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Reduced Virulence of *Candida albicans PHR1* Mutants

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***Candida albicans* mutants lacking *PHR1* exhibit a pH-dependent morphogenic defect which is expressed at pH 7.5, a pH comparable to that of mammalian blood (S. M. Saporito-Irwin, C. E. Birse, P. S. Sypherd, and W. A. Fonzi, Mol. Cell. Biol. 15:601–613, 1995). The in vivo relevance of this expression pattern was tested in a mouse model of systemic candidiasis. A *phr1*/*phr1* mutant was found to be less virulent than an isogenic Phr1$^+$ strain and exhibited altered morphological development in vivo. These results indicate that *PHR1* contributes to the virulence of *C. albicans*.**

While a great deal has been learned about the molecular basis of bacterial pathogenesis, a comparable understanding of fungal virulence determinants is lacking, in part, because of inherent difficulties in genetic manipulation of these organisms. The development of genetic transformation methods and well-defined auxotrophic strains of *Candida albicans* has provided the potential for defining pathogenic determinants of this organism via reverse genetics (3, 7). Using this approach, Kirsch and Whitney (6) demonstrated the importance of adenine, uracil, and heme biosynthesis to the virulence of *C. albicans*. Although indirect evidence has implicated adherence, protease secretion, extracellular phospholipases (5), and hypha formation in the virulence of *C. albicans* (2), the genetic determinants of these attributes have not been defined.

We recently identified a *C. albicans* gene, *PHR1*, that is regulated in response to the pH of the culture medium and functions in morphogenesis (11). The deletion of *PHR1* resulted in a mutant strain exhibiting a pH-dependent morphological defect. At an alkaline pH, which results in high levels of *PHR1* expression, the mutant strain was unable to conduct apical growth of either yeast or hyphal forms. As the phenotype was expressed at pH 7.5, a pH comparable to the physiological pH of mammalian blood, we hypothesized that this gene is expressed in vivo during systemic candidiasis and is required for pathogenesis.

As a first test of this hypothesis, the survival of BALB/c mice following intravascular injection with a Phr1$^-$ or isogenic Phr1$^+$ strain was examined. Strains CAS10 (Δ*phr1*/Δ*phr1*) and CAS11 (*PHR1*/Δ*phr1*) (11) were grown to stationary phase at 30°C in Sabouraud dextrose broth (Difco Laboratories) adjusted to pH 4.5. Following a second passage in this medium, the blastoconidia were washed twice in calcium- and magnesium-free phosphate-buffered saline (Mediatech) and suspended to a density of 3×10^6 cells per ml on the basis of hemocytometer counts. Control plating experiments demonstrated equivalent viabilities for these two strains. Ten BALB/c mice (18 to 20 g each; Harlan-Sprague-Dawley, Inc., San Diego, Calif.) were each injected in the lateral tail with 0.5 ml of cell suspension of either CAS10 or CAS11. Within 48 h of infection, all 10 of the CAS11 (Phr1$^+$)-injected mice exhibited the ruffled fur and morbidity characteristic of candidal infection, and severe ocular candidiasis was also evident. In contrast, CAS10 (Phr1$^-$)-infected mice appeared to be normal. The survival of CAS11-infected mice was short, with several succumbing by day 3 and all of them succumbing by day 4 (Fig. 1). After 5 days, all of the CAS10-infected mice were alive and free of pathological symptoms, demonstrating a striking difference in the virulence of these strains. Since strain CAS11 was derived from Phr1$^-$ strain CAS10 by reintroduction of a wild-type *PHR1* allele (11), the altered pathogenic properties of CAS10 can be attributed to its lack of *PHR1*.

Similar to results obtained with chitin synthase mutants of *C. albicans* (1), the reduced virulence associated with the loss of *PHR1* was not the result of rapid clearance of host tissue. Fifteen mice were infected as in the previous experiment with either CAS10 or CAS11, and five mice in each group were sacrificed by cervical dislocation after 24, 48, or 72 h. One mouse in the CAS11 group died within 72 h; thus, the 72-h sample of this group consists of only four mice. The brain, liver, and kidney of each mouse were aseptically removed, weighed, and homogenized in 5 ml of saline. Dilutions of the homogenate were spread on Sabouraud dextrose agar (Difco Laboratories), and the numbers of CFU were determined after 24 h of incubation at 30°C. As seen in Table 1, a notable number of CAS10 cells were present in the brain, liver, and kidney tissues of infected animals. However, the fungal burden in these animals differed from that in the Phr1$^+$ controls in several respects. In all samples except the 24-h liver samples, the burden due to CAS10 infection was at least 10-fold lower than that due to CAS11, and in all samples, the number of CAS10-colonizing cells was significantly lower ($P \leq 0.05$; Kruskal-Wallis analysis of variance for censored data). Although parallel changes occurred in the brain tissues of both groups, with a slight increase between 48 and 72 h, a divergent pattern was observed in liver and kidney samples. In these tissues, the level of CAS10 remained unchanged over the 72-h time course while the Phr1$^+$ control group exhibited a greater than 10-fold increase in the kidney fungal burden and a lesser, but statistically significant ($P \leq 0.05$), increase in the liver

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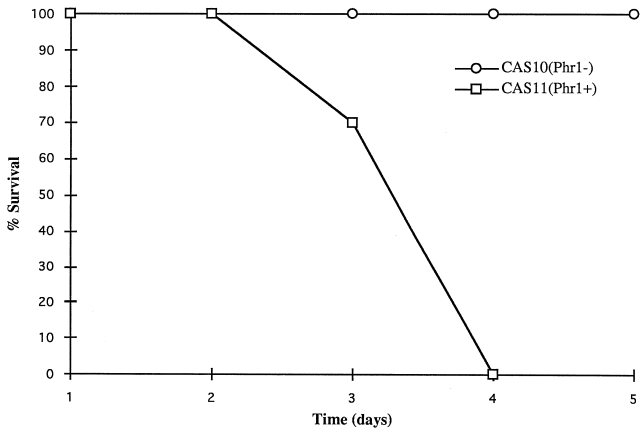

FIG. 1. Survival of mice following intravenous challenge with 1.5×10^6 blastoconidia of a $Phr1^+$ strain, CAS11, or a $Phr1^-$ strain, CAS10.

fungal burden. The latter is interesting in that previous studies have reported the clearance of *C. albicans* from the livers of immunocompetent mice (4, 8). In keeping with previous studies of candidal infection, both strains colonized the kidney more heavily than they did the brain or liver (4, 10).

