A functional analysis of the Candida albicans homolog of Saccharomyces cerevisiae VPS4

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Abstract

To investigate the role of the prevacuolar secretion pathway in the trafficking of vacuolar proteins in Candida albicans, the C. albicans homolog of the Saccharomyces cerevisiae vacuolar protein sorting gene VPS4 was cloned and analyzed. Candida albicans VPS4 encodes a deduced AAA-type ATPase that is 75.6% similar to S. cerevisiae Vps4p, and plasmids bearing C. albicans VPS4 complemented the abnormal vacuolar morphology and carboxypeptidase missorting in S. cerevisiae vps4 null mutants. Candida albicans vps4A null mutants displayed a characteristic class E vacuolar morphology and multilamellar structures consistent with an aberrant prevacuolar compartment. The C. albicans vps4A mutant degraded more extracellular bovine serum albumin than did wild-type strains, which implied that this mutant secreted more extracellular protease activity. These phenotypes were complemented when a wild-type copy of VPS4 was reintroduced into its proper locus. Using a series of protease inhibitors, the origin of this extracellular protease activity was identified as a serine protease, and genetic analyses using a C. albicans vps4A prc1Δ mutant identified this missorted vacuolar protease as carboxypeptidase Y. Unexpectedly, C. albicans Sap2p was not detected in culture supernatants of the vps4Δ mutants. These results indicate that C. albicans VPS4 is required for vacuolar biogenesis and proper sorting of vacuolar proteins.

Introduction

Candida species are the fourth most common cause of hospital-acquired bloodstream infections, and the third most common cause of nosocomial urinary tract infections. Despite improvements in antifungal therapy, the high attributable mortality rate due to Candida infections is no better than two decades ago. Thus, the ability to prevent, diagnose, and treat Candida infections still needs to be greatly improved. Candida albicans is a versatile commensal organism which possesses a number of attributes which enhances its ability to survive in diverse environments and enables it to transition from harmless commensal to invasive pathogen. Recently, substantial effort has been invested to understand the molecular mechanisms of Candida virulence and pathogenesis. Determinants associated with virulence include surface adhesins, agglutinin-like molecules encoded by the ALS family of genes, the integrin-like surface protein Int1p, extracellularly secreted degradative enzymes (in particular, secreted aspartyl proteases, phospholipases, and lipases), yeast-to-hyphal transition, phenotypic switching, and the expression of pH- and other environmentally responsive genes (reviewed in Ghannoum, 2000; Calderone & Fonzi, 2001; Naglik et al., 2003).

Recent molecular studies of C. albicans have investigated the role of the vacuole and vacuolar protein sorting (VPS) genes in filamentation and pathogenesis. Candida albicans vps34Δ mutants, which are vacuolar class ‘E’ mutants lacking the phosphatidylinositol 3-kinase Vps34p, are sensitive to temperature and osmotic stresses, are defective in filamentation, and have impaired adhesion (Bruckmann et al., 2000). In vivo, C. albicans vps34Δ mutants are avirulent in a mouse model of disseminated candidiasis (Eck et al., 2000). Candida albicans vps11Δ mutants, which are vacuolar class ‘C’ mutants defective in multiple protein sorting pathways to the PVC and vacuole, are also sensitive to temperature and osmotic stresses and are defective in...
Materials and methods

Strains and media

The S. cerevisiae vps4 null mutant strain YPR173C was from the American Type Culture Collection (Manassas, VA). Candida albicans strains used are indicated in Table 1. Candida albicans strains SC5314 (wild-type, from W. Fonzi, Georgetown University) and its derivative strain BWP17 (ura3Δ::imm434/ura3Δ::imm434 his1::hisG/His1::hisG arg4::hisG/arg4::hisG, from A. Mitchell, Columbia University) were grown at 30 °C in YPD (1% yeast extract, 2% peptone, 2% glucose) supplemented with uridine (80 μg mL⁻¹), or in minimal glucose [0.67% yeast nitrogen base without amino acids (YNB), 0.1 mg uridine mL⁻¹, and 0.7 mg FOA mL⁻¹]. Plasmids were expanded in Escherichia coli DH5α or TOP10F cells grown in LB medium +ampicillin (100 μg mL⁻¹) at 37 °C. Solid media were prepared by adding 2% agar. To assay filamentation, Lee’s media (Lee et al., 1973), Spider media (Liu et al., 1994), and Milk–Tween agar (Jitsurong et al., 1993) were prepared as described, and RPMI 1640 was obtained from Gibco BRL (Carlsbad, CA).

