Phosphorylation of the Transcription Factor PHO4 by a Cyclin-CDK Complex, PHO80-PHO85

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Induction of the yeast gene *PHO5* is mediated by the transcription factors PHO2 and PHO4. *PHO5* transcription is not detectable in high phosphate; it is thought that the negative regulators PHO80 and PHO85 inactivate PHO2 and PHO4. Here it is reported that PHO80 has homology to yeast cyclins and interacts with PHO85, a p34$^{cdc2/CDC28}$-related protein kinase. The PHO80-PHO85 complex phosphorylates PHO4; this phosphorylation is correlated with negative regulation of *PHO5*. These results demonstrate the existence of a cyclin-cdk complex that is used for a regulatory process other than cell-cycle control and identify a physiologically relevant substrate for this complex.

The *PHO5* gene encodes a secreted acid phosphatase in *Saccharomyces cerevisiae* whose transcription is repressed when yeast are grown in high concentrations of inorganic phosphate. It is induced up to ~1000-fold in response to phosphate starvation (*1*). Genetic studies have identified three positive regulators (PHO2, PHO4, PHO81) and two negative regulators (PHO80, PHO85) of *PHO5* (*2–4*). PHO2 and PHO4, required for the activation of *PHO5* (*1*), are transcription factors that bind to the *PHO5* promoter (*5*); PHO2 is a homeodomain protein (*6, 7*) and PHO4 has a basic helix-loop-helix (bHLH) DNA-binding motif (*6, 8*). Both PHO80 and PHO85 are required for negative regulation; a deletion of either gene results in constitutive expression of *PHO5* (*2*). Genetic studies suggest that PHO4 may be the target of the negative regulators PHO80 and PHO85 (*9, 10*).

One clue to the function of the negative regulators comes from sequence analysis, which reveals that PHO85 is greater than 50% identical to CDC28 (*11*). CDC28 is a member of the highly conserved cyclin-dependent protein kinase (cdk) family whose members are involved in cell-cycle progression in all eukaryotes (*12*). To be active as protein kinases, cdk's require association with a cyclin subunit (*12*). The similarity between PHO85 and CDC28, and the requirement of both PHO80 and PHO85 for negative regulation, led us to the hypothesis that PHO80 might serve as a cyclin (a positive regulatory subunit required for activity) for the kinase PHO85.

To test whether PHO80 might be a cyclin, we reexamined its sequence. This analysis revealed that PHO80 is homologous to two yeast cyclins, HCS26 (*13*) and OrfD (*14*), over a ~120–amino acid cyclin homology region that contains residues conserved in all cyclins (*15*). PHO80 is 33% identical to either HCS26 or OrfD in this region (Fig. 1). In addition, PHO80, HCS26, and OrfD have similarity in regions within the cyclin homology domain that are not conserved in other cyclins, suggesting that they may form a cyclin subfamily.

Cyclin-cdk complexes have characteristic biochemical properties: The cyclin and cdk form a complex, and the complex, but not either protein alone, has protein kinase activity (*12*). To test biochemically whether PHO80 and PHO85 can form a functional cyclin-cdk complex, we first determined whether these two proteins can interact. Immunoprecipitation studies demonstrate that a PSTAIRE-reactive band presumed to be PHO85 (*16*) can be coimmunoprecipitated with epitope-tagged PHO80 (Fig. 2A, lane 2). In extracts derived from strains deleted for *PHO80* or *PHO85*, no PHO80-PHO85 complex is observed (Fig. 2A, lanes 3 and 4). In addition, the PHO80-PHO85 complex can interact with purified, recombinant PHO4 (Fig. 2A, lanes 1 and 7). In contrast, no interaction of PHO80 and PHO4 is observed under these experimental conditions in extracts derived from a strain lacking the *PHO85* gene (Fig. 2A, lane 6). Thus, PHO80 and PHO85 form a complex, and this complex, but not PHO80 alone, can interact with the transcription factor PHO4.

