The PHOA and PHOB Cyclin-Dependent Kinases Perform an Essential Function in Aspergillus nidulans

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ABSTRACT

Unlike Pho85 of Saccharomyces cerevisiae, the highly related PHOA cyclin-dependent kinase (CDK) of Aspergillus nidulans (Busink and Osmani 1998) is a member of the Pho85 family of cyclin-dependent kinases. PHO85 was isolated in Saccharomyces cerevisiae due to its involvement in regulation of phosphate-scavenging enzymes (Uesono et al. 1987; Toh-e et al. 1988) and has been implicated in numerous other cellular processes, including stress adaptation, glycogen storage, and cell cycle progression (see Andrews and Meadany 1998; Nishizawa et al. 2001; Carroll and O’Shea 2002 for reviews and further references). Pho85 has been shown to bind to 10 cyclin partners (Meadany et al. 1997), which are thought to target the kinase activity of Pho85 to numerous substrates involved in these processes. However, PHO85 is not an essential gene nor is the highly related Pef1 kinase of the fission yeast Schizosaccharomyces pombe (Tanaka and Okayama 2000).

PHOA of A. nidulans is not apparently involved in regulation of phosphate-scavenging enzymes but, when compared to isogenic wild-type strains, lack of PHOA causes a switch from asexual to sexual development (at pH 8.0) or the absence of development altogether (at pH 6.0) under limiting phosphate growth conditions. A. nidulans PHOA is therefore required to integrate environmental conditions with developmental fate to allow ordered differentiation of asexual and sexual cell types under varying growth conditions (Busink and Osmani 1998). Deletion of the kinase does not cause lethality or developmental defects under nonrestrictive growth conditions.

We have isolated a second CDK with 77% identity to PHOA and report the consequences of its deletion in combination with a lack of PHOA in recombinant germinating ascospores. A temperature-sensitive allele of phoa was also generated, which caused growth defects in combination with deletion of phob. The ability of human Cdk5 to complement loss of PHOA/PHOB function was also determined. The data demonstrate that PHOA and PHOB function redundantly and have an essential function involved in cell growth and nuclear division.

MATERIALS AND METHODS

Strains and medium: The A. nidulans strains used in this study are listed in Table 1. Minimal, yeast extract glucose (YG), and malt extract glucose (MAG) media with supplements were prepared as described previously (Pontecorvo 1953; Busink and Osmani 1998).

Dominant-negative phoa mutagensis: Plasmid pPAP (M47) containing phoa under control of the alcohol dehydrogenase promoter was mutated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Lysine 39 of phoa was replaced by an arginine codon using oligonucleotides.
**TABLE 1**

**Strains used in this study**

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<tr>
<td>SO51</td>
<td>urA2</td>
<td>Osmani <em>et al.</em> (1994)</td>
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<td>urA2; nimT23</td>
<td>Ye <em>et al.</em> (1997)</td>
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<td>urA3; pyroA4</td>
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<td>urA3; ΔphoA (phoA::pyG); pyroA4</td>
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<tr>
<td>916</td>
<td>Ascospores obtained by crossing HB9 × AZ16</td>
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*The phoA2 allele contains a mutation that changes codon 39 from lysine to arginine.*

PHOALU-1 (5′-GAA CTT GTC GCC CTG AGG GAA ATC CAC CTC-3′) and PHOALU-2 (5′-GAG GTG CAT TTT CCT CAG GGC GAC AAG ATG C-3′). The plasmid with mutant phoA was transformed into A. nidulans strain GR5 and transformants were streaked to single colonies three times before replica plating on both inductive (ethanol) and repressive (glucose) media to identify any strains inhibited by dominant-negative PHOA.

