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PHR1, a pH-Regulated Gene of *Candida albicans*, Is Required for Morphogenesis

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***Candida albicans*, like many fungi, exhibits morphological plasticity, a property which may be related to its biological capacity as an opportunistic pathogen of humans. Morphogenesis and alterations in cell shape require integration of many cellular functions and occur in response to environmental signals, most notably pH and temperature in the case of *C. albicans*. In the course of our studies of differential gene expression associated with dimorphism of *C. albicans*, we have isolated a gene, designated *PHR1*, which is regulated in response to the pH of the culture medium. *PHR1* expression was repressed at pH values below 5.5 and induced at more alkaline pH. The predicted amino acid sequence of the *PHR1* protein was 56% identical to that of the *Saccharomyces cerevisiae* Gpg1/Gas1 protein, a highly glycosylated cell surface protein attached to the membrane via glycosylphosphatidylinositol. A homozygous null mutant of *PHR1* was constructed and found to exhibit a pH-conditional morphological defect. At alkaline pH, the mutant, unlike the parental type, was unable to conduct apical growth of either yeast or hyphal growth forms. This morphological aberration was not associated with defective cytoskeletal polarization or secretion. The results suggest that *PHR1* defines a novel function required for apical cell growth and morphogenesis.**

Fungi exhibit morphological plasticity, a property integral to their biology. *Saccharomyces cerevisiae* typically grows as an ellipsoidal yeast, but extends mating projections in response to mating factors and can adopt a pseudohyphal morphology as a means of foraging in response to nitrogen limitation (31, 33, 42). Haploid cells of the phytopathogen *Ustilago maydis* are yeast in form and noninfectious, virulence being associated with the filamentous dikaryon phase (37). Dimorphism is similarly characteristic of many human fungal pathogens, including *Candida albicans* (50). *C. albicans* can adopt a number of morphologies, including a yeast form, termed a blastospore, a true hyphal form, and a range of intermediate, or pseudohyphal, shapes (50). The pseudohyphae/hyphae exhibit increased adherence to host tissue and increased invasiveness, properties which may contribute to the pathogenic process (50).

Fungal morphogenesis is a consequence of polarized cell growth, which requires integration of multiple cellular functions, including bud site selection, bud site assembly, cytoskeletal assembly, and polarized secretion (reviewed in references 23 and 46). Morphogenesis of *C. albicans* has been studied almost exclusively in the context of dimorphism, but the molecular basis of the process and its regulation are essentially unknown. In vitro, the organism responds to a number of environmental signals, pH and temperature being the predominant determinants of cell morphology (11, 50). The yeast phase is favored at 25°C and acidic pH; conversely, hyphal growth is favored at 37°C and a pH near neutrality (11, 50). Medium composition, particularly the presence of serum or other inducing compounds, can favor growth as hyphae (50). The actin cytoskeleton is polarized toward the growing tip of both yeasts and hyphae and is required for polarized growth of the cells (3, 67). Cell wall biosynthesis has received considerable study, and structural differences in the alkali-insoluble glucan fraction of the cell wall have been associated with the different morphologies (59). The chitin content of the cell wall is increased in hyphae (15), and chitin synthesis, which occurs predominantly at the hyphal tip (9), is required for morphogenesis (34). The genes encoding chitin synthases I and II have been isolated (4, 17), and although *CHS2* expression is increased in hyphae (17), it is not required for hypha formation (32).

A classical genetic approach has identified mutations in several complementation groups affecting cell morphology (30, 47); however, the diploid genome and asexual life cycle of *C. albicans* have stymied extensive genetic characterization of the process. More recently, our laboratory has identified a number of genes differentially expressed during morphogenesis (7); this effort, in conjunction with the development of more appropriate auxotrophic strains and improvements in reverse genetic techniques (26), promises to yield insights into the molecular basis of morphogenesis. In this report, we describe characterization of the gene *PHR1*, which is differentially regulated in response to the pH of the growth medium. *PHR1* encodes a putative cell surface glycoprotein anchored to the membrane by glycosylphosphatidylinositol (GPI). Deletion of *PHR1* results in a pH-conditional defect in morphogenesis.

MATERIALS AND METHODS

Strains and growth conditions. The *C. albicans* strains used in this study are listed in Table 1. The strains were routinely cultured on YPD medium (60) at 30°C. Cell growth and morphology were typically monitored following incubation in medium 199 containing Earle's salts and glutamine but lacking sodium bicarbonate (Gibco BRL). The medium was buffered with 150 mM *N*-2-hydroxyeth-

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TABLE 1. *C. albicans* strains used in this study

Strain	Parent	Genotype	Reference
SGY243		Δ*ura3*::*ADE2*/Δ*ura3*::*ADE2*	34
SC5314		Clinical isolate	34
CAF3-1	SC5314	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	25
CAS5	CAF3-1	Δ*phr1*::*hisG-URA3-hisG*/*PHR1* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	This work
CAS6	CAS5	Δ*phr1*::*hisG*/*PHR1* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	This work
CAS7	CAS6	Δ*phr1*::*hisG*/*PHR1*-pUC18-*URA3*-Δ*phr1* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	This work
CAS8	CAS7	Δ*phr1*::*hisG*/Δ*phr1* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	This work
CAS10	CAS8	Δ*phr1*::*hisG*/Δ*phr1* Δ*ura3*::*imm434*/*URA3*	This work
CAS11	CAS8	Δ*phr1*::*hisG*/*PHR1*-pUC18-*URA3*-Δ*phr1* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	This work
CAS12	CAS8	Δ*phr1*::*hisG*/Δ*phr1* Δ*ura3*::*imm434*/Δ*ura3*::*imm434*-pBSK-*EF1*::*PHR1-URA3*	This work

ylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) to various pHs as indicated in Results. With inclusion of the buffer, the pH of the medium did not vary by more than 0.2 pH units over the course of the experiments. Cultures were inoculated to a density of 2.5×10^6 to 5×10^6 cells/ml into prewarmed medium and incubated at either 25 or 37°C with vigorous aeration on a rotary shaker. Stationary-phase cells grown at 25°C in unbuffered YPD medium were used as an inoculum. Cell morphology was also examined following growth in YNB medium (2% glucose, 1× Difco yeast nitrogen base), in YPD, or in the medium of Lee et al. (40), each buffered at pH 7.5 or 8.0 with 150 mM HEPES. All media were supplemented with uridine (25 μg/ml) for the growth of Ura$^-$ strains. For induction of extracellular β-*N*-acetylglucosaminidase activity, medium 199 was supplemented with 25 mM *N*-acetylglucosamine (13).

Isolation of the *PHR1* gene. A cDNA clone corresponding to a pH-responsive gene was isolated by differential hybridization screening of a cDNA library. Construction of the cDNA library and differential hybridization screening of the library were described previously (7). One of the cDNA clones contained a 1.0-kb insert that hybridized with a pH-regulated transcript. The insert from this clone was used for hybridization screening of a λGEM-12 genomic library (7) as described by Carlock (14). Positive plaques were characterized by restriction endonuclease mapping and Southern blot hybridization. A 2.1-kb *Eco*RI genomic DNA fragment which hybridized with the cDNA clone was subcloned into pUC18 to generate plasmid pSMS-20 and into pBSK$^+$ (Stratagene) to generate plasmid pSMS-24.

Southern and Northern (RNA) blot analysis. Southern and Northern blot hybridizations were conducted as previously described (56). *C. albicans* genomic DNA was prepared by the method of Scherer and Stevens (58). RNA was prepared by the method of Langford and Gallwitz (39). Transcript size was determined by comparison with rRNA species. The 2.1-kb *Eco*RI genomic DNA fragment of *PHR1* was used as a hybridization probe. The *C. albicans* actin gene used in control hybridizations was kindly provided by W. S. Riggsby (44).

DNA sequence analysis. Portions of the genomic insert in plasmid pSMS-20 were subcloned into the M13 cloning vector mp18 or mp19. The nucleotide sequence was determined by the dideoxy-chain termination method (55), using T7 polymerase (U.S. Biochemical Corp.) (63) and either M13 universal sequencing primers or custom-synthesized oligodeoxribonucleotide primers. Nucleotide sequence analyses were performed with the Wisconsin Genetics Computer Group sequence analysis software package, version 7.0 (21). Homology searches of the GenBank database were conducted with the FASTA program of Pearson and Lipman (51).

