

The Vacuolar Ca^{2+} Exchanger Vcx1 Is Involved in Calcineurin-Dependent Ca^{2+} Tolerance and Virulence in *Cryptococcus neoformans*^{∇†}

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***Cryptococcus neoformans* is an encapsulated yeast that causes a life-threatening meningoencephalitis in immunocompromised individuals. The ability to survive and proliferate at the human body temperature is an essential virulence attribute of this pathogen. This trait is controlled in part by the Ca^{2+} -calcineurin pathway, which senses and utilizes cytosolic calcium for signaling. In the present study, the identification of the *C. neoformans* gene *VCXI*, which encodes a vacuolar calcium exchanger, is reported. The *VCXI* knockout results in hypersensitivity to the calcineurin inhibitor cyclosporine A at 35°C, but not at 30°C. Furthermore, high concentrations of CaCl_2 lead to growth inhibition of the *vcx1* mutant strain only in the presence of cyclosporine A, indicating that Vcx1 acts in parallel with calcineurin. The loss of *VCXI* does not influence cell wall integrity or capsule size but decreases secretion of the major capsular polysaccharide glucuronoxylomannan (GXM) in culture supernatants. Vcx1 also influences *C. neoformans* phagocytosis by murine macrophages and is required for full virulence in mice. Analysis of cellular distribution by confocal microscopy confirmed the vacuolar localization of Vcx1 in *C. neoformans* cells.**

Calcium (Ca^{2+}) is an intracellular messenger that controls numerous cellular processes. Two essential mediators of calcium signals in eukaryotic cells are the Ca^{2+} binding protein calmodulin and the Ca^{2+} /calmodulin-activated serine/threonine protein phosphatase calcineurin (4, 21). When intracellular calcium levels are low, calcineurin is inactive. An increase in calcium levels in the external environment leads to elevate cytosolic calcium. This process is sensed by calmodulin, which binds the C-terminal region of calcineurin. Then, activated calcineurin prompts transcription of genes whose products allow the cell to survive under stress conditions and maintain calcium homeostasis (3, 5). In mammalian T cells, calcineurin activates the transcription factor NFAT (nuclear factor of activated T cells). This pathway is the target of the immunosuppressive drugs cyclosporine A (CsA) and FK506, which inhibit T-cell activation (16).

An *in silico* comparative analysis of fungal Ca^{2+} signaling components showed that fungi share well-conserved key regulators of Ca^{2+} signaling. These include Ca^{2+} channels, pumps,

transporters, and exchangers, calmodulin, calmodulin-dependent kinases, and calcineurin. The Ca^{2+} signaling apparatus of *Saccharomyces cerevisiae* regulates the cell cycle, mating, sensing of glucose starvation, resistance to salt stress, and cell survival (41). The stress response in *S. cerevisiae* leads to calcineurin-mediated dephosphorylation and activation of the transcription factor Crz1, which regulates the transcription of more than 160 genes (30, 36). In the filamentous fungi *Neurospora crassa* and *Magnaporthe grisea*, there is evidence for the involvement of Ca^{2+} in many physiological processes, including the cell cycle, sporulation, spore germination, and hyphal growth (41).

The Ca^{2+} -calcineurin signaling pathway in the human pathogenic fungus *Cryptococcus neoformans* has been characterized (reviewed in reference 19). In this fungus calcineurin is required for mating, morphogenesis, growth at 37°C, and virulence (7, 13, 33). *C. neoformans* calmodulin is essential for viability and acts in response to high temperatures by two distinct mechanisms. Only one of these pathways, however, involves Ca^{2+} and calcineurin (22). Furthermore, two important components of the *C. neoformans* Ca^{2+} signaling network have been described (10, 24). The first is Cch1, a Ca^{2+} -permeable channel that mediates calcium entry in *C. neoformans* cells. This plasma membrane calcium channel is required for calcium uptake in low-calcium environments (24). The second is Eca1, a sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase that participates in stress tolerance (10). Both components,

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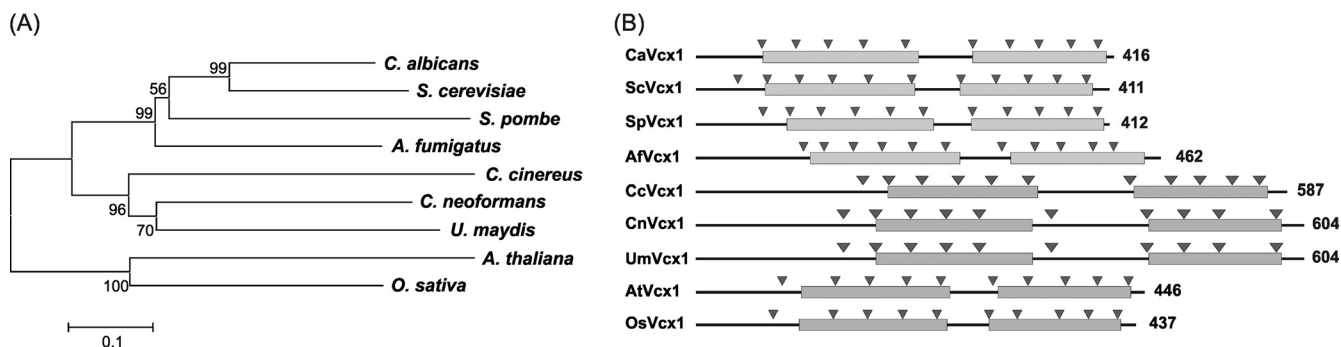


