Candida albicans VPS11 Is Required for Vacuole Biogenesis and Germ Tube Formation

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Received 21 October 2002/Accepted 14 February 2003

The Candida albicans vacuole has previously been observed to undergo rapid expansion during the emergence of a germ tube from a yeast cell, to occupy the majority of the parent yeast cell. Furthermore, the yeast-to-hypha switch has been implicated in the virulence of this organism. The class C vps (vacuolar protein sorting) mutants of Saccharomyces cerevisiae are defective in multiple protein delivery pathways to the vacuole and prevacuole compartment. In this study C. albicans homologues of the S. cerevisiae class C VPS genes have been identified. Deletion of a C. albicans VPS11 homologue resulted in a number of phenotypes that closely resemble those of the class C vps mutants of S. cerevisiae, including the absence of a vacuolar compartment. The C. albicans vps11Δ mutant also had much-reduced secreted lipase and aspartyl protease activities. Furthermore, vps11Δ strains were defective in yeast-hypha morphogenesis. Upon serum induction of filamentous growth, mutants showed delayed emergence of germ tubes, had a reduced apical extension rate compared to those of control strains, and were unable to form mature hyphae. These results suggest that Vps11p-mediated trafficking steps are necessary to support the rapid emergence and extension of the germ tube from the parent yeast cell.

The fungal vacuole is an acidic, membrane-bound compartment containing a variety of hydrolytic enzymes. The functions of the fungal vacuole have been well defined for model fungi such as Saccharomyces cerevisiae and Aspergillus nidulans. These include degradation of cellular proteins for the recycling of amino acids and storage of cellular metabolites such as amino acids, phosphate, and metal ions (25). A functional vacuole is necessary for cytoplasmic homeostasis, and several S. cerevisiae mutants lacking an intact vacuole are sensitive to external pH and osmotic pressure (4). Regulated uptake and release of stored nutrients by the vacuole also help to maintain stable nutrient concentrations within the cytoplasm (24, 31). While the vacuoles’ functions are not essential for vegetative growth, they are of increased importance, and often essential, for survival during periods of stress such as starvation or growth at elevated temperatures (4, 45). In addition, the vacuole may play a central role during processes of differentiation; for example, S. cerevisiae mutants defective in vacuolar hydrolase activity are unable to undergo the process of sporulation (51). This suggests that the vacuole may function in adaptation to new environments.

To date little has been reported about the specific functions of the vacuole in pathogenic species. It was postulated that the vacuole is likely to play a key role during adaptation of a pathogenic fungus to different host environments. Furthermore, vacuolar functions may be required for processes of cellular differentiation. An intensively studied example for such a differentiation process is the yeast-hypha transition in the fungal pathogen Candida albicans, which has been strongly implicated in the pathogenicity of this organism (10). Previous work by Gow and Gooday (20) studied the morphology of the C. albicans vacuole through the yeast-hypha transition. It was observed that germ tube emergence is accompanied by a rapid increase in vacuolar volume (20). The rapid expansion of the vacuole coincides with migration of parental cytoplasmic material into the hyphal tip. As the germ tube extends, subapical compartments can also be identified; these are composed almost entirely of vacuole and contain little protoplasm, whereas the protoplasm migrates with the hyphal tip. After a delay period of apparent inactivity, the highly vacuolated compartments regenerate cytoplasm and the vacuoles recede. Formation of secondary germ tubes from mother cells or of branches from subapical compartments occurs only after cytoplasmic regeneration, when the cytoplasm again migrates into the newly synthesized hyphal tip, leaving behind a highly vacuolated parent cell. At present the mechanism by which vacuole expansion occurs during germ tube formation and its regulation remain obscure. However, one major advantage of this mechanism may be that it requires minimal amounts of de novo cytoplasmic biosynthesis while allowing C. albicans to germinate rapidly and explore its environment or forage for nutrients under limiting conditions. Such a developmental model is unusual in filamentous fungi, and this mechanism may be unique to C. albicans.

