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30. The following cell lines were used: Mutant KI-8 has reduced guanylyl cyclase activity and is derived from wild-type strain XP55 (*18*); the myosin II null mutant *mhc*$^-$ (strain HS2205); transformant *mhc*^{wt} in which wild-type myosin II is expressed in *mhc*$^-$ cells (strain HG1554); and transformant *mhc*^{AAA} expressing in *mhc*$^-$ cells a mutant myosin II with the three threonine phosphorylation sites mutated to alanine residues (strain HG1555) (*9*). Cells were cultivated in tissue culture plate in HL5 defined medium (*mhc*$^-$, *mhc*^{wt}, *mhc*^{AAA}, and AX2) (*7*) or on 1/3 SM agar plates [0.3% glucose, 0.3% peptone, 1.5% agar, and 40 mM KH_2PO_4-Na_2HPO_4 (pH 6. 0)] with *Escherichia coli* B/r (KI-8 and XP55). The cells were removed from the plates with phosphate buffer [10 mM K_2HPO_4-NaH_2PO_4 (pH 6. 5) (Fig. 1, A and B)] or MES buffer [20 mM MES, 2 mM $MgSO_4$, 0. 2 mM $CaCl_2$ (pH 6.15) (Fig. 1C)]. Cells were shaken in these buffers at a density of 10^7 cells per milliliter for 1 hour, washed three times, and resuspended in the respective buffers at 10^7 cells per milliliter (Fig. 1A) or 10^8 cells per milliliter (Fig. 1, B and C), and then stimulated with 300 mM glucose. Survival was measured by 1000-fold dilution of cells at the time indicated, followed by plating 200 cells on a 1/3 SM plate with *E. coli* and counting colonies several days later. Intracellular accumulation of cGMP was measured by isotope dilution assay (*27*). Phosphorylation of myosin II heavy chain was determined by labeling cells with ^{32}P, lysis of the cells at the time indicated, followed by immunoprecipitation of myosin with monoclonal antibody JIG-3 to myosin II as described (*15*).
31. In (A), cells were incubated with 300 mM glucose for 30 min in the absence or presence of 1 mM 8Br-cGMP. The survival of XP55, KI-8, *mhc*^{wt}, *mhc*$^-$, and *mhc*^{AAA} cells was determined as described in Fig. 1. In (B), phosphorylation of myosin II heavy chain was induced in XP55 and KI-8 cells upon incubation with 300 mM glucose with or without 1 mM 8Br-cGMP.
32. Cells were starved for 1 hour in phosphate buffer, incubated in the presence of 300 mM glucose for 10 min and 30 min, and fixed with 15% picric acid and 2% formaldehyde in 10 mM Pipes-HCl (pH 6.0) (*28*). The fixed cells were incubated simultaneously with fluorescein isothiocyanate–conjugated phalloidin to stain actin filaments and with monoclonal antibody 396 to myosin II [which recognizes both myosin II monomers and filaments (*29*)] plus tetramethyl rhodamine isothiocyanate–conjugated goat antibody to mouse immunoglobulin G. Confocal sections of cells were taken with a 40× Plan-Neofluar objective on a Zeiss LSM 410 fluorescence laser-scanning microscope; the images were color-labeled to red (myosin II) and green (actin).
33. We thank P. C. Newell for monoclonal antibody JIG-3 and M. Oyama for useful comments. The *mhc*^{AAA} transformant was produced by D. Vielmetter in collaboration with J. A. Spudich, and KI-8 was isolated by H.K. in collaboration with S. Ishida. Supported in part by grant SFB266/D7 of the Deutsche Forschungsgemeinschaft.

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Regulation of PHO4 Nuclear Localization by the PHO80-PHO85 Cyclin-CDK Complex

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PHO4, a transcription factor required for induction of the *PHO5* gene in response to phosphate starvation, is phosphorylated by the PHO80-PHO85 cyclin-CDK (cyclin-dependent kinase) complex when yeast are grown in phosphate-rich medium. PHO4 was shown to be concentrated in the nucleus when yeast were starved for phosphate and was predominantly cytoplasmic when yeast were grown in phosphate-rich medium. The sites of phosphorylation on PHO4 were identified, and phosphorylation was shown to be required for full repression of *PHO5* transcription when yeast were grown in high phosphate. Thus, phosphorylation of PHO4 by PHO80-PHO85 turns off *PHO5* transcription by regulating the nuclear localization of PHO4.

