The yeast cyclins Pc16p and Pc17p are involved in the control of glycogen storage by the cyclin-dependent protein kinase Pho85p

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Abstract Pho85p is a yeast cyclin-dependent protein kinase (Cdk) that can interact with 10 cyclins (Pcls) to form multiple protein kinases. The functions of most of the Pcls, including Pc16p and Pc17p, are poorly defined. We report here that Pc16p and Pc17p are involved in the metabolism of the branched storage polysaccharide glycogen under certain conditions and deletion of *PCL6* and *PCL7* restores glycogen accumulation to a *snf1 pcl8 pcl10* triple mutant, paradoxically activating both glycogen synthase and phosphorylase. Pho85p thus affects glycogen accumulation through multiple Cdks composed of different cyclin partners. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Glycogen; Cyclin-dependent protein kinase; *PHO85*; *SNF1*; *PCL6*; *PCL7*

1. Introduction

Cyclin-dependent protein kinases (Cdks) are hetero-dimeric enzymes that contain a kinase catalytic subunit associated with a regulatory cyclin partner (reviewed in [1]). These enzymes were first identified because of their roles in cell cycle progression and the cyclins were named because their levels fluctuated during the course of cell division [1–3]. Pho85p, one of five Cdks in the budding yeast *Saccharomyces cerevisiae*, appears to have pleiotropic functions [4] presumably due to its ability to interact with 10 different Pho85 cyclins or Pcls [5–7]. The Pcls have been placed into two groups, one (composed of 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 [8]. Pho80p is implicated in the control of gene expression in response to limitation of inorganic phosphate [9–11]. Pcl1p, Pcl2p and Pcl9p are thought to have roles in controlling cell cycle progression [6,12,13] and Pcl1p/Pho85p phosphorylates the Cdk inhibitor Sic1p in vitro [14]. Pcl9p and Pcl2p interact with Rvs167p providing a possible link between cell cycle controls and the actin cytoskeleton [15]. Pcl8p and Pcl10p have been implicated in the regulation of glycogen accumulation, and Pcl10p has been shown to be a targeting subunit that directs Pho85p to phosphorylate Gsy2p, the major glycogen synthase isoform in yeast [16,17]. Glycogen is a major polysaccharide storage form of glucose whose synthesis and utilization are intricately controlled by nutritional status [18,19]. Very little is known of the functions of Pcl6p and Pcl7p. Lee et al. [20] 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. Some defects in carbon source utilization were also reported.

SNFl encodes the yeast homologue of the mammalian AMP-activated protein kinase and has been studied extensively for its regulation of the expression of glucose-repressed genes (reviewed in [21–23]). In addition, *snfl* mutants do not accumulate glycogen, but this defect appeared to be linked to hyperphosphorylation, and inactivation, of glycogen synthase rather than the modest, two-fold reduction in enzyme level that was observed [24]. Thus, we used a *snfl* mutant to screen for second site suppressors of the glycogen defect with the goal of identifying glycogen synthase kinases. This resulted in our demonstration that Pho85p was a component of a glycogen synthase kinase [25]. We went on to establish that Pcl8p and Pcl10p directed Pho85p to the control of glycogen synthase phosphorylation [16]. However, when we examined *snfl pcl8 pcl10* mutants, we unexpectedly found that this strain, unlike *snfl pho85* double mutants, failed to store glycogen.

2. Materials and methods

2.1. Strains, media and genetic methods

The *S. cerevisiae* strains used are listed in Table 1. Rich medium contains 1% (w/v) yeast extract, 2% (w/v) Bacto peptone, and 2% (w/v) of the indicated carbon source (glucose in YPD medium). Synthetic complete medium contains 0.67% (w/v) yeast nitrogen base, 2% (w/v) of the indicated carbon source (glucose in SD) and complete supplement mix (Bio 101 Inc.). Synthetic selective medium consists of 0.67% (w/v) yeast nitrogen base, 2% (w/v) of the indicated carbon source (glucose in SD) and the indicated complete supplement mix lacking appropriate amino acids. For analysis of glycogen accumulation on plates, aliquots (5–10 μl) were spotted onto plates and cells were grown for the indicated time before detection of glycogen by exposing plates to iodine vapor. Plasmids were maintained in *Escherichia coli* DH5α. Standard methods of yeast genetic analysis and transformation were used [26]. To generate *snfl pcl8 pcl10 pcl6 pcl7* mutant cells, strain ZWS2 was crossed with DH106-91. The diploid cells were then sporulated and tetrads were analyzed. Null mutants were scored according to the marker genes *TRPl* or *URA3*, and the null mutants were further conformed by PCR.

