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Pho85 and signaling environmental conditions

Adam S. Carroll and Erin K. O'Shea

Through its association with a family of ten cyclins, the Pho85 cyclin-dependent kinase is involved in several signal transduction pathways in the yeast *Saccharomyces cerevisiae*. The responses mediated by Pho85 include cell-cycle progression and metabolism of nutrients such as phosphate and carbon sources. Although these responses require the phosphorylation of different substrates, and have different mechanistic consequences as a result of this phosphorylation, all appear to be involved in responses to changes in environmental conditions. Few of the activating signals or regulated targets have been unambiguously identified, but the kinase activity of Pho85 appears to inform the cell that the current environment is satisfactory.

Microorganisms such as the budding yeast *Saccharomyces cerevisiae* must elicit coordinated, accurate and robust responses to changes in a complex extracellular environment. They can coordinate such responses by re-using individual factors and motifs for related responses, although they must then ensure that the correct response is triggered by the appropriate stimulus. The cyclin-dependent kinase (CDK) Pho85 is an example of a kinase that is shared between several pathways, having multiple functions and cyclin-binding partners [1].

The past 15 years have seen the extensive characterization of a wide variety of CDKs. These signaling molecules have proven to be central to several cellular processes in addition to regulation of the cell cycle, the context in which they were first identified. Our basic understanding of CDKs and their activity is by no means complete. We understand that the binding of a cyclin partner confers substrate specificity on the kinase, but we have no systematic way to predict the substrates,

the regulators or the physiological function targeted by a given cyclin-CDK complex. This is certainly true of the budding yeast CDK Pho85, which has proven useful for approaching these general questions because of its non-essential nature, the number of cyclin partners with which it associates, and the experimental tractability of yeast in general.

As we learn more about the functions of Pho85, one theme emerges repeatedly. Many functions of Pho85 appear to be involved in transducing signals related to changes in the extracellular environment (Table 1). Furthermore, the kinase activity of the relevant Pho85 complex appears to signal that the current environmental situation is satisfactory; when environmental conditions become stressful, the relevant kinase activities of Pho85 are switched off, resulting in activation of the appropriate response.

Pho80 and phosphate metabolism

Pho85 is best known for its pivotal role in the *PHO* pathway, a signaling pathway that coordinates the responses of yeast to phosphate starvation [2,3]. Pho85 is directed to this function by its association with the cyclin Pho80 [4]. Pho80-Pho85 kinase activity is regulated in response to phosphate levels by the CDK inhibitor (CKI) Pho81, which remains bound to Pho80-Pho85 in both high and low phosphate conditions [5,6]. When high levels of inorganic phosphate are present in the environment, the Pho80-Pho85 kinase is active, phosphorylating and inactivating the transcription factor, Pho4 [7].

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Table 1. Pho85 cyclins and their functions^a

Cyclin	Expression	Substrate(s)	Functions
Pcl1	Peaks in late G1	Sic1 Gcn4	Sic1 stability, cell cycle progression? Gcn4 stability? Morphogenesis
Pcl2	Peaks in late G1	Rvs167	Morphogenesis Sic1 stability, cell cycle progression?
Pho80	Constant	Pho4	Pho4 localization, phosphate-dependent gene expression
Clg1	Constant		Morphogenesis
Pcl5	Constant?		Morphogenesis
Pcl6	Constant		Carbon source utilization
Pcl7	Peaks in mid to late S		Carbon source utilization Activity regulated by Pho81 and levels of P_i
Pcl8	Constant	Gsy2	Glycogen metabolism
Pcl9	Peaks in late M, early G1		Morphogenesis
Pcl10	Constant	Gsy2	Glycogen metabolism

^aAbbreviations: G, growth phase of cell cycle; M, mitotic phase of cell cycle; P_i , inorganic phosphate; S, DNA synthesis phase of cell cycle.

