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Functional Domains of Pho81p, an Inhibitor of Pho85p Protein Kinase, in the Transduction Pathway of P_i Signals in *Saccharomyces cerevisiae*

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The *PHO81* gene is thought to encode an inhibitor of the negative regulators (Pho80p and Pho85p) in the phosphatase (*PHO*) regulon. Transcription of *PHO81* is regulated by P_i signals through the same *PHO* regulatory system. Elimination of the *PHO81* promoter or its substitution by the *GAL1* promoter revealed that stimulation of the *PHO* regulatory system requires both increased transcription of *PHO81* and a P_i starvation signal. The predicted Pho81p protein contains 1,179 amino acids (aa) and has six repeats of an ankyrin-like sequence in its central region. The minimum amino acid sequence required for Pho81p function was narrowed down to a 141-aa segment (aa 584 to 724), which contains the fifth and sixth repeats of the ankyrin-like motif. The third to sixth repeats of the ankyrin-like motif of Pho81p have significant similarities to that of $p16^{INK4}$, which inhibits activity of the human cyclin D-CDK4 kinase complex. Deletion analyses revealed that the N- and C-terminal regions of Pho81p behave as negative and positive regulatory domains, respectively, for the minimal 141-aa region. The negative regulatory activity of the N-terminal domain was antagonized by a C-terminal segment of Pho81p supplied in *trans*. All four known classes of $PHO81^c$ mutations that show repressible acid phosphatase activity in high-P_i medium affect the N-terminal half of Pho81p. An in vitro assay showed that a glutathione *S*-transferase–Pho81p fusion protein inhibits the Pho85p protein kinase. Association of Pho81p with Pho85p or with the Pho80p-Pho85p complex was demonstrated by the two-hybrid system.

Transcription of the genes encoding three isozymes (p60, p58, and p56) of a repressible acid phosphatase (EC 3.1.3.2) (rAPase), a repressible alkaline phosphatase (EC 3.1.3.1) (rALPase), and a P_i-transporter in *Saccharomyces cerevisiae* is repressed coordinately by the P_i concentration of the medium (for reviews, see references 16, 25, and 26). P_i signals from the medium are conveyed to these genes, *PHO5* (encoding the p60 rAPase), *PHO10* (p58), *PHO11* (p56), *PHO8* (rALPase), and *PHO84* (P_i-transporter), through a *PHO* regulatory system consisting of products of at least five genes: *PHO2* (also known as *BAS2*/*GRF10*), *PHO4*, *PHO80*, *PHO81*, and *PHO85*. A current model proposes that a specific DNA-binding protein, Pho4p, encoded by *PHO4*, is indispensable for transcription of the structural genes of all of these enzymes. The *PHO2* gene, which bears a homeodomain (5), is also required for transcription of the *PHO* system and various other genes. In high-P_i medium, the two negative regulators, Pho80p and Pho85p, form a complex similar to that formed by a cyclin and cyclin-dependent protein kinase, which is thought to inhibit Pho4p function by causing its hyperphosphorylation (18). When the P_i concentration of the medium is sufficiently low, Pho81p inhibits the Pho80p-Pho85p complex, allowing Pho4p (with Pho2p) to transcribe the structural genes (18, 32).

The *PHO4* (42), *PHO80* (38), and *PHO85* (21) genes are transcribed constitutively at low levels. The *PHO2* gene is also transcribed at a low level but is self-regulated (42). Transcription of *PHO81*, however, is under the regulation of P_i through the *PHO* regulatory system discussed above (7, 23, 43). Thus, the *PHO* regulatory system forms a positively regulating feedback loop for enzyme synthesis.

Most mutations occurring in the *PHO81* locus are recessive and result in the $rAPase^-$ phenotype. However, a dominant mutation, $PHO81^c$, conferring a low but substantial level of expression of rAPase in high-P_i medium, had been isolated (37). Creacy et al. (7) also isolated seven mutants conferring the same phenotype as the $PHO81^c$ mutation and mapped the mutations to three separate regions in the *PHO81* locus.

PHO81 DNA was cloned independently by Coche et al. (6) and by ourselves (41). Its putative product was shown to be a 134-kDa protein containing six repeats of an ankyrin-like motif (23), which have been suggested to function in protein-protein interactions (1, 35). It was reported recently that human $p16^{INK4}$, an inhibitor of cyclin-dependent protein kinase 4 (CDK4), also contains the ankyrin repeats (33).

Here, we report that both enhanced transcription of *PHO81* by the positive feedback regulation and a P_i starvation signal are necessary for proper expression of *PHO5*. Complementation experiments with various deletion fragments of *PHO81* DNA revealed that a 141-amino-acid (aa) middle region is sufficient for Pho81p function. The N- and C-terminal regions of Pho81p antagonize each other negatively and positively, respectively, for Pho81p function. Pho81p was shown to associate with Pho85p and to inhibit its protein kinase activity.

MATERIALS AND METHODS

Organisms, plasmids, DNAs, and sera. The wild-type *PHO81* DNA fragment and various derivatives of it were obtained during a previous study (23). *S. cerevisiae* NBD81-6D (*MAT***a** *pho3-1* Δ*pho81*::*HIS3* [here described as *pho81-Δ1*] *ade2 can1 leu2-3,112 his3-532 trp1-289 ura3-1,2* [4]), which has a disrupted *pho81* allele, and the $PHO81^c$*-1* mutant, O106-M30 (*MAT***a** *pho3-1* $PHO81^c$*-1 leu1 his5 gal4*), isolated during a previous study (37), were used. An *S. cerevisiae* strain, YAT1565 (*MAT*α *pho3 his3 ura3 leu2*::[*LEU2 GAPp-PHO80*]) harboring plasmid pKOM1 or pGST-PHO85 (11), was obtained from A. Toh-e and used for the preparation of glutathione *S*-transferase (GST) and the GST-Pho85p fusion protein, respectively. Expression of the *GST-PHO85* construct of the pGST-PHO85 plasmid was driven by a promoter fragment of the glyceraldehyde-3-

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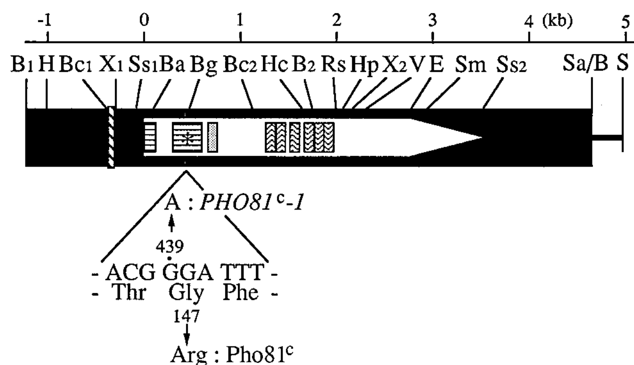