More than 40 S. cerevisiae vacuolar protein sorting (vps) mutants, which missort the vacuolar hydrolase carboxypeptidase Y (CPY) to the cell surface, have been identified (3, 35, 36, 37). The corresponding protein products define components required for the delivery of vacuolar hydrolases from the Golgi apparatus to the vacuole. The class C vps mutants, vps11 (pep5), vps16, vps18 (pep3), and vps33, exhibit the most severe phenotypic defects of all vps mutants (3, 35). The class C VPS

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genes of *S. cerevisiae* encode large hydrophilic proteins, which physically interact in a complex on the cytosolic surface of the vacuolar membrane (32). These proteins are required for the delivery of proteins to the vacuole from the Golgi apparatus, from the cell surface via endocytosis, from the cytoplasm via the cytoplasm-to-vacuole trafficking route, and through autophagy (32, 35, 49). The class C Vps complex mediates the docking of transport vesicles with the vacuolar membrane through the regulation of vacuolar t-SNARE (target soluble N-ethylmaleimide-sensitive factor attachment protein receptors) (Vam3p and Vam7p) and transport vesicle vehicular (v)-SNARE (Vti1p) interaction, to form the trans-SNARE complex (40), a critical step leading to membrane fusion. Furthermore, it has been shown that Vps11p interacts with Vps39p, the guanine nucleotide exchange factor for Ypt7p, a Rab GTPase that mediates the initial "tethering" of vesicle and target membranes (50). Thus, the Class C Vps complex has an essential function in coordinating the vesicle fusion machinery at the vacuolar surface.

In addition, Vps11p and Vps18p have been shown to function at earlier steps in the CPY and endocytotic delivery pathways (42). Woolford et al. (49) uncovered a genetic interaction between *VPS11* and *VPS8*, a gene known to have functions in the recycling transport step from the prevacuole compartment (PVC) to the Golgi apparatus. This was later supported by analysis of *vps11* and *vps18* temperature-sensitive mutant strains (42). Synthetic interactions were also observed between *vps11* and *vps18* alleles and genes known to function in Golgi-to-PVC transport, including *PEP12* and *PEP7* (42), which encode an endosomal t-SNARE and SNARE regulator, respectively. The same study revealed that *VPS18* function was required for endocytotic transport from the cell surface to the PVC. As such, deletion of the class C *VPS* genes results in a range of secondary phenotypes, including the missorting of multiple vacuolar hydrolases as inactive precursors, an inability to grow at 37°C, and sensitivity to osmotic stress (4, 5, 14, 30, 42). The class C mutants also show reduced activity of Kex2p (34), an endopeptidase localized to a trans-Golgi compartment, which proteolytically cleaves secreted peptides such as the α-factor precursor (17). Class C *vps* mutants lack a recognizable vacuole compartment and instead accumulate a range of different-sized vesicles, presumed to be transport intermediates destined for the vacuole (4, 32). In summary, both *Vps11p* and *Vps18p* function at multiple steps in vesicle-mediated transport to the vacuole and are essential for vacuolar biogenesis. Since this project was undertaken, a *C. albicans* strain with a deletion of a *vps* gene (*C. albicans VPS34*) was reported as defective in filamentation (8). This supports the hypothesis that *vps* trafficking pathways may be required for filamentation.

The aims of this study were, first, to generate a *C. albicans* mutant strain defective in vacuolar protein delivery pathways and vacuole biogenesis and, second, to assess how germ tube emergence is affected by these defects.

### MATERIALS AND METHODS

**Strains.** The *C. albicans* strains used in this study are listed in Table 1. Strain BW17 was provided by Aaron Mitchell (Columbia University) (48). Strain YJB6284 (prototrophic strain derived from BW17) was provided by Judy Bereman (University of Minnesota) (6). *C. albicans* was transformed using the lithium acetate procedure (18). The PCR-based gene disruption method (48) was utilized to construct *VPS11* heterozygous and null strains. Oligonucleotides (11D5

### TABLE 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAEND1</td>
<td>CTAACCATTCCTCCCTTGTCCCG</td>
</tr>
<tr>
<td>CAEND2</td>
<td>CTCCACCATGAAACCAACCCC</td>
</tr>
<tr>
<td>CAPEP3</td>
<td>GATCAAGCCTTAGAAGACTTGC</td>
</tr>
<tr>
<td>CAPEP32</td>
<td>CCTGTGAGAACCATGAGCC</td>
</tr>
<tr>
<td>11D5</td>
<td>TCTTTCCAGGACAATATCATTCTCATTATCATTATACTATCGG</td>
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<tr>
<td>11D3</td>
<td>ATAGTGATCTTCATACCTACCCATTTACAAATATATAAGTTCGCTT</td>
</tr>
<tr>
<td>11PB5</td>
<td>CCGACTTCCATCATTTCCACCTACAGAAGAAGAATCAATCTAC</td>
</tr>
<tr>
<td>11PB3</td>
<td>CTCGAGATTCAGCCCAATCATACGTGAGTG</td>
</tr>
<tr>
<td>11R3</td>
<td>TCAGGATTCCATGGAAGTCGACACTCC</td>
</tr>
<tr>
<td>GEMHISR</td>
<td>CTCCCGGCGCCGCTG</td>
</tr>
<tr>
<td>11HISR</td>
<td>ACTGGGTGCTGATTCTTTC</td>
</tr>
<tr>
<td>HIS3AMP</td>
<td>GTTGGTGGCCCAAGAC</td>
</tr>
</tbody>
</table>