**phoB deletion:** A phoB bacterial artificial chromosome (BAC) clone was obtained by screening an A. nidulans genomic BAC library (obtained by W. Choi, H. Zhu and R. A. Dean, Genomics Institute, Clemson University). *Escherichia coli* strain DY380 (Lee *et al.* 2001; Swaminathan *et al.* 2001) was transformed with BAC DNA harboring the phoB gene. A pyrA/Zeo gene cassette was amplified from plasmid pZD with primers PPA (5′-GAG ACC CTG TTT TCT TGT ATT CGT ATG CAT ATT TTT ATT CAT TTT CGT AAC TCC TCC TCC T-3′) and PZP (5′-TTC TTT GTT GTC ACC TGT CGA ATC ATA GCA GGA GAT GTC GAG CCC CAC AGT TGA GCC TGA GAC CAA T-3′). Plasmid pZD was generated by cloning the Zeo gene from pCD421 as an EcoRI-BamHI fragment into plasmid pHL containing the pyrA gene of A. nidulans. The amplified pyrA/Zeo gene cassette was transformed into the above BAC-transformed *E. coli* strain DY380 competent cells to promote in vivo homologous recombination as described (Lee *et al.* 2001; Swaminathan *et al.* 2001). The successful replacement of phoB in the BAC by the pyrA/Zeo cassette was checked with PCR amplification using primers anchored out of the phoB gene deletion area. The phoB-deleted BAC DNA was prepared and used to transform A. nidulans strain GR5, as described (Osmani *et al.* 1997). The deletion of phoB was confirmed by PCR amplification as above and Western blot analysis as described (Bussink and Osmani 1998) using antibodies raised against PHOA that also detect PHOB. The phoB null allele was named ΔphoB.

**A. nidulans crossing and growth conditions:** Crosses were completed as previously described (PonTECORYO 1953) and mature cleistothecia were collected and cleaned on 4% agar plates and broken into 0.25% Tween 80 solution. Ascospore germination was checked on minimal medium with supplements as indicated and grown at 37°C. The test of sexual differentiation on phosphate-limited media, growth sensitivity or resistance to hydroxyurea, high NaCl and sucrose media, and the activity test of three phosphate-regulated extracellular phosphatases (alkaline phosphatase, acid phosphatase, and phosphodiesterase) were as described (Bussink and Osmani 1998).

**The construction of the phoA3+ΔphoB strain:** phoA3 was mutated in plasmid pRG3 using oligonucleotides phoAW337H (5′-GGC GCT CTG CAG CAT CCA CAC TTC CAT GAC CTTPHOA) and phoAW337HR (5′-CTG CGG AAG GTC ATG CAT AGT CAG CAC AAG CAC T-3′) as described (Osmani *et al.* 1997) to generate the W337H mutation. The resulting plasmid DNA was used to transform a ΔphoB strain (AZ16). Transformants were streaked to single colonies three times and conidial spores were harvested and spread on minimal medium uridine and uracil (UU) plates plus 1% 5-fluoroorotic acid (5-FOA) at a density of 4000–6000 spores/plate. Viable colonies on FOA plates were streaked to single colonies three times on minimal media plus uridine and uracil and checked for growth at 32°C and 42°C. The mutations in those colonies showing temperature sensitivity were confirmed by sequencing genomic DNA amplified using PCR.

**Human CDK5 complementation:** Human CDK5 gene was a kind gift from B. Andrews, which was cloned into the *BamH*I site of the pAL5 expression plasmid under the control of the *alcA* regulatable promoter. The phoA3+ΔphoB strain XD1 was transformed with the CDK5 expression plasmid and successful transformation was confirmed by PCR amplification using CDK5-specific primers.

**4′,6-Diamidino-2-phenylindole staining and determination of the spindle mitotic index:** Nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI) staining and the spindle mitotic index was determined after immunofluorescence staining of microtubules as described (de Souza *et al.* 2000) using mouse anti-α-tubulin antibody (TAT1, Amersham, Buckinghamshire, UK) and a second antibody (AlexA Fluro 595 goat anti-mouse IgG, Molecular Probes, Eugene, OR). A total of 500 ascospore germlings were counted for each strain to determine its mitotic index. The spindle mitotic index of phoA3+ΔphoB strain at 32°C or 42°C was obtained by directly counting spindle bars in strain XG1. XG1 strain was obtained by crossing XD1 (phoA3+ΔphoB) with LO1022 (α-tubulin-green fluorescent protein (GFP) tag).
Identification of a second nonessential Pho85-like kinase in *A. nidulans*: Two lines of evidence suggest that *A. nidulans* may encode a second Pho85-like kinase in addition to the previously isolated PHOA kinase. First, polyclonal antiserum raised against recombinant PHOA detects not only PHOA but also an additional band of a size predicted for a CDK (lysine 39 was converted into arginine) when grown on low- 