FIG. 1. Restriction map of *PHO81* DNA. The closed box and thick line indicate DNA fragments of the *S. cerevisiae* DNA bearing the *PHO81* gene and a 276-bp *Bam*HI-*Sal*I fragment of the pBR322 DNA. The open arrow and the box with diagonal stripes indicate the *PHO81* ORF and the Pho4p protein binding site (23), respectively. The two boxes with horizontal lines, the dotted box, and the six boxes with wavy lines in the ORF indicate regions similar to Pho87p (our unpublished results), the asparagine-rich region, and ankyrin-like repeats (23), respectively. The site of the *PHO81^c-1* mutation is indicated by an asterisk, and its nucleotide and deduced amino acid sequences are shown below the map. The restriction sites are indicated as follows: B, *Bam*HI; Ba, *Bal*I; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; Hp, *Hpa*II; Rs, *Rsa*I; S, *Sal*I; Sa, *Sau*3AI; Sm, *Sma*I; Ss, *Ssp*I; V, *Eco*RV; and X, *Xho*I. Two or more identical restriction sites in the map are distinguished by numerical suffixes. Other *Eco*RI, *Hin*cII, *Hin*dIII, *Hpa*II, *Rsa*I, and *Sau*3AI sites are present in the 6.2-kb *Bam*HI$_1$-*Sal*I fragment but are not shown for simplicity.

phosphate dehydrogenase gene (*GAPp*) as part of the *GAPp-PHO80* fusion gene. Another *S. cerevisiae* strain, YAT1514 (*MAT*α *ura3 pho3 pho80 pho85*) harboring the pGST-PHO85 plasmid, was also provided by A. Toh-e and was used for the preparation of GST-Pho85p in the *pho80*-disrupted cells. A yeast host, Y190 (*MAT***a** *leu2-3,112 ura3-52 trp1-901 his3-Δ200 ade2-101 gal4Δ gal80Δ* [*URA3 GAL-lacZ*] [*LYS2 GAL-HIS3*] *cyh^r*), and two plasmids, pAS1 and pACTII, for the two-hybrid system (10), were gifts from S. J. Elledge (8, 9, 13). These pAS1 and pACTII plasmids bear the DNA-binding domain and transcriptional activation domain, respectively, of *GAL4*. The plasmid vectors for *S. cerevisiae* were YCp50 (27), YEp13 (27), YEp131 (YEp13 with an inversion of a 2.2-kb *LEU2* DNA [24]), YEp241 (constructed by insertion of a 2.2-kb *Eco*RI fragment of the B form of 2μm plasmid prepared from YEp24 [27] into the *Eco*RI site of YIp5 [27]), and pBM150 (a YCp50-based plasmid bearing the *GAL1* promoter of *S. cerevisiae* [17, 27]). A 1.0-kb *Hin*dIII-*Xho*I fragment of the *ACT1* gene used as a hybridization probe was prepared from pYA301 (12). A YCp-type plasmid, pSB32-GAL4, bearing the wild-type *GAL4* DNA and marked with the *LEU2* gene (30), was obtained from S. A. Johnston.

Escherichia coli DH5α (31), for the manipulation of DNA, and GM33 (*dam*$^-$) (22), for the preparation of plasmid DNA that was digested with *Bcl*I, were used. Plasmid pUC18 (40) was used as an *E. coli* vector for DNA manipulation. Another *E. coli* plasmid, pGEX-2T, purchased from Pharmacia (Uppsala, Sweden), was used for expression of GST-fusion proteins in *E. coli*. Rabbit sera containing anti-GST and anti-Pho4p antibodies were raised against the GST (see below) and Pho4p (23) proteins, respectively, as previously described (14).

To investigate the positive feedback loop of the *PHO* regulon, we constructed a plasmid, pAC879, bearing the *PHO81* open reading frame (ORF) region directly downstream of the *GAL1* promoter (*GAL1p*) from nucleotide +4 of *GAL10* to +55 of *GAL1*, relative to the respective transcription start sites, by inserting a 5.1-kb *Ssp*I$_1$-*Sal*I fragment of *PHO81* DNA (nucleotides −76 to +4656 relative to the ATG codon of *PHO81* with a 276-bp fragment of pBR322 [Fig. 1]) into the small *Bam*HI-*Sal*I gap of plasmid pBM150 after connecting an 8-bp *Bam*HI linker (Takara Shuzo, Kyoto, Japan) at the *Ssp*I-cleaved end of the *PHO81* DNA. Another plasmid, pAC882, constructed by the insertion of a 6-kb *Hin*dIII-*Sal*I fragment of the wild-type *PHO81* DNA (Fig. 1) into the small *Hin*dIII-*Sal*I gap of YCp50, was used as the control. To detect interaction of Pho81p and Pho85p by the two-hybrid system (10), four YEp-type plasmids were constructed by the insertion of a 4.8-kb *Bal*I-*Sal*I fragment of the *PHO81* DNA (nucleotides +104 to +4656 of *PHO81* and the pBR322 fragment [Fig. 1]) or a 2.6-kb *Xho*I-*Sal*I fragment of the *PHO85* DNA (nucleotides +246 to approximately +2300 with the 276-bp fragment of pBR322) from plasmid pNF1 (39) into the *Bam*HI-*Sal*I gap of pAS1 (8) or the *Bam*HI-*Xho*I gap of pACTII (8, 9) at their cloning sites. Coding frames of the *GAL4-PHO81* and *GAL4-PHO85* fusion genes in these plasmids were adjusted with appropriate linker insertions and/or a one-base deletion at the cloning site (details not shown).

Media and genetic and analytical methods. The synthetic high-P$_i$ (containing 1,500 mg of KH_2PO_4 per liter with appropriate additional nutrients but lacking uracil and/or leucine) and low-P$_i$ (containing 30 mg of KH_2PO_4 per liter but the same concentrations of other components as high-P$_i$ medium) media were prepared as described previously (42). Reported methods were used for the determination of rAPase activity of yeast cell suspensions and colonies (37) and for the preparation and purification of Pho4p protein (23). Routine methods were used for genetic manipulation, transformation, and β-galactosidase assay with *S. cerevisiae* (29); preparation, modification, sequencing, and analyses of DNA; and Northern (RNA) blotting and Western blotting (immunoblotting) analyses (31).

