A cysteine residue in helix\textsubscript{II} of the bHLH domain is essential for homodimerization of the yeast transcription factor Pho4p

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ABSTRACT

The yeast transcription factor Pho4p is required for expression of the phosphate-repressible acid phosphatase encoded by the PHO5 gene. Functional studies have shown that the molecule is composed of an N-terminal acidic activation domain, a central region which is necessary for interaction with a negative regulatory factor (the cyclin Pho80) and a C-terminal basic helix–loop–helix domain, which mediates DNA binding and homodimerization. In this study the homodimerization domain maps specifically to helix\textsubscript{II} of this region and a cysteine residue within this region is essential for this function. Experiments support the role of an intermolecular disulfide bond in stabilization of homodimerization, which is critical for DNA binding.

INTRODUCTION

The PHO4 gene of the yeast \textit{Saccharomyces cerevisiae} encodes a transcriptional activator protein that is indispensable for derepression of transcription of the phosphate-repressible acid phosphatase (\textit{rAPase}) encoded by the \textit{PHO5} gene. Expression of \textit{PHO5}, which is regulated in response to the concentration of inorganic phosphate (P\textsubscript{i}), is primarily controlled by regulation of Pho4p activity. Pho4p functions in combination with the homeodomain molecule Pho2 to initiate \textit{PHO5} expression under Pi-depleted conditions. In Pi-rich medium \textit{PHO5} expression is repressed as Pho4p activity is inhibited due to phosphorylation by the Pho80/Pho85 kinase complex (1). Phosphorylation of Pho4p was recently shown to control the activity of this region and a cysteine residue within this region is necessary for this function. Experiments support the role of an intermolecular disulfide bond in stabilization of homodimerization, which is critical for DNA binding.

Materials and Methods

Yeast strains and media

Yeast strains CY99 (MAT\textalpha, ura3-52, lys2-801, leu2-\Delta1, ade2-101, trp1-910, his3-\Delta200, gal4-542, gal80-538, LEU2::GAL1–LacZ, pho4::LEU2), CY105 (MAT\textalpha, ura3-52, lys2-801, leu2-\Delta1, ade2-101, trp1-\Delta1, his3-\Delta200, pho4::LEU2, pho80::LEU2) and 4L-14 (MAT\textalpha, ura3-52, lys2-801, leu2-\Delta1, ade2-101, trp1-\Delta1, his3-\Delta200, pho4::LEU2) were utilized in this study. Yeast cells were grown at 30°C in either YPD (1% yeast extract, 2% bactopeptone, 2% dextrose) or minimal medium (0.67% yeast nitrogen base, 2% dextrose) or minimal medium (0.67% yeast nitrogen base, 2% dextrose or 2% raffinose) supplemented with the appropriate amino acids or bases as required.

Plasmid construction

Plasmids 316-GAL4\textsubscript{147}-Pho4p and 316-GAL4\textsubscript{74}-Pho4p were constructed by ligation of a fragment containing the \textit{ADH1} promoter fused to either amino acids 1–147 of Gal4p (as a \textit{BamHI–SalI}

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fragment) or amino acids 1–74 of Gal4p (as a BamHI–XhoI fragment) to a XhoI–XhoI fragment containing amino acids 20–312 of the wild-type PHO4 gene in the centromeric URA3 vector pRS316 (11). Plasmid 316-Pho4p contains a 1.4 kbp HindIII–XhoI fragment with the wild-type PHO4 gene (including the PHO4 promoter) in the same vector. Deletions or point mutations of PHO4 or PHO4–c-Myc chimeras were fused to ADH1–GAL4147 or ADH1–GAL4145 or the PHO4 promoter either in the same manner or by substitution of a unique Mlu–XhoI fragment of PHO4. For Western analysis the coding sequences of PHO4 or various pho4 mutants were fused to the CYC1 promoter on the centromeric plasmid pRS316 and used to transform strain 4L-14. Extracts were prepared, analyzed on 12.5% polyacrylamide gels and subjected to Western analysis using a mouse polyclonal anti-Pho4p antibody and the Chemiluminescence Reagent Plus system (Du Pont NEN).