* The introduced *BamHI* site is underlined.
and 11D3 [Table 2]) for disruption were designed so that each primer contained 20 bp homologous to PCR disruption plasmids flanked with 70 bp of sequence to direct homologous integration into the VPS11 open reading frame (ORF). The primer set was used in PCR amplifications with plasmids pRS-ARGΔ5 sper and pGEM-HIS1 (promoter fragment of VPS11) to amplify with forward and reverse primers, each at a 1 μM concentration, by using Life Technologies Taq polymerase and reagents according to the manufacturer’s instructions or by using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech). Amplification conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min/kb, with a final step of 72°C for 10 min. The resulting PCR product was purified with Gentrap® PCR purification beads (Promega) and ligated into the SalI site of pGEM-URA3 to produce plasmid pGP11. Plasmid pGP11 was digested with unique SalI and the 3.8-kb product was ligated into pGEM-TEasy (Promega) to prepare template plasmid pGEM-HIS1. Two nanograms of plasmid DNA was amplified by using Ready-To-Go DNA Labeling Beads [32P]dCTP by using Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech). Amplification products were separated in 4% agarose gels, and the 607-nt VPS11 fragment and the 393-nt VPS18 fragment were visualized and quantified by a method modified from the work of Jones (22). The CPY-specific substrate N-benzoyl-l-tyrosine p-nitroanilide (NTPNA) was diluted 1.5 from 2.5 mg/ml stock solution in dimethyl formamide with 0.1 M Tris-HCl (pH 7.5). A 190-μl volume of assay buffer was added to the wells of a microtiter plate, and 5 × 106 cells suspended in 10 μl of sterile distilled H2O were added to each well. Assay plates were incubated at 37°C before absorbance at 405 nm was measured at 5 and 20 h. Controls with cells but no NTPNA were also measured, and these values were subtracted from those for reactions with NTPNA. Each experiment was performed in triplicate, and statistical analysis was performed using the r test. Data are given as mean Aact ± standard deviation.

Vascular morphology. The vascular marker dye 5-(and 6-) carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA; Molecular Probes, Inc.) was used to visualize vascular morphology according to the manufacturer’s instructions. Cells grown at 30°C overnight in liquid YPD medium were harvested and resuspended at 105/ml in 50 mM sodium citrate buffer, pH 5, containing 2% glycerol. Carboxy-DCFDA was added to a final concentration of 10 μM, and the assay was incubated for 20 min in the dark. Cells were then washed twice in sterile distilled H2O before staining with 4′,6′-diamidino-2-phenylindole (DAPI), as described by Burke et al. (9).

Identification of C. albicans class C VPS genes. Initial attempts to isolate C. albicans class C VPS homologues through the functional complementation of the four S. cerevisiae class C VPS mutants were unsuccessful. BLAST searches of the C. albicans sequence database (http://alces.med.umich.edu/databases) identified the University of Wisconsin Genetics Computer Group (GCG) sequence analysis software programs (13). Nucleotide database searches were performed using BLAST (1). PCR was performed under routine conditions unless otherwise described. The sequences of oligonucleotides used in PCR amplifications are listed in Table 2. Putative protein sequences were analyzed in BLAST searches and with other programs including GCG (13) and PSORT2 (http://bioweb.pasteur.fr/seqanal/interfaces/psort2.html).

