Essential Role for Vacuolar Acidification in Candida albicans Virulence*

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Background: V-ATPase is responsible for establishing electrochemical gradients through energy-dependent proton transport. Results: In Candida albicans, mutation of vph1 resulted in defective phenotypic switching and avirulence in a murine septicemia model. Conclusion: The vacuolar specific isoform Vph1 is essential for vacuolar acidification and virulence in Candida albicans. Significance: Acidification of the vacuole plays a unique role in fungal virulence and growth phenotype.

Fungal infections are on the rise, with mortality above 30% in patients with septic Candida infections. Mutants lacking V-ATPase activity are avirulent and fail to acidify endomembrane compartments, exhibiting pleiotropic defects in secretory, endosomal, and vacuolar pathways. However, the individual contribution of organellar acidification to virulence and its associated traits is not known. To dissect their separate roles in contribution of organellar acidification to virulence and its endosomal, and vacuolar pathways. However, the individual compartments, exhibiting pleiotropic defects in secretory, ATPase activity are avirulent and fail to acidify endomembrane

Infectious microbes have evolved a surprising array of strategies to identify and infect host cells for optimal pathogen survival and propagation. Critical to the process of microbial pathogenesis is the manipulation and response to pH. For example, Salmonella typhimurium evades host defense mechanisms by synchronizing the construction of the type III secretion system with macrophage lysosomal acidification (1). The facultative intracellular fungal pathogen Histoplasma capsulatum survives and replicates within macrophages by inhibiting phagolysosomal fusion and regulating phagosomal pH to acquire nutrients, including iron (2). In pathogenic yeasts, such as Candida albicans, pH has been implicated in proliferation, dimorphic switching between budding and hyphal forms, and virulence (3). C. albicans is also a successful commensal, surviving in host niches with ambient pH ranging from highly acidic (pH <2) to alkaline (pH >10) (4, 5).

Central to each of these processes is the V-ATPase, a multi-subunit, evolutionarily conserved proton pump that maintains pH gradients across the endomembranes of the secretory, vacuolar, and endocytic pathways in the fungal cell. The role of the V-ATPase in fungal physiology is far-reaching, impacting diverse cellular pathways ranging from protein processing and degradation, endocytic trafficking, pH-driven exocytosis, to the transport and sequestration of metabolites, ions, and toxic drugs (6). Impairment of V-ATPase activity can cripple many processes important for infection: secretion of virulence factors, filamentation, and host tissue invasion, biofilm formation, countering host immunity, and tolerance to antifungal drugs (3, 7). Not surprisingly, C. albicans vma7 mutants, with complete loss of V-ATPase activity, are avirulent (8).

The pleiotropic effects of vma mutants lacking all V-ATPase activity, do not offer a means to dissect the individual contribution of organellar acidification to virulence and associated traits. In the budding yeast Saccharomyces cerevisiae, only the V-ATPase subunit a is expressed as two organelle-specific isoforms: Stv1p and Vph1p, that localize to the Golgi and secretory pathway, or to the vacuolar membranes, respectively. Extensive studies in this non-pathogenic model yeast have clarified the individual roles of these distinct secretory and vacuolar V-ATPase isoforms in the development of pH gradients across endomembrane compartments. The ~100 kDa a subunit is an integral part of the membrane embedded V0 domain where it

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forms two hemichannels constituting the proton conduction pathway and provides the essential positively charged counterion to facilitate dissociation of bound protons. Stv1p containing V-ATPase complexes differ from Vph1p containing enzymes in having a lower coupling efficiency of the ATP hydrolytic activity with proton transport, and lower assembly efficiency with the cytoplasmic V1 ATPase domain (9). Consequently, in vph1-null mutants, Stv1p-containing V-ATPase is unable to efficiently acidify the vacuolar compartment (10, 11). In contrast, Vph1p-containing V-ATPase complexes appear to compensate for loss of the secretory isoform in stv1-null mutants, presumably in transit to the vacuolar membrane (12).

The unique role of the Vph1p isoform offers an approach to specifically evaluate the role of vacuolar acidification in the development of virulence-associated traits in C. albicans. S. cerevisiae vph1-null mutants display only a partial vma phenotype, with no loss of function reported in a number of cellular pathways where Vph1p has a redundant role with Stv1p (12). This functional redundancy offers the potential to mask the contribution of these pathways to virulence in C. albicans. With these goals in mind, we identified the two subunit α orthologs in C. albicans and confirmed their subcellular localization to the secretory and vacuolar compartments. We describe distinct phenotypes of C. albicans stv1 and vph1 null mutants and establish an essential role for vacuolar acidification in virulence. These novel findings extend and clarify the in vitro findings of Raines et al. (13) who recently demonstrated overlapping functions of Stv1p and Vph1p in C. albicans. Given the urgent need to expand the arsenal of antifungal drugs against the growing threat of candidiasis and other fungal infections, our findings validate the importance of the V-ATPase, and specifically, vacuolar acidification as a drug target (7, 14).

