The budding yeast Glc7 serine/threonine protein phosphatase-1 is regulated by Glc8, the yeast ortholog of mammalian protein phosphatase inhibitor-2. In this work, we demonstrated that similarly to inhibitor-2, Glc8 function is regulated by phosphorylation. The cyclin-dependent protein kinase, Pho85, in conjunction with the related cyclins Pcl6 and Pcl7 comprise the major Glc8 kinase in vivo and in vitro. Several glc7 mutations are dependent on the presence of Glc8 for viability. For example, glc7 alleles R121K, R142H, and R198D are lethal in combination with a glc8 deletion. We found that glc7-R121K is lethal in combination with a pho85 deletion. This finding indicates that Pho85 is the sole Glc8 kinase in vivo. Furthermore, glc7-R121K is also lethal when combined with deletions of pcl6, pcl7, pcl8, and pcl10, indicating that these related cyclins redundantly activate Pho85 for Glc8 phosphorylation in vivo. In vitro kinase assays and genetic results indicate that Pho85, Pcl6 and Pcl7 comprise the predominant Glc8 kinase.

A major fraction of mammalian protein phosphatase-1 exists in a complex with inhibitor-2 (I-2)\(^1\) (1, 2). I-2 appears to change the conformation of the protein phosphatase-1 catalytic subunit (PP1c). When I-2 is phosphorylated in an inactive PP1c-I-2 complex on Thr-72, the PP1c becomes activated. This activation is thought to occur by the transference of a phosphorylation-dependent I-2 conformational change to PP1c. This idea is supported by data showing that phosphorylated I-2 mixed with inactive PP1c cannot activate it (3, 4). Autodephosphorylation of phosho-I-2 within the active PP1c-I-2 complex deactivates PP1c, even though PP1c can be removed from the complex in an active state. Therefore, I-2 can both inactivate and activate PP1c by changing its conformation in a phosphorylation-dependent fashion. I-2 interaction with denatured recombinant PP1c, and subsequent phosphorylation yields an active enzyme, which has inspired the notion that I-2 is a PP1c molecular chaperone (5).

Several identified protein kinases phosphorylate I-2. Glycogen synthase kinase-3 (GSK3), extracellular signal-regulated kinase-2, and cyclin-dependent kinase-5 (CDK5) phosphorylate I-2 Thr-72 in vitro (6–9). Casein kinase-2 (CK2) phosphorylates I-2 on three residues, Ser-86, Ser-120, and Ser-120, but does not change its activity. CK2 phosphorylation at these sites cooperatively enhances GSK3 phosphorylation at Thr-72 (10). Whether any of these kinases actually phosphorylate I-2 in vivo is unknown.

Glc8 is the budding yeast ortholog of I-2. Mutations in glc8 were discovered because they reduce yeast glycogen accumulation (11). Glc8 inhibits mammalian PP1c, and yeast PP1\(_c\), Glc7, in vitro (12). I-2 has several PP1\(_c\)-binding sites in addition to the inhibitory region (13). Alignments of I-2 and Glc8 sequences indicate that Glc8 may lack some PP1\(_c\)-binding sites found in I-2. In contrast to PP1, in mammalian cells, the majority of Glc7 in yeast is bound to Sds22 (14, 15). In comparison with other yeast Glc7-binding proteins, the affinity for Glc8 is weak (16).

Glc7, like mammalian PP1\(_c\), regulates many physiological processes: glycogen metabolism, transcription, translation initiation, membrane fusion, sporulation, and mitosis (17). In regard to its mitotic function, evidence indicates that Glc7 dephosphorylates proteins phosphorylated by the Ipl1 protein kinase, which regulates chromosome bi-orientation on the spindle (18, 19). Indeed, overexpression or deletion of GLC8 can suppress ipl1 mutations (12). These data suggest that Glc8 can activate or inhibit Glc7 activity in vivo.