FIG. 1. Results of the *in silico* analysis of the *C. neoformans* Vcx1 ortholog. (A) Phylogenetic analysis results, applying the neighbor-joining method and including Vcx1 sequences from distinct eukaryotic organisms, as follows: *S. cerevisiae* (NCBI accession number NP_010155.1), *C. albicans* (NCBI accession number XP_711893.1), *A. fumigatus* (NCBI accession number XP_750174.2), *S. pombe* (NCBI accession number O59768), *C. cinereus* (Broad Institute accession number CC1G_02539), *U. maydis* (Broad Institute accession number UM_05132), *A. thaliana* (NCBI accession number Q945S5), *O. sativa* (NCBI accession number NP_00105030.1), and *C. neoformans* (Broad Institute accession number CNAG_00025.1). The bar marker indicates the genetic distance, which is proportional to the number of amino acid substitutions. Bootstrap values obtained with 1,000 resamplings are shown at the nodes. (B) Domain architecture in the Vcx1 orthologs of different eukaryotic organisms (with two-letter species abbreviations for each Vcx1; species are the same as those listed in panel A). The sodium/calcium exchanger superfamily (*Na₂Ca₁ex*; pfam01699) domain, a characteristic domain in Ca²⁺ exchangers, is represented by gray boxes. The transmembrane domains are represented by arrowheads. The lengths (in amino acids) of each of the sequence proteins are indicated on the right.

Cch1 and Eca1, are involved in *C. neoformans* virulence (10, 24), emphasizing the importance of calcium signaling in this pathogen.

Ca²⁺ exchangers regulate the concentration of cytosolic Ca²⁺ and its transport to storage organelles. This process is achieved by exchanging positive ions across membranes (41). *S. cerevisiae* possesses one such previously identified H⁺/Ca²⁺ exchanger, named Vcx1 (ScVcx1), which localizes to the vacuolar membrane (9, 32). ScVcx1 is negatively regulated by calcineurin, acting in Ca²⁺ tolerance and Ca²⁺ sequestration efficiently when calcineurin is inactivated (9). Additionally, ScVcx1 may also function in Cd²⁺ transport (35). Here we report the identification of the *C. neoformans* gene *VCX1*, which encodes a vacuolar calcium exchanger. The *VCX1* knockout results in hypersensitivity to the calcineurin inhibitor cyclosporine A at 35°C, but not at 30°C. Growth analysis of the *vcx1* mutant strain with high concentrations of CaCl₂ in the presence or absence of cyclosporine A indicated that Vcx1 confers a much larger degree of calcium tolerance when calcineurin has been inhibited. Importantly, Vcx1 influences *C. neoformans* phagocytosis by murine macrophages and is required for full virulence in mice.

MATERIALS AND METHODS

Fungal strains, plasmids, and media. *C. neoformans* serotype A strain H99 was the recipient for construction of the mutant strain. Strains were maintained on YPD medium (1% yeast extract, 2% peptone, 2% dextrose, and 1.5% agar). YPD plates with hygromycin (200 µg/ml) were used to select *C. neoformans* *VCX1* deletion transformants (*vcx1* strain). YPD plates with nourseothricin (100 µg/ml) were used to select *C. neoformans* *VCX1* complementation transformants (*vcx1::VCX1* strain). Plasmid pJAF15 (14) was the source of a hygromycin resistance cassette, and pAI4 (17) was the source of a nourseothricin resistance cassette. Plasmids were maintained in *Escherichia coli* grown at 37°C in LB broth or on agar supplemented with 50 µg/ml of kanamycin.

***In silico* analysis of the *C. neoformans* *VCX1* ortholog.** The putative *C. neoformans* *VCX1* gene sequence was identified by a BLAST search of the *C. neoformans* var. *grubii* strain H99 genomic database at the Broad Institute by using the Vcx1 sequence of *S. cerevisiae* (GenBank accession number NP_010155.1). The amino acid sequences of Vcx1 orthologs from *S. cerevisiae*, *Candida albicans*, *Aspergillus fumigatus*, *Schizosaccharomyces pombe*, *Coprinus cinereus*, *Ustilago*

maydis, *Arabidopsis thaliana*, *Oryza sativa*, and *C. neoformans* were aligned using Clustal X2 (23). Mega4 was utilized for phylogenetic analysis, applying the neighbor-joining method, and the tree architecture was inferred from 1,000 bootstraps (37). A search for conserved domains in the ortholog proteins was performed using the Pfam database (<http://pfam.sanger.ac.uk/>). Prediction of putative transmembrane segments was conducted by using the TMHMM server (<http://www.cbs.dtu.dk/services/TMHMM/>).

Disruption and complementation of *C. neoformans* *VCX1*. Disruption of *VCX1* was achieved with DNA constructs generated by the Delsgate methodology (15). A Gateway cloning system donor vector (Invitrogen) carrying the hygromycin-selectable marker for *C. neoformans* transformation was constructed. A 2.2-kb PCR product encompassing the hygromycin marker cassette was amplified from plasmid pJAF15 and cloned into the EcoRV site of pDONR201 (Invitrogen), resulting in a vector named pDONRHYG. The 5' and 3' *VCX1* flanks (900 bp each) were PCR amplified and gel purified using the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare). Approximately 300 ng of vector pDONRHYG and 30 ng of each PCR product were submitted to a BP clonease reaction, according to the manufacturer's instructions (Invitrogen). This reaction mixture was transformed into *E. coli* OmniMAX 2-T1 cells. After confirmation of the correct deletion construct, the plasmid was linearized by I-SceI digestion prior to *C. neoformans* biolistic transformation (38). The transformants were screened by colony PCR, and the deletion was confirmed by Southern blot analysis and semiquantitative reverse transcription-PCR (RT-PCR). For complementation of the mutation, a 4.4-kb genomic PCR fragment carrying the wild-type *VCX1* gene was cloned into the SmaI site of vector pAI4. The resulting plasmid was used for transformation of the *vcx1* mutant strain, and the transformants were selected in the presence of nourseothricin (200 µg/ml). Random genomic insertion of the complemented gene was confirmed by Southern blot analysis and semiquantitative RT-PCR. The primers used in these constructions are listed in Table S1 of the supplemental material.