2.2. Plasmids

The *PCL6* and *PCL7* coding sequences were amplified by PCR from yeast genomic DNA and cloned into the TA vector (Invitrogen). To generate *PCL6* with an NH_2-terminal Flag tag, the following

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primers, 5′-TCCCGGGGGAATTCatgGACTACAAAGACGATGACGACAAGtctacaaaggtgattccccc and 3′-ACTCGAGctacatgcccgtaactag, were used to add an *Eco*RI site, the tag and a *Xho*I site (upper case). To generate *PCL7* with an NH_2-terminal Flag tag, the following primers, 5′-TGGATCCatgGACTACAAAGACGATGACGACAAGgagctaagttcaccatcaaaaaaaacc and 3′-TCTCGAGctactggtcgttccagaattt, were used to add a *Bam*HI site, the tag and a *Xho*I site (upper case). The PCR product was cloned into the pPCR2.1 vector and checked by sequencing. An *Eco*RI/*Xho*I fragment containing the *PCL6* open reading frame was cloned into the p425-TEF vector between the *Eco*RI and *Xho*I sites. A *Bam*HI/*Xho*I fragment containing *PCL7* was cloned into the p425-TEF vector between the *Bam*HI and *Xho*I sites.

2.3. Enzyme and other assays

For the assay of glycogen synthase and glycogen phosphorylase activity in yeast cell extracts, cultures were grown in 150 ml of the indicated medium in 500 ml flasks at 30°C. Aliquots of 7 ml were removed at the indicated time points, and cells were harvested by centrifugation (2 min, room temperature, 1500×*g*) in a clinical bench-top centrifuge. The cell pellet was frozen on dry ice and stored at −80°C prior to analysis. The frozen cells were thawed on ice and resuspended in 400 μl homogenization buffer (50 mM Tris–HCl, 1 mM EDTA, 5 mM dithiothreitol, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone, 5 mM benzamidine, 0.25 μg/ml leupeptin, and 0.5 μg/ml aprotinin, pH 7.4). The cells were broken with glass beads as described previously [24].

Glycogen synthase was assayed by the method of Thomas et al. [27], as described by Hardy et al. [24]. The total activity of glycogen synthase is measured in the presence of 7.2 mM glucose-6-P. The −/+ glucose-6-P activity ratio is defined as the activity measured in the absence of glucose-6-P divided by the activity measured in its presence. Each measurement was the average of duplicate assays.

Glycogen phosphorylase was assayed in the direction of glycogen synthesis by published methods with minor modification [28,29]. Each measurement was the average of duplicate assays.

2.4. Measurement of glycogen, ATP and glucose-6-P

Quantitative determination of glycogen content of yeast cells was performed as described previously [30]. Aliquots (∼1–2×10^7 cells) of cells grown in SD-Leu medium to stationary phase were used to inoculate fresh SD-Leu liquid medium. Aliquots of culture were collected at the indicated time points and glycogen content was determined.

For measurement of ATP and glucose-6-P, yeast cells were grown for the indicated times and harvested by rapid filtration. Cells were rapidly frozen in liquid nitrogen and stored at −80°C until measurement. The assay of glucose-6-P and ATP was carried out as described [31].