The observation that PHO4 can interact with the PHO80-PHO85 complex, and data demonstrating that PHO85 can phosphorylate casein (*17*), led us to test whether PHO4 might be a substrate for the PHO80-PHO85 complex. The immunopurified PHO80-PHO85 complex was assayed for protein kinase activity in vitro with the use of purified, recombinant PHO4 as a substrate. The PHO80-PHO85 complex efficiently phosphorylates PHO4 in vitro (Fig. 2B, lane 1). In contrast, immunoprecipitates derived from strains lacking either *PHO80* (Fig. 2B, lane 3) or *PHO85* (Fig. 2B, lane 5) have only background kinase activity (*18*). Thus, PHO80 and PHO85 have properties characteristic of cyclin-cdk complexes: They associate stably and the resulting complex has protein kinase activity that requires both subunits.

Our biochemical studies indicate that PHO80 and PHO85 act as a cyclin-cdk complex that phosphorylates the transcription factor PHO4 in vitro. We wished to determine whether the PHO80-PHO85 complex could also phosphorylate PHO4 in vivo. Genetic studies predict that the negative regulators PHO80 and PHO85 will act under repressing conditions (high phosphate), but not under inducing conditions (low phosphate) (*1*). To test the hypothesis that PHO80 and PHO85 might phosphorylate PHO4 under repressing conditions, we examined the phosphorylation state of PHO4 in vivo in our wild-type strain grown in low and high phosphate. Because hyperphosphorylated PHO4 exhibits retarded mobility in an SDS-acrylamide gel, the phosphorylation state of PHO4 can be assayed by immunoprecipitation followed by immunoblotting with an antibody to PHO4. We found that PHO4 is moderately phosphorylated in vivo even under induc-

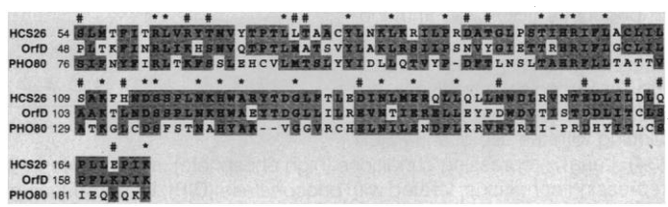

Fig. 1. PHO80 is similar to two yeast cyclins over a ~120–amino acid region in which all cyclins share homology. PHO80 is 33% identical when compared to OrfD and HCS26 at each position. Residues that are identical in all three proteins are marked (*), and those positions where PHO80 is identical to either OrfD or HCS26 are indicated (#). Similarity between the proteins is indicated by shading (*29*). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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ing conditions (low phosphate) (Fig. 3A, lane 3, and Fig. 3B, lane 5). However, when yeast cells are grown under repressing conditions (in high phosphate), PHO4 exhibits retarded mobility in an SDS-acrylamide gel, indicating that it becomes hyperphosphorylated (Fig. 3A, lane 1). This retarded mobility is the result of phosphorylation, because treatment with alkaline phosphatase restores the mobility of PHO4 to the same position as PHO4 isolated from cells grown under inducing conditions (Fig. 3A, lane 2). These data are consistent with the hypothesis that the PHO80-PHO85 complex phosphorylates PHO4 in vivo when yeast are grown under conditions that result in repression of *PHO5* transcription. Hyperphosphorylation of PHO4 in vivo under repressing conditions is *PHO80*- and *PHO85*-dependent; PHO4 protein isolated from strains deleted for either *PHO80* (Fig. 3B, lane 5) or *PHO85* (Fig. 3B, lane 10) is not hyperphosphorylated.

To determine whether the PHO4 phosphorylations observed in vitro are the same as those correlated with negative regulation of *PHO5* in vivo, we examined the phosphorylation state of PHO4 with the use of ^{32}P metabolic labeling experiments. Studies of the phosphorylation state of PHO4 in vivo with this method are complicated because efficient ^{32}P labeling requires that the yeast be grown in low-phosphate media, conditions that lead to induction of the *PHO5* gene in wild-type yeast strains. To circumvent this problem and study phosphorylation in the repressed state, we have made use of genetic work demonstrating that a strain deleted for the positive regulator *PHO81* is unable to induce *PHO5*, even when starved for phosphate (4). Genetic studies suggest that *pho81* mutants may be defective in sensing or transmitting the low-phosphate signal to the negative regulators *PHO80* and *PHO85* (*4*, *19*). To mimic the induced state, we used a strain that is constitutive for *PHO5* transcription because it lacks the *PHO80* gene. As assayed by mobility shift, the PHO4 protein derived from the *pho81* deletion strain (repressed for *PHO5* transcription), but not the *pho80* deletion strain (constitutive for *PHO5* expression), is hyperphosphorylated (*20*).