RESULTS

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importance. Lupas's algorithm (26) detected heptad repeats for endoplasmic reticulum translocation, this is unlikely to be of importance for Vps11p (KLPN), but given the lack of a signal peptide for protein is predicted to have transmembrane domains or N-CX14CXHX2HX2CX5CX2C sequence near its C terminus. Similarly, the putative Vps18p has a CX2CXHX3HX2CX4CX2C sequence near its C terminus. C. albicans Vps11p and Vps18p have conserved C-terminal RING domains. C-terminal sequences from S. cerevisiae VPS18 and VPS33-like ORF fragments were designed for specific amplification of each of the ORFs (accession numbers AJ492193 and AJ289080, respectively).

The C. albicans VPS11 ORF encodes an 1,100-amino-acid protein that shares 35% identity and 45% similarity with its S. cerevisiae homologue. A putative cysteine-rich RING (really interesting new gene) domain has been identified in the C-terminal regions of both C. albicans Vps11p and Vps18p (7, 14, 30, 34), and it is likely that these mediate protein-protein interactions to form large complexes. Site-directed mutagenesis has demonstrated that at least the first cysteine residue of the proposed Vps18p motif is required for normal function (34). Notably, many of the cysteine and histidine residues of the previously proposed RING domain were conserved in the C. albicans homologues (Fig. 1). The putative C. albicans VPS11 gene product is predicted to possess a CX2CX12CXHX3HX2CX4CX2C sequence near its C terminus. Similarly, the putative C. albicans Vps11p has a CX2CX12CXHX3HX2CX4CX2C sequence near its C terminus. Both of these sequences conform to the H2 type RING domain consensus CX2CX3CX4CX5CX6 (7). Neither protein is predicted to have transmembrane domains or N-terminal signal peptides, suggesting that they do not enter the secretory pathway, and likely reside in the cytoplasm. A possible vacuolar targeting motif was identified at amino acid 418 of Vps11p (KLPN), but given the lack of a signal peptide for endoplasmic reticulum translocation, this is unlikely to be of importance. Lupas's algorithm (26) detected heptad repeats likely to form α-helices within residues 530 to 558 of Vps11p and 722 to 750 of Vps18p, which through interaction with other similar motifs may participate in the formation of coiled-coil structures (41). S. cerevisiae Vps11p is predicted to form a similar helical region (residues 850 to 914), while S. cerevisiae Vps18p is not. The functional significance of the S. cerevisiae Vps11p heptad repeat has not yet been reported, but it may mediate protein-protein interactions. Similar helical domains present on v- and t-SNAREs are known to mediate the tight interaction of these proteins through the formation of coiled-coils in the trans-SNARE complex (46). Through interaction with similar structures on v- and t-SNAREs, the heptad repeats present on Vps11p and Vps18p may regulate the formation of trans-SNARE complexes and thereby regulate vesicle-target membrane fusion.

More recently, the sequences of ORFs with homology to all four class C VPS genes have been made available through the C. albicans genome sequencing project (http://www-sequence.stanford.edu/group/candida).

Construction of C. albicans vps11Δ mutant. The PCR-based gene disruption method described by Mitchell and colleagues (48) was utilized for construction of a vps11Δ null strain as described in Materials and Methods. Correct integration of either marker at the VPS11 chromosomal locus should result in the deletion of 3,144 bp of the 3,300-bp ORF (Fig. 2A). Initially the ARG4 cassette was transformed into the BWP17 recipient strain (arg4Δ::ARG4 his1Δ::HIS3 ura3Δ::URA3), and Arg+ transformants were screened by Southern blot hybridization for the desired integration event by using a VPS11-specific probe (Fig. 2B). VPS11::ARG4 “single mutants” (GPS1 to GPS4) were then transformed with the vps11::URA3 cassette, and Arg+ Ura+ transformants were screened for a second integration event, resulting in vps11Δ::ARG4::vps11Δ::URA3 “double mutants” (GPD1 to GPD3) (Fig. 2B).

In order to confirm that the loss of VPS11 function was responsible for any observed phenotypes, a “wild-type” copy of VPS11 was reintroduced into the his1 locus of the double mutant GPD1 on plasmid pGEM-HIS1 to form revertant strains GPR1 to GPR3. Plasmid control strains were also constructed by transforming GPD1 with linearized pGEM-HIS, generating strains GPH1 to GPH3.