EXPERIMENTAL PROCEDURES

Animal Studies—All animal work was conducted at The Johns Hopkins University according to guidelines established by the private Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The Johns Hopkins University’s Animal Care and Use Committee approved all animal procedures conducted under the protocol entitled, “Isolation of Virulence Genes in Candida” (protocol number MO10M234; approval renewed on 11/20/2012).

Yeast Strains and Plasmids—SN152 (arg4Δ/arg4Δ, leu2Δ/leu2Δ, his1Δ/his1Δ, URA3/ura3Δ) was generously donated by Susan Noble (UCSF) (23). This background was used to create homozygous null mutants in STV1 and VPH1, as well as STV1-GFP and VPH1-GFP fusion strains. An isogenic pair of wild type and homozygous vma7-null mutant was generously donated by Raimund Eck (Hans Knöll Institute, Germany) (8). pGEM-HIS plasmids containing C. albicans STV1 and VPH1 were kindly donated by Karlett Parra (University of New Mexico) (13).

Generation of GFP-tagged Yeast Strains—STV1-GFP and VPH1-GFP strains were created by transforming SN152 with their respective fusion cassettes. First, a synthetic GFP gene optimized for expression in C. albicans was inserted into pJK1027 downstream of the ClonNat cassette to generate plasmid pZR15.5. pJK1027 is an integration vector carrying nourseothricin resistance and regions homologous to the actin promoter. pJK1027 was constructed by excising a cassette containing the CaNAT1 gene flanked by the Ashbya gossypii TEF1 transcriptional promoter and terminator from pJK795 (31) with EcoRV and KpnI. The fragment was blunted with Klenow and ligated into pÅU34 (32), which had been digested with NdeI and treated with Klenow. Next, the GFP-ClonNat fusion fragment was amplified from pZR15.5 with a 45 bp linker sequence attached upstream of the GFP sequence. Then the STV1 open reading frame (ORF)3 (stop codon removed) plus ~500 bp upstream (5′-UTR-STV1) and ~500 bp immediately downstream of the ORF (3′-UTR) were amplified separately. Next the three PCR amplicons (5′-UTR-STV1, linker-GFP-ClonNat, and 3′-UTR) were fused together by a one-step PCR. Finally this fusion PCR cassette was transformed to SN152 to replace one copy of the wild type STV1 gene and generate the STV1-GFP strain YZR226. The VPH1-GFP strain was generated similarly.

Generation of Null Mutants—STV1 and VPH1 null strains were generated as described previously (33) and confirmed by PCR amplification. Briefly, gene disruption cassettes containing either Candida dubliniensis HIS1 or Candida maltosa LEU2 flanked by ~350 upstream and downstream of the STV1 and VPH1 genes were constructed by fusion PCR. Homozygous deletion strains were constructed by transformation of SN152 with a HIS1-marked gene disruption cassette; His+ transformants were screened by colony PCR for the presence of expected 5′ and 3′ junctions of the integrated DNA. Homozygous gene disruption strains were constructed by transformation of the heterozygous knockout strain with a LEU12-marked gene disruption cassette; His+ Leu+ transformants were screened for expected 5′ and 3′ junctions of the second disrupted allele, absence of the original target ORF, and the presence of CdhHis1 and CmLEU12 ORF. The following null strains were generated: YZR218 (arg4Δ/arg4Δ, leu2Δ/leu2Δ, his1Δ/his1Δ, URA3/ura3Δ, stv1Δ::Leu2/stv1Δ::His1) and YZR219 (arg4Δ/arg4Δ, leu2Δ/leu2Δ, his1Δ/his1Δ, URA3/ura3Δ, vph1Δ::Leu2/vph1Δ::His1).

Generation of Reintegrants—VPH1 ORF and flanking regions were cloned into pJK1027, an integration vector carrying nourseothricin resistance and regions homologous to the actin promoter. The plasmids were linearized by restriction digestion with BsrI and subsequently transformed into the appropriate knock-out strains. Transformants were selected on YPD supplemented with nourseothricin (100 μg/ml). Confirmation of reintegration was by PCR amplification and by showing a reversal of zinc hypersensitivity.

Yeast Growth—C. albicans strains were maintained on YPD plates unless specified otherwise. Sensitivity to Zn2+ was monitored in synthetic complete (SC) or YPD medium supplemented with ZnCl2 to 0.5 mM. Sensitivity to acidic pH was conducted in SC medium buffered with 50 mM sodium citrate and adjusted to pH 2.5. Sensitivity to alkaline pH was conducted in SC medium, which was buffered with 50 mM MOPS and adjusted to pH 8. Respiration deficiency was assessed in YPEG

3 The abbreviations used are: ORF, open reading frame; UTR, untranslated region; LDH, lactate dehydrogenase.
which contained 1% Bacto-yeast extract, 2% Bacto-peptone, 3% glycerol, and 2% ethanol. Calcium homeostasis deficiency was assessed in SC medium at pH 6 supplemented with FK506 to 2 μg/ml. Sensitivity to calcofluor white was assessed on YPD supplemented with calcofluor white to 20 μg/ml.

pH Measurements—Vacular pH was measured with BCECF-AM (Molecular Probes, Eugene, OR), a pH-sensitive fluorophore that accumulates in the yeast vacuole (27, 34). Yeast strains were grown to mid-logarithmic phase in YPD medium. Cells were collected by centrifugation and incubated in SC containing 50 μM BCECF-AM for 25 min then washed twice before being resuspended in SC to OD 2 and transferred to a 96-well plate. Fluorescence emission at 485 nm and 450 nm in a Fluostar Optima plate reader. All measurements were taken from samples in triplicate. Vacular pH was calculated using a calibration curve covering pH from 4 to 8.5 as described previously (34).

Hyphal Development—Late exponential-phase cultures grown at 30 °C were diluted 10-fold into fresh YPD supplemented with 10% (w/v) FCS at 37 °C as described previously (8) to induce hyphal growth in liquid medium. Hyphal growth in solid media was induced on Spider plates as described previously (8).

Cell Adhesion and Damage—C. albicans were grown for 6 h from a starter culture, sonicated in a water bath to disperse clumps, and 5000 cells were applied to the monolayer of Caco-2 cells grown in a 35-mm dish for 10 days. After 30 min, culture supernatant was collected, diluted, and plated to YPD to estimate the number of non-adherent yeast cells. The Caco-2 monolayer was washed briefly five times with PBS and then cells were scraped in 1 ml PBS and the lysate was plated on YPD to calculate the number of adherent yeast cells. Data represented shows the number of adherent cells compared with the total number of viable cells used. Data shown are an average of three experiments with error bars displaying the standard error of the mean. p values were calculated using the Student’s t test.

J774A.1 macrophages were grown as confluent monolayer in high glucose DMEM without sodium pyruvate supplemented with 10% FBS in 24-well plates. They were infected with the indicated C. albicans strains at multiplicity of infection of 5.

The following morning, cellular medium was aspirated and fresh medium was applied to the monolayer. After 4 h, medium was collected, and lactate dehydrogenase activity was measured as per manufacturer’s protocol (Promega CytoTox 96 Nonradioactive Cytotoxicity Assay).

Systemic Infection—Virulence assays were performed as modified from Noble and Johnson (23). In short, C. albicans were grown overnight at 30 °C and then used to inoculate at second culture (by a 1:30 dilution). After 5 h, optical densities were determined. Cells were pelleted at 2000 rpm for 5 min and then washed once with normal saline and serially diluted to achieve 8 × 10⁸ cells/ml for tail vein injections. 0.1 ml was injected into each of Balb/c female mice that were 8–10 weeks old. We injected 10 mice per strain and monitored death over 25 days. Mice that were moribund, showing weight loss, hunched posture, failure to groom, and/or motor deficits, were euthanized. Survival curves were analyzed using the log rank test.