We used two-dimensional tryptic phosphopeptide analysis to map the changes in PHO4 phosphorylation that accompany growth in high phosphate. For each labeling reaction, ^{32}P-labeled PHO4 was digested with trypsin, the resulting peptides were separated by electrophoresis and thin-layer chromatography, and the phosphorylated peptides were identified by autoradiography (*21*). When *PHO5* is constitutively expressed in vivo (*pho80* deletion strain), PHO4 is phosphorylated predominantly on two tryptic peptides (Fig. 4A, peptides 4 and 5). In the strain that is repressed for *PHO5* transcription (*pho81* deletion strain), PHO4 contains four additional tryptic phosphopeptides (Fig. 4B, peptides 1, 2, 3, and 6). These four peptides are also observed in the tryptic map produced from

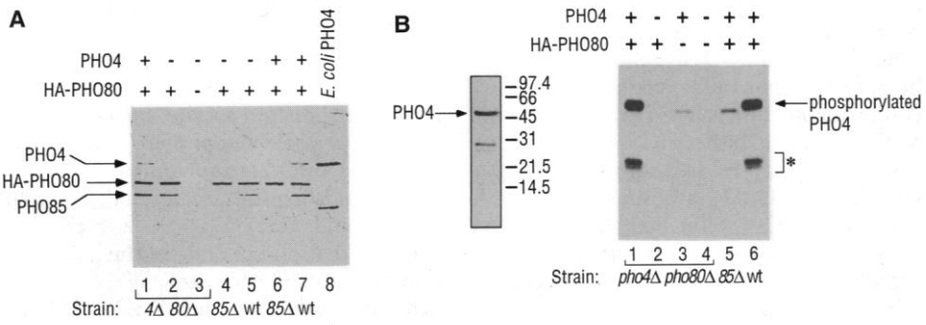

Fig. 2. PHO80 and PHO85 form a complex that phosphorylates PHO4. (**A**) Protein immunoblot of HA-PHO80 (*30*) immunoprecipitates (*27*) derived from lysates of the indicated strains (*31*). The blot was probed with anti-PHO4, anti-HA, and anti-PSTAIRE (*32*). Lane 1, *pho4Δ pho80Δ* overexpressing HA-PHO80 + purified, recombinant PHO4 (*26*); lane 2, *pho4Δ pho80Δ* overexpressing HA-PHO80 (no PHO4 added); lane 3, *pho4Δ pho80Δ* (no PHO4 added); lane 4, *pho85Δ* overexpressing HA-PHO80 (no PHO4 added); lane 5, wild-type overexpressing HA-PHO80 (no PHO4 added); lane 6, *pho85Δ* overexpressing HA-PHO80 + PHO4; lane 7, wild-type overexpressing HA-PHO80 + PHO4; lane 8, 200 ng of purified, recombinant PHO4. (**B**) HA-PHO80 was immunoprecipitated from the indicated strains and a kinase assay was performed on the immunoprecipitate (*33*) with purified, recombinant PHO4 as a substrate. A 12% SDS-acrylamide gel of purified, recombinant PHO4 (2.5 μg) stained with Coomassie blue is shown to the left of the kinase assays (*26*). Lane 1, *pho4Δ pho80Δ* overexpressing HA-PHO80 + purified, recombinant PHO4; lane 2, *pho4Δ pho80Δ* overexpressing HA-PHO80 (no PHO4 added); lane 3, *pho4Δ pho80Δ* + PHO4; lane 4, *pho4Δ pho80Δ* (no PHO4 added); lane 5, *pho85Δ* overexpressing HA-PHO80 + PHO4; lane 6, wild-type overexpressing HA-PHO80 + PHO4. The asterisk marks the position of a PHO4 truncation product that is phosphorylated.

