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Eca1, a Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase, Is Involved in Stress Tolerance and Virulence in *Cryptococcus neoformans*▽

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The basidiomycetous fungal pathogen *Cryptococcus neoformans* is adapted to survive challenges in the soil and environment and within the unique setting of the mammalian host. A *C. neoformans* mutant was isolated with enhanced virulence in a soil amoeba model that nevertheless exhibits dramatically reduced growth at mammalian body temperature (37°C). This mutant phenotype results from an insertion in the *ECA1* gene, which encodes a sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+}-ATPase (SERCA)-type calcium pump. Infection in murine macrophages, amoebae (*Acanthamoeba castellanii*), nematodes (*Caenorhabditis elegans*), and wax moth (*Galleria mellonella*) larvae revealed that the *eca1* mutants are virulent or hypervirulent at permissive growth temperatures but attenuated at 37°C. Deletion mutants lacking the entire *ECA1* gene were also hypersensitive to the calcineurin inhibitors cyclosporin and FK506 and to ER and osmotic stresses. An *eca1*Δ *cna1*Δ mutant lacking both Eca1 and the calcineurin catalytic subunit was more sensitive to high temperature and ER stresses than the single mutants and exhibited reduced survival in *C. elegans* and attenuated virulence towards wax moth larvae at temperatures that permit normal growth in vitro. Eca1 is likely involved in maintaining ER function, thus contributing to stress tolerance and virulence acting in parallel with Ca^{2+}-calcineurin signaling.

Nonobligate human pathogens confront and must survive diverse environmental conditions. The ability to survive and proliferate in various challenging environmental niches, including in environmental microbial predators, is hypothesized to contribute to the ability of *Cryptococcus neoformans*, a basidiomycete fungus of global distribution, to cause disease in humans (15, 37, 49). The success of this pathogen is also attributed to its ability to survive at mammalian body temperature, which is an uncommon trait among fungal species. Tolerance to thermal stress (caused by body temperature) and oxidative stresses (from mammalian host defenses) is critical for successful infection. *C. neoformans* mutants with increased sensitivity to oxidative or nitrosative challenge, including those lacking superoxide dismutase Sod1 or Sod2 (18, 35), alternative oxidase Aox1 (2), flavohemoglobin denitrosylase Fhb1 (23), thiol peroxidase Tsa1 (48), or thioredoxin Trx1 (47), exhibit attenuated virulence. Temperature-sensitive mutants have been isolated in *C. neoformans*, including *cna1* (calcineurin catalytic subunit), *cnb1* (calcineurin regulatory subunit), *cpa1* (cyclophilin A), and *ras1* strains, and all of these have reduced virulence in mammalian host models (3, 29, 55, 73).

Calmodulin and calcineurin are central mediators of calcium signaling and respond to a multiplicity of stresses. Calmodulin is a Ca^{2+} sensor that undergoes conformational changes upon Ca^{2+} binding and transduces this Ca^{2+} signal to other proteins, such as calcineurin and Ca^{2+}/calmodulin-dependent protein kinases (CaMK), via protein-protein interactions. Calcineurin is a highly conserved Ca^{2+}/calmodulin-activated serine/threonine protein phosphatase that exists as a heterodimer of a catalytic A subunit and a regulatory B subunit. It is the target of the immunosuppressive drugs cyclosporin (CsA), which binds to cyclophilin A, and FK506, which binds to FKBP12. Calcineurin transduces Ca^{2+} signaling by activating the transcription factors NFAT in mammalian T cells and Crz1 in the yeasts *Saccharomyces cerevisiae* and *Candida albicans* (21, 36, 39). In *C. neoformans*, calcineurin is required for mating, morphogenesis, growth at 37°C, and virulence in murine models (20, 29, 55). However, the downstream signaling events regulated by calcineurin and the factors affecting calcineurin function are still largely unknown in *C. neoformans* and other pathogenic fungi.

