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Regulation of yeast glycogen phosphorylase by the cyclin-dependent protein kinase Pho85p

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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.
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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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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.

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