The regulatory Thr-72 in I-2 corresponds to Glc8 Thr-118. Previous work indicated that this site must be a phosphorylatable residue for in vivo Glc8 function (12). In this study, we identify the Glc8 kinase as the cyclin-dependent kinase, Pho85, which is orthologous with mammalian CDK5. We also demonstrate that Pho85 phosphorylates Glc8 in vivo and that is the sole Glc8 kinase in budding yeast. Moreover, four redundant Pho85 cyclins are required for in vivo Glc8 phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Strains and General Methods**—Rich (YEPL) and oxiom (SC) media used for growth of *Saccharomyces cerevisiae* and LB broth have been previously described (24, 25). Low adenine medium contained 0.6% adenine (w/v). Glycogen accumulation was assayed qualitatively by exposure of yeast colonies grown on YEPD plates to iodine crystals for 3 min at room temperature (11). Wild-type strains stain brown, and glycogen-deficient strains stain yellow.

Most strains of *S. cerevisiae* and *Escherichia coli* used in this study are listed in Table I. Additional strains included BY4743 diploids with *kanMX4*-marked deletions (20). Strains in Table I with these deletions were made congenic to JC746–9D by six or more backcrosses. The *grb11ΔHis3* mutation was made by transformation with a PCR fragment containing HIS3 flanked by 40 bases from the 5′ and 3′ ends of PCL8. This fragment was made by amplification of HIS3 from pRS316 (21) with primers 466 (5′-AAGATTTTGT TTGAGGTTTT GCAGATAAAC) and 317 (5′-AAGATTTTGT TTGAGGTTTT GCAGATAAAC).

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§ The abbreviations used are: I-2, inhibitor-2; PP1c, protein phosphatase-1 catalytic subunit; GSK3, glycogen synthase kinase-3; CDK5, cyclin-dependent kinase-5; CK2, casein kinase-2; GST, glutathione S-transferase.
AAGAACGAGT CTCGTCGTTTACACGGC-3′) and 467 (′-TATTAGCGAAA CTCATGTTT TCTTAAAGCC TGTGTTGAT AGATTG TAATGAGTGCAC-3′). The p1841 plasmid from peace was transferred from Drosophila melanogaster V82B4 (22). The glc-7′-R121K mutation was scored by a DraI restriction site polymorphism. The DraI site present in the mutant allele was test in a 1476-bp GLC7 fragment amplified by colony PCR (23) using primers 462 (′-ATGACGAGTG ATGATTGCATC-3′) and (23) using primers 462 (′-ATGACGAGTG ATGATTGCATC-3′) and 378 (′-AATTCTCATGTTTGA-3′). The p1841 fragment from p1841, containing the initiating methionine codon is underlined) (27). The TACTGAGAGTGCAC-3′ mutation was scored by a DraI site in p1841, coming from a yeast DNA (33), mutations were found in many of the fusion genes (Table III). We assume that these mutations do not adversely affect the biochemistry of the fusion proteins. For the Pcl1 and Pho81 pools, an authentic GST fusion plasmid could not be found.

**Glc8 Preparation—** E. coli strain BL21(DE3) (34) transformed with either pPM1539, pYT114, or pYT116 was grown to early exponential phase in LB broth containing 100 μg/ml ampicillin at 37 °C. Isopropyl-β-D-thiogalactopyranoside was added to a 6 mM final concentration, and the culture was grown an additional 5 h. The harvested cell pellets were resuspended in 1/20th culture volume of sonication buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 1.0 mM phenylmethylsulfonyl fluoride) and sonicated three times for 1-min intervals on ice with a 250 μl sonicating tip. The final supernatant was heated at 95 °C for 15 min. The denatured proteins were removed by centrifugation at 15,000 × g for 15 min, and the supernatant proteins were heated at 95 °C for 15 min. The denatured proteins were removed by centrifugation. Contaminating high molecular weight substances were removed by passing supernatant through a 300,000 nominal molecular weight limit cellulose membrane (Millipore). The final supernatant containing Glc8 was stored at −20 °C.

**In Vitro Glc8 Kinase Assays—** Yeast crude extracts were prepared by vortexing exponential phase cells 4 × 30 s at 4 °C in the presence of glass beads and an equal volume of homogenization buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 10 mM NaF, 1× complete protease inhibitor mixture (Roche Molecular Biochemicals) followed by a 10-min centrifugation at 4 °C. To remove contaminating endogenous ATP, the

