displace radiolabeled insulin binding to IRs expressed in intact CHO.IR cells, nor did it affect the affinity of insulin for the receptor (*12*). Third, L-783,281, but not L-767,827, increased IRTK activity of recombinant IR in vitro (*18*) (Fig. 5B). Finally, the partial proteolysis pattern of the IR intracellular domain (48 kD) was altered in the presence of L-783,281 (Fig. 5C). A different pattern of proteolysis was observed when the 48-kD protein was incubated with an adenosine 5′-triphosphate (ATP) analog (ATP-γ-S) that affects IR kinase conformation (*5*). Yet another pattern was observed when the 48-kD protein was incubated with both L-783,281 and ATP-γ-S. Of particular interest was a ~30-kD band produced when the 48-kD protein was incubated with L-783,281 followed by partial digestion with trypsin (lane 2, asterisk). In the absence of L-783,281, a 10 to 50 times higher concentration of trypsin was required to produce the ~30-kD product. NH_2-terminal peptide sequencing of the ~30-kD band revealed the sequence Thr^{1031}-Val-Asn-Glu-Ser-Ala-Ser-Leu (*19*). This peptide is immediately adjacent to Lys^{1030}, the residue involved in ATP binding to the active site of the IRTK domain (*2*, *20*). Thus, interaction of L-783,281 with the IR kinase domain appears to alter the conformation of the protein in the region encompassing the ATP binding site, resulting in the exposure of tryptic recognition site (or sites) adjacent to Lys^{1030}. On the basis of published crystal structures, conformational change in the kinase domain is required for the activation of the receptor (*4*, *5*). The results of our studies suggest that interaction of L-783,281 with IRTK alters the conformation of IRTK, leading to its activation.

The discovery of L-783,281 demonstrates that a small, nonpeptidyl molecule is capable of mimicking the in vitro and in vivo function of a protein hormone by interacting with and activating its receptor. Vanadate is another orally active compound that can function in vivo as an insulin mimetic agent (*21*). However, unlike vanadate, which augments tyrosyl phosphorylation of a wide variety of cellular proteins and functions in vitro as an inhibitor of protein tyrosine phosphatases (PTPases) (*22*), L-783,281 was selective for the IR and did not inhibit selected PTPases in vitro (*12*). Selective IR activators, as exemplified by L-783,281, may lead to the development of a novel class of antidiabetic agents.

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11. L-783,281 was isolated from liquid cultures of a strain of the fungus *Pseudomassaria* sp. (American Type Culture Collection 74411), which was recovered from leaves of an undetermined plant collected near Kinshasa, Democratic Republic of Congo. For production of the compound, the strain was grown in a medium containing (per liter) D-mannitol (100 g), NZ-Amine (type E) (Quest International, Norwich, NY) (33 g), Difco–Yeast Extract (Becton Dickinson, Franklin Lakes, NJ) (10 g), ammonium sulfate (5 g), and K_2HPO_4 (9 g). An organic extract of the fermentation broth was prepared by gel chromatography and two subsequent cycles of reversed-phase high-performance liquid chromatography. The title compound crystallized as dark purple needles from ethyl acetate:hexane when stored at 4°C. Spectroscopic data (^1H, ^{13}C, and two-dimensional nuclear magnetic resonance data and high-resolution fast atom bombardment mass spectroscopy) were collected on the pure compound, which was identified as demethylasterriquinone B-1 (L-783,281).
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25. We thank G. Bills, R. Schwartz, and L. So for characterization of the L-783,281–producing organism; D. Zink for mass spectroscopy data on L-783,281; T. Doebber, M. Wu, and J. Ryder for assistance with biological characterization; and M. Turner and S. Gould for invaluable support.

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Roles of Phosphorylation Sites in Regulating Activity of the Transcription Factor Pho4

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Transcription factors are often phosphorylated at multiple sites. Here it is shown that multiple phosphorylation sites on the budding yeast transcription factor Pho4 play distinct and separable roles in regulating the factor's activity. Phosphorylation of Pho4 at two sites promotes the factor's nuclear export and phosphorylation at a third site inhibits its nuclear import. Phosphorylation of a fourth site blocks the interaction of Pho4 with the transcription factor Pho2. Multiple phosphorylation sites provide overlapping and partially redundant layers of regulation that function to efficiently control the activity of Pho4.