RESULTS

Localization of Vₜ Subunit α Isoforms to Vesicular (Stv1p) and Vacular (Vph1p) Compartments in C. albicans—Two C. albicans ORFs have been annotated as V-ATPase subunit a STV1 and VPH1 (www.candidagenome.org). These two paralogs have 48.2% amino acid identity with one another. The putative CaVph1p (orf19.6863) and CaStv1p (orf19.1190) proteins share identities of 74.9 and 73.5% to their respective Saccharomyces orthologs. Neither ORF contains the N-terminal WKY motif that has been identified as targeting sequence for secretory pathway localization in Saccharomyces ortholog Stv1p (15), although putative CaStv1p does contain a WKY motif within a loop region. FXFXD motifs at positions 221 and 755 were previously described as the putative localization sequences for Golgi retention of ScStv1p (12). Putative CaStv1p also contains a FXFXD motif (822–826, FNFGD) whereas CaVph1p does not. To confirm these in silico predictions, we tagged each ORF with C-terminal GFP at the chromosomal locus in wild type C. albicans strain SN152. Consistent with bioinformatics data, live fluorescence imaging of transformed yeast showed punctate labeling for CaStv1-GFP (Fig. 1B), reminiscent of secretory

FIGURE 1. CaStv1p and CaVph1p localize to the secretory pathway and the vacuole, respectively. A, diagram of budding yeast showing expected localization of Vₜ α isoforms to the vacuole (Vph1p) and the Golgi and post-Golgi compartments (Stv1p), based on distribution of S. cerevisiae orthologs. Each C. albicans candidate ORF was cloned and C-terminal GFP fusion constructs were expressed in WT cells. B, C. albicans ORF19.1190-GFP showed a punctate distribution consistent with secretory pathway morphology in yeasts. C, GFP-tagged ORF19.6863 localized to the vacuole as marked by the vacuolar dye FM4–64.
pathway staining of Golgi, endosomes, and prevacuolar compartments in *S. cerevisiae*. CaVph1-GFP localized to the vacuolar membrane marked by the vacuolar dye FM4–64 in live cells (Fig. 1C), similar to *S. cerevisiae* Vph1-GFP (12).

Redundant Roles for Stv1p and Vph1p Revealed by Lack of Some vma Phenotypes—As expected for its diverse role in pH and ion homeostasis, loss of V-ATPase function is accompanied by pleiotropic defects that include the growth sensitivity to acid or alkaline stress, calcium signaling, cell wall integrity, and cell respiration. To assess the functional redundancy of a subunit isoforms Stv1p and Vph1p to these processes, we generated homozygous null mutant strains *stv1*Δ/

*stv1*Δ and *vph1*Δ/

*vph1*Δ isogenic to *C. albicans* SN152. Vma7p is an essential subunit of the V-ATPase that drives the rotary catalytic mechanism (16). Therefore, we used the homozygous null mutant *vma7*Δ/

*vma7*Δ along with its isogenic control, to elicit the *vma* phenotype (8). While all strains grew well in YPD medium buffered to pH 4.0, the homozygous *vma7*Δ mutant showed characteristic growth sensitivity at extremes of acid (pH 2.5) and alkaline (pH 8.0) media as has been reported for *S. cerevisiae* *vma* mutants. Mutants lacking Stv1p or Vph1p, however, showed normal or near normal growth (Fig. 2A). Calcium homeostasis defects in *vma* mutants are exacerbated by loss of calcineurin signaling, resulting in synthetic lethal phenotype (17, 18). Addition of the calcineurin inhibitor, FK506, under conditions of mild alkaline stress (pH 6.0) resulted in nearly complete inhibition of growth in the *vma7*Δ mutant, whereas neither *stv1*Δ nor *vph1*Δ mutants were affected (Fig. 2B). Furthermore, *vma* mutants show signs of oxidative stress and defects in cellular respiration (12, 19). We show that *C. albicans* *vma7*Δ mutant is unable to grow on glycerol as a non-fermentable carbon source (YPEG, Fig. 2C). However, deletion of neither of the two *vo* subunit isoforms revealed any growth sensitivity on this medium (Fig. 2C). Similarly, slow growth of *vma7*Δ mutant on YPD plates was further impaired by calcofluor white, an agent previously shown to cause cell wall stress, but this was not observed in *vph1*Δ- or *stv1*Δ-null mutants (Fig. 2D). We conclude that the two isoforms of subunit *a* play redundant roles in a wide range of functions attributed to the V-ATPase.