| Strain | Genotype | Source |
|--------|-----------|--------|
| BL21(DE3) | E. coli F- ompT hsdS (lambda with lacOP-T7 polymerase) | Ref. 34 |
| BY4743 | MATa his3D1 leu2D0 lys2D0 ura3D0 | Ref. 20 |
| CJC46-9D | MATa can1 his3 leu2 trp1 ura3 | Ref. 11 |
| CJC860B | MATa ura3 leu2 can1 trp1 his3 met8 cgl-8 HIS3 | Ref. 11 |
| CJC945-5C | MATa glc8- HIS3 ade2 ade3 leu2 trp1 ura3 | This study |
| CJC950 | MATa glc7-RI121K glc8- HIS3 ade2 ade3 can1 his3 leu2 trp1 ura3 | This study |
| CJC1327-11A | MATa leu2 ura3 his3 glc7-RI121K URA3 | This study |
| CJC1328-5A | MATa pho85- hisMX4 sup85 ura3 his3 leu2 | This study |
| CJC1338-20A | MATa prc17- : : canMX4 ura3 his3 leu2 | This study |
| CJC1338-20C | MATa prc17- : : canMX4 ura3 his3 leu2 | This study |
| CJC1344-12A | MATa prc17- : : canMX4 prc17- : : canMX4 pc18- : : HIS3 ura3 his3 leu2 | This study |
| CJC1344-12B | MATa prc17- : : canMX4 prc17- : : canMX4 pc18- : : HIS3 glc7-RI121K URA3 ura3 his3 leu2 | This study |
| CJC1347-12D | MATa prc17- : : HIS3 p1841 | This study |
| CJC1347-20B | MATa prc17- : : canMX4 pc18- : : HIS3 p1841 | This study |
| CJC1349-17C | MATa prc17- : : canMX4 pc18- : : HIS3 p1841 | This study |
| CJC1350-5A | MATa prc17- : : canMX4 pc18- : : canMX4 p1841 | This study |

**Table I**

| Name | Vector | Description | Source |
|------|--------|-------------|--------|
| p1814 | YEp50 | CEN URA3 GLC8 | Ref. 11 |
| p1841 | YEp352 | 2μ URA3 GLC8 | This study |
| p1855 | YEp352 | 2μ URA3 GLC7 | This study |
| p215 | pRS316 | CEN URA3 GLC8 | Ref. 11 |
| p2087 | pTVS30A | 2μ ADE3 LEU2 GLC8 | This study |
| p2462 | pYEX4T-1 | 2μ URA3 LEU2d GST-PCL6 | Ref. 32 |
| p2465 | pYEX4T-1 | 2μ URA3 LEU2d GST-PCL2 | Ref. 32 |
| p2466 | pYEX4T-1 | 2μ URA3 LEU2d GST-PH080 | Ref. 32 |
| p2467 | pYEX4T-1 | 2μ URA3 LEU2d GST-PCL7 | Ref. 32 |
| p2468 | pYEX4T-1 | 2μ URA3 LEU2d GST-PCL9 | Ref. 32 |
| p2470 | pYEX4T-1 | 2μ URA3 LEU2d GST-CG1 | Ref. 32 |
| p2475 | pYEX4T-1 | 2μ URA3 LEU2d GST-GC8 | Ref. 32 |
| p2476 | pYEX4T-1 | 2μ URA3 LEU2d GST-PCL10 | Ref. 32 |
| p2085 | pYEX4T-1 | 2μ URA3 LEU2d GST-PH085 | Ref. 32 |
| pPM1539 | pT7-7 | T7p-GLC8 | This study |
| pYT114 | pT7-7 | T7p-glc8-T118A | This study |
| pYT115 | pRS316 | CEN URA3 glc8-T118A | This study |