Purification of the GST-Pho81p fusion protein. A 1.8-kb *Bgl*II-*Eco*RV fragment of the wild-type *PHO81* DNA (Fig. 1) was inserted into the *Bam*HI-*Sma*I gap of plasmid pGEX-2T, and the resulting plasmid pETI136 was introduced into *E. coli* DH5α. The transformant, selected by ampicillin resistance, was grown at 37°C to an optical density at 600 nm of about 0.6 in broth supplemented with 50 μg of ampicillin per ml. Isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM, and the culture was incubated for an additional 2 h. A crude extract was prepared from a 1.5-liter culture as described previously (24), except that phosphate-buffered saline (150 mM NaCl in sodium phosphate buffer, pH 7.3) was used instead of buffer A. GST-Pho81p protein bearing a 614-aa portion of the Pho81p protein from aa 159 to 772 was purified with a glutathione-Sepharose affinity column (Pharmacia) as a 105-kDa fraction determined by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE [31]) at 5 V/cm according to the predicted molecular weight of GST-Pho81p. The same procedure was used for the purification of GST protein from *E. coli* DH5α(pGEX-2T). The putative GST-Pho81p band was confirmed to react with rabbit anti-GST serum.

Preparation and protein kinase assay of the GST-Pho85p fusion protein. Protein kinase was assayed as described by Reed et al. (28). The yeast transformants YAT1565(pGST-PHO85) (*PHO80*$^+$) (11), YAT1565(pKOM1) (*PHO80*$^+$) (11), and YAT1514(pGST-PHO85) (*pho80*$^-$) (36) were grown to an optical density at 660 nm of about 0.8 in 10 ml of synthetic high-P$_i$ or low-P$_i$ medium depleted of uracil at 30°C. The cultures were then chilled on ice, and all subsequent purification steps were performed at 0°C or on ice. The cells were harvested and washed with lysis buffer (1 mM sodium PP$_i$, 1% Triton X-100, 1% deoxycholate, 0.1% SDS in 50 mM Tris-HCl buffer [pH 7.5]). The cell pellet was resuspended in 0.3 ml of lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 μg each of aprotinin, leupeptin, and pepstatin A per ml and shaken vigorously for 30 min with 0.3 g of glass beads (diameter, 0.6 mm; Toshinriko, Tokyo, Japan) in a microtube mixer (model 5432; Eppendorf, Fremont, Calif.). The cell lysate was centrifuged for 10 min at 18,500 × *g*, and the supernatant was used as a cell extract. Samples of 1 ml of the cell extract containing 1 mg of protein were mixed with 3 μl of anti-GST serum and incubated at 0°C for 1.5 h or more. The extract was then mixed with 50 μl of protein A-Sepharose beads (Pharmacia) equilibrated with radioimmunoprecipitation assay buffer (lysis buffer supplemented with 300 mM NaCl) for 1.5 h or more. The supernatant was then removed, and the Sepharose beads were washed at least four times with 1 ml of radioimmunoprecipitation assay buffer and then with 0.5 ml of reaction buffer (20 mM Tris-HCl buffer [pH 7.5] with 10 mM $MgCl_2$). The beads were suspended in 40 μl of reaction buffer containing 1.65 pmol of [γ-^{32}P]ATP (5 μCi; ICN Biomedicals, Costa Mesa, Calif.) and 0.12 μg of Pho4p prepared from *E. coli* or κ-casein (Sigma Chemicals, St. Louis, Mo.) and incubated at 30°C for 30 min. A portion (30 μl) of the reaction mixture was then mixed with 6 μl of 5× loading buffer (250 mM Tris-HCl buffer [pH 6.8] containing 500 mM dithiothreitol, 10% SDS, 50% glycerol, and 0.5% bromophenol blue) and heated in boiling water for 5 min. Samples (15 μl) of the mixture were subjected to SDS–10% PAGE, and then the gel was dried and exposed to X-ray film. A portion (5 μl) of the same loading sample was subjected to SDS-PAGE as described above. Protein bands on the gel were transferred onto a polyvinylidene difluoride filter, and the filter was subjected to Western analysis.

For assay of the inhibitory activity of GST-Pho81p against Pho85p kinase, the method described above was modified as follows. The yeast cell extract (1 ml) containing 2 mg of protein was prepared from cells of YAT1565(pGST-PHO85) (*PHO80*$^+$) cultivated in high-P$_i$ medium and immunopurified with 6 μl of anti-GST serum and 100 μl of protein A-Sepharose beads. After being washed with the reaction buffer, the beads were resuspended in 300 μl of the same reaction buffer containing 13 pmol of [γ-^{32}P]ATP, and the mixture was divided into 30-μl portions. Each portion of the mixture was combined with 5 μl of the reaction buffer containing 60 ng of the Pho4p sample and various amounts of GST-Pho81p. The reaction mixture was incubated at 30°C for 30 min as described above.

RESULTS

Stimulation of the *PHO* regulatory system requires enhanced expression of *PHO81* and low P$_i$ concentration. Genetic analysis (25) suggested that *PHO81* encodes a negative regulator of the negative regulator Pho80p (and Pho85p) and functions at the most hypostatic position in the *PHO* regulatory system. In addition, *PHO81* transcription was shown to be regulated by the *PHO* regulatory system (7, 23, 41, 43). To investigate this positive feedback loop, plasmid pAC879 bear-

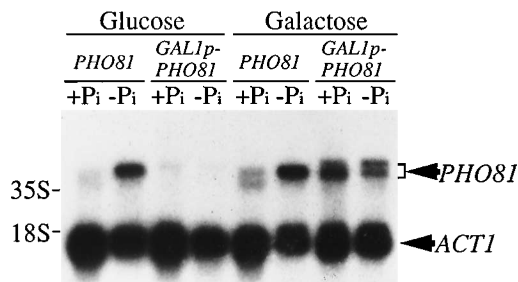

FIG. 2. Transcription from the *GAL1p-PHO81* gene fusion. Transformant cells NBD81-6D(pAC879) (bearing the *GAL1p-PHO81* gene) and NBD81-6D(pAC882) (*PHO81*$^+$) were inoculated into high-P_i ($+P_i$) and low-P_i ($-P_i$) media without the addition of uracil and containing 2% glucose or 3% galactose as the carbon source after precultivation in the same high-P_i glucose medium. The cultures were shaken at 30°C for 12 h for glucose medium or 20 h for galactose medium. Polyadenylated RNA fractions (1 μg) prepared from the cells were subjected to electrophoresis in agarose gel (1%) in the presence of formaldehyde and transferred onto a nylon filter. The RNA blots were hybridized with a mixture of the ^{32}P-labeled 1.2-kb *Bam*HI$_2$-*Sma*I fragment of *PHO81* DNA and a ^{32}P-labeled 1.0-kb *Hin*dIII-*Xho*I fragment of the *ACT1* DNA (12).

ing the *PHO81* ORF directly downstream of the *GAL1* promoter was introduced into the *pho81-Δ1* strain, NBD81-6D, and the rAPase activities of Ura$^+$ transformants were examined. We confirmed that the *GAL1p-PHO81* fusion gene in NBD81-6D(pAC879) cells was transcribed in galactose medium, irrespective of the P_i concentration, but not in media containing glucose (Fig. 2). The NBD81-6D(pAC879) cells showed rAPase activity in low-P_i galactose medium but not in high-P_i galactose medium (Fig. 3). These results indicate that *PHO5* derepression requires both high-level transcription of *PHO81* and low P_i concentration.

We reported previously (23) that the *PHO81* DNA segment responsive to P_i signals is located in the region between nucleotides −385 and −291 and that the specific sequence, CAC GTG, for binding the positive regulatory protein Pho4p, is located at nucleotide −344. We next constructed two YCp-type plasmids bearing the *PHO81*$^+$ or *PHO81*^{c} (see below) alleles but lacking the promoter region bearing the upstream activating sequence (UAS) from the −291 position (the *Xho*I$_1$ site [Fig. 1]). The resultant ΔUAS *PHO81* fragments could not complement the *pho81-Δ1* mutation when it was ligated into a low-copy-number vector, YCp50, but could do so slightly when it was ligated into a high-copy-number vector, YEp131 (Fig. 3). These results suggest that a residual amount of Pho81p was produced from ΔUAS *PHO81* DNA, that host cells with an increased dose of this DNA fragment could derepress *PHO5* slightly, and that the Pho81p activity was affected by the P_i concentration of the medium. Thus, both enhanced expression of *PHO81* and a low-P_i signal from the medium are necessary for activation of the *PHO* regulatory system.

Characterization of *PHO81*^{c} mutations. The *PHO81*^{c} mutation is dominant over the wild-type allele and results in an rAPase$^+$ phenotype in high-P_i medium (37). Northern blot analysis showed that the *PHO81*^{c} gene is transcribed in high-P_i medium at the same level as that for the wild-type cells cultivated in low-P_i medium (data not shown). Since the enhanced transcription of *PHO81* was not sufficient for *PHO5* derepression, constitutive synthesis of rAPase in the *PHO81*^{c} cells might be due to a modification of the Pho81p protein.

To characterize the *PHO81*^{c} mutation, various DNA fragments of the *PHO81*^{c}*-1* mutant, O106-M30, were cloned into pUC18 and selected by colony hybridization with *E. coli* DH5α as a host (details not shown). When a 0.37-kb *Bal*I-*Bgl*II region in the cloned *PHO81*^{c}*-1* DNA was substituted for the corresponding region of the wild-type *PHO81* DNA, the resultant chimeric 6-kb *Hin*dIII-*Sal*I fragment conferred the Pho81^c phenotype on *pho81-Δ1* cells. No other portion of the 5.9-kb *Bam*HI$_1$-*Sau*3AI *PHO81*^{c} DNA exchanged for the corresponding wild-type region conferred the Pho81^c phenotype. Only one base substitution, A for G at nucleotide +439, was found in the 0.37-kb *PHO81*^{c} fragment. This mutation should result in a substitution of arginine for Gly-147 (Fig. 1), and it is

DNA fragment	Gene structure	rAPase activity (mU/OD660/ml)					
		High copy		Low copy			
		Glucose		Glucose		Galactose	
		+Pi	-Pi	+Pi	-Pi	+Pi	-Pi
PHO81	H1 X1 Ss1 Sa1/B S; UAS*PHO81*	4.3	116	0.69	131	<0.5	57.7
PHO81 c	H1 S; *	ND	ND	189	405	ND	ND
GAL1p-PHO81	E B(Ss1) S; *GAL1p*	ND	ND	<0.5	1.37	0.62	62.7
ΔUAS *PHO81*	X1 S	<0.5	9.90	<0.5	0.63	ND	ND
ΔUAS *PHO81* c	X1 S; *	<0.5	9.40	<0.5	0.88	ND	ND
None (vector)		<0.5	<0.5	<0.5	<0.5	<0.5	0.50

FIG. 3. Effects of *PHO81* expression on synthesis of rAPase. The *PHO81* fragments with modification at their promoter or with the *PHO81*^{c} mutation were ligated into the small *Hin*dIII-*Sal*I gap of YEp131 (high-copy vector) and YCp50 (low-copy vector) plasmids. The shaded box (*GAL1p*) indicates the DNA fragment of *GAL1p*, and the other symbols and abbreviations are the same as those described in the legend to Fig. 1. Cells of strain NBD81-6D (*pho81-Δ1*) harboring these plasmids were precultivated in high-P_i medium containing 2% glucose without leucine or uracil, inoculated into high-P_i ($+P_i$) and low-P_i ($-P_i$) media containing 2% glucose or 3% galactose without leucine or uracil, and shaken at 30°C for 16 h for glucose medium or 24 h for galactose medium. Then the cells were harvested and their rAPase activities were determined at appropriate intervals, and the highest value attained in each culture was recorded. Values of rAPase activities are means for triplicate cultures. The standard deviation did not exceed 10% of each value. ND, Not determined; OD_{660}, optical density at 660 nm.

identical to one of the three *PHO81*^{c} mutations reported by Creasy et al. (7). The other two *PHO81*^{c} mutations reported by Creasy et al. (7) resulted in the substitution of aspartic acid for Gly-4 and of lysine for Glu-79. These observations suggest that the N-terminal portion of Pho81p from aa 4 to 158 inhibits the function of Pho81p.

We also obtained another *PHO81*^{c} mutation by the insertion of an 8-bp *Bam*HI linker at the *Bgl*II site (nucleotide position, +472) after filling in with the Klenow fragment. This linker insertion resulted in the insertion of four amino acid residues, SGSG, between aa 158 and 159. The resultant DNA fragment conferred the Pho81^{c} phenotype on the *pho81-Δ1* mutant even though the fragment was ligated into YCp50 (data not shown).