Since *PHR1* is required for proper morphogenesis in vitro, histological examinations of kidney tissues were conducted to determine if a similar requirement was expressed in vivo. Mice were infected as in the preceding experiments and sacrificed by cervical dislocation 48 h postinfection. Kidneys were aseptically removed, fixed in 10% formalin, dehydrated, embedded in paraffin, and sectioned in 4-μm slices. As seen in Fig. 2, silver staining of tissues from CAS11-infected animals revealed extensive hyphal and pseudohyphal development and invasion of renal tubules and glomerules. In contrast, sections from animals infected with $Phr1^-$ strain CAS10 contained relatively few organisms, and these exhibited yeast-like, perhaps slightly aberrant, morphology. No hyphal forms were found. Thus, there was a clear distinction in the morphological development of these two strains.

The results of these experiments implicate *PHR1* in the pathogenesis of *C. albicans* and additionally illustrate the feasibility and utility of using reverse genetic techniques in dissecting the pathogenic properties of this fungus. These data also indirectly suggest that *PHR1* is expressed in vivo. Although the morphology of CAS10 cells in host tissue was not as aberrant as that observed in vitro (11), this may simply indicate that the local pH at the infection site is not sufficiently alkaline to result in a severe morphological phenotype. A variation of half a pH unit can have a strong effect on the morphology of a *phr1* mutant (11). This assumes that the expression of *PHR1* in the host is controlled in response to pH; though this assumption is likely, we cannot exclude the possibility that alternate signals endogenous to the host environment modulate its expression. This may be a significant issue in relation to the virulence mechanisms of fungal pathogens. The expression of many bacterial virulence determinants is regulated in response to environmental signals intrinsic to the host niche, including pH (9). The results reported in this study may indicate that fungal pathogens have adopted a similar approach in regulating virulence factor expression and may suggest a more directed approach in identifying such factors on the basis of their regulation (9).

TABLE 1. Candida census in infected tissues

Time post-infection (h)	Strain	Tissue census (log [CFU/g] ± SD)[a]		
		Brain	Liver	Kidney
24	CAS10	4.83 ± 0.15	4.48 ± 0.11	5.50 ± 0.34
	CAS11	5.99 ± 0.16	4.82 ± 0.26	6.88 ± 0.19
48	CAS10	4.83 ± 0.29	4.43 ± 0.21	5.75 ± 0.17
	CAS11	5.88 ± 0.25	5.35 ± 0.29	7.85 ± 0.35
72	CAS10	5.16 ± 0.17	4.79 ± 0.12	5.53 ± 0.26
	CAS11	6.64 ± 0.23	5.78 ± 0.22	7.64 ± 0.18

[a] For each time point and tissue sample, the difference between the CAS10 and CAS11 values was statistically significant ($P \leq 0.05$; Kruskal-Wallis analysis of variance for censored data).

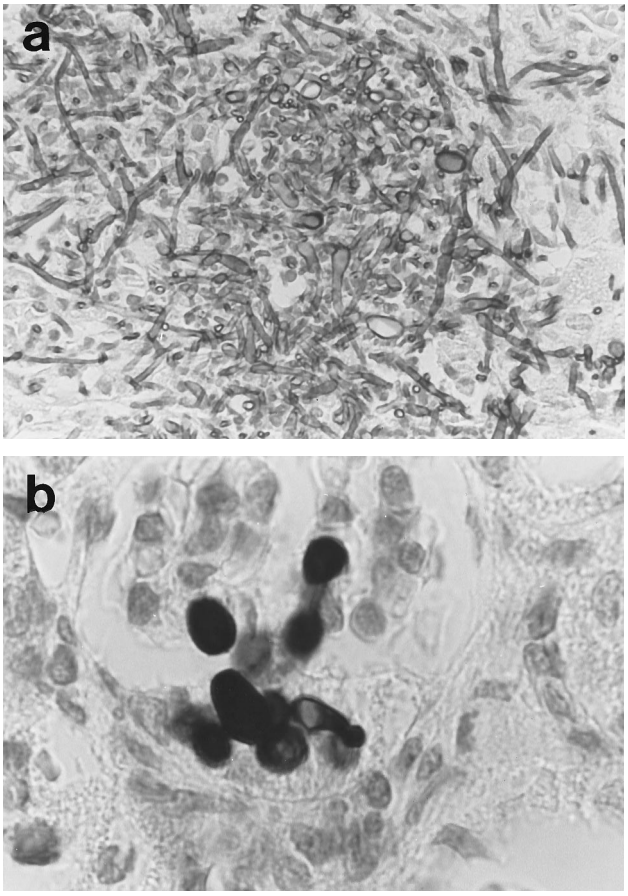

FIG. 2. Silver-stained sections of kidney tissues 48 h postinfection with strain CAS11 (a) or strain CAS10 (b). Magnification, ×200 (A) and ×500 (B).

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