Many signaling pathways rapidly and reversibly convert extracellular signals into changes in gene expression. Phosphorylation of a transcription factor, often at multiple sites, is a common mechanism for responding to signaling events (*1*). This modification can lead to changes in transcription factor concentration or activity in the nucleus (*2*). However, the role of multiple phosphorylation sites in regulating the activity of a protein is not well understood.

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To study how multiple phosphorylation sites control protein activity, we focused on the regulation of Pho4, a transcription factor in budding yeast that activates expression of genes induced in response to phosphate starvation (*3*). When yeast cells are grown in phosphate-rich conditions, Pho4 is phosphorylated by the Pho80/Pho85 cyclin-cyclin–dependent kinase (CDK) complex (*4*) and exported to the cytoplasm (*5*), thereby terminating expression of phosphate-responsive genes. The kinase Pho80/Pho85 phosphorylates Pho4 on five Ser-Pro (SP) dipeptides, referred to as SP1, SP2, SP3, SP4, and SP6 (*6*). When yeast cells are starved for phosphate, the CDK inhibitor Pho81 inactivates Pho80/Pho85 (*7*),

leading to the accumulation of unphosphorylated Pho4 in the nucleus (*6*) and the subsequent transcription of phosphate-responsive genes.

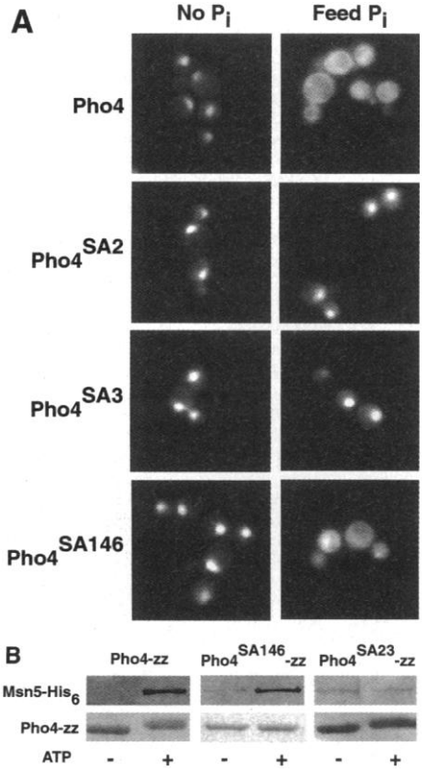

Fig. 1. Phosphorylation of sites 2 and 3 promotes nuclear export of Pho4. **(A)** Localization of wild-type Pho4-GFP, or the indicated Pho4 mutants fused to GFP, in cells grown in no phosphate medium. For the Feed P_i sample, phosphorylation and nuclear export of Pho4-GFP were triggered by addition of phosphate to a culture that had been grown in no phosphate medium (*25*). **(B)** Wild-type Pho4 and the indicated Pho4 mutants, joined to two IgG-binding z domains derived from protein A (Pho4-zz), were phosphorylated (+ATP) or mock phosphorylated (−ATP) in vitro, immobilized on IgG-Sepharose, and binding to Msn5-His_6 was measured (*26*). (Top) The amount of bound Msn5-His_6 was analyzed on a silver-stained SDS-PAGE (polyacrylamide gel electrophoresis) gel. (Bottom) The amount of immobilized Pho4-zz was analyzed on a Coomassie-stained SDS-PAGE gel.

Addition of phosphate to a phosphate-starved culture causes rapid phosphorylation and nuclear export of Pho4 fused to the green fluorescent protein (*5*) (Fig. 1A). Export of Pho4 requires phosphorylation by Pho80/Pho85; Pho4 is localized to the nucleus and fully active transcriptionally in strains lacking Pho80 or Pho85 (*6*). Additionally, the nonphosphorylatable mutant $Pho4^{SA12346}$ (containing Ser→Ala substitutions at the five sites of phosphorylation) is constitutively localized to the nucleus and partially active transcriptionally (*6*). To determine which of the five phosphorylation sites are required for the export of Pho4, we tested the ability of Pho4 mutants to be exported from the nucleus. $Pho4^{SA1}$-GFP, $Pho4^{SA4}$-GFP, and $Pho4^{SA6}$-GFP, containing an individual Ser→Ala substitution at phosphorylation site 1, 4, or 6, had no defect in nuclear export (*8*). However, $Pho4^{SA2}$-GFP and $Pho4^{SA3}$-GFP, containing a Ser→Ala substitution at sites 2 and 3, respectively, could not be exported (Fig. 1A). Additionally, $Pho4^{SA146}$-GFP, a mutant that can only be phosphorylated on sites 2 and 3, was exported from the nucleus upon addition of phosphate (Fig. 1A). Thus, phosphorylation of sites 2 and 3 is necessary and sufficient for nuclear export of Pho4.