A 141-aa middle region of Pho81p is sufficient for Pho81p function. In a previous study (41), we observed that a 2.8-kb *Bam*HI$_2$-*Sau*3AI fragment encoding the C-terminal half of the *PHO81* ORF could complement a *pho81* mutation when ligated into YEp13, suggesting that the functional domain of Pho81p is encoded by the C-terminal half. For further investigation of this finding, various *pho81* mutant fragments bearing internal deletions but with the native *PHO81* promoter were constructed (Fig. 4) and ligated into YCp50 and YEp131. These plasmids were introduced into NBD81-6D, and the rAPase activities of the Ura^{+} and Leu^{+} transformants were determined in high- and low-P$_i$ media. Three fragments, the region from aa 376 to 1179 (376–1179), 551–1179, and 584–1179, producing Pho81p with N-terminal deletions complemented the *pho81-Δ1* mutation when ligated into YEp131, but fragments 666–1179, 773–1179, and 983–1179 did not. Thus, the region from aa 35 to 583 of Pho81p is dispensable for Pho81p function. The 584–1179 fragment covers almost the same region as the truncated *PHO81* DNA isolated by Yoshida et al. (41).

To determine the minimum functional region of Pho81p, sequential C-terminal deletions were constructed from the 584–1179 fragment. We found that fragments 584–982, 584–772, and 584–724 conferred substantial levels of rAPase activity on the *pho81-Δ1* mutant when ligated into YEp131, whereas the 584–690 fragment did not (Fig. 4). Thus, the minimal portion for Pho81p function is a 141-aa sequence encoded by the 584–724 fragment, which contains two copies of the ankyrin-like motif.

Various Pho81p derivatives with C-terminal deletions but an intact N-terminal half were constructed (Fig. 4). Surprisingly, two deletion fragments, 1–982 and 1–772, both retaining the 141-aa minimal sequence, hardly complemented the *pho81-Δ1* mutation, while fragment 1–1175 did. The 1–584 fragment could not complement the *pho81-Δ1* mutation at all. These results suggest that the N-terminal region of Pho81p has a negative effect on the minimal Pho81p segment and that the C-terminal region may antagonize the inhibition.

The 81^{c} 1–982 and 81^{c} 1–772 fragments bearing the *PHO81*^{c} mutation in the N-terminal segment conferred only trace activities of rAPase; this was similar to the cases for the 1–982 and 1–772 fragments, respectively, having the wild-type N-terminal segment (Fig. 4). This fact indicates that the function of the C-terminal segment is necessary for expression of the Pho81^{c} phenotype.

Interaction between the N- and C-terminal halves. To examine the antagonistic effects of the N- and C-terminal regions, a YEp241-based plasmid (marked with *URA3*) carrying the 773–1179 fragment (Fig. 4) was introduced into *pho81-Δ1* cells harboring another plasmid constructed by ligation of the 1–772 or 1–982 fragment (Fig. 4) into YEp131 (marked with *LEU2*). The resultant Ura^{+} Leu^{+} transformants exhibited the rAPase^{+} phenotype in low-P$_i$ medium (Fig. 5). These results

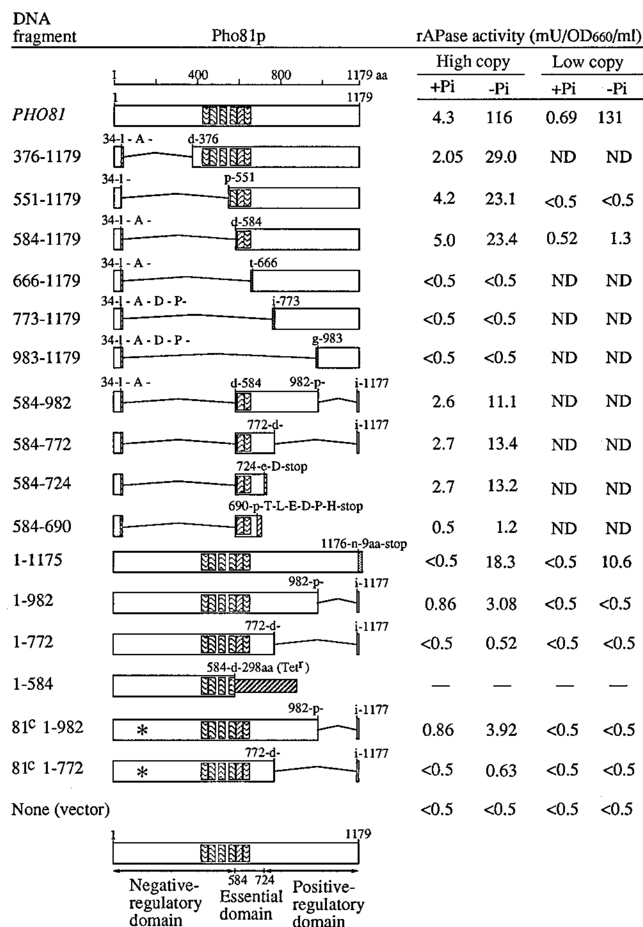

FIG. 4. Effects of modified Pho81p on rAPase synthesis. DNA fragments 376–1179, 551–1179, 584–1179, 666–1179, 773–1179, and 983–1179 were constructed by deleting the *Bal*I-*Bcl*I$_2$, *Bal*I-*Hin*cII, *Bal*I-*Bam*HI$_2$, *Bal*I-*Rsa*I, *Bal*I-*Eco*RV, and *Bal*I-*Sma*I regions, respectively (Fig. 1). The 584–982, 584–772, 584–724, and 584–690 fragments were constructed from the 584–1179 fragment by deletion of the *Sma*I-*Ssp*I$_2$, *Eco*RV-*Ssp*I$_2$, *Xho*I$_2$-*Ssp*I$_2$, and *Hap*II-*Ssp*I$_2$ regions, respectively. For adjustment of the coding frames at the deletion points of fragments 376–1179, 584–1179, 666–1179, 773–1179, and 983–1179, an 8-bp *Bam*HI linker (indicated by a thin shaded box) was ligated at these joints. For joining the *Xho*I$_2$-restricted end to the *Ssp*I$_2$ end of the 584–724 fragment, an 8-bp *Xho*I linker was ligated. The deletion gap of the 584–690 fragment was connected with a 16-bp *Acc*I-*Sma*I fragment of pUC18 DNA as a linker. The 1–1175 fragment was constructed by insertion of the 8-bp *Xho*I linker at the *Ssp*I$_2$ site of the full-length *PHO81* DNA. Fragments 1–982 and 1–772 were constructed similarly to fragments 584–982 and 584–772, respectively, but from the 6.2-kb wild-type *PHO81* DNA, and fragments 81^{c}1–982 and 81^{c}1–772 were similarly constructed from the same 6.2-kb fragment of the *PHO81*^{c} DNA. Fragment 1–584 was prepared by cloning the 3-kb *Bam*HI$_1$-*Bam*HI$_2$ fragment. All the linkers inserted at the indicated sites created amino acid insertions as illustrated for each ligation site by the single-letter amino acid code (uppercase letters) between two amino acids of Pho81p shown in lowercase letters or "stop" for the termination codon, except for the 1–1175 and 1–584 fragments. The 1–1175 fragment encodes nine additional amino acid residues, PRGLICKVL, following Asn-1176, and the Pho81p coding region of the 1–584 fragment was fused with the Tet^{r} gene of YEp131 or YCp50 (box with diagonal stripes). The boxes with wavy lines indicate the ankyrin-like motif, and the asterisk marks the site of the *PHO81*^{c} mutation. Each *Hin*dIII-*Sal*I *PHO81* fragment containing the above-described modifications was inserted into a short *Hin*dIII-*Sal*I gap of YEp131 or YCp50 and introduced into cells of *S. cerevisiae* NBD81-6D (*pho81-Δ1*). Cells of the transformants precultivated overnight in high-P$_i$ medium without leucine or uracil were inoculated into high-P$_i$ (+P$_i$) and low-P$_i$ (−P$_i$) media without leucine or uracil and shaken for 16 h at 30°C. Then the cells were harvested, and their rAPase activities were determined. Values shown for the enzyme activities are means for triplicate cultures. The standard deviation did not exceed 10% of each value. The dash indicates that no rAPase activity was detected when colonies of the transformants were stained for rAPase activity. ND, not determined; OD$_{660}$, optical density at 660 nm.

