I-1 (*32*), I-2 (*33*, *70*), NIPP1 (*35*, *71*), and MYPT1/M110 (*72*, *73*) have points of contact with PP1c in addition to the V/IXF binding site. Mutants in Glc7 that contain amino acid substitutions outside the hydrophobic channel have specific defects in Gac1 or Reg1 binding (*11*, *46*, *55*). Therefore, it is not surprising that some amino acid substitutions within the hydrophobic channel differentially influence subunit binding. For example, Glc7-L288A and I168A are defective in their interaction with Gac1 but interact normally with Reg1. These differences suggest that although all V/IXF-motif containing proteins may bind to the single hydrophobic channel, the precise molecular contacts for each targeting subunit may be unique.

If some of the *GLC7* mutants identified in this study reduce binding by all VXF-containing targeting subunits then the phenotype of these mutants may indicate additional physiological pathways that are regulated by VXF-containing targeting subunits. Defects in glycogen accumulation and glucose repression can be accounted for by the failure of the mutants to bind Gac1 and Reg1, respectively. It is also likely that a failure to bind Gip1, a VXF-containing targeting subunit with roles in sporulation (*60*), could account for some of the sporulation defects. However, no VXF-containing subunits have been identified whose binding defect could account for the temperature or salt sensitivity of *glc7-F256A* and *glc7-F292A*. The fact that high level, enforced expression of Gac1 induces a similar CsCl sensitive phenotype (*69*) provides further evidence for an as yet uncharcterized targeting subunit that directs Glc7 to a role in ion homeostasis.

PP1 is highly resistant to trypsin, a property used to unmask PP1c from inhibitory binding proteins (*64*). We used trypsin treatment to determine if the low level of phosphorylase phosphatase activity observed in some of our mutants was due to binding of inhibitory subunits. Unexpectedly, we found that the two mutants were sensitive to trypsin. The resistant core segments of Glc7-F256A and Glc7-F292A are less stable than that of the wild-type protein, an indication that the mutant proteins have an altered conformation. It is therefore possible that the failure of Gac1 and Reg1 to bind to these Glc7 variants could be due to nonspecific changes in Glc7 structure rather than to the loss of specific contacts in the hydrophobic channel. At this time, however, we do not know if the conformational change is the cause or the effect of the binding defect. The conformation change may prevent targeting subunit binding but alternatively, the loss of subunit binding may induce the change in structure. Analysis of purified proteins will be necessary to distinguish between these alternatives. In any case, it is important to note that the conformational changes noted for Glc7-F256A and Glc7-292A have subtle effects on Glc7 function. The steady state levels of these proteins are similar to that of the wild type Glc7. Both mutants retain normal activity against MBP, indicating that substrate specificity rather than total activity is altered, and only the ability to bind V/IXF-containing proteins is reduced in the mutants. The ability to associate with Sds22, a non-V/IXF containing protein, is not altered.

Targeting subunits have been shown to alter PP1c activity by influencing both the K_m and K_{cat}. The reduced K_m can be explained by a scaffolding role for the targeting subunits whereby substrate and catalytic subunit are tethered together. However, the effect on K_{cat} is less well understood. In the case of myosin phosphatase targeting subunit 1 (MYPT1/M110), sequences adjacent to the V/IXF domain are required to activate PP1c toward myosin (*74*). Evidence for an effect on PP1 activity independent of a scaffolding role also comes from a functional analysis of NH_2-terminal fragments of Gac1 that contain only the Glc7 binding domain. Peptides as small as 93-amino acid residues partially complement the glycogen defect of a *gac1* null mutant (*69*). This unexpected phenomenon could be explained if the targeting subunit has an important role in regulating the activity of Glc7 independent of any targeting role. Also, Ramaswamy et al. have identified *GLC7* mutants that fail to accumulate glycogen but retain the ability to bind Gac1 (*11*). These mutants could be unable to adopt a conformation necessary for acting on glycogen synthase even in the presence of the targeting subunit Gac1. An effect of targeting subunit binding on K_{cat} toward specific substrates could explain the apparent change in substrate specificity observed in many of our Glc7 mutants. Seven of 11 mutants displayed a dramatic drop in phosphorylase phosphatase activity yet retained activity against MBP. Immunoprecipitated Glc7 was used for our phosphatase assays so we do not know if the activity differences we observed are due to associated proteins or are intrinsic differences in the Glc7 mutants. However, if the loss in phosphorylase phosphatase activity is an intrinsic property of some Glc7 mutants then it suggests that perturbations of the V/IXF-binding domain can influence substrate specificity. Perhaps the hydrophobic channel is part of an intramolecular switch that regulates phosphatase activity. Specific targeting subunits could help configure activity toward specific substrates. A more detailed biochemical analysis of purified Glc7 mutants and their interaction with targeting subunits will be necessary to distinguish between these alternative models of regulation.

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