Strain constructions. To construct a *PHR1* null mutant, plasmid pSMS-24 was digested at the unique *Pst*I-*Hin*dIII sites within the polylinker region to release the 2.1-kb insert containing the *PHR1* gene. This fragment was cloned into the *Hin*dIII and *Pst*I sites of pUC18. From the resulting plasmid, pSMS-25, a 841-bp *Cla*I fragment was deleted and replaced with the 3.95-kb *Bam*HI-*Bgl*II *hisG-URA3-hisG* fragment from pCUB6 (26) to construct plasmid pSMS-23. The DNA fragments were blunt-end ligated following an end-filling reaction with Klenow DNA polymerase (54). The *Cla*I sites lie within the *PHR1* open reading frame and encompass codons 132 to 431. Plasmid pSMS-23 was digested with *Hin*dIII and *Pvu*II, releasing a DNA fragment containing the *PHR1* gene deletion/disruption. Approximately 15 μg of this DNA fragment was used to transform the Ura$^-$ *C. albicans* strain CAF3-1 (26) by the method of Kelly et al. (36). Transformed cells were selected as Ura$^+$, and integration of the transforming DNA at the *PHR1* locus was verified by Southern blot analysis. One of the heterozygous disruptants recovered was designated strain CAS5. Spontaneous Ura$^-$ derivatives of this strain were selected on medium containing 5-fluoroorotic acid (8). These clones were analyzed by Southern blot hybridization to identify those which had undergone intrachromosomal recombination. One of these Ura$^-$ derivatives was designated CAS6 and used for mutagenesis of the second allele of *PHR1*.

A null mutation was introduced into the remaining functional allele of *PHR1* by a pop-in/pop-out replacement (53). The 841-bp *Cla*I fragment was deleted from plasmid pSMS-20 by digestion with *Cla*I followed by intramolecular ligation generating plasmid pSMS-30. A 1.4-kb *Xba*I-*Sca*I fragment containing the *C. albicans URA3* gene (43) was ligated into the *Xba*I-*Sma*I sites of the polylinker region of pSMS-30 to create plasmid pSMS-31. Plasmid pSMS-31 was linearized at the unique *Sac*II site within the *PHR1* gene and used to transform strain CAS6. Transformed cells were selected as Ura$^+$, and integration into the undisrupted *PHR1* allele was verified by Southern blot analysis. Spontaneous Ura$^-$ segregants were selected by resistance to 5-fluoroorotic acid. Those Ura$^-$ segregants having lost the wild-type copy of *PHR1* and retaining the deletion mutation were identified by Southern blot analysis. One of these *PHR1* null mutants, designated CAS8, was used in subsequent studies.

Strain CAS10, a Ura$^+$ derivative of CAS8, was constructed by transformation of CAS8 with a 4.9-kb *Pst*I-*Bgl*II fragment containing *URA3* (36). Strain CAS11, a Phr$^+$ derivative of strain CAS8, was generated by transformation of CAS8 with plasmid pSMS-54. Plasmid pSMS-54 was constructed by ligation of a 1.36-kb *Pst*I-*Xba*I fragment containing the *URA3* gene into the *Pst*I-*Xba*I sites of plasmid pSMS-20. The *Pst*I-*Xba*I *URA3* fragment was generated by subcloning the *Sca*I-*Xba*I fragment of *URA3* into the *Sma*I-*Xba*I sites of pBSK$^+$, which places a *Pst*I site adjacent to the *Sca*I end of the fragment. pSMS-54 DNA was digested with *Sac*II to target integration to the *PHR1* locus and used to transform strain CAS8 to Ura$^+$. The site of integration and structure of the locus were verified by Southern blot analysis.

Strain CAS12, a mutant with constitutive expression of *PHR1*, was constructed by transformation of strain CAS8 with plasmid pSMS-43. To construct plasmid pSMS-43, the 0.75-kb *Eco*RI-*Nla*III fragment containing the promoter region of the *C. albicans* EF1α-2 gene (62) was blunt ended with Klenow polymerase and ligated into the *Eco*RV site of plasmid pBSK$^+$ (Stratagene). Plasmid pSMS-24 was cut at the *Nci*I site located 72 bp 5′ of the *PHR1* start codon, blunt ended with Klenow DNA polymerase, and digested with *Pst*I. The fragment containing the *PHR1* gene was cloned into the *Eco*RI-*Pst*I sites located 3′ to the EF1α promoter. The *Eco*RI site was filled in with Klenow DNA polymerase prior to ligation. The resulting plasmid, pSMS-28, was digested with *Xba*I and ligated with the 4.4-kb *Xba*I fragment from plasmid pUR3 (36) containing the *URA3* gene to generate pSMS-43. This plasmid was linearized at the unique *Hpa*I site within the *URA3* fragment and used to transform strain CAS8. The site of integration and structure of the integrated DNA were verified by Southern blot analysis, and constitutive expression of *PHR1* was verified by Northern blot analysis.

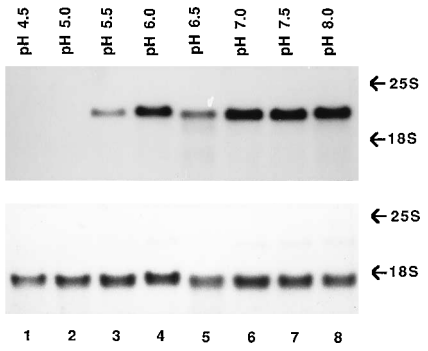

FIG. 1. Northern blot analysis of the *PHR1* transcript. Strain CAF3-1 was inoculated into TC199 medium adjusted to the indicated pHs and incubated at 37°C for 1 h. RNA isolation and Northern blot hybridization were performed as described in Materials and Methods. The upper panel displays the results of hybridization with *PHR1*, and the lower panel displays the results of hybridization with actin DNA. The electrophoretic positions of the rRNAs are indicated on the right.