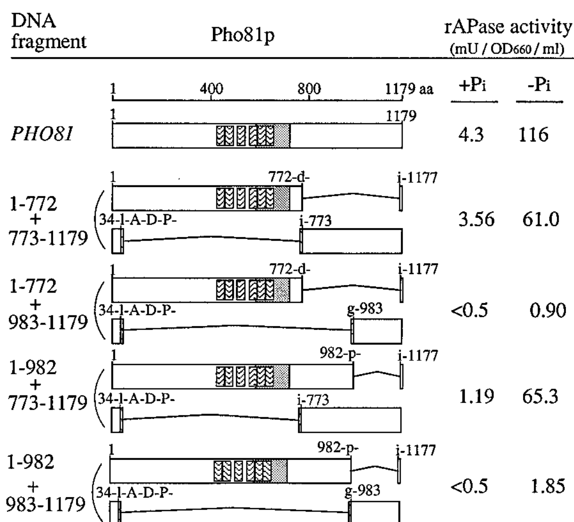

FIG. 5. Complementation between two truncated *PHO81* fragments. The 773–1179 and 983–1179 fragments having the *Hin*dIII and *Sal*I restriction ends (Fig. 4) were inserted into a short *Hin*dIII-*Sal*I gap of YEp241 marked with *URA3* and introduced into NBD81-6D (*pho81*-Δ*1 leu2 ura3*) harboring the same YEp131 plasmids (marked with *LEU2*) ligated with the 1–772 or 1–982 fragment as described in the legend to Fig. 4. The symbols and amino acid residues coded by the linker sequences of the DNA fragments, cultivation methods, and rAPase determination of the resultant Leu$^+$ Ura$^+$ transformants were as described in the legend to Fig. 4. Dotted box, the 141-aa minimum functional region.

were not due to recombination between the 773–1179 and 1–772 or 1–982 fragments, because the transformants frequently segregated Leu$^+$ Ura$^-$ and Leu$^-$ Ura$^+$ clones on culturing in nutrient medium, and these segregants showed the rAPase$^-$ phenotype. Thus, the products of the 773–1179 fragment function in *trans* with those of the 1–772 and 1–982 fragments. These observations support the idea that intramolecular interactions of the N- and C-terminal regions competitively modulate the function of the Pho81p central region by their inhibitory and stimulatory functions. Moreover, these observations negate the possibility that the barely detectable rAPase activities conferred by the 1–982 and 1–772 fragments (Fig. 4) were due to the low level of expression from these truncated fragments or to the low level of stability of their products. The 983–1179 fragment, however, hardly complemented the 1–983 and 1–772 fragments (Fig. 5). Thus, the C-terminal function was destroyed by splitting the segment between aa positions 982 and 983.

Pho81p is an inhibitor of Pho85p protein kinase. Genetic evidence suggests that Pho81p functions as an inhibitor, in response to low P_i concentration, of the Pho80p protein or the Pho80p-Pho85p complex. To test this possibility, we examined the inhibitory activity of Pho81p against the Pho85p protein kinase. The protein kinase activity of Pho85p in vitro has been demonstrated by Fujino et al. (11) and Kaffman et al. (18). A sample of GST-Pho85p protein was prepared from *S. cerevisiae* YAT1565(pGST-PHO85) (11) expressing both the *GST-PHO85* construct and the *PHO80* gene under the control of the *GAPp* promoter and was immunopurified with anti-GST serum. The GST-Pho85p protein phosphorylated Pho4p (50 kDa, as determined by SDS-PAGE analysis [23]) in vitro much more effectively than κ-casein (Fig. 6A), as previously described (11, 18), whereas the GST protein prepared from the yeast transformant with pKOM1 (without the *PHO85* gene) did not phosphorylate Pho4p. The identity of the phosphorylated protein in the reaction mixture was confirmed by Western blotting analysis of the sample with anti-Pho4p serum (Fig. 6B). Western blotting analysis also showed that the faster-migrating bands shown in Fig. 6A were probably degradation products of Pho4p. Although we did not have direct evidence for the presence of Pho80p in the GST-Pho85p sample prepared from the *PHO80*$^+$ cells without anti-Pho80p serum, it presumably contained Pho80p, because the Pho85p kinase reaction is detectable only in the presence of Pho80p (18, 36). This was supported by the observation that no Pho4p phosphorylation activity of Pho85p was detected in the cell extract prepared from the *pho80* disruptant, YAT1514(pGST-PHO85) (Fig. 6A; lanes 3 and 4). No differences were observed between