Cellular Ca^{2+} homeostasis is maintained through the coordinated actions of Ca^{2+} pumps on the plasma, vacuolar, Golgi apparatus, and endoplasmic reticulum (ER) membranes. The sarcoplasmic/endoplasmic Ca^{2+}-ATPases (SERCAs) are a family of ER Ca^{2+} pumps that are highly conserved in eukaryotic organisms. The ER is the cellular compartment for protein posttranslational processing, such as folding, glycosylation, assembly of protein complexes, and transport to the Golgi apparatus. ER function requires the maintenance of high Ca^{2+} concentrations, an oxidative milieu in the lumen, and the activity of molecular chaperones (reviewed in reference 12). The ER is also a major site of Ca^{2+} storage, with release of Ca^{2+} into the cytosol in response to certain stimuli. SERCAs are important in replenishing Ca^{2+} levels in the ER by transport-

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ing Ca^{2+} from the cytosol to the ER and thereby play critical roles in maintaining Ca^{2+} homeostasis in both the ER and the cytosol. In mammals, SERCAs are important for muscle relaxation and deficiencies in SERCAs are linked to multiple genetic diseases (46, 74). In the plant *Arabidopsis thaliana*, the *ECA1* gene encodes a SERCA that is required for growth under low Ca^{2+} or high Mn^{2+} concentrations (75). In the parasite *Leishmania mexicana amazonesis*, a SERCA-type Ca^{2+} pump (Lmaa1) is involved in virulence (59). In the basidiomycete plant pathogen *Ustilago maydis*, *ECA1* encodes a SERCA required for growth at high temperature and hyphal development (1).

Forward genetic screens are a powerful approach to identify new gene functions. Recently we identified a component in the Ca^{2+} signaling pathway of *C. neoformans* (i.e., calmodulin) through an insertional mutagenic screen of temperature-sensitive mutants (42). Here we report the identification of the *C. neoformans* gene *ECA1*, encoding a SERCA-type Ca^{2+} pump, by screening a collection of insertion mutants in an amoeba model of virulence. Genetic analysis indicated that *ECA1* is involved in tolerance to multiple stresses and is required for growth at 37°C. Inhibition or mutation of the Ca^{2+}-activated protein phosphatase calcineurin aggravates *eca1*Δ mutant phenotypes. Infection in four host models suggests that Eca1 contributes to virulence in a temperature-dependent manner: at 37°C, the *eca1* mutants are hypovirulent, yet at temperatures permissive for in vitro growth the *eca1* mutants have wild-type or enhanced virulence. Our results further extend our understanding of the connections between Ca^{2+} signaling with stress tolerance and virulence and suggest that the maintenance of virulence of *C. neoformans* in the environment is a trait of greater complexity than simple positive selection in microbial predators.

MATERIALS AND METHODS

Strains and growth conditions. The *C. neoformans* strains used in this study are listed in Table 1. The serotype A *MAT*α strain H99 and congenic *MAT*α KN99α and *MAT***a** KN99**a** strains were used as the wild type (53, 57). The calcineurin subunit A *cna1*Δ deletion strains were as reported previously (40). The collection of ~590 insertional mutants was generated previously by biolistic transformation of strain H99 with plasmid pCH233 conferring resistance to nourseothricin (38). *C. neoformans* was grown in YPD (1% yeast extract, 1% Bacto peptone, 2% dextrose) liquid or solid (supplemented with 2% agar) medium. For treatments, compounds were added after the medium was autoclaved. The macrophage cell line J774A.1 (ATCC TIB-67) is derived from a murine (BALB/c, haplotype *H-2*^{d}) reticulum sarcoma (58). Macrophage cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1% nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% NCTC-109 medium (Invitrogen). The cell line was used between passages 4 and 15. The amoeba *Acanthamoeba castellanii* (ATCC 30234) was maintained at 22°C in PYG medium (ATCC medium 712) following ATCC instructions. *Galleria mellonella* (wax moth) larvae were obtained from Vanderhorst, Inc. (St. Marys, OH) and maintained in wood shavings at room temperature (22°C). The standard *Caenorhabditis elegans* strain N2 Bristol was propagated on *Escherichia coli* strain OP50 lawns on nematode growth medium (NGM) at 25°C.