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        1       .       .       .       .       .       .       .       .
        GAATTCATCGACGCCTCATTCATACTAATATAATAGGTGTTGCAACATCAAAATCATTTCATCGCTAATACAAACATCCGA
82      .       .       .       .       .       .       .       .       .
GATTAATCTTGTCCATTGGCAATATAATTAACACCACTGCCAATACATATAAATCAAATCAGCATTAACTAATAGTATTTATTAATTACT
172     .       .       .       .       .       .       .       .       .
TAGAAAAGCCGGGCTGCGTTTTTTTGTTTCATTCATAGATTTACTTATTTCCATTTAACATACCAAACTACAGGTTGAAGCCAAAAAAAA
262/1   .       .       .       .       .       .       .       .       .
ATGTATTCATTAATCAAATCATTGGCCACATTTGCCACACTCTTTTCATTAACTTTAGCCAAGTTTGAATCGTCCACCCCACCAGTTGAA
 M  Y  S  L  I  K  S  L  A  T  F  A  T  L  F  S  L  T  L  A  K  F  E  S  S  T  P  P  V  E
352/31  .       .       .       .       .       .       .       .       .
GTTGTTGGTAACAAATTTTATTTTTCCAATAATGGGTCTCAGTTTTTAATCAGGGGTATCGCCTATCAGCAAGATGCCGCGGGCTCAGTT
 V  V  G  N  K  F  Y  F  S  N  N  G  S  Q  F  L  I  R  G  I  A  Y  Q  Q  D  A  A  G  S  V
442/61  .       .       .       .       .       .       .       .       .
TCCTCCGGTTACGACGCCGATCCTAATAGAAAATACAATGATCCTTTAGCCGATCGTGACGCTTGTAAACGTGACGTCAAGTATTTCAAA
 S  S  G  Y  D  A  D  P  N  R  K  Y  N  D  P  L  A  D  R  D  A  C  K  R  D  V  K  Y  F  K
532/91  .       .       .       .       .       .       .       .       .
GAATCAAACACCAATACTTTGAGAGTTTATGCTATTGACCCAGATAAGGATCATGAAGAGTGTATGAAAATTTTCAGTGACGCTGGTATT
 E  S  N  T  N  T  L  R  V  Y  A  I  D  P  D  K  D  H  E  E  C  M  K  I  F  S  D  A  G  I
622/121 .       .       .       .       .       .       .       .       .
TACATTGTTGCTGATTTATCAGAACCAACTGTATCGATTAACAGAAACAACCCAGAATGGAACTTGGATTTATACAAACGTTATACAAAA
 Y  I  V  A  D  L  S  E  P  T  V  S  I  N  R  N  N  P  E  W  N  L  D  L  Y  K  R  Y  T  K
712/151 .       .       .       .       .       .       .       .       .
GTCATTGATAAGATGCAAGAATATTCTAATGTTTTGGGATTTTTTGCTGGTAACGAAGTAACTAATAATCGTTCAAATACCGATGCTTCT
 V  I  D  K  M  Q  E  Y  S  N  V  L  G  F  F  A  G  N  E  V  T  N  N  R  S  N  T  D  A  S
802/181 .       .       .       .       .       .       .       .       .
GCATTTGTTAAGGCTGCCATTAGAGATATGAAGAAATACATCAAGGAGTCTGATTATAGACAAATTCCTGTTGGTTATTCATCCAATGAT
 A  F  V  K  A  A  I  R  D  M  K  K  Y  I  K  E  S  D  Y  R  Q  I  P  V  G  Y  S  S  N  D
892/211 .       .       .       .       .       .       .       .       .
GACGAAGAAATTAGAGTCGCCATTGCCGATTATTTCTCTTGTGGTTCATTAGATGATCGTGCTGATTTCTTTGGTATCAATATGTATGAA
 D  E  E  I  R  V  A  I  A  D  Y  F  S  C  G  S  L  D  D  R  A  D  F  F  G  I  N  M  Y  E
982/241 .       .       .       .       .       .       .       .       .
TGGTGTGGCAAATCAACTTTCGAAACCTCAGGTTACAAGGACAGAACTGAAGAAATCAAGAACTTGACTATCCCAGCCTTCTTCTCCGAA
 W  C  G  K  S  T  F  E  T  S  G  Y  K  D  R  T  E  E  I  K  N  L  T  I  P  A  F  F  S  E
1072/271.       .       .       .       .       .       .       .       .
TATGGATGTAATGCTAACCGTCCACGTTTGTTCCAAGAAATTGGTACCTTGTATTCCGATAAGATGACTGATGTTTGGTCCGGAGGTATT
 Y  G  C  N  A  N  R  P  R  L  F  Q  E  I  G  T  L  Y  S  D  K  M  T  D  V  W  S  G  G  I
1162/301.       .       .       .       .       .       .       .       .
GTTTATATGTATTTTGAAGAGGCTAACAAATACGGTTTGGTTCTGGTTGATGGTAATTCGGTCAAGACATTATCTGACTACAACAATTAC
 V  Y  M  Y  F  E  E  A  N  K  Y  G  L  V  L  V  D  G  N  S  V  K  T  L  S  D  Y  N  N  Y
1252/331.       .       .       .       .       .       .       .       .
AAATCAGAAATGAACAAAATAAGCCCATCCCTTGCCCATACTTCAACATTATCCAGTTCTGACGCCAGCAAGACTTTGCAATGTCCAGGA
 K  S  E  M  N  K  I  S  P  S  L  A  H  T  S  T  L  S  S  S  D  A  S  K  T  L  Q  C  P  G
1342/361.       .       .       .       .       .       .       .       .
ACTGCTGCTAGCACTTGGAAAGCTGCAACTAATTTGCCACCAACTCCAGATGAAAGTTACTGTGATTGTATTTCCAAGTCATTAGAATGT
 T  A  A  S  T  W  K  A  A  T  N  L  P  P  T  P  D  E  S  Y  C  D  C  I  S  K  S  L  E  C
1432/391.       .       .       .       .       .       .       .       .
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 V  V  A  D  D  V  D  K  E  D  Y  G  D  L  F  G  Q  V  C  G  Y  I  D  C  S  A  I  S  A  D
1522/421.       .       .       .       .       .       .       .       .
GGTAGCAAAGGTGAATATGGTGTTGCTTCCTTCTGTTCTGATAAAGATCGTTTGTCATATGTGTTGAACCAGTATTACCTTGACCAAGAC
 G  S  K  G  E  Y  G  V  A  S  F  C  S  D  K  D  R  L  S  Y  V  L  N  Q  Y  Y  L  D  Q  D
1612/451.       .       .       .       .       .       .       .       .
AAGAAATCCAGTGCTTGTGATTTCAAAGGCAGTGCTTCAATCAATAGCAAGGCTAGTGCTAGTGGCAGCTGCAAAGCTGTTAGTGGAGTA
 K  K  S  S  A  C  D  F  K  G  S  A  S  I  N  S  K  A  S  A  S  G  S  C  K  A  V  S  G  V
1702/481.       .       .       .       .       .       .       .       .
GCTACTGGTAAGGCATCTTCCTCTGGTGGAAGCTCCAAATCTGGATCTTCCTCTGCATCTGCTTCTGGATCATCAAGCAGCAGCACCAGC
 A  T  G  K  A  S  S  S  G  G  S  S  K  S  G  S  S  S  A  S  A  S  G  S  S  S  S  S  T  S
1792/511.       .       .       .       .       .       .       .       .
TCTGGGTCCAGCTCAAGCTCTGGAGTTAAAGCAACTCAACAAATGTCTATGGTCAAATTGGTTTCAATTATTACTATTGTTACTGCATTT
 S  G  S  S  S  S  S  G  V  K  A  T  Q  Q  M  S  M  V  K  L  V  S  I  I  T  I  V  T  A  F
1882/541.       .       .       .       .       .       .       .       .
GTTGGTGGTATGTCCGTTGTTTTTTAAATCAATGAAGACTAACCGATATGAAGGGTTCTGTTGTATAGGACGAATATATATAGTTTAATA
 V  G  G  M  S  V  V  F
1972    .       .       .       .       .
AGAGTTCATTATTTAAAAGTTCCAATTTGAAATCAACTTGGTACACATATCTATT
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FIG. 2. Nucleotide sequence of *PHR1* and sequence of the deduced protein.

Photomicroscopy. Cells were examined with an Olympus BH microscope equipped with Nomarski optics and photographed with Polaroid P/N55 film. Actin distribution was visualized by immunofluorescence staining with rhodamine-conjugated phalloidin (Molecular Probes, Inc.). Cells were fixed and stained as described by Amatruda et al. (2). Stained samples were examined on a Nikon Labophot epifluorescence microscope and photographed with Kodak Tri-X-Pan film.

β-*N*-Acetylglucosaminidase assay. Extracellular β-*N*-acetylglucosaminidase activity was assayed by a modification of the method of Cannon et al. (13). Cells from 10 ml of culture were collected by centrifugation, washed three times with 0.5 ml of 125 mM sodium citrate (pH 4.5), and suspended in 1 ml of citrate buffer. Then 250 μl of cell suspension was mixed with 250 μl of 10 mM *para*-nitrophenol-β-*N*-acetylglucosaminide in citrate buffer. The cell samples and substrate were preequilibrated to 37°C prior to mixing. The reaction mixtures were incubated with shaking at 37°C. After 30 min, the reaction was stopped by the addition of 0.5 ml of 2% sodium carbonate. The cells were removed by filtration, and the A_{420} was measured. One unit of enzyme is defined as that which hydrolyzes 1 μmol of substrate per min.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank under accession number M90812.

RESULTS

Isolation of a pH-responsive gene, *PHR1*. Previously, we isolated a number of cDNA clones corresponding to genes that were differentially expressed during morphogenesis of *C. albicans* (7). These clones were identified by a differential hybridization screen sensitive to differences in gene expression related not only to morphology but also to differences in the environmental conditions used to control morphology, temperature, and pH. One of the cDNA clones isolated in this screen hybridized to a transcript that varied quantitatively with the pH of the external medium (Fig. 1). The transcript was not detectable in RNA prepared from cells grown at either pH 4.5 or pH 5.0; however, the cDNA clone hybridized to an approximately 2-kb transcript in RNA samples from cells grown at pH 5.5. Above pH 5.5, each incremental increase in pH resulted in increased levels of *PHR1* mRNA as normalized to actin mRNA. At pH 6.5, the level was approximately three times the amount in the pH 5.5 sample, and at pH 8.0, the level was ninefold higher. The transcript was not detected in stationary-phase cells but was rapidly induced upon subculturing cells into fresh medium of appropriate pH (data not shown). The pH-dependent expression was observed in several other media and was unaffected by the temperature of incubation or the morphology of the cells (data not shown). The induction pattern was also strain independent, as evidenced from an examination of strains SC5314, CAF3-1, and SGY243 (data not shown). We designated this pH-responsive gene *PHR1*.

Sequence analysis of *PHR1*. A 2.1-kb genomic DNA fragment containing the *PHR1* gene was isolated and sequenced as described in Materials and Methods. We identified a single, uninterrupted open reading frame of 1,644 nucleotides which could encode a 548-residue peptide with a theoretical molecular weight of 59,530. The nucleotide sequence of *PHR1* and deduced amino acid sequence are shown in Fig. 2.

A FASTA search of the GenBank and EMBL databases (51) revealed that the *PHR1* protein was 56% identical to the protein encoded by the *S. cerevisiae GGP1*/*GAS1* gene (49, 64) (Fig. 3). No other significant homologies were found. *GGP1*/*GAS1* encodes a cell surface glycoprotein anchored to the membrane by GPI (49, 64). The two proteins were homologous along their entire lengths, and several functionally significant features were conserved (Fig. 3). These include a hydrophobic amino terminus characteristic of secretory signal sequences, three consensus N-glycosylation sites, a 32-amino-acid region composed of over 70% serine residues located near the carboxy terminus, and, as characteristic of GPI-linked proteins (22, 25), a hydrophobic carboxy terminus. In addition, the

