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Analysis of respiratory mutants reveals new aspects of the control of glycogen accumulation by the cyclin-dependent protein kinase Pho85p

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Abstract The *PHO85* gene of *Saccharomyces cerevisiae* encodes a cyclin-dependent protein kinase that can interact with 10 different cyclins (Pcls). In conjunction with Pcl8p and Pcl10p, Pho85p phosphorylates and regulates glycogen synthase. Respiratory-deficient strains, such as *coq3* mutants, have reduced glycogen stores and contain hyperphosphorylated and inactive glycogen synthase. We show here that *pho85 coq3* mutants have dephosphorylated and active glycogen synthase yet do not maintain glycogen reserves. In contrast, deletion of *PCL8* and *PCL10* in the *coq3* mutant background partially restores glycogen accumulation. This suggested the existence of inputs from Pho85p into glycogen storage, independent of Pcl8p and Pcl10p, and acting antagonistically.

Key words: Glycogen; Cyclin-dependent protein kinase; *PHO85*; COQ3; Respiratory mutant

1. Introduction

In the yeast *Saccharomyces cerevisiae*, glycogen synthesis is initiated in response to nutrient limitation, such as the approach to stationary phase [1]. The control of glycogen biosynthesis involves regulation of both the activity and the abundance of glycogen synthase, which in yeast is encoded by two genes, *GSY1* and *GSY2* [2]. *GSY2* encodes the major, nutritionally regulated form of the enzyme, and transcription of *GSY2* is increased on the approach to stationary phase by a mechanism dependent upon cyclic AMP-dependent protein kinase (PKA) [2–4].

In addition to transcriptional control, glycogen synthase is regulated by covalent phosphorylation which occurs at C-terminal sites and is inhibitory [5]. However, full activity can be restored to the phosphorylated enzyme by addition of the allosteric activator, glucose 6-phosphate. This property forms the basis for the −/+ glucose-6-P activity ratio that is used as an index of activation state. The phosphorylation of glycogen synthase is catalyzed by at least two distinct kinases [6]. We have shown that one of these kinases comprises a complex of the cyclin-dependent protein kinase Pho85p and the cyclin Pcl10p [7,8]. A related cyclin, Pcl8p, is also implicated in the control of glycogen synthase, although its precise role is less well defined [7]. Deletion of either *PHO85* or both *PCL8* and *PCL10* results in dephosphorylation and activation of glycogen synthase which is accompanied, in rich media, by hyperaccumulation of glycogen [6,7,9]. Ten different cyclin subunits have been identified for Pho85p [10] and, recently, we have established that the cyclins encoded by the *PCL6* and *PCL7* genes can also influence glycogen accumulation under certain circumstances [11]. Furthermore, PKA contributes to post-translational control of glycogen synthase activity although it is most likely not by direct phosphorylation [3].

It has long been known that there is a link between the ability of yeasts to respire and their ability to accumulate glycogen [12]. When respiratory mutants are deprived of fermentable carbon, they accumulate less glycogen than do wild type cells. In addition to reduced glycogen, these mutants have lowered ATP levels under the same conditions [12]. It was therefore proposed that respiratory mutants failed to accumulate glycogen due to insufficient energy reserves. More recently an alternative explanation was offered. Yang et al. [13] suggested that strains containing mutations in genes that are required for oxidative growth, such as *COQ3*, which is required for the synthesis of coenzyme Q, failed to accumulate glycogen due to hyperphosphorylation and inactivation of glycogen synthase [13]. In this work, the authors showed that the glycogen-deficient phenotype of *coq3* mutant cells could be suppressed by the expression of non-phosphorylatable mutants of glycogen synthase, or by mutations that inactivated PKA. However, deletion of the *PHO85* gene in *coq3* mutant cells did not restore glycogen accumulation. Therefore, the authors concluded that the phosphorylation and inactivation of glycogen synthase in respiratory-deficient cells occurred by a mechanism that was independent of Pho85p function [13].

Here we show that phosphorylation of glycogen synthase in respiratory mutants, as in wild type cells, is controlled by Pho85p. Deletion of *PHO85* in *coq3* mutants led to dephosphorylation and activation of glycogen synthase, but the resulting *pho85 coq3* mutant cells remained glycogen-deficient like *coq3* mutants. In contrast, deletion of the *PCL8* and *PCL10* genes in a *coq3* mutant background was found to partially suppress the glycogen deficiency, demonstrating that Pho85p has inputs into glycogen storage that are independent of Pcl8p and Pcl10p.

