tumor sets (*8*, *11*, *18*, *19*). The proportion of incidental *BRCA1* LOH attributable to linked genes awaits isolation and characterization of these putative 17q tumor suppressor loci. Also, a proportion of the allelic losses observed may be explained by the background of random aneuploidy in breast tumors, which averages as much as 15% (*20*).

If confirmed by further studies, the absence of somatic *BRCA1* mutations implies that there may be a fundamental difference between the genesis of sporadic tumors and those in genetically predisposed *BRCA1* carriers. For example, mutations in *BRCA1* may only have an effect on tumor formation when present at a specific stage early in breast and ovarian development, a possibility consistent with a primary role for *BRCA1* mutations in premenopausal breast cancer. However, no clinical or pathological differences in familial versus sporadic breast and ovary tumors, other than age of onset, have been described (*21*). On the other hand, the finding of an increased frequency of *TP53* mutation and microsatellite instability in breast tumors from patients with a family history of breast cancer (*22*) is consistent with an etiologic difference between familial and sporadic tumors. The absence of somatic *BRCA1* mutations may reflect the existence of multiple genes that function in the same pathway of tumor suppression as *BRCA1* but that collectively represent a more favored target for mutation in sporadic tumors.

The data from primary tumors support a role for *BRCA1* in early-onset breast cancer and ovarian cancer and raise the possibility that *BRCA1* may have only a minor role in sporadic breast and ovarian tumor formation. Of paramount concern is the function of wild-type BRCA1 protein in breast and ovarian epithelial cells. The identification of a zinc finger domain in the BRCA1 polypeptide, a motif found in several DNA binding proteins, indicates that *BRCA1* may regulate gene expression (*12*). For example, the function of *BRCA1* in hormone-sensitive tissues suggests that *BRCA1* may mediate hormonal signals in the breast and ovary. Ultimately, it will be important to identify the other genes in the pathway of tumor suppression in which *BRCA1* participates. These genes may play a significant role in breast and ovarian carcinogenesis and may represent alternative avenues for therapeutic intervention.

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14. Tumor DNA samples from Memorial Sloan-Kettering Cancer Center (MSKCC) were sequenced with a Cyclist cycle sequencing kit (Stratagene, La Jolla, CA) (*12*) and those from Duke University were sequenced with an ABI 373 automated fluorescent sequencer with PRISM dye terminators according to the manufacturer's suggestions. Sequences were analyzed with the Sequence Navigator software package (ABI, Foster City, CA). PCR-generated templates from genomic DNAs of the Duke University tumor samples as well as complementary DNA (cDNA) templates from 9 of the breast and all 12 of the ovarian tumors were examined by SSCA as described (*23*). Sequences of intron-based PCR primers used to amplify each of the 23 exons of *BRCA1* and PCR conditions are available by anonymous FTP at the following internet address: Morgan.Med.utah.edu in the directory pub/BRCA1 or by fax at the following number: 801-584-3650. The GenBank accession number for the *BRCA1* sequence is U14680.
15. The mutations found in BT098 and BT106 were absent from 162 Caucasian control chromosomes, the mutation found in OV24 was absent in 128 Caucasian chromosomes, and the mutation in MC44 was absent in 116 African American and 48 Caucasian control chromosomes.
16. Two intronic polymorphisms were detected. PM4 is located 143 bp upstream of exon 12 in intron 11. The A and C alleles were detected by allele-specific oligonucleotide hybridization in 116 and 56 chromosomes, respectively. PM5 is located 49 bp downstream of exon 18 in intron 18. The G and A alleles were detected in 123 and 55 chromosomes, respectively.
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Phosphate-Regulated Inactivation of the Kinase PHO80-PHO85 by the CDK Inhibitor PHO81

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A complex consisting of the cyclin-dependent kinase (CDK) PHO85 and the cyclin PHO80 phosphorylates and is thought to inactivate the transcription factor PHO4 when yeast cells are grown in medium containing high concentrations of phosphate. The CDK inhibitor PHO81 inhibits the kinase activity of the PHO80-PHO85 complex when *Saccharomyces cerevisiae* cells are grown in medium depleted of phosphate. A region of PHO81 with similarity to the mammalian CDK inhibitor p16^{INK4} is sufficient for inhibition in vitro. These studies demonstrate that CDK inhibitors are used to regulate kinases involved in processes other than cell cycle control and suggest that the ankyrin repeat motif may be commonly used for interaction with cyclin-CDK complexes.