**Table II**

**Strain list**

**Plasmid list**

| Name | Vector | Description | Source |
|------|--------|-------------|--------|
| p1814 | YEp50 | CEN URA3 GLC8 | Ref. 11 |
| p1841 | YEp352 | 2μ URA3 GLC8 | This study |
| p1855 | YEp352 | 2μ URA3 GLC7 | This study |
| p215 | pRS316 | CEN URA3 GLC8 | Ref. 11 |
| p2087 | pTVS30A | 2μ ADE3 LEU2 GLC8 | This study |
| p2462 | pYEX4T-1 | 2μ URA3 LEU2d GST-PCL6 | Ref. 32 |
| p2465 | pYEX4T-1 | 2μ URA3 LEU2d GST-PCL2 | Ref. 32 |
| p2466 | pYEX4T-1 | 2μ URA3 LEU2d GST-PH080 | Ref. 32 |
| p2467 | pYEX4T-1 | 2μ URA3 LEU2d GST-PCL7 | Ref. 32 |
| p2468 | pYEX4T-1 | 2μ URA3 LEU2d GST-PCL9 | Ref. 32 |
| p2470 | pYEX4T-1 | 2μ URA3 LEU2d GST-CG1 | Ref. 32 |
| p2475 | pYEX4T-1 | 2μ URA3 LEU2d GST-GC8 | Ref. 32 |
| p2476 | pYEX4T-1 | 2μ URA3 LEU2d GST-PCL10 | Ref. 32 |
| p2085 | pYEX4T-1 | 2μ URA3 LEU2d GST-PH085 | Ref. 32 |
| pPM1539 | pT7-7 | T7p-GLC8 | This study |
| pYT114 | pT7-7 | T7p-glc8-T118A | This study |
| pYT115 | pRS316 | CEN URA3 glc8-T118A | This study |
extracts were further purified through P25 spin columns (Bio-Rad). The eluants were collected, and the protein concentrations were quantitated using bovine serum albumin as a standard (35). Glc8 kinase assays were performed by mixing 10 μg of crude yeast extract or the indicated amounts of purified GST fusion protein, 2.5 μg of Glc8, 5 μl of ATP mixture (1 mM ATP, 25 mM MgCl₂, 1200 cpm/pmol [γ-³²P]ATP), and homogenization buffer without protease inhibitors, in a final volume of 25 μl. The reactions were incubated for 30 min at 30 °C. Adding equal volumes of 2× SDS loading buffer (36) stopped the reactions. The samples were then heated for 3 min at 95 °C and resolved with SDS-PAGE (36). The gels were dried and exposed to Kodak XAR5 film or to a Kodak phosphorimaging screen. The data on phosphorimaging screens were read on Molecular Imager FX and analyzed with Quantity One software (Bio-Rad).

**Isolation of Glc8-dependent Mutants**—The strain JC945-8Cp2087 was mutagenized with ethyl methanesulfonate to 10% survival and plated on YEPD (37). A colony sectorsing assay was used to recognize and isolate GLC8-dependent mutants (38–40). Putative GLC8-dependent mutants were initially selected by their inability to sector. True GLC8-dependent mutants regained their ability to sector when transformed with p1855. By this method we isolated 11 independent GLC8-dependent mutants after screening 2 × 10⁶ colonies. All of the mutations were complemented by CEN GLC7 plasmids and were genetically linked to the GLC7 locus.

We retrieved the GLC7 locus from one of the mutant strains, JC968-1, by plasmid gap repair (11, 41). Transformation of JC968-1 with p1855 deleted for a 1.1-kb fragment yielded sectoring and nonsectoring transformants. The sectoring transformants had re-paired the p1855 gap to wild-type GLC7, whereas the nonsectoring ones did not contain wild-type GLC7. Sequencing of DNA of the retrieved GLC7 locus from two independent nonsectoring transformants revealed a G to A transition in codon 121, which converted the wild-type Glc7 Arg-121 to Lys (R121K). Amino acid Arg-121 of Glc7 corresponds to Thr-118 in Glc8 was previously shown to be important for Glc8 function in vivo (12). Consequently, we examined whether Glc8 Thr-118 was actually phosphorylated. Indeed, yeast crude extracts efficiently phosphorylated Glc8 in vitro (Fig. 1A). In contrast, a mutant Glc8 in which alanine is substituted for Thr-118 (Glc8-T118A) was not phosphorylated (Fig. 1A, lane 4). I-2 is phosphorylated at additional sites by CK2 (7, 44). Although yeast contains CK2 and our reaction conditions were favorable for its activity, Glc8-T118A was not phosphorylated in vitro. This is consistent with the fact that sites that correspond to I-2 CK2 phosphorylation are not found in Glc8. Therefore, Thr-118 is the major site of Glc8 phosphorylation under the conditions of our in vitro reactions.