2. Materials and methods

2.1. Yeast strains and media

The yeast strains used in this study are detailed in Table 1. Standard methods for yeast culture and genetic manipulation were used

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Table 1
Yeast strains used in this study

Strain	Genotype	Source or reference
EG328-1A	*MATα leu2 trp1 ura3-52*	K. Tatchell
RY51	*MATα leu2 trp1 ura3-52 coq3::URA3*	[13]
RY71	*MATα leu2 trp1 ura3-52 pho85::URA3 coq3::TRP1*	[13]
RY213	*MATα leu2 trp1 ura3-52 pcl8::TRP1 pcl10::URA3 coq3::URA3*	R.C. Wek
WW10	*MATα leu2 trp1 ura3-52 pcl8::TRP1 pcl10::LEU2*	[8]
WW11	*MATα leu2 trp1 ura3-52 pho85::TRP1*	[8]
WW13-28d	*MATα leu2 trp1 ura3-52 pho85::URA3 pcl8::URA3 pcl10::URA3 coq3::TRP1*	This study

The wild type strain used was EG328-1A and all strains listed are isogenic.

[14]. Yeast cells were grown in synthetic complete medium (SC; containing 0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose, and complete supplement mix (Bio 101)). For solid media, agar was added to 2% (w/v).

2.2. Growth of yeast in liquid culture

Cultures were grown overnight in SC medium. These cultures were then used to inoculate fresh SC medium such that, after 24 h of growth, a cell density of approximately 1×10^8 cells/ml was reached. At this time, and again at 48 h post inoculation, aliquots were removed for the determination of glycogen content or glycogen synthase and glycogen phosphorylase activity as described below.

2.3. Enzyme assays

Glycogen synthase was assayed in extracts prepared from yeast cells by lysis with glass beads as described previously [3,15]. 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 modification of published procedures [16,17].

2.3.1. Glycogen determination. Glycogen was assessed qualitatively by growing patches of cells on SC plates for 48 h and then exposing the plates to iodine vapor. Cells stain brown in proportion to the amount of glycogen they contain. Glycogen was determined enzymatically as described previously [18].

2.3.2. Protein determination. The protein content was estimated by the method of Bradford, using bovine serum albumin as a standard [19].

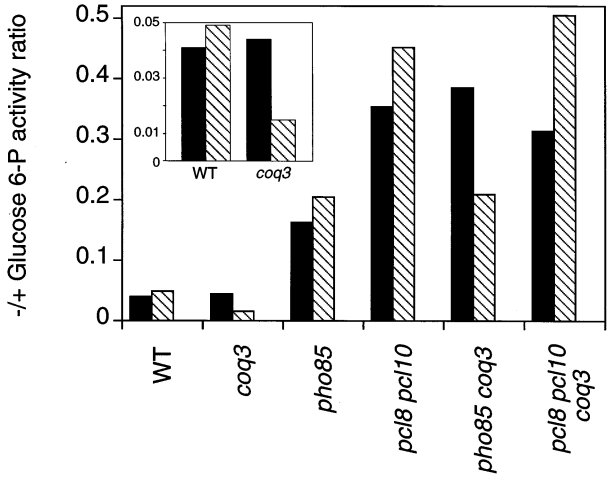

Fig. 1. Glycogen synthase activity ratio in different strains. The indicated strains were cultured in SC medium. Cultures were inoculated such that they reached saturation ($\sim1\times10^8$ cells/ml) after 24 h of growth. Samples were withdrawn at this 24 h time point (solid bars) and again after 48 h growth (cross-hatched bars) and the glycogen synthase −/+ glucose-6-P activity ratio determined. The inset shows the data for wild type and the *coq3* mutant, expanded for clarity. The data are the average of two independent experiments, each of which was performed in duplicate. Strains used: wild type (EG328-1A), *coq3* (RY51), *pho85* (WW11), *pcl8 pcl10* (WW10), *pho85 coq3* (RY71), *pcl8 pcl10 coq3* (RY213).