The *PHO5* gene encodes a secreted acid phosphatase in *Saccharomyces cerevisiae* whose transcription is regulated in response to extracellular concentrations of inorganic phosphate (*1*). *PHO5* expression is dependent on the activity of the transcription factor PHO4. When yeast cells are grown in medium containing a high concentration of phosphate, PHO4 is phosphorylated by the PHO80-PHO85 cyclin-cyclin–dependent kinase (CDK) complex and transcription of *PHO5* is repressed (*2*). In medium depleted of phosphate, PHO4 is underphosphorylated and transcription of *PHO5* is induced. PHO4 might become activated upon phosphate starvation as a result of induction of a PHO4 phosphatase or inactivation of the PHO80-PHO85 complex.

To investigate the mechanism of PHO4 activation, we purified the PHO80-PHO85 complex from yeast cells grown in low or high concentrations of phosphate and assayed its kinase activity in vitro with PHO4 as a substrate. The PHO80-PHO85 complex was isolated by immunoprecipitation from a yeast strain expressing PHO80 tagged with three copies of the hemagglutinin epitope (HA_3-PHO80) (*3*). The kinase activity of the PHO80-PHO85 complex isolated from yeast cells grown in medium containing a high concentration of phosphate was approximately five times higher than that of the complex isolated from yeast cells grown in medium depleted of phosphate (Fig. 1, lanes 1 and 2). Protein immunoblotting indicated that the observed difference in kinase activity was not

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a result of different amounts of HA_3-PHO80 (Fig. 1A) or PHO85 (Fig. 1C) in the immunoprecipitates. Thus, the kinase activity of the PHO80-PHO85 complex is regulated in response to the concentration of extracellular inorganic phosphate; its activity is high when yeast cells are grown in the presence of high concentrations of phosphate and decreased when they are starved for phosphate.

Genetic studies suggest that PHO81 may regulate or antagonize PHO80-PHO85 activity (*1*). PHO81 is required for induction of *PHO5* transcription (*4*); overexpression of PHO81 causes constitutive expression of *PHO5* (*5*, *6*), and epistasis studies indicate that PHO81 acts upstream of PHO80 and PHO85 (*7*). To determine if PHO81 is involved in controlling PHO80-PHO85, we tested whether PHO80-PHO85 activity was phosphate-regulated in a *pho81* deletion strain (*pho81*Δ). The PHO80-PHO85 complex isolated by immunoprecipitation from a *pho81* deletion strain grown in the presence of low or high concentrations of phosphate had similar amounts of kinase activity, which suggests that the decrease in PHO80-PHO85 activity observed when yeast cells are starved for phosphate is PHO81-dependent (Fig. 1A, lanes 4 and 5) (*8*). HA_3-PHO80 immunoprecipitates from a wild-type strain grown in the presence of low or high concentrations of phosphate contained similar amounts of PHO81 (Fig. 1C), which indicates that PHO81 is associated with the PHO80-PHO85 complex under both conditions. Thus, PHO81 may directly participate in the phosphate-dependent modulation of the kinase activity of PHO80-PHO85.

To investigate the requirements for the interaction of PHO81 with the PHO80-PHO85 complex, we examined immunoprecipitates of PHO81. Py_2-PHO81, an epitope-tagged version of PHO81 containing two copies of a polyoma medium T epitope tag (*9*), was immunoprecipitated from yeast whole-cell lysates containing overexpressed HA-PHO80 (*2*) and Py_2-PHO81, and the samples were analyzed on silver-stained SDS-polyacrylamide gels. The PHO80-PHO85 complex was associated with Py_2-PHO81 (Fig. 2A, lane 4) (*10*–*12*).

We next examined if the interaction of PHO81 with the PHO80-PHO85 complex is mediated primarily through the cyclin or CDK subunit. Py_2-PHO81 was immunoprecipitated from a strain that contains overexpressed HA-PHO80 (*2*) but lacks PHO85 (*pho85*Δ) (Fig. 2A, lane 3) or from a strain that contains overexpressed PHO85-HA (*13*) but lacks PHO80 (*pho80*Δ) (Fig. 2A, lane 5). Py_2-PHO81 interacted with the cyclin HA-PHO80 in the absence of the CDK PHO85, but not with PHO85-HA in the absence of PHO80 (*10*). PHO85 was not displaced when PHO81 interacted with the PHO80-PHO85 complex; in Py_2-PHO81 immunoprecipitates, both HA-PHO80 and PHO85 were observed (Fig. 2A, lane 4) and in HA_3-PHO80 immunoprecipitates both PHO85 and PHO81 were present (Fig. 1C). Thus, PHO81 associates specifically with the PHO80-PHO85 complex, and much of this interaction is mediated through the PHO80 cyclin subunit. These biochemical results are consistent with genetic data that suggest that the defect in *PHO5* transcription observed in yeast cells carrying a dominant, constitutive allele of PHO81 can be partially corrected by overexpressing PHO80 but not PHO85 (*6*).

