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Biochemical and Biophysical Research Communications 329 (2005) 161–167
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Regulation of yeast glycogen phosphorylase by the cyclin-dependent protein kinase Pho85p

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Received 4 January 2005

Abstract

Yeast accumulate glycogen in response to nutrient limitation. The key enzymes of glycogen synthesis and degradation, glycogen synthase, and phosphorylase, are regulated by reversible phosphorylation. Phosphorylation inactivates glycogen synthase but activates phosphorylase. The kinases and phosphatases that control glycogen synthase are well characterized whilst the enzymes modifying phosphorylase are poorly defined. Here, we show that the cyclin-dependent protein kinase, Pho85p, which we have previously found to regulate glycogen synthase also controls the phosphorylation state of phosphorylase.

Keywords: Pho85p; Glycogen; Phosphorylase; Regulation; Yeast

Yeast synthesize glycogen, a branched polymer of glucose, in response to nutrient limitation, such as upon the approach to stationary phase. This compound then serves as a reserve of carbon and energy during diauxic shift or after prolonged starvation [1,2]. The onset of glycogen synthesis is accompanied by the induction of the required biosynthetic enzymes, including glycogen synthase (encoded by *GSY1* and *GSY2*) [3]. Paradoxically, glycogen phosphorylase (encoded by *GPH1*), which mediates glycogen degradation, is also induced [4,5] but futile cycling between synthesis and degradation is suppressed by other layers of control. For example, glycogen synthase can be inactivated by phosphorylation at three COOH-terminal Ser and Thr residues [6]. The phosphorylation of glycogen synthase is catalyzed by at least two distinct protein kinase activities [7], one of which is a complex of the cyclin-dependent protein kinase (Cdk) Pho85p and the cyclin Pcl10p [8]. Dephosphorylation and activation of glycogen synthase is catalyzed by a complex of the yeast type 1 protein phosphatase, Glc7p, and the targeting subunit, Gac1p [5,9], although there also appears to be some role for type 2A phosphatases [10–13]. Glycogen phosphorylase, Gph1p, is also controlled by phosphorylation, modification of Thr31 causing activation of the enzyme [14,15]. Although cyclic AMP-dependent protein kinase can phosphorylate Gph1p in vitro, the nature of the physiological phosphorylase kinase in yeast has never been established [15,16]. Similarly, it is unclear what phosphatase(s) dephosphorylate and inactivate glycogen phosphorylase, although there is evidence that Glc7p plays a role here too [17].

The starting point for our current work was the observation that cells in which the *PHO85* gene was deleted had increased glycogen phosphorylase activity [18]. We wanted to determine the mechanism by which Pho85p regulates phosphorylase. Pho85p interacts with some 10 different Pho85p cyclins or Pcls namely Pcl1p, Pcl2p, Pho80p, Clg1p, and Pcl5p–Pcl10p (reviewed in [19]). Different Pho85p/Pcl complexes have been shown

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doi:10.1016/j.bbrc.2005.01.106

to have distinct cellular functions and Measday et al. [20] originally placed the Pcls into two groups, one (Pcl1p, Pcl2p, Pcl5p, Pcl9p, and Clg1p) postulated to function in cell cycle controls and the other (Pho80p, Pcl6p, Pcl7p, Pcl8p, and Pcl10p) in metabolic regulation. Pcl8p and Pcl10p have been implicated in the regulation of glycogen accumulation [21] and Pcl10p, as mentioned above, specifically targets Pho85p to phosphorylate Gsy2p, the major yeast glycogen synthase isoform [8].

Relatively little is known of the functions of Pcl6p and Pcl7p which, based upon sequence alignments among the Pcls, are most similar to each other. Lee et al. [22] have shown that a Pcl7p/Pho85p complex has protein kinase activity and also that *PCL7* expression fluctuates during the cell cycle, with a maximum in mid- to late S phase. The same study reported that *pcl6* and *pcl7* mutants had some defects in carbon source utilization and glycogen storage. Furthermore, we have previously demonstrated that deletion of *pcl6* and *pcl7* led to increased phosphorylase activity, at least under certain circumstances [23].

Here, we show that the increase in phosphorylase activity that occurs upon deletion of *PHO85* or *PCL6* and *PCL7* is due to a reduction in phosphorylase phosphatase activity. The control of phosphorylase phosphatase by Pho85p requires the cyclin Pcl6p, perhaps with a contribution from Pcl7p. The phosphorylase phosphatase is a type 1 phosphatase and the Glc7p regulatory proteins Glc8p and Shp1p are required for maximum activity.

Materials and methods

Yeast strains and media. The yeast strains used are listed in Table 1. Standard methods for yeast culture and genetic manipulation were used [24]. Gene deletions were carried out using a polymerase chain reaction strategy [25] and the pRS series of vectors as templates for marker cassette amplification [26]. Yeast cells were grown in rich medium (YPD, containing 2% bacto peptone, 1% yeast extract, and 2% glucose).

Phosphorylase assay. Glycogen phosphorylase activity was measured in the direction of glycogen synthesis by monitoring the incorporation of [^{14}C]glucose from [^{14}C]glucose-1-phosphate into glycogen using a slight modification of published procedures [4,27]. Briefly, cells were resuspended in buffer comprising 50 mM Tris–HCl, pH 7.0, 100 mM NaF, and 1 mM EDTA. Protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine HCl, 0.1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and dithiothreitol (1 mM) were added immediately before use. Cells were homogenized by vortex mixing with glass beads and cell debris was removed by low-speed centrifugation (1500*g* for 1 min). The supernatant was removed and desalted by passage over a 1 ml spin column of Sephadex G25. Aliquots were incubated with 100 mM [^{14}C]glucose-1-phosphate (~20–30 dpm/nmol), 100 mM KF, and 1 mM EDTA for 20 min at 30 °C. Samples were withdrawn, spotted to 31ET chromatography paper (Whatman), and washed with 66% ethanol to remove unincorporated glucose-1-phosphate. The papers were rinsed briefly with acetone, dried, and the radioactivity incorporated into glycogen was determined by liquid scintillation counting.

Phosphorylase phosphatase assay. Cell extracts were prepared and desalted as described above, except that the lysis buffer comprised 50 mM Tris–HCl, pH 7.0, 100 mM NaCl, 1 mM dithiothreitol, and the protease inhibitors listed above. The extracts were incubated for 15 min at 30 °C either with or without the addition of a mixture of $MgCl_2$ and ATP (12.5 mM $MgCl_2$ and 0.5 mM ATP). Aliquots were then removed and assayed for glycogen phosphorylase activity as described above. The amount of glycogen phosphorylase phosphatase activity is expressed as the ratio of the phosphorylase activity measured without magnesium and ATP present in the preincubation reaction to the activity measured with magnesium and ATP present.

Protein determination. Protein concentrations were determined by the method of Bradford, with bovine serum albumin as a standard [28].

Results

Loss of Pho85p increases phosphorylation of glycogen phosphorylase

We sought to understand why *pho85* mutants had elevated phosphorylase activity (Fig. 1). Since Gph1p is active only in the phosphorylated state [15], one potential explanation for the increased phosphorylase activity in the *pho85* strain was increased phosphoryla-

Table 1
Yeast strains used

Strain	Genotype	Source or reference
EG328-1A	*MATα trp1 leu2 ura3-52*	K. Tatchell
DH4-101[a]	*MAT**a** trp1 thr4 ura3-52*	[42]
WW10	*MATα trp1 leu2 ura3-52 pcl8::TRP1 pcl10::LEU2*	[8]
WW11	*MATα trp1 leu2 ura3-52 pho85::TRP1*	[8]
DH105-1523	*MATα trp1 leu2 ura3-52 pho80::URA3*	This laboratory
DH106-91	*MAT**a** trp1 thr4 ura3-52 pcl6::URA3 pcl7::TRP1*	[23]
DH109-41	*MAT**a** trp1 thr4 ura3-52 pcl6::URA3*	This laboratory
DH120-14	*MAT**a** trp1 thr4 ura3-52 pcl7::TRP1*	This laboratory
BY4743[b]	*MAT**a**/MATα his3/his3 leu2/leu2 lys2/LYS2 MET15/met15 ura3/ura3*	Research genetics

[a] DH4-101 and EG328-1A are congenic. All DH and WW strains were derived from EG32 8-1A and DH4-101 by PCR-mediated gene disruption and mating.

[b] BY4743 is the wild type background in which the *Saccharomyces* Genome Deletion Project consortium generated the diploid deletion series.

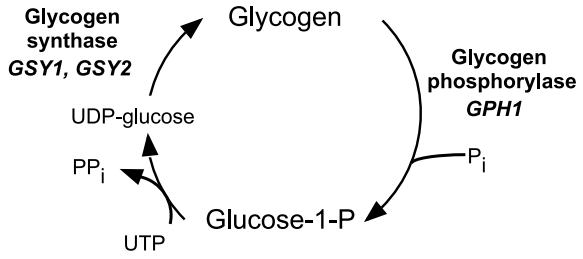

Fig. 1. Simplified scheme of glycogen synthesis and degradation. Glycogen is synthesized from UDP-glucose. Glycogen synthase (encoded by the *GSY1* and *GSY2* genes), in conjunction with a branching enzyme, catalyzes bulk glycogen synthesis. Glycogen degradation is catalyzed by glycogen phosphorylase, encoded by the *GPH1* gene, acting together with a debranching enzyme. In the standard assay, phosphorylase is driven backwards and activity is assessed by monitoring the incorporation of radioactive glucose from glucose-1-phosphate into glycogen.

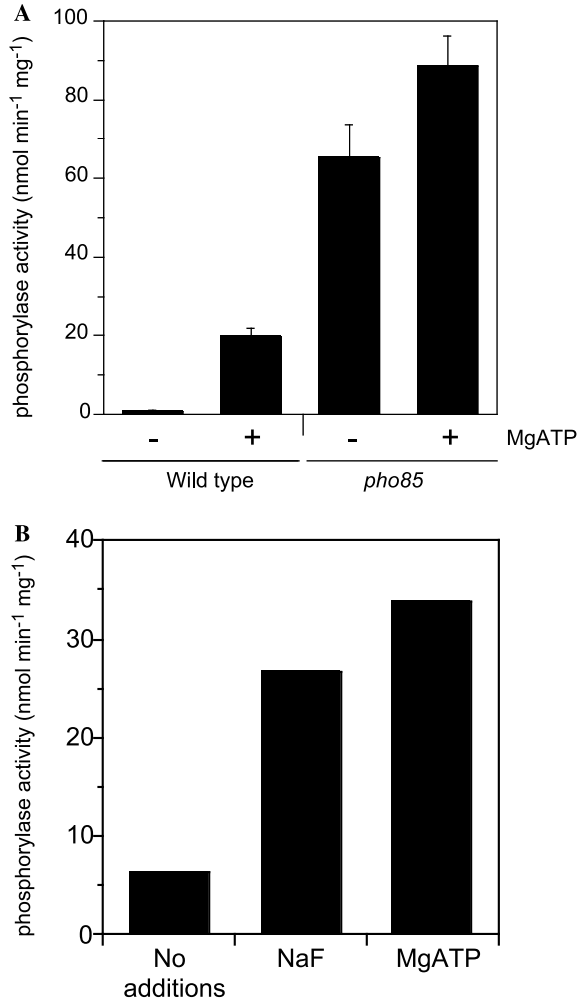

Fig. 2. Phosphorylase activity in wild type and *pho85* mutant yeast after 15 min pre-incubation. Cell lysates were prepared from wild type (EG328-1A) and *pho85* mutants (WW11) in the absence of phosphatase inhibitors and desalted by gel filtration. Aliquots were incubated for 15 min either with or without the addition of MgATP prior to the measurement of phosphorylase activity. The results are shown as means ± SEM for three independent determinations.

tion of phosphorylase. Yeast extracts were prepared in a buffer lacking both EDTA and NaF such that endogenous kinases and phosphatases were active. The extracts were then incubated prior to analysis in the standard phosphorylase assay (Fig. 2A). With wild type cell extracts, very little phosphorylase activity was detected (Fig. 2A). This inactivation was blocked by either 50 mM sodium fluoride (Fig. 2B) or 1 μM okadaic acid (not shown), indicating that it represented dephosphorylation of phosphorylase. Pre-incubation with MgATP, which would promote phosphorylase kinase activity or might inhibit the phosphatase, led to much higher activity (Fig. 2A). In extracts from *pho85* cells, there was high basal phosphorylase activity and inclusion of MgATP led to only a small further increase (Fig. 2A). The high activity in the absence of MgATP is indicative of low phosphorylase phosphatase activity, suggesting that Pho85p positively regulates a phosphorylase phosphatase.

The action of Pho85p on glycogen phosphorylase is mediated principally by Pcl6p

Since Pho85p control of phosphorylase is presumably mediated by specific cyclins, we questioned whether Pcls from the "metabolic" group were responsible. Of particular interest were the cyclins Pcl8p and Pcl10p, due to their known role in the control of glycogen synthase, and Pcl6p and Pcl7p since we previously established that deletion of *pcl6* and *pcl7* led to increased phosphorylase activity in a *snf1* mutant background [23].

As noted above, incubation of yeast extracts in the absence of phosphatase inhibitors leads to a loss of glycogen phosphorylase activity that can be prevented by the inclusion of MgATP. We used this effect as the basis for a phosphorylase phosphatase assay. Cell extracts were incubated without phosphatase inhibitors and in the presence or absence of MgATP. The ratio of the phosphorylase activity measured without and with MgATP (±MgATP ratio) in the pre-incubation forms an index of phosphorylase phosphatase activity, with higher values indicating reduced phosphorylase phosphatase activity.

Analysis of the ±MgATP activity ratio showed that deletion of *PCL6* alone led to a high value, close to that seen in *pho85* mutants (Fig. 3). Although loss of *PCL7* alone had little effect, the *pcl6 pcl7* double mutant had a significantly higher activity ratio than *pcl6* mutants ($p < 0.05$). In contrast, in *pho80* or *pcl8 pcl10* mutants, the activity ratio was close to wild type. Therefore, we conclude that Pho85p controls phosphorylase activity through negative regulation of a phosphorylase phosphatase. The predominant protein kinase is Pcl6p/Pho85p with a lesser possible role for Pcl7p/Pho85p.

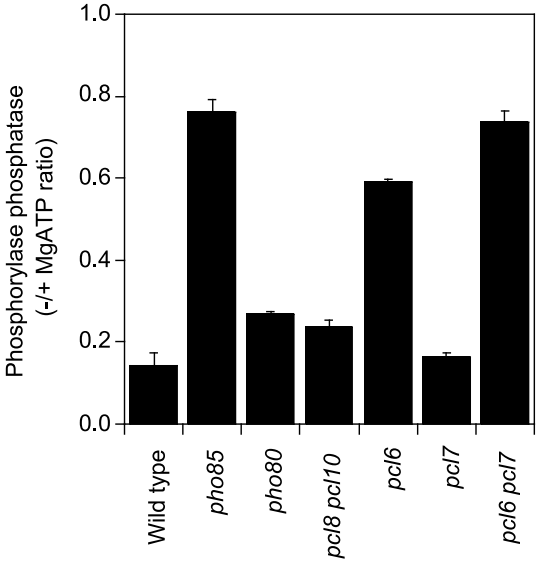

Fig. 3. Phosphorylase phosphatase activity in mutants lacking particular Pho85p cyclins. Cell lysates were prepared, desalted, and incubated for 15 min either with or without addition of MgATP. Phosphorylase activity was then measured. The graph shows the ratio of phosphorylase activity obtained without MgATP in the preincubation to that obtained when MgATP was present. *Low* values indicate a higher requirement for MgATP to maintain phosphorylase activity and, therefore, the presence of active phosphorylase phosphatase. Conversely, *high* values indicate little effect of added MgATP, implying low levels of phosphorylase phosphatase activity. The results shown are means ± SEM ($n = 3$). Wild type (EG328-1A), *pho85* (WW11), *pho80* (DH105-1523), *pcl8 pcl10* (WW10), *pcl6* (DH109-41), *pcl7* (DH120-14), and *pcl6 pcl7* (DH106-91).

Glc8p and Shp1 are involved in the control of phosphorylase dephosphorylation

In an effort to define the phosphatases that dephosphorylate and inactivate glycogen phosphorylase, we used the yeast strains constructed by the *Saccharomyces* Genome Deletion Project [29,30]. Type 1 protein phosphatases have long been implicated in the control of glycogen accumulation, in both yeast and mammals, and so we elected to begin by analyzing this group of phosphatases. In yeast, there is but one type 1 protein phosphatase catalytic subunit, encoded by the *GLC7* gene [31,32]. Type 1 phosphatases are believed to carry out their many functions by forming complexes with distinct targeting subunits [33]. There are 11 known or potential Glc7p regulatory subunits listed by the MIPS Comprehensive Yeast Genome Database: Gac1p, Gip1p, Gip2p, Glc8p, Pig1p, Pig2p, Red1p, Reg1p, Reg2p, Sds22p, and Shp1p. Of these 11, we examined 9 (*SDS22* is an essential gene and *gip2* was not present in the strains from the *Saccharomyces* Genome Deletion Project). We measured phosphorylase phosphatase activity (as the phosphorylase ±MgATP activity ratio) in the nine deletion mutants (Fig. 4). Of the strains examined, the largest reduction in phosphorylase phosphatase activity was

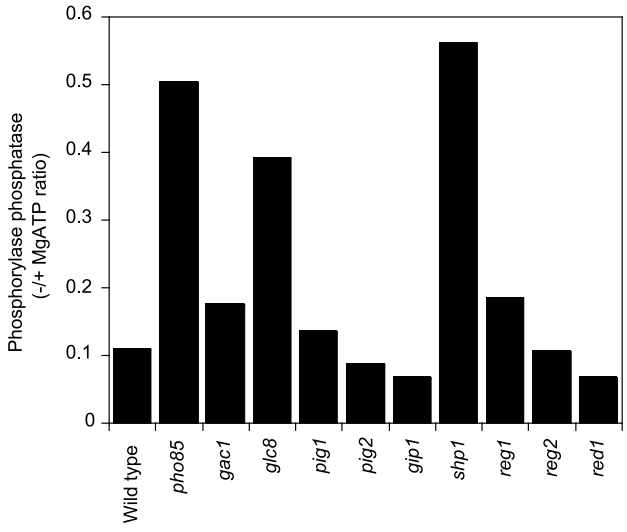

Fig. 4. Phosphorylase phosphatase activity in mutants lacking particular type 1 phosphatase targeting subunits. Phosphorylase phosphatase activity was assessed in a selection of mutants from the *Saccharomyces* Genome Deletion Project that lacked particular Glc7p associated or targeting proteins. Only the *glc8* and *shp1* mutants had significantly reduced phosphorylase phosphatase activity, confirmed by subsequent experiments. The mean values ± SEM were: wild type (BY4743) 0.127 ± 0.009, *glc8* 0.390 ± 0.041, and *shp1* 0.622 ± 0.059 ($n = 3$).

seen in the *glc8* and *shp1* mutants. The *GLC8* gene encodes a small, heat stable protein that is related to mammalian inhibitor-2, originally isolated as an inhibitor of type 1 protein phosphatase [34,35]. Little is known regarding the function of Shp1p, although deletion of *SHP1* has been reported to reduce Glc7p activity in vitro [36].

We also measured phosphorylase phosphatase activity in 28 different protein phosphatase catalytic subunit mutants available in the deletion series. None of these mutants were found to have reduced glycogen phosphorylase phosphatase activity (not shown).

Discussion

The starting point for this study was the observation that deletion of *PHO85* led to increased glycogen phosphorylase activity. We wanted to determine both the reason for the increase in activity and the particular cyclin partners for Pho85p that were involved in this process. Gph1p is a phosphoprotein and is active only in the phosphorylated form. Extracts from *pho85* mutant yeast showed reduced capacity to dephosphorylate and inactivate phosphorylase than did extracts from wild type cells, indicating that Pho85p was a positive regulator of a phosphorylase phosphatase activity.

In order to identify the individual cyclins involved in the regulation of phosphorylase by Pho85p, we measured phosphorylase phosphatase activity in extracts

from strains in which genes encoding different Pcls had been deleted. We concentrated on the 'metabolic' family of Pcls, Pcl6p, Pcl7p, Pcl8p, Pcl10p, and Pho80p [20]. We were particularly interested in Pcl6p and Pcl7p because we had noted earlier that *pcl6 pcl7* mutant strains exhibited elevated glycogen phosphorylase activity, at least in certain mutant backgrounds [23]. The *pcl6 pcl7* mutant behaved very similarly to the *pho85* mutant and both strains were impaired in the ability to dephosphorylate and inactivate phosphorylase in cell extracts. Therefore, Pho85p, in conjunction with the cyclins Pcl6p and Pcl7p, is a positive regulator of phosphorylase dephosphorylation and inactivation.

We took a systematic genomics approach to identify the phosphatase(s) responsible for dephosphorylation of glycogen phosphorylase. We assayed all 28 viable phosphatase catalytic subunit deletion mutants and 9 of the 10 viable type 1 protein phosphatase targeting/interacting subunit deletion mutants for reduced ability to inactivate phosphorylase. Of these 37 strains, only the *glc8* and *shp1* mutants were defective in phosphorylase phosphatase activity.

Loss of function mutants of *GLC8* were recovered by Cannon et al. [34] in a screen for cells with altered glycogen levels and *glc8* strains show reduced glycogen compared to wild type. Glc8p is the yeast ortholog of mammalian inhibitor-2. The inactive complex of the type 1 protein phosphatase catalytic subunit and inhibitor-2 can be converted into an active phosphatase by phosphorylation of Thr72 in inhibitor-2 [37], which is catalyzed by glycogen synthase kinase 3 [38]. Glc8p functions as an in vitro inhibitor of Glc7p but whether this is the role in vivo is unclear since both deletion and overexpression of *GLC8* appear to lead to inhibition of Glc7p [39]. This observation led to the hypothesis that Glc8p might actually be an activator of Glc7p and that inhibition occurred as an artifact of the high levels of overexpression [39]. Like inhibitor-2, Glc8p is a phosphoprotein, and phosphorylation is required for the in vivo function of Glc8p [39].

Our data indicate that Glc8p acts to promote dephosphorylation and inactivation of Gph1p. The Pho85p/Pcl6p (or Pcl7p) kinase complex is also required to stimulate dephosphorylation of Gph1p and could conceivably achieve this by direct phosphorylation of Glc8p. The phosphorylation site in Glc8p is Thr118, corresponding to Thr72 in inhibitor-2 [39], which lies in the sequence KTPY and Pho85p is a proline-directed protein kinase. During the course of our study, two relevant pieces of work were published by Cannon's group. First, deletion of *GLC8* was shown to reduce Glc7p activity, measured towards the non-physiological substrate, mammalian phosphorylase *a* [40]. Second, Pho85p, in complex with either Pcl6p or Pcl7p (and, perhaps to a lesser degree, Pcl8p or Pcl10p), was shown to phosphorylate Glc8p on Thr118, placing Pho85p upstream of Glc8p, acting positively to increase Glc7p/Glc8p activity [41]. These results are consistent with the work presented here and indicate that the effect we observe on phosphorylase phosphatase activity by deletion of *PHO85* or *PCL6* and *PCL7* likely occurs due to a failure to activate the Glc7p/Glc8p phosphatase in these mutants.

In contrast to Glc8p, very little is known about Shp1p. Loss of function mutations in *SHP1* were obtained in screens for suppressors of the lethality which is brought about by the overexpression of *GLC7* [36]. Furthermore, deletion of *SHP1* was found to decrease the activity of Glc7p (assayed using mammalian phosphorylase *a* as a substrate) without having any effect upon the level of Glc7p [36]. Therefore, Shp1p is either a direct or indirect positive regulator of Glc7p activity. Unlike Glc8p and the other known Glc7p regulators such as Gac1p, Shp1p has not been shown to bind to Glc7p and its mode of action remains undefined. We reasoned that, like Glc8p, Shp1p might be phosphorylated by Pho85p. Indeed, Shp1p is somewhat proline-rich, containing three SP motifs (Ser108, 315, and 322) and one TP motif (Thr331). We amplified the *SHP1* gene by PCR and expressed it in *E. coli* from the pET-28a vector. The recombinant Shp1p was soluble and we could obtain 5–10 mg of pure Shp1p per liter of culture (not shown). Incubation of purified, recombinant Shp1p with yeast lysates in the presence of [γ-^{32}P]ATP resulted in only weak phosphorylation of Shp1p that was unaffected by deletion of the *PHO85* gene (not shown).

In summary, consideration of the available data gives us the model shown (Fig. 5). The complex of Pho85p

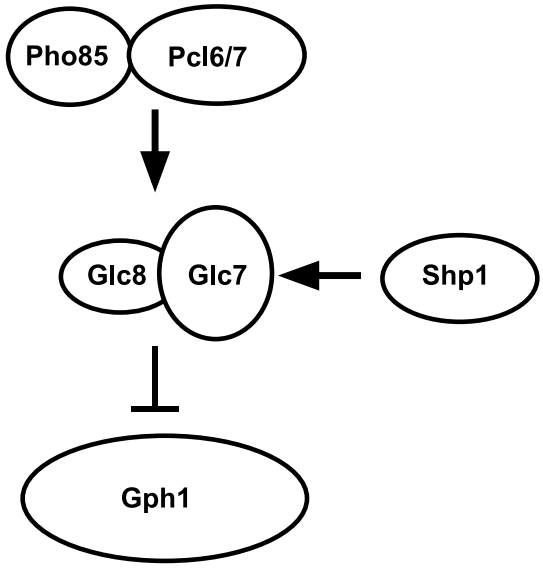

Fig. 5. Model for control of glycogen phosphorylase by Pho85p and Glc7p. Pho85p and the cyclin Pcl6p (and perhaps Pcl7p) phosphorylates Glc8p, activating the Glc7p/Glc8p phosphatase which dephosphorylates and inactivates glycogen phosphorylase. Shp1p is a positive regulator of bulk Glc7p phosphatase activity, operating independently of Pho85p.

and the cyclin Pcl6p (and perhaps Pcl7p) phosphorylates Glc8p and this leads to activation of the Glc7p/Glc8p phosphatase that dephosphorylates and inactivates glycogen phosphorylase. Shp1p may well be a regulator of bulk Glc7p phosphatase activity and operates independently of Pho85p. By using the *Saccharomyces* deletion series, we were able to take a systematic approach to identify phosphorylase phosphatase activities, analyzing all viable phosphatase mutants. Such a targeted use of the deletion series is a powerful analytical tool, likely to be of widespread use.

Acknowledgments

This work was supported by the National Institutes of Health Grant DK20542 (to P.J.R.) and by a grant from the Indiana University School of Medicine Biomedical Research Committee (to W.A.W.). Thanks to Drs. Anna DePaoli-Roach and Bart Pederson for many helpful discussions.

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