**VPH1** Is Required for Virulence in Candida albicans—The acidic pH established by the H+ pumping activity of the V-ATPase is central to the function of the vacuole or lysosome in eukaryotic cells. We used the ratiometric pH indicator BCECF to measure the pH of the yeast vacuole. The acetoxymethyl form of BCECF is taken into the cell and de-esterified in the yeast vacuole, where it becomes trapped (Fig. 3A, inset) allowing quantitative estimation of pHv in live yeast. As expected, vacular pH in wild type yeast was acidic, ranging
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Hyphal formation can also be visualized at the peripheral margins of yeast colonies growing on solid medium. In solid Spider medium, we observed significant differences in hyphal outgrowth between vph1 and wild type, but not for stv1 mutant (Fig. 5A, top panel). These differences are evident at 25× magnification of the colony periphery (Fig. 5A, bottom panel) and suggest that Vph1p is important for hyphal formation on solid media whereas Stv1p is largely dispensable. To confirm this, we also examined hyphal formation on solid YPD medium supplemented with serum. Again, the vph1 mutant was defective in filamentation seen at the periphery of colonies, like the vma7-null strain (Fig. 5B, top and bottom panels). Taken together, our observations point to a role for Vph1p and vacuolar acidification in hyphal formation.

Host Cell Interaction Differs among V-ATPase Mutants—The intestinal mucosal barrier plays a critical role in determinig virulence of C. albicans (21). To be retained on the intestinal surface, yeast cells must adhere to epithelial membranes. Therefore, we tested whether V-ATPase mutants were defective in adhesion to a polarized monolayer of Caco-2 epithelial cells. Late log phase C. albicans were applied to a polarized, differentiated epithelial monolayer and allowed to attach for 30 min. Subsequently, yeasts remaining in the supernatant were collected and plated to determine if any loss in viability occurred during incubation at pH 7.4. After five washes, Caco-2 were scraped and resuspended in PBS for plating to YPD. Like vma7, the stv1 mutant had a modest although statistically significant reduction in adhesion, suggesting a potential role for V-ATPase in secretion of adhesion factors (Fig. 6A). Adhesion of vph1 mutants was not reduced to statistically significant levels. However, adherence does not appear to be a defining feature in the relative virulence of the strains tested. Thus, host cell damage in stv1 mutants was indistinguishable from wild type, as monitored by release of lactate dehydrogenase from Caco-2 monolayers exposed to yeast cells for 16 h. In comparison, loss of Vph1p did reduce cellular damage to Caco-2 cells, although this was not as drastic as seen with vma7 mutant strain, which has a complete loss of V-ATPase activity (Fig. 6B).

In systemic infection by Candida, professional phagocytes, including macrophages, constitute the first line of host defense. Fungal cells are internalized into the phagosome, which matures into an acidic phagolysosome where hydrolytic enzymes like cathepsin B are activated. In response, environmental cues such as CO2 concentration and pH trigger filamentation by C. albicans, followed by piercing of the macrophage membrane and escape. Here, we evaluate the interaction of V-ATPase mutants with cultured murine macrophage–like cells, J774A.1 (22). Following an overnight incubation at a multiplicity of infection of 5, wild type strains SN152 and CNC44 were able to filament robustly (Fig. 6C), and kill macrophage cells as evidenced by release of LDH (Fig. 6D). In contrast, vma7 mutants failed to form filaments and escape macrophages, and were unable to elicit LDH release above that of control, uninfected macrophages. We observed no requirement for Stv1p in filamentation and no deficit in the ability to cause host cell damage. However, both filament formation and LDH release from macrophages were significantly attenuated in vph1 mutants (Fig. 6, C and D).

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from pH 5.12 ± 0.088 in SN152 and 5.54 ± 0.053 for CNC44. Loss of Vma7p resulted in significant vacuolar alkalization by 1.43 pH units. Similarly, pH, was increased by 1.44 pH units in the vph1 null mutant but did not alter significantly in stv1 mutant (pH5, 5.5). This confirms extensive studies in S. cerevisiae showing isofrom specificity of subunit a in vacuolar acidification. We conclude that, as in S. cerevisiae, C. albicans Vph1p is required for robust vacuolar acidification, and that Stv1p is not capable of compensating for loss of Vph1p.

The acidic vacuole is the major site of ion and metabolite sequestration, driven by the H+ gradient established by V-ATPase. Thus, the vma phenotype includes hypersensitivity to metal ions, including Co2+, Mn2+, Cu2+, Ni2+, and Zn2+ (8). We show increased sensitivity to elevations in extracellular zinc in vph1 and vma7 null strains, relative to their isogenic controls (Fig. 3B), consistent with the absence of vacuolar acidification. Loss of Stv1p did not alter Zn2+ sensitivity, similar to findings in S. cerevisiae (15).