To determine if the association of PHO81 with PHO80-PHO85 affects its function, we immunoprecipitated the PHO80-PHO85 complex from yeast cells containing either endogenous or overexpressed levels of PHO81 and assayed its kinase activity. The amount of PHO4 phosphorylated by the PHO80-PHO85 complex isolated from yeast cells containing overexpressed PHO81 was reduced compared to that in similar samples derived from strains with endogenous levels of PHO81 (Fig. 2B, lanes 1 and 4). It is unlikely that PHO81 is a PHO4 phosphatase because PHO4 phosphorylated by the PHO80-PHO85 complex was not dephosphorylated when incubated with immunoprecipitated PHO81 (Fig. 2B, lane 2). Moreover, adenosine triphosphate and PHO4 incubated with the PHO81-PHO80-PHO85 complex could still be used by the PHO80-PHO85 complex, which suggests that PHO81 does not act as an adenosine triphosphatase or PHO4 protease (Fig. 2B, lane 5). These data suggest that PHO81 is an inhibitor of the kinase activity of PHO80-PHO85.

To test this model directly, we purified Py_2-PHO81 (9) from yeast cells and assayed

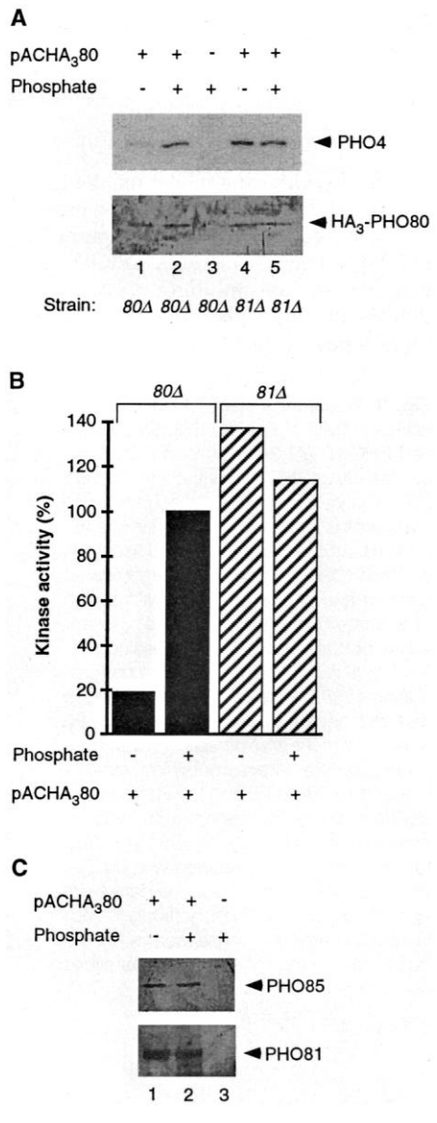

Fig. 1. The kinase activity of the PHO80-PHO85 complex is regulated in response to phosphate in a PHO81-dependent manner. (**A**) HA_3-PHO80 (*3*) immunoprecipitates (*30*) from lysates of the indicated strains (*33*), grown in medium with either a low or high phosphate concentration (*34*), were divided into two samples and either assayed for kinase activity with PHO4 as a substrate (top) (*2*) or boiled in sample buffer and assayed for the presence of HA_3-PHO80 by protein immunoblot analysis with antibodies to HA (bottom) (*23*). A *pho80*Δ strain harboring the $pACHA_3$80 vector (*3*) (lanes 1 and 2) or no vector (lane 3) was grown in medium with a low phosphate concentration (lane 1) or a high phosphate concentration (lanes 2 and 3); a *pho81*Δ strain harboring $pACHA_3$80 (lanes 4 and 5) was grown in medium with a low phosphate concentration (lane 4) or a high phosphate concentration (lane 5). (**B**) Quantitation of the kinase activity data from (A) with a PhosphorImager (Molecular Dynamics). Kinase activity in (A), lane 2, was set to 100%. (**C**) HA_3-PHO80 immunoprecipitates (*30*) from lysates of a *pho80*Δ strain grown in medium with a low or high phosphate concentration (*34*) were assayed for the presence of PHO85 (top) and PHO81 (bottom) by protein immunoblot analysis with antibodies to PHO85 (*31*) and PHO81 (*16*), respectively (*35*). A *pho80*Δ strain harboring the $pACHA_3$80 vector (lanes 1 and 2) or no vector (lane 3) was grown in medium with a low phosphate concentration (lane 1) or a high phosphate concentration (lanes 2 and 3).

it for its ability to inhibit the kinase activity of the immunopurified PHO80-PHO85 complex. Py_2-PHO81 purified to apparent homogeneity migrated at approximately 130 kD (Fig. 3A), close to its predicted molecular size of 132 kD (*14*), and was immunoreactive with antibodies to polyoma medium T antigen (*15*) and antibodies to PHO81 (*12*, *16*). An acid phosphatase plate assay indicated that this epitope-tagged version of PHO81 was functional in yeast, as overexpression caused constitutive *PHO5* expression (Fig. 3B). Purified Py_2-PHO81 inhibited the kinase activity of PHO80-PHO85 in a dose-dependent manner (Fig. 3C), and approximately 1 nM Py_2-PHO81 is required for 50% inhibition (IC_{50}) (*17*).