3. Results

3.1. Deletion of PCL6 and PCL7 restores glycogen accumulation to a snf1 pcl6 pcl8 mutant

Glycogen accumulation is conveniently assessed by staining colonies grown on plates with iodine vapor, darker staining correlating with increased glycogen levels (Fig. 1). The lack of glycogen storage in *snf1* mutants, noted in Section 1, and its restoration by the additional deletion of *PHO85* are also shown. However, *snf1 pcl8 pcl10* triple mutants did not accumulate glycogen under these conditions (Fig. 1). Therefore, *PHO85* exerts an influence over glycogen accumulation that is independent of *PCL8* and *PCL10* but presumably mediated by other Pho85p cyclins. Reasoning that the responsible Pcls would belong to the so-called metabolic group, we constructed the mutant *snf1 pcl6 pcl7 pcl8 pcl10* and analyzed glycogen accumulation. This quintuple mutant accumulated glycogen (Fig. 1). The rationale for mutating both *PCL6* and *PCL7* was based on the fact that these two Pcls are more closely related to each other than other Pcls and may constitute a gene pair with functional overlap. Indeed, the quadruple mutants, *snf1 pcl6 pcl8 pcl10* and *snf1 pcl7 pcl8 pcl10*, had somewhat lower glycogen accumulation and the increased accumulation in the quintuple mutant was difficult to assign to loss of either gene individually (data not shown). Mutation of *PCL6* and *PCL7*, in a wild-type (not shown) or a *snf1* (Fig. 1) background, also did not elevate glycogen levels. Therefore, Pho85p kinases containing both Pcl8/10p and Pcl6/7p are necessary to permit glycogen accumulation in the absence of Snf1p.

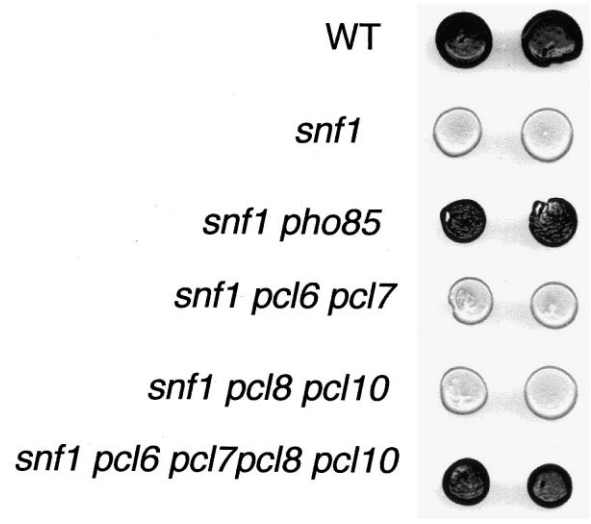

Fig. 1. Glycogen accumulation by various *snf1* and *pcl* mutants. Strains were grown for 48 h on SC plates and glycogen accumulation assessed by exposure to iodine vapor for 2 min. Strains used: wild-type (EG328-1A), *snf1* (EG353-1C), *snf1 pho85* (DH29), *snf1 pcl6 pcl7* (ZW32-1), *snf1 pcl8 pcl10* (ZWS2), *sfn1 pcl6 pcl7 pcl8 pcl10* (ZW33-54).

3.2. Deletion of PCL6 and PCL7 activates glycogen synthase and phosphorylase

In order to explore the mechanism by which Pcl6/7 affects glycogen accumulation, the activities of the synthetic and deg-

Table 1
Yeast trains used in this study

Strain	Genotype	Reference or source
DH106-91	*MAT a trp1 leu2 ura3-52 pcl6::URA3 pcl7::TRP1*	This laboratory
DH29	*MAT α trp1 leu2 ura3-52 snf1::LEUu2 pho85::URA3*	This laboratory
EG328-1A	*MAT α trp1 leu2 ura3-52*	K. Tatchell
EG353-1C	*MAT α trp1 leu2 ura3-52 snf1::URA3*	K. Tatchell
ZWS2	*MAT α trp1 leu2 ura3-52 snf1::TRP1 pcl8::URA3 pcl10::URA3*	This laboratory
ZW33-54	*MAT α trp1 leu2 ura3-52 snf1::TRP1 pcl8::URA3 pcl10::URA3 pcl6::URA3 pcl7::TRP1*	This laboratory
ZW32-1	*MAT α trp1 leu2 ura3-52 snf1::TRP1 pcl6::URA3 pcl7::TRP1*	This laboratory

All strains are isogenic to EG328-1A.