3. Results

3.1. Inactivation of glycogen synthase in respiratory mutants is dependent on Pho85p function

Respiratory mutants, such as *coq3* cells that are defective in the synthesis of ubiquinone, are deficient in glycogen storage. Recently, this was attributed to hyperphosphorylation and inactivation of glycogen synthase [13]. The *PHO85* gene encodes the catalytic subunit of a cyclin-dependent protein kinase which phosphorylates glycogen synthase. We were intrigued that deletion of *PHO85* was reported not to suppress the glycogen deficit of *coq3* mutants, even though expression of mutant glycogen synthase lacking the Pho85p phosphorylation sites did. Although glycogen accumulation was examined in *pho85 coq3* mutants, the activation state of glycogen synthase was not. It thus remained a possibility that deletion of *pho85* did activate glycogen synthase in *coq3* mutants but that *pho85 coq3* mutants were unable to make glycogen for other reasons. To address this issue, we measured the −/+ glucose-6-P activity ratio in extracts made from wild type, *coq3*, *pho85* and *pho85 coq3* cells at two different points during growth. Cultures were inoculated so that they reached stationary phase (a density of $\sim1\times10^8$ cells/ml) after 24 h of growth, and samples were withdrawn for assay. Growth was continued for a further 24 h and samples were again taken. As reported by Yang et al. [13], *coq3* mutants had an abnormally low −/+ glucose 6-P activity ratio late in growth (Fig. 1). However, in *pho85 coq3* mutants, this activity ratio was restored and was even higher than wild type cells, resembling that of *pho85* mutants (Fig. 1). There was no significant change in the total amount of glycogen synthase activity between the various strains (not shown). Consistent with previous results, when patches of either *coq3* or *pho85 coq3* mutant cells were grown on synthetic medium and stained with iodine vapor, both *coq3* and *pho85 coq3* cells were glycogen-deficient (Fig. 2). Note that growth in synthetic medium results in higher glycogen accumulation by wild type cells than does growth in rich medium, such that *pho85* mutant cells do not appear to hyperaccumulate glycogen under these conditions.

Previously, we had demonstrated that the Pho85p cyclins, Pcl8p and Pcl10p, direct Pho85p to phosphorylate glycogen synthase [7] but their role in glycogen accumulation by *coq3* mutants had not been investigated. Therefore, we analyzed a *pcl8 pcl10 coq3* triple mutant. The −/+ glucose-6-P activity ratio of glycogen synthase was elevated, as in *pho85 coq3* mutants (Fig. 1), but in this case, glycogen storage was also partially restored (Fig. 2).

The fact that the glycogen phenotype of *pho85 coq3* and *pcl8 pcl10 coq3* mutants differed suggests that *PHO85* must

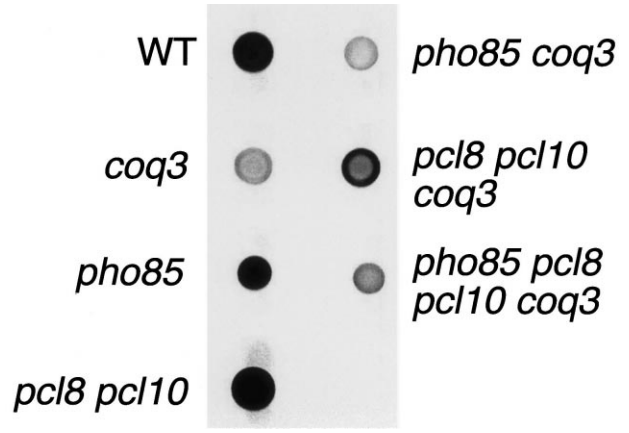

Fig. 2. Glycogen levels in different yeast strains. The indicated strains were grown for 48 h on SC plates. Glycogen accumulation was then assessed by exposing the agar plate to iodine vapor. Cells stain brown in proportion to the amount of glycogen present. Strains used: wild type (EG328-1A), *coq3* (RY51), *pho85* (WW11), *pcl8 pcl10* (WW10), *pho85 coq3* (RY71), *pcl8 pcl10 coq3* (RY213), *pho85 pcl8 pcl10 coq3* (WW13-28d).

have other inputs to control glycogen metabolism beyond the known negative role of inactivating glycogen synthase. Furthermore, there must be some positive input that either increases glycogen synthesis or inhibits glycogen degradation. Presumably these different functions would be carried out by Pho85p in association with cyclins other than Pcl8p and Pcl10p. This hypothesis predicts that the quadruple mutant *pho85 pcl8 pcl10 coq3* would have the glycogen phenotype characteristic of *pho85 coq3* cells rather than *pcl8 pcl10 coq3* cells and this was indeed observed (Fig. 2).