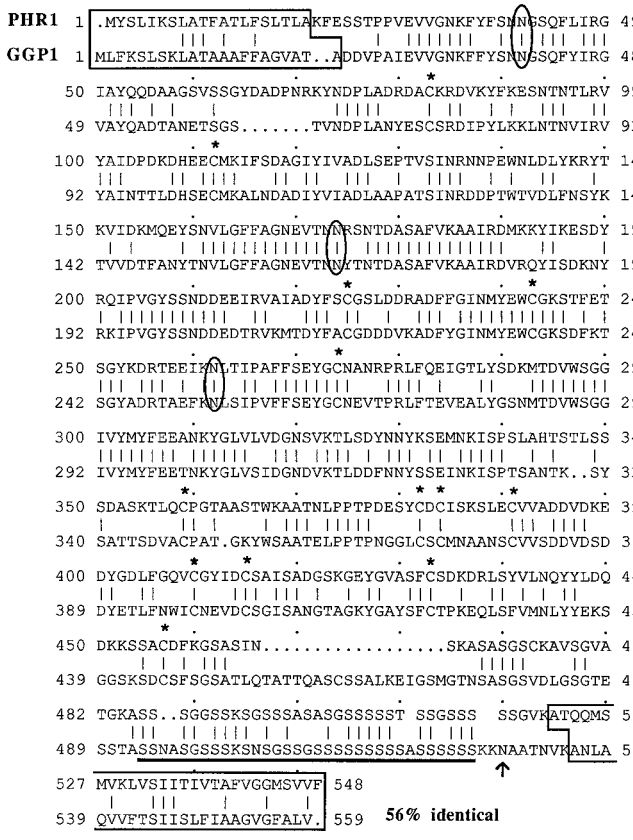

FIG. 3. Comparison of predicted amino acid sequences of *PHR1* and *GGP1*/*GAS1*. Identical residues are indicated by vertical bars. The hydrophobic amino and carboxy termini are boxed. Conserved N-glycosylation consensus sites are circled, and the serine-rich region is underlined. Conserved cysteine residues are indicated by asterisks. The arrows indicate proposed GPI attachment sites.

positions of 13 of 14 cysteine residues were conserved in these cysteine-rich proteins. The only feature not conserved was the Asn-506 residue identified as the protein cleavage and GPI attachment site of the *S. cerevisiae* protein (49).

Construction of a *PHR1* null mutant. *S. cerevisiae GGP1*/*GAS1* null mutants exhibit pleiotropic defects including morphological aberrations (49, 52). This latter observation is of particular interest since the pH of the culture medium is an important determinant of *C. albicans* morphology and *PHR1* was most abundantly expressed at pHs conducive to hyphal/pseudohyphal development (11). Therefore, a *phr1* null mutant was constructed to determine the role, if any, of *PHR1* morphogenesis.

One allele of *PHR1* was mutated by using a strategy originally developed for *S. cerevisiae* (1) and modified for use in *C. albicans* (26). This method uses a cassette consisting of the *C. albicans URA3* gene flanked by direct repeats of the *S. typhimurium hisG* gene. This cassette was used to replace 841 bp of the *PHR1* coding region as illustrated in Fig. 4. A DNA fragment consisting of the cassette flanked by the remaining *PHR1* sequences was used to transform strain CAF3-1 to Ura^+. Ten of the resulting Ura^+ isolates were examined, and each contained the desired insert at the *PHR1* locus (data not shown). Southern blot analysis of a representative isolate, CAS5, is shown in Fig. 5. DNA from strain CAS5 exhibited two *PHR1*-hybridizing bands, a 4.2-kb *Spe*I fragment characteristic of the parental strain and an additional fragment of 7.3 kb (Fig. 5).

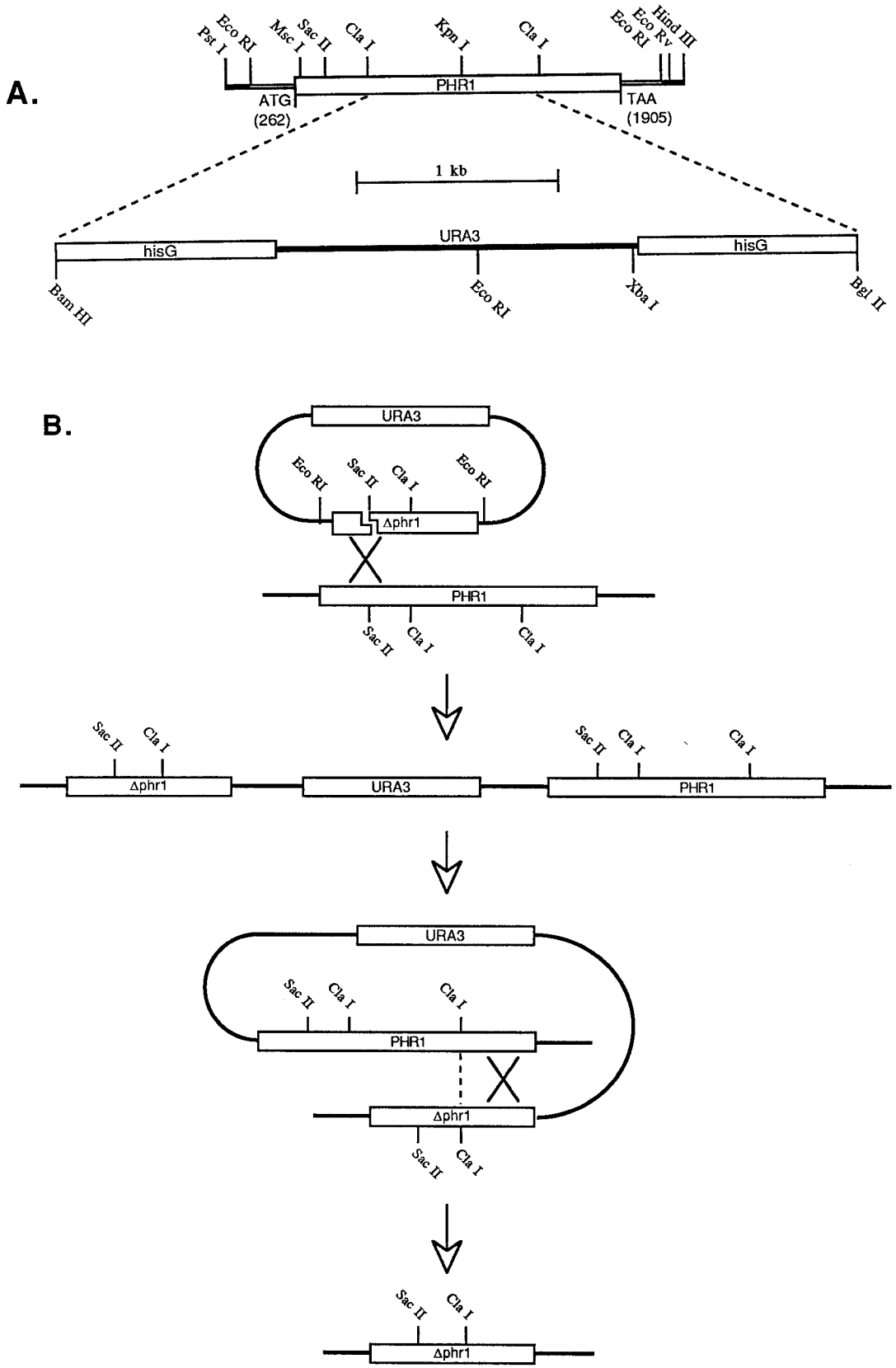

FIG. 4. (A) Construction of the Δ*phr1*::*hisG-URA3-hisG* allele. (B) The pop-in/pop-out strategy for introducing a deletion into *PHR1*.

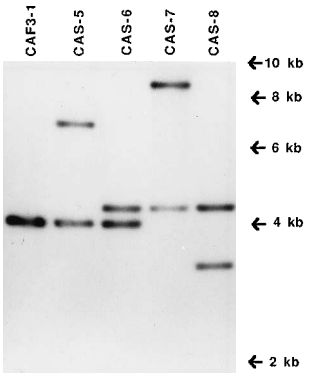

FIG. 5. Southern blot analysis of *PHR1* mutants. Genomic DNA from the indicated strains was digested with *Spe*I and hybridized with *PHR1* DNA. The electrophoretic positions of DNA size markers are indicated on the right.

The size of the latter fragment is consistent with replacement of one allele of *PHR1* with the transforming DNA.

The advantage of using the *hisG-URA3-hisG* cassette is that intrachromosomal recombination between the *hisG* sequences results in excision of the *URA3* marker. This returns the cells to a Ura$^-$ phenotype and permits repeated use of *URA3* as a selectable marker gene. Ura$^-$ segregants of strain CAS5 were selected on 5-fluoroorotic acid-containing medium (8) and examined by Southern blot analysis. Of the 10 independent segregants examined, 8 had undergone intrachromosomal recombination and 2 had experienced an interchromosomal recombination event, reverting to the parental genotype (data not shown). Southern blot analysis of a representative intrachromosomal recombinant, strain CAS6, is shown in Fig. 5. The 7.3-kb *Spe*I fragment seen in strain CAS5 and containing the Δ*phr1*::*hisG-URA3-hisG* disruption was absent, and a new hybridizing fragment, 4.5 kb in length, was present. The size of this latter fragment is consistent with the desired event, loss of *URA3* and one copy of *hisG*.