Phosphorylation of Pho4 inhibits its transcriptional activity by preventing its association with the transcription factor Pho2 and the nuclear import receptor Pse1, and by promoting association with the nuclear export receptor Msn5 [8–10]. Each of these associations depends on a particular phosphorylation site or sites but, in general, Pho4 phosphorylation causes it to be localized predominantly to the cytoplasm, where it is unable to activate transcription of the phosphate-starvation-responsive genes [10,11]. When phosphate becomes limiting, the kinase activity of Pho80–Pho85 is inactivated, permitting the activation of Pho4 and causing the transcription of genes that are involved in the survival response to phosphate starvation [5]. This inhibition of kinase activity is accomplished through a novel inhibitory domain in Pho81 that allows the CKI to bind stably to Pho80–Pho85 in high and low phosphate conditions, yet it only inhibits when yeast are starved for phosphate [12]. The intracellular signal that causes this change in Pho81, and the molecular nature of the change, are not yet known.

Phenotypes of the *pho85Δ* strain

Pho85 has multiple functions, as suggested by the pleiotropic phenotype caused by its deletion. Deletion of *PHO85* causes not only the constitutive expression of phosphate-starvation-dependent genes [2], but also slow growth with a G1 delay on rich media, poor growth on non-fermentable carbon sources, hyperaccumulation of glycogen, abnormal morphology, irregular budding, and sporulation defects [13–16]. Additionally, Pho85 has been proposed to function as a negative regulator of starvation-induced autophagy [17], and also seems to be required when proline is the only available nitrogen source [18]. Cells lacking *PHO85* are hypersensitive to several chemical treatments, including hydroxyurea [19], hygromycin B and G418 [20], and salt [21]. Furthermore, there are synthetic lethal interactions between *PHO85* and several other genes involved in the cell cycle, morphogenesis and transcription [19,22]. These genes include the

G1 cyclins *CLN1* and *CLN2*, the bud emergence gene *BEM2*, and the transcription factors *SPT7*, *GCR1* and *SRB5*. In some cases, the aspect of the phenotype can be reproduced by deletion of cyclins, but we do not have a comprehensive understanding of which cyclins account for which aspects of the *pho85Δ* phenotype.

The Pcls

Currently, Pho85 is known to have ten cyclin partners, called Pcls for Pho85 cyclin (Table 1). The Pcls were identified primarily through sequence homology and two-hybrid screens [15,23,24]. They have been grouped by sequence homology into two subfamilies, each with five members: the Pcl1,2 subfamily of Pcl1, Pcl2, Clg1, Pcl5 and Pcl9; and the Pho80 subfamily of Pho80, Pcl6, Pcl7, Pcl8 and Pcl10 [15] (Fig. 1). Of these ten cyclins, four (*PCL1*, *PCL2*, *PCL7* and *PCL9*) demonstrate a pattern of cell-cycle-regulated expression [23–26]. These patterns suggest that at least some functions of Pho85 have cell-cycle dependence or relevance. Transcriptional regulation could be important for those *PCLs* whose expression is not controlled by the cell cycle. In fact, several *PCLs* showed divergent patterns of expression in a comparison of genome transcript profiling experiments done under different conditions of stress [27,28]. Such expression data might provide clues to the function and regulation of Pcls with no known molecular functions, and allow us to distinguish differences between seemingly redundant cyclins.

The pleiotropic phenotype produced by deletion of *PHO85* provides many possible interpretations for the function of individual Pcl–Pho85 complexes. One long-term goal is to ascribe each aspect of the *pho85Δ* phenotype to the loss of function of a particular Pcl, or group of Pcls, and to a resulting change in the activity of the relevant substrate. This review will focus on recent advances in our understanding of the functions of the non-Pho80 Pcls, with particular emphasis on those with proposed molecular functions or substrates.