Isolation and analysis of C. albicans VPS4

A C. albicans gene homologous to S. cerevisiae VPS4 was identified by searching the Stanford C. albicans genome sequencing database. The coding sequence and c. 300 bp of upstream and downstream flanking sequence was amplified from C. albicans SC5314 genomic DNA using TaqHiFi DNA polymerase and primers V4-5 and V4-3 (Table 2). DNA sequencing confirmed the accuracy of the C. albicans VPS4 ORF. Standard methods were used for restriction mapping, subcloning, Southern hybridization, plasmid production,

Table 1. Candida albicans strains used in this study

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<th>Strain</th>
<th>Parent</th>
<th>Relevant genotype</th>
<th>Source/Reference</th>
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<td>This study</td>
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<td>This study</td>
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DNA sequencing, and lithium acetate transformation of \textit{S. cerevisiae} mutants.

**Targeted disruption of \textit{C. albicans VPS4}**

To disrupt both chromosomal alleles of \textit{C. albicans VPS4}, a PCR-based gene disruption strategy was used which employs \textit{cis}-recombination to recycle a \textit{URA3} marker (Wilson \textit{et al.}, 2000), thus preserving this auxotrophy for additional studies described below. PCR-generated amplicons were generated using the synthetic oligonucleotides shown in Table 2 and plasmid pDDB57 (from A. Mitchell, Columbia University) as the template. \textit{Candida albicans} BWP17 was transformed directly with the PCR reaction mixtures using the lithium acetate method (Ausubel \textit{et al.}, 1987). Uridine prototrophs were selected and purified on synthetic media lacking uracil and uridine, genomic DNA was extracted by vortexing the transformants with glass beads in phenol–chloroform, and the homologous integration of each gene-targeting cassette was first verified by allele-specific PCR, using one primer upstream and one primer downstream of the ORF and outside of the targeting region of the disruption cassette (Table 2). Next, \textit{C. albicans} genomic DNA was digested with HhaI and transferred to nylon membranes. The membranes were hybridized in 4 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C with a 417 bp digoxigenin-labeled PCR product generated with primers VPS4-5SOU and VPS4-3SOU using SC5314 genomic DNA as a template, after which the membranes were washed in 0.2 $\times$ SSC-0.1% sodium dodecyl sulfate (SDS) at 65°C and analyzed using the DIG DNA detection kit (Boehringer Mannheim) according to the manufacturer’s instructions.

To disrupt the second \textit{VPS4} allele, selected \textit{C. albicans VPS4}/vps4\textsubscript{D}::dpl200-URA3::dpl200 mutants were expanded in YPD with uridine to permit loss of \textit{URA3} by \textit{cis} recombination between the flanking \textit{dpl200} repeats, uracil auxotrophs were selected on FOA medium, and these strains’ genotypes were determined by PCR and by Southern hybridization as described above. The resulting \textit{C. albicans VPS4}/vps4\textsubscript{D}::dpl200 strains were transformed again with the PCR-generated gene disruption cassette, uracil prototrophs were selected, and their genotypes were analyzed by PCR and by Southern hybridization to identify strains with a vps4\textsubscript{D}::dpl200/\textit{vps4}\textsubscript{D}::dpl200 genotype.

In order to confirm that the phenotypic results observed were a direct result of loss of \textit{VPS4} function, one copy of wild-type \textit{VPS4} was subcloned into pGEM-URA3 (from A. Mitchell, Columbia University), digested with NotI, and transformed into the \textit{vps4}\textsubscript{D}::dpl200-URA3::dpl200/\textit{vps4}\textsubscript{D}::dpl200 strain. Correct integration of the wild-type \textit{VPS4} gene was confirmed by allele-specific PCR in multiple independent transformants.
Targeted disruption of *C. albicans* APR1 and PRC1

Strains bearing a deletion of *C. albicans* APR1, which encodes a homolog of the *S. cerevisiae* vacuolar aspartyl protease Pep4p, were generated in *C. albicans* BWP17 and a *C. albicans* vps4Δ null mutant (ura3- strain SAL2-4F; Table 1) using PCR-based gene disruption with primers APR1-5DRB and APR1-3DRB as described above, except that the second allele was disrupted utilizing the ARG4 marker in plasmid pRS-ARG4ASpeI (from A. Mitchell, Columbia Univ.) (Wilson et al., 1999). Strains bearing a deletion of *C. albicans* PRC1, which encodes a homolog of *S. cerevisiae* carboxypeptidase Y, were generated in *C. albicans* BWP17 and *C. albicans* vps4Δ null mutant strain SAL2-4F using PCR-based gene disruption with primers PRC1-5DRB and PRC1-3DRB in analogous fashion. The genotypes of the resulting mutants and their wild-type parents were verified by allele-specific PCR using genomic DNA as a template and resulting mutants and their wild-type parents were verified by allele-specific PCR using genomic DNA as a template and primers APR1-5DetA and APR1-3DetC, and PRC1-5Det and PRC1-3Det, respectively (Table 2).