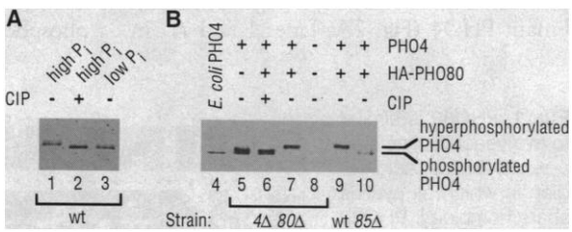

Fig. 3. Phosphorylation of PHO4 in vivo is *PHO80*- and *PHO85*-dependent and correlates with negative regulation of *PHO5*. (**A**) Endogenous PHO4 was immunoprecipitated from a wild-type strain (*31*) grown under repressing or inducing conditions for *PHO5* and analyzed by immunoblotting with the use of anti-PHO4 (*34*). Lane 1, repressing conditions (high phosphate), mock-treated with phosphatase (*35*); lane 2, repressing conditions, treated with phosphatase (CIP); lane 3, inducing conditions (low phosphate). (**B**) PHO4 was immunoprecipitated from the indicated strains (*31*) grown in high phosphate and was analyzed by immunoblotting with affinity-purified anti-PHO4 (*28*). Lane 4, purified, recombinant PHO4; lane 5, *pho4Δ pho80Δ* overexpressing PHO4; lane 6, *pho4Δ pho80Δ* overexpressing PHO4 and HA-PHO80, treated with phosphatase (CIP) (*35*); lane 7, *pho4Δ pho80Δ* overexpressing PHO4 and HA-PHO80, mock treated with phosphatase; lane 8, *pho4Δ pho80Δ*; lane 9, wild-type overexpressing PHO4 and HA-PHO80; lane 10, *pho85Δ* overexpressing PHO4 and HA-PHO80.

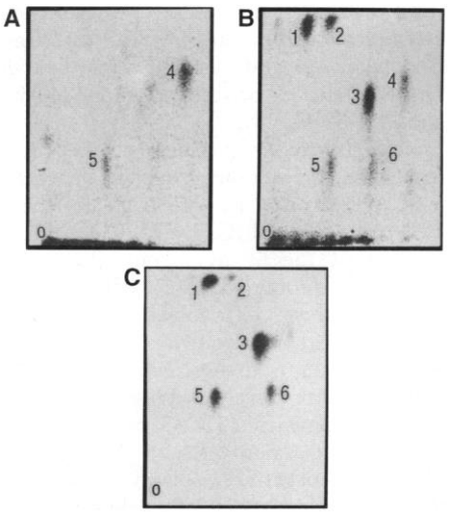

Fig. 4. Two-dimensional tryptic phosphopeptide analysis (*36*) indicates that the *PHO80*-dependent phosphorylations of PHO4 produced by the PHO80-PHO85 complex are similar in vivo and in vitro. (**A**) PHO4 from strain constitutive for *PHO5* expression (*pho4Δ pho80Δ* overexpressing PHO4). (**B**) PHO4 from strain repressed for *PHO5* expression (*pho81Δ* overexpressing HA-PHO80 and PHO4). (**C**) PHO4 phosphorylated in vitro with the immunopurified PHO80-PHO85 complex. Electrophoresis was performed in the horizontal dimension and chromatography in the vertical dimension. The origin is indicated (o).

PHO4 phosphorylated in vitro with the immunopurified PHO80-PHO85 complex (Fig. 4C). Thus, *PHO5* repression is correlated with hyperphosphorylation of PHO4 by the PHO80-PHO85 complex.

Our biochemical data, combined with previous genetic studies, suggest the following model for negative regulation of *PHO5* (Fig. 5). Under repressing conditions (high phosphate), PHO80 and PHO85 form a cyclin-cdk complex that phosphorylates, and presumably inactivates, the transcription factor PHO4. This model explains the observation that negative regulation requires both *PHO80* and *PHO85* (*1, 2*). It is also consistent with genetic data suggesting that PHO80 and PHO85 may interact (*22*) and that PHO4 is the target of PHO80 (*9, 10*). It is unknown how phosphorylation affects the function of PHO4; it could prevent DNA binding or affect the interaction of PHO4 with other regulators of *PHO5* or with the transcription preinitiation complex.