Isoform-specific Differences in the Role of V o Subunit a in Hyphal Development—The vacuole is critical in germ tube formation and for invasive hyphal growth in C. albicans. Sub-apical vacuoles have been shown to rapidly fuse and enlarge, crowding out the cytoplasm, which remains confined to the advancing apical tip of the hypha (20). The critical importance of cellular pH homeostasis in hyphal development was indicated by a nearly complete inability of vma7 mutants to generate filaments in liquid Spider medium, which has mannitol as sole carbon source (Fig. 4) (8). However, the relative contribution of transmembrane pH gradients in the secretory and vacuolar compartments in hyphal formation is unclear. Interestingly, the stv1 mutant showed significant reduction of nearly 40% in filamentation, with larger reduction (85%) observed in the vph1-null strain (Fig. 4). Because hyphal formation is reduced but not completely abolished in the absence of either subunit a isoform, these results suggest that Stv1p and Vph1p both contribute to germ tube formation in liquid cultures, but can compensate, at least in part, for each other.

FIGURE 3. Isoform-specific role for CaVph1p in vacuolar acidification and zinc tolerance. A, vacuolar pH was measured using the ratiometric fluorescent dye BCECF-AM, which is de-esterified and accumulates inside the vacuoles of live yeast (inset), and pH, was calculated as described in “Experimental Procedures.” Loss of Stv1p did not significantly alter acidification of the vacuole. In contrast, loss of Vph1 resulted in large (1.4 pH unit) vacuolar alkalization, similar to that seen in the vma7 mutant. B, vacuolar sequestration of toxic cations was assessed by evaluating Zn2+ toxicity in SC medium. Growth of homozygous null mutant strains is presented as a percentage of the isogenic wild type strain. Both vma7 and vph1 strains were hypersensitive to elevated zinc concentrations (0.5 mM) whereas stv1 was similar to WT.

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Vph1p Is Required for Virulence in Candida albicans

Previously, vma7 mutants lacking all V-ATPase activity were shown to be avirulent in a mouse model of systemic infection (8). The distinct contributions of Vph1p and Stv1p in vacuolar acidification provided a unique opportunity to distinguish between the relative roles of vacuolar and secretory pathway acidification by the V-ATPase in virulence. We generated a reintegrant of VPH1 in the null strain that was verified by PCR amplification and complementation of the Zn²⁺-sensitive phenotype (Fig. 7, A and B). Freshly grown, viable cultures (8 × 10⁵ cells) of each of homozygous null, VPH1 reintegrant and corresponding wild type strain SN152 strains were injected into the tail vein of 8–10 week old female Balb/c mice (n = 10 each) and survival was scored over 25 days (Fig. 7C). The reintegrant was not statistically different in virulence from wild type (p value 0.168, log rank test) (23). The stv1-null mutant retained virulence and was also similar to wild type (p value 0.5), whereas the vph1-null strain failed to kill any of the mice over the course of the observed period (p value 1.7e-09).

DISCUSSION

As a master regulator of intracellular pH, the fungal V-ATPase is critical for a diverse range of cellular functions including vacuole acidification, vesicular transport, and trafficking, pH-dependent growth, metal ion homeostasis, hyphal growth, and pathogenicity. Not surprisingly, complete loss of V-ATPase activity in the vma7 mutant of C. albicans leads to loss of virulence (8), although it is not clear which of these many functions is critical for virulence. Here, we have confirmed and extended the phenotypes of vma7 mutants. In addition to previously known defects in alkaline pH sensitivity, endocytosis defects, and metal ion sensitivity, we also demonstrate impaired ability to deal with acid stress, calcium homeostasis, growth in non-fermentable carbon sources, and cell wall stress. Thus, the pleiotropic phenotypes of vma7 mutants observed in the model yeast S. cerevisiae readily extend to the pathogenic yeast C. albicans. Importantly, we provide new observations showing that C. albicans vma7 mutants fail to colonize Caco-2 intestinal epithelia and are efficiently eliminated by macrophages. Mucosal barrier damage as well as neutropenia are required.

FIGURE 4. Impairment of morphogenic switching in V-ATPase mutants. C. albicans was cultured in liquid spider medium for four hours and the number of cells showing hyphal development was determined by microscopic examination. In the wild type strain SN152, the majority of buds showed emergent hyphae (89%). Hyphal formation was decreased in stv1 to 55% and in vph1 to 15% of yeast cells. Nearly complete absence of morphogenic switching was observed in vma7 (0.3% compared with 83% in control strain CNC44).

FIGURE 5. Hyphal growth on solid medium is impaired in vph1 mutants. Colony morphology of V-ATPase mutants was monitored on A, solid Spider, and B, solid YPD plates supplemented with FBS. In both conditions, peripheral hyphae were not observed in colonies of the vph1 strain, similar to vma7, after a period of 14 days.