Msn5, a member of the β-importin family of nuclear transport receptors, is the export receptor for Pho4 (*5*). In vitro, Msn5 and the small guanosine triphosphatase (GTPase) Ran (in its GTP-bound state) form a stable complex with phosphorylated Pho4, but not with unphosphorylated Pho4 (*5*). We examined whether phosphorylation of sites 2 and 3 is also required for an interaction with Msn5 in vitro. $Pho4^{SA146}$ and $Pho4^{SA23}$ were tagged with two immunoglobulin G (IgG)–binding "z" domains derived from protein A ($Pho4^{SA146}$-zz and $Pho4^{SA23}$-zz), phosphorylated in vitro (*9*), and assayed for Msn5 binding in the presence of Gsp1Q71L, a yeast Ran mutant locked in the GTP-bound form. $Pho4^{SA23}$-zz failed to interact with Msn5 in either its phosphorylated or unphosphorylated form (Fig. 1B). By contrast, $Pho4^{SA146}$-zz interacted with Msn5 only when phosphorylated (Fig. 1B). Thus, phosphorylation of Pho4 at sites 2 and 3 is necessary and sufficient to promote binding to Msn5 (*10*).

Pse1, another member of the β-importin family of transport receptors, is the import receptor for Pho4 (*11*). Phosphorylation of Pho4 inhibits its interaction with Pse1. Because phosphorylation site 4 is contained within the nuclear localization signal (NLS) of Pho4 (*11*), phosphorylation of this site might inhibit the interaction between Pho4 and Pse1. $Pho4^{SA4}$-zz (a mutant that can be phosphorylated on all sites except site 4) and $Pho4^{SA1236}$-zz (a mutant that can only be phosphorylated on site 4) were phosphorylated in vitro and assayed for binding to Pse1. Phosphorylated $Pho4^{SA4}$-zz bound to Pse1, whereas $Pho4^{SA1236}$-zz failed to bind Pse1 when phosphorylated (Fig. 2A). Thus, phosphorylation of Pho4 at site 4 is necessary and sufficient to disrupt the association of Pho4 and Pse1.

We examined the role of phosphorylation of site 4 in regulating import of Pho4 in vivo. We used a mutant that cannot be exported, because export of Pho4 and a block in its import both lead to its cytoplasmic accumulation. Because phosphorylation of Pho4 by Pho80/Pho85 occurs in the nucleus (*5*), we attempted to mimic phosphorylation of site 4 by substituting Ser with Asp. $Pho4^{SA1236SD4}$-zz (containing Ser→Ala substitutions at sites 1, 2, 3, and 6 and a Ser→Asp substitution at site 4) failed to bind Pse1 in vitro (Fig. 2A). To examine the effect of the Asp substitution on import of Pho4 in vivo, we induced expression of $Pho4^{SA12346}$ and $Pho4^{SA1236SD4}$ fused to three tandem copies of GFP (GFP_3) (*12*) and monitored the localization of these proteins by fluorescence microscopy. One-and-one-half hours after induction, $Pho4^{SA12346}$-GFP_3 remained nuclear, whereas $Pho4^{SA1236SD4}$-GFP_3 was mainly cytoplasmic (*13*, *14*) (Fig. 2B). Thus, phosphorylation at site 4 inhibits nuclear import of Pho4.