Phenotypic characterization assays. For phenotypic characterization, wild-type (WT), *vcx1* mutant, and complemented strains were grown on YPD medium for 16 h, washed, and adjusted to a cell density of 10⁷ cells/ml. The cell suspensions were serially diluted 10-fold, and 3 µl of each dilution was spotted onto YPD agar supplemented with CaCl₂ (50, 100, 150, or 200 mM), MnCl₂ (2, 4, 6, or 8 mM), CdCl₂ (20 or 50 µM), and/or cyclosporine A (100 µg/ml). The plates were incubated for 2 days at 30°C, 35°C, or 37°C and photographed. Fungal cells were also grown at 37°C in 5% CO₂ in YPD medium with alkaline, neutral, or acidic pH (8.5, 7, and 4, respectively), or in the presence of Congo Red (0.5%). Melanin production was examined on niger seed agar plates containing 0.1% glucose. Capsule formation was examined by microscopy after incubation for 24 h at 30°C in a minimal medium (12) and staining with India ink. Relative capsule size was defined as the distance between the cell wall and the capsule outer border by cell diameter. ImageJ software was utilized to determine capsule measurements of 100 cells of each strain. The content of extracellular GXM in

these culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) according to a protocol previously described (11). The content of extracellular GXM in culture supernatants in the presence of cyclosporine A (25 to 150 $\mu\text{g/ml}$) was also determined. Phenotypic analysis of the cell surface architecture was performed after incubation of yeast cells with the wheat germ lectin (WGA), calcofluor white, and the monoclonal antibody (MAb) 18B7 (12). These probes were used to visualize, by fluorescence microscopy, the surface distribution of *N*-acetylglucosamine (GlcNAc) oligomers (with WGA), cell wall chitin (with calcofluor), and GXM (with 18B7), following a previously described protocol (12).

Virulence assay. Virulence studies were conducted according to a previously described intranasal inhalation infection model (6) using eight female BALB/c mice (approximately 5 weeks old) for each strain tested. Fungal cells were cultured in 50 ml of YPD medium at 30°C overnight with shaking, washed twice, and resuspended in phosphate-buffered saline (PBS). Mice were infected with 10^7 yeast cells suspended in 50 μl PBS and monitored daily. Kaplan-Meier analysis of survival was performed using GraphPad Prism software. Animal studies were approved by the Federal University of Rio Grande do Sul Ethics Committee.

Macrophage infection assay. The susceptibility of fungal cells to the antifungal action of phagocytes was determined by counting CFU after interaction of WT, *vcx1* mutant, and *vcx1::VCX1*-complemented strains with the murine macrophage-like cell line RAW 264.7. Murine cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS). Fungal cells were opsonized with monoclonal antibody 18B7 (1 $\mu\text{g/ml}$). Macrophages were seeded at a concentration of 10^5 cells/well in a 96-well cell culture plate and incubated overnight. Then, 10^6 fungal cells were inoculated in each well, and after 1 h the wells were washed to remove unattached, extracellular fungal cells. After 20 h of incubation, infected cultures were again washed, and sterile ice-cold distilled H_2O was added to each well to promote macrophage lysis. Fungal viability was measured by plating the lysates on YPD for CFU determination after cultivation of the plates for 48 h at 30°C. The assay was performed in triplicate sets for each strain. Student's *t* test was used to determine the statistical significance of differences in fungal survival. To determine the rate of phagocytosis of WT, *vcx1* mutant, and complemented cells during interaction with RAW 264.7 cells, yeast cells were incubated with fluorescein isothiocyanate (FITC) at 0.5 mg/ml in PBS for 10 min at room temperature. Then, the cells were washed with PBS and incubated with macrophage-like cells for 1 h at a 5:1 fungus/host cell ratio, followed by extensive washing with PBS for removal of nonadherent fungi. The fluorescence intensity of the macrophage-like cells was therefore a function of association with FITC-labeled *C. neoformans*. The infected cells were then detached from tissue culture plates by scraping, fixed with 4% paraformaldehyde, and analyzed in a FACScalibur (BD Biosciences). The data were analyzed using the winMDI 2.9 software.

Construction of *C. neoformans* Vcx1-mCherry fusion strain. In order to assess the subcellular localization of Vcx1 in *C. neoformans* cells, a cassette carrying a Vcx1-mCherry fusion was constructed. Briefly, a 2-kb fragment encompassing the *VCX1* promoter and coding sequences without the stop codon was amplified using genomic DNA from strain H99 as template. The pLKB25 plasmid (18) was utilized as a template to amplify a second fragment containing the mCherry-GPD1 terminator and the neomycin resistance gene. These fragments, which overlap by ~40 bp, were combined and utilized as template for the overlap PCR. The product of the overlap PCR was cloned into the pCRTOP02.1 vector. This final construct was transformed in the *C. neoformans* *vcx1* mutant strain by biolistic transformation (38). Transformants (Vcx1-mCherry strains) were selected in YPD plates with 200 $\mu\text{g/ml}$ of neomycin. These cells were visualized and photographed using an Olympus FluoView 1000 confocal laser scanning microscope.