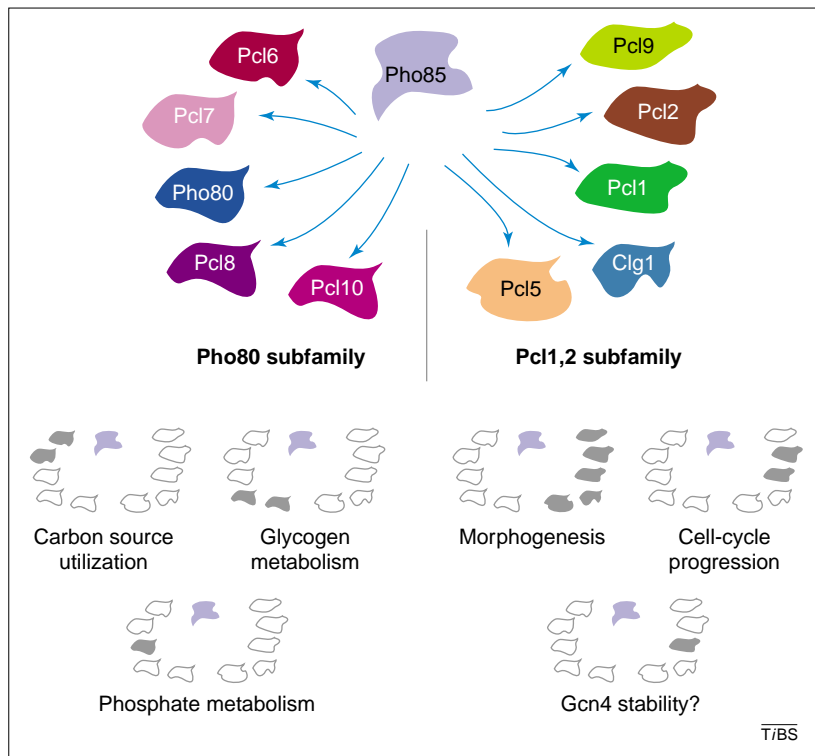


Fig. 1. The Pho85 cyclins and their functions. The large graphic denotes which cyclins belong to which subfamily, as determined by sequence homology. Each small graphic indicates which of the cyclins participate in the listed function. The filled-in gray cyclins participate, whereas the outlined ones do not.

Molecular functions of Pho85

The cell cycle

There is genetic and gene expression data to suggest that some Pcl–Pho85 kinases play a role in the cell cycle. The first identified synthetic lethal interaction for *PHO85* was the inviability of *cln1Δ cln2Δ pho85Δ* and *cln1Δ cln2Δ pcl1Δ pcl2Δ* strains [23,24]. *CLN1* and *CLN2* encode cyclins that associate with Cdc28, the yeast CDK required for cell-cycle progression [29]. Cln1, Cln2, Pcl1 and Pcl2 are all expressed during the G1 phase of the cell cycle, and it is thought that each of these cyclins contributes information to the decision to pass START and commit to the cell cycle, perhaps through the sharing of substrates between Cdc28-containing and Pho85-containing complexes. Deletion of *PCL1* and *PCL2* recapitulates this synthetic lethal phenotype of the *pho85Δ* strain, thereby suggesting that these cyclins might be the only *PCLs* required for this function of Pho85 (see also Ref. [19]). So far, the best model to explain this observation arises from the demonstration that the *in vivo* stability of the CKI Sic1 depends on *PHO85* (see below).

Regulation of Sic1 stability

Sic1 is responsible for repressing the activity of Clb cyclin-containing Cdc28 complexes until the cell is prepared to leave G1 and commit to progression through the cell cycle [29]. Sic1 degradation has been previously demonstrated to be predominantly dependent on the G1 Cln–Cdc28 kinases [30,31].

A role for Pho85 in controlling Sic1 stability might explain the lethality of the *cln1Δ cln2Δ pho85Δ* and *cln1Δ cln2Δ pcl1Δ pcl2Δ* strains. If Sic1 were hyperstabilized by loss of both Cdc28- and Pho85-dependent mechanisms for its degradation, then the cell would not be able to exit G1.

In vivo, the instability of Sic1 depends on the presence of *PHO85*, and this Pho85-dependent stability depends on a particular phosphorylation site on Sic1 [32]. *In vitro*, phosphorylation studies have shown that coexpression of Pho85 with the cyclin Pcl1 produces more activity towards Sic1 than does Pho85 coexpression with Pcl2, Pho80, or the Cdc28 cyclins Clb2 and Clb5 [32]. However, at this point there is no evidence to show that Pcl1 (or Pcl2) is the actual cyclin required *in vivo* for the observed stabilization of Sic1. The predicted (and parsimonious) explanation for the observed synthetic lethality would suggest that both Pcl1 and Pcl2 control Sic1 stability, but no concrete link for these cyclins has been established. If such a link is established, it will require an explanation for how cyclins that function primarily in the cytoplasm [33,34] act on the predominantly nuclear protein Sic1 [29].