**Mutagenesis**

Deletion analysis of PHO4 was performed using PCR and specific oligonucleotide primers. Plasmid pGem11Z-Pho4p (XhoI–XbaI fragment described above) served as the template and amplification utilized specific primers and the T7 promoter primer to produce fragments with the desired end points. Site-directed mutagenesis was performed utilizing a two step PCR as described elsewhere (12). PCR products were purified using Wizard PCR prep (Promega), digested with XhoI and XbaI, cloned into pBluescriptII and sequenced.

**Protein purification**

Several PHO4 derivatives were subcloned into a His6 expression vector (Qiagen). Recombinant proteins produced in *Escherichia coli* were purified under denaturing conditions using a Ni–NTA column according to the manufacturer’s specifications. Purified proteins were dialyzed against DNA binding reaction buffer (see below) and concentrated. Proteins were subsequently analyzed by SDS–PAGE under reducing (4.0 mM β-mercaptoethanol) or non-reducing conditions or used in DNA binding reactions.

Purified proteins were mixed with 32P-labeled oligonucleotides containing the Pho4p binding site in reaction buffer [15.0 mM Tris, pH 8.0, 65.0 mM NaCl, 7.5% glycerol, 1.25 mM DTT (except where indicated), 1.8 mM EDTA and 3.75 mg/ml BSA]. Samples were incubated at room temperature for 45 min, electrophoresed on a 4% polyacrylamide gel for 3 h at 15 V/cm at 4°C and subsequently exposed to X-ray film.

**Enzyme assays**

To measure β-galactosidase individual transformants were grown in minimal medium supplemented with the appropriate amino acids and bases and 2% raffinose as carbon source. Aliquots were removed and the OD 420 of the supernatant measured. This value was plotted versus the OD 600 of the culture.

RESULTS AND DISCUSSION

The first 74 amino acids of the transcription factor Gal4p have been identified as the DNA binding domain, however, efficient DNA binding requires that the dimerization domain (residues 75–147) is present (14). We have taken advantage of this property of Gal4p to test whether Pho4p contains a region that is able to facilitate dimerization. Two centromeric plasmids using either amino acids 1–147 of Gal4p (Gal4p147) or amino acids 1–74 of Gal4p (Gal4p74) fused to Pho4p were constructed. In this system homodimerization between two Gal4p–Pho4p fusion molecules results in activation of GAL1–LacZ expression. Failure of the molecules to dimerize would result in a failure to efficiently bind DNA and hence lower the level of GAL1–LacZ expression. As shown in Figure 1A, when linked to Gal4p147 either Pho4p (residues 20–312) or truncated Pho4p (residues 20–291) activated LacZ expression at nearly equivalent levels. Conversely, when linked to Gal4p74 Pho4p (residues 20–312) activated expression at similar levels to the Gal4p147–Pho4p fusions, however, Pho4p (residues 20–291) lacking the putative dimerization
domain activated expression poorly (there is an ~10-fold difference in the levels of β-galactosidase production). These results suggest that the C-terminal region of Pho4p (residues 291–312) is critical for dimerization. Therefore, this assay provides a convenient assay to study Pho4p dimerization, i.e. when linked to Gal4p74 the Gal4–Pho4p fusion will activate expression only if dimerization mediated by the Pho4p bHLH occurs efficiently. Additionally, removal of helix_2 from Pho4p results in a molecule that is unable to activate PHO5 expression (Fig. 1B), presumably due to the inability of the molecule to homodimerize.

As depicted in Figure 2, the bHLH motif of Pho4p shows considerable primary sequence homology and even more significant secondary structural homology to the bHLH domain of c-Myc. To further confirm the key role of this region in homodimerization of Pho4p we constructed a Gal4–Pho4p–c-Myc chimera without the HLH domain of Pho4p. In this chimera residues 359–402 of c-Myc have been used to replace the corresponding residues (265–312) of Pho4p (see Fig. 2). As shown in Figure 1A, when Pho4p–c-Myc was linked to Gal4p_74 GAL1–LucZ expression was ~10-fold lower than when Pho4p–c-Myc was fused to Gal4p_147, suggesting that when the HLH domain of Pho4p is replaced by the corresponding region of c-Myc the molecule fails to homodimerize. This is consistent with the observation that c-Myc does not form a homodimer, but rather in many cases heterodimerizes with Max in order to function (15,16). Figure 1B and previous studies (17) have shown that this Pho4p–c-Myc fusion failed to activate PHO5 expression.