3.2. Deletion of PHO85 increases glycogen phosphorylase activity

To characterize better the glycogen phenotype of the *coq3*, *pho85 coq3* and *pcl8 pcl10 coq3* mutants, we performed quantitative glycogen measurements after 24 h and 48 h growth in liquid culture. At the 24 h time point, both wild type cells and *coq3* mutants accumulated equivalent amounts of glycogen (Fig. 3). However, whilst wild type cells largely maintained this store, *coq3* mutants were deficient in glycogen by the 48 h time point, consistent with a recent report from the group of Francois [20]. Similarly, *pho85 coq3* mutant cells synthesized glycogen but had degraded it by the 48 h time point whilst *pcl8 pcl10 coq3* mutants were at least partially capable of maintaining glycogen stores.

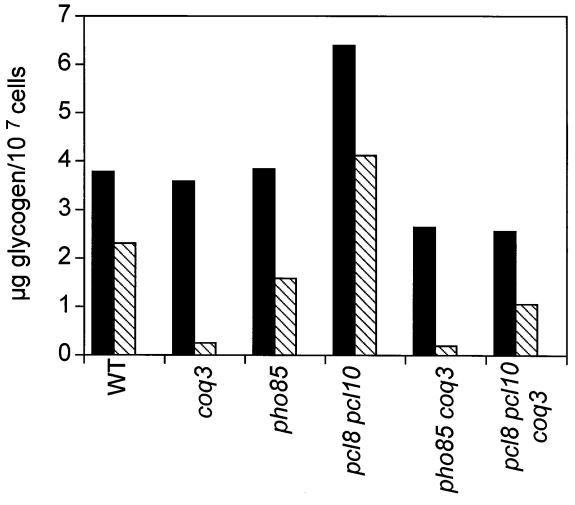

Fig. 3. Glycogen accumulation early and late in saturated culture. The indicated strains were cultured in SC medium as described in the legend to Fig. 1. Samples were withdrawn at this 24 h time point (solid bars) and again after 48 h growth (cross-hatched bars) and the glycogen content was determined. The data shown are the average of two independent experiments, each of which was performed in duplicate. Strains used: wild type (EG328-1A), *coq3* (RY51), *pho85* (WW11), *pcl8 pcl10* (WW10), *pho85 coq3* (RY71), *pcl8 pcl10 coq3* (RY213).

Because *coq3* and *pho85 coq3* mutants exhibited accelerated glycogen degradation relative to *pcl8 pcl10 coq3* cells, we measured the activity of glycogen phosphorylase in the various strains at the 24 h and 48 h time points. Glycogen phosphorylase activity was found to be substantially increased in both *pho85* and *pho85 coq3* mutant cells compared to either wild type, *coq3*, *pcl8 pcl10* or *pcl8 pcl10 coq3* cells, and this was most obvious at the 48 h time point (Fig. 4). Therefore, whilst deletion of *PHO85* leads to dephosphorylation and activation of glycogen synthase, it also results in an increase in glycogen phosphorylase activity. The increased glycogen phosphorylase activity is not seen in *pcl8 pcl10* or *pcl8 pcl10 coq3* mutants and thus, the control which Pho85p exerts over glycogen phosphorylase is achieved via interaction with other cyclins.

4. Discussion

Over 30 years ago Chester [12] reported that the inability of yeast cells to respire correlated with an inability to store glycogen. This observation was attributed to the fact that respiratory-deficient cells growing in glucose-depleted medium had decreased ATP levels, and hence the diminished energy supply failed to support glycogen synthesis. Recently, an alternative explanation was offered [13]. It was shown that glycogen synthase was hyperphosphorylated and inactivated in respiratory mutants, such as *coq3* strains. Furthermore, deletion of *PHO85* in a *coq3* strain did not restore glycogen accumula-

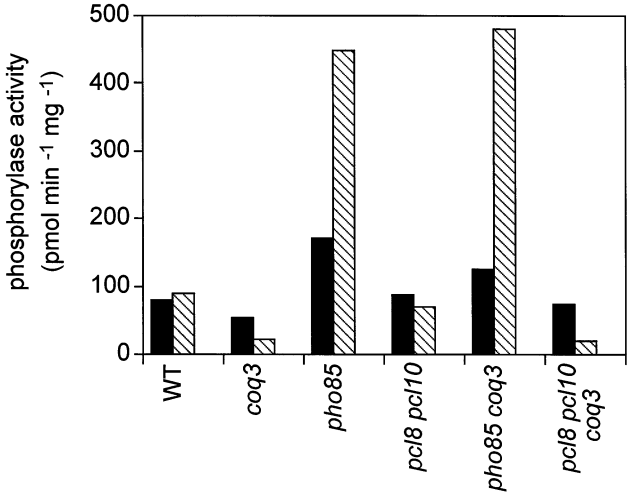

Fig. 4. Glycogen phosphorylase activity early and late in saturated culture. The indicated strains were grown as described in the legend to Fig. 1. Glycogen phosphorylase activity was measured after 24 h (solid bars) and 48 h (cross-hatched bars) growth. The data shown are the average of two independent experiments, each of which was performed in duplicate.

tion, leading to the conclusion that the phosphorylation and inactivation of glycogen synthase in respiratory-deficient cells occurred by a mechanism that was independent of Pho85p function [13]. We show here that the defective glycogen storage exhibited by *coq3* mutants is not impaired synthesis but rather an inability to maintain glycogen once it is made. Early in stationary phase, both wild type cells and *coq3* mutants have accumulated equivalent levels of glycogen. However, whereas wild type cells maintained this glycogen for an extended period of time, the store was more rapidly depleted in *coq3* mutants. We confirmed the report that deletion of *PHO85* was unable to suppress the glycogen defect of *coq3* mutants but found additionally that deletion of the Pho85p cyclins encoded by the *PCL8* and *PCL10* genes could partially suppress the glycogen deficit of *coq3* mutants. Furthermore, in either *pho85 coq3* or *pcl8 pcl10 coq3* mutants, the glycogen synthase −/+ glucose-6-P activity ratio was greatly elevated, resembling *pho85* or *pcl8 pcl10* mutants. Therefore, the phosphorylation state of glycogen synthase in respiratory mutants, as in wild type cells, is regulated by Pho85p and the cyclins Pcl8p and Pcl10p, and the failure of *coq3* mutants to maintain glycogen cannot be explained solely in terms of the glycogen synthase activation.

Our view is that the results of Yang et al. [13], and our own results, are best explained as follows. The amount of glycogen at any time is a function of the rates of synthesis and breakdown. Expression of non-phosphorylatable glycogen synthase or deletion of *PCL8* and *PCL10* both increase glycogen synthesis because they bypass the phosphorylation control of glycogen synthase. Thus, whatever the mechanism that leads to increased phosphorylation of glycogen synthase in *coq3* mutants, it is blocked in these cells, the balance is tipped in favor of glycogen synthesis and glycogen accumulates. However, *coq3* mutants cannot utilize non-fermentable carbon sources, such as ethanol, for growth and the glycogen reserves are consumed in order to support growth once glucose is depleted from the medium. Therefore, even when phosphorylation of glycogen synthase is blocked, cells with the *coq3* mutation cannot maintain glycogen stores in the long term. This is apparent from Fig. 2 where the older *pcl8 pcl10 coq3* cells at the center of the colony have lost glycogen but the younger cells at the periphery still retain the polysaccharide. Similar conclusions have been reached by Francois's group [20]. Additionally, Enjalbert et al. [20] reported an activation of glycogen phosphorylase in respiratory mutants which they attributed to decreased cellular glucose-6-P. Glucose-6-P is both an inhibitor of glycogen phosphorylase and an activator of glycogen synthase, making this scheme logical. However, in our strain background and with the growth conditions used in the present work, we could not detect any increase in phosphorylase activity in the *coq3* mutants when compared to wild type. However, if glycogen synthesis is decreased, be it by increased phosphorylation of glycogen synthase, a reduction in glucose-6-P or some other mechanism, then, as long as there is some glycogen phosphorylase activity, the net result will be decreased glycogen storage.

The observation that glycogen accumulation in *pho85 coq3* mutants resembled *coq3* mutants, whilst *pcl8 pcl10 coq3* mutants were not so severely deficient, led us to conclude that Pho85p had inputs into glycogen storage which were independent of Pcl8p and Pcl10p. Since the *coq3* and *pho85 coq3* mutants exhibited accelerated glycogen degradation relative

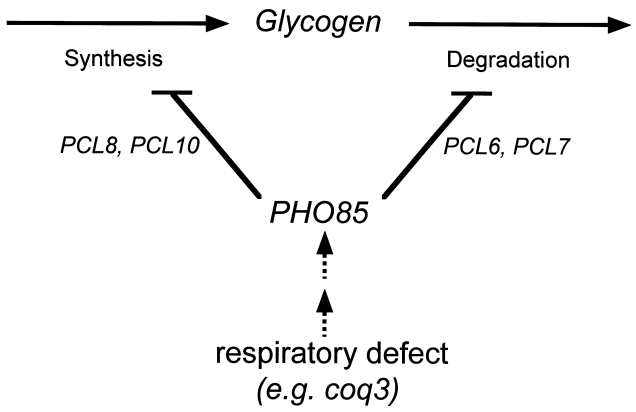

Fig. 5. Multiple inputs of Pho85p into the regulation of glycogen accumulation. The model shows the relationships between the genes and alleles studied. *PHO85* regulates glycogen storage both positively and negatively via interaction with different cyclin subunits. In association with Pcl8p or Pcl10p, Pho85p phosphorylates and inactivates glycogen synthase, promoting reduced glycogen storage. However, Pho85p, in complex with Pcl6p, Pcl7p or an undefined Pcl protein, also negatively regulates glycogen phosphorylase activity, leading to increased glycogen storage. Although the Pho85p catalytic subunit is common to the different kinase complexes, their specificity is defined by the associated Pcl protein and each kinase complex could be regulated independently in response to different signals. When glucose is depleted, respiratory mutants such as *coq3*, which are unable to utilize non-fermentable carbon sources, begin to starve and show increased phosphorylation of glycogen synthase that can be blocked by mutation of *PHO85* or *PCL8* and *PCL10*. Therefore, a signal that activates Pho85p/Pcl8,10p is probably generated in response to energy limitation.

to *pcl8 pcl10 coq3* mutants or wild type cells, an obvious possibility was that deletion of *PHO85* led to activation of glycogen phosphorylase. We found there was about five-fold higher phosphorylase activity in *pho85* and *pho85 coq3* mutants compared to either wild type or *pcl8 pcl10 coq3* cells. Thus, the positive input which Pho85p has to glycogen storage appears to be a downregulation of phosphorylase activity (Fig. 5).

We recently obtained a separate body of evidence linking Pho85p to the control of glycogen phosphorylase [11]. We found that glycogen storage could be restored to *snf1* mutants, in which glycogen synthase was phosphorylated and inactive, by deletion of *PHO85*, but not by deletion of *PCL8* and *PCL10*. We went on to show that deletion of both *PCL6* and *PCL7* in a *snf1 pcl8 pcl10* mutant resulted in a restoration of glycogen accumulation. We do not believe that control of glycogen phosphorylase by Pho85p occurs solely via Pcl6p and Pcl7p since deletion of *PCL6* and *PCL7* in wild type cells increases phosphorylase activity only about two-fold, compared to the about five-fold increase when *PHO85* is deleted (Wilson and Roach, unpublished observation). Currently, experiments are under way to address precisely how Pho85p regulates glycogen phosphorylase activity.

Although it might at first sight seem teleologically illogical to have a single kinase catalytic subunit, Pho85p, involved in both the positive and negative regulation of glycogen storage, it is important to realize that the functional identity of this kinase is conferred by the nature of the associated regulatory subunit. We have previously suggested that a pool of the Pho85p free catalytic subunit is always present and is re-

cruited, by individual cyclins, to specific tasks as conditions demand [8]. Consistent with this view, different Pho85p/Pcl complexes can be subject to different types of regulation and be responsive to distinct signals.

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