If hyperstabilization of Sic1 is the cause of the synthetic lethality, then deletion of *SIC1* should restore viability. One group has reported that deletion of *SIC1* suppresses the synthetic lethality of a *cln1Δ cln2Δ pho85Δ* strain, albeit with a substantial decrease in the viability of the strain [32]. However, it has also been reported that *cln1Δ cln2Δ pcl1Δ pcl2Δ sic1Δ* strains were as inviable as a *cln1Δ cln2Δ pcl1Δ pcl2Δ* strain [19], suggesting the existence of an additional function for Pho85 that is independent of Sic1. The resolution of these apparently conflicting genetic differences awaits further investigation. Although deletion of a CDK can certainly have a very different phenotype from deletion of a subset of its cyclins, differences in strain background could provide a simple explanation for these differences in synthetic lethal interactions.

Pcl8, Pcl10 and glycogen metabolism

In most strain backgrounds, deletion of *PHO85* causes hyperaccumulation of glycogen during late log-phase growth [14,25]. This hyperaccumulation occurs because Pho85 phosphorylates and downregulates Gsy2 [35,36], the predominant glycogen synthase isoform in yeast [37]. The cyclin partners responsible for directing Pho85 to this well-characterized function are Pcl8 and Pcl10. Deletion of these two cyclins produces the same glycogen hyperaccumulation phenotype and hyperactive Gsy2 enzyme as is observed in *pho85Δ* cells [35]. Furthermore, the Pcl10–Pho85 complex is capable of phosphorylating recombinant Gsy2 *in vitro* and demonstrates a 50-fold *in vitro* specificity for Gsy2 over Pho4 [35]. A similar *in vitro* activity for Pcl8–Pho85 complexes has not yet been demonstrated. Neither the *in vivo* mechanism of

regulation of this glycogen synthase kinase activity nor the physiological relevance of this regulation in responding to particular nutritional conditions has been elucidated. The activity of Pcl10–Pho85 does demonstrate that not all Pcl–Pho85 complexes will directly regulate transcription factors and gene expression.

The participation of Pcl8 and Pcl10 in the regulation of reserve carbohydrate levels suggests that these cyclins might be responsible for the phenotype of the *pho85Δ* strain that causes poor growth on non-fermentable carbon sources. Strangely, this is not the case; only *pcl6Δ* and *pcl7Δ* strains have demonstrated this phenotype [25,35]. These are the closest homologs of Pcl8 and Pcl10, but their regulation, particularly that of Pcl7, is complex, as described below [25].

Morphogenesis

There are three lines of evidence suggestive of a role for Pho85 in controlling cellular morphogenesis. First, *pho85Δ* strains are larger, with more elongated buds and a wider bud neck, than isogenic wild-type strains; this effect is more pronounced in the diploid than in the haploid [15]. The cyclins important for this morphogenetic phenotype are the Pcl1,2 subfamily, as deletion of all five members of this subfamily causes a phenotype very similar to that observed in the *pho85Δ* strain [15]. Also, the *pho85Δ* strain and a strain lacking all five members of the *PCL1,2* subfamily display random patterns of budding [16]; this phenotype is probably related to the abnormal morphology and actin localization observed in those strains [21]. Second, several genes involved in morphogenesis, including *BCK1*, *MPK1* and *BEM2*, display a synthetic lethal phenotype when mutated in strains lacking *PHO85* [19,22]. Pcl1 and Pcl2 are the cyclins relevant to this phenotype, as deletion of *PCL1* and *PCL2* also causes synthetic lethality with mutations in *BCK1*, *MPK1*, *BEM2* and *CDC42*. In the case of *BEM2*, deletion of only *PCL1* produces the synthetic lethal phenotype, indicating that there are non-redundant functions for these highly homologous cyclins [19]. Third, Pcl2 and Pcl9 interact physically with Rvs167, the yeast amphiphysin homolog known to be involved in organization of the actin cytoskeleton, endocytosis, and survival during starvation conditions [21]. Deletion of *PHO85*, similar to deletion of *RVS167*, causes defects in this set of processes [21]. It is thought that Rvs167 is phosphorylated *in vivo* by Pcl2–Pho85, as maximal *in vivo* phosphorylation of Rvs167 depends on the presence of Pho85 and the Pcl1,2 subfamily [21]. However, it is not clear whether this effect is direct, and no effect on the activity or intermolecular associations of Rvs167 have been attributed to loss of regulation by Pho85. Although all this evidence points towards a morphogenetic function for the Pcl1,2 subfamily and Pho85, the precise molecular mechanism of the function remains unknown.