RNA prepared from strains BWP17, GPS1, GPD1 and -2, GPR1 to -3, GPH1 to -3, and YJB6284 (a prototroph derived (A)
from BWP17) was subjected to Northern blot analysis with a \textit{VPS11}-specific probe (Fig. 2C). This resulted in the detection of two low-abundance transcripts of approximately 3.6 and 3.0 kb in the parental strain BWP17 (Fig. 2C, leftmost lane) and the prototrophic control strain YJB6284 (Fig. 2C, rightmost lane). The 3.0-kb band comigrated with the larger rRNA species and may result from “trapping” of the \textit{VPS11} transcript in the rRNA. It is unlikely that the second transcript is the product of a second \textit{VPS11} homologue, since neither transcript was detected in our \textit{vps11} strains. The same species were detected in the heterozygous strain GPS1 (data not shown) and in each of three revertant strains (GPR1, GPR2, and GPR3) (Fig. 2C). As expected, this transcript was not detected in the null strains GPD1 and -2 (data not shown) and GPH1 to -3 (Fig. 2C), even when a threefold increase in RNA was probed (30 ng instead of 10 ng).

**Deletion of \textit{C. albicans} \textit{VPS11} causes defects in vacuole function and biogenesis.** \textit{C. albicans} strains with deletions in both \textit{VPS11} alleles grew significantly more slowly than parental strains (the generation time was approximately twice that of the parental strain) under all conditions tested. Furthermore, the mutant was more heterogeneous in cell size, with approximately one-third of cells appearing oversized when grown in YPD broth at 30°C. In contrast, when grown in the less-rich YNB medium, mutant cells were smaller than Vps11\textsuperscript{+} cells.

Initially the \textit{C. albicans} \textit{vps11}\textsuperscript{Δ} mutant strains were examined for phenotypes indicative of defective vacuole function. The double mutants exhibited an inability to grow on YPD agar supplemented with 2.5 M glycerol or 1 M NaCl\textsubscript{2} (Fig. 3), indicating sensitivity to osmotic stress. The \textit{VPS11} null mutant was also sensitive to growth on YNB medium supplemented with 400 μM CuSO\textsubscript{4} (data not shown), consistent with defects in metal ion homeostasis. The parental and heterozygous strains grew well at either 30 or 37°C and grew less abundantly at 42°C. However, while the double mutant grew well at either 30 or 37°C, it was completely unable to grow at 42°C (Fig. 3). Each of these phenotypes closely resembles those of the \textit{S. cerevisiae} class C \textit{vps} mutants (4, 5, 28, 30), with the exception that the \textit{S. cerevisiae} mutants are sensitive to growth at 37°C rather than 42°C. Furthermore, each phenotype was fully complemented by the reintroduction of a wild-type copy of \textit{VPS11} (GPR1 to -3) but not by that of the pGEM-HIS1 vector alone (GPH1 to -3) (Fig. 3). These observed phenotypes are consistent with defects in multiple vacuolar functions.

The \textit{S. cerevisiae} class C \textit{vps} mutants are defective in the delivery, processing, and activity of several vacuolar hydrolases including CPY (21, 35). CPY activity was assayed to provide an indication of vacuolar hydrolase function in the \textit{C. albicans} \textit{vps11}\textsuperscript{Δ} mutant. The \textit{vps11}\textsuperscript{Δ} mutant, GPH1, showed a threefold reduction in CPY activity (0.189 ± 0.057 [mean \textit{A}_{405} ± standard deviation]) compared to the prototrophic control strain YJB6284 (0.618 ± 0.105; \textit{P} < 0.01). The revertant, GPR1, showed restored CPY activity and actually had higher levels of CPY activity (1.126 ± 0.163) than YJB6284.

The vacuole morphology of the prototrophic strains YJB6284, GPR1, and GPH1 was determined by using carboxy-DCFDA (Molecular Probes, Inc), a fluorescent dye which specifically accumulates within the vacuole (33). The control strain YJB6284 had typical wild-type vacuole morphology, with one to three well-defined, brightly stained subcellular compart-
the double mutant GPH1 did not localize the fluorophore to any distinct subcellular compartment (Fig. 4B), instead exhibiting staining of a lower intensity dispersed throughout the cell. This diffuse staining pattern confirms the absence of an intact vacuole in the C. albicans vps11Δ mutant. A normal nucleus morphology was observed for each strain by DAPI staining (data not shown), indicating that nuclear structure is unaffected in the vps11Δ mutant.