The transcription of *PHO5*, which encodes a secreted acid phosphatase, is tightly repressed when *Saccharomyces cerevisiae* are grown in phosphate-rich medium, and its induction is more than 100-fold increased when yeast are starved for phosphate (*1*). Transcriptional induction of *PHO5* requires the transcription factor PHO4, and correlative evidence suggests that PHO4 activity is negatively regulated by phosphorylation (*2*). When yeast are grown in phosphate-rich medium, the PHO80-PHO85 cyclin-CDK (cyclin-dependent kinase) complex phosphorylates PHO4 (*2*) and transcription of *PHO5* is repressed. When yeast are starved for phosphate, the kinase activity of the PHO80-PHO85 complex is down-regulated by the CDK inhibitor PHO81 (*3*). This reduced activity results in the appearance of an underphosphorylated form of PHO4 (*2*) that, in combination with a second transcription factor, PHO2, binds to the *PHO5* promoter and activates *PHO5* transcription (*4*).

We wished to determine how changes in phosphate availability affect PHO4 function. Phosphate starvation does not have a large effect on PHO4 stability; no difference in the amount of PHO4 is observed between yeast grown in low versus high phosphate medium (*2*). Because PHO4 occupies binding sites in the *PHO5* promoter in vivo under inducing, but not repressing, conditions (*5*), the phosphate signal is likely to affect PHO4 function at the level of DNA binding or some prior step, such as nuclear localization. Preliminary data suggest that phosphorylated and unphosphorylated PHO4 have a similar affinity for DNA (*6*).

We therefore examined the subcellular localization of PHO4 in wild-type cells grown in low or high phosphate medium. PHO4 is concentrated in the nucleus when yeast are starved for phosphate and is largely cytoplasmic when yeast are grown in phosphate-rich medium (Fig. 1, A to D). In *pho80*Δ and *pho85*Δ strains, in which PHO4 is not phosphorylated (*2*) and which express *PHO5* constitutively (*1*), PHO4 was concentrated in the nucleus, even when the strains were grown in phosphate-rich medium (*6*). In contrast, PHO4 was predominantly cytoplasmic in a *pho81*Δ strain grown in high or low phosphate conditions (*6*). In this strain, PHO4 is phosphorylated even when yeast are starved for phosphate (*2*),

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and *PHO5* expression is uninducible (*1*). These data demonstrate that the PHO signal transduction pathway is required for properly regulated nuclear localization of PHO4.

Our next goal was to determine whether phosphorylation was required for the regulation of PHO4 activity. We therefore wished to identify the residues of PHO4 that are phosphorylated by PHO80-PHO85. Several observations guided our efforts to identify these sites of phosphorylation: (i) Phosphoamino acid analysis indicated that PHO4 is phosphorylated exclusively on serine residues (Fig. 2A); and (ii) PHO4 has no perfect matches to the CDK phosphorylation site consensus Ser/Thr-Pro-X-Lys/Arg (*7*) (where X is any amino acid), but does contain six Ser-Pro dipeptide sequences (Fig. 2B). We tested whether these six Ser-Pro dipeptides are sites of phosphorylation by PHO80-PHO85. The observation that the PHO80- and PHO85-dependent phosphorylation sites observed in vitro and in vivo are similar (*2*) allowed us to use PHO4 phosphorylated in vitro by PHO80-PHO85 for the experiments described here. We generated a mutated version of PHO4 with six serine to alanine changes in the Ser-Pro dipeptides, called $PHO4^{SA1-6}$. We analyzed this mutant by tryptic phosphopeptide analysis, which revealed that all of the major phosphopeptides were missing from the $PHO4^{SA1-6}$ map (Fig. 2C; compare $PHO4^{SA1-6}$ with $PHO4^{WT}$). These data suggest that the major sites of phosphorylation on PHO4 are among the six Ser-Pro dipeptides.

To determine which among these six dipeptide sequences are sites of phosphorylation, we analyzed a series of six mutants, each with a single serine residue mutated to alanine ($PHO4^{SA1}$ to $PHO4^{SA6}$). Comparison of the tryptic phosphopeptide map of wild-type PHO4 with those of mutants $PHO4^{SA1}$ and $PHO4^{SA5}$ indicated that no peptides were missing from the phosphopeptide maps of these mutants (Fig. 2C). In contrast, the tryptic phosphopeptide maps of mutants $PHO4^{SA2}$, $PHO4^{SA3}$, $PHO4^{SA4}$, and $PHO4^{SA6}$ (Fig. 2C) had at least one altered or missing phosphopeptide (*8*), suggesting that Ser-Pro dipeptides 2, 3, 4, and 6 are sites of phosphorylation by PHO80-PHO85 and that mutation of serine to alanine prevents phosphorylation of that site. An alternative explanation is that mutation of serine to alanine at one or more of these sequences affects phosphorylation of a different serine.