Quantitative real time RT-PCR analysis. For RNA extraction, cultures of WT and *vcx1* mutant cells were grown overnight in YPD medium at 37°C with shaking. Three independent sets of RNA samples from independent experiments were prepared using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. After DNase treatment, RNA preparations were purified using RNeasy minicolumns (Qiagen), and reverse transcription reactions were performed. Real-time PCRs were performed in an Applied Biosystems 7500 real-time PCR system. PCR thermal cycling conditions were an initial step at 95°C for 5 min followed by 40 cycles at 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s. Platinum SYBR green qPCR Supermix (Invitrogen) was used as reaction mix, supplemented with 5 pmol of each primer and 2 μl of the cDNA template in a final volume of 25 μl . All experiments were performed in three independent cultures, and each cDNA sample was analyzed in duplicate with each primer pair. Melting curve analysis was performed at the end of the reaction to confirm a single PCR product. Data were normalized to actin cDNA levels amplified in each set of PCR experiments. Relative expression was determined by the $2^{-\Delta\Delta\text{CT}}$

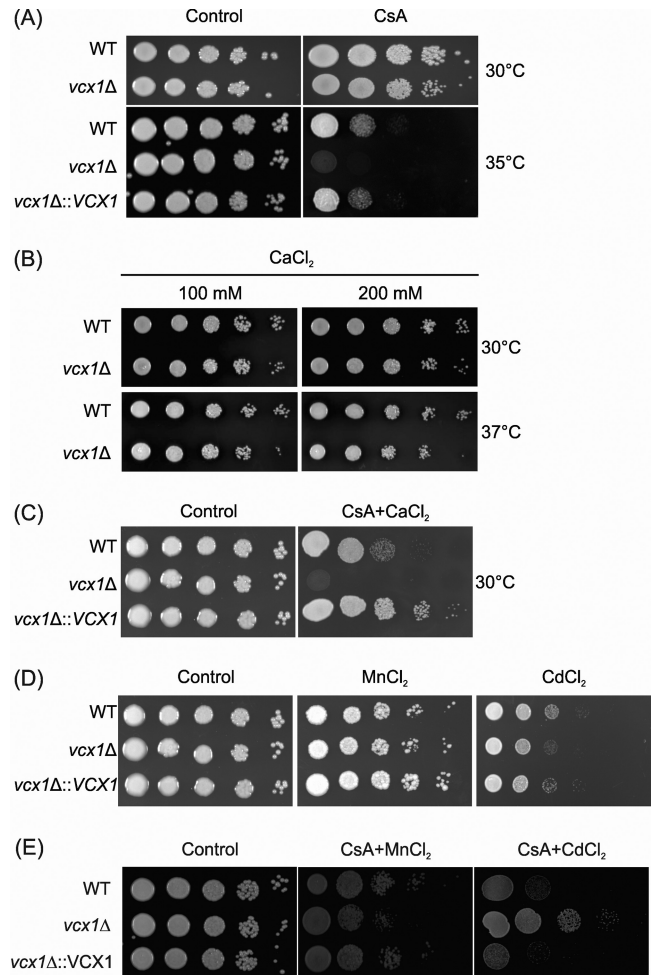


FIG. 2. The *vcx1* mutant displays calcineurin-dependent Ca^{2+} sensitivity. Ten-fold serial dilutions of WT H99, *vcx1* mutant (*vcx1Δ*), and *vcx1::VCX1*-complemented (*vcx1Δ::VCX1*) cells were plated in YPD agar containing 100 $\mu\text{g/ml}$ of CsA (A), 100 or 200 mM CaCl_2 (B), 50 mM CaCl_2 amended with 100 $\mu\text{g/ml}$ of CsA (C), 4 mM MnCl_2 or 50 μM CdCl_2 (D), or 4 mM MnCl_2 or 50 μM CdCl_2 amended with 100 $\mu\text{g/ml}$ of CsA (E). The plates were incubated for 2 days at 30°C, 35°C, or 37°C, as indicated. As control, cells were grown in YPD agar only.

method (25). The primers utilized in these experiments are listed in Table S1 of the supplemental material.

Determination of relative levels of intracellular calcium concentration. The relative intracellular free calcium concentration was determined using the acetoxymethyl ester of Fura-2 (Fura-2-AM; Invitrogen). Briefly, WT and *vcx1* mutant cells were cultured in YPD medium overnight with shaking. Then, 10^7 cells of each strain were incubated for 1 h in fresh YPD amended or not with 100 mM CaCl_2 . The cells were washed three times with PBS and loaded with 10 μM Fura-2-AM for 30 min at 37°C. After extensive washing, Fura-2 fluorescence was measured by alternating the excitation wavelengths at 340 and 380 nm with an emission wavelength fixed at 505 nm. The relative intracellular calcium concentration is expressed as the ratio between fluorescence intensities with excitation wavelengths at 340 and 380 nm. All data presented are representative of three independent experiments.

RESULTS

Identification of the vacuolar calcium exchanger Vcx1 ortholog in *C. neoformans*. The *VCX1* gene was identified in the