A different approach was tested in mutating the remaining functional allele of *PHR1*. The frequency of intrachromosomal recombination in *C. albicans* (26) and facility with which intrachromosomal recombinants can be selected suggested that pop-in/pop-out replacements (53, 57) would provide another useful mutagenic strategy for manipulating the genome of *C. albicans*. Figure 4 illustrates the pop-in/pop-out strategy as applied to deletion of *PHR1*. To test this approach, plasmid pSMS-31 was constructed. This plasmid contained *URA3* for selection and a mutated copy of *PHR1* with an internal 841-bp deletion. The plasmid was linearized at a unique *Sac*II site to direct integration to the *PHR1* locus and used to transform the heterozygous strain CAS6. The *Sac*II site is located in the *PHR1* sequence 226 bp 5′ of the deletion endpoint. Of the 16 Ura$^+$ transformants examined, 1 contained pSMS-31 integrated adjacent to the wild-type allele, 6 contained the plasmid integrated adjacent to the *hisG* disrupted allele, and the other 9 had undergone more complex recombinational events (data not shown). Southern blot analysis of the desired transformant, strain CAS7, demonstrated the loss of the 4.2-kb *Spe*I fragment characteristic of the wild-type allele and the appearance of a predicted 9.5-kb hybridizing fragment (Fig. 5). Thus, one chromosome of strain CAS7 contains a nontandem repeat of *PHR1*, one copy being the wild-type allele and one being the mutated allele.

To complete the replacement, Ura$^-$ segregants of strain CAS7 were selected on medium containing 5-fluoroorotic acid. Loss of *URA3* can occur either by interchromosomal recombination with the *hisG* disrupted allele or by intrachromosomal recombination between the adjacent wild-type and mutated alleles. Intrachromosomal recombination can either restore the wild-type locus or replace the locus with the deletion allele. Recombination 5′ of the mutation site regenerates the wild-type allele, while recombination 3′ of the deletion site replaces the wild-type allele with the mutated allele. Of the 12 Ura$^-$ segregants examined, one had become homozygous for the Δ*phr1*::*hisG* allele by interchromosomal recombination, 8 retained the wild-type allele, and 3 exhibited replacement of the wild-type allele with the mutated copy of *PHR1* (data not shown). Southern blot analysis of a representative isolate of the latter class of segregants, strain CAS8, is shown in Fig. 5. In this strain, the 9.5-kb *Spe*I fragment indicative of the nontandem *PHR1* repeats in strain CAS7 is absent and a new fragment of 3.3 kb is present. The 3.3-kb fragment was of the predicted size for the deletion allele and demonstrated the feasibility of pop-in/pop-out replacements in *C. albicans.*

Loss of *PHR1* results in morphological defects. Hyphal growth of *C. albicans* is initiated from blastospores by the emergence of a cylindrical projection termed a germ tube (50). Germ tube emergence is optimal at 37°C but is affected also by the pH of the induction medium (11). Stationary-phase blastospores inoculated into fresh induction medium at pH 5.0 grow exclusively by budding (11). As the pH is adjusted progressively higher, an increasing fraction of the population gives rise to germ tubes (11). This pattern of induction parallels the pattern of *PHR1* expression. To test the significance of this correlation, germ tube induction of the parental and Δ*phr1* mutant strain was examined at various pHs.

Despite the affect of pH on the frequency of germ tube formation, the germ tubes formed by the parental strain, CAF3-1, were similar in morphology irrespective of pH (Fig. 6). At pH 6.0, the Phr$^-$ strain, CAS8, was indistinguishable from CAF3-1 in both frequency and morphology of the germ tubes produced. Although germ tube emergence from CAS8 was evident at higher pH values, progressive increases in pH resulted in a corresponding reduction in the length of the germ tube and lateral expansion of the germ tube apex (Fig. 6). At pH 8.0, the pattern culminated with the emergence of distorted growth projections, many cells adopting aberrant shmoo and dumbbell- or peanut-shaped morphologies (Fig. 6), similar to what has been found for some mating-defective mutants of *S. cerevisiae* (16). No further distortion of cell morphology was noted at higher pH values (data not shown). The phenotype of the mutant suggested that the pH of the medium alters the ability of the cell to sustain or conduct apical growth.

Extended incubation of CAF3-1 cultures resulted in a typical mixture of pseudohyphal and yeast morphologies (Fig. 7). The Δ*phr1* mutant also adopted mixed morphologies at pH 6.0 to 7.0, although the cells became progressively less elongated with increased pH (Fig. 7). At pH 7.0, the culture was composed exclusively of chains of yeast cells (Fig. 7). A dramatic change was noted in the pH 7.5 and 8.0 cultures. The cells became multibudded, rounded, and much enlarged, with a large central vacuole (Fig. 7), a morphology similar to that of *S. cerevisiae GGP1*/*GAS1* null mutants (49, 64). Cell enlargement did not occur during the first several cell divisions and became evident only after six or seven generations of growth at the restrictive pH (data not shown).

Since expression of *PHR1* mRNA was strictly pH dependent and not affected by cell morphology, the role of *PHR1* in morphogenesis may not be restricted to hyphal cells. This possibility was tested by growing the parental and mutant strains at 25°C, which promotes growth in the yeast morphology. The

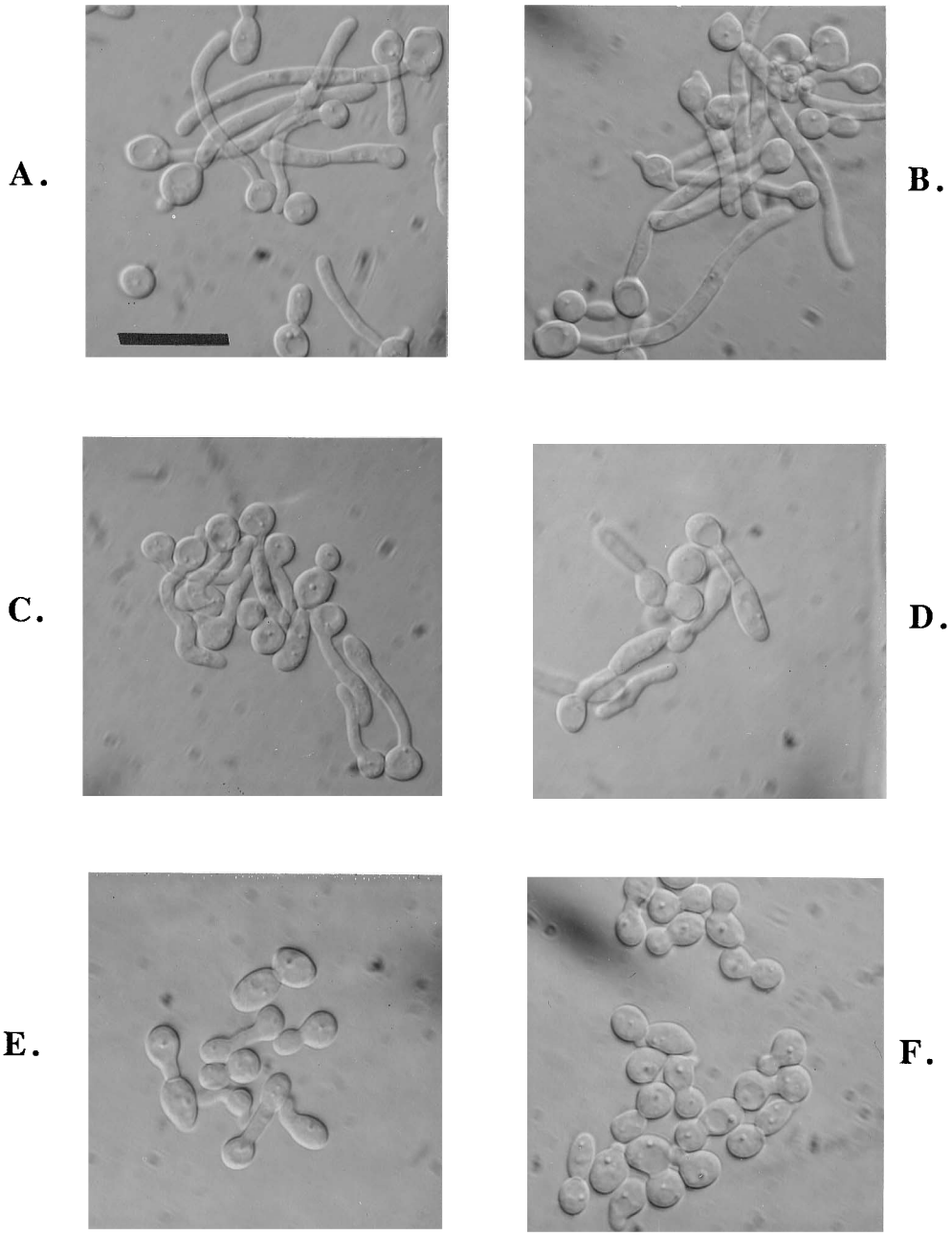

FIG. 6. Effect of pH on germ tube morphology. Stationary-phase yeast cells of the parental strain CAF3-1 or the Δ*phr1* mutant CAS8 were inoculated into buffered medium 199 as described in Materials and Methods. After 2 h of incubation at 37°C, the cultures were chilled to 4°C and photographed. (A) Representative sample of strain CAF3-1 incubated at pH 8.0. (B to F) Germ tube morphology of CAS8 incubated at pH 6.0, 6.5, 7.0, 7.5, and 8.0, respectively. The bar represents 10 μm.

parental strain exhibited a normal yeast morphology at all pH values tested (Fig. 8). In contrast, the *phr1* null mutant again exhibited morphological aberrations when grown at alkaline pH, becoming rounded and enlarged after extended growth at pH 7.5 or 8.0 (Fig. 8). Shorter periods of incubation resulted in more subtle morphological changes. Rather than being ellipsoidal in shape, which is typical of normal buds, the buds of the mutant strain were more wide than long and appeared flattened compared with buds of the parental strain (see Fig. 10 for an example). Despite the aberrant morphology, the cells continued to exhibit polarized budding. These morphological aberrations were not specific to the growth medium; similar effects were observed when the mutant was grown at alkaline pH in YPD, YNB, or the medium of Lee et al. (40). There was no evidence of osmotic instability in the mutant at the restrictive pH, and inclusion of 1 M sorbitol in the medium had no effect on morphology. Thus, *PHR1* was required for apical growth regardless of which morphological form the cells were stimulated to adopt, and this requirement was a function of pH alone.