Vph1p Is Required for Virulence in a Murine Systemic Infection Model—Previously, vma7 mutants lacking all V-ATPase activity were shown to be avirulent in a mouse model of system infection (8). The distinct contributions of Vph1p and Stv1p in vacuolar acidification provided a unique opportunity to distinguish between the relative roles of vacuolar and secretory pathway acidification by the V-ATPase in virulence. We generated a reintegrant of VPH1 in the null strain that was verified by PCR amplification and complementation of the Zn²⁺-sensitive phenotype (Fig. 7, A and B). Freshly grown, viable cultures (8 × 10⁵ cells) of each of homozygous null, VPH1 reintegrant and corresponding wild type strain SN152 strains were injected into the tail vein of 8–10 week old female Balb/c mice (n = 10 each) and survival was scored over 25 days (Fig. 7C). The reintegrant was not statistically different in virulence from wild type (p value 0.168, log rank test) (23). The stv1-null mutant retained virulence and was also similar to wild type (p value 0.5), whereas the vph1-null strain failed to kill any of the mice over the course of the observed period (p value 1.7e-09).

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for dissemination in a mouse model of GI to bloodstream transport (24, 25).

Unlike mammalian orthologs of V-ATPase, the fungal enzyme shows little isoform diversity of the structural subunits, with the exception of the V_{o} subunit a encoded by the orthologous genes \textit{VPH1} and \textit{STV1} in both \textit{S. cerevisiae} and \textit{C. albicans}. The distinct functional properties and organellar distribution of V-ATPase complexes containing either Vph1p or Stv1p offer a unique approach to dissect the relative contributions of vacuolar and secretory pathway acidification to virulence and associated traits such as the morphogenic switch from budding cells to hyphae. Although a null mutant of Vph1p was previously examined in the fungal pathogen \textit{Cryptococcus neoformans} and shown to be avirulent (26), the absence of additional a subunit isoforms in this organism meant that the lone \textit{VPH1} isoform was essential for all V-ATPase functions.

As a starting point for our analysis, we localized two \textit{C. albicans} open reading frames to the vacuole and secretory pathway and evaluated their null phenotypes. We show that the vacuole-specific subunit a isoform, Vph1p, is required for vacuolar acidification and that this function cannot be adequately performed by the Stv1p isoform, consistent with similar observations in the non-pathogenic yeast \textit{S. cerevisiae}. While this study was underway, Raines et al. (13) also generated \textit{stv1} and \textit{vph1} null mutants to show that vacuolar alkalinization in \textit{C. albicans} required Vph1p. Indeed, ATP hydrolysis and proton pumping rates were severely reduced in isolated vacuolar membranes from \textit{vph1} mutants, but were normal in the \textit{stv1} mutant (13). Consistent with these observations, functions tightly linked to vacuolar acidification are disrupted in \textit{vph1} mutants. This includes tolerance to metal ion toxicity (this work) and delivery or retention of the membrane-bound dye FM4–64 to the vacuole (13). However, many other cellular phenotypes are largely unaffected in the \textit{vph1-} and \textit{stv1-}null mutants, due to the functional redundancy of the two isoforms. Thus, we demonstrate characteristic \textit{vma} phenotypes that are readily observed in the

**FIGURE 6.** \textbf{V-ATPase mutants have different effects on host cell infectivity in vitro.} A, infection from \textit{C. albicans} is initiated by mucosal damage or invasion. V-ATPase mutant strains were tested for defects in host adhesion by applying 5000 yeast cells to Caco-2 monolayers for 30 min. The percentage of adherent cells was calculated relative to the total number of viable cells as described under “Experimental Procedures.” During this time ~31% (SN152) or 27% (CNC44) of WT cells adhered to the intestinal epithelial cells. The percentage of adhering cells in \textit{stv1} (23%) and \textit{vma7} (17%) strains was decreased, but the reduction was not statistically significant in \textit{vph1} mutants (27%). B, host cell damage was evaluated by measuring release of the cytosolic enzyme LDH after overnight incubation of Caco-2 monolayers with \textit{C. albicans}. LDH release was significantly reduced in \textit{vph1} (to 60.9% of WT) and \textit{vma7} (to 11% of WT) but not \textit{stv1} mutants. Control cells were not exposed to yeast. C, ability of \textit{C. albicans} to escape phagocytosis by macrophages was monitored in V-ATPase mutants and their wild type controls after incubation of yeast cells with J774A.1 cells as described under “Experimental Procedures.” D, LDH release from J774A.1 cells after overnight incubation with \textit{C. albicans} yeast was reduced in \textit{vph1} (41.1% of WT) and \textit{vma7} (9.4% of WT) mutants, consistent with defects in the ability to damage and escape from macrophages.
VPH1 Is Required for Virulence in Candida albicans