When *PHO5* is induced by phosphate starvation, we observed that PHO4 is not hyperphosphorylated by the PHO80-PHO85 complex. Two simple models could explain this observation. (i) The PHO80-PHO85 complex could be inactivated when yeast are starved for phosphate. This inactivation could occur as a result of posttranslational modification of PHO85 or degradation of PHO80 (*23*). Alternatively, a molecule that inactivates the PHO80-PHO85 complex could be induced upon phosphate starvation. One candidate for such a gene is the positive regulator *PHO81* (Fig. 5). Epistasis studies indicate that *PHO81* lies upstream of *PHO80* and *PHO85* (*1, 4*). In addition, *PHO81* is the only *PHO* regulatory gene whose transcription is induced in response to phosphate starvation (*9, 19*). Thus, *PHO81* could function by inactivating the PHO80-PHO85 complex, for example, by binding to, modifying, or disrupting it. (ii) The activity of the PHO80-PHO85 complex may be unaffected by phosphate levels, and a phosphatase that dephosphorylates PHO4 could be induced when cells are starved for phosphate. In this model PHO80-PHO85 is constitutively active, and induction of *PHO5* is triggered by a phosphatase that dephosphorylates PHO4. PHO81 is a candidate for such a phosphatase, although it does not appear to have homology to known phosphatases (*19*).

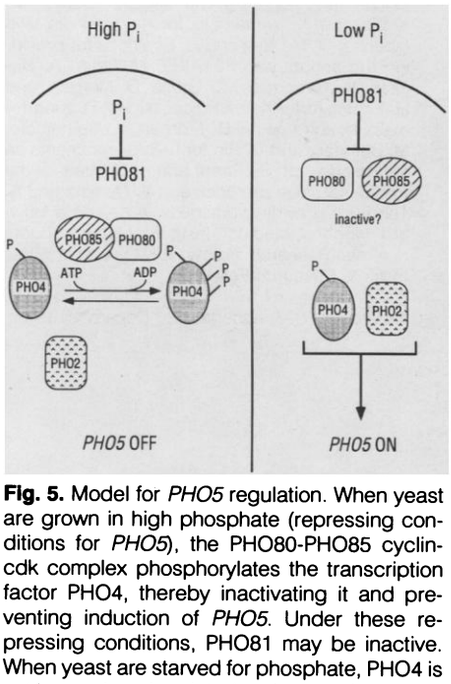

Fig. 5. Model for *PHO5* regulation. When yeast are grown in high phosphate (repressing conditions for *PHO5*), the PHO80-PHO85 cyclin-cdk complex phosphorylates the transcription factor PHO4, thereby inactivating it and preventing induction of *PHO5*. Under these repressing conditions, PHO81 may be inactive. When yeast are starved for phosphate, PHO4 is not hyperphosphorylated and can activate transcription of *PHO5* in conjunction with PHO2. Under inducing conditions, PHO81 may inactivate the PHO80-PHO85 complex.

The extended homology between PHO80 and the cyclins HCS26 and Orf D is intriguing and may reflect functional similarity; preliminary data suggest that HCS26 may also use PHO85 as its catalytic subunit (*24*). Thus, PHO85 appears to be the catalytic subunit for two cyclins involved in different regulatory processes: PHO80, used for phosphate metabolism, and HCS26 (*13*), a cyclin that appears to be involved in some aspect of cell-cycle regulation. Perhaps PHO85 provides a link between cell-cycle regulation and phosphate metabolism. For example, if the activity of the PHO80-PHO85 complex is controlled by modification of PHO85, this modification may also affect the activity of the HCS26-PHO85 complex. Many cdk and cyclin-like molecules of unknown function have been identified in eukaryotic cells; like the PHO80-PHO85 complex, some of these cyclin-cdk complexes probably regulate processes other than the cell cycle (*25*).