To demonstrate that the morphological defect was a direct consequence of the mutation in *PHR1* and not due to extra-

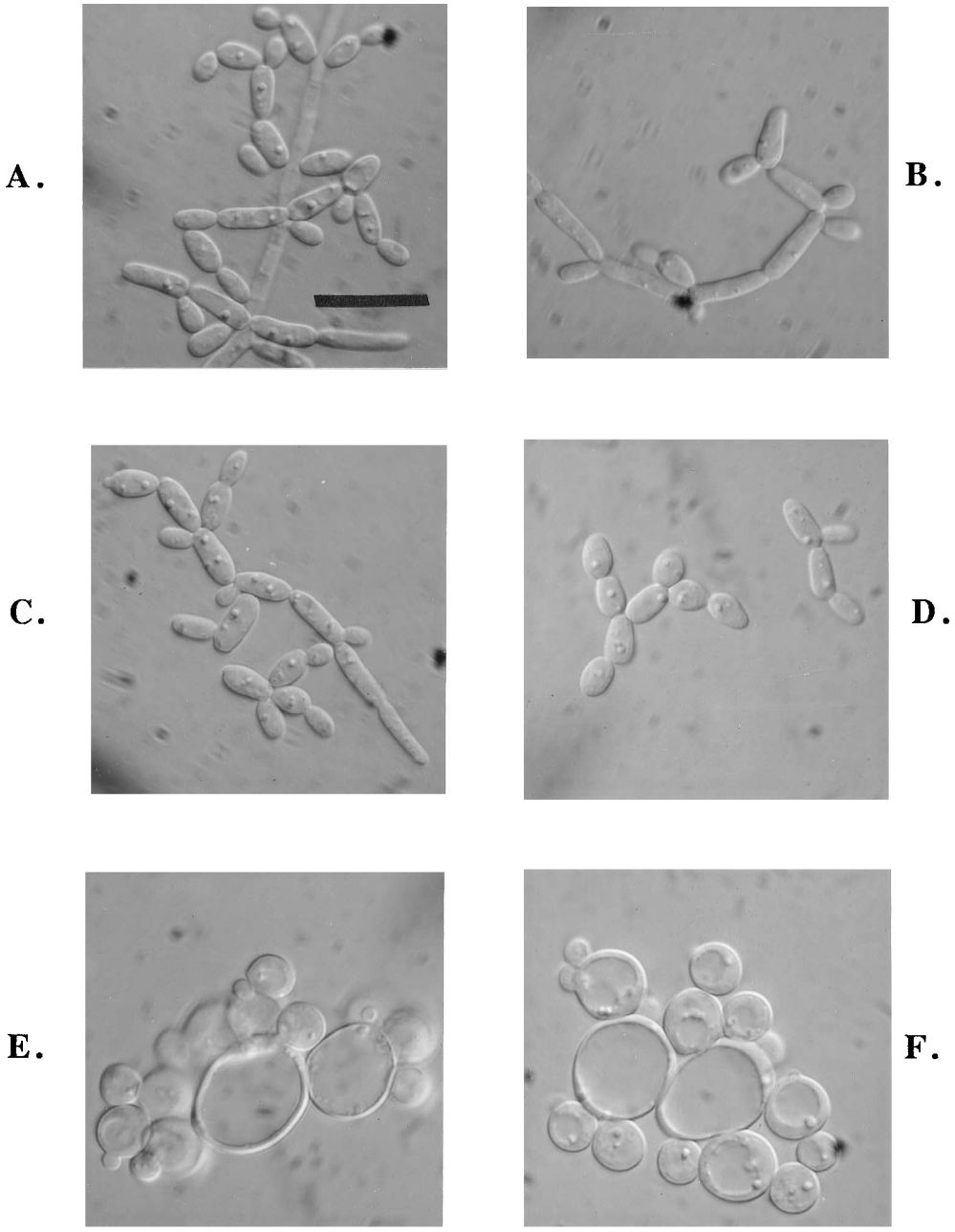

FIG. 7. Effect of pH on cell morphology after prolonged growth. Cultures of the parental strain CAF3-1 and the Δ*phr1* mutant CAS8 were prepared as described for Fig. 6 and incubated for 16 h. (A) Representative sample of strain CAF3-1 incubated at pH 8.0. (B to F) Morphology of CAS8 incubated at pH 6.0, 6.5, 7.0, 7.5, and 8.0, respectively. The bar represents 10 μm.

neous mutations, complementation of the Δ*phr1* allele by the wild-type allele was tested. Strain CAS11, containing a single copy of the wild-type allele, was generated by transformation of the null mutant CAS8 with plasmid pSMS-54 as described in Materials and Methods. CAS11 was morphologically normal under all growth conditions (data not shown). Complementation of the morphological defect was not due to the simultaneous introduction of *URA3* into CAS11, since introduction of *URA3* alone, as in strain CAS10, did not complement the morphological phenotype (data not shown). The results verify that the pH-dependent morphological defect is due to loss of *PHR1* and verify also the recessive nature of the mutation. While *PHR1* is necessary for apical growth, it is not sufficient to drive the process. Strain CAS12, which expressed *PHR1* constitutively from the EF1α promoter, exhibited normal yeast-like morphology when grown at acidic pH even though the level of expression from this promoter was at least 60% of that derived from the native promoter at pH 7.0 (data not shown).

Actin distribution. Extensive genetic evidence has been accumulated demonstrating that proper assembly and polarization of the actin cytoskeleton are essential for polarized fungal cell growth (6). Indirect evidence suggests a similar role in *C. albicans* (3, 67). The morphogenic defect associated with loss of *PHR1* may result from a failure to properly assemble the

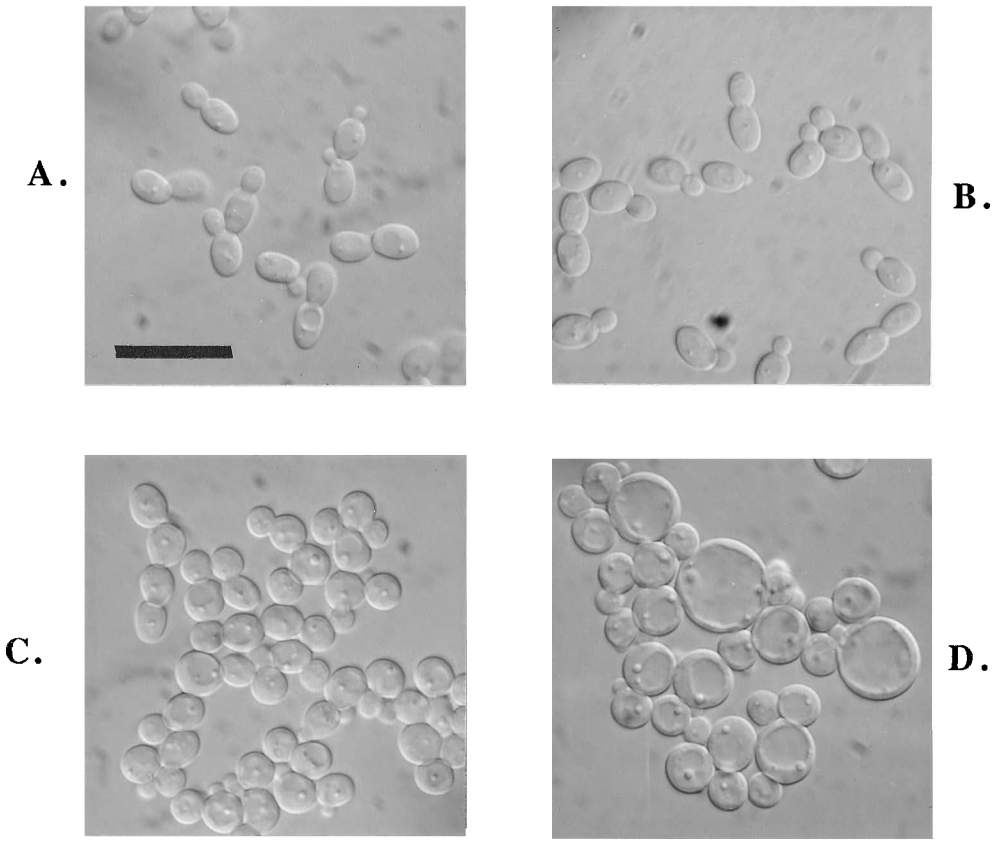

FIG. 8. Effect of pH on yeast morphology. Cultures of the parental strain CAF3-1 and the Δ*phr1* mutant CAS8 were prepared as described for Fig. 6 and incubated for 16 h at 25°C. (A) Sample of strain CAF3-1. (B to D) Morphology of CAS8 incubated at pH 7.0, 7.5, and 8.0, respectively. The bar represents 10 μm.