If control of nuclear localization is the only mechanism by which phosphorylation regulates the activity of Pho4, then Pho4 that is localized to the nucleus should activate transcription of phosphate-responsive genes

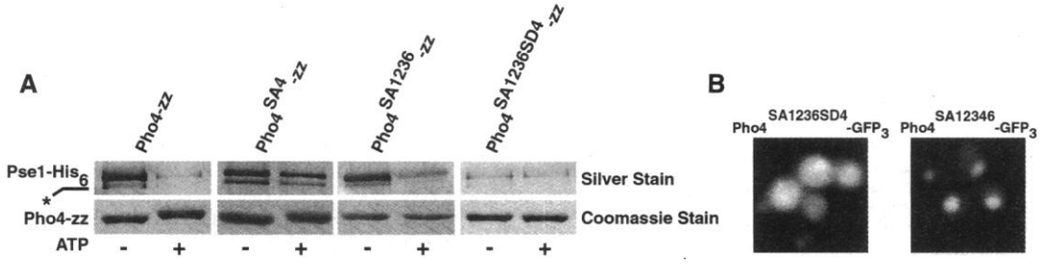

Fig. 2. Phosphorylation of site 4 inhibits nuclear import of Pho4. **(A)** Wild-type Pho4-zz and the indicated Pho4-zz mutants were phosphorylated (+ATP) or mock phosphorylated (−ATP) in vitro, immobilized on IgG-Sepharose, and binding to Pse1-His_6 was measured (*11*). The amount of bound Pse1-His_6 was analyzed on a silver-stained SDS-PAGE gel. The amount of immobilized Pho4-zz was analyzed on a Coomassie-stained SDS-PAGE gel. The band below Pse1-His_6 is an NH_2-terminally truncated form of the protein (indicated by the asterisk). **(B)** Expression of $Pho4^{SA12346}$-GFP_3 or $Pho4^{SA1236SD4}$-GFP_3 was induced, and localization was monitored by fluorescence microscopy (*27*).

in both high- and low-phosphate conditions. Therefore, we measured production of the secreted acid phosphatase Pho5 in a strain expressing Pho4^{SA1234}, a mutant containing Ser→Ala substitutions at sites 1, 2, 3, and 4 (*15*) that was constitutively localized to the nucleus (Fig. 3A). Although expression of acid phosphatase was elevated in yeast expressing Pho4^{SA1234} grown in phosphate-rich medium (*16*), it was further induced in response to phosphate starvation (Fig. 3B). Additionally, an *msn5*Δ strain, in which Pho4 is constitutively localized to the nucleus because it cannot be exported, produces high levels of acid phosphatase when starved for phosphate (*8*), but not when grown in phosphate-rich medium (*5*). Thus, another mechanism, distinct from control of its localization, regulates the activity of Pho4.

The only site that can be phosphorylated in the Pho4^{SA1234} mutant is site 6. We constructed a mutant Pho4 that could not be phosphorylated on site 6 by making a Pro→Ala substitution in the Ser-Pro dipeptide corresponding to phosphorylation site 6 (Pho4^{PA6}) (*17*). We did not use a Ser→Ala substitution to prevent phosphorylation of site 6 because the Pho4^{SA6} mutant is not fully functional in activating transcription of acid phosphatase (*18*). Localization of Pho4^{PA6}-GFP was regulated in response to phosphate levels (Fig. 3A), and Pho4^{PA6} was fully functional as a transcriptional activator (Fig. 3B). We combined the mutations that cause Pho4 to be constitutively localized to the nucleus with the Pro→Ala mutation at site 6 to create Pho4^{SA1234PA6} (Fig. 3A). In contrast to a strain expressing Pho4^{SA1234}, a strain expressing Pho4^{SA1234PA6} produced acid phosphatase at nearly fully induced levels when grown in high-phosphate medium (*19*) (Fig. 3B). Additionally, a strain lacking the export receptor Msn5 and expressing Pho4^{PA6} produced high levels of acid phosphatase when grown in phosphate-rich medium (*8*). Thus, phosphorylation of site 6 provides an additional mode for regulating the activity of Pho4. These observations suggest that phosphorylation by Pho80/Pho85 is the primary mode of regulating Pho4 in response to phosphate availability (*20*).