It is likely that PHO80-PHO85 and HCS26-PHO85 will have different substrate specificities (*24*). The identification of a physiologically relevant substrate for the PHO80-PHO85 complex provides an experimental opportunity to study the enzymology of a cyclin-cdk complex.

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16. On the basis of the following criteria the PSTAIRE-reactive band is presumed to be PHO85: (i) PHO85 and CDC28 are the only PSTAIRE-containing kinases known in yeast; (ii) the band is the appropriate size to be PHO85 (the band migrates at ~33 to 34 kD and the predicted molecular size of PHO85 is 34,898 daltons); (iii) the PSTAIRE-reactive band is missing in the *pho85* deletion strain (Fig. 2A, lanes 4 and 6).
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18. The background kinase activity observed (Fig. 2B, lanes 3 and 5) is nonspecifically associated with the protein A–Sepharose beads. We have not been able to eliminate this background by preincubating with protein A–Sepharose.
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23. S. I. Reed, *Annu. Rev. Cell. Biol.* **8**, 529 (1992); M. J. Solomon, *Curr. Opin. Cell Biol.* **5**, 180 (1993).
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26. *Escherichia coli* (BL21) harboring the PHO4 expression vector T7-PHO4 were grown in 200 ml of LB containing ampicillin (0.1 mg/ml) to an absorbance at 600 nm (A_{600}) of ~0.4 and induced with 0.4 mM isopropyl β-D-thiogalactopyranoside for 2 hours. Cells were harvested, washed with RB 0.1 buffer [RB buffer: 20 mM tris-acetate (pH 7.9), 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol (DTT), 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF) + 0.1 M potassium acetate], and lysed by resuspending in 5 ml of RB 0.1 and sonicating. Debris was sedimented in an SS34 rotor at 10,000 rpm for 20 min at 4°C, and the supernatant was loaded at a flow rate of 40 ml/hour onto a 10-ml DEAE FF Sepharose column equilibrated with RB 0.1. The column was washed with 40 ml of RB 0.1, and PHO4 was eluted with RB 1.0 buffer (RB + 1.0 M potassium acetate). Fractions containing PHO4 were collected as S Sepharose load. The conductivity of the S Sepharose load was adjusted to match RB 0.3 (RB + 0.3 M potassium acetate) and was loaded at 20 ml/hour onto a 5-ml SP Sepharose FF column equilibrated with RB 0.3. The column was washed with 20 ml of RB 0.3 and eluted with 20 ml of RB 1.0. Fractions containing PHO4 were collected to give a total of ~2 mg of PHO4. The PHO4 is greater than 80% pure as judged by SDS–polyacrylamide gel electrophoresis (Fig. 2B). Although the calculated molecular size of PHO4 is 33,856 daltons, purified, recombinant PHO4 migrates with an apparent molecular size of ~49 kD in an SDS gel (Fig. 2B). The identity of the purified

PHO4 was confirmed by both NH_2-terminal sequencing (which indicates that the NH_2-terminal Met is cleaved off) and electrospray mass spectrometry [calculated molecular weight (MW), 33,856; observed MW, 33,857].