Regulation of Gcn4 stability

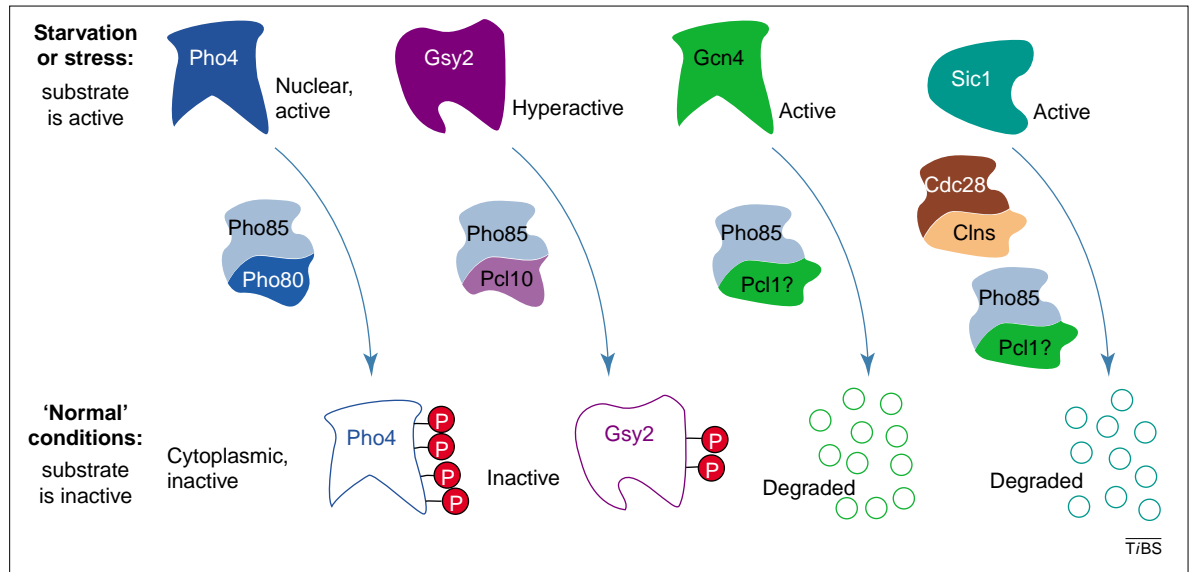
Pho85 has also been implicated in controlling the stability of the transcription factor Gcn4. This function of Pho85 appears to be similar to its role in Sic1 regulation, as both Sic1 and Gcn4 undergo phosphorylation- and ubiquitination-dependent degradation [30,31,38]. Gcn4 is required for the increased expression of amino acid biosynthetic genes, such as *HIS4*, upon amino acid starvation [39,40]; such starvation causes an increase in the half-life of the protein, as measured by *in vivo* pulse-chase experiments [41]. Similarly, deletion of *PHO85* causes a significant increase in the half-life of Gcn4 [41]. Conditions that stabilize Gcn4 also produce a lower kinase activity in immunoprecipitated Pcl1–Pho85 complexes, although no corresponding control measurement of Pcl1 and Pho85 protein levels was obtained [41]. Gcn4 can also be efficiently phosphorylated *in vitro* by Pho80–Pho85 [41]. Furthermore, no *in vivo* consequences on Gcn4 stability were found for the *pcl1Δ* strain nor for several unspecified strains containing deletions of *PCLs* distinct from *PCL1* [41]. Further studies are required to establish which Pcl is responsible for this stabilization *in vivo*.