Pi medium while little pigment is formed in the wild-type strain (Bussink and Osmani 1998). Other highly related CDKs include *S. pombe* Pef1 (69%) and human Cdk5 (56%; Figure 1).
pigment production under these conditions and they responded exactly like the wild-type control strain with no visible pigment being formed (Figure 3A). A ΔphoB strain was also tested for sensitivity or resistance to hydroxyurea, high NaCl, and sucrose media with no defects being observed (data not shown).

Because Pho85 is involved in regulation of phosphate-scavenging enzymes in S. cerevisiae and a similar system appears to function in Neurospora crassa (Kang and Metzenberg 1993; Peleg et al. 1996a,b), we considered the possibility that phoB may have a similar function. A ΔphoB strain was therefore tested for activity of three phosphate-regulated extracellular phosphatases (alkaline phosphatase, acid phosphatase, and phosphodiesterase) by activity staining of colonies grown in the presence or absence of inorganic phosphate (P) observed (data not shown). This indicates that phoB, like phoA, plays no obvious or important role in the regulation of enzymes involved in phosphorous acquisition.

It has also been shown that lack of phoA affects development in confluent plate cultures depending on pH, inoculation density, and phosphate concentration. A ΔphoA strain was therefore checked under similar growth conditions and its development compared to a wild-type and a phoA-deleted strain (Figure 3B). It is clear that deletion of phoA caused no defects in the differentiation programs of A. nidulans in response to these environmental conditions. This is in marked contrast to the defects caused by lack of phoA (Figure 3B), a CDK with 77% identity to PHOB.

**Deletion of both phoA and phoB causes lethality:** To see if deletion of both phoA and phoB would cause lethality or other marked phenotypes, strains deleted for phoA or phoB were crossed together to see if viable ΔphoA ΔphoB progeny could be generated. To do this, two strains were crossed in which phoA and phoB were deleted using two different nutritional marker genes. The genetic makeup of the cross was arranged so that plaques on selective media would allow only the double-deleted recombinants the chance to germinate and grow. However, if the double mutant were inviable, no colony formation would occur on selective media.

For this analysis we used strain HB9, which contains phoA deleted with pyrG+ (previously termed phoA1 by Bussink and Osmani 1998, but referred to here as ΔphoA). HB9 also carries the pyroA nutritional marker and therefore requires pyridoxine (pyro). HB9 has no spore color mutations and produced wild-type green asexual spores. The pertinent genotype for HB9 is ΔphoA; pyroA4. HB9 was crossed to strain AZ16, which has phoB deleted with pyroA4 term ΔphoB. This strain contains the pyrG89 nutritional marker and so requires UU for growth and carries the white spore color marker wa3. The pertinent genotype for AZ16 is ΔphoB; pyrG89. Crosses were completed using the forcing nutritional markers pyroA4 and pyrG89. Ascospores isolated from fruiting bodies (cleistothecia) were plated on nonselective media (+pyridoxine +uridine +uracil) to allow colony formation. Crossed HB9 × AZ16 cleistothecia were identified because they generated ascospores that formed both green and white recombinant colonies. Self-
crossed cleistothecia produced green (HB9) or white (AZ16) colonies.

Ascospores (~500) from HB9 (ΔphaA) and AZ16 (ΔphaB) self-crosses and from HB9 × AZ16 (ΔphaA × ΔphaB) crosses were spotted onto supplemented media (+Pyro +UU) and all generated colonies (Figure 4A). Media selective for either the pyrG89 (−UU) or pyroA4 (−Pyro) nutritional markers allowed the expected strains to grow. As expected, recombinants from the ΔphaA × ΔphaB cross could also grow on these media. Most importantly, however, no recombinants from the ΔphaA × ΔphaB cross were able to form colonies on media selective for both pyrG89 and pyroA4 markers (Figure 4A, − −). This indicates that the ΔphaA ΔphaB double mutants, even though prototrophic (ΔphaA pyrG89 ΔphaB: pyroA4), are not viable (Figure 4A, − −). This genetic analysis demonstrates that phaA and phaB have a redundant but essential function.