To distinguish between these explanations, we used a second approach that exploits the observation that CDKs phosphorylate threonine as well as serine (*7*). Six PHO4 mutants were generated, each containing a single serine to threonine change in Ser-Pro dipeptides 1 through 6 ($PHO4^{ST1}$ to $PHO4^{ST6}$), and analyzed by phosphoamino acid analysis (Fig. 2A). Wild-type PHO4 was phosphorylated only on serine (Fig. 2A); thus, if a phosphorylation site is mutated to threonine, phosphoamino acid analysis should reveal both phosphoserine and phosphothreonine. Phosphothreonine was present in $PHO4^{ST1}$, $PHO4^{ST2}$, $PHO4^{ST3}$, $PHO4^{ST4}$, and $PHO4^{ST6}$, but not in $PHO4^{ST5}$ (Fig. 2A). Thus, both the tryptic phosphopeptide analysis and phosphoamino acid analysis suggest that Ser-Pro dipeptides 2, 3, 4, and 6 are sites of phosphorylation. In the case of Ser-Pro dipeptide 1, some phosphothreonine was detected in the phosphoamino acid analysis of mutant $PHO4^{ST1}$, yet no difference was observed between the tryptic phosphopeptide maps generated with $PHO4^{SA1}$ and wild-type PHO4 (*9*).

To determine whether Ser-Pro dipeptide 1 is a site of phosphorylation, we generated two additional mutants, $PHO4^{SA2346}$ and $PHO4^{SA12346}$, in which four or five of the serine residues were changed to alanine. These two mutants, in addition to $PHO4^{SA1-6}$ and wild-type PHO4, were subjected to phosphorylation in vitro by PHO80-PHO85, and the total amount of phosphate incorporated into each protein was determined and compared with that incorporated into wild-type PHO4 (Fig. 3). $PHO4^{SA12346}$ and $PHO4^{SA1-6}$ each incorporated 4% of the phosphate incorporated by wild-type PHO4, whereas $PHO4^{SA2346}$

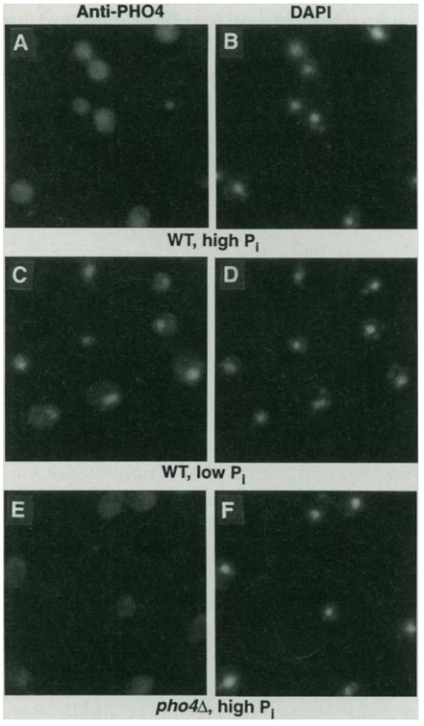

Fig. 1. Localization of the PHO4 protein. Indirect immunofluorescence was performed with PHO4 antiserum to determine the subcellular localization of PHO4 (*17*). Each pair of panels shows the same field; the left panel of each pair shows the PHO4 staining pattern, and the right panel shows DAPI staining (to visualize nuclei). The genotype of each strain (*18*) and the type of medium (*19*) in which it was grown are as follows: (**A** and **B**) a wild-type strain grown in phosphate-rich medium; (**C** and **D**) a wild-type strain grown in phosphate-depleted medium; (**E** and **F**) a *pho4*Δ strain grown in phosphate-rich medium.