An interesting future direction for this line of research would be to determine whether Pho85 function has an impact on the native promoters of genes known to be under Gcn4 control. One such experiment has shown that deletion of *PHO85* causes a 2.5-fold increase in expression from a derivative of a *HIS4* promoter that is exclusively dependent on Gcn4 [41]. Subsequent experiments have demonstrated that the CDK Srb10 also plays a role in Gcn4 stability [38]. The role of Srb10 is apparently distinct from that of Pho85, as the simultaneous deletion of both *SRB10* and *PHO85* results in a stabilization of Gcn4 greater than that observed for either mutant alone. When both *SRB10* and *PHO85* are inactivated, Gcn4 might be sufficiently stabilized to enable expression of Gcn4-dependent genes. Alternatively, if transcription of Gcn4-dependent genes is unaffected, the stabilization could be important for establishment of a sensitized state that augments the ability of the yeast to respond to further environmental insult.

Interaction with Swi5

Pho85 has also been implicated in regulating the transcription factor Swi5 [42]. Swi5 regulates transcription of several cell-cycle-dependent genes, including *PCL9* and *PCL2* [16,26]. Interestingly, Swi5 and Pcl2 proteins interact in a two-hybrid screen and also when translated *in vitro* [42]. Although Pcl2–Pho85 will phosphorylate Swi5 *in vitro*, several results call into question the *in vivo* relevance of this observation. First, Pho80–Pho85 will also phosphorylate Swi5 *in vitro* to a similar level as does Pcl2–Pho85 [42]. Second, the only apparent effect of Pho85 on Swi5 activity *in vivo* is a mild transcriptional defect in two Swi5-dependent genes [42]. To observe

Fig. 2. The consequences of phosphorylation by Pho85. Four well-characterized substrates are shown, with their active condition shown on the top. The question marks in the cyclin cartoon indicate that a requirement for Pcl1 has not been shown for this activity *in vivo*. The degradation of the substrates is phosphorylation-dependent; Pho85 does not actually degrade these substrates directly.



this effect, the partially redundant transcription factor Ace2 must also be deleted from the strain [42]. Unlike that of Sic1 and Gcn4, the stability of Swi5 is unchanged by deletion of *PHO85*, in either synchronized or asynchronized cultures [42]. It is possible that the binding of Swi5 to Pcl2 serves a function other than substrate targeting.

The environmental stress response

The ability of the Pho80–Pho85 complex to signal a response to the stress of phosphate limitation is well documented. Recently, a more general role for Pho85 in producing a generic response to many types of cellular stress has been suggested by studies using a chemical genetic approach [43]. The rapid loss of Pho85 activity in the absence of any cellular stress causes the induction of a diverse set of genes that have recently been characterized as comprising a generic response to stressful conditions known as the environmental stress response (ESR) [27,28]. It is not clear whether such induction results from loss of Pho85 function involved directly in induction of the ESR or whether loss of a particular non-Pho4-dependent Pho85 function creates ESR-inducing conditions. If Pho85 is directly involved in the ESR signaling pathway, it will be interesting to determine what signal is detected and how it is transduced to Pho85. To resolve this issue, the Pho85 cyclins responsible for this effect must be identified. It is important to note that these ESR genes are not constitutively expressed when *PHO85* is deleted from the genome, suggesting that activation of the ESR is eventually repressed. Some aspects of the pleiotropic phenotype caused by deletion of *PHO85* can be explained by the adaptive state that corrects for this induction of the ESR. The constitutive expression of ESR genes, such as *CTT1* and *GSY2*, resulting from *PHO85* deletion in some strain backgrounds [44], might be a consequence of the failure of these strains to adapt to induction of the ESR.

Regulation of Pcl–Pho85-containing complexes

At this juncture, substantially more effort has been directed towards the identification of substrates for Pcl–Pho85 complexes than towards the identification of regulatory factors that control Pcl–Pho85 complexes. As described above, Pho80–Pho85 complexes are bound and regulated by the CKI Pho81 [5]. Pho81 also binds Pcl7–Pho85 and regulates its activity based on extracellular phosphate levels, as determined by the ability of immunoprecipitated complexes to phosphorylate Pho4 *in vitro* [25]. Assessing the relevance of this regulation will require identification of a *bona fide in vivo* substrate targeted by Pcl7. As Pcl7 is regulated by both the cell cycle and phosphate levels, and is involved in carbon-source metabolism, its functions are probably complex [25]. Strangely, Pcl6, the closest homolog of Pcl7, shares only the carbon source utilization phenotype with Pcl7 [25].