Defects in the ΔphaA ΔphaB double mutant: Although the ΔphaA ΔphaB mutant was unable to form visible colonies after 2 days, we examined very early growth of the mutant to investigate the defect caused by lack of phaA and phaB. Ascospores from a ΔphaA × ΔphaB cross were grown over a 24-hr period along with ascospores of a control GR5 (pyrG89; pyroA4) self-cross. To allow growth of the GR5 spores, they were plated on media with added pyridoxine, uridine, and uracil. To identify the double ΔphaA ΔphaB recombinants, the ascospores from the ΔphaA × ΔphaB cross were plated on minimal media lacking pyridoxine, uridine, and uracil. As phaA is deleted with pyrG89, and phaB is deleted with pyroA4, these conditions will allow germination of only the double ΔphaA:pyrG89 ΔphaB:pyroA4 recombinants. Over the 24-hr period of growth, GR5 ascospores germinated and formed small colonies visible without magnification. Over the same time period no visible colonies were formed from the ΔphaA × ΔphaB cross.

GR5 germlings could be observed after 6 hr of incubation and had grown to form a mat of cells by 12 hr (Figure 4B). Only 20% of the ascospores from the ΔphaA × ΔphaB cross were able to germinate and form germlings. Taking spore viability into account, this number fits into the theoretic expectation that one-fourth of the spores are phaA and phaB wild type, one-fourth are phaA wild type and ΔphaB, one-fourth are ΔphaA and phaB wild type, and one-fourth are ΔphaA and ΔphaB. Although the ΔphaA ΔphaB ascospores could germinate, they failed to continue to grow and arrested with cells ~50 μm in length (Figure 4B, two germlings indicated by arrows and quantified in Figure 4C). This indicates that the ΔphaA ΔphaB ascospores are able to break dormancy and start normal growth processes but are unable to sustain growth.

A potential problem with the analysis is that other recombinant progeny from the ΔphaA × ΔphaB cross could perhaps germinate and form germlings. Three other classes of progeny are expected from this cross: (1) ΔphaA; pyroA4, (2) ΔphaB; pyrG89, and (3) pyrG89; pyroA4. We therefore allowed PHO17 (ΔphaA; pyroA4) and AZ16 (ΔphaB; pyrG89) to undergo self-crosses and
Figure 4.—PHOA and PHOB play an essential function. (A) Ascospores of (a) a cross between a strain with deletion of \( \text{phoA} \) to a strain with deletion of \( \text{phoB} \) (\( \Delta \text{phoA}:\text{pyrG}; \text{pyrG}4 \times \Delta \text{phoB};\text{pyrA}; \text{pyrG}89 \)), (b) self-cross of \( \Delta \text{phoA};\text{pyrG}; \text{pyrG}4 \), (c) self-cross of wild type, (d) self-cross of \( \Delta \text{phoB};\text{pyrA}; \text{pyrG}89 \) plated on minimal media with supplements as indicated. pyro, pyridoxine that complements the \( \text{pyrG}4 \) mutation; UU, uridine and uracil that complement the \( \text{pyrG}89 \) mutation. Plates were incubated for 2 days at 37°C. (B) Ascospores from a wild-type (GR5; left, top) self-cross plated on nonselective media or ascospores from a \( \Delta \text{phoA};\text{pyrG}; \text{pyrG}4 \times \Delta \text{phoB};\text{pyrA}; \text{pyrG}89 \) cross (left, bottom) plated on selective media for \( \text{pyrG} \) and \( \text{pyrA} \) for the time indicated. (Right) Ascospores from self-crossed \( \Delta \text{phoA};\text{pyrG}; \text{pyrG}4 \) and \( \Delta \text{phoB};\text{pyrA}; \text{pyrG}89 \) strains. The media employed were minimal media supplemented with uridine/uracil and pyridoxine unless otherwise indicated. Insets show higher magnification of arrowed cells. Bar, 50 µm. (C) The growth rates of wild-type strain ascospores (GR5, dashed line) and double \( \Delta \text{phoA} \Delta \text{phoB} \) mutant ascospores (916, solid line). (D) Nuclear division in germinating ascospores from a wild-type self-cross and a \( \Delta \text{phoA} \times \Delta \text{phoB} \) cross after incubation at 37°C for 6.5 or 24 hr.