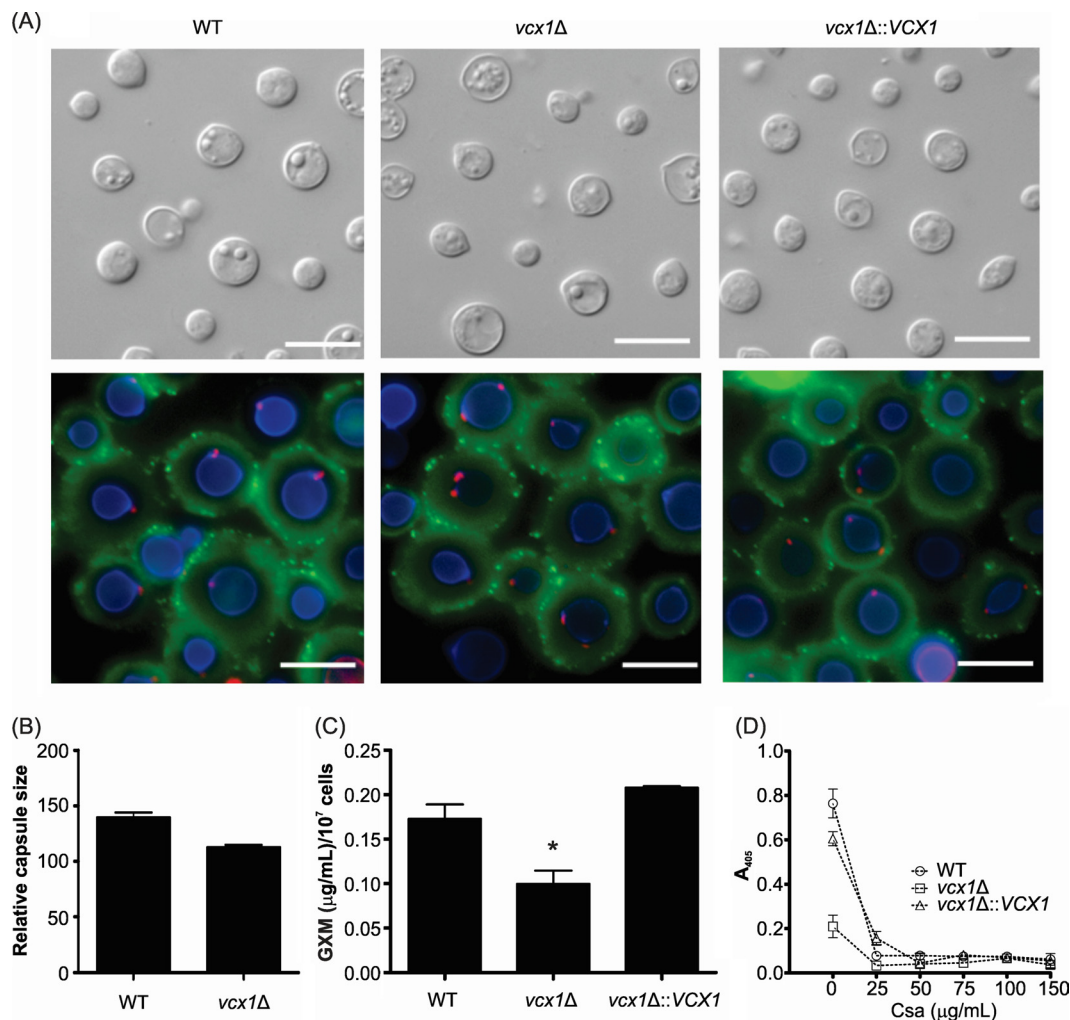


FIG. 3. Disruption of *VCX1* does not influence capsule size but decreases extracellular GXM secretion. (A) Fluorescence microscopy of WT, *vcx1* mutant, and *vcx1::VCX1*-complemented mutant cells stained with the lectin WGA (red spots), calcofluor white (blue staining), and the monoclonal antibody 18B7 (green), to visualize GlcNAc oligomers, cell wall, and GXM, respectively. Bars, 10 μ m. (B) Relative capsule sizes of WT and *vcx1* mutant cells. (C) Content of extracellular GXM in culture supernatants of WT, *vcx1* mutant, and *vcx1::VCX1*-complemented mutant cells, as determined by ELISA. *, $P < 0.05$. (D) Content of extracellular GXM in culture supernatants of WT, *vcx1* mutant, and *vcx1::VCX1*-complemented mutant cells incubated with different concentrations of CsA, as determined by ELISA.

C. neoformans var. *grubii* H99 genomic database of the Broad Institute (accession number CNAG_00025.1) (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html), based on its similarity to the vacuolar calcium exchanger Vcx1 from *S. cerevisiae*. The *C. neoformans* *VCX1* coding region is 2,399 bp long, contains nine introns, and encodes a putative 604-amino-acid protein. Ca^{2+} exchanger proteins are characterized by the presence of calcium/hydrogen antiporter (*Cha4*; COG0387), calcium/proton exchanger (*caca2* [TIGR00846], *cax* [TIGR00378]), and sodium/calcium exchanger superfamily (*Na_Ca_ex*; pfam01699) domains (41). BLAST search using the Conserved Domain Database at NCBI (<http://www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml>) revealed that these domains are also present in the *C. neoformans* Vcx1 ortholog. Furthermore, a phylogenetic analysis including Vcx1 sequences from distinct eukaryotic organisms was performed (Fig. 1A). *C. neoformans* Vcx1 has higher similarity to Vcx1 of *U. maydis* (36% identity and 52% similarity) and lowest similarity to that in *A. thaliana* (21% identity and 39% similarity) when the amino acids of

each protein are compared. The phylogeny tree splits into two clades, corresponding to fungal and plant Vcx1 orthologs. As expected, *C. neoformans* Vcx1 clusters with other basidiomycete fungal Vcx1 proteins. The domain architecture of the *C. neoformans* Vcx1 was compared to the orthologs herein analyzed. All of them possess two well-conserved domains related to the sodium/calcium exchanger superfamily (*Na_Ca_ex*; pfam01699). Moreover, prediction of transmembrane (TM) regions revealed that all the Vcx1 proteins analyzed had 10 or 11 predicted TM domains (Fig. 1B), in agreement with known Ca^{2+} exchangers (41).

The *vcx1* mutant displayed calcineurin-dependent Ca^{2+} sensitivity. In order to investigate the functional role of *VCX1* in *C. neoformans*, knockout and complemented strains were constructed. Deletion and complementation of *VCX1* were confirmed by Southern blot analysis and semiquantitative RT-PCR (see Fig. S1 in the supplemental material). The ability to grow at the human body temperature (36° to 37°C) is one of the most important virulence factors of *C. neoformans* (28).