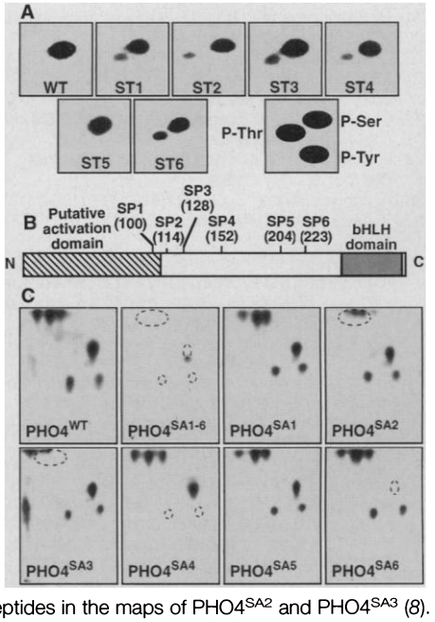

Fig. 2. Identification of the sites of phosphorylation on PHO4. (**A**) Phosphoamino acid analysis (*20*) of wild-type PHO4 or mutant PHO4 proteins with single serine to threonine substitutions at putative phosphoacceptor residues. Analysis was performed with protein phosphorylated in vitro by PHO80-PHO85. Shown in the lower right is a schematic diagram representing ninhydrin visualization of the migration of standards used for phosphoamino acid analysis: phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr). (**B**) Schematic diagram of the PHO4 protein. The amino acid position of the serine residue in each Ser-Pro dipeptide is indicated (SP1 to SP6) (*21*). The basic helix-loop-helix domain, which contains both the DNA binding and dimerization domains of PHO4, consists of residues 251 to 309 (*22*). A putative transcription activation domain consists of residues 1 to 109 (*23*). (**C**) Tryptic phosphopeptide analysis of wild-type PHO4 or mutant PHO4 proteins with serine to alanine substitutions at putative phosphoacceptor residues. Proteins were phosphorylated in vitro by PHO80-PHO85 (*24*). Ser-Pro dipeptides 2 and 3 are contained in the same tryptic peptide, which results in a complicated pattern of phosphopeptides in the maps of $PHO4^{SA2}$ and $PHO4^{SA3}$ (*8*). Dashed lines indicate missing or altered phosphopeptides; the large dashed ellipse marks where the tryptic peptides derived from Ser-Pro dipeptides 2 and 3 were located in the wild-type map. The origin is in the lower left corner of each panel. Samples were electrophoresed in the horizontal dimension and chromatography was performed in the vertical dimension.

incorporated 9%. From these data, we conclude that Ser-Pro dipeptide 1 is a site of phosphorylation and that Ser-Pro dipeptides 1, 2, 3, 4, and 6 represent the predominant sites of phosphorylation by PHO80-PHO85 (*10*). This result is consistent with mass spectrometry data that demonstrate that a considerable fraction of wild-type PHO4 phosphorylated in vitro contains five phosphate groups (*11*).

To test directly whether phosphorylation of PHO4 is required for it to be localized in the cytoplasm, we examined the subcellular localization of the $PHO4^{SA12346}$ mutant expressed from a plasmid in a *pho4*Δ strain grown in phosphate-rich medium (Fig. 4, A and B). We observed that all $PHO4^{SA12346}$ visible above background levels was concentrated in the nucleus. By contrast, wild-type PHO4 expressed from a plasmid in the same strain was predominantly cytoplasmic (6). These data demonstrate that phosphorylation of PHO4 is required for it to be localized in the cytoplasm under repressing (high phosphate) conditions.

To determine whether the regulated nuclear localization of PHO4 is sufficient for proper regulation of *PHO5* expression, we examined acid phosphatase activity in yeast expressing the $PHO4^{SA12346}$ mutant. This strain expresses *PHO5* constitutively in phosphate-rich medium (Fig. 4C), though the level of *PHO5* expression is only 10% of the fully induced level observed with wild-type PHO4 in phosphate-depleted medium (*12*). We also examined the ability of $PHO4^{SA12346}$ to induce *PHO5* expression under conditions of phosphate starvation. This mutant form of PHO4 activated *PHO5* expression to 42% of the level observed for wild-type PHO4 (Fig. 4C), suggesting that this mutated form of PHO4 is mildly defective as a transcriptional activator. These data suggest that whereas phosphorylation of PHO4 is important for full repression of *PHO5*, the PHO pathway may also control another aspect of PHO4 activity or *PHO5* regulation.