The C. albicans vps11 mutant has reduced secreted proteinase and lipase activities. S. cerevisiae vps11 and vps18 mutants are defective in Kex2p-mediated maturation of the mating pheromone α-factor (34). In C. albicans the Kex2p endopeptidase is required for the maturation and full activity of SAP (27). A C. albicans kex2 mutant strain has been described as having reduced SAP activity and furthermore is defective in yeast-hypha morphogenesis (27). We therefore examined SAP activity in our C. albicans vps11Δ mutant as an indicator of Kex2p activity by observing BSA hydrolysis at the peripheries of colonies grown on solid BSA-plus-yeast extract agar. Colonies of the prototrophic control (YJB6284) and revertant (GPR1) strains had large opaque zones at their peripheries, indicating SAP-mediated BSA hydrolysis (Fig. 5A). The double mutant (GPH1) had no visible opaque zone, indicating a loss of SAP activity (Fig. 5A). The reduction in SAP activity was much more severe than that for the C. albicans kex2 mutant (27); thus, loss of Kex2p activity alone cannot account for the SAP defects in GPH1. This suggests that there are either different or additional defects in the processing or secretion of SAP in GPH1. C. albicans is also known to secrete a number of lipase enzymes (16). As a further marker of secretion, secreted lipase activity was assessed on egg yolk medium. The absence of a white precipitation zone around strain GPH1 (Fig. 5B) is indicative of little or no secreted lipase activity in the vps11Δ mutant. The revertant GPR1 had opaque zones of a size similar to that of the YJB6284 zones (Fig. 5B). Reduced secreted lipase activity was confirmed by the severely retarded growth of the vps11Δ mutant on YNB medium containing Tween 20 as a carbon source (data not shown).

The C. albicans vps11 null mutant is defective in the yeast-hypha switch. The vps11Δ strain was examined for defects in morphogenesis under both solid agar and liquid inducing conditions. Mutant strains GPD1 and -2 and GPH1 to -3 failed to filament on either M199 (Fig. 6A) or 10% FCS (data not shown) solid medium at 37°C, conditions which resulted in extensive filamentation of the parental strain (BWP17), control strain (YJB6284), and revertant strains (GPR1 to -3) (Fig. 6A).

Yeast-hypha morphogenesis was next examined in liquid culture. Each strain was grown to saturation in YPD medium at 30°C, then subcultured into fresh YPD supplemented with 10% (vol/vol) FCS, and incubated at 37°C to induce filamentation. Under these conditions the majority of both the control (YJB6284) and revertant (GPR1) cells rapidly formed germ tubes, clearly visible after 60 min (Fig. 6B). By 120 min, the majority of germ tubes had elongated to form parallel walled hyphae with some branches. Some pseudohyphae were also observed (Fig. 6B). In contrast, few cells of the double-mutant strain GPH1 were observed to form true germ tubes after 60 or even 120 min of serum induction (Fig. 6B). A proportion of GPH1 cells were observed in elongated forms, but many of these elongated forms had visible constrictions along the cell wall (Fig. 6B), characteristic of pseudohyphal growth, or else
formed aberrant short projections which tapered toward the end. Of the true germ tubes that were present, none were observed of more than two or three cell compartments in length, even after 18 h. By 180 min, YJB6284 and GPR1 hyphae had begun to aggregate and form clumps that were visible to the naked eye at 240 min. In contrast, no clumping was observed in the GPH1 culture, even after 18 h of growth in serum culture.

Thus, upon serum induction of filamentous growth, only a small proportion (<10%) of cells of the C. albicans vps11Δ mutant strain were able to produce true germ tubes. In addition, there was a significant delay in the emergence of these germ tubes, which had a reduced apical extension rate compared to those of parental and revertant strains. Finally, the mutant cells were unable to sustain filamentous growth to form mature hyphae.

FIG. 4. C. albicans VPS11 is involved in vacuole biogenesis. Strains YJB6284 (VPS11/VPS11), GPH1 (vps11Δ/vps11Δ), and GPR1 (vps11Δ/vps11Δ/VPS11) were stained with the vacuole-specific dye carboxy-DCFDA to analyze vacuole morphology. (A) YJB6284 cells have one to three distinctly stained subcellular vacuole compartments. (B) The double mutant GPH1 exhibits diffuse staining throughout the cytoplasm, indicating the absence of a vacuole compartment. (C) The revertant strain GPR1 has a staining pattern similar to that of YJB6284, indicating that vacuole biogenesis is restored.