inoculated the resulting ascospores on selective media (Figure 4B, right two panels). Neither set of ascospores was able to form germlings although the \( \Delta \text{phoB}; \text{pyrG}89 \) spores from the AZ16 self-cross swelled enough to split the two shells of the ascospore casings (Champe and Simon 1992). However, in no instance were germ tubes similar to those seen in the \( \Delta \text{phoA} \times \Delta \text{phoB} \) cross observed (Figure 4B). We can therefore say with confidence that the ascospores that are able to form germ tubes from the \( \Delta \text{phoA} \times \Delta \text{phoB} \) cross are indeed the \( \Delta \text{phoA} \Delta \text{phoB} \) mutant recombinants.

One potential factor involved in lack of continued growth of the \( \Delta \text{phoA} \Delta \text{phoB} \) ascospores could be defects in nuclear division as previously observed for tempera-
Figure 5.—(A) Nuclear morphology in ΔphoA ΔphoB germinating ascospores. Germinating ascospores from a WT were incubated for 6.5 hr and a ΔphoA ΔphoB ascospore (MT) was incubated at 37°C for 12 hr. Cells were stained with DAPI to visualize nuclear number and morphology. Bar, 5 μm. (B) The percentage of cells in mitosis as determined by staining microtubules and scoring of the spindle mitotic index. A total of 500 cells of each strain at each time point were counted.

ture-sensitive cell cycle mutants (Morris 1976). The number of nuclear divisions of ΔphoA ΔphoB and control ascospores was therefore determined during 24 hr of growth (Figure 4D). Unlike uninucleate asexual spores, ascospores of A. nidulans are binucleate (Champe and Simon 1992). After 6.5 hr of germination, the majority of wild-type germinals had undergone at least one nuclear division, whereas the ΔphoA ΔphoB germinals remained largely at the two-nuclear-division stage, having undergone no nuclear divisions (Figure 4D). The rate of initial germ-tube extension for the ΔphoA ΔphoB mutant compared to that for GR5 controls was not markedly different (Figure 4C) although their abilities to complete the first cell cycle were notably different. This indicates a potential cell-cycle-specific defect in the ΔphoA ΔphoB mutant. Even after 24 hr of growth, half the ΔphoA ΔphoB germinals were still arrested with two nuclei. Only 38% underwent one mitosis and 12% underwent more than one nuclear division. In contrast, the control cells had grown to visible colonies at this time with uncountable numbers of nuclei (Figure 4D).

Another defect of the ΔphoA ΔphoB cells was a marked effect on nuclear structure. As revealed by DAPI staining, A. nidulans nuclei are normally oval in appearance and contain a domain occupied by the nucleolus with diminished DAPI staining. This defect becomes more pronounced after 12 hr of growth with nuclear DNA becoming quite condensed and sometimes fragmented (Figure 5A, MT). We considered that these defects could be associated with defective mitosis, but virtually no spindles could be detected in the ΔphoA ΔphoB germinals for the first 9 hr of germination. In contrast, the wild-type spindle mitotic index increased to ~4% after 5 hr of growth (Figure 5B). Therefore, no spindles could be seen at a time when the nuclear DNA of the ΔphoA ΔphoB germinals was condensed.
and nuclei lacked a visible nucleolus, these changes are unlikely to result from defective mitosis.