27. Fifty milliliters of cell culture grown to an A_{600} of ~1 were harvested and washed with 5 ml of phosphate-buffered saline (PBS). All subsequent steps were carried at 4°C. Cells were resuspended in 350 μl of HSB buffer [45 mM Hepes-KOH (pH 7.5), 400 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5% NP-40, 2 mM DTT, 2 mM benzamidine, 1 mM PMSF, leupeptin (1 μg/ml), pepstatin (1 μg/ml), 80 mM β-glycerophosphate, 10 mM NaF, 10 nM calyculin A], and acid-washed glass beads were added to bring the final volume to 1.2 ml. Cells were lysed on a horizontal bead beater (Eppendorf Mixer 5432) for 30 min and the supernatant was transferred to a new tube and spun in a microfuge for 5 min. Supernatant was transferred again to a new tube and spun in a microfuge for 15 min. One hundred and fifty microliters of lysate (~2.5 mg of total protein) were incubated for 1 hour either with or without 800 ng of PHO4 purified from *E. coli* (*26*). The reaction was then incubated with 20 μl of protein A–Sepharose coupled to antibody to HA (anti-HA) for 1 hour. The beads were washed four times with 200 μl of PBS [150 mM NaCl, 10 mM Na phosphate (pH 7.4)] + 1% NP-40 and once with 200 μl of PBS. SDS sample buffer containing 2-mercaptoethanol was added, and the samples were boiled for 5 min and then loaded onto a 12% SDS-acrylamide gel. After transfer to nitrocellulose, the blot was probed sequentially with anti-PSTAIRE, anti-HA, and affinity-purified anti-PHO4 in TBST [10 mM tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20, 0.25 mM EDTA] containing 3% milk. Alkaline phosphatase coupled to anti-rabbit or anti-mouse immunoglobulin G was used as secondary antibody.
28. Cell lysate was prepared (*27*), and 300 μl of lysate (~5 mg of total protein) were incubated with 30 μl of protein A–Sepharose coupled to anti-PHO4 for 1 hour. The beads were washed four times with 200 μl of washing buffer [0.1% NP-40, 1 mM DTT, 2 mM benzamidine, 1 mM PMSF, leupeptin (1 μg/ml), pepstatin (1 μg/ml) in PBS] and once with 200 μl of PBS. Samples were prepared, loaded on a 10% polyacrylamide minigel (Hoefer SE250), and run at 15-mA constant current with circulating cooling water. After transfer to nitrocellulose, the immunoblot was probed with affinity-purified anti-PHO4 in TBST containing 3% milk. Protein A coupled to peroxidase was used as the secondary antibody.
29. The rules used for determining similarity are as follows: A, V, I, L, M, and F, hydrophobic residues; F, Y, and W, aromatic residues; T and S, polar neutral residues; R and K, basic residues; D and E, acidic residues.
30. HA-PHO80 consists of the PHO80 sequence [A. Toh-e and T. Shimauchi, *Yeast* **2**, 129 (1986)] preceded by the hemagglutinin epitope tag [I. Wilson *et al.*, *Cell* **37**, 767 (1984)] and a factor Xa cleavage site (the sequence IEGR). The NH_2-terminal sequence of HA-PHO80 reads MGYPY-DVPDYAIEGRHM-PHO80. Expression of HA-PHO80 is driven by the *GPD* promoter on a 2μ plasmid carrying *URA3* [M. Schena, D. Picard, K. R. Yamamoto, *Methods Enzymol.* **194**, 389 (1991)]. HA-PHO80 complements a *pho80Δ* strain for the *PHO5* phenotype, as assayed by phosphatase plate assay (*4*).
31. All strains are isogenic derivatives of Y57 (*mat***a** *ura3-52 trp1-Δ63 leu2 his3-Δ1 prb1-1122 pep4-3 prc1-407 GAL*$^+$), which is protease-deficient but wild type with respect to the *PHO* genes. The *PHO5* gene is induced in Y57 by phosphate starvation (*20*), as assayed by primer extension and by phosphatase plate assay (*4*). Deletion strains were constructed by one-step gene replacement techniques [R. Rothstein, *Methods Enzymol.* **194**, 281 (1991)].
32. Anti-HA is the monoclonal antibody 12CA5 [I. Wilson *et al.*, *Cell* **37**, 767 (1984)] (obtained from BAbCo, Inc., Richmond, CA). Anti-PHO4 is polyclonal serum derived from rabbits immunized with purified, recombinant PHO4 protein (*26*). Anti-PHO4 was affinity-purified from serum with the use of a PHO4 column made by coupling of purified, recombinant PHO4 to Affigel-10 (Bio-Rad Laboratories). Anti-PSTAIRE is anti-peptide antibody directed against the PSTAIRE sequence that is highly conserved in cdk's [S. K. Hanks and A. M. Quinn, *Methods Enzymol.* **200**, 38 (1991)].