FIG. 5. The C. albicans vps11Δ mutant has reduced secreted lipase and protease activities. Shown are results for strains YJB6284 (VPS11/VPS11), GPH1 (vps11Δ/vps11Δ), and GPR1 (vps11Δ/vps11Δ/VPS11). (A) SAP expression was induced in liquid culture using BSA as a nitrogen source before SAP activity was assessed on BSA-plus-yeast extract agar. The white precipitation zone indicates SAP-mediated BSA hydrolysis. (B) Cell suspensions were spotted onto egg yolk medium in order to detect lipase activity, indicated by a white precipitation zone at the colony periphery.
DISCUSSION

In this study, we have demonstrated that Vps11p is required for vacuole biogenesis, SAP and secreted lipase activities, and normal yeast-hypha morphogenesis in C. albicans. As expected, the C. albicans vps11Δ mutant lacked normal vacuole morphology and exhibited a diffuse staining pattern with the vacuole-specific dye carboxy-DCFDA. This contrasts with the
observed to undergo rapid expansion upon serum-induced
S. cerevisiae
in vps11
or in the ability of carboxy-DCFDA to localize to small vesicles
staining pattern with similar dyes (30, 44). This could be due to
fill a physical requirement, perhaps to aid the rapid exten-
growth in serum culture (28). It is possible that this expansion
expression increased severalfold following induction of hyphal
logues may be required to facilitate vacuolar expansion upon
vacuole in the yeast form, the
class C homo-
C. albicans
function of the vacuole and/or PVC, rather than their
mutants are also
defective in hyphal formation (27). Thus, the filamentation
defects of our vps11Δ mutant could result from a depletion of
activity in the trans-Golgi compartment. More gener-
Albicans “donor” may be perturbed or compromised in our vps11Δ mutant due to the absence of
PVC or vacuole “recipient” compartment.
Secondly, S. cerevisiae Vps11p and Vps18p are known to function at multiple transport steps in vacuolar protein deliv-
and of these proteins’ activities results in defects in
defects in multiple PVC and vacuolar functions. It is likely that the C. albicans homologues described here operate at equivalent
steps in this organism. Thus, a further possibility is that a
specific function of the vacuole and/or PVC, rather than their
physical expansion, is required for filamentation. The C. albicans
strain described in this study was defective in a
range of vacuolar functions that resulted in temperature and
osmotic sensitivity. S. cerevisiae class C vps mutants are also
known to be defective in the processing of multiple vacuolar
hydrolases and to missort these hydrolases as inactive precur-
sors (35). Vacular hydrolase activity is essential for the pro-
cess of sporulation in S. cerevisiae (51), an example of cellular
differentiation. It therefore remains possible that it is the
reduced hydrolase activity of C. albicans vps11Δ strains rather
than a lack of vacuolar expansion which accounts for the yeast-
hypha differentiation defects. This explanation of the defects
observed in the Candida vps11Δ mutant is less favored by us,
mainly because the yeast-hypha transition is a very rapid pro-
cess in C. albicans, whereas the degradation mediated by the
vacuolar hydrolases during sporulation and autophagy in S.
cerevisiae occurs much more slowly, over many hours (15).
Thirdly, S. cerevisiae vps11 and vps18 mutants are also
defective in Kex2p-dependent maturation of α-factor (34). Kex2p
is an endopeptidase localized to a trans-Golgi compartment
(17, 23). Ordinarily it transits to the PVC via the CPY trans-
port pathway before returning to the Golgi apparatus in a
retrograde transport step (29). Due to the transport defects in
the vps11 and vps18 mutants, Kex2p is unlikely to be returned
to the Golgi apparatus, resulting in depletion of Kex2p activity
in the trans-Golgi compartment (47). The loss of SAP activity
in our C. albicans vps11Δ mutant could be due in part to similar
defects in Kex2p function. This is of particular interest in light of
the fact that a C. albicans kex2Δ mutant has been reported
developmental defects in hyphal formation (27). Thus, the filamentation
defects of our vps11Δ mutant could result from a depletion of
Kex2p activity in the trans-Golgi compartment. More gener-
ally, the function of the Golgi “donor” may be perturbed or compromised in our vps11Δ mutant due to the absence of
PVC or vacuole “recipient” compartment.
Additional studies are required to elucidate which of the
possibilities discussed above accounts for the morphogenesis
defects observed in our vps11Δ mutant. At present it is not
clear which of the Vps11p-mediated trafficking steps are
required for filamentation. The construction of C. albicans
mutants defective in individual transport steps to the vacuole will
begin to identify which transport steps, and by inference which
compartment functions, are required for filamentation.
Somewhat unexpectedly, the C. albicans vps11Δ mutant ex-
hibited very little or no SAP activity in the BSA plate assay.
This defect was more severe than that of a kex2Δ mutant (27),
so it is unlikely to be accounted for by Kex2p function alone.
This could suggest a defect in either SAP biosynthesis or
secretion. A similar loss of secreted lipase activity was also
demonstrated on egg yolk medium. The almost total loss of these
activities could be caused by defects in the secretory pathway
from Golgi apparatus to cell surface. The secretory pathway
of S. cerevisiae vps11 mutants is apparently unaffected, as judged
by invertase and α-factor secretion as well as CPY mislocal-
ization to the cell surface (34, 35, 37). This may indicate that C.
albicans’ Vps11p functions as part of a general fusion complex
required for multiple delivery pathways from the Golgi appa-
ratus. Alternatively, the SAP and lipase defects may be a sec-
ondary consequence of the absence of a vacuolar
compartment. This may result in the mislocalization of proteolytic
enzymes to the cell surface, as is the case for S. cerevisiae vps11
mutants (35), potentially causing degradation of secreted pro-
teins. However, further investigation is required to determine
the cause of the SAP and lipase defects.
The importance of vacuolar inheritance during hyphal de-
velopment has been the subject of a separate investigation (C.
Barella, R. Mathias, C. Gaillardin, N. Gow, and A. Brown,
albicans vac8Δ mutant was constructed and shown to be
defective in vacuole inheritance. Surprisingly, this has little effect
on germ tube formation or extension rate but results in an
increase in branching frequency. Although these mutants do
not inherit vacuolar material from the parent cell, after a short
delay they are able to generate a new vacuole compartment.
These results suggest that vacuole protein-sorting pathways,
rather than inheritance, are important during the yeast-hypha
switch.
The observations made by Gow and Gooday (19) raise several important questions. First, from where is the extra membrane derived which is incorporated into the vacuole during germination? Is there a “reservoir” of membrane vesicles that fuse to the vacuole upon hyphal induction, or is membrane material derived from other membrane-bound organelles such as the PVC, Golgi apparatus, or endoplasmic reticulum? How is this process of vacuolar expansion regulated? This may prove more difficult to investigate and may be resolved only with further elucidation of the signal transduction pathways that regulate morphogenesis. While the present study was in progress, it was reported in the literature that a C. albicans VPS34 homologue had been disrupted (8). The Candida vps34Δ mutant is phenotypically very similar to the vps11Δ strain described here. The vps34Δ strain is hypersensitive to temperature and osmotic stress, it shows delayed emergence of germ tubes, and only a small proportion of cells form true germ tubes in response to serum induction (8). However, there are several differences between the vps34Δ mutant and the vps11Δ mutant analyzed in this study. First, it was not reported if the vps34Δ strain has a reduced germ tube extension rate. Also, no defects in CPY, SAP, or secreted lipase activity were reported. Moreover, while the vps11-deficient strain lacks a central vacuole, the vps34-null strain has a grossly enlarged vacuole, occupying approximately 80% of cell volume (8). The C. albicans vps34Δ mutant is predicted to have defects in anterograde and retrograde traffic between the Golgi apparatus and the vacuole. Thus, upon induction of hyphal growth, it is predicted that the vacuole would neither expand nor contract. This may account for the observed defects in filamentous growth. The similarities between the C. albicans vps34Δ and vps11Δ mutants could suggest that it is not just the presence of a vacuole but the trafficking pathways to the vacuole that are important for morphogenesis. It is likely that these pathways mediate vacuole expansion, which may be required for germination (19).

The results of this study demonstrate that one or more of the trafficking steps mediated by Vps1p are required for normal filamentation in the pathogen C. albicans. However, further investigation is required to identify which of the transportation steps affected in our vps11Δ mutant accounts for the defect in filamentous growth.

ACKNOWLEDGMENTS

The work at Leicester University was supported through a BBSRC postgraduate studentship. Work at Georgetown University and the LSUHSC School of Dentistry was supported by NIH grant NIAID AI46142, awarded to J.S.

We thank Peter Meacock (Leicester University) for providing the C. albicans genomic DNA library used in these studies. We also thank A. P. Mitchell, W. A. Fonzi, and J. Berman for providing plasmids and C. albicans strains.

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