Defects in a phoA<sup>TS</sup> allele in a ΔphoB strain: Because of potential endowment of PHOA and PHOB to ascospores when crossing ΔphoA to ΔphoB strains to look at the phenotypes caused by lack of phoA and phoB, we tried to generate a temperature-sensitive (ts<sup>-</sup>) allele of phoA. Previously, ts<sup>-</sup> alleles of nimX<sup>cd2</sup> of A. nidulans were generated on the basis of mutations causing temperature sensitivity in cdc2 of S. pombe (Osmani et al. 1994). On the basis of protein sequence alignment among NIMX<sup>cd2</sup>, CDK5, Pho85, PHOA, and PHOB, three similar point mutations were introduced into phoA and used to replace the wild-type allele of phoA in a ΔphoB strain. In vitro mutagenesis was used to generate the individual mutations in phoA in a plasmid vector. A two-step gene replacement was completed and ts<sup>-</sup> strains were identified after replica plating at permissive and restrictive temperatures. Of the three mutations introduced to phoA (F255 was replaced by L, G257 by S, and W337 by H), only the W337H mutation caused a ts<sup>-</sup> phenotype (Figure 6). This mutation was called phoA<sup>TS</sup> and was recessive to phoA.<br>

The phenotypes caused by phoA<sup>TS</sup> were investigated by germinating phoA<sup>TS</sup>/ΔphoB strains in liquid minimal media at both permissive and restrictive temperatures. Even after 48 hr of incubation at 42°, very few conidia were able to send out a germ tube (Figure 7A) and 99% were unable to undergo any mitotic division arresting with a single nucleus per cell (Figure 7B). At the permissive temperature of 25° the vast majority of cells had undergone germ-tube emergence (Figure 7A) and mitotic divisions after 13 hr of growth.

Surprisingly, the terminal phenotype of the phoA<sup>TS</sup>/ΔphoB strains was modified on richer YG media at restrictive temperature, even though the cells were still markedly temperature sensitive. After 48 hr of germination in YG media, only 22% of phoA<sup>TS</sup>/ΔphoB conidia were able to send out a germ tube and, even though the rest were able to germinate and undergo some nuclear divisions, they were incapable of establishing polarized growth but instead expanded to form large, round-shaped cells (Figure 7, A and B). Those cells that were able to form a germ tube were abnormal compared to wild-type cells at 42° or the phoA<sup>TS</sup>/ΔphoB cells grown at 32°, typically being thicker (Figure 7B). The ability of the YG media to modify the phenotype of the phoA<sup>TS</sup>/ΔphoB strain was found to reside in the yeast extract component of this medium, which was active even when yeast extract was added to minimal media at 1/50 dilution (Figure 7A) and even down to a 1/200 dilution (data not shown).

It has previously been shown that the polarity defect caused by the mpkA mitogen-activated protein kinase was remediated by growth on high-osmolality media (Bussink and Osmani 1999). However, unlike deletion of mpkA, the polarity defect caused by phoA<sup>TS</sup>/ΔphoB was not remediated by growth in 1 m sucrose-supplemented YG media (data not shown).

Expression of mammalian CDK5 can complement the lethality caused by phoA<sup>TS</sup>/ΔphoB: The generation of the phoA<sup>TS</sup>/ΔphoB temperature-sensitive strain allowed
us the opportunity to test for similarities in function between higher eukaryotic CDK5 and \( \text{pho}^{A} / B \) function in \( A. \text{nidulans} \) by complementation. Expression of mammalian CDK5 was placed under control of the inducible \( \text{alc}^{A} \) promoter and introduced into a \( \text{alc}^{A} \)-repressing media at 32\({}^\circ\). (B) \( \text{alc}^{A} \)-repressing media at 32\({}^\circ\). (C) \( \text{alc}^{A} \)-repressing media at 42\({}^\circ\). (D) \( \text{alc}^{A} \)-inducing media at 42\({}^\circ\). Strains: 1, wild type (SO51); 2, control ts strain (SO53); 3 and 4, \( \text{pho}^{A}/\Delta\text{pho}^{B} \) strain XD1; 5–8, XD1 transformed with mammalian CDK5 under control of the inducible \( \text{alc}^{A} \) promoter.