Several CDK inhibitors involved in cell cycle control have been cloned (*18*, *19*). PHO81 has similarity to $p16^{INK4}$ (Fig. 4A), an inhibitor of mammalian CDK4 (*19*) that may be a tumor suppressor (*20*). The similarity between PHO81 and $p16^{INK4}$ is limited to a region of PHO81 containing six copies of the ankyrin repeat motif (*14*), a 33–amino acid sequence postulated to participate in protein-protein interactions (*21*). To test directly if the ankyrin repeat–containing region of PHO81 is functional as a CDK inhibitor, we expressed this region of PHO81 (amino acids 400 to 720) in *Escherichia coli*, purified it, and assayed it for its ability to inhibit the kinase activity of PHO80-PHO85 in vitro (Fig. 4, B and C). The ankyrin repeats of PHO81, but not equivalent fractions from *E. coli* lacking the protein (*12*), inhibited the kinase activity of PHO80-PHO85 (Fig. 4C). The concentration of His-PHO81 ankyrin repeats required for IC_{50} is approximately 60 nM (*17*).

PHO81 was associated with PHO80-PHO85 when yeast cells were grown in either low or high concentrations of phosphate, yet PHO81 associated with PHO80-PHO85 is inactive as an inhibitor when yeast cells are grown in the presence of high phosphate concentrations (Fig. 1). In contrast, overexpressed Py_2-PHO81 purified from yeast cells grown in the presence of high concentrations of phosphate is active as an inhibitor in vitro (Figs. 2B and 3). One model that accounts for these data is as follows: A molecule or enzyme may exist that inactivates PHO81 when yeast cells are grown in medium containing high concentrations of phosphate. When PHO81 is overexpressed, such a molecule may become limiting, and as a consequence, most of the PHO81 would escape inactivation. Previous studies are consistent with the idea that the activity of PHO81 may be controlled after translation; *PHO5* mRNA induction is unaffected by cycloheximide treatment (*22*).

Our findings and previous genetic data lead to the following model for the induction of *PHO5* transcription. In the presence of high concentrations of phosphate, PHO81 is bound to PHO80-PHO85 but is inactive as an inhibitor. The kinase PHO80-PHO85 phosphorylates PHO4, thereby inactivating it and preventing transcription of *PHO5*. In medium depleted of phosphate, PHO81 is bound to and inhibits the kinase activity of PHO80-PHO85; inhibition of PHO80-PHO85 allows underphosphorylated PHO4 to activate transcription of *PHO5*.