Phosphorylation site 6 lies within a region of Pho4 involved in binding to the transcription factor Pho2 (*21*). Pho2 is required for transcription of *PHO5* (*3*), interacts with Pho4, and binds cooperatively with Pho4 to the *PHO5* promoter (*22*). To determine if phosphorylation of site 6 modulates the interaction between Pho4 and Pho2, we phosphorylated a Pho4-zz fusion protein in vitro and assayed for its binding to Pho2. Pho2 bound to unphosphorylated Pho4-zz, but not to phosphorylated Pho4-zz, indicating that phosphorylation of Pho4 inhibits its interaction with Pho2 (Fig. 3C). Pho4^{SA1234}-zz, which can only be phosphorylated on site 6, bound to Pho2 when unphosphorylated, but not when phosphorylated (Fig. 3C). Additionally, Pho4^{PA6}-zz, a mutant that can be phosphorylated on all sites except site 6, bound to Pho2 independent of its phosphorylation state (Fig. 3C). Thus, phosphorylation of site 6 is necessary and sufficient to inhibit interaction of Pho4 with Pho2 (*23*).

Regulation of nuclear localization and regulation of the interaction with Pho2 provide partially redundant levels of regulation to control the activity of Pho4; yeast expressing either Pho4^{PA6} (regulated only by nuclear localization) or Pho4^{SA1234} (regulated only by control of the interaction with Pho2) induce transcription of the acid phosphatase Pho5 in response to phosphate starvation (Fig. 3B). Although overlapping, both levels of regulation are required for complete repression of Pho5 expression, because acid phosphatase expression is not completely repressed in yeast expressing Pho4^{SA1234} or Pho4^{PA6} (Fig. 3B). Therefore, multiple phosphorylation sites may exist to ensure complete shutoff of transcription.

The phosphorylation events that modify Pho4 have unique and separable roles in regulating the protein's export, import, and ability to activate transcription in the nucleus (Fig. 4). Multiple levels of regulation cooperate to control Pho4 in a switchlike manner. Many transcription factors, CDK inhibitors, and other regulatory proteins are phosphorylated on multiple sites, but the role of these phosphorylation events is not well understood. Phosphorylation may provide multiple levels of control that are important for efficient regulation of proteins other than Pho4.

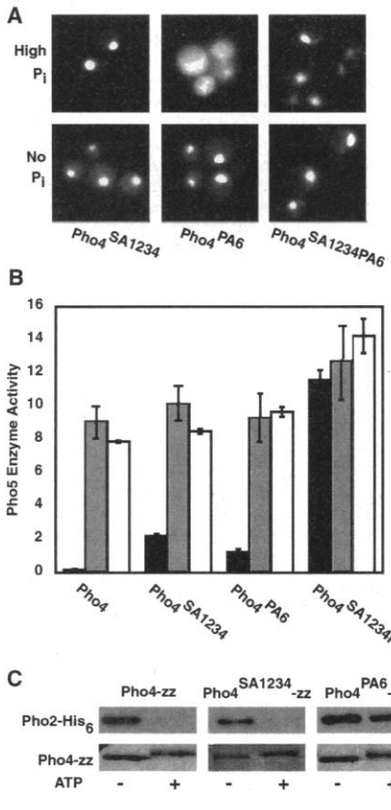

Fig. 3. Pho4 is regulated by a mechanism distinct from control of its nuclear localization. (**A**) Localization of the indicated Pho4 mutants fused to GFP in cells grown in no or high-phosphate medium (*25*). (**B**) Measurement of Pho5 acid phosphatase enzyme activity in either *pho4*Δ *pho3*Δ or *pho4*Δ *pho80*Δ *pho3*Δ yeast strains (*24*) transformed with a low-copy plasmid expressing the indicated Pho4 mutant (*28*). The *pho4*Δ *pho3*Δ strain expressing the indicated Pho4 mutant was grown in high- (black boxes) or low-phosphate (white boxes) medium and the *pho4*Δ *pho80*Δ *pho3*Δ strain was grown in high-phosphate medium (gray boxes). (**C**) Wild-type Pho4-zz and the indicated Pho4-zz mutants were phosphorylated (+ATP) or mock phosphorylated (−ATP) in vitro, immobilized on IgG-Sepharose, and binding to Pho2-His$_6$ was measured (*29*). (Top) The amount of bound Pho2-His$_6$ was analyzed by SDS-PAGE followed by protein immunoblotting with anti-Pho2. (Bottom) The amount of immobilized Pho4-zz was analyzed on a Coomassie-stained SDS-PAGE gel.