This trait is controlled in part by the Ca^{2+} -calcineurin pathway, which senses and utilizes cytosolic calcium for signaling (19). We then asked whether the *C. neoformans* Vcx1 participates in this signaling pathway during growth at high temperatures. Our results demonstrated that Vcx1 is required for *C. neoformans* growth at 35°C (semipermissive temperature) only in the presence of cyclosporine A, a calcineurin inhibitor (Fig. 2A). Furthermore, the role of *VCX1* in Ca^{2+} tolerance was evaluated by monitoring growth of the *vcx1* mutant strain in YPD agar plates supplemented with increasing concentrations of CaCl_2 amended or not with cyclosporine A (Fig. 2B and C). The growth of the *vcx1* mutant strain was inhibited only in the presence of cyclosporine A, indicating that Vcx1 confers tolerance to a wider range of calcium concentrations when calcineurin is inhibited (Fig. 2C). Together, these results suggest that Vcx1 influences the Ca^{2+} -calcineurin signaling pathway in *C. neoformans*. Since *S. cerevisiae* calcineurin regulates the transport of cations by variants of Vcx1 (35), we evaluated the Cd^{2+} and Mn^{2+} tolerance of the *C. neoformans* *vcx1* mutant strain. No differences in fungal growth were observed under conditions of a high concentration of CdCl_2 or MnCl_2 (Fig. 2D). However, when calcineurin was inhibited by cyclosporine A, the *vcx1* mutant strain exhibited a slightly increased resistance against high concentrations of CdCl_2 compared to the WT and complemented strains (Fig. 2E).

Disruption of *VCX1* does not influence capsule size, but it decreases extracellular GXM secretion. The *vcx1* mutant strain was tested for its ability to grow at 37°C, for capsule size, and for melanin production, since these traits are thought to be the major virulence factors in *C. neoformans* (31). The *VCX1* knockout apparently did not interfere with any of these features, compared to WT cells (Fig. 3 and data not shown). Moreover, the deletion of *VCX1* also did not cause defects in cell wall integrity, as assessed by staining of chitin and GlcNAc oligomers (Fig. 3A) and by a spot growth assay on plates containing Congo red (see Fig. S2 in the supplemental material). However, mutant cells showed an impaired ability to produce extracellular GXM, as concluded from the lower polysaccharide contents in culture supernatants of the *vcx1* mutant strain than in WT or complemented cells ($P < 0.05$) (Fig. 3C). We believe that the antibody-based lower GXM detection in supernatants of *vcx1* mutant cells reflects a decreased production of this extracellular polysaccharide rather than the production of a structurally modified GXM, since the surface polysaccharide was regularly recognized by MAb 18B7. This observation, which echoed previous findings with a secretion mutant of *C. neoformans* (34), suggests a role for Vcx1 in the release of capsular polysaccharides to the extracellular environment. The levels of extracellular GXM in culture supernatants of WT, *vcx1* mutant, and complemented cells treated with cyclosporine A were also quantified. Different concentrations of cyclosporine A (ranging from 25 to 150 $\mu\text{g/ml}$) completely inhibited GXM secretion by all strains tested (Fig. 3D).

***VCX1* influences *C. neoformans* phagocytosis by macrophages and is required for full virulence in animal cryptococcosis.** The susceptibility of WT, *vcx1* mutant, and *vcx1::VCX1*-complemented strains to the antimicrobial action of macrophage-like cells was evaluated *in vitro*. In comparison to WT and complemented cells, the *vcx1* deletion mutant showed decreased rates of survival after interaction with the

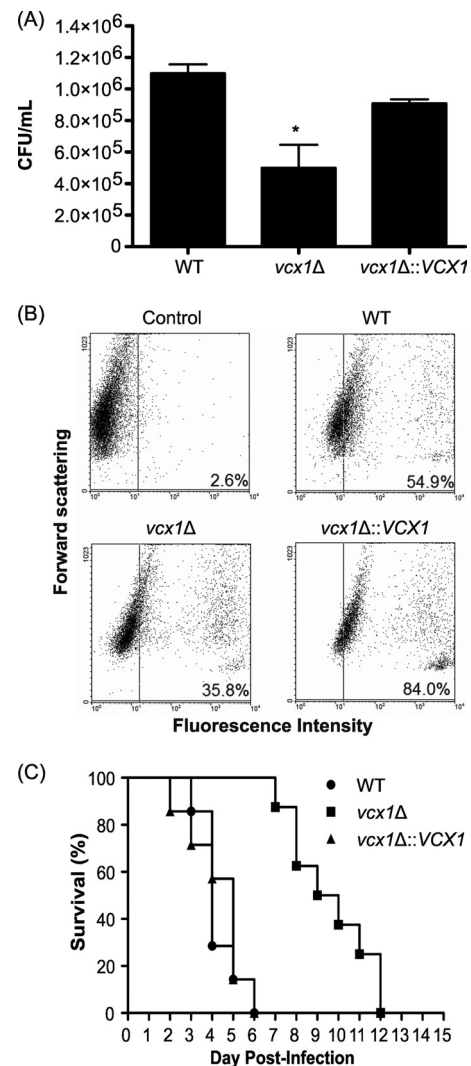


FIG. 4. *VCX1* influences *C. neoformans* phagocytosis by macrophages and is required for full virulence in mice. (A) CFU counts after macrophage infection with WT, *vcx1* mutant, or *vcx1::VCX1*-complemented strains. *, $P < 0.05$. (B) Rates of phagocytosis of WT, *vcx1* mutant, and complemented cells as measured by flow cytometry. (C) Virulence assay of WT, *vcx1* mutant, and *vcx1::VCX1*-complemented strains in an intranasal inhalation infection model with BALB/c mice.

phagocytes (Fig. 4A) ($P < 0.05$). To exclude the possibility that the lower rate of *vcx1* mutant survival inside macrophages was due to its hypersensitivity to extreme pH or to 5% CO_2 , fungal cells were grown in YPD medium with alkaline, neutral, or acidic pH (8.5, 7, and 4, respectively) or in 5% CO_2 . However, no growth differences were observed under these conditions (see Fig. S2 in the supplemental material). The lower CFU counts for the *vcx1* mutant could be due to a decreased survival within the macrophages (lower viability or slower proliferation within the macrophages) or, alternatively, they may be due to a lower rate of phagocytosis. To test this hypothesis, the rates of phagocytosis of WT, *vcx1* mutant, and complemented cells were measured by flow cytometry. In fact, the rate of phagocytosis of the *vcx1* mutant cells was significantly lower than in