Our results indicate that PHO81 is a

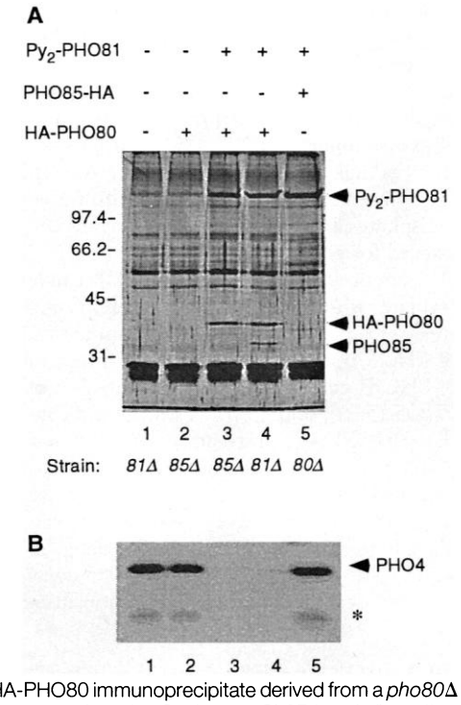

Fig. 2. Interaction of PHO81 with the PHO80-PHO85 complex inhibits the kinase activity of PHO80-PHO85. (**A**) Silver-stained SDS-polyacrylamide gel (9%) of Py_2-PHO81 (*9*) immunoprecipitates (*30*) derived from lysates of the indicated strains grown in medium with a high phosphate concentration. Lane 1, *pho81*Δ; lane 2, *pho85*Δ overexpressing HA-PHO80 (*2*); lane 3, *pho85*Δ overexpressing HA-PHO80 and Py_2-PHO81 (*9*); lane 4, *pho81*Δ overexpressing HA-PHO80 and Py_2-PHO81; lane 5, *pho80*Δ overexpressing PHO85-HA (*13*) and Py_2-PHO81. (**B**) HA-PHO80 was immunoprecipitated (*30*) from the indicated strains grown in medium with a high phosphate concentration, and a kinase assay (*2*) was performed on the immunoprecipitate with purified PHO4 as a substrate. The soluble portion of each kinase reaction was transferred to a second immunoprecipitate (*36*) and incubated before electrophoretic separation on an SDS-polyacrylamide gel (12%) and autoradiography (*37*). An HA-PHO80 immunoprecipitate derived from a *pho80*Δ strain overexpressing HA-PHO80 (*2*) was transferred to Sepharose CL2B beads (lane 1) or to an HA-PHO81 immunoprecipitate derived from a *pho81*Δ strain overexpressing HA-PHO81 (*38*) (lane 2). A control immunoprecipitate derived from a *pho4*Δ*pho80*Δ strain lacking HA-PHO80 was transferred to Sepharose CL2B beads (lane 3). An HA-PHO80 immunoprecipitate derived from a *pho80*Δ strain overexpressing HA-PHO80 and PHO81 (*26*) was transferred to Sepharose CL2B beads (lane 4) or to an HA-PHO80 immunoprecipitate derived from a *pho80*Δ strain overexpressing HA-PHO80 (lane 5). The asterisk marks the position of a truncated PHO4 protein that is phosphorylated.

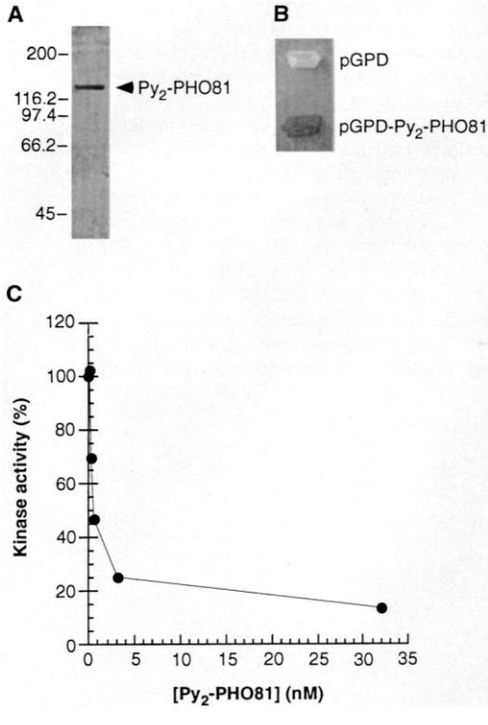

Fig. 3. Dose-dependent inhibition of the kinase activity of PHO80-PHO85 by purified PHO81. (**A**) Silver-stained SDS-polyacrylamide gel (8%) of purified Py_2-PHO81 (*32*). The position of the molecular weight standards is indicated on the left in kilodaltons. (**B**) Acid phosphatase plate assay (*4*) of strain NBW3 (*5*) carrying the indicated plasmid and grown on plates with a high phosphate concentration [top, vector alone; bottom, vector overexpressing Py_2-PHO81 (*9*)]. Yeast cells that were not producing *PHO5* appeared white and those that did appeared dark. (**C**) HA-PHO80 was immunoprecipitated from a *pho4*Δ*pho80*Δ strain containing overexpressed HA-PHO80, and the immunoprecipitates were incubated with various amounts of purified Py_2-PHO81 (*17*, *32*). Kinase activity was measured with purified PHO4 as a substrate (*39*). The data were quantitated with a PhosphorImager (Molecular Dynamics). Kinase activity in the buffer control (no Py_2-PHO81) was set to 100%.