We have described an important mechanism by which the activity of the transcription factor PHO4 is regulated by the PHO80-PHO85 cyclin-CDK complex in response to extracellular phosphate levels. A different mechanism was proposed, based on data obtained from two-hybrid analysis of PHO4 and PHO80 (*13*), in which PHO80 binds to PHO4 and masks its transcription activation domain. We believe that although the PHO4-PHO80 interaction may regulate PHO4 activity under certain conditions, it is not a physiologically relevant mechanism for the following reasons. (i) PHO4 is not bound to its sites in the *PHO5* promoter in vivo when yeast are grown in high-phosphate medium (*5*). If PHO4 is not bound to the *PHO5* promoter under these conditions, an interaction that masks its activation domain is not likely to be relevant for *PHO5* regulation. (ii) The two-hybrid experiments were performed with highly overexpressed PHO4, a condition that results in accumulation of PHO4 in the nucleus (6) and in constitutive expression of *PHO5* in high phosphate medium (*14*). We believe that the masking model may be relevant only under conditions that result in the accumulation of PHO4 in the nucleus in high phosphate medium, such as when PHO4 is overexpressed or in yeast expressing the $PHO4^{SA12346}$ mutant.

This type of masking may be responsible for the incomplete derepression of *PHO5* (only 10%) observed with yeast expressing $PHO4^{SA12346}$ grown in phosphate-rich medium. Furthermore, the ability of PHO80 to interact with PHO4 could be regulated by the CDK inhibitor PHO81, which would explain the observation that yeast expressing $PHO4^{SA12346}$ are still partially responsive to phosphate starvation.

The mechanism regulating PHO4 localization is similar to the mechanism by which the yeast transcription factor SWI5 is regulated by the CDK CDC28 (*15*), suggesting that CDKs may regulate the subcellular localization of many different proteins by phosphorylation. In contrast to PHO4, the physiological relevance of regulated nuclear localization of SWI5 is unclear, because a mutated form of SWI5 that is in the nucleus at all stages of the cell cycle does not cause inappropriate expression of the SWI5 target gene *HO* (*15*).

A lack of physiologically relevant cyclin-CDK substrates has hampered efforts to determine the mechanisms by which these important kinases regulate cell cycle events. This description of how PHO80-PHO85 acts to regulate the activity of PHO4 provides an important insight into how phosphorylation by cyclin-CDK complexes can affect protein function.

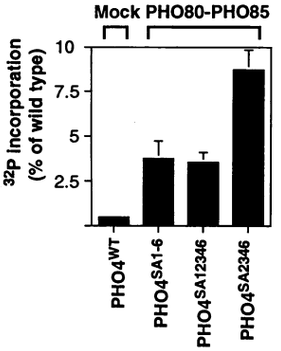

Fig. 3. $PHO4^{SA12346}$ is not efficiently phosphorylated by the PHO80-PHO85 cyclin-CDK complex. The amount of phosphate incorporated into $PHO4^{SA1-6}$, $PHO4^{SA12346}$, and $PHO4^{SA2346}$ was compared relative to that in wild-type PHO4 in an in vitro kinase assay performed with immunopurified PHO80-PHO85 kinase (*25*). For the negative control, wild-type PHO4 protein was used as substrate in a kinase assay performed with a mock PHO80-PHO85 immunopurification derived from an extract that did not contain epitope-tagged PHO80.

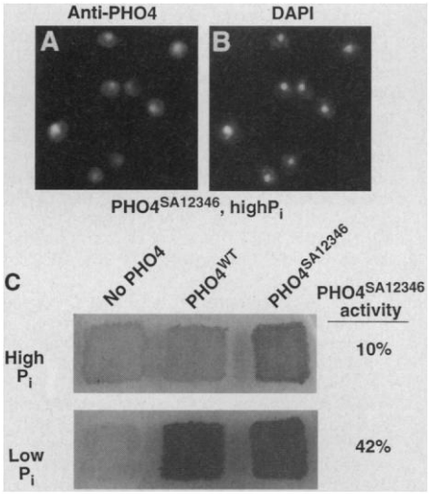

Fig. 4. $PHO4^{SA12346}$ is localized to the nucleus in phosphate-rich medium and causes partially constitutive expression of *PHO5*. (**A**) Indirect immunofluorescence (*17*) performed with PHO4 antiserum on a *pho4*Δ strain transformed with a low-copy plasmid that expresses $PHO4^{SA12346}$ under the control of the *PHO4* promoter [YCp400^{SA12346} (*26*)] grown in phosphate-rich medium (*19*). (**B**) DAPI staining of the field shown in (A) permits visualization of nuclei (*17*). (**C**) Yeast strains expressing no PHO4, wild-type PHO4, or $PHO4^{SA12346}$ (*27*) were grown on plates containing either standard synthetic medium (high phosphate, upper panel) or phosphate-depleted medium (low phosphate, lower panel) and then overlaid with agar containing a chromagenic phosphatase substrate (*28*). Yeast strains expressing *PHO5* turn red by this assay. Acid phosphatase activity was also measured by means of a quantitative liquid phosphatase assay (*29*). The acid phosphatase activity for $PHO4^{SA12346}$ is given as a percentage of that observed with yeast expressing wild-type PHO4 grown in phosphate-depleted medium.