actin cytoskeleton, particularly since the progressive isotropic expansion seen with the mutant cells is similar to that of *S. cerevisiae* cells with defective actin or actin-binding proteins (6). Visualization of actin distribution within the cells by using rhodamine-conjugated phalloidin showed the expected polarized fluorescence pattern in the parental cells undergoing germ tube formation. Patches of fluorescence were concentrated in the germ tube apex, with faintly visible actin cables extending back into the mother cell (Fig. 9). At the restrictive pH, 8.0, the Δ*phr1* mutant exhibited a polarized distribution of actin, despite the failure to form germ tubes (Fig. 9). Similarly, when grown as a yeast for two generations at 25°C and pH 8.0, the mutant exhibited a polarized distribution of actin similar to that found in the control cells, even though an aberrant bud morphology was apparent (Fig. 10). However, after six to seven generations, the resulting enlarged, rounded cells exhibited a very faint, diffuse pattern of fluorescence with no evidence of actin polarization (Fig. 10). This pattern is characteristic of *S. cerevisiae* mutants with cytoskeletal defects (6). The actin staining patterns suggested that the initial defect in morphogenesis of the mutant is not a consequence of altered cytoskeletal assembly. However, cumulative effects of the mutation during subsequent cell growth eventually lead to disorganization of the cytoskeleton.

β-*N*-Acetylglucosaminidase secretion. Despite the appropriate organization of actin, the Δ*phr1* mutation could affect utilization of the cytoskeleton for polarized secretion or compromise secretion per se. Sullivan et al. (61) previously demonstrated the presence of an *N*-acetylglucosamine-inducible, extracellular β-*N*-acetylglucosaminidase in *C. albicans*. To determine if generalized secretion was affected by the Δ*phr1* mutation, we examined secretion of inducible β-*N*-acetylglucosaminidase at the restrictive pH. Parental and mutant cells were inoculated into medium 199 (pH 8.0) at 25°C with or without *N*-acetylglucosamine, and the amount of extracellular β-*N*-acetylglucosaminidase was measured during growth of the cultures. In the absence of inducer, the parental and mutant strains exhibited similar constant levels of extracellular β-*N*-acetylglucosaminidase (Fig. 11). In the presence of inducer, enzyme activity was induced severalfold (Fig. 11). The strains were indistinguishable in either the rate or the extent of induction. From these results, it appears that *PHR1* is not required for generalized secretion.

DISCUSSION

PHR1 was identified as a differentially expressed gene regulated in response to the pH of the culture medium. Sequence analysis of *PHR1* demonstrated that it was homologous to the *GGP1*/*GAS1* gene of *S. cerevisiae* (49, 64), which encodes one of the most abundant GPI-anchored proteins of the *S. cerevisiae* cell surface (18, 65). The protein is both N glycosylated and O glycosylated, which accounts, in part, for the difference between the native molecular mass, reported as 115 (65) or 125 (18) kDa, and the molecular mass of approximately 60 kDa predicted from the nucleotide sequence (49, 64). Conserved structural features of the Phr1 protein sequence suggest that it is similarly processed. The deduced protein contained a hydro-

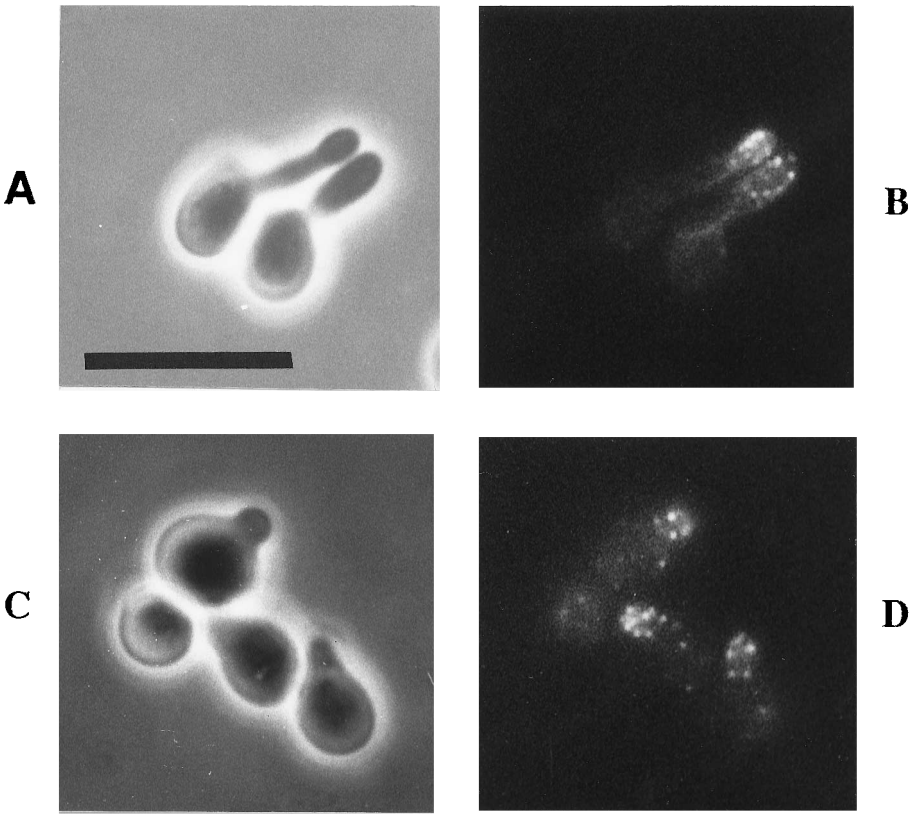

FIG. 9. Actin distribution during germ tube formation. Stationary-phase yeast cells of the parental strain CAF3-1 or the Δ*phr1* mutant CAS8 were inoculated into medium 199 buffered at pH 8.0 and incubated at 37°C. After 75 min, the cultures were harvested and stained with rhodamine-conjugated phalloidin as described in Materials and Methods. (A and B) Phase-contrast and fluorescence micrographs, respectively, of parental strain CAF3-1. (C and D) Analogous photomicrographs of the Δ*phr1* mutant CAS8. The bar represents 10 μm.

phobic signal sequence at the amino terminus, consistent with its presumptive secretion. The leader sequence was 20 amino acids in length with a predicted cleavage site after Ala-20 (66), only two amino acids shorter than the Ggp1/Gas1 signal sequence (49). N glycosylation of the Phr1 protein is predicted on the basis of the presence of several consensus sites which are positionally conserved in the *GGP1/GAS1* sequence (49, 64). *PHR1* also contains a serine-rich domain of 32 amino acids near the carboxy terminus which could provide sites for O glycosylation. Such domains are characteristic of many extracellular proteins that are O glycosylated (35).

Addition of a GPI anchor requires a C-terminal hydrophobic domain which is cleaved from the protein concomitant with GPI attachment. This requirement has been demonstrated both for mammalian cells (22, 25) and for the Ggp1/Gas1 protein of *S. cerevisiae* (49). GPI modification occurs at residue Asn-506 of the mature Ggp1/Gas1 protein, corresponding to Asn-528 of the primary amino acid sequence (49). While the Phr1 sequence contained the requisite hydrophobic C-terminal domain, the Asn-506 residue was not conserved. However, Ser-516 of the Phr1 sequence has the proper location and context to serve as an equally acceptable GPI attachment site. Serine residues are among several residues that provide good acceptor, or ω (19, 48), sites, and the adjacent Ser-517 Gly-518 would satisfy the requirement for residues with small side chains at the ω+1 and ω+2 positions (19, 28, 48). These features suggest that the Phr1 protein will prove to be modified by GPI addition, and this will likely be of functional significance. Mutations in *GGP1/GAS1* which prevent GPI addition result in a null phenotype (49).

The function of *PHR1* was investigated by construction of a homozygous null mutant. As expected from its pH-conditional expression, genetic inactivation of *PHR1* resulted in a pH-dependent phenotype. At the restrictive alkaline pH, the mutant was unable to form normal germ tubes or yeast buds. The morphology of the cells was suggestive of a defect in the ability of the cells to conduct or maintain apical growth. This defect was evident within the first generation of growth at the restrictive pH, and subsequent cell growth led to a secondary morphological aberration, with the cells becoming rounded, highly enlarged, and multibudded. Thus, *PHR1* has an essential role in morphogenesis, but its function is not form specific and is not involved in the dimorphic decision process.