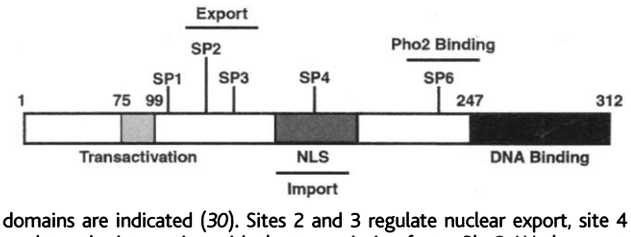

Fig. 4. Phosphorylation events regulate Pho4 by distinct and separable mechanisms. Sites of phosphorylation consist of five Ser-Pro dipeptides labeled SP1, SP2, SP3, SP4, and SP6 (amino acids 100, 114, 128, 152, and 223) (*6*). The activation and DNA binding domains are indicated (*30*). Sites 2 and 3 regulate nuclear export, site 4 regulates import, and site 6 regulates the interaction with the transcription factor Pho2. We have not been able to determine a function for phosphorylation site 1 (*15*).

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9. Previous studies demonstrate that Pho4 mutants containing Ser→Ala substitutions of a subset of the phosphorylation sites can be efficiently phosphorylated in vitro (*6*).
10. Phosphorylation of sites 2 and 3 cannot be mimicked by Ser→Asp mutations. $Pho4^{SA146SD23}$-GFP, containing Ser→Ala substitutions at sites 1, 4, and 6, and Ser→Asp substitutions at sites 2 and 3, is not exported from the nucleus (*8*).
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12. Three tandem copies of GFP were used in order to (i) enhance the fluorescence signal and (ii) slow down the import of the two proteins to facilitate detection of a difference in their nuclear import.
13. The following observations suggest that $Pho4^{SA1236SD4}$-GFP_3 is not cleaved by proteolysis to release cytoplasmic GFP and is a functional transcription factor: (i) Both $Pho4^{SA1236SD4}$-GFP and $Pho4^{SA12346}$-GFP are localized to the nucleus at steady state when expressed constitutively from the *PHO4* promoter (on a *CEN/ARS* plasmid) (*8*). (ii) When grown in phosphate-rich medium, a *pho4*Δ strain containing $Pho4^{SA1236SD4}$-GFP or $Pho4^{SA12346}$-GFP expresses acid phosphatase at slightly elevated levels compared with a *pho4*Δ strain containing wild-type Pho4-GFP (*8*). (iii) Protein immunoblotting analysis of extracts derived from strains expressing either $Pho4^{SA1236SD4}$-GFP_3 or $Pho4^{SA12346}$-GFP_3 indicate there is a similar amount of each full-length protein and a minor population of degraded protein (*8*).
14. Substitution of site 4 with Glu slowed import of Pho4 in response to phosphate starvation. $Pho4^{SE4}$-GFP expressed under the control of the *PHO4* promoter on a *CEN/ARS* plasmid was still cytoplasmic after 2 hours of phosphate starvation. In contrast, wild-type Pho4-GFP was imported into the nucleus in less than 30 min after phosphate starvation (*8*). Additionally, a *pse1-1* strain, defective for import of Pho4, cannot induce expression of acid phosphatase in response to phosphate starvation.
15. In mapping the five sites of phosphorylation, site 1 was found to be phosphorylated poorly in vitro (*6*). Thus, this phosphorylation site may not play a physiologically relevant role in regulating Pho4 activity. Additionally, the $Pho4^{SA1}$-GFP mutant has no defect in localization or regulation of acid phosphatase expression (*8*).
16. A *pho4*Δ strain expressing $Pho4^{SA2}$-GFP or $Pho4^{SA3}$-GFP, which cannot be exported from the nucleus, produced elevated levels of acid phosphatase in high-phosphate conditions (*8*). By contrast, yeast expressing $Pho4^{SA4}$-GFP, which can be exported but whose import cannot be inhibited by phosphorylation, regulated acid phosphatase expression appropriately (*8*).
17. We believe that the Pro→Ala mutation at site 6 prevents phosphorylation because (i) phosphorylation of substrates by CDKs requires a Pro preceding the residue of phosphorylation [R. B. Pearson and B. E. Kemp, *Methods Enzymol.* **200**, 62 (1991)], and (ii) $Pho4^{SA1234PA6}$ does not undergo a shift in its electrophoretic mobility after incubation with Pho80/Pho85 and adenosine 5′-triphosphate (ATP), suggesting that it cannot be phosphorylated (*8*).