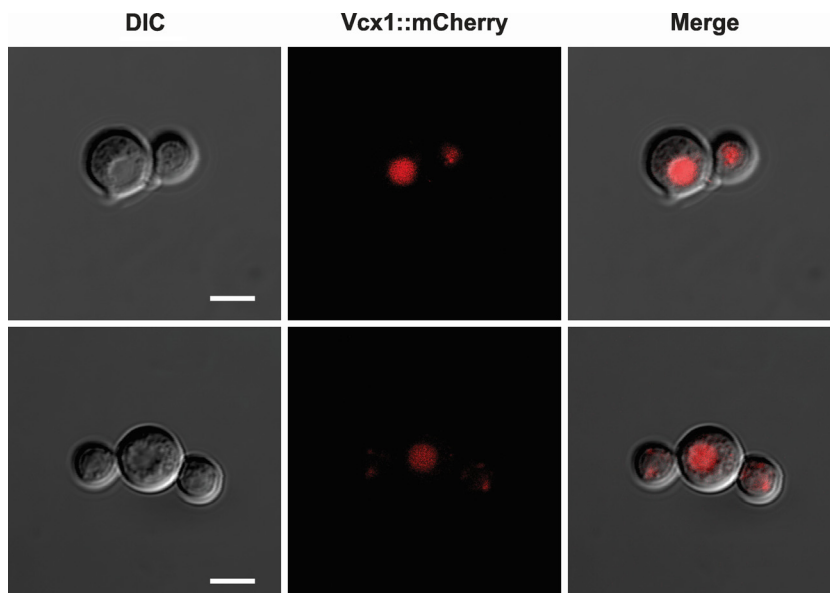


FIG. 5. Vcx1 localizes to the vacuoles of *C. neoformans* cells, as shown via confocal microscopy of Vcx1-mCherry fusion strain cells. The mCherry-tagged Vcx1 (red) colocalizes with vacuole organelles, which appear as a depression in the DIC image. Bars, 5 μ m.

WT or complemented cells (Fig. 4B), indicating that *VCX1* is required for efficient phagocytosis and perhaps has some minor role in survival within the macrophages.

These observations encouraged us to test the role of Vcx1 in the pathogenesis of *C. neoformans* in a mouse inhalation model of cryptococcosis. Mice inoculated with WT or complemented strains had mean survival times of 4 and 5 days, respectively. Statistical analysis revealed no significant difference between mortality rates caused by these two strains ($P = 0.79$). In contrast, mice inoculated with *vcx1* mutant cells survived longer, with a mean survival time of 9.5 days ($P = 0.002$) (Fig. 4C). This result demonstrates that Vcx1 is required for the virulence of *C. neoformans* in animal cryptococcosis.

Vcx1 localizes to the vacuole of *C. neoformans* cells. In order to confirm the predicted vacuolar localization of Vcx1 in *C. neoformans* cells, an mCherry-tagged Vcx1 strain was constructed. Figure 5 illustrates the colocalization of mCherry-tagged Vcx1 with vacuole organelles, which appear as a depression in the differential interference contrast (DIC) image. This result confirmed that Vcx1 localizes to the vacuole of *C. neoformans* cells.

Disruption of *VCX1* influences the expression of other calcium transporters in *C. neoformans*. Because *C. neoformans* Cch1 and Eca1 are also calcineurin pathway calcium transporters, the transcript levels of these genes were evaluated in WT and *vcx1* mutant strains. Disruption of *C. neoformans* *VCX1* led to a decrease in expression of *ECA1*, but the *CCH1* transcription level was not influenced. Furthermore, we also analyzed the expression of two putative orthologs of well-characterized calcium transporters of *S. cerevisiae*, *PMCI*, a vacuolar calcium ATPase, and *PMRI*, a Golgi apparatus calcium ATPase (8, 29). Interestingly, the transcript levels of the *C. neoformans* *PMCI* ortholog significantly increased in *vcx1* mutant cells ($P < 0.05$). A slight increase in *PMRI* ortholog expression was also observed in *vcx1* mutant cells (Fig. 6).

Loss of *VCX1* influences the relative level of intracellular calcium concentration in *C. neoformans* cells.

In order to determine the relative concentration of free calcium in WT and *vcx1* mutant strains, the calcium-sensitive dye Fura-2-AM was utilized. This fluorescent calcium indicator can passively diffuse across cell membranes. Once inside the cell, the esters are cleaved by intracellular esterases to yield cell-impermeant fluorescent indicators. Upon binding Ca^{2+} , Fura-2 exhibits an absorption shift from 380 to 340 nm of excitation. Therefore, the relative Ca^{2+} concentration was obtained based on the fluorescence ratio after dual-wavelength excitation. The *vcx1* mutant strain had an increased relative level of intracellular calcium concentration compared to the WT strain (Fig. 7). This increase was more pronounced when an excessive amount of CaCl_2 was added to the medium, indicating that the loss of

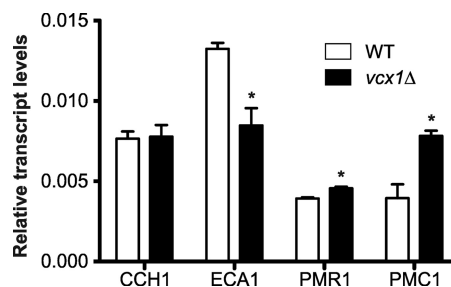


FIG. 6. Disruption of *VCX1* influences expression of other calcium transporters in *C. neoformans*. The relative expression levels of different *C. neoformans* calcium transporters (*CCH1*, *ECA1*, *PMRI*, and *PMCI*) in WT and *vcx1* mutant cells were quantified by RT-PCR. The measured quantity of the mRNA in each of the samples was normalized using the threshold cycle (C_T) values obtained for the actin gene. The accession numbers for the *C. neoformans* orthologs of *PMRI* and *PMCI* from the Broad Institute database are CNAG_05135.2 and CNAG_01232.2, respectively. Data shown are means \pm standard deviations. *, $P < 0.05$.