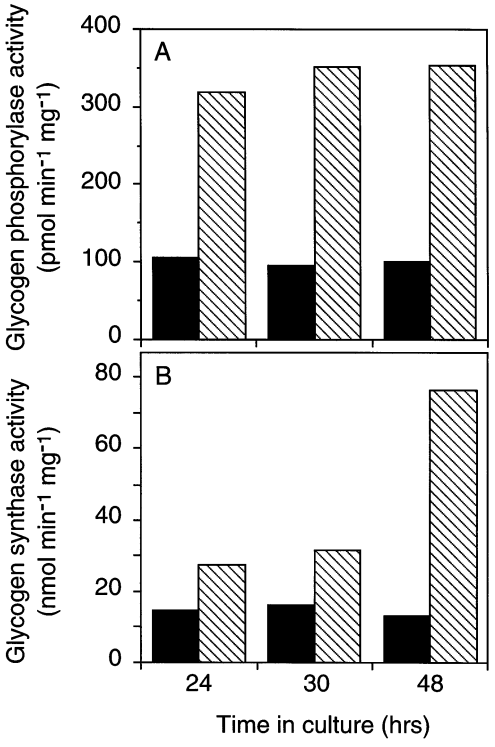

Fig. 2. Glycogen phosphorylase and glycogen synthase activity in *snf1 pcl6 pcl7* and *snf1 pcl6 pcl7 pcl8 pcl10*. Strains were grown in SC medium. At the indicated times, samples were withdrawn and glycogen phosphorylase (A) and glycogen synthase (B) activities determined. Strains used: *snf1 pcl8 pcl10* (ZWS2, solid bar), *snf1 pcl6 pcl7 pcl8 pcl10* (ZW33-54, hatched bar).

radative enzymes were analyzed in *snf1 pcl8 pcl10* and *snf1 pcl6 pcl7 pcl8 pcl10* strains. Glycogen phosphorylase, the enzyme primarily responsible for glycogen degradation, was actually increased (Fig. 2) in the strain lacking *PCL6* and *PCL7*, which would simplistically be expected to correlate with decreased glycogen accumulation. However, the two strains also differed in the level of the biosynthetic enzyme and total glycogen synthase activity was two-fold higher at 24–30 h and five-fold at 48 h. The activation state of the glycogen synthase, which is determined by its phosphorylation state, was relatively constant over this period. We conclude therefore that the balance of the effects on glycogen synthase and phosphorylase was in favor of synthesis, especially at the later times. Glucose-6-P concentration, which can affect both phosphorylase and glycogen synthase, was not different between the two strains (data not shown).

3.3. *PCL6 and PCL7 overexpression reduces glycogen accumulation*

Either *PCL6* or *PCL7* was overexpressed in a *snf1 pcl6 pcl7 pcl8 pcl10* mutant strain from a high copy plasmid p425-TEF [32] under control of the TEF promoter (Fig. 3). Compared to a control with empty vector, the expression of either *PCL6* or *PCL7* significantly diminished glycogen levels and this was sustained late into stationary phase. In addition, the result was similar whether *PCL6* or *PCL7* was expressed, suggesting that both have similar roles as relates to glycogen metabolism.

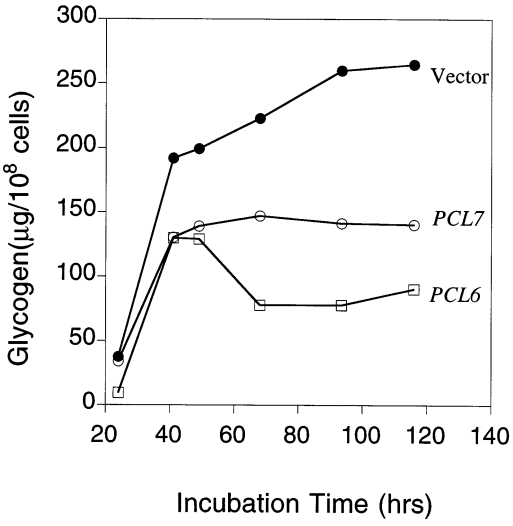

Fig. 3. Overexpression of Pcl6p or Pcl7p in *snf1 pcl8 pcl10 pcl6 pcl7* mutant cells decreases glycogen accumulation. *snf1 pcl8 pcl10 pcl6 pcl7* mutants (ZW33-54) harboring different plasmids (p425-TEF, ●; p425-TEF-PCL6, □; or p425-TEF-PCL7, ○) were grown in SD-Leu medium. At the indicated times, samples were withdrawn and glycogen content was measured. The data are the average from two independent transformants.