The conditional nature of the mutant phenotype is interesting in that it implies that the cellular function performed by *PHR1* either is not required at acidic pH, occurs spontaneously, or is conducted by a functional homolog active at low pH. This last possibility is supported by preliminary studies with monospecific antisera which indicate the presence of a *PHR1* homolog (27). Assuming the presence of a *PHR1* homolog, then the progressive, pH-conditional phenotype of the *phr1* mutant might reflect the pH sensitivity of homolog expression or function and *PHR1* might normally complement this deficiency as the external pH rises. It is of interest that *PHR1* is required for normal morphogenesis at pH 7.5, a value

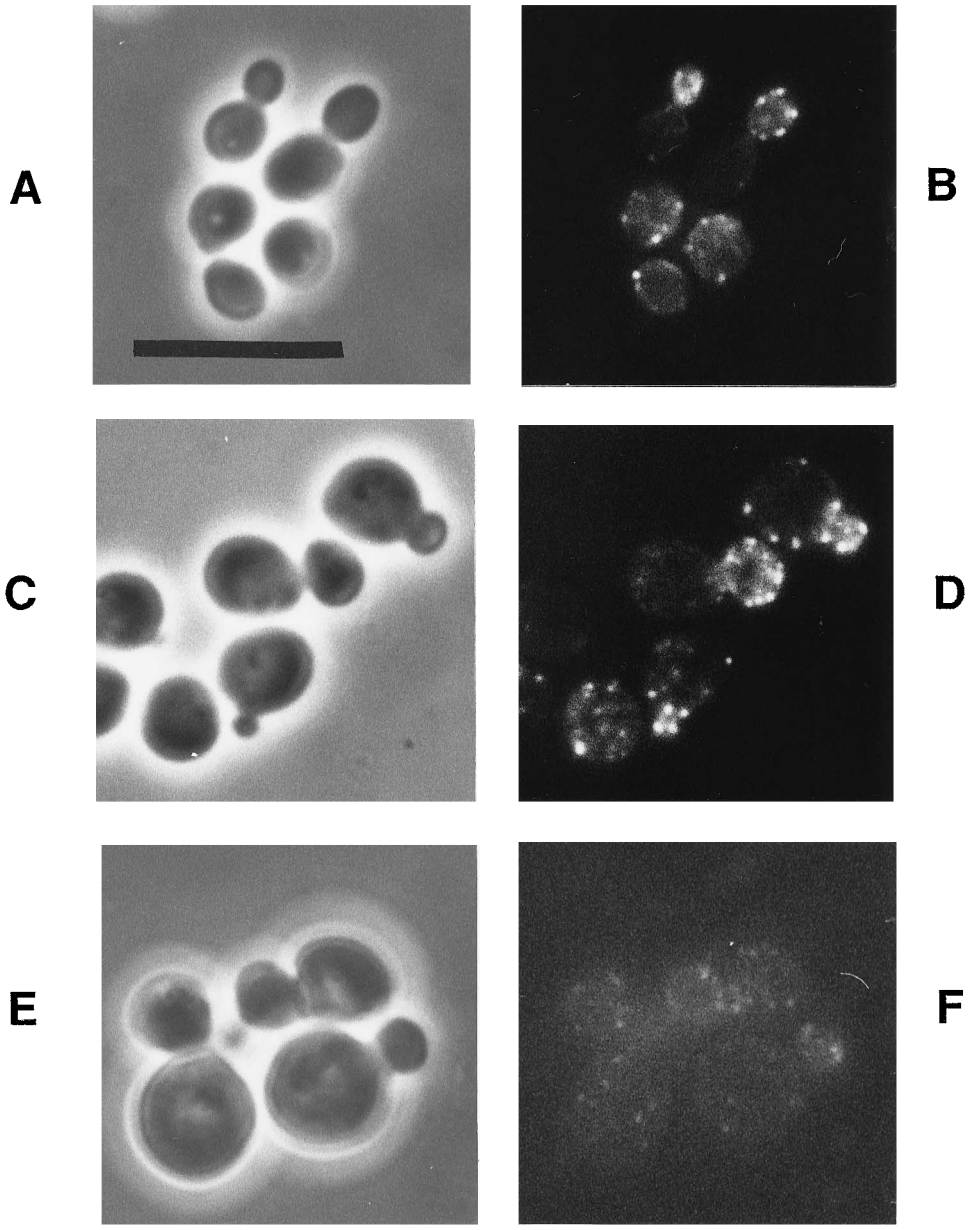

FIG. 10. Actin distribution in yeast cells. Stationary-phase yeast cells of the parental strain CAF3-1 or the Δ*phr1* mutant CAS8 were inoculated into medium 199 buffered at pH 8.0 and incubated at 25°C. Samples were collected after 6 h (A to D) or 16 h (E and F) and stained with rhodamine-conjugated phalloidin as described in Materials and Methods. (A and B) Phase-contrast and fluorescence micrographs, respectively, of parental strain CAF3-1. (C to E) Photomicrographs of the Δ*phr1* mutant CAS8. The bar represents 10 μm.

close to the physiological pH of the human host. This finding suggests that *PHR1* may be integral to the pathogenic ability of *C. albicans*.

Although the function of *GGP1*/*GAS1* is unknown, it too is involved in cellular morphogenesis, consonant with its structural similarity to *PHR1*. Deletion of *GGP1*/*GAS1* results in cells which are enlarged, rounded, and multibudded (52, 64). In addition, these cells exhibit delocalized chitin deposition (52), a phenotype associated with cytoskeletal mutations (6). However, cell enlargement and actin disorganization appear to be secondary consequences of mutations in *PHR1*, as evidenced by the temporal lag in their expression. The conditional nature of the *phr1* mutant phenotype makes this distinction possible, whereas the constitutive nature of *GGP1*/*GAS1* expression in *S. cerevisiae* may obscure the primary and secondary effects of *ggp1*/*gas1* mutations. Thus, it is not clear whether the primary defect associated with *ggp1*/*gas1* mutations is analogous to that of *phr1*. In this context, Nuoffer et al. (49) demonstrated altered expression of several proteins in *ggp1*/*gas1* mutants and pointed out that this observation raised the possibility that the phenotype of the null mutants may be an indirect consequence of alterations in proteins other that Ggp1/Gas1. Functional differences between *PHR1* and *GGP1*/*GAS1* are suggested by differences in their regulation

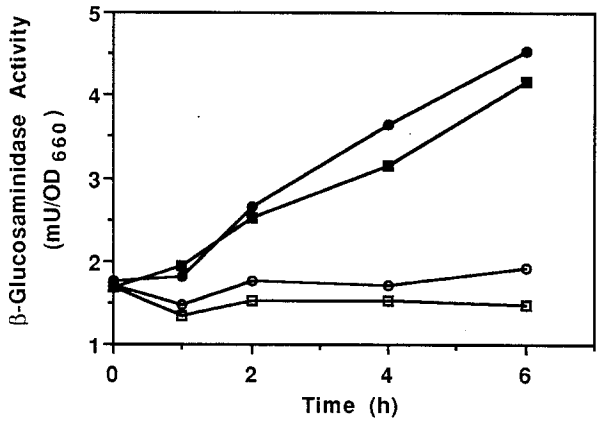

FIG. 11. Induction of extracellular β-*N*-acetylglucosaminidase. The parental strain CAF3-1 (squares) and the mutant strain CAS8 (circles) were inoculated into medium 199 (pH 8.0) at 25°C without inducer (open symbols) or supplemented with 25 mM *N*-acetylglucosamine (closed symbols). Culture samples were collected at the indicated times and assayed for β-*N*-acetylglucosaminidase activity as described in Materials and Methods. OD_{660}, optical density at 660 nm.

and by the apparent presence of a *PHR1* homolog in *C. albicans*, a *GGP1*/*GAS1* homolog being absent in *S. cerevisiae* (64).

The morphogenic defects associated with loss of *PHR1* suggest that *PHR1* serves a novel role in morphogenesis. The Δ*phr1* cells continued to bud normally, indicating that bud site selection and assembly were unaffected. Deletion of *PHR1* was of no immediate consequence to cytoskeletal organization or secretion per se, and therefore, *PHR1* does not appear to be involved in these processes. If the Phr1 protein is a GPI-anchored cell surface protein, then it likely functions either during secretion or after reaching the cell surface. It could have a highly specific role in targeting secretion to the apex of the cell. This suggestion stems from the observations that the Ggp1/Gas1 protein has a unique GPI anchor (24), GPI addition is essential to its function (49), and GPI serves as a localization signal for targeted secretion in mammalian cells (10, 41, 68). Alternatively, an extracellular location of the Phr1 protein is consistent with a role in cell wall biosynthesis or assembly which is also required for proper morphogenesis (5, 12, 45). It should also be noted that some GPI-linked proteins from heteromeric complexes with transmembrane proteins (20), and in this way, *PHR1* could function in transmembrane signalling or regulation of pH/ion gradients. The pH-dependent expression of *PHR1* is of interest in light of observations demonstrating the requirement for a cytosolic pH gradient during apical growth of algal cells (29). Assuming a similar requirement in *C. albicans*, *PHR1* may be required to establish or maintain such a gradient to facilitate apical cell growth.

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