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8. Several factors may contribute to the complex pattern of phosphopeptides in the phosphopeptide maps of mutants $PHO4^{SA2}$ and $PHO4^{SA3}$ (Fig. 2C). Ser-Pro dipeptides 2 and 3 are contained in the same tryptic peptide, which could result in the appearance of

phosphoisomers (*16*) in the wild-type map if the protein is not fully phosphorylated. Furthermore, there is only a single trypsin cleavage site between Ser-Pro dipeptide 1 and Ser-Pro dipeptide 2 that, if not fully cleaved, could complicate the pattern. Two phosphopeptides are missing from the map of PHO^{SA4} (Fig. 2C). Loss of these two peptides is probably a result of incomplete trypsin digestion, because the seven trypsin cleavage sites COOH-terminal to Ser-Pro dipeptide 4 are all in amino acid contexts that are not efficiently cleaved by trypsin (*16*).

9. The most likely explanation for this discrepancy is that Ser-Pro dipeptide 1 is used as a site and that tryptic phosphopeptide analysis of $PHO4^{SA1}$ was uninformative because of anomalous migration of the phosphopeptide containing Ser-Pro dipeptide 1 or insolubility of this particular peptide in the buffer used to prepare the samples (*16*).
10. Serines 100, 114, 128, 152, and 223 of PHO4 are phosphorylated by PHO80-PHO85. Four of the five serine residues phosphorylated by PHO80-PHO85 are found in a similar amino acid context, conforming to the consensus Ser-Pro-X-Ile/Leu. The fifth site has a threonine instead of a hydrophobic amino acid at the third residue following the phosphoacceptor.
11. E. K. O'Shea, D. A. Jeffery, D. King, unpublished data.
12. This difference in *PHO5* expression is not due to a difference in the level of PHO4 protein, as protein immunoblotting indicates that wild-type PHO4 and $PHO4^{SA12346}$ are expressed at comparable levels in yeast grown in low or high phosphate medium (*6*). Additionally, $PHO4^{SA12346}$ isolated from yeast grown in high phosphate medium and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) does not have the reduced electrophoretic mobility that is characteristic of phosphorylated wild-type PHO4 (*2*). This result suggests that the difference in *PHO5* expression is not likely to be due to residual phosphorylation of $PHO4^{SA12346}$. Also, we have observed no difference in the ability of wild-type PHO4 and $PHO4^{SA12346}$ to interact with PHO80-PHO85 in vitro (*6*), suggesting that the observed *PHO5* phenotype is not due to an inability of PHO80-PHO85 to associate with the mutant PHO4. We have also tested the phenotype of yeast expressing $PHO4^{SA1}$ and $PHO4^{SA2346}$ (*6*) and observed that they did not express *PHO5* constitutively. The observation that expression of $PHO4^{SA2346}$ does not cause constitutive *PHO5* expression lends further support to the idea that Ser-Pro dipeptide 1 is a functionally important phosphorylation site.
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17. Yeast cultures (25 ml) were grown as described (*19*) to an optical density at 600 nm (OD_{600}) of 0.5 to 1.5, at which time cells were fixed by addition of 3.5 ml of 37% formaldehyde directly to the culture medium. Fixation was allowed to proceed for 2 to 4 hours at room temperature, and cells were washed twice with water. Spheroplasts were prepared as follows. Fixed cells were incubated at room temperature for 10 min in 1 ml of 100 mM tris-HCl (pH 7.5) containing 25 mM 2-mercaptoethanol, washed once with phosphate-buffered saline (PBS), and resuspended in 25 μl of PBS per OD_{600} of cells. Zymolyase 100T (2 μl; 0.5 mg/ml) per OD_{600} of cells was added, and the cells were incubated at 37°C until 80 to 90% of cells appeared dark when visualized by phase contrast microscopy. Zymolyase treatment was stopped by washing three times in 1 M sorbitol with low-speed (3000 rpm) centrifugation in an Eppendorf 5415C microcentrifuge. Spheroplasts were resuspended in 25 μl of 1 M sorbitol per OD_{600} of cells. Spheroplasts were placed on glass microscope slides that were first treated with poly-L-lysine (1 mg/ml) for 