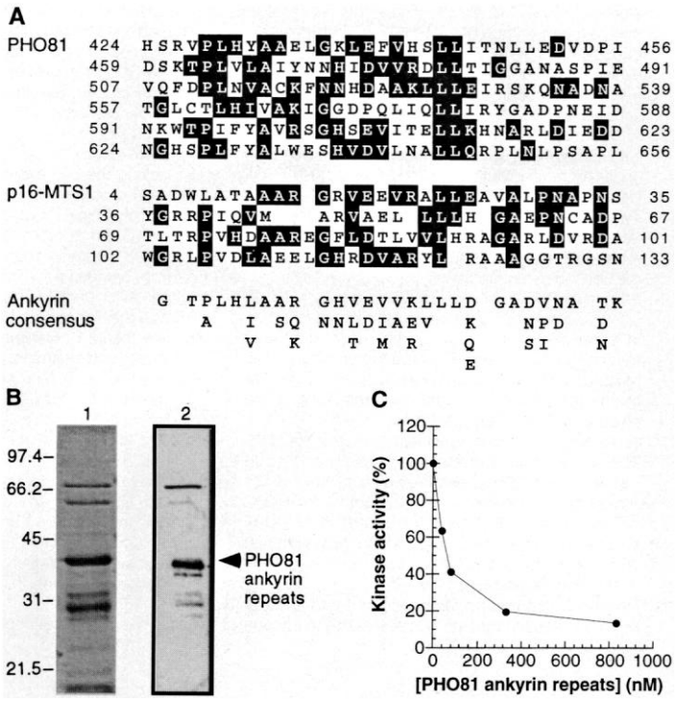

Fig. 4. Similarity of the ankyrin repeat domain of PHO81 to that of $p16^{INK4}$ and inhibition of PHO80-PHO85 kinase activity in vitro. (**A**) Primary sequence comparison of the ankyrin repeat region of PHO81 (*14*) with $p16^{INK4}$ (*19*) and with an ankyrin repeat consensus sequence (*21*, *24*). The shaded regions denote amino acids of $p16^{INK4}$ and PHO81 that match the ankyrin repeat consensus (*21*). (**B**) Purified PHO81 ankyrin repeats (*28*) were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie staining (lane 1) or by protein immunoblotting with antibodies to PHO81 (lane 2) (*35*). The position of the molecular weight standards is indicated on the left in kilodaltons. (**C**) HA-PHO80 immunoprecipitates were incubated with various amounts of purified PHO81 ankyrin repeats (*17*, *28*) and assayed for kinase activity with purified PHO4 as a substrate (*39*). The data were quantitated with a PhosphorImager (Molecular Dynamics). Kinase activity in the buffer control (no His-PHO81 ankyrin repeats) was set to 100%.

CDK inhibitor for the kinase PHO80-PHO85 and suggest that the activity of PHO81 is controlled in response to extracellular phosphate levels by a posttranslational mechanism. In addition to contributing to an understanding of the mechanisms of *PHO5* regulation, this system provides an opportunity to study the function and regulation of a cyclin-CDK complex, its substrate, and a CDK inhibitor in a physiologically relevant context.

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10. The identity of each labeled band was confirmed by protein immunoblotting with antibody to HA (*23*), Py (polyoma virus medium T) (*15*), or PSTAIRE (*24*). The PSTAIRE sequence is conserved in many CDKs, including PHO85 [S. K. Hanks and A. M. Quinn, *Methods Enzymol.* **200**, 38 (1991)].
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17. The concentration of Py_2-PHO81 was estimated by comparison to molecular weight standards of known concentration on a silver-stained SDS-polyacrylamide gel. The concentration of His-PHO81 ankyrin repeats was similarly estimated with a Coomassie-stained gel. The error in these measurements is estimated to be approximately fivefold.
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26. The vector for overexpressing untagged PHO81 (GPD-PHO81) consists of the *GPD* promoter (*25*) followed by the entire PHO81 open reading frame (*14*).
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28. The purified PHO81 ankyrin repeats consist of PHO81 amino acids 400 to 720, preceded by a polyhistidine tag. The predicted amino acid sequence of the protein produced with the vector T7 His-PHO81 ankyrin repeats reads MGHHHHHHHHHH-SSGHIEGRMLDEAL-PHO81-KLR (*24*). *Escherichia coli* strain BL21 (DE3) harboring the T7 His-PHO81 ankyrin repeats vector was grown to an absorbance at 600 nm (A_{600}) of ~0.5 in 2 liters of Luria broth with ampicillin and induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 90 min at 25°C. Cells were harvested, resuspended in 30 ml of histidine-binding buffer (HBB) [20 mM tris-Cl (pH 8.0), 5 mM imidazole, 500 mM NaCl, 0.1% NP-40, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by sonication. All subsequent steps were done at 4°C. Debris was sedimented in an SS34 rotor for 20 min at 15,000 rpm, and the supernatant was loaded onto a 0.7-ml iminodiacetic acid column (Sigma) that had been loaded with 0.2 M $NiSO_4$ and equilibrated with HBB. The column was washed with 50 ml of HBB followed by 50 ml of imidazole wash buffer [60 mM imidazole, 500 mM NaCl, and 20 mM tris-Cl (pH 8.0)]. The PHO81 ankyrin repeats were eluted with imidazole elution buffer [1 M imidazole, 500 mM NaCl, and 20 mM tris-Cl (pH 8.0)], and fractions containing the purified protein were pooled and dialyzed overnight in 2 liters of storage buffer [500 mM NaCl, 20 mM tris-Cl (pH 8.0), 10% glycerol, and 1 mM PMSF]. The final yield of purified protein was estimated to be ~300 μg after dialysis. Mock purification of the PHO81 ankyrin repeats was done in exactly the same manner except that the starting material was 2 liters of BL21 (DE3) cells lacking the expression vector and grown in Luria broth without ampicillin.
29. E. Harlow and D. Lane, Eds., *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988), pp. 522–523.
30. Lysate preparation and immunoprecipitation were as