Although Raines et al. (13) did not investigate virulence of vph1 and stv1 mutants, they found that loss of Vph1p resulted in deficits in hyphae formation in some media (M199) but not in others (Spider, YPD + 10% serum). The reason for the difference from our findings is unclear and may lie within strain variations or experimental protocols since a vma mutant was not included for comparison. Inducing media stimulate multiple signaling pathways leading to morphogenic switching, and liquid medium is a stronger inducer in many C. albicans mutant strains when compared with solid medium (28). It would be interesting to know if the vph1 mutant reported by Raines et al. retained virulence in an animal model. Further clarification could be derived from the hyphal phenotype of a vma mutant in the same background as their vph1 mutant. The role of Stv1p remains elusive given the absence of specific and strong vma phenotypes in both model and pathogenic yeasts. The unique role of Stv1p may be unmasked under specific conditions such as glucose starvation, known to cause preferential dissociation of Vph1p containing V-ATPase complexes (9). Indeed, we did observe a significant decrease in hyphal formation in the stv1 mutant grown in liquid Spider medium. Recently, Kane and coworkers used a Golgi-localized pH reporter to show that luminal pH becomes more acidic rather than alkaline upon loss of S. cerevisiae Stv1p (29). This unexpected acidification was even more pronounced in vma mutants with complete loss of V-ATPase activity, suggesting compensatory changes in organelar pH homeostasis that remain poorly understood.

Why is pHi important for fungal pathogenesis? We know that pH homeostasis is crucial for the ability to sense and adapt to pH changes in various host niches, generate or transduce morphogenetic signals for yeast to hyphal differentiation, and to maintain vital secretory and endocytic pathways necessary for the secretion of virulence factors. V-ATPase may be responsible for assisting in metabolic signaling events that trigger hyphal inducing genes, as suggested by Poltermann et al. (8) who attributed differences in hyphal phenotype induced by Spider and serum media to unique contributions from MAPK or CAM signaling pathways. It is known that the Rim/Pal proteins that are required for ambient pH signaling and alkaline pH response interact with ESCRT/Vacuolar Protein Sorting components Vps28 and Vps32 (35). Deletion of VPS28 and VPS32 alters pHi (36), confers sensitivity to alkaline pH growth, and reduces virulence of C. albicans (37), suggestive of a role for pHi in the Rim/Pal signaling pathway. Alternatively, V-ATPase may be necessary for tip growth to establish polarity and germ tube formation. This is supported by Neurospora crassa vma-1 mutants that exhibit a hyperfilamentation phenotype where the hyphae have increased branching and decreased persistence at polarity growth sites (30). Furthermore, there is consistent evidence that antifungal drugs that disrupt pHi also block hyphal formation. Thus, fluconazole blocks yeast-to-hyphal transition (38), and inhibits V-ATPase by depleting membranes of ergosterol, leading to alkalization of the vacuole (3). Similarly, amiodarone exerts its antifungal effects by alkalinizing the vacuole, and at the same concentrations, also inhibits hyphal formation in C. albicans (39). The molecular mechanism underlying this phenotype was linked to transcriptional changes in genes regulating hyphal growth. Specifically, UME1, an inducer of hyphal development, showed no expression in the presence of amiodarone whereas the transcription factors TUP1, NRG1, and RFG1, that act as negative regulators of the morphogenetic switch, were all up-regulated in amiodarone (39). Future work may identify specific contributions of pHi and distinguish between potential roles in pH sensing and response.

FIGURE 7. Vph1p is required for murine systemic infection. A, PCR analysis of genomic DNA showing deletion of VPH1 and STV1 in the null strains, and reinteg rant of VPH1 as indicated. B, reversal of Zn2+ sensitivity in the VPH1 reinteg rant. C, C. albicans blastoconidia (8 × 105), from the indicated strains, were injected into the tail vein of female Balb/c mice and virulence (defined by death or moribund phenotype) was monitored over a period of 25 days. Virulence of C. albicans was not decreased in stv1 relative to WT. In contrast, vph1 was avirulent. Virulence was restored by reintegration of VPH1 gene (vph1R).
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