18. The observation that $Pho4^{SA6}$ is not fully functional in activating transcription of acid phosphatase helps to explain our previously published results that $Pho4^{SA12346}$ is a weak activator of transcription (*6*).
19. A *pho4*Δ *pho80*Δ strain expressing $Pho4^{SA1234}$ also produces fully induced levels of acid phosphatase when grown in phosphate-rich medium (Fig. 3B). Thus, the additional mode of regulation requires Pho80, as expected if the regulation involves phosphorylation.
20. Our new studies of $Pho4^{SA1234PA6}$ (Fig. 3B) and our previous studies of $Pho4^{SA12346}$ (*6*) indicate that a phosphorylation-independent mode of regulation of Pho4 exists, but it makes a minor contribution to the regulation of Pho5. We estimate the contribution of the phosphorylation-independent regulation to be less than twofold—this is the ratio of the level of acid phosphatase produced by a strain expressing $Pho4^{SA1234PA6}$ [or $Pho4^{SA12346}$ (*8*)] grown in low-phosphate conditions (or in a strain lacking Pho80), divided by the level of acid phosphatase produced when this strain is grown in high-phosphate conditions. In contrast, the contribution of the phosphorylation-dependent regulation is ~70-fold—this is the ratio of the level of acid phosphatase produced in a strain expressing a $Pho4^{SA1234PA6}$, divided by the level of acid phosphatase produced in a strain expressing wild-type Pho4, both grown in high-phosphate conditions. Our previous studies (*6*) underestimated the phosphorylation-dependent contribution to Pho5 regulation for two reasons: (i) These studies were performed with a strain producing the constitutively expressed acid phosphatase Pho3, which makes it difficult to accurately measure the levels of Pho4-dependent acid phosphatase production in high-phosphate conditions. (ii) The previous studies measured the activity of $Pho4^{SA12346}$, which is not completely functional transcriptionally because of the SA6 mutation (*18*). A source of phosphorylation-independent regulation has been proposed—it has been suggested that Pho80 can negatively regulate Pho4 in high-phosphate conditions by directly binding to and masking its activation domain [P. S. Jayaraman, K. Hirst, C. R. Goding, *EMBO J.* **13**, 2192 (1994)]. Our conclusions rely on the assumption that the PA6 mutation does not affect the phosphorylation-independent mode of regulation. For the following reasons we do not believe that the PA6 mutation renders Pho4 defective in its ability to interact with and be masked by Pho80: (i) Export of $Pho4^{PA6}$-GFP occurs with the same kinetics as wild-type Pho4 (*8*), suggesting that the ability of this mutant to interact with and be phosphorylated by Pho80 is similar to that of wild-type Pho4. (ii) Phosphorylation site 6 is in a domain involved in interaction with Pho2 (*21*), not with Pho80 (P. S. Jayaraman *et al.*, above).
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23. $Pho4^{SA6}$ and $Pho4^{SD6}$ did not bind efficiently to Pho2 and did not efficiently activate expression of acid phosphatase (*8*). A *pho4*Δ strain expressing $Pho4^{SN6}$, containing a Ser→Asn mutation at site 6, produced acid phosphatase and bound Pho2 at a level between that of the $Pho4^{SA6}$ and $Pho4^{PA6}$ mutants (*8*). Additionally, unphosphorylated $Pho4^{SA1234}$ and $Pho4^{SA1234PA6}$ interacted efficiently with Pho2 (Fig. 3C) (*8*) and activated transcription of acid phosphatase (Fig. 3B). Thus, Ala substitutions at Ser 1, 2, 3, and 4 do not affect the interaction with Pho2, and the Ser at site 6 is important for the interaction between Pho4 and Pho2.
24. All yeast strains are derived from K699 *MAT***a** [K. Nasmyth, G. Adolf, D. Lydall, A. Seddon, *Cell* **62**, 631 (1990)]. *PHO3* encodes an acid phosphatase expressed in phosphate-rich conditions [A. Toh-e, Y. Ueda, S.-I. Kakimoto, Y. Oshima, *J. Bacteriol.* **113**, 727 (1973)].