Analysis of growth and stress tolerance

Growth was assessed in liquid media by measuring OD_{600\,nm} at intervals after strains and control strains SC5314, BWP17, and DAY185 were washed and transferred to fresh media, and diluted to a starting OD_{600\,nm} of 0.1. Four hundred-microliters cultures of each strain were grown in triplicate at 30 °C for 48 h in a Honeywell-100 plate with an automated Bioscreen C Analyzer (Thermo Labsystems, Waltham, MA). Shaking of the micro-cultures was performed at high intensity with irregular rotation every 3 min for 20 s and ODs were measured every hour. Growth curves were generated automatically using the BIOLINK software (Thermo Labsystems, Waltham, MA). Automated growth curves were confirmed with manual dilutions and spectrophotometry at selected time points.

Visualization of vacuolar morphology

The fluorescent dye FM4-64 (Molecular Probes, Eugene, OR) was used to visualize endocytic and vacuolar membranes, as previously described (Vida & Emr, 1995). Additionally, vacuolar staining with quinacrine was performed as follows: log-phase yeast cells were harvested, resuspended with 400 μL of phosphate-buffered YEPD containing 200 μM quinacrine in a microfuge tube, and incubated for 5 min at room temperature (Conibear & Stevens, 2002). Cells were harvested by a 5-s centrifugation, washed once in 500 μL of YPD buffered to pH 7.6 with 50 mM Na_{2}HPO_{4}, and viewed directly using differential interference contrast (DIC) and fluorescence microscopy with an Olympus BH50 epifluorescence microscope equipped with a Retiga digital camera (QImaging, Vancouver, Canada), and a ×60 PlanApo oil immersion objective. Green fluorescence filters (excitation filter 460–500 nm, barrier 510–560 nm) were used to visualize structures stained with quinacrine, yellow fluorescence filters (excitation filter 490–510 nm, barrier 520–550 nm) were used to visualize YFP, and red fluorescence filters (excitation filter 533–588 nm, barrier 608–683 nm) (all from Chroma, Rockingham, VT, USA), were used to visualize structures stained with FM4-64. Digitized images were processed with Adobe Photoshop 5.0.

The ultrastructural morphology of *C. albicans* cells was determined by harvesting 10 ml of culture by centrifugation, fixation in 2% (w/v) KMnO_{4}, washed five times in water, and postfixed for 1 h with buffered 1% OsO_{4} in 0.1 M KH_{2}PO_{4}/Na_{2}HPO_{4} buffer (pH 7.0). Three washes in H_{2}O were followed by 0.5% uranylacetate staining for 21 h and washing cells as described (Weber, 1979).

Enzyme assays

Carboxypeptidase secretion in *S. cerevisiae* strains was detected using the colony immunoblot method, as described by Conibear & Stevens (2002). Sap expression by *C. albicans* was assayed using bovine serum albumin (BSA) plate assays, as previously described (Crandall & Edwards, 1987). In addition, Sap secretion was examined in 0.34% YNB without ammonium sulfate and with 0.2% BSA, 0.2% yeast extract, and 2% glucose (glucose–BSA–YE). Sap expression was induced by growing the *C. albicans* transformants to stationary phase in minimal glucose, after which the cells were washed and resuspended at OD_{600\,nm} 10 in 0.2% BSA with appropriate amino acid supplementation. The cell suspensions were shaken at 30 °C, and cell-free supernatants obtained after 2–48 h were tested for residual BSA by SDS-polyacrylamide gel electrophoresis with Coomassie blue staining and for immunoreactive Sap by Western blotting, using anti-Sap2p polyclonal antibody (from M. Monod, University of Lausanne), after transferring to nitrocellulose membranes (Biorad, Hercules, CA), and detection using chemiluminescence (ECL Detection Kit, Amersham Biosciences, Pittsburgh, PA) and autoradiography.

Protease inhibitor experiments were performed by incubating strains in liquid BSA as previously described, except with or without the addition of a complete protease inhibitor cocktail (Roche, Indianapolis, IN) or alternatively individual serine, cysteine, and other protease inhibitors (Roche, Indianapolis, IN). The following protease inhibitors with differing specificities were tested: Pefabloc SC (a serine protease inhibitor), Aprotinin (a serine protease inhibitor), Leupeptin (a serine and cysteine protease inhibitor), and Antipain dihydrochloride (a papain and trypsin inhibitor). In brief, strains were cultured overnight in YPD, after which they were washed, and resuspended in BSA or BSA plus...
protease inhibitor for 6 h at 30 °C. Cultures were centrifuged at high speed to collect cell-free supernatant followed by analysis with reducing SDS-PAGE and visualization with Coomassie blue stain (BioRad, Hercules, CA).

Results

A C. albicans VPS4 homolog complements the vps4 null mutation in S. cerevisiae

A search of the C. albicans genome database revealed a 1320-bp intronless ORF, the deduced protein product of which was a 340-aa AAA-type ATPase that was 75.6% identical to S. cerevisiae Vps4p. To determine if this C. albicans gene was functionally homologous to S. cerevisiae VPS4, the C. albicans gene of interest was cloned by PCR, this C. albicans gene or S. cerevisiae VPS4 was ligated into low- and high-copy yeast shuttle plasmids, and the resulting plasmids were introduced into the S. cerevisiae vps4 null mutant strain YPR173C.

Saccharomyces cerevisiae vps4 null mutants are 'class E' vacuolar mutants and have abnormally large vacuoles that can be visualized by light microscopy or by fluorescence microscopy after staining with quinacrine or FM4-64 (Vida & Emr, 1995). It was found that low-copy shuttle plasmids encoding either S. cerevisiae VPS4 or the C. albicans VPS4 homolog corrected the abnormal vacuolar morphology observed by light or fluorescence microscopy in S. cerevisiae YPR173C cells, whereas the empty plasmid vector did not (Fig. 1a). Saccharomyces cerevisiae vps4 null mutants also missort the vacuolar enzyme carboxypeptidase Y (CPY) out of the cell (Bryant & Stevens, 1998). When assayed for extracellular CPY by colony immunoblot, the S. cerevisiae vps4 null mutant bearing only an empty vector missorted CPY out of the cell as expected, in contrast to the wild-type S. cerevisiae strain which does not. Plasmids bearing S. cerevisiae VPS4 and the C. albicans VPS4 homolog mostly corrected the abnormal extracellular carboxypeptidase sorting observed in S. cerevisiae vps4 null mutants, thus indicating complementation of CPY missorting (Fig. 1b). Since these results implied that the C. albicans VPS4 homolog is both structurally and functionally homologous to S. cerevisiae VPS4, this gene is referred to as C. albicans VPS4 below.

Effects of the vps4Δ null mutation on growth and morphology

The functions of C. albicans VPS4 were examined by constructing and analyzing vps4Δ null mutants. Homologous gene targeting was used to delete both chromosomal VPS4 alleles in C. albicans BPW17 and to reintroduce a wild-type VPS4 allele back into its native locus in a vps4Δ null mutant, and gene-specific PCR and genomic Southern hybridizations were used to verify the genotypes of the resulting strains (Supplementary material and Table 1). Candida albicans strain SAL2-4 bearing the vps4Δ null mutation had minimal effects on growth rates or final cell densities when the C. albicans vps4Δ mutants and C. albicans control strains SC5314, DAY185, and BPW17 were grown in rich media at 30 °C (Fig. 2) or 37 °C (data not shown). When the strains of interest were grown in conditions of high osmolar stress (2.5 M glycerol, shown in Fig. 2a, or 1 M NaCl, data not shown), the C. albicans vps4Δ null mutant strain had mildly slower growth and slightly reduced final cell densities than the control strains, and this abnormality was corrected when a wild-type VPS4 allele was reintroduced (Fig. 2a and b). No differences in filamentation were observed when the vps4Δ null mutant and wild-type controls were grown in liquid media (RPMI, 10% fetal calf serum, Lee’s medium) or solid media (Spider’s media, Milk–Tween agar) (data not shown).

Fluorescence microscopy of cells stained with FM4-64 or quinacrine showed that the C. albicans vps4Δ null mutants had aberrant large vacuoles with discrete areas of intense staining at their peripheries, which is similar to the ‘class E’ vacuolar morphology in S. cerevisiae vps4 null mutants (Fig. 3a). Also, thin-section transmission electron microscopy showed that the C. albicans vps4Δ null mutants contained multiple stacks of curved cisternal membranes (Fig. 3b) that closely resembled the aberrant prevacuolar compartment seen in S. cerevisiae vps4 and other ‘class E’ vacuolar sorting mutants (Babst et al., 1997). All of these morphologic abnormalities were corrected by reintegrating a single wild-type VPS4 allele, thereby demonstrating that these vacuolar morphology phenotypes were due to the vps4Δ null mutation and not to extraneous mutations elsewhere in the genome.

Effects of the vps4Δ null mutation on total extracellular proteases

In S. cerevisiae, the vps4 null mutation interferes with normal trafficking of the soluble secretory proteins carboxypeptidase Y, invertase and acid phosphatase (Bryant & Stevens, 1998). Since the Saps are the most extensively studied C. albicans secretory proteins (Naglik et al., 2003), the abilities of wild-type C. albicans strains SC5314, DAY185, BPW17, C. albicans vps4Δ null mutant strain SAL2-4, and C. albicans VPS4 reintegrant strain SAL3-1 to secrete catalytically active protease out of the cell were compared. When these strains were spotted on BSA agar (Crandall & Edwards, 1987), the vps4Δ null mutant produced larger zones of proteolysis than did wild-type C. albicans SC5314, the VPS4 reintegrant strain (Fig. 4a), BPW17, and DAY185 (data not shown). Similarly, when these strains were incubated in liquid YNB-glucose+BSA, SDS-PAGE analyses of culture supernatants showed that the vps4Δ mutant degraded more extracellular BSA than did C. albicans SC5314 or the VPS4 reintegrant strain (Fig. 4b).
Comparison to protease secretion by \textit{S. cerevisiae} \textit{vps1}, \textit{vps4}, \textit{vam3} and \textit{pep12} mutants

Next it was assessed whether the increased extracellular proteolytic activity was specifically a consequence of the \textit{vps4} \textit{D} mutation or would also result from other vacuolar pathway sorting mutations in \textit{C. albicans}. For comparison, first the abilities of \textit{S. cerevisiae} strains with loss-of-function mutations in several classes of vacuolar protein sorting pathway genes to degrade extracellular BSA were assayed. When \textit{S. cerevisiae} \textit{vps1}, \textit{vps4}, \textit{vam3}, and \textit{pep12} mutants were plated on BSA agar, these strains all produced zones of proteolysis, whereas wild-type \textit{S. cerevisiae} strain BY4742 did not (Supplementary material). However, the wild-type \textit{S. cerevisiae} strain BY degraded extracellular BSA (data not shown). Next, the phenotype of a \textit{C. albicans} \textit{vam3} \textit{D} mutant on BSA plates was assessed. This strain, which bears a deletion in the gene encoding the vacuolar t-SNARE Vam3p which mediates fusion of prevacuolar vesicles to the vacuole, also secreted increased extracellular proteolytic activity compared with \textit{C. albicans} control strains (S. Lee, unpublished data). Taken together, these results suggested that a missorted vacuolar protease or proteases are responsible for this increased extracellular proteolysis.

Biochemical identification of the missorted vacuolar protease in \textit{C. albicans} \textit{vps4} \textit{D} mutants

Because \textit{S. cerevisiae} \textit{vps1}, \textit{vps4}, \textit{vam3}, and \textit{pep12} mutants but not wild-type \textit{S. cerevisiae} also degraded extracellular BSA on BSA plate assays, it was hypothesized that a missorted vacuolar protease was responsible for the
Statistical significance was calculated according to the F-test. Experiments were performed independently three times; the mean OD₆₀₀ nm values and corresponding error bars are shown in the graph. All experiments were performed independently three times; the mean OD₆₀₀ nm values and corresponding error bars are shown in the graph. 

Effects of the vps₄Δ mutation on Sap2p secretion

Although the results summarized above implied that missorted CPY was responsible for the increased extracellular protease activity seen in the wild-type and complemented strains (Fig. 5d). These results indicate that the increased proteolytic phenotype of the C. albicans vps₄Δ mutant is due to a missorted vacuolar protease.

Genetic identification of the missorted vacuolar protease in C. albicans vps₄Δ mutants

Since vacuolar protease A (PrA), encoded by S. cerevisiae PEP₄, is an aspartyl protease, and is known to be missorted to the extracellular space in S. cerevisiae vps₄ and other vps mutants, it was reasoned that missorting of PrA may have caused the increased extracellular BSA degradation observed in the C. albicans vps₄Δ mutant. To test this hypothesis, homologous gene targeting was used to disrupt both chromosomal alleles of C. albicans APR₁, which is the homolog of S. cerevisiae PEP₄, in a C. albicans vps₄Δ mutant and in control strain BWP17, and these strains’ genotypes were verified by allele-specific PCR (data not shown). When the resulting strains’ abilities to degrade BSA were tested, it was found that deletion of APR₁ did not reverse the abnormal extracellular protease activity observed in the vps₄Δ mutant (Fig. 6a). Thus, missorting of Apr₁p out of the cell did not explain the increased extracellular protease activity observed in C. albicans vps₄Δ mutants.

Since a missorted serine protease was identified in the biochemical analysis of this abnormal proteolytic activity, the role of carboxypeptidase Y (CPY) in this phenotype was investigated next. Since CPY is a serine protease, and is known to be missorted extracellularly in S. cerevisiae vps₄ and other vps mutants, it was reasoned that increased secreted aspartyl protease activity could be responsible for this increased proteolytic phenotype in the C. albicans vps₄Δ mutant. Therefore BSA degradation was assayed in the presence of the aspartyl protease inhibitor pepstatin. No reduction was seen in the increased proteolytic phenotype of the vps₄Δ mutant, although partially reduced Sap activity was seen in the wild-type and complemented strains (Fig. 5d). These results indicate that the increased proteolytic phenotype of the C. albicans vps₄Δ mutant is due to a missorted vacuolar protease.

Increased extracellular protease activity. In order to identify the specific vacuolar protease, a series of protease inhibitor experiments were performed in which C. albicans cultures were incubated in media containing a protease inhibitor cocktail, and a series of individual protease inhibitors. It was found that a cocktail of protease inhibitors (Roche, Indianapolis, IN), and a specific serine protease inhibitor, Pefabloc SC (Roche, Indianapolis, IN), inhibited the increased extracellular protease activity seen in the vps₄Δ mutant (Fig. 5a and b), whereas other individual protease inhibitors had no effect (Fig. 5c). Another possibility considered was that increased secreted aspartyl protease activity could be responsible for this increased proteolytic phenotype in the C. albicans vps₄Δ mutant. Therefore BSA degradation was assayed in the presence of the aspartyl protease inhibitor pepstatin. No reduction was seen in the increased proteolytic phenotype of the vps₄Δ mutant, although partially reduced Sap activity was seen in the wild-type and complemented strains (Fig. 5d). These results indicate that the increased proteolytic phenotype of the C. albicans vps₄Δ mutant is due to a missorted vacuolar protease.

Fig. 2. In vitro growth of C. albicans vps₄Δ mutants compared with wild-type and reintegrant strains. (a) Wild-type strains SC5314, DAY185, and BWP17, C. albicans vps₄Δ null mutant, and VPS₄ reintegrant strains were grown overnight in rich media, washed with sterile water, and resuspended to an OD₆₀₀nm of 0.1 in YPD supplemented with uridine. 400-µL cultures of each strain were grown in triplicate at 30 °C for 48 h in a Honeywell-100 plate with an automated Bioscreen C Analyzer (Thermo Labsystems), and optical densities were measured every hour. All experiments were performed independently three times; the mean OD₆₀₀nm values and corresponding error bars are shown in the graph. (b) Strains SC5314, DAY185, and BWP17, C. albicans vps₄Δ null mutant, and VPS₄ reintegrant strains were grown overnight in rich media, washed with sterile water, and resuspended to an OD₆₀₀nm of 0.1 in YPD supplemented with uridine and 2.5 M glycerol, and growth was assessed as before. All experiments were performed independently three times; the mean OD₆₀₀nm values and corresponding error bars are shown in the graph. Statistical significance was calculated according to the F-test. * denotes significance as P < 0.05 for the vps₄Δ null mutant.
Fig. 3. *Candida albicans* vps4Δ mutants have abnormal ‘class E’ vacuolar morphology. (a) Overnight cultures of strains grown in rich media were shifted to fresh media and examined during late exponential phase and stained with the endocytic and vacuolar dye FM4-64 and quinacrine. Live cells were examined by epifluorescence and DIC microscopy. The *C. albicans* vps4Δ mutant displays a ‘class E’ vacuolar morphology, characterized by an enlarged vacuole with surrounding areas of hyperintense signal [arrows] thought to represent the aberrant prevacuolar compartment. (b) The ultrastructural morphology of *C. albicans* cells was determined by thin section electron microscopy. The *C. albicans* vps4Δ mutant has an abnormal multilamellar structure [PVC] which represents an aberrant prevacuolar compartment, as seen in *S. cerevisiae* vps4 mutants. For reference, the nucleus (N) and vacuole (V) are indicated in a control strain.
protease activity observed in the *C. albicans vps4Δ* mutant, the abilities of the *C. albicans vps4Δ* mutant to secrete Sap2p were also examined, since these BSA assays are traditionally used to assay the degree of extracellular Sap secretion. When extracellular supernatants of the strains of interest were analyzed by Western blotting with polyclonal antibodies to Sap2p (from M. Monod, University of Lausanne), it was unexpectedly found that wild-type *C. albicans* SC5314 and the VPS4 reintegrant strain both secreted abundant amounts of immunoreactive Sap2p out of the cell, whereas the *vps4Δ* mutant did not (Fig. 7).

**Discussion**

The major goals of this study were to determine (i) if the pathogenic fungus *C. albicans* has a prevacuolar pathway analogous to the pathway described in *S. cerevisiae* and (ii) if this pathway is required for proper trafficking of vacuolar and other proteins with a role in pathogenesis. When a search of the *C. albicans* genome database revealed a close structural homolog of the *S. cerevisiae* vacuolar protein sorting gene VPS4, two general approaches to study this gene’s functions were used. First, the vacuolar morphology and CPY sorting phenotypes of a *S. cerevisiae vps4 null* mutant were corrected by low-copy plasmids encoding *C. albicans* VPS4, but not by the empty plasmid vector. Thus, although *C. albicans* VPS4 contains one CUG codon, it appears to be functional when expressed in *S. cerevisiae*. Second, disruption of both chromosomal VPS4 alleles in *C. albicans* resulted in a ‘class E’ vacuolar morphology, and this abnormal phenotype was corrected by reintroducing a wild-type *C. albicans* VPS4 allele. In addition to a defect in vacuolar biogenesis, the *C. albicans vps4Δ* mutant showed a mild defect in osmotolerance. Taken together, these observations confirmed that the gene designated *C. albicans* VPS4 is both a structural and functional homolog of *S. cerevisiae* VPS4.

Next, the role of *C. albicans* VPS4 in vacuolar trafficking and secretion was studied. Several lines of evidence suggested that the increased extracellular protease activity observed in the *C. albicans vps4Δ* mutant was due to mis-sorting of vacuolar proteases. First, several different classes of *S. cerevisiae* vacuolar protein sorting mutants (i.e. *vps1*, *vps4*, *pep12*, and *vam3*) had the same increased ability to degrade extracellular BSA as was observed in the *C. albicans vps4Δ* mutant. Second, increased extracellular protease activity in a *C. albicans vam3Δ* vacuolar t-SNARE mutant (Lee et al., unpublished data) was observed. Since *S. cerevisiae* strains with mutations in several vacuolar protein sorting genes are known to missort the vacuolar enzymes protease A, protease B, and carboxypeptidase to the extracellular space, the results above constitute good indirect evidence that *C. albicans* vps4Δ mutants behave similarly. Direct evidence that the *C. albicans* vps4Δ mutation caused misorting of vacuolar enzymes out of the cell was provided by the protease inhibitor experiments and analysis of the double deletion mutants. Studies using a series of protease inhibitors suggested that a missorted serine protease, such as CPY, was responsible for the increased extracellular proteolytic activity on BSA plates. This hypothesis was confirmed by analysis of a *vps4Δ prc1Δ* mutant, which rescued the increased proteolytic phenotype of the *vps4Δ* mutant. The results summarized above are consistent with those obtained in earlier studies of the effects of the *S. cerevisiae vps4 null* mutation on secretion of soluble proteins such as PrA and CPY (Babst et al., 1997).

In contrast, the observation that *C. albicans* vps4Δ mutants did not secrete Sap2p out of the cell was unexpected. In *S. cerevisiae*, the conditionally expressed secretory
proteins invertase and acid phosphatase are missorted from high-density to low-density vesicles in \textit{vps1}, \textit{vps4}, and \textit{pep12} mutants (Harsay & Bretscher, 1995; Gurunathan \textit{et al}, 2002; Harsay & Shekman, 2002). While these missorted proteins still reach the cell surface in \textit{S. cerevisiae}, it was not possible to detect Sap2p in the culture supernatant of the \textit{C. albicans} \textit{vps4} \textit{D} mutant. In previous studies, it has been shown that interrupting the general secretory pathway at key ER-Golgi and post-Golgi steps interferes with Sap secretion (Mao \textit{et al}, 1999; Lee \textit{et al}, 2001) and other virulence-related proteins (Lee \textit{et al}, 2005), but it is not known how prevacuolar secretion pathway genes affect Sap secretion.

**Fig. 5.** Protease inhibitor analysis of secreted extracellular protease activity. (a) Overnight cultures of \textit{C. albicans} wild-type SC5314, \textit{vps4} \textit{D} null, and \textit{vps4} \textit{D} + \textit{VPS4} reintegrant strains were washed and then shifted to liquid BSA media either with or without the addition of a complete protease inhibitor cocktail (Roche, Indianapolis, IN) for 6 h at 30°C. Samples of culture supernatant were then analyzed by reducing SDS-PAGE followed by staining with Coomassie blue. These strains were also spotted on BSA plates containing complete protease inhibitor cocktail at 0.5 × concentration. (b) Overnight cultures of \textit{C. albicans} SC5314, \textit{vps4} \textit{D} null, and \textit{vps4} \textit{D} + \textit{VPS4} reintegrant strains were washed and then shifted to liquid BSA media either with or without the addition of an individual protease inhibitor for 6 h at 30°C, after which samples were analyzed by reducing SDS-PAGE and Coomassie blue staining. These strains were also spotted on BSA plates containing the serine protease inhibitor Pefabloc at 1 mg mL\textsuperscript{-1}. (c) Overnight cultures of the \textit{vps4} \textit{D} \textit{null} mutant were washed and then shifted to liquid BSA media either with or without the addition of the individual protease inhibitors shown for 6 h at 30°C, after which samples were analyzed by reducing SDS-PAGE and Coomassie blue staining. (d) Overnight cultures of \textit{C. albicans} SC5314, \textit{vps4} \textit{D} null, and \textit{vps4} \textit{D} + \textit{VPS4} reintegrant strains (5-2) were washed and then shifted to liquid BSA media either with or without the addition of pepstatin at a concentration of 15 μg mL\textsuperscript{-1} for 24 h at 30°C, after which samples were analyzed by reducing SDS-PAGE and Coomassie blue staining. These strains were also spotted on BSA plates containing pepstatin at 30 μg mL\textsuperscript{-1}.
Interestingly, *C. albicans* class C vps11Δ mutants do not appear to secrete increased extracellular protease activity when assayed by a BSA plate assay, as no zone of extracellular proteolysis is detected (Palmer *et al.*, 2003), perhaps because *VPS11* may regulate late Golgi trafficking in addition to its well-described ‘class C’ function at the stage of vesicle-vacuolar fusion. Furthermore, it has been recently reported that *C. albicans* vps34Δ mutants have impaired Sap secretion, when assayed by an ELISA-based method (Kitanovic *et al.*, 2005).

There are a number of potential mechanisms which could explain the lack of extracellular Sap2p in the vps4Δ mutant strain. Saps are encoded by a multi-gene family of 10 SAP genes, which are differentially secreted extracellularly depending on strain and environmental conditions, including temperature and pH (Hube & Naglik, 2001). Saps are
induced transcriptionally by protein nitrogen sources, such as hemoglobin and albumin. *In vitro*, SAP1, SAP2, and SAP3 are expressed predominantly in yeast cells, whereas SAP 4–6 are expressed predominantly in hyphal forms. Under many growth conditions, such as those used in these experiments, Sap2p is the dominant isoenzyme that is produced and secreted in *vitro* (White & Agabian, 1995). Thus, another possibility to be considered is that lack of VPS4 may influence transcription of other SAP genes, thereby indirectly affecting Sap2p secretion. For example, if deletion of VPS4 leads to abnormally increased expression of another SAP gene or genes, SAP2 expression could potentially be reduced. Other important possibilities include posttranslational degradation of extracellular Sap2p due to missorting of vacuolar proteases, impaired transcription of Sap2p due to impaired pH regulation or other indirect cause, or a trafficking defect due to lack of VPS4. The molecular basis of these VPS-related Sap-deficient phenotypes has not yet been defined, and the authors are currently studying the mechanisms leading to absence of extracellular Sap2p in the *vps4A* mutant background, including detailed analyses of the cell biology of Sap2p trafficking.

These studies indicate that *C. albicans* VPS4 is required for vacuolar biogenesis and proper trafficking of vacuolar proteins, however, it does not appear to be required for filamentation *in vitro*. Detailed studies are currently underway to define the role of VPS4 and other prevacuolar genes in Sap trafficking and secretion.

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**References**


Candida albicans VPS4

985


Supplementary material

The following supplementary material is available for this article:

Fig. S1-S3. Targeted disruption of C. albicans VPS4.

Fig. S4. Ca APR1 gene disruption in BWP17 and vps4Δ mutant background.

Fig. S5. Ca PRC1 gene disruption in BWP17 and vps4Δ mutant background.

Fig. S6. Saccharomyces cerevisiae vps mutants secrete increased extracellular protease activity.

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