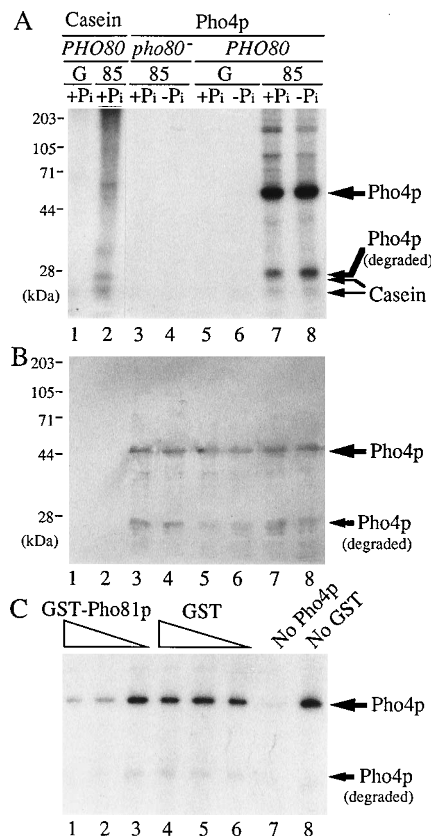

FIG. 6. Inhibitory effect of Pho81p on protein kinase activity of Pho85p. (A) GST-Pho85p phosphorylation of Pho4p. The GST (G) and GST-Pho85p (85) protein immunopurified from yeast cells of YAT1565(pKOM1) (*PHO80*), YAT1565(pGST-PHO85) (*PHO80*), and YAT1514(pGST-PHO85) (*pho80*$^-$) cultivated in high-P_i ($+P_i$) and low-P_i ($-P_i$) media were mixed with [γ-^{32}P]ATP and 0.12 μg of either κ-casein or Pho4p. After incubation at 30°C for 30 min, the reaction mixtures were subjected to SDS-PAGE and exposed to X-ray film for 12 h (lanes 1 and 2) or 2 h (lanes 3 to 8). The migration distances of the radioactive bands of Pho4p and κ-casein (Casein) coincided with those obtained by staining with Coomassie blue on an SDS-PAGE gel. (B) Western blotting analysis of the kinase reaction products. The same reaction mixture as that described above for panel A was subjected to SDS-PAGE and Western blotting analysis with anti-Pho4p serum as a primary antibody and anti-rabbit immunoglobulin G conjugated with alkaline phosphatase as a secondary antibody. Bands were stained with a mixture of nitroblue tetrazolium (Promega, Madison, Wis.) and 5-bromo-4-chloro-3-indolyl phosphate (Promega). (C) Inhibitory effect of Pho81p on Pho85p kinase activity. Immunopurified GST-Pho85p fractions prepared from 100 μl of yeast extract of YAT1565(pGST-PHO85) cultivated in high-P_i medium were mixed with 1.3 pmol of [γ-^{32}P]ATP and 60 ng of Pho4p in the presence of 18 (lane 1), 1.8 (lane 2), 0.18 (lane 3), or 0 (lane 8) μg of the GST-Pho81p protein or 18 (lane 4), 1.8 (lane 5), or 0.18 (lane 6) μg of the GST protein. The mixture in lane 7 was the same as that in lane 1 but without Pho4p. The reaction mixture (total volume, 40 μl) was incubated at 30°C for 30 min, subjected to SDS-PAGE, and exposed to X-ray film.

TABLE 1. β-galactosidase expression by association of Pho81p and Pho85p in the two-hybrid system[a]

pAS1 insert	pACTII insert	β-Galactosidase activity (U/mg of protein)[b]	
		High-P_i medium	Low-P_i medium
None	None	33.8 ± 9.9	8.8 ± 2.2
PHO81	None	32.4 ± 1.2	14.2 ± 2.5
None	*PHO85*	3.6 ± 0.7	6.0 ± 3.0
PHO81	*PHO85*	284 ± 64.3	332 ± 93.2
PHO85	None	28.4 ± 2.8	26.1 ± 10.0
None	*PHO81*	36.4 ± 19.5	22.0 ± 4.7
PHO85	*PHO81*	861 ± 122	524 ± 168
pSB32-GAL4[c]	pSB32-GAL4[c]	1,226 ± 201	866 ± 140

[a] Transformant cells of Y190 harboring pAS1 and pACTII plasmids ligated with or without (None) the indicated *PHO81* and *PHO85* DNA fragments were cultured in high- and low-P_i media lacking tryptophan and leucine. β-galactosidase activities were determined during the cultivation.

[b] Values for enzyme activities represent the average and the standard deviation of at least three independent cultivations.

[c] Y190 cells harboring pSB32-GAL4 (30) were cultured in the same media as those used for the other transformants, but the media were supplemented with tryptophan.

the GST-Pho85p proteins prepared from cells grown in high- and low-P_i media (Fig. 6A), as reported by Fujino et al. (11). These yeast cells did not show any rAPase activity, probably because of overproduction of the Pho80p and Pho85p (i.e., GST-Pho85p) proteins by the *GAPp* promoter.

Inhibition of the Pho85p protein kinase by Pho81p was examined by adding GST-Pho81p protein, which contains a 614-aa portion of Pho81p (residues 159 to 772) prepared in *E. coli* cells, to the reaction mixture of GST-Pho85p (presumably with Pho80p) and Pho4p. A significant reduction in the amount of phosphorylated Pho4p was observed as the amount of GST-Pho81p was increased (Fig. 6C), whereas no change was observed upon addition of a GST sample. Phosphorylated GST-Pho81p was not detected in the reaction mixture in the absence of Pho4p (Fig. 6C). These observations support the hypothesis that Pho81p is not a substrate of Pho85p protein kinase. Thus, Pho81p is an inhibitor of Pho85p, a cyclin (Pho80p)-dependent protein kinase. We cannot, however, exclude the possibility that Pho81p is a protein phosphatase specific for phosphorylated Pho4p, protease, or ATPase.

Pho81p associates with Pho85p in vivo. To examine the association of Pho81p and Pho85p proteins in vivo, we employed the two-hybrid system (10) by connecting the *PHO81* and *PHO85* DNAs to two separate DNA fragments of *GAL4*, encoding the DNA-binding domain in plasmid pAS1 and the transcriptional activation domain in pACTII, respectively, as described in Materials and Methods. *S. cerevisiae* Y190 (*gal4*Δ *gal80*Δ *GALp-lacZ* [13]) was transformed with various combinations of two of the pAS1, pACTII, pAS1::*PHO81*, pAS1::*PHO85*, pACII::*PHO81*, and pACII::*PHO85* plasmids. It is evident that Y190 cells transformed with pAS1::*PHO81* (pAS1 bearing the *PHO81* DNA) in combination with pACTII::*PHO85* or with a combination of pAS1::*PHO85* and pACTII::*PHO81* showed significant levels of β-galactosidase activity, irrespective of the P_i concentration in the medium (Table 1). These results indicate that Pho81p binds with Pho85p or with the Pho80p-Pho85p complex.