25. Pho4-GFP (*11*) and the Pho4 mutants fused to GFP were expressed under the control of the *PHO4* promoter on the *CEN/ARS* plasmid pRS316 in a *pho4*Δ strain (*24*). Phosphate starvation experiments and microscopy were performed as described (*11*). For the phosphate feed assay, KH_2PO_4 was added to a final concentration of 20 mM. Photos were taken 5 to 10 min after the addition of phosphate.
26. Phosphorylation of Pho4-zz and binding to Msn5-His_6 were performed essentially as described (*5*) except that 5 μM His_6-Gsp1Q71L was used in place of 1 μM Gsp1. His_6-Gsp1Q71L was purified as described for Pse1-His_6 (*11*).
27. A *pho4*Δ strain (*24*) expressing $Pho4^{SA12346}$-GFP_3 or $Pho4^{SA1236SD4}$-GFP_3 (*12*) under the control of the *GAL1-10* promoter was grown in synthetic raffinose medium at 30°C to log phase. No fluorescence is visible under noninducing conditions when cells are grown in raffinose. Expression of each fusion protein was induced by addition of galactose to a final concentration of 2%, and localization was monitored as a function of time by fluorescence microscopy (*11*). The photo was taken 1.5 hours after the addition of galactose.
28. Pho4 and each Pho4 mutant (not fused to GFP) were expressed in *pho4*Δ *pho3*Δ or *pho4*Δ *pho80*Δ *pho3*Δ yeast strains (*24*) under the control of the *PHO4* promoter on the *CEN/ARS* plasmid YCp50 [M. Johnston and R. W. Davis, *Mol. Cell. Biol.* **4**, 1440 (1984)]. Five-milliliter cultures were grown in synthetic dextrose media lacking uracil for 12 to 16 hours and diluted to an optical density at 600 nm (OD_{600}) of 0.1. For high-phosphate experiments, the diluted cultures were grown to an OD_{600} of ~1.0. For phosphate starvation experiments the diluted cultures were grown to an OD_{600} of 0.5, centrifuged, washed, resuspended in low-phosphate synthetic medium (*11*), and grown for 6 hours. Liquid acid phosphatase assays were performed essentially as described [A. Toh-e *et al.* (*24*)]. One-tenth and 1/20 of the cultures were used, and the assay was performed in a volume of 820 μl. The units are the ratio of OD_{420} to OD_{600} values.
29. Twenty microliters of protein G–Sepharose beads were incubated with 30 μg of 12CA5 antibody to hemagglutinin A (anti-HA) for 45 min and then washed twice with phosphate-buffered saline (PBS) [150 mM NaCl, 10 mM sodium phosphate (pH 7.4)] + 0.1% NP40. Antibody beads were incubated with 750 μg of yeast extract overexpressing HA-Pho80 (*4*) for 1 hour. The immunoprecipitates were washed three times with PBS + 0.1% NP40, once with PBS, and once with kinase buffer [20 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$]. Five micrograms of Pho4-zz, 5 μl of 10× kinase buffer, and 5 μl of 10 mM ATP were added to each reaction, and the final volume was adjusted to 50 μl with IgG buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20, 5 mM $MgCl_2$]. ATP was omitted from the mock phosphorylation reactions. Reactions were carried out at room temperature for 1 hour, and phosphorylated or mock-phosphorylated Pho4-zz was obtained by collecting the supernatant. Binding of Pho4-zz to Pho2-His_6 was performed essentially as described for Pse1-His_6 (*11*). The plasmid expressing Pho2-His_6 and its purification from *Escherichia coli* have been described elsewhere [R. M. Brazas and D. J. Stillman, *Mol. Cell. Biol.* **13**, 5524 (1993)]. Pho2-His_6 was used at a concentration of 6 nM for each reaction. Ten percent of the 1 M $MgCl_2$ elutions were run on 7.8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Pho2 was detected by protein immunoblotting with polyclonal anti-Pho2.
30. P. C. McAndrew, J. Svaren, S. R. Martin, W. Hörz, C. R. Goding, *Mol. Cell. Biol.* **18**, 5818 (1998); N. Ogawa and Y. Oshima, *ibid.* **10**, 2224 (1990).

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