**RESULTS**

In Vivo Phosphorylation of Glc8 on Thr-118—The homology between Glc8 and mammalian I-2 is greatest surrounding I-2 Thr-72, the residue that is phosphorylated by GSK3, extracellular signal-regulated kinase-2, and CDK5 (6–9, 11). The corresponding Thr-118 in Glc8 was previously shown to be important for Glc8 function in vivo (12). Consequently, we examined whether Glc8 Thr-118 was actually phosphorylated. Indeed, the reactions were incubated for 30 min at 30 °C. Adding equal volumes of 2× SDS loading buffer (36) stopped the reactions. The samples were then heated for 3 min at 95 °C and resolved with SDS-PAGE (36). The gels were dried and exposed to Kodak XAR5 film or to a Kodak phosphorimaging screen. The data on phosphorimaging screens were read on Molecular Imager FX and analyzed with Quantity One software (Bio-Rad).
were purified from 12 pools of 10 strains each and used for which yeast protein kinase phosphorylates Glc8, we used a Glc8 kinase activity (Fig. 1B). Next, GST protein kinases were in vitro Glc8 kinase assays. Protein from pool 6 had the greatest phosphorylation.

Because Glc8-T118A protein is not phosphorylated glc8-T118A two traits: the glc7-R121K and that some other alleles Pcl6 and Pcl7 are closely related and belong to a family that includes Pcl8 and Pcl9, and the Glc8 kinase activity was assayed. The Glc8 kinase activity of GST-Pho85 was uniquely diminished when it was purified from the pcl6 deletion strain (Fig. 1D).

In the second experiment, GST fusions to each of the Pho85 cyclins were purified from EJ758 yeast. Pho85 binds to each of these cyclins and hence should co-purify with them (45). Affinity purification of GST-Pcl7 from yeast consistently (n = 4) yielded the greatest Glc8 kinase activity (Fig. 3). Although equivalent masses of GST-cyclin/Pho85 were used in these assays, the relative affinity of Pho85 to these GST-cyclin fusions will temper the activity we measured. Therefore, we only used the above data to indicate that the Pho85 cyclins Pcl6 and Pcl7 contribute to the Glc8 kinase in vivo. Genetic analyses (see below) were employed to confirm whether this contention was true in vivo. Based on amino acid sequence comparison, Pcl6 and Pcl7 are closely related and belong to a family that includes Pcl8 and Pcl10 (45, 46).

Pho85 affinity with its substrates Pho4 and Gsy2 is sufficient to detect the interaction by co-precipitation or two-hybrid methods (46, 47). In our analysis, we found that GST-Glc8 purified from yeast co-purified Glc8 kinase activity (Fig. 3). Glc8 affinity for Pho85 was independently reported while this work was in progress (48). Our results show that the Pho85 associated with Glc8 is catalytically active. GST-Glc8 was also phosphorylated somewhat in the reaction, which indicates that GST-Glc8 isolated from yeast was partially dephosphorylated.

Pho85/Pcl7 Is the Major Glc8 Kinase in Vitro—To determine which yeast protein kinase phosphorylates Glc8, we used a collection of yeast strains that each express a GST fusion to a yeast protein kinase (32). Initially, GST fusion proteins were purified from pools of cells. The GST protein kinase fusions were purified from 12 pools of 10 strains each and used for in vitro Glc8 kinase assays. Protein from pool 6 had the greatest Glc8 kinase activity (Fig. 1B). Next, GST protein kinases were purified and assayed from the ten component strains in pool 6. GST-Pho85 had the greatest Glc8 kinase activity (Fig. 1C). Many protein kinases could phosphorylate Glc8 in these in vitro reactions. However, we assumed that the in vivo Glc8 kinase would demonstrate the best phosphorylation kinetics and yield the greatest Glc8 phosphorylation in these assays. Therefore, we disregarded protein kinases such as Hall5, which phosphorylate Glc8 greater than the average protein kinase.

The strain containing GST-Pho85 may contain a mixture of plasmids because of the way the GST fusions were constructed (32). Therefore, yeast DNA was purified from the GST-Pho85 containing yeast strain and retrieved by E. coli transformation, and individually isolated DNAs were used to transform EJ758. Of the eight DNAs analyzed, seven had a restriction map expected for a GST-Pho85 plasmid. The DNA sequence from one of these isolates was identical to PHO85 with the exception of an A to T transition in codon 61, which converts a lysine to an isoleucine (Table III). This GST-Pho85 plasmid expressed high Glc8 kinase activity when the GST-Pho85 was purified from EJ758 transformants. Together, these data identify Pho85 as an in vitro Glc8 kinase. Although there were other protein kinases that phosphorylate Glc8 in vitro, Pho85 was most active, and as our genetic analyses demonstrate below, it is the only in vivo Glc8 kinase.