There are several genes in the yeast genome with significant homology to Pho81; it remains to be seen whether any of these have a role in regulation of Pcl–Pho85 complexes. These putative regulatory domains might be easier to identify given the recent definition of the minimum domain of Pho81 required for Pho80–Pho85 regulation in response to phosphate levels [12]. It is possible that some Pcl–Pho85 complexes are regulated by other means; for example, by CKIs unrelated to Pho81 or by phosphorylation of the CDK subunit [45]. Structure and function studies have returned conflicting results regarding the functional consequences of Pho85 phosphorylation. Earlier studies showed that a conserved serine residue in the T-loop, phosphorylation of which is required for activity in other CDKs, is required for Pho85 activity [46]; however, phosphorylation of this residue has not been demonstrated *in vivo*. More recently, experiments have suggested that this same serine residue is dispensable for Pho85 activity [47]. The more recent study also demonstrated that phosphorylation of an N-terminal tyrosine, which is conserved among CDKs and is

usually involved in regulation of the kinase activity, regulates specificity of cyclin binding to Pho85 [47].

Conclusions

Conclusively demonstrating that a protein is an *in vivo* substrate of a Pho85 complex is not an easy task. Given that more than half of the proteins in the yeast genome contain at least one SP or TP dipeptide – the Pho85 target site – the range of *in vitro* substrates of Pho85 kinases could be large. The range of significant *in vivo* activities is probably much smaller. A strong indication that an *in vitro* substrate of a Pcl–Pho85 complex might have some *in vivo* relevance is a preference for phosphorylation by that specific Pcl–Pho85 complex over other Pcl–Pho85 complexes. At present, seven out of the ten Pcl–Pho85 complexes have been assayed for kinase activity and all are capable of phosphorylating Pho4 *in vitro* [4,23–26,35]. By contrast, only Pho80–Pho85 has any *in vivo* relevance for Pho4 regulation [4]. For example, Pcl10–Pho85 exhibits a 50-fold *in vitro* preference favoring its *in vivo* substrate Gsy2 over the purely *in vitro* substrate Pho4 [35]. The experiments that can conclusively demonstrate that an *in vitro* substrate is also an *in vivo* substrate – a tryptic phosphopeptide map of *in vivo* phosphorylated material and comprehensive Ser/Thr substitution mutation analysis – have so far only been performed with Pho4 [4].

The remaining challenges in the study of the functions of Pcl–Pho85 complexes include understanding issues of specificity, redundancy and regulation, and identifying a theme that links these functions together. All these functions might be thematically related to changes in the extracellular environment, possibly to the specific stress of starvation for an essential nutrient such as phosphate or a carbon source. We suggest that all known

functions of Pho85 (summarized in Table 1) involve the kinase activity signaling that the current conditions are satisfactory. Perhaps all the activities of Pho85 will exhibit constitutive kinase activity under 'normal' conditions in this manner, or perhaps some will require induction of Pho85 kinase activity by the presence of a particular activating signal. We do not yet know whether this trend will also apply to the Pcls that are additionally regulated by their abundance through the cell cycle. Furthermore, it is possible that signals activating or repressing certain Pcl–Pho85 complexes could be generated intracellularly, in the absence of any extracellular perturbation. It is also clear from the existing data that many downstream effects of Pho85-mediated phosphorylation are possible. Such phosphorylation can regulate the subcellular localization, enzymatic activity, cofactor association, and stability of the targets (Fig. 2). Two of the known Pho85 targets are transcription factors and, although the activities of Pho85 might have profound transcriptional consequences, there is no guarantee that such effects need be direct.

In the future, proteomic approaches could prove fruitful for addressing the abundant unresolved issues concerning Pho85. *In vitro* kinase assays might enable the identification of novel substrates of Pcl–Pho85 complexes, as well as providing information about substrate specificity and its mechanisms. Eventually, we might be able to assign a function of Pho85 to every Pcl, and correlate this assignment with a mechanistic understanding of effects on the substrate. Furthermore, we hope to understand the mechanism of regulation and to identify the activating signal for each Pcl–Pho85 complex. Such a comprehensive catalog of functions could reveal the advantages behind Pho85 being involved in multiple processes related to changes in the environment.

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