To further delineate the function of this 21 amino acid region several residues located within this region which are highly conserved in all bHLH domains were subjected to site-directed mutagenesis (K292, E297, C300 and R304 were changed to Thr and Q307 was changed to a stop codon). All mutations were introduced into both Gal4p_147 and Gal4p_74 constructs, described above, were fused to the PHO4 promoter and assayed for ability to complement the pho4::LEU2 allele in strain CY105 (see Materials and Methods for strain genotype). Three independent isolates were grown in high phosphate medium and assayed at various time points for ß-galactosidase activity using a whole cell assay (see Materials and Methods). A graph of average enzyme activity versus cell growth is depicted.

A truncated wild-type or C300A mutant Pho4p (residues 119–312) was expressed in E.coli as a His_6 fusion protein. Proteins were purified using a Ni–NTA column under denaturing conditions, dialyzed, resolved by SDS–PAGE under reducing or non-reducing conditions and subjected to Western blotting using a polyclonal anti-Pho4p antiserum. As seen in Figure 4A, under reducing conditions both wild-type and mutant Pho4p proteins migrated as a monomer and a higher molecular weight protein. Proteins were purified using a Ni–NTA column under denaturing conditions, dialyzed, resolved by SDS–PAGE under reducing or non-reducing conditions and subjected to Western blotting using a polyclonal anti-Pho4p antiserum. As seen in Figure 4A, under reducing conditions both wild-type and mutant Pho4p proteins migrated as a monomer and a higher molecular weight band with the approximate size of a dimer (lanes 3 and 4). To demonstrate that dimer formation was critical for DNA binding a gel mobility shift assay was performed (Fig. 4B) using a PHO5 oligonucleotide and the E.coli-produced proteins described above. Wild-type Pho4p protein bound to the oligonucleotide (lane 6) and binding was competed by a 50-fold excess of unlabeled oligonucleotide (lane 7), while the C300A mutant protein failed to bind to DNA (lane 8). Furthermore, completion of the binding reaction in the presence of 10-fold higher concentrations of DTT (1.25 mM...
His-tagged recombinant Pho4p, either wild-type or the C300A mutant (residues 119–312), were purified from extracts of E.coli as described in Materials and Methods. (A) SDS–PAGE of the samples under reducing (left) or non-reducing (right) conditions. (B) DNA binding assay of the recombinant proteins (see Materials and Methods) using a 32P-labeled PHO5 UAS oligonucleotide. DNA binding assays performed with no exogenously added protein (lane 1), added wild-type Pho4p (lane 2), added wild-type Pho4p in the presence of 50-fold excess unlabeled binding site oligonucleotide (lane 3) or added pho4pC300A (lane 4). DNA binding assay of the recombinant proteins performed in the absence of added Pho4p (lane 5) or in the presence of Pho4p incubated in either 1.25 (lane 6) or 12.5 mM DTT (lane 7), essentially as described above. After the incubation period 25 mM iodoacetamide was added to each of the reactions prior to electrophoresis.

As discussed above, the Cys residue of Pho4p is not conserved in the homologous helix II of c-Myc. To assess the role that the Cys residue plays in dimer formation we have replaced the corresponding Thr residue of c-Myc with a Cys residue by site-directed mutagenesis in the context of Gal4p147 and Gal4p74 and the Pho4p–c-Myc chimera described above. As seen in Figure 5A, the presence of the Cys residue increases the ability of the Gal4–Pho4p–c-Myc molecule to activate GAL1–LacZ expression ∼6-fold as compared with the corresponding molecule containing a Thr residue. Gal4p147 molecules containing either residue activate expression equally well. Furthermore, the presence of the Cys residue in the Pho4p–c-Myc fusion supports partial complementation of a pho4::LEU2 allele in activating PHO5 expression (Fig. 5B). Western analysis (Fig. 5C) demonstrates that all three of these molecules are expressed in yeast cells at near equivalent levels. From these results we conclude that an intermolecular disulfide bond formed between Cys residues in helixII of the bHLH domain of Pho4p is critical for homodimerization of the molecule and is necessary for the molecule to bind DNA.