5 min, washed twice with water, and air-dried. Attached spheroplasts were treated with blocking buffer (PBS + 0.1% Triton X-100, 1% bovine serum albumin) for 30 min at room temperature and incubated with preadsorbed primary antibody (diluted 1:200 in blocking buffer) overnight at 4°C. Spheroplasts were then washed three times with PBS, incubated with preadsorbed secondary antibody (diluted 1:200 in blocking buffer) for 1 hour at room temperature, washed three times with PBS, and mounted in Fluoromount G (Southern Biotechnology Associates, Inc.) + 4′,6′diamidino-2-phenylindole dihydrochloride (DAPI; 22.5 ng/ml). Primary antibody was polyclonal rabbit antiserum directed against PHO4 protein (*2*). Secondary antibody was rhodamine-conjugated goat antibody to rabbit immunoglobulin G (Boehringer Mannheim). Both secondary and primary antibodies were preadsorbed as described (*30*) with spheroplasts of either a wild-type strain or a strain deleted for the *PHO4* gene, respectively.
18. The wild-type strain used in this study was K699 *MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3* (*30*). *PHO4*, *PHO80*, *PHO81*, and *PHO85* were each deleted in this strain by standard gene replacement techniques [R. Rothstein, *Methods Enzymol.* **194**, 281 (1991)] to generate strains EY0130 (*pho4*Δ*::TRP1*), EY0134 (*pho80*Δ*::HIS3*), EY0150 (*pho81*Δ*::TRP1*), and EY0140 (*pho85*Δ*::LEU2*), respectively.
19. High phosphate cultures were grown in standard synthetic complete (SC) medium (*31*), and low phosphate cultures were grown in phosphate-depleted SC medium (*32*). Low phosphate cultures were inoculated at an OD_{600} of 0.05 to 0.2 with yeast from a log phase culture that had been grown in standard SC medium and then washed two times with sterile water to remove phosphate. High and low phosphate cultures were grown at 30°C with shaking for 8 to 16 hours to a final OD_{600} of 0.5 to 1.5 before analysis.
20. The procedures used for preparation of recombinant PHO4 (either wild-type or mutant) and in vitro phosphorylation of PHO4 with immunopurified PHO80-PHO85 complex are described (*2*). Plasmids T7-$PHO4^{ST1}$ to T7-$PHO4^{ST6}$ (*26*) were used to express the mutant versions of PHO4 in *Escherichia coli*. Phosphoamino acid analysis was performed as described (*16*).
21. 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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24. The procedures used for preparation of recombinant PHO4 (either wild-type or mutant), in vitro phosphorylation of PHO4 with immunopurified PHO80-PHO85 complex, and tryptic phosphopeptide analysis are described (*2*). Plasmids T7-$PHO4^{SA1}$ to T7-$PHO4^{SA6}$, and T7-$PHO4^{SA1-6}$ (*26*) were used to express the mutant versions of PHO4 in *E. coli*.
25. The procedures used for preparation of recombinant PHO4 (either wild-type or mutant) and in vitro phosphorylation of PHO4 with immunopurified PHO80-PHO85 complex were essentially as described (*2*), with two changes: (i) a version of PHO80 containing two copies of an epitope tag from the polyoma virus medium T antigen [sequence MEYMPME (*21*); T. Grussenmeyer, K. H. Scheidtmann, M. A. Hutchinson, W. Eckhart, G. Walter, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7592 (1985)] at its NH_2-terminus was used instead of the hemagglutinin tag; and (ii) protein G–Sepharose (Pharmacia Biotech) was used instead of protein A–Sepharose. Phosphorylated proteins were then resolved by SDS-PAGE. An equivalent amount of substrate, as judged by Coomassie blue staining and protein concentration determination, was used in each reaction. Quantitation was performed with the use of a PhosphorImager (Molecular Dynamics) and is expressed as a percentage of phosphate incorporated into wild-type PHO4 phosphorylated in vitro by PHO80-PHO85. Values represent an average of three to five experiments. Error bars represent SDs. Plasmids T7-$PHO4^{SA2346}$ and T7-$PHO4^{SA12346}$ (*26*) were used to express the mutant versions of PHO4 in *E. coli*.