DISCUSSION

Genetic analyses have suggested that Pho81p is an inhibitor of the negative regulatory factors Pho80p and/or Pho85p (25). Results of in vitro phosphorylation experiments (Fig. 6) are in accord with this idea. A minimal domain sufficient for Pho81p function was delimited to the 141-aa sequence from aa 584 to 724 (Fig. 4). The N-terminal region outside this domain appears to inhibit Pho81p function, while the C-terminal half is stimulatory, perhaps by competing with the N-terminal half (Fig. 5). The fact that all four types of *PHO81*^{c} mutations so far examined affect the N-terminal half supports the view that this domain is inhibitory. The minimal Pho81p domain bears two of the six copies of the ankyrin-like motif.

The *PHO81* gene is under the control of Pho4p. The *PHO* regulatory system thus includes a positive feedback loop. Experiments with modified *PHO81* promoters revealed that enhanced *PHO81* transcription and the P_i starvation signal are both essential for activation of the *PHO* regulatory circuit and indicated that the feedback loop is important for proper regulation of the *PHO* regulon. This fact is contradictory to the early observation from a physiological study of Lemire et al. (19) that de novo protein synthesis is not required for *PHO5* transcription. This view is, however, limited to an early phase of rAPase derepression, and their data also support the feedback and autoregulatory mechanisms in the *PHO* regulon which is completely inhibited by cycloheximide.

Pho80p protein and Pho85p protein kinase are analogous to G_1 cyclins, encoded by *CLN1* and *CLN2*, and cyclin-dependent protein kinase (encoded by *CDC28*) in *S. cerevisiae*, respectively (18). The G_1 cyclin-Cdc28p complex is hypothesized to activate Swi4p and Swi6p by phosphorylation, and the activated Swi4p-Swi6p complex transcribes the *CLN1* and *CLN2* genes (15). Thus, these genes and proteins also form a positive feedback loop. Enhanced expression of the *CLN* genes by the signals of nutritional conditions and/or mating-type pheromones is important for the enhancement of G1 cyclin-Cdc28p kinase activity because of the instabilities of Cln1p and Cln2p, but the stability of Pho81p is unknown.

Ankyrins were initially found in erythrocytes as proteins forming a high-affinity ternary complex with the spectrin skeleton or anion exchangers (1). They are now known to constitute a family of domains that have the striking feature of a repeated 33-aa motif, the ankyrin repeats. This structure is widely distributed in various proteins, including regulatory proteins for gene transcription (1, 2, 35). These facts strongly suggest that the ankyrin-like motif of Pho81p might participate in interacting with target proteins, such as Pho85p, or with an anion-exchange protein, perhaps the P_i-transporter proteins Pho84p (4), Pho86p (our unpublished results), Pho87p (also called Ycr524p [34]) (our unpublished results), and/or Gtr1p (3). One possibility is that Pho81p senses P_i signals, perhaps via the P_i-transporter complex, and then transfers these signals to the nucleus. The yeast P_i-transporter proteins do not, however, exhibit similarities to anion-exchange proteins such as band 3 or AE1 of human erythrocytes (20).

Pho81p exhibits similarity to a protein, p16^{INK4}, that inhibits activity of the human cyclin D-CDK4 complex (33). p16^{INK4} contains a 148-aa sequence bearing four copies of an ankyrin-like motif in its N-terminal region which has significant similarities to that of Pho81p, especially with each consecutive three copies of the ankyrin-like motifs (repeats III to VI in Pho81p and II to IV in p16^{INK4} [Fig. 7]). Since p16^{INK4} was shown to bind with CDK4 directly, probably through its ankyrin-like motif, the ankyrin-like motif of Pho81p might have a function for binding with Pho85p (Table 1). Pho81p,

```
                    .         .         .
Pho81p   III 507-VQFDPLNVACKFNNHDAAKLLLEIRSKQNADNA-539
                   :* *  *      : :::***:    ** *
p16INK4    I    3-PSADWLATAAARGRVEEVRALLEAVALPNAPNS-35
                        .         .         .

                    .         .         .         .
Pho81p    IV 557-TGLCTLHIVAKIGGDPQLIQLLIRYGADPNEIDGF-591
                        * :   *   :::**:  **:** :* :
p16INK4   II     36-YGRRPIQVMMMGSARVAELLLLHGAEPNCADPA-68
                       .         .         .

                         .         .         .
Pho81p     V 592-NKWTPIFYAVRSGHSEVITELLKHNARLDIEDD-624
                     *:  *:* *  : :  * :  ****: *
p16INK4  III   69-TLTRPVHDAAREGFLDTLVVLHRAGARLDVRDA-101
                  .         .         .         .

                      .         .         .
Pho81p    VI 625-NGHSPLFYALWESHVDVLNALLQRPLNLPSAPL-657
                  *: *:  *    * **:  *
p16INK4   IV 102-WGRLPVDLAEELGHRDVARYLRAAAGGTRGSNH-134
                         .         .         .
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FIG. 7. Alignment of amino acid sequences of the ankyrin domain in Pho81p and p16^{INK4}. The p16^{INK4} sequence is from Serrano et al. (33). Roman numerals represent the order of each ankyrin-like motif from the N-terminal side in the respective proteins. Identical and similar residues are indicated by asterisks and double dots, respectively.

however, binds with Pho85p through the cyclin subunit, Pho80p, of the Pho80p-Pho85p complex, as described by Schneider et al. (32) recently.

Regarding the role of Pho81p in the *PHO* regulon, p105 protein of NF-κB (2) is particularly interesting. It bears the p50 moiety in its N-terminal half, and its C-terminal half contains seven copies of the ankyrin-like motif, which is thought to act as an anchor to retain p105 in cytoplasm. p105 is localized in the cytoplasm, but its processed form, p50, moves to the nucleus to form the active heterodimer, p65-p50 (i.e., NF-κB). Pho81p mediates the P_i signals from the cell surface to the nucleus. Pho81p may bind with one or more proteins of the P_i-transporter complex, consisting of Pho84p, Pho86p, Pho87p, and/or Gtr1p. The P_i starvation signal, detected by most probably the P_i-transporter complex, may change the tertiary structure of Pho81p, perhaps allowing the C-terminal domain to antagonize the inhibitive function of the N-terminal domain and allowing Pho81p to be translocated into the nucleus similarly to p105. Then it can inhibit the function of Pho85p protein kinase and allow Pho4p to transcribe the phosphatase genes. It is noteworthy that Pho81p exhibits substantial similarity to Pho87p in two separate amino acid sequences: aa 1 to 41 and 103 to 197 of Pho81p show 34 and 24% identity to aa 1 to 39 and 275 to 362 of Pho87p, respectively (our unpublished results) (Fig. 1). Gly-147, which is replaced by arginine in the *PHO81^c-1* mutant, is located in the 103–197 region.

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