4. Discussion

The cellular functions of Pcl6/7p are poorly understood and an immediate outcome of this study is to establish a link between *PCL6* and *PCL7* and glycogen accumulation. Such a relationship had been suggested in the study of Lee et al. [20] who reported, however, that disruption of *PCL6* and *PCL7* in a wild-type background resulted in a reduced glycogen level. In our experiments, a *pcl6 pcl7* mutant had normal glycogen accumulation whereas deletion of *PCL6* and *PCL7* in a *snf1 pcl8 pcl10* background had the opposite effect, restoring glycogen storage. The present study also sheds light on this apparent discrepancy. Pho85p appears to be involved in a complex regulation of different steps of glycogen metabolism, not all of which are conceptually coherent (Fig. 4). For example, via control of the activities of glycogen synthase and phosphorylase, there are negative inputs both to glycogen syn-

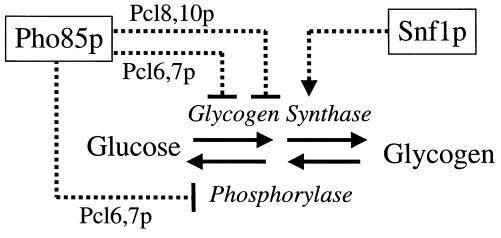

Fig. 4. Multiple inputs of Pho85p into the regulation of glycogen accumulation. The Pho85p kinase regulates glycogen storage both positively and negatively via interaction with different cyclin subunits. In conjunction with the cyclins Pcl8p and Pcl10p, Pho85p phosphorylates and inactivates glycogen synthase, leading to a reduction in glycogen stores. The complex of Pho85p and Pcl6/7p inactivates glycogen phosphorylase and negatively regulates the level of glycogen synthase. Arrows indicate positive regulation, bars indicate negative regulation.

thesis and glycogen breakdown. This is reminiscent of cyclic AMP-mediated control of gene expression whereby both glycogen synthase and phosphorylase are induced as cells approach stationary phase [24,29,33,34]. Presumably, other signals must normally be layered over *PHO85* control of glycogen synthesis and degradation. These signals may in turn depend on factors like growth conditions or genetic background. Such considerations may underlie the observation that in our usual strain background, deletion of *PHO85* causes much greater glycogen hyperaccumulation in cells grown on rich medium than synthetic medium. In addition, the genetic background may influence the consequences of *PHO85* mutation. For example, *PHO85* deletion restores normal glycogen levels to *snf1* mutants whether grown in YPD or SC. The same assessment may extend to less well defined genetic differences between strains. Disruption of *PHO85* in some strain backgrounds, such as NBD85A-1 from the collection of the Brown laboratory, does not cause glycogen hyperaccumulation (Fujino and Roach, unpublished results). Likewise, Goding and colleagues reported that elimination of *PHO85* does not affect glycogen storage in a W303 strain whereas isogenic *pcl6* or *pcl7* mutants hypoaccumulated glycogen [20]. When a single gene product is a constituent of multiple regulatory enzymes that have opposing effects on a process of interest, the consequences of its deletion are hard to predict and of necessity depend on other controls and conditions.

We recently found that autophagy, which is a process whereby cytosol and organelles are delivered to the vacuole for recycling, is a determinant of glycogen accumulation in yeast and is under the control of *SNF1* and *PHO85* [30]. Defective autophagy correlates with the inability to maintain glycogen and, although this occurred without effects on the activities of glycogen synthase and phosphorylase, we wanted to test whether *PCL6* and *PCL7* were connected indirectly with glycogen accumulation through a role in controlling autophagy. We analyzed autophagy in a *pcl6 pcl7* double mutant and found normal induction as the cells entered into stationary phase (Wang and Roach, unpublished results). Therefore, the effects of *PCL6* and *PCL7* are likely mediated by changes in metabolic enzymes, although the exact mechanism remains to be determined.

Pho85p and its associated Pcls are an example of an enzyme family in which a common catalytic subunit is utilized in multiple functionally distinct complexes. It is important in considering such enzyme systems to recognize that the functional identity of the enzyme complex is conferred by the regulatory component(s), the Pcl for Pho85p kinases, which can be subject to its own specific controls. We have previously proposed a model in which an excess of free Pho85p forms a reservoir from which individual Pcl/Pho85p kinases are formed as needed [17]. Although a global control, through the Pho85p catalytic subunit, is formally possible, this seems not to be true since Pho85p is not regulated by phosphorylation and its level is relatively constant under a variety of conditions [17,35]. The present study re-emphasizes the need to view each Pcl/Pho85p as a distinct entity and brings the added complexity that different Pcl/Pho85p kinases can exert opposing controls over the same process.

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