26. To generate plasmids with individual serine to threonine substitutions (T7-$PHO4^{ST1}$ to T7-$PHO4^{ST6}$) or serine to alanine substitutions (T7-$PHO4^{SA1}$ to T7-$PHO4^{SA6}$), mutations were introduced into T7-PHO4 (*2*) by site-directed mutagenesis [J. Geisselsoder, F. Witney, P. Yuckenberg, *BioTechniques* **5**, 786 (1987)]. Plasmids containing mutations of multiple phosphorylation sites were generated by performing site-directed mutagenesis with multiple oligonucleotides simultaneously or by using standard molecular biology techniques to combine mutants, or both. The plasmid YCp400 was generated by ligating a 3.2-kb Hind III–Hpa I fragment from pAC312 (*22*) into YCp50 (*33*) that had been digested with Bam HI, end-filled, and then digested with Hind III. $YCp400^{SA12346}$ was generated by replacing a fragment in YCp400 with the appropriate fragment from T7-$PHO4^{SA12346}$.
27. The three yeast strains used were generated by transforming the *pho4*Δ strain (*18*) with each of the following three plasmids: (i) YCp50, an empty *URA3*-marked ARS-CEN vector (*33*); (ii) YCp400 (*26*), a plasmid expressing wild-type PHO4 under the control of the *PHO4* promoter; or (iii) $YCp400^{SA12346}$ (*26*), a plasmid expressing $PHO4^{SA12346}$ under the control of the *PHO4* promoter.
28. Each strain was patched onto a plate containing standard SC medium (*31*) and allowed to grow overnight at 30°C. This plate was then replica-plated, both back onto standard SC medium and onto phosphate-depleted medium (*32*). These two plates were allowed to grow at 30°C for 12 hours and then were overlaid with agar containing a chromagenic phosphatase substrate (*34*).
29. Liquid acid phosphatase assays were performed with a modification of the procedure described in (*34*). Strains were grown until they reached an OD_{600} of between 0.5 and 1.5, at which point 1 ml of culture was harvested and resuspended in 200 μl of water. Ten microliters of this suspension was added to 90 μl of water and 400 μl of substrate [*p*-nitrophenyl phosphate (56.2 mg/ml) in 0.1 M sodium acetate (pH 4.2)]. The reaction was allowed to proceed for 10 min at room temperature. Reactions were stopped by addition of 720 μl of saturated sodium carbonate. Cells were removed from the reactions by centrifugation, and the absorbance at 420 nm (A_{420}) was measured. One unit of acid phosphatase is defined to be a change of 1.0 in A_{420} per milliliter of yeast at an OD_{600} of 1.0. The strain used in these assays is wild type for *PHO3*, a second, constitutively expressed acid phosphatase gene. The presence of PHO3 results in a low level of background phosphatase activity, which was subtracted before the percentage of wild-type PHO5 activity was calculated. The actual values determined for the acid phosphatase activity (expressed as units per OD_{600}) of these strains are as follows: in high phosphate medium, YCp50 = 4.1 ± 1.1, YCp400 = 3.6 ± 0.6, and $YCp400^{SA12346}$ = 8.8 ± 0.8; in low phosphate medium, YCp50 = 2.6 ± 0.8, YCp400 = 48.3 ± 4.8, and $YCp400^{SA12346}$ = 22 ± 5.6. For comparison, the acid phosphatase activity of a *pho80*Δ strain is 51.3 ± 10.2 in high phosphate medium and 59.9 ± 14.8 in low phosphate medium.
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35. We thank D. King for assistance with mass spectrometry; D. Jeffery for preparation of phosphorylated PHO4 used for mass spectrometry; W. Lau for constructing the $PHO4^{SA1-6}$ mutant; S. Sanders and N. Valtz for advice regarding immunofluorescence; Y. Oshima for providing plasmids; and I. Herskowitz, M. Lenburg, M. Peter, M. Maxon, D. Jeffery, R. Tjian, and K. Schneider for comments on the manuscript. E.M.O. was supported by a National Institutes of Health (NIH) postdoctoral training grant awarded to the Department of Biochemistry and Biophysics at the University of California, San Francisco (UCSF) and by the Jane Coffin Childs Memorial Fund for Medical Research. Support for this work was provided to E.K.O. by the NIH, the Lucille P. Markey Foundation, the David and Lucile Packard Foundation, and the UCSF Simon Fund.

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