Identification and disruption of *ECA1*. The hypervirulent mutant AI-5C9 was identified in a collection of insertion mutants by screening for altered virulence in amoebae, using the virulence assay as described previously (65) and below. The genome insertion junction of AI-5C9 was cloned by inverse PCR and sequenced. The *ECA1* gene was identified in serotype A and D strains by performing BLASTn searches of the Duke University *C. neoformans* serotype A genome database (http://cneo.genetics.duke.edu) and The Institute for Genomic Research (TIGR) serotype D database (45) with the sequence of the insertion junction. Targeted replacement of the *ECA1* open reading frame in strain H99 was achieved by biolistic transformation with an overlap PCR product in which a *NAT* cassette was flanked by a 1-kb fragment from upstream of the *ECA1* start codon and a 1-kb fragment from downstream of the *ECA1* stop codon. Transformants were selected on YPD agar medium plates containing 100 mg/liter nourseothricin at 30°C and then screened by PCR for an *eca1*Δ strain, and the deletion was confirmed by Southern and Northern blot analyses. The *eca1*Δ mutation was complemented by reintroducing a wild-type version of the gene. *ECA1* was amplified from strain JEC21 and cloned into plasmid pPZP-NEO1 (72). *Agrobacterium tumefaciens* strain EHA105 was transformed with this plasmid by electroporation and used to transform *C. neoformans* to neomycin resistance using methods reported previously (38).

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

Strain	Genotype	Parent strain/background	Source or reference
H99	Wild-type *MAT*α		57
KN99α	Wild-type *MAT*α		53
KN99**a**	Wild-type *MAT***a**		53
AI-5C9	*eca1*::*NAT MAT*α	H99	38
WF04-11/*eca1-11*	*eca1*::*NAT MAT*α	H99	This study
WF04-26/*eca1-26*	*eca1*::*NAT MAT*α	H99	This study
AI137	*eca1*::*NAT MAT***a**	WF04-11 × KN99**a**	This study
AI150	*eca1*::*NAT ECA1-NEO MAT*α	WF04-11	This study
KK8	*cna1*::*NEO MAT***a**	KN99**a**	40
WF06-1	*cna1*::*NEO eca1*::*NAT MAT*α	WF04-11 × KK8	This study

Generation of an *eca1*Δ *cna1*Δ double mutant. The *eca1*Δ mutant (NAT^R) in the *MAT*α background (parent strain H99) was crossed with a *cna1*Δ (Neo^R) mutant in the *MAT***a** background (parent strain KN99**a**) on V8 agar medium. Basidiospores were dissected 9 days after mating and were allowed to germinate on YPD medium at 30°C. The isolates were then tested for the presence of both nourseothicin and neomycin resistance markers, and the presence of both *eca1* and *cna1* mutations in these strains was confirmed by PCR analysis.

RNA extraction and Northern blot analysis. Total RNA was extracted from *C. neoformans* with the TRIzol reagent (Invitrogen), separated by electrophoresis in a 1.2% denaturing agarose gel, and transferred to nylon membrane (Hybond H+; Amersham Biosciences, United Kingdom). The blot was hybridized with ^{32}P-labeled probes in the ULTRAhyb buffer (Ambion, Austin, TX) according to the manufacturer's instructions. Autoradiograph images were analyzed with NIH *Image* software.

Virulence assays. Virulence assays were conducted in four model systems as described previously: amoeba *A. castellanii* (65), murine macrophage cell line J774A.1 (26, 27), the nematode *C. elegans* (50, 71), and *G. mellonella* (wax moth) larvae (52). For most assays, *C. neoformans* cells were cultured in YPD medium at 30°C to mid-to-late log phase, and the cells were washed three times with phosphate-buffered saline (PBS) and resuspended in PBS to the desired concentrations as the inocula. Cell numbers were determined by counting with a hemocytometer. For *C. elegans* assays, *C. neoformans* strains were cultured overnight and spread onto brain heart infusion (BHI) agar to create lawns.

For the amoeba virulence assay, *A. castellanii* was cultured in PYG medium in 96-well microtiter plates to a density of 1×10^5 cells/well, and *C. neoformans* cells were added to the amoebae at a multiplicity of infection (MOI) of 2:1. The amoeba-yeast coculture was incubated at either 30 or 37°C for 24 or 48 h before being lysed with ice-cold H_2O containing 0.05% sodium dodecyl sulfate. Serial dilutions of the lysate were plated on YPD, and the CFU served as an indicator of *C. neoformans* virulence.

For the macrophage assay, J774A.1 cells were grown in 96-well microtiter plates to a density of 1×10^5 cells/well, and *C. neoformans* cells were added to the macrophