Candida albicans PEP12 Is Required for Biofilm Integrity and In Vivo Virulence

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To investigate the role of the prevacuolar secretion pathway in biofilm formation and virulence in Candida albicans, we cloned and analyzed the C. albicans homolog of the Saccharomyces cerevisiae prevacuolar trafficking gene PEP12. C. albicans PEP12 encodes a deduced t-SNARE that is 28% identical to S. cerevisiae Pep12p, and plasmids bearing C. albicans PEP12 complemented the abnormal vacuolar morphology and temperature-sensitive growth of an S. cerevisiae pep12 null mutant. The C. albicans pep12Δ null mutant was defective in endocytosis and vacuolar acidification and accumulated 40- to 60-nm cytoplasmic vesicles near the plasma membrane. Secretory defects included increased extracellular proteolytic activity and absent lipolytic activity. The pep12Δ null mutant was more sensitive to cell wall stresses and antifungal agents than the isogenic complemented strain or the control strain DAY185. Notably, the biofilm formed by the pep12Δ mutant was reduced in overall mass and fragmented completely upon the slightest disturbance. The pep12Δ mutant was markedly reduced in virulence in an in vitro macrophage infection model and an in vivo mouse model of disseminated candidiasis. These results suggest that C. albicans PEP12 plays a key role in biofilm integrity and in vivo virulence.

In Saccharomyces cerevisiae, distinct secreted marker proteins are trafficked differentially through a prevacuolar compartment (PVC) prior to exocytosis (14). Furthermore, prevacuolar protein sorting genes play an important role in cargo transport in the prevacuolar branch of the exocytic pathway in S. cerevisiae (13, 15). By isolating dense- and light-vesicle populations in S. cerevisiae vps1 sec6-4, vps4 sec6-4, and pep12 sec6-4 mutants, it was observed that mutants blocked in this prevacuolar pathway missort marker proteins that are normally found in high-density post-Golgi compartment vesicles into low-density vesicles (15). Gurunathan et al. (13) also demonstrated these findings for vps1 and pep12 mutants with a late secretory mutant (snc1) background similar to that of the sec6-4 strains. These results indicate that some exocytic cargo, including the conditionally regulated soluble secretory proteins invertase and acid phosphatase, are differentially sorted through a PVC prior to exocytosis in the model yeast S. cerevisiae.

To study the prevacuolar branch of exocytosis in Candida albicans and its role in virulence, we have previously cloned and analyzed the C. albicans prevacuolar trafficking genes VPS1 and VPS4. We demonstrated that C. albicans VPS4 is required for extracellular secretion of Sap2p and Sap4-6p and for virulence in an in vivo model of disseminated candidiasis (19, 20). C. albicans VPS1 is required for Sap2p secretion and biofilm formation (4). Interestingly, although the C. albicans null mutant lacking VPS4 forms a biofilm that is denser than that formed by the isogenic reintegrant strain, the conditional mutant lacking VPS1 expression forms a patchy biofilm of reduced density (4, 34). Thus, it appears that interference with normal prevacuolar trafficking affects both the secretion of virulence-associated proteins and biofilm formation.

S. cerevisiae PEP12 encodes a 288-amino-acid syntaxin which regulates docking of Golgi compartment-derived transport vesicles at the PVC (3). Pep12p interacts with the v-SNARE Vti1p, and overexpression of Pep12p suppresses extracellular missorting of carboxypeptidase in the vti1 mutant (37). The S. cerevisiae pep12 null mutant displays a temperature-sensitive growth defect and is characterized by an enlarged vacuole with morphology defined as class D (3). A search of the C. albicans genome database identified a structural homolog of S. cerevisiae PEP12. Thus, the experiments described below were designed to determine whether the C. albicans PEP12 homolog is functionally homologous to S. cerevisiae PEP12 and to investigate its role in secretion, biofilm formation, and virulence.

MATERIALS AND METHODS

Strains and media. The S. cerevisiae pep12 null mutant strain (ATCC 4001812; YOR036W BY4741) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). C. albicans strains used in this study are listed in Table 1. Strains 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 medium (0.67% yeast nitrogen base without amino acids [YNB], 2% glucose) supplemented with uridine (80 μg ml−1) or in minimal glucose medium (0.67% yeast nitrogen base without amino acids [YNB], 2% glucose) supplemented with appropriate amino acids according to auxotrophic requirements. Filamentation was assayed on Spider agar medium (21), medium 199 (M199) containing Earle’s salts (Invitrogen) supplemented with l-glutamine and buffered with 150 mM HEPES to pH 7.5, and 10% (vol/vol) fetal calf serum (FCS) in YPD. Biofilms were assayed in liquid RPMI 1640 supplemented with l-glutamine (Gibco BRL). Liquid complete synthetic medium (CSM) supplemented with uridine and buffered to pH 4.0 with 150 mM HEPES was used for growth in acidic medium. YPD agar supplemented with uridine and buffered to pH...
pH 8.0 with 50 mM sodium succinate–50 mM NaH₂PO₄ was used for growth in alkaline medium. Solid medium was prepared by adding 2% agar.

Preparation of plasmid and genomic DNA. Plasmids were expanded in *Escherichia coli* DH5α cells grown in Luria-Bertani medium with ampicillin (100 µg ml⁻¹) at 37°C. Plasmid DNA was prepared from *E. coli* strains using a FastPlasmid mini kit according to the instructions of the manufacturer (Eppendorf). Genomic DNA was extracted from fungal cells using a MasterPure yeast DNA purification kit (Epicentre Biotechnologies) according to the manufacturer’s instructions, with the exception of a further incubation step (1 h on ice) performed after the addition of the MasterPure Complete protein precipitation reagent.

Isolation and analysis of *C. albicans* PEP12. The *C. albicans* homolog of *S. cerevisiae* PEP12 was identified by searching the Candida Genome Database (http://www.candidagenome.org/) and CandidaDB (http://genolist.pasteur.fr/CandidaDB). The coding sequence and 603 bp of upstream and 556 bp of downstream flanking sequences were amplified from *C. albicans* BWP17 genomic DNA by using Platinum Taq high-fidelity DNA polymerase (Invitrogen) and primers SacI-5PEP12 and MluI-3PEP12 (Table 2), and the amplified products were cloned using the TOPO-TA cloning kit (Invitrogen). PCR mixtures were typically heated to 94°C for 3 min and subjected to 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min 30 s, with a 10-min final extension at 72°C. DNA sequencing of the forward and reverse strands confirmed that no mutations had been introduced into the cloned gene’s open reading frame compared to the sequence in the Candida Genome Database. Next, the *C. albicans* PEP12 gene was ligated into the yeast shuttle vector pRS316 for use as a null mutant strain was transformed with the resulting plasmid. This study

| Day185 | ura3ΔΔ::imm46*ura3ΔΔ::imm34 his3::G418HIS1-his1::hisG arg4::hisG/ARG4::URA3::arg4::hisG PEP12/PEP12 |
|--------|-----------------------------------------------------------------------------------------------------|
| BWP17  | ura3Δ::arg4::arg4/arg4 his1Δ::his1::PEP12/PEP12                                                                 |
| APSK5  | ura3Δ::arg4::arg4/arg4 his1Δ::his1::PEP12/PEP12                                                     |
| APSK8-8| ura3Δ::arg4::arg4/arg4 his1Δ::his1::PEP12::dpl200-URA3::dpl200 ARG4                                   |
| APSK8-8H| ura3Δ::arg4::arg4/arg4 his1Δ::his1::HIS1 pep12::dpl200-URA3::dpl200 ARG4                           |
| APSK8-8R| ura3Δ::arg4::arg4/arg4 his1Δ::his1::HIS1::PEP12::dpl200-URA3::dpl200 pep12::dpl200 ARG4          |

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

| Strain | Genotype | Reference |
|--------|----------|-----------|
| DAY185 | ura3Δ::imm46*ura3Δ::imm34 his3::G418HIS1-his1::hisG arg4::hisG/ARG4::URA3::arg4::hisG PEP12/PEP12 | 9 |
| BWP17  | ura3Δ::arg4::arg4/arg4 his1Δ::his1::PEP12/PEP12                                                                 | 39 |
| APSK5  | ura3Δ::arg4::arg4/arg4 his1Δ::his1::PEP12/PEP12                                                     | This study |
| APSK8-8| ura3Δ::arg4::arg4/arg4 his1Δ::his1::PEP12::dpl200-URA3::dpl200 ARG4                                   | This study |
| APSK8-8H| ura3Δ::arg4::arg4/arg4 his1Δ::his1::HIS1 pep12::dpl200-URA3::dpl200 ARG4                           | This study |
| APSK8-8R| ura3Δ::arg4::arg4/arg4 his1Δ::his1::HIS1::PEP12::dpl200-URA3::dpl200 pep12::dpl200 ARG4          | This study |

216 bp downstream of the PEP12 open reading frame (Table 2). To disrupt the second allele, selected PEP12/pep12::dpl200-URA3::dpl200 mutants were transformed with the PCR-generated gene disruption cassette by using pRS-ARG4SpeI as the template. Homologous integration of the gene disruption cassette into the second allele was again verified with primers PEP12-5DET and PEP12-3DET. Histidine prototrophy was restored after transforming the pep12::dpl200 null mutant strain with NruI-linearized pGEM-HIS1 (39). An isogenic PEP12 reintegrant strain was generated by PCR-based cloning of the *C. albicans* PEP12 gene along with 603 bp upstream and 556 bp downstream of the open reading frame, and SacI and MluI restriction sites were added to the 5’ and 3’ ends, respectively. The PCR product was cloned into pGEM-HIS1, and selected pep12::dpl200-URA3::dpl200 pep12::dpl200 ARG4 null mutant strains were transformed with the plasmid construct after linearization of the construct with NruI. Correct integration of pGEM-HIS1 and pGEM-HIS1-PEP12 was confirmed by allele-specific PCR using primers GEMHISR and HIS3AMP (Table 2) by the strategy described by Palmer et al. (23).

Correct strain construction was subsequently confirmed by Southern blotting. In brief, genomic DNA prepared from candidate strains was digested with EcoRI and HindIII (New England Biolabs) and run on a 0.8% (wt/vol) agarose gel. A digoxigenin-labeled probe was prepared from the yeast shuttle vector plasmid pRS314 with primers PEP12-5Sou2 and PEP12-3Sou2 (Table 2) and reagents supplied in the PCR digoxigenin probe synthesis kit (Roche); Southern blotting was carried out by following standard protocols (2).

Analysis of growth and stress tolerance. Growth in liquid CSM with uridine (80 µg ml⁻¹) was assessed by measuring the optical density at 600 nm (OD₆₀₀) at fixed intervals. The strains were grown overnight at 37°C in YPD, washed, transferred into fresh CSM, and diluted to a starting OD₆₀₀ of 0.1. Cultures of each strain (400 ml) were grown in triplicate in a microtiter plate at 30, 37, or 40°C for 30 h in an automated Bioscreen C analyzer (Thermo Labsystems). Shaking of the microcultures was performed at high intensity with irregular rotation every 3 min for 20 s, and ODs were measured every half hour. Growth curves were generated automatically using BioLink software (Thermo Labsystems).

Growths were analyzed under conditions of high osmolar stress (in the presence of 2.5 M glycerol or 1 M NaCl) or under acidic (pH 4.0) or alkaline (pH 8.0) growth conditions on YPD agar plates supplemented with uridine (80 µg ml⁻¹) at 30°C. Strains were also grown on YPD agar plates supplemented with uridine (80 µg ml⁻¹) and subinhibitory concentrations of the antifungal agents amphotericin B, caspofungin, and flucytosine (5FC) and the cell wall-stress agents Congo red, calcofluor white, and sodium dodecyl sulfate (SDS).

Visualization of vacuolar morphology. The fluorescent dye FM4-64 (Molecular Probes) was used to visualize endocytic and vacuolar membranes, as described previously (36). Following incubation with FM4-64, the cells were harvested by 5 s of centrifugation, washed with CSM, and viewed directly by phase-contrast and fluorescence microscopy using a Nikon epifluorescence microscope equipped with a Hamamatsu camera with a 60X PlanApo oil immersion objective. Red fluorescence filters (excitation filter, 535 to 588 nm; barrier, 560 nm) were used to visualize structures stained with FM4-64. To assess vacuolar acidification, a 500-µl sample of cells from an overnight culture was processed, stained, and visualized after quinacrine staining according to published methods (29).

Enzyme and adhesion assays. Extracellular protease expression by *C. albicans* was assayed using bovine serum albumin (BSA) plate assays, as described previously (7). Lipase activity was assayed on YNB agar containing 2.5% (vol/vol) Tween 80 or on 10% egg yolk agar (10). Adhesion to polystyrene was assessed as described previously (31) with slight modifications. An inoculum of 1.0 x 10⁶ cells ml⁻¹ in phosphate-buffered saline (PBS) or RPMI 1640 was prepared, and 150-µl aliquots were added to individual wells of a 96-well microtiter plate. Samples of each strain identical to those in the wells of the plate were dispensed into individual microcentrifuge tubes for use as the unwashed controls to indicate...
the total number of adherent and nonadherent cells. Following a 2-h incubation period at 37°C, nonadherent cells were removed by washing the wells of the microtiter plate, while cells incubated in microcentrifuge tubes were pelleted at high speed on a benchtop centrifuge. The 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay (26) was then used to determine the amount of adhered cells and the total amount of cells in each well and microcentrifuge tube, respectively. After incubation with the XTT-menadione substrate at 37°C for 3 h, 75 μl of colored formazan was transferred into a fresh microtiter plate and absorbance at 490 nm was read. The adherence capacity of each strain was calculated as the mean XTT value for the washed cells relative to the mean XTT value for the unwashed cells. Experiments were performed at least twice, each with eight replicates per strain tested. Statistical significance was assessed with an analysis of variance (ANOVA) among all strains, compared using Prism 5.0 (GraphPad Software, Inc.).

**Analysis of fungal biofilms.** Analysis of the formation of *C. albicans* biofilms and the XTT reduction assay were performed as described previously (26). *C. albicans* biofilm mass was measured according to previously published methods, with slight modifications (6, 18). In brief, cells in 5-ml aliquots of RPMI 1640 containing 10^6 cells ml^-1 were grown in a six-well culture plate at 37°C for 24 h. The biofilms were scraped using a sterile scraper and transferred onto preweighed cellulose nitrate filters (pore size, 0.45 μm; diameter, 25 mm). The biofilms (dry weights) were measured in four separate experiments, each performed in quadruplicate.

**Visualization of fungal biofilms.** *C. albicans* biofilms were formed as described above on 15-mm-diameter sterile coverslips (Thermanox; Nalge Nunc International) in a six-well plate. After incubation at 37°C for 24 h, the coverslips were gently washed twice with 2% d-(+)-glucose and 10 mM Na-HEPES (pH 7.2), stained with 10 μM FUN 1 (Molecular Probes), and visualized using an LSM 510 confocal laser scanning microscope (Carl Zeiss, Inc.). Serial sections in the xy plane were obtained along the z axis. Three-dimensional reconstructions of imaged biofilms were obtained using associated software (SlideBook 5.0; Leeds Precision Instruments, Inc.). The images were processed for display using Photoshop (Adobe Systems, Inc.).

**Macrophage and Candida survival assay.** The macrophage killing assay was performed as described by Palmer et al. (24). The murine macrophage cell line J774A.1 was purchased from the ATCC and propagated in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% FCS. Next, 2.0 x 10^6 J774A.1 cells in a volume of 0.75 ml were seeded into Lab-Tek chambered slides (Nalge Nunc) and incubated overnight at 37°C with 5% CO₂. *C. albicans* strains, diluted and grown as described previously (24), were coincubated with the adhered macrophages at a multiplicity of infection of 2 for specified time periods. Following coincubation, the cells were washed twice with PBS and viability was assessed using 0.2 μM calcein AM and 4 μM ethidium bromide homodimer from a LIVE/DEAD viability/cytotoxicity kit according to the instructions of the kit manufacturer (Invitrogen). Live macrophages from four fields of each chamber were counted, and statistical differences among the average values were assessed using ANOVA followed by Tukey’s multiple comparison of means.

*Candida* survival was assessed using an end point dilution assay as described previously (24, 30). Results are presented as the values obtained by dividing the numbers of colonies in the presence of macrophages by the numbers of colonies

**FIG. 1.** Complementation of an *S. cerevisiae* pep12 null mutant. (A) *C. albicans* PEP12 complements the temperature sensitivity of an *S. cerevisiae* pep12 null mutant. *S. cerevisiae* pep12 mutant strains transformed with a vector bearing either *C. albicans* PEP12 (CaPEP12) or *S. cerevisiae* PEP12 (ScPEP12) or the vector alone were incubated for 48 h at 30 and 40°C. (B) *C. albicans* PEP12 complements the *S. cerevisiae* pep12 null mutant vacuolar phenotype. Overnight cultures of an *S. cerevisiae* pep12 null mutant transformed with empty vector pRS316 or a vector containing *C. albicans* PEP12 or *S. cerevisiae* PEP12 were shifted into fresh medium. Vacuolar morphology was examined by FM4-64 staining of live cells at exponential phase and observation by epifluorescence and light microscopy. *S. cerevisiae* wild-type strain BY4741 (WT) was used as a positive-control strain. (C) *C. albicans* PEP12 complements the *S. cerevisiae* pep12 null mutant vacuolar acidification defect. Vacuolar acidification was assessed by quinacrine staining of live cells.
in the absence of macrophages and multiplying by 100. Each experiment was set up in quadruplicate, and \( P \) values were determined using ANOVA.

**Murine model of disseminated candidiasis.** In order to assess virulence in a standard mouse tail vein model of invasive candidiasis, *C. albicans* strains (DAY185, the pep12\(^{-}/\)H9004 null mutant, and the PEP12 reintegrant) were grown to mid-log phase in YPD at 30°C and yeast-phase cells were harvested, washed, counted, and resuspended in sterile 0.9% (wt/vol) NaCl. Next, groups of 10 BALB/c female mice (from Charles River Laboratories) were injected intravenously with 0.2 ml of each cell suspension at 1.0 \( \times \) 10\(^6\) cells per animal, and survival over 30 days was assessed.

**RESULTS**

**Complementation of a *S. cerevisiae* pep12 mutant.** A search of the *C. albicans* genome database revealed an 831-bp intron-less open reading frame (orf.19.4292) whose deduced protein product is a 276-amino-acid t-SNARE that is 28% identical to *S. cerevisiae* Pep12p. Further bioinformatic analysis suggested that *C. albicans* orf.19.4292 is a structural homolog of *S. cerevisiae* PEP12 (http://bioinformatics.mpibpc.mpg.de/snare/) (17). The open reading frame contains four CUG codons (corresponding to amino acid positions 52, 90, 152, and 162). To determine if the *C. albicans* PEP12 homolog is functionally homologous to *S. cerevisiae* PEP12, *C. albicans* PEP12 was subcloned into the low-copy-number yeast shuttle vector pRS316 to generate pCaPEP12. Since *S. cerevisiae* pep12 mutants display a temperature-sensitive growth phenotype, we next transformed an *S. cerevisiae* pep12 null mutant with pCaPEP12 or with a plasmid bearing *S. cerevisiae* PEP12 and assayed growth at permissive (30°C) and restrictive (40°C) temperatures. Strains bearing *C. albicans* PEP12 or wild-type *S. cerevisiae* PEP12 grew at the restrictive temperature, in contrast to strains bearing an empty vector alone, which did not (Fig. 1A).

The *S. cerevisiae* pep12 null mutant exhibits class D vacuolar morphology, characterized by an enlarged central vacuole. Thus, we next examined vacuolar morphology in these strains. *S. cerevisiae* pep12 mutants bearing either *C. albicans* or *S. cerevisiae* PEP12 grew at the restrictive temperature, in contrast to strains bearing an empty vector alone, which did not (Fig. 1B).

The **FIG. 2. Growth, vacuolar morphology, and vacuolar function of the *C. albicans* pep12\(^{-}\) null mutant.** (A) *In vitro* growth of the *C. albicans* pep12\(^{-}\) mutant, wild-type (DAY185), and isogenic PEP12 reintegrant strains. Independent experiments were performed in triplicate three times; the mean OD\(_{600}\) values and corresponding error bars are indicated. (B) Characterization of vacuolar morphology. Vacular morphology was visualized by FM4-64 staining and bright-field and epifluorescence microscopy. (C) Ultrastructural vacuolar and cellular morphology. Thin-section transmission electron microscopy analysis of the pep12\(^{-}\) null mutant demonstrated the accumulation of 40- to 60-nm secretory vesicles near the plasma membrane (arrows). Bars representing 10 \( \mu \)m are shown. (D) Functional assessment of endocytosis in the pep12\(^{-}\) mutant. Investigation of the endocytic pathway using FM4-64 staining revealed that FM4-64 failed to stain the vacuole of the pep12\(^{-}\) mutant, indicating a defect in endocytosis. (E) Assessment of vacuolar acidification in the pep12\(^{-}\) mutant. Quinacrine staining revealed a defect in vacuolar acidification of the pep12\(^{-}\) mutant.
normally, indicating complementation of the vacuolar acidification defect (Fig. 1C).

Taken together, these results suggest that *C. albicans* PEP12 complements the *S. cerevisiae* pep12 mutant and is functionally homologous to the corresponding *S. cerevisiae* gene.

**Construction and phenotypic analysis of a *C. albicans* pep12/H9004 null mutant.** We next generated a *C. albicans* pep12/H9004 homozygous null mutant by PCR-mediated gene disruption and then constructed an isogenic complemented strain (data not shown). The *C. albicans* pep12/H9004 null mutant grew similarly to control strain DAY185 and the isogenic PEP12 reintegrant strain in rich medium at 30, 37, 40, or 42°C (Fig. 2A and data not shown). The mean doubling times ± standard deviations of DAY185, the pep12/H9004 null mutant, and the PEP12 reintegrant strain were 2.25 ± 0.03, 2.23 ± 0.02, and 2.19 ± 0.03 h, respectively, at 30°C.

**Vacuolar morphology of the *C. albicans* pep12/H9004 null mutant.** Class D yeast vacuolar mutants are characterized by a single enlarged vacuole, abnormalities in mother-to-daughter vacuolar inheritance, and defects in vacuolar H^+^-ATPase assembly (28). Like other class D vacuolar mutants, the *S. cerevisiae* pep12 mutant has an enlarged class D vacuole which can be visualized using vacuolar staining and fluorescence microscopy (3). In contrast, the *C. albicans* pep12/H9004 null mutant did not have clearly enlarged vacuoles as observed by light microscopy and failed to stain with the membrane dye FM4-64 (Fig. 2B).

Thin-section electron microscopy demonstrated that, near the plasma and vacuolar membranes, the *C. albicans* pep12/H9004 mutant accumulated vesicles of 40 to 60 nm (Fig. 2C), resembling the small, 40- to 50-nm vesicles present in the *S. cerevisiae* pep12 null mutant (3).

We next analyzed the endocytic pathway using FM4-64 staining. FM4-64 failed to stain the vacuole of the *C. albicans* pep12Δ mutant at any time point (Fig. 2D); in contrast, FM4-64 reached the vacuoles of the control strain DAY185 and the isogenic PEP12 reintegrant by 60 to 90 min. Functionally, the *S. cerevisiae* pep12 mutant’s vacuolar pH is only slightly

**FIG. 3.** Cell wall integrity and antifungal susceptibility of the *C. albicans* pep12Δ null mutant. Serial dilutions (1 × 10^6, 2 × 10^6, 4 × 10^6, and 8 × 10^6 cells ml^-1) of DAY185 (WT), the pep12Δ null mutant, and the PEP12 reintegrant strain were spotted onto agar containing the cell wall-stressing reagents SDS, Congo red, and calcofluor white (A) and the antifungal agents amphotericin B (AMB), caspofungin (CAS), fluconazole (FLU), and 5FC (B) and grown at 30°C for 2 days. The *C. albicans* pep12Δ mutant was more sensitive to cell wall-stressing agents than DAY185 or the isogenic PEP12 reintegrant strain. The pep12Δ mutant was also more sensitive to the cell wall-targeting agents amphotericin B and caspofungin and the ergosterol synthesis inhibitor fluconazole, but not the DNA synthesis inhibitor 5FC.

**FIG. 4.** Secretion of proteolytic and lypolytic enzymes by the *C. albicans* pep12/H9004 null mutant. Overnight cultures were spotted onto BSA, Tween 80, and egg yolk agar plates, incubated at 30 and 37°C, and visualized on a daily basis. The relative amounts of extracellular protease and lipolytic activities are indicated by the halo or zone of precipitation surrounding the fungal colony. The *C. albicans* pep12/H9004 null mutant produced an increased zone of extracellular proteolysis compared to DAY185 or the isogenic PEP12 reintegrant strain on BSA agar. In contrast, the pep12/H9004 null mutant produced less phospholipase and extracellular lipase activities than the control strains on Tween 80 and egg yolk agars.
more alkaline than that of the wild-type strain, but unlike wild-type controls, the mutant vacuole fails to stain with quinacrine (25). Therefore, we performed quinacrine staining to assess vacuolar acidification. The vacuoles of the DAY185 and PEP12 reintegrant strains stained as expected, but that of the C. albicans pep12/H9004 mutant failed to stain, suggesting a defect in vacuolar acidification (Fig. 2E).

Response of the C. albicans pep12/H9004 mutant to cell wall synthesis inhibitors and antifungal agents. When C. albicans pep12/H9004 and the corresponding control strains were grown at acidic or alkaline pH (pH 4 or 8, respectively) or under conditions of osmolar stress (in the presence of 2.5 M glycerol or 1 M NaCl), no differences in growth were observed. However, compared to DAY185 and PEP12 reintegrant strains stained as expected, but that of the C. albicans pep12Δ mutant failed to stain, suggesting a defect in vacuolar acidification (Fig. 2E).

Secreted aspartyl protease and lipase secretion from the C. albicans pep12Δ mutant. We next tested for extracellular secreted aspartyl protease by using a BSA plate assay. The C. albicans pep12Δ null mutant produced a large zone of extracellular proteolysis compared to control strain DAY185 and the PEP12 reintegrant strain (Fig. 4). We next tested lipolytic activity on Tween 80 agar plates and egg yolk agar plates; the C. albicans pep12Δ null mutant did not produce any extracellular lipolytic activity on Tween 80 agar, and there was substantial reduction of phospholipase activity on egg yolk agar compared to the activities of DAY185 and the reintegrant strain (Fig. 4). These results are similar to those obtained with the C. albicans vps4Δ mutant in previous studies (19), suggesting that prevacuolar secretion plays a role in the secretion of aspartyl proteases and lipases.

Filamentation of the C. albicans pep12Δ null mutant. When the C. albicans pep12Δ null mutant strain and control strains were grown at 37°C in 10% FCS, there was a decrease in the percentage of cells of the pep12Δ mutant that had filamented at 60 min compared to control strains; however, it appeared to be comparable to the control strains at 90 min. (*, *P < 0.0046 by ANOVA) (C) Overnight cultures of the DAY185, pep12Δ null mutant, and PEP12 reintegrant strains were spotted onto Spider medium, M199, and 10% FCS–YPD agar and incubated at 37°C for the times indicated. Filamentous structures emerging from the edges of each colony were observed for all the strains; however, the pep12Δ mutant appeared to filament slower than the control strains on M199 plates.
tures were formed from each colony, but the *C. albicans* pep12Δ mutant also was delayed in filamentation on solid medium (Fig. 5C). Similar results were seen with Spider agar and 10% FCS plates (Fig. 5C).

**Adhesion and biofilm formation by the *C. albicans* pep12Δ null mutant.** We examined the adherence of the *C. albicans* pep12Δ mutant by using a simple assay for adhesion to polystyrene. There was no statistically significant difference in adherence among DAY185, the pep12Δ mutant, and the PEP12 reintegrant strain (data not shown). Next, we examined the role of *C. albicans* PEP12 in biofilm formation in vitro. The *C. albicans* pep12Δ mutant formed a biofilm that was significantly reduced in metabolic activity compared to control biofilms when measured by the XTT assay (Fig. 6A) and, strikingly, was completely fragmented and detached from the underlying surface (Fig. 6B).

Because most of the biofilm of the pep12Δ mutant was fragmented and largely nonadherent to the polystyrene well, we next measured the dry weight of the entire biofilm (including the nonadherent fragments) collected onto filter paper. The biomasses from DAY185 (mean ± standard deviation, 0.0944 ± 0.0041 g), the pep12Δ mutant (0.0606 ± 0.0048 g), and the PEP12 reintegrant (0.0930 ± 0.0068 g) were measured, and the biofilm formed by the pep12Δ null mutant had approximately one-third less biomass than that formed by DAY185 or the PEP12 reintegrant strain (Fig. 6C).

To characterize the kinetics of biofilm formation in the pep12Δ mutant, a time course experiment was conducted to identify when the biofilm fragments and detaches from the plate. The *C. albicans* pep12Δ null mutant biofilm detached from the plate at 6 h with gentle tapping of the microtiter plate, in contrast to DAY185 and PEP12 reintegrant strain biofilms, which remained firmly attached and intact as expected (Fig. 7). By 8 h, the biofilm of the pep12Δ mutant lifted off the polystyrene well without any disturbance. Taken together, these results suggest that *PEP12* plays an important role in normal biofilm integrity.

**Visualization of *C. albicans* pep12Δ null mutant biofilm structure.** We next used confocal laser scanning microscopy (CLSM) to identify the structural characteristics of the pep12Δ biofilm compared to the biofilm of the control strain. The *C. albicans* pep12Δ mutant formed a biofilm that was much more

FIG. 6. Biofilm formation by the *C. albicans* pep12Δ mutant. (A) Assessment of biofilm metabolic activity. Biofilms were formed in RPMI 1640 as described in the text, and biofilm metabolic activity was analyzed using the XTT reduction assay. Experiments were performed three times independently, and each experiment included eight replicates. Statistical significance was analyzed by ANOVA. Minimal XTT activity was observed in the pep12Δ mutant strain (*, P < 0.001), although this is likely an artifact due to loss of the fragmented, nonadherent biofilm during washing. (B) Characterization of biofilm gross morphology. Biofilm morphology was visualized after growing the inoculum at 37°C for 24 h in a six-well plate before and after gentle motion. Complete fragmentation of the pep12Δ biofilm occurred after gentle motion. (C) Measurement of biofilm dry mass. Biofilm dry mass was assessed by growing the biofilms for 24 h in a six-well plate. Quadraplicate experiments were performed independently four times. The biofilms were scraped, filtered using a 0.22-μm-pore-size nitrocellulose membrane, washed with PBS, and dried on the filter at 37°C for 48 h. Thus, loose fragments of the biofilm were collected along with the intact biofilm. A total reduction in the mass of the pep12Δ mutant biofilm compared to those of control biofilms was seen (*, P < 0.0002).
disorganized than the biofilm formed by control strain DAY185 (Fig. 8A and B). Overall, the pep12Δ biofilm was much thicker (~180 μm) than the DAY185 biofilm (84 μm) when analyzed by CLSM, although this finding is due most likely to an artifact of the detached, fragmented structure of the pep12Δ biofilm.

Detailed comparison of air-dried biofilms using scanning electron microscopy, which preserves the biofilm extrapolymeric substance (EPS), revealed that the *C. albicans* pep12Δ mutant had a greatly reduced amount of EPS compared to control strain DAY185 (Fig. 8C).

**Assessment of virulence of the *C. albicans* pep12Δ mutant in an *in vitro* macrophage model.** To determine whether deletion of PEP12 affects the virulence of *C. albicans*, we first used an *in vitro* macrophage model of virulence. At 24 h, most of the macrophages had been killed by control strain DAY185 and the isogenic *PEP12* reintegrand strain; however, there was a 30-fold increase in the number of surviving macrophages when the cells were incubated with the pep12Δ null mutant (Fig. 9A and B). These survival data are similar to those obtained previously with a *C. albicans* *vps11Δ* mutant by using the same assay (24). We next measured *Candida* survival within the macrophages. Similarly, there was a statistically significant (*P* < 0.0001) reduction in the survival rate of the pep12Δ null mutant compared to those of the control strains (Fig. 9C).

**Virulence of the *C. albicans* pep12Δ null mutant in a mouse model of disseminated candidiasis.** We next assessed the role of PEP12 in virulence in an *in vivo* mouse tail vein model of hematogenously disseminated candidiasis. Mice infected with control strain DAY185 and the *PEP12* reintegrand had a 100% mortality rate by the fifth and sixth days, respectively. In contrast, 90% of the mice infected with the pep12Δ mutant survived at 30 days (*P* < 0.0001) (Fig. 10).

**DISCUSSION**

The major goals of this study were to determine the contribution of the *C. albicans* prevacuolar secretion pathway gene PEP12 to key pathogenesis-related phenotypes *in vitro*; biofilm formation, and virulence *in vivo*. When a search of the *C. albicans* genome database revealed a close structural homolog of the *S. cerevisiae* vacuolar protein sorting gene PEP12, we used a complementation approach to study gene function. First, the temperature sensitivity, vacuolar morphology, and vacuolar acidification phenotypes of a *S. cerevisiae* pep12Δ mutant were corrected by plasmids bearing *C. albicans* PEP12 but not by an empty vector. Taken together, these observations suggested that the gene designated *C. albicans* PEP12 is both a structural and a functional homolog of *S. cerevisiae* PEP12. However, it should be noted that overexpression of *S. cerevisiae*...
PEP12 suppresses the mutant phenotypes of the *S. cerevisiae* vam3 null mutant and that overexpression of *S. cerevisiae* VAM3 suppresses the mutant phenotypes of the *S. cerevisiae* pep12 null mutant (8, 12, 33). Moreover, Pep12p is structurally similar to the related t-SNAREs Vam3p and Tlg2p, and all of these proteins have transmembrane domains containing 18 amino acids, with high degrees of sequence identity (1). Thus, it remains a possibility that our gene of interest is not the *C. albicans* homolog of *S. cerevisiae* PEP12 but instead a closely related t-SNARE gene. However, the *C. albicans* vam3Δ mutant has been identified and characterized previously (35), and we have generated a null mutant of the putative *C. albicans* TLG2 homolog (unpublished data).

The *C. albicans* pep12Δ mutant accumulated 40- to 60-nm vesicles, as seen in the *S. cerevisiae* pep12 mutant, but unlike its *S. cerevisiae* counterpart, did not appear to have a characteristic class D vacuole. Similar to the *S. cerevisiae* pep12 mutant, the *C. albicans* pep12Δ mutant was defective in vacuolar acidification.

Deletion of *C. albicans* PEP12 resulted in increased susceptibility to cell wall-stressing compounds such as Congo red and calcifluor white. The *C. albicans* pep12Δ null mutant had increased sensitivity to amphotericin B, caspofungin, and fluconazole, also suggesting a defect in cell wall or plasma membrane integrity. Overall, these results suggest that intact prevacuolar secretion may be required for normal cell wall and/or plasma membrane integrity. Despite increased sensitivity to cell wall-stressing agents and these specific antifungal agents, growth of the *C. albicans* pep12Δ mutant was not different from that of control strains in response to acidic or alkaline pH, general osmotic stress, or high temperature.

Like the *C. albicans* prevacuolar vps4Δ secretory mutant, the *C. albicans* pep12Δ null mutant produces an increased amount of extracellular proteolytic activity on BSA plates. Although we have not identified the origin of this increased extracellular proteolysis, this secretion phenotype is similar to that of the *C. albicans* vps4Δ mutant, an avirulent strain in a mouse tail vein model of disseminated candidiasis (20). Using a series of bio-

FIG. 8. Structural analysis of the *C. albicans* pep12Δ null mutant biofilm. (A and B) Biofilms were examined using CLSM. Biofilms of control strain DAY185 and the pep12Δ null mutant were formed in RPMI 1640 as described previously, stained with FUN 1 (Invitrogen), and subsequently visualized using a Zeiss LSM 510 META confocal laser scanning microscope with a 20× objective. Images were processed to obtain a three-dimensional view by compilation of xy optical sections taken across the z axis to produce a lateral view in order to determine biofilm thickness (A) and a rotated view to present a global perspective (B). The *C. albicans* pep12Δ mutant produced a thicker and more disorganized biofilm than the wild type, although this outcome was due likely to an artifact of fragmentation of the biofilm. (C) Biofilm ultrastructure was examined using scanning electron microscopy. Biofilms were formed on a plastic coverslip and air dried overnight to leave the EPS intact. The biofilm formed by DAY185 was clearly encased in a dense matrix of EPS mixed with filamentous cells. In contrast, the biofilm formed by the pep12Δ mutant had very little EPS, despite having a dense network of filamentous cells.
chemical inhibitors to study the increased extracellular proteolytic activity of the \( \text{vps4} / \text{H9004} \) mutant, we identified this activity as serine protease activity. Using a genetic approach, we demonstrated that this increased proteolysis is due likely to missorted vacuolar carboxypeptidase, as a \( \text{vps4}\Delta \text{prc1}\Delta \) mutant demonstrated wild-type extracellular proteolytic activity. In addition, the \( \text{C. albicans pep12}\Delta \) null mutant had reduced phospholipase activity on Tween 80 and egg yolk agars, also similar to the \( \text{vps4}\Delta \) mutant secretion phenotype (20).

The role of secretory genes in biofilm formation has been studied only in limited fashion. \( \text{C. albicans VPS1} \) is important for adhesion and biofilm formation, as a \( \text{VPS1} \) conditional mutant forms only a sparse biofilm composed predominantly of pseudohyphal and yeast cells when gene expression is repressed (4). In this study, the \( \text{C. albicans pep12}\Delta \) mutant cells is substantially reduced compared to those of \( \text{DAY185} \) and \( \text{PEP12} \) reintegrant cells (\( \ast, P < 0.0001 \)).

FIG. 9. In vitro macrophage model of \( \text{C. albicans} \) virulence. (A) Quantification of live macrophages after infection with \( \text{C. albicans} \) strains. The numbers of live macrophage cells per field (with four fields per strain) at 24 h were determined. The data indicate an average of the number of live macrophages from two individual experiments measuring live and dead macrophage cells after 24 h. A large number of macrophages remained alive when coincubated with the \( \text{pep12}\Delta \) mutant, but most were nonviable when coincubated with the fully virulent control strain \( \text{DAY185} \) or the isogenic reintegrant \( \text{PEP12} \) strain (\( \ast, P < 0.0001 \)). (B) The \( \text{C. albicans DAY185, pep12}\Delta \) null mutant, and \( \text{PEP12} \) reintegrant strains were coincubated with macrophage cells for 1 h, 5 h (data not shown), and 24 h and stained for analysis of macrophage viability. Viable macrophages are stained green, and dead cells are stained red. (C) \( \text{C. albicans} \) survival was assessed using an end point dilution assay. Results are presented as percentages of surviving \( \text{C. albicans} \) cells, and the significance of the data was calculated using Student’s unpaired \( t \) test. The percentage of surviving \( \text{C. albicans pep12}\Delta \) mutant cells is substantially reduced compared to those of \( \text{DAY185} \) and \( \text{PEP12} \) reintegrant cells (\( \ast, P < 0.0001 \)).

FIG. 10. Assessment of virulence in a mouse model of disseminated candidiasis. The \( \text{C. albicans DAY185, pep12}\Delta \) null mutant, and \( \text{PEP12} \) reintegrant strains were tested in a mouse tail vein model of disseminated candidiasis. Survival of the mice infected with \( 10^6 \) cells of each \( \text{Candida} \) strain was monitored for 30 days. Mice infected with \( \text{DAY185} \) and the \( \text{PEP12} \) reintegrant strain had a 100% mortality rate by days 5 and 6, respectively. In contrast, mice infected with the \( \text{pep12}\Delta \) null mutant had a 10% mortality rate at 30 days (\( \ast, P < 0.0001 \)).
studies of biofilm detachment using an in vitro flow model, Sellam et al. (32) observed a clear phenotype of detachment at 6 h, with complete detachment at 8 h. However, no change in PEP12 expression was seen in their transcriptional profiling studies of this event.

A number of molecular studies have indicated a role for the vacuole in \textit{C. albicans} virulence. For example, loss of Vps21p, Ypt12p, Vps11p, and Vps4p has led to attenuated virulence in mouse models of disseminated candidiasis (11, 16, 19, 23). The contribution of the vacuole to \textit{Candida} virulence has also been assayed using an in vitro macrophage model (5, 22, 30). The importance of \textit{C. albicans} PEP12 in virulence was apparent in our macrophage experiment using \textit{J774A.1} cells; the pep12Δ null mutant was clearly defective in macrophage killing. Next, we found that the pep12Δ null mutant was markedly hypovirulent in a mouse tail vein model of invasive candidiasis, thus providing additional data suggesting that normal vacuolar function is important for \textit{Candida} virulence.

In these experiments, we have demonstrated that the \textit{C. albicans} PEP12 homolog is important for normal endocytosis and vacuolar acidification. \textit{C. albicans} PEP12 also appears to play a major role in biofilm integrity, although the mechanism of the dramatic phenotype of the pep12Δ mutant biofilm is unknown. Finally, it appears that \textit{C. albicans} PEP12 is required for wild-type virulence in an in vitro macrophage model of pathogenesis and in a standard in vivo mouse model of disseminated candidiasis. We are currently pursuing further studies of PEP12 within a biofilm flow model, as well as genomic and proteomic analyses of the changes in the pep12Δ mutant biofilm, in order to help define the molecular mechanisms responsible for the dramatically fragmented biofilm phenotype.

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