Pho85 is a cyclin-dependent kinase that is regulated by 10 alternative cyclins (45). The purified GST-Pho85 fusion presumably had one or more of these cyclins associated with it. Two biochemical experiments delineated which Pho85 cyclins comprise the active Glc8 kinase. In the first experiment, GST-Pho85 was purified from BY4743 diploids with homoygous deletions of Pho85 cyclins (pho80, clg1, pcl1, pcl2, pcl6, pcl7, pcl8, and pcl9), and the Glc8 kinase activity was assayed. The Glc8 kinase activity of GST-Pho85 was uniquely diminished when it was purified from the pcl6 deletion strain (Fig. 1D).

In the second experiment, GST fusions to each of the Pho85 cyclins were purified from EJ758 yeast. Pho85 binds to each of these cyclins and hence should co-purify with them (45). Affinity purification of GST-Pcl7 from yeast consistently (n = 4) yielded the greatest Glc8 kinase activity (Fig. 3). Although equivalent masses of GST-cyclin/Pho85 were used in these assays, the relative affinity of Pho85 to these GST-cyclin fusions will temper the activity we measured. Therefore, we only used the above data to indicate that the Pho85 cyclins Pcl6 and Pcl7 contribute to the Glc8 kinase in vivo. Genetic analyses (see below) were employed to confirm whether this contention was true in vivo. Based on amino acid sequence comparison, Pcl6 and Pcl7 are closely related and belong to a family that includes Pcl8 and Pcl10 (45, 46).

Pho85 affinity with its substrates Pho4 and Gsy2 is sufficient to detect the interaction by co-precipitation or two-hybrid methods (46, 47). In our analysis, we found that GST-Glc8 purified from yeast co-purified Glc8 kinase activity (Fig. 3). Glc8 affinity for Pho85 was independently reported while this work was in progress (48). Our results show that the Pho85 associated with Glc8 is catalytically active. GST-Glc8 was also phosphorylated somewhat in the reaction, which indicates that GST-Glc8 isolated from yeast was partially dephosphorylated.

Pho85/Pcl6 Is the Major in Vivo Glc8 Kinase—The above experiments show that Pho85, in association with certain cyclins, can phosphorylate Glc8 in vitro. We wished to test whether Pho85 is the sole Glc8 kinase in vivo. The glc7-R121K mutation is lethal in combination with glc8 null mutations. Because Glc8 function requires Thr-118 phosphorylation, any mutation that compromises in vivo Glc8 phosphorylation should be lethal in combination with glc7-R121K. Therefore, we generated strains with a pho85 glc7-R121K genotype to test their viability. Congenic strains were used for these analyses to eliminate the influence of cryptic background mutations. Control crosses with either pho85 or glc7-R121K strains showed good spore viability. However, pho85 by glc7-R121K crosses yielded inviable double mutant spores. Typical tetratype tetrads from these crosses are shown in Fig. 4A, in which the pho85 slow growth and missing pho85 glc7-R121K spores were apparent. The pho85 glc7-R121K spores germinated but
stopped dividing at the −16 cell stage. Therefore, this result demonstrates that Pho85 is the sole Glc8 kinase in vivo.