![Image](https://example.com/image.png)

**Figure 8.**—Mammalian CDK5 can complement the \( \text{pho}^{A}/\Delta\text{pho}^{B} \) mutations. Strains were replica plated and incubated for 2 days before photography. (A) \( \text{alc}^{A} \)-repressing media at 32\({}^\circ\). (B) \( \text{alc}^{A} \)-repressing media at 32\({}^\circ\). (C) \( \text{alc}^{A} \)-repressing media at 42\({}^\circ\). (D) \( \text{alc}^{A} \)-inducing media at 42\({}^\circ\). Strains: 1, wild type (SO51); 2, control ts strain (SO53); 3 and 4, \( \text{pho}^{A}/\Delta\text{pho}^{B} \) strain XD1; 5–8, XD1 transformed with mammalian CDK5 under control of the inducible \( \text{alc}^{A} \) promoter.

Temperature-sensitive inactivation or deletion of \( \text{nim}X^{\Delta \text{a}2} \) causes cell cycle arrest and lack of colony formation, demonstrating that it is an essential gene. The second CDK, PHOA, is not essential but plays a role in developmental fate in response to environmental conditions, including phosphorous concentrations. PHOA is most similar to the Pho85 class of CDKs, which includes \( \text{PHO}^{85} \) of \( S. \text{cerevisiae} \) and \( Pef1 \) of \( S. \text{pombe} \). This particular class of CDKs performs a wide range of functions in different species, but neither of these yeast CDKs are essential and strains carrying null alleles are viable. Neither yeast genomes encode a second CDK in the Pho85 family.

Although \( A. \text{nidulans} \) \( \text{pho}^{A} \) is a nonessential gene, we were surprised to find that expression of a dominant-negative version of \( \text{phoa} \) caused inhibition of growth. This indicated that \( \text{phoa} \) could potentially have a redundant essential function shared by a second unknown CDK. Previous Western blot analysis, using antiserum raised against PHOA, also indicated that a second PHOA-like kinase may be present in \( A. \text{nidulans} \) (Buss-Sink and Osmani 1998). This potential was confirmed using BLAST searches of the genome of \( A. \text{nidulans} \) for a gene encoding a \( \text{phoa} \)-related kinase. This kinase, with 77\% identity to PHOA, was isolated and termed \( \text{phob} \).

To analyze the function of \( \text{phob} \), a null allele was generated using homologous recombination to replace the coding domain of \( \text{phob} \) with the \( \text{pyro}^{A} \) nutritional marker. Analysis of protein from deleted strains demonstrated that \( \text{phob} \) does encode the second PHOA-related kinase previously seen by use of Western blotting (Buss-Sink and Osmani 1998). No phenotype could be attributed to the deletion of \( \text{phob} \) under any growth conditions tested, including those that markedly affect development in a \( \text{phoa} \)-deleted strain. Thus, like \( \text{phoa} \), \( \text{phob} \) is a nonessential gene.

\( \text{phob} \) apparently has no functions that cannot be fulfilled by \( \text{phoa} \). However, because deletion of \( \text{phob} \) causes marked developmental defects under specific growth conditions, and lack of \( \text{phob} \) does not cause such defects, it is clear that \( \text{phoa} \) has some functions during development that cannot be fulfilled by \( \text{phob} \). These two kinases, however, do have some common functions because deletion of both caused lethality. This was revealed by crossing haploid strains having deletions of either \( \text{phoa} \) or \( \text{phob} \) using complementing genetic markers. In this way we could ask if germination and growth of the \( \Delta \text{phoa} \) \( \Delta \text{phob} \) mutant recombinant ascospores could occur on media selective for the two nutritional markers used to delete the genes. In the \( \Delta \text{phoa} \Delta \text{phob} \) mutant ascospores, germination was able to take place but no colony formation was observed. This is because the double mutants had a limited capacity to undergo continued hyphal growth and nuclear division. These cells also displayed abnormal nuclear morphology and thinner germ tubes compared to controls at later times of germination. This indicates that neither \( \text{phoa} \) nor \( \text{phob} \) is required for

**DISCUSSION**

Previous work has identified two cyclin-dependent kinases in the filamentous fungus \( A. \text{nidulans} \). The first, \( \text{nim}X^{\Delta \text{a}2} \), is a functional homolog of the cell-cycle-specific Cdc2 kinase of fission yeast (Osmani et al. 1994).
breaking ascospore dormancy or short-term growth, but they are required to undergo the first mitosis during germination, suggestive of a cell-cycle-specific defect. However, unlike mutations in genes that are specifically required for cell cycle progression (Morris 1976), the ΔphoAΔphoB germlings did not continue normal growth while preventing nuclear divisions. Instead, germlings became thin and nuclear structure was compromised.