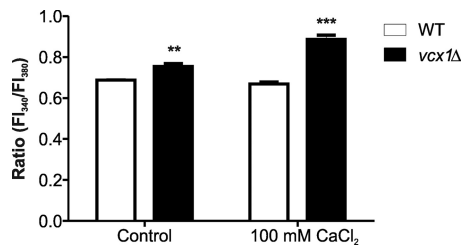


FIG. 7. Disruption of *Vcx1* influences the relative levels of intracellular calcium concentration in *C. neoformans*. The relative levels of intracellular calcium in WT and *vcx1* mutant cells were determined using the calcium-sensitive dye Fura-2-AM. The relative Ca²⁺ concentration was determined based on the fluorescence ratio after dual-wavelength excitation (fluorescence intensity at 340 nm [F₁₃₄₀]/F₁₃₈₀). The control was fresh YPD medium. Data shown are means \pm standard deviations. **, $P < 0.05$; ***, $P < 0.01$.

Vcx1 influences the relative level of intracellular calcium concentration. This phenotype is probably due to a calcium transport defect of vacuoles in the *vcx1* mutant strain, because Ca²⁺ exchangers regulate the concentration of cytosolic Ca²⁺ and its transport to storage organelles (41).

DISCUSSION

The calcium-calcineurin signaling pathway in *C. neoformans* is fundamental to sense and to adapt to the human host milieu. In addition to its importance for high-temperature growth, calcineurin is also essential for cell wall integrity, mating, and monokaryotic fruiting (7, 13, 19, 20, 28, 33). In the present study *Vcx1*, a newly recognized component of the *C. neoformans* Ca²⁺ signaling network, was identified. This vacuolar calcium exchanger is part of a conserved family of Ca²⁺ exchangers that regulate cytosolic calcium concentration and transport into Ca²⁺ storage organelles (i.e., vacuoles) in distinct eukaryotes (41).

First, we investigated if *C. neoformans* *Vcx1* acts in the Ca²⁺-calcineurin pathway under host temperature growth conditions. In fact, *Vcx1* is required for *C. neoformans* growth at 35°C only when calcineurin has been inhibited. Moreover, *Vcx1* participates in Ca²⁺ tolerance in a calcineurin-dependent manner. These findings suggest that *Vcx1* influences the Ca²⁺-calcineurin signaling pathway in *C. neoformans*, in agreement with findings reported for the *S. cerevisiae* *Vcx1* ortholog (9, 35). Calcineurin significantly affects *Vcx1* Ca²⁺/H⁺ exchange activity in *S. cerevisiae*. The negative regulation of Sc*Vcx1* by calcineurin is thought to be posttranslational and independent of the transcription factor Crz1, which regulates genes encoding ion pumps and cell wall biosynthesis enzymes (9, 30, 36). No ortholog of the *CRZ1* gene has been identified in *C. neoformans*. It is possible that *C. neoformans* contains a different transcription factor or more than one transcription factor responsive to calcineurin (19).

The loss of *C. neoformans* *Vcx1* by knockout did not cause defects in cell wall integrity, melanin production, or capsule size, but the *vcx1* mutant showed a reduced ability to produce extracellular GXM. Although the concentration of extracellular GXM is usually related to capsule enlargement, Panepinto and colleagues recently demonstrated that a *sec6* mutant of *C. neoformans* produced a normal capsule even under conditions

in which GXM secretion was decreased (34). Our result suggests that, although yeast cells lacking *Vcx1* expression are able to assemble a normal capsule, the protein is somehow required for polysaccharide release.

A key feature of cryptococcal pathogenesis is its ability to survive and replicate inside macrophages, in a process that requires GXM release for further accumulation in cytoplasmic vesicles (39). GXM is toxic for the macrophages, which implies that polysaccharide secretion reduces the antimicrobial activity of phagocytes. Furthermore, *C. neoformans* possesses mechanisms that allow cell-to-cell spread and extrusion from infected macrophages (1, 2, 26, 27, 39, 40). These processes supposedly require production of GXM. Therefore, capsular polysaccharide interferes with macrophage function at multiple levels (reviewed in reference 40). In this context, the defective ability to produce extracellular GXM that was observed in the *vcx1* mutant could be related to its reduced rate of phagocytosis and survival after macrophage infection.

In our study, *Vcx1* was required for full virulence during infection. This same phenotype was observed for *C. neoformans* mutants lacking expression of *Cch1* and *Eca1*, a plasma membrane calcium channel and a sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase, respectively (10, 24). These results emphasize the key role of calcium signaling for cryptococcal virulence. Calcineurin is essential for *C. neoformans* survival in the host environment (19, 33). Our results showed that in the presence of the calcineurin inhibitor cyclosporine A, the *vcx1* mutant failed to grow at the high temperature, suggesting that *Vcx1* acts in parallel with calcineurin in response to the host's temperature. *C. neoformans* *Vcx1* also influences the expression of other calcium transporters. The transcript level of the *PMC1* ortholog, a putative vacuolar calcium ATPase, was significantly increased in the *vcx1* mutant compared to the WT strain, probably due to a compensatory effect, since *PMC1* also transports calcium into vacuoles (8).

In conclusion, we have shown that *Vcx1*, a vacuolar calcium transporter, influences *C. neoformans* phagocytosis by macrophages and is required for full virulence in animal infection. Additionally, *Vcx1* is involved in calcineurin-dependent Ca²⁺ tolerance, acts in the Ca²⁺-calcineurin signaling pathway in *C. neoformans*, and influences the relative intracellular calcium concentration. Further studies are necessary to address the role of *Vcx1* in the release of GXM to the extracellular environment and to completely understand how this component of the Ca²⁺-calcineurin pathway contributes to the pathogenesis of cryptococcosis.

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