described (*2*), except that cells were washed with H_2O instead of phosphate-buffered saline (PBS) [10 mM sodium phosphate (pH 7.4) and 150 mM NaCl] before lysis, and immunoprecipitates were washed with PBS containing 0.1% NP-40. Immunoprecipitation was done with 25 μl of either protein A–Sepharose coupled (*29*) to antibodies to HA (*23*) or protein G–Sepharose coupled (*29*) to antibodies to Py (*15*).

31. Polyclonal serum to PHO85 was derived from rabbits immunized with purified glutathione-S-transferase (GST)–PHO85 protein. The plasmid producing GST-PHO85 consists of PHO85 DNA coding for amino acids 4 to 306 (*27*) cloned into pGEX-2T (Pharmacia). Antibodies to PHO85 were affinity-purified from serum with a GST-PHO85 column made by coupling the purified protein to AffiGel-10 (Bio-Rad).
32. To purify Py_2-PHO81, we grew 4 liters of the strain Y57 *pho81*Δ carrying the GPD-Py_2-PHO81 expression vector (*9*) to a value of A_{600} of ~0.9 in synthetic medium containing a high concentration of phosphate. Cells were harvested and resuspended in 50 ml of ice-cold Py_2 buffer [20 mM tris Cl (pH 8.0), 100 mM NaCl, 10% glycerol, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM PMSF, 2 mM benzamidine-HCl, 80 mM β-glycerophosphate, 10 mM NaF, and 10 nM calyculin A (LC Laboratories, Woburn, MA)]. All subsequent steps except the elution were done at 4°C. Acid-washed glass beads were added, and the cells were lysed in a bead beater (Biospec Products, Bartlesville, OK) by beating for seven 30-s pulses with 1-min interludes on ice. Beads were pelleted by spinning the lysate for 5 min at 2000 rpm in a Sorvall RC-3B rotor. Debris was removed by spinning the supernatant in a Beckman Ti70 rotor for 90 min at 60,000 rpm. The supernatant contained ~40 mg of protein in ~40 ml. *N*-Octylglucoside (Boehringer Mannheim) was added to the supernatant to a final concentration of 0.1%, and PMSF was added to a final concentration of 1 mM; the solution was spun again for 10 min in an SS34 rotor at 10,000 rpm. The supernatant was combined with 750 μl of a 1:1 slurry of protein G–Sepharose coupled (*29*) to antibodies to Py (*15*) and mixed on a rotating wheel for 60 min. The resin was washed twice with 15 ml of Py_2-buffer containing 0.1% *N*-octylglucoside, then twice with 15 ml of Py_2-buffer containing 0.5% NP-40 and 1 mM DTT. Finally, the resin was washed once with 10 ml of PBS with 0.1% *N*-octylglucoside and then eluted by incubation for 10 min at room temperature with 250 μl of PBS containing 0.1% *N*-octylglucoside and 100 μg/ml Py peptide (EYMPME) (*24*). The eluted protein was approximately 10 to 20 μg of highly purified Py_2-PHO81.
33. Unless noted, all strains are isogenic derivatives of Y57 (*MAT***a** *ura3-52 trp1-Δ63 leu2 his3-Δ1 prb1-1122 pep4-3 prc1-407*), which is protease-deficient but wild-type with respect to the *PHO* genes (*2*). Disruption strains were constructed by one-step gene replacement techniques [R. Rothstein; *Methods Enzymol.* **194**, 281 (1991)].
34. Five-milliliter cultures of either Y57 *pho81*Δ or Y57 *pho80*Δ harboring the pACHA$_3$80 vector (*3*) were grown overnight to saturation in synthetic medium lacking uracil but with a high phosphate concentration. The cells were harvested, resuspended in an equal volume of H_2O, and then inoculated into 100 ml of selective medium that had been depleted of inorganic phosphate [K. F. O'Connell and R. E. Baker, *Genetics* **132**, 63 (1992)] and supplemented with either KH_2PO_4 (1.5 mg/ml, high phosphate concentration) or KCl (1.5 mg/ml, low phosphate concentration). These cultures were grown for approximately 15 hours to a value of A_{600} of 0.5 to 1.0. Liquid whole-cell phosphatase assays were performed (*22*) to compare the levels of acid-phosphatase activity in these cultures. The relative amounts of such activity normalized to A_{600} were pACHA$_3$80/*pho80*Δ (low phosphate concentration), 105; pACHA$_3$80/*pho80*Δ (high phosphate concentration), 31; pACHA$_3$80/*pho81*Δ (low phosphate concentration), 7; and pACHA$_3$80/*pho81*Δ (high phosphate concentration), 17. We believe that the relatively modest induction that we observed in low phosphate concentrations in the *pho80*Δ strain carrying the complementing pACHA$_3$80 plasmid is due at least in part to the presence of the *PHO3* constitutive acid phosphatase gene [W. Bajwa *et al.*, *Nucleic Acids Res.* **12**, 7721 (1984)]. Lysates were prepared from these cultures (*30*) and processed as described above.
35. After transfer to nitrocellulose, the blot was probed with either affinity-purified antibodies to PHO81 (*16*) or affinity-purified antibodies to PHO85 (*31*) in TBST [10 mM tris-Cl (pH 8.0), 150 mM NaCl, 0.05% Tween-20, and 0.25 mM EDTA] containing 3% nonfat milk. Peroxidase coupled to donkey antibody to rabbit immunoglobulin G (Amersham) was used as the secondary antibody.
36. After immunoprecipitation (*30*) and a kinase assay (*2*) were done, the beads were sedimented for 2 min in a microfuge, and 20 μl of the supernatant was transferred to a second immunoprecipitate. This mixture was then incubated for an additional 5 min at room temperature before the reactions were stopped with SDS sample buffer.
37. Silver-stained SDS-polyacrylamide gels of HA-PHO80 (*2*) immunoprecipitation reactions done in parallel indicated that each reaction contained approximately the same amount of both HA-PHO80 and PHO85 (*12*). This gel also indicated that PHO81 is present in the HA-PHO81 immunoprecipitation and in the HA-PHO80 immunoprecipitations from strains overexpressing PHO81.
38. HA-PHO81 consists of amino acids 36 to 1179 of PHO81 preceded by a single copy of the HA epitope (*23*). The NH_2-terminal sequence of the construct reads MGYPYDVPDYAIEGRHTIP-PHO81 (*24*). The expression of this construct is under the control of the *GPD* promoter on a CEN-ARS plasmid (*25*).
39. HA-PHO80 was immunoprecipitated (*2*) from a whole-cell extract [M. Woontner *et al.*, *Mol. Cell Biol.* **11**, 4555 (1991)] made from a *pho4*Δ*pho80*Δ strain overexpressing HA-PHO80 or from the same cells lacking the overexpressed HA-PHO80. The immunoprecipitates were washed with kinase buffer [20 mM tris Cl (pH 7.5) and 10 mM $MgCl_2$] and incubated for 10 min at room temperature with either purified PHO81 ankyrin repeats (*28*), equivalent fractions from bacteria not expressing the PHO81 ankyrin repeats, or purified Py_2-PHO81 (*32*). A kinase assay was then done with PHO4 as the substrate (*2*).
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Interaction of the Protein Kinase Raf-1 with 14-3-3 Proteins