To further characterize the role of phoA/B, a temperature-sensitive allele of phoA was generated in a ΔphoB background. This approach also allowed characterization of the phoA phoB null phenotype in rich media as we did not need to impose nutritional limitations on cells to identify the double mutant as dictated by crossing the ΔphoA to ΔphoB strains. The phenotype caused by temperature inactivation of phoAΔphoB in germinating conidia on minimal media was more dramatic than that seen in ΔphoA ΔphoB ascospores germinated in similar media. These differences could be attributed to differences in the germination properties of conidia vs. ascospores and/or to the potential for endowment of wild-type phoA function during ascospore formation. However, in both cases inactivation of phoA phoB function allows some features of germination (such as swelling of spores and germ-tube emergence for ΔphoA ΔphoB ascospores), but not cell cycle progression.

Surprisingly, the phenotype of phoAΔphoB was significantly affected by low levels of yeast extract (down to 1/200 of YG medium that contains 0.5% yeast extract). The main effect of the yeast extract was to allow germination and limited cell cycle progression. Importantly, the majority of conidia that were able to germinate were unable to switch to polarized growth but instead continued isotropic growth. This phenotype can also be caused by actin depolymerization using cytochalasin, suggesting that perhaps phoA phoB function may be required for normal actin function, as has been proposed for Pho85 (Lee et al. 1998) and CDC5 (Smith and Tsai 2002).

The fact that very low levels of yeast extract can modify the terminal phenotype caused by lack of PHO85 function further implicates these kinases as mediators of responses to extracellular conditions. At this time, however, it is unclear what particular component of yeast extract is responsible for modifying the phenotype.

The lethality caused by lack of both phoA and phoB demonstrates that in A. nidulans an essential function exists for this class of CDK, whereas in both budding and fission yeast neither PHO85 nor pef1 is essential. In S. cerevisiae six cyclin-dependent kinases are known. Of these, CDC28, KIN28, and BUR1 are essential genes whereas PHO85, CTK1, and SRB10 are nonessential. It is therefore possible that phoA phoB may fulfill functions carried out by one of the essential CDKs in S. cerevisiae (CDC28, KIN28, and BUR1). It is known that nimX and CDC28 are functional homologs. We therefore searched by BLAST analysis for potential CDKs in the genomes of both A. nidulans (http://www.genome.wi.mit.edu/annotation/fungi/aspergillus/index.html), which has not yet been annotated, and another fully sequenced filamentous fungus, N. crassa (Galagan et al. 2003; http://www.genome.wi.mit.edu/annotation/fungi/neurospora/). This analysis indicates that in A. nidulans and in N. crassa, there is a complement of six CDKs similar to that encoded in S. cerevisiae. It is therefore unlikely that the reason that the PHO8A-PHOB pair is essential is due to lack of one of the essential CDKs found in S. cerevisiae. Interestingly, unlike A. nidulans, only one PHO8/B-like kinase is found in N. crassa and it will be interesting to determine if this is an essential gene.

The question therefore remains, Why are PHO85s in S. cerevisiae and pef1 in S. pombe nonessential genes whereas phoA and phoB together play an essential role in A. nidulans? In vertebrates the nearest CDK to PHO85/PHOB based upon primary amino acid sequence identity is CDK5. Interestingly, CDK5 is an essential gene in mice where homozygous nulls die in utero or soon after birth (Ohshima et al. 1996). This is due to lack of normal neuronal migration and subsequent failure of normal brain development. It has been recently hypothesized that perhaps CDK5 plays a role in intracellular trafficking (Smith and Tsai 2002). Our results showing that a high percentage of phoAΔphoB conidia germinated at restrictive temperature are unable to establish polarized growth is consistent with phoA/phoB perhaps having a role in regulating intracellular trafficking. Further suggesting a similar role for phoA/phoBand CDK5 is the ability of CDK5 to complement the lethality caused by lack of phoA/phoB function.

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