Transcription factors bearing the bHLH motif have been identified in various tissues and cells. This class of proteins form either homo- or heterodimers to regulate their activities and sequence preferences, which expand the potential functional repertoire of these proteins. The yeast transcription factor Pho4p contains a bHLH motif that is homologous to many mammalian proteins bearing the bHLH domain. In this report we demonstrate the critical role of helixII of the bHLH domain in facilitating homodimerization of Pho4p. These results are consistent and extend the work of others implying a role of the bHLH domain of Pho4p in dimerization (3–5,8–10). Studies by Ogawa and Oshima (4) have implicated a second region of Pho4p (residues 203–227) as being critical for Pho4p oligomerization. This may represent a higher order structure formed by Pho4p (in association with Pho2p), as this region is not required for dimer formation. The crystal structure of a dimer of only the C-terminal 85 amino acids (residues 228–312) of Pho4p bound to DTT, lane 10 versus 12.5 mM DTT, lane 11) blocked the ability of Pho4p to bind to the binding site oligonucleotide.

Figure 4. Site-directed mutagenesis of Thr394 of c-Myc. (A) The mutants were fused to either amino acids 1–147 or 1–74 of Gal4p and assayed for their ability to activate GAL1–LacZ expression as described in Materials and Methods. Activities represent the average of three independent isolates assayed in duplicate at two different growth time points. (B) Wild-type Pho4p, the Pho4p–c-Myc chimera or the Pho4p–c-MycT394C mutant chimera were fused to the PHO4 promoter and assayed for ability to complement the pho4::LEU2 allele in strain CY105 (see Materials and Methods for strain genotype). Three independent isolates were grown in high phosphate medium and assayed at various time points for rAPase activity using a whole cell assay (see Materials and Methods). A graph of average enzyme activity versus cell growth was determined and the enzyme activity at a cell growth corresponding to OD600 = 0.5 was calculated. (C) Western analysis using a mouse polyclonal anti-Pho4p antibody of the steady-state protein levels in strain 4L-14 (pho4::LEU2, lane 1) transformed with either wild-type PHO4 (lane 2), pho4–c-Myc (lane 3) or pho4–c-MycT390C (lane 4).
DNA has been determined (18). Furthermore, a Cys residue present within this region was found to be necessary for dimerization, suggesting that an intramolecular disulfide bond is formed in the Pho4p homodimer. Biochemical evidence confirmed the genetic observations concerning formation of the disulfide bond and its role in DNA binding. This result is consistent with the previous findings that a Cys which resides in helix I of the E2A protein mediates disulfide bond formation in the E2A homodimer under oxidizing conditions (19). In E2A this disulfide bond stabilizes the E2A homodimer, preventing E2A from forming heterodimers with the MyoD or Id proteins in B cells. However, in muscle cells an activity reduces the disulfide bond, facilitating heterodimerization with MyoD or Id. The mammalian bHLH transcription factor USF contains a Cys residue at the homologous site to Pho4p but also has a second Cys residue within the loop region. Pognonec et al. (20) have shown that an intermolecular disulfide bridge has little effect on USF function, whereas an intramolecular disulfide bond dramatically decreases DNA binding and USF function. These observations provide a novel mechanism for regulating protein activity via dimerization status. Pho4p, however, has not been shown to heterodimerize with any other protein to date or to be regulated at the level of dimerization status, which is mediated by a change in the redox state of the molecule. Nevertheless, oxidative or reducing activity or other post-translational modifications within helix I may regulate Pho4p activity by altering its ability to dimerize.

c-Myc shares the same DNA binding as Pho4p and there is considerable sequence homology within the bHLH motif (Fig. 2), but the Cys residue is not conserved between the two molecules. The Myc proteins homodimerize and bind DNA poorly at physiological levels (15). When the HLH region of Pho4p is replaced with that of c-Myc the chimera fails to form a homodimer, suggesting that this region of c-Myc is not able to facilitate dimerization as seen with Pho4p. This may suggest why the Myc–Max heterodimer is found to be the preferred species in vivo (13). However, mutation of the corresponding Thr of c-Myc to Cys does facilitate dimerization in the yeast system (Fig. 5) and suggests that if such a mutation would occur in c-Myc the molecule might acquire new properties, i.e. the ability to homodimerize.

In conclusion, the activity of the Pho4p molecule is regulated by subcellular localization (2) and protein conformation (16) and may be regulated at the level of dimerization status, which is mediated by an intermolecular disulfide bond and is required for DNA binding.

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