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Members of a family of highly conserved proteins, termed 14-3-3 proteins, were found by several experimental approaches to associate with Raf-1, a central component of a key signal transduction pathway. Optimal complex formation required the amino-terminal regulatory domain of Raf-1. The association of 14-3-3 proteins and Raf-1 was not substantially affected by the activation state of Raf.

Raf-1, a mitogen-stimulated serine-threonine protein kinase, functions in the control of cell growth, transformation, and differentiation (*1*). Binding of ligands to tyrosine kinase receptors at the cell surface leads to an increase in the amount of the active [guanosine triphosphate (GTP)–bound] form of Ras (*2*). Activated Ras interacts directly with the NH_2-terminal regulatory domain of Raf-1 (*3*), resulting in the recruitment of Raf-1 to the plasma membrane (*4*, *5*). There, Raf-1 is activated by an unknown mechanism that is independent of Ras. Because Raf-1 exists as a large (300- to 500-kD) complex (*5*, *6*), identification and analysis of proteins that interact with Raf-1 are crucial for understanding how it is activated in the plasma membrane.

To search for proteins that directly participate in Raf function, we immunoprecipitated Raf-1 from ^{35}S-methionine–labeled NIH 3T3 cells stably expressing human Raf-1 in amounts about five times greater than the amount of endogenous Raf-1. When Raf immune complexes were analyzed on two-dimensional isoelectric focusing–SDS-polyacrylamide gels, four major protein spots (of 27, 29, 50, and 90 kD) were found that were absent in preimmune controls (Fig. 1, A and B) (*7*). The 90-kD and 50-kD proteins are the molecular chaperones hsp90 and p50 (*6*). The migration of the p27 and p29 proteins on these gels was similar to that of 14-3-3 proteins, which had been identified previously as polyoma-

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