To determine which Pho85 cyclin promotes Glc8 in vivo phosphorylation, additional crosses were performed. We focused on the role of the related Pcl6, Pcl7, Pcl8, and Pcl10 cyclins because they were implicated by the in vitro analyses above. No individual pcl mutation was lethal in combination with glc7-R121K. However, strains in which pcl6 pcl7 double mutations were combined with glc7-R121K showed slow spore germination (data not shown) and temperature sensitivity (Fig. 5). The pcl6 pcl7 glc7-R121K cells arrested as large budded cells with replicated DNA at the nonpermissive temperature. When PCL6 was the sole wild-type gene in this set, glc7-R121K did not inhibit growth at 37 °C (JC1349–17C; Fig. 5). In contrast, when PCL7 was the sole wild-type gene in this set, glc7-R121K caused a detectable temperature sensitivity (JC1347–20B; Fig. 5). Strains with pcl6 and pcl7 combined with glc7-R121K showed great temperature sensitivity, which was further compromised by elimination of pcl8. These results are consistent with redundant functions of Pcl6, Pcl7, and Pcl8 for Glc8 kinase activity with the strength of function increasing in the order: Pcl8, Pcl7, and Pcl6. Although the above results did not address the contribution of Pcl10, spores with a pcl6 pcl7 pcl8 pcl10 glc7-R121K genotype were uniquely inviable (Fig. 4B). Therefore, temperature sensitivity results from the significant reductions of Glc8 phosphorylation in a pcl6 pcl7 pcl8 glc7-R121K strain, but elimination of the four related Pcl6, Pcl7, Pcl8, and Pcl10 prevented growth of glc7-R121K strains at any temperature. In conclusion, Pcl6, Pcl7, Pcl8, and Pcl10 function redundantly as Pho85 cyclins for Glc8 phosphorylation in vivo with Pho85/Pcl6 providing the majority of the Glc8 kinase activity.

DISCUSSION

From the primary sequence alignment of Glc8 with its homologs, Thr-118 was anticipated to be phosphorylated (11, 12). Indeed, Tung et al. (12) showed that a high copy Glc8-T118A mutant protein was defective in ipl1 suppression (12). Mammalian protein kinases GSK3, extracellular signal-regulated kinase-2, or CDK5 phosphorylate the homologous I-2 residue in vitro (6–9). Budding yeast has at least one homolog to each of these protein kinases (49). Using a collection of GST fusions to yeast protein kinases, we discovered that Pho85 was the prevailing Glc8 kinase in vitro (Fig. 1C). However, this collection of gene fusions is known to be incomplete (50), so there was the possibility that other protein kinases could also phosphorylate Glc8. Independently, we know that none of the four yeast GSK3 homologs contribute Glc8 kinase activity because extracts from the quadruple mds1 mck1 mkk1 yol128c mutant strain have the same Glc8 kinase specific activity as wild-type strains (data not shown). We used a glc7 mutant that requires Glc8 to grow to test whether Pho85 phosphorylates Glc8 in vivo. The invia-
bility of glc7-R121K pho85 double mutants not only demonstrated that Pho85 phosphorylates Glc8 in vivo, but it also illustrates that Pho85 must be the only Glc8 kinase. This result is consistent with a single Glc8 kinase resolved by sedimentation velocity centrifugation and by several chromatographic steps (data not shown). Note that the in vivo phosphorylation of I-2 by the mammalian kinases has not always been demonstrated.

While this work was in progress, mammalian CDK5, the ortholog of Pho85, was reported to phosphorylate I-2 in vitro (9). Furthermore, CDK5 binds directly to PP1, (9). This binding suggests that I-2 in the PP1-I-2 complex would be predominately active because of its phosphorylation by CDK5 and hence exhibit PP1 activity. The CDK5 region that binds to PP1, RVRLLDDDD, weakly corresponds to the Pho85 sequence EKKLDSEE, matching only two of eight residues. In particular the Pho85 sequence does not have the canonical (R/K)(V/I) X(F/Y) sequence that many PP1-binding proteins possess (51). Therefore, Pho85 probably does not bind directly to Glc8. However, others and we found that Pho85 binds to Glc8 (Fig. 3A and Ref. 49). Our data additionally show that the Pho85 when bound to Glc8 is active for transphosphorylation. In Fig. 3 the added Glc8 was predominantly phosphorylated; however, there was a small amount of phospho-GST-Glc8 detectable. Therefore, Glc8 would be highly phosphorylated in vivo as long as the redundant Pcl6, Pcl7, Pcl8, and Pcl10 levels were high.

Pho85 cyclins are functionally partitioned; subsets of them promote Pho85 phosphorylation of substrates involved in specific processes. For example, the related Pcl1 and Pcl2 cyclins direct Pho85 to phosphorylate the Cdc-Cdc28 kinase inhibitor, Sic1, and thus provide additional Cdc28 in regulation of the G1-specific processes. For example, the related Pcl1 and Pcl2 cyclins promote Pho85 phosphorylation of substrates involved in spore tetrads from JC1327.

Glc8 would be highly phosphorylated in vivo as long as the redundant Pcl6, Pcl7, Pcl8, and Pcl10 levels were high.

Pho85 cyclins are functionally partitioned; subsets of them promote Pho85 phosphorylation of substrates involved in specific processes. For example, the related Pcl1 and Pcl2 cyclins direct Pho85 to phosphorylate the Cdc-Cdc28 kinase inhibitor, Sic1, and thus provide additional Cdc28 in regulation of the G1 to S phase transition (52–54). In this work we found that in vivo Glc8 kinase activity was predominantly derived from Pcl6 and Pcl7-associated Pho85. These cyclins are members of the “metabolic regulation” group of Pho85 cyclins because they regulate glycogen metabolism and prevent growth on alternative carbon sources (47, 55). The redundant Pcl6 and Pcl7 influence both glycogen phosphorylase and glycogen synthase activity, whereas Pcl8 and Pcl10 only regulate glycogen synthase. Glc8, through its regulation of Glc7, was previously recognized as a regulator of glycogen metabolism. Therefore, the metabolic group of Pho85 cyclins influences glycogen metabolism on several different levels.

Although glycogen synthesis is not essential for yeast viability, glc8 glc7-R121K cells are inviable. This indicates that Glc8 may modulate the functions of Glc7 essential for viability. An essential function of Glc7 seems to be modulation of the kine-tocho during mitosis (56). Because activity of Pho85/Pcl7 is maximized during the S phase of the cell cycle, Pcl7 may also modulate cell cycle functions (46). Two-hybrid results connect Pcl6 to the kine-tocho-associated protein Spc24 via YLR190W (58–60). Therefore, Glc8 phosphorylation may change during the cell cycle, perhaps close to the kine-tocho, and thereby regulate Glc7 phosphatase activity at the kine-tocho.

The temporal expression of Pcl7 can explain the discrepancy in the determination of the Pho85 cyclin in the in vitro Glc8 kinase. The panel of assays in Fig. 1D clearly shows a reduction in the Glc8 kinase activity from the pcl6 mutant strain. We interpret this to show that Pcl6 is the major Pho85 cyclin for Glc8 kinase activity. Because we used asynchronous cells to prepare the extracts for these assays, few cells would be in the S phase, and thus little Pcl7 would be provided. In contrast, when GST-Pcl7/Pho85 was purified for the assays shown in Fig. 3, it was no longer subject to the normal PCL7 transcriptional control and perhaps could overcome post-translational influences on Pcl7 levels because of its great expression. The comparably low Glc8 kinase activity mustered by GST-Pcl6/Pho85 in this assay probably reflects poor affinity of Pho85 for Glc8 and some other glc7 alleles confer a dependence on Glc8 for viability is unknown. Reduction of Glc7 activity causes cell cycle arrest at the spindle assembly checkpoint (61, 62). Depletion of Glc8 in glc7-R121K cells appears to cause the same block in the cell cycle because they arrest as large budded cells with replicated chromosomes. The hyperactivity of Pho85/Pcl7 in S phase phosphorylating Glc8 would activate Glc7 for mitotic activity. If Glc8 mimics the I-2 phosphorylation-dependent chaperone function (5), then Glc8-deendent glc7 alleles may encode proteins that fold poorly or fail to adopt a conformation suitable to dephosphorylate critical substrates without Glc8. We know that the Glc8-dependent glc7 alleles neither encode enzymes with uniformly low protein phosphatase activity nor exhibit a trend in Glc8 affinity (16, 63). Furthermore, they affect residues unnecessary for catalytic activity.

Mammalian I-2 is found in two complexes that contain PP1
protein phosphatase and a protein kinase. The first is the CKD5 complex described above, which is involved in neuronal development (9). The second is a Nek2 protein kinase complex, which regulates the duplication and separation of centrosomes (57). I-2 promotes centrosome separation by its inhibition of PP1 in this complex with concomitant increase of Nek2 activity (57). Spindle pole body (equivalent to centrosome) duplication occurs in yeast in late G1, but separation continues through the S phase. The arrest of pcl6 pcl7 glc7-R121K cells in metaphase at the nonpermissive temperature that we find is inconsistent with phospho-Glc8 promoting spindle pole dynamics, but it is consistent with kinetochore activity required in the metaphase to anaphase transition (56). Therefore, I-2 orthologs are found in kinase-phosphatase complexes in these two eukaryotic species and play roles in cell cycle regulation, but the specific processes regulated are different.

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