33. Cell lysates were prepared and HA-PHO80 was immunoprecipitated with anti-HA coupled to protein A–Sepharose (*27*). The immunoprecipitate was washed with 400 μl of kinase buffer [20 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$]. Twenty microliters of kinase cocktail [20 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 μCi of [γ-^{32}P]adenosine triphosphate (ATP), 100 μM ATP, purified PHO4 (20 ng/μl)] were added to each reaction and incubated at room temperature for 20 min. Reactions were stopped by the addition of SDS sample buffer, boiled for 5 min, and loaded onto a 12% SDS-acrylamide gel. Bands were visualized with the use of a PhosphorImager (Molecular Dynamics).
34. A 5-ml culture of wild-type strain Y57 was grown overnight in high P_i-YD {YEPD media depleted for inorganic phosphate [G. M. Rubin, *J. Biol. Chem.* **248**, 3860 (1973)] and supplemented with 11 mM KH_2PO_4} or in 3/5 high P_i-YD (3 ml of high P_i-YD, 2 ml of P_i-depleted YEPD). Seventy microliters of 3/5 high P_i-YD overnight were used to inoculate 100 ml of P_i-depleted YEPD, and 30 μl of high P_i-YD overnight were used to inoculate 100 ml of high P_i-YD. The cultures were grown for ~20 hours to an A_{600} of ~1, and liquid phosphatase assays were performed [J. M. Lemire, T. Willcocks, H. O. Halvorson, K. A. Bostian, *Mol. Cell. Biol.* **5**, 2131 (1985)] to compare the levels of acid phosphatase activity in low and high phosphate media. A ratio of ~1:14 was obtained (Y57 grown in high P_i as compared with low P_i media). Lysates were prepared as described (*27*) except that cells were washed with H_2O instead of PBS before lysis. Samples were prepared and electrophoresed on a 10% SDS-acrylamide gel (*28*).
35. Samples to be treated with calf intestinal phosphatase (CIP) were washed with 200 μl of CIP buffer [50 mM tris-HCl (pH 8.8), 1 mM $ZnCl_2$, 2 mM DTT, 1 mM PMSF, pepstatin (1 μg/ml), leupeptin (1 μg/ml)] and resuspended in 100 μl of the same buffer. Forty units of CIP (Boehringer Mannheim, 20 U/μl) were added to the samples indicated, and the reactions were incubated at 37°C for 30 min. Mock treated samples were incubated at 37°C, but without added CIP. Samples were prepared and electrophoresed on a 10% SDS-acrylamide gel (*28*).
36. One hundred–milliliter cultures of each strain (*pho4Δ pho80Δ* overexpressing PHO4, *pho81Δ* overexpressing HA-PHO80 and PHO4) were grown in selective media containing high phosphate to an A_{600} of ~0.7. Cells were harvested, washed once with phosphate-free media (*4*), resuspended in 100 ml of phosphate-free selective media, and incubated at 30°C for 30 min. Cells were harvested, washed once with phosphate-free media, resuspended in 5 ml of phosphate-free media plus 5 mCi of [^{32}P]orthophosphate, and incubated at 30°C for 2 hours. Cells were harvested, washed once with PBS, and lysed as described (*27*). PHO4 was immunoprecipitated from these lysates (*28*) and electrophoresed on a 12% SDS-acrylamide gel. PHO4 was eluted from the gel, digested with 10 μg of sequencing-grade trypsin for 4 to 8 hours at 37°C in 50 mM ammonium bicarbonate (5 μg of trypsin was then added and the samples were digested for 4 to 8 hours more), and samples were prepared and analyzed by two-dimensional phosphopeptide analysis (*21*). The samples were electrophoresed for 45 min at 1000 V on Kodak Chromagram cellulose thin-layer chromatography plates (Kodak 13255) in pH 1.9 buffer (22 ml of 88% formic acid, 78 ml of acetic acid, 900 ml of H_2O). Chromatography was performed in isobutyric acid buffer (65 ml of isobutyric acid, 2 ml of butanol, 5 ml of pyridine, 3 ml of acetic acid, 29 ml of H_2O) until the buffer front was ~1 inch from the top of the plate. Plates were dried and phosphorylated peptides were detected with the use of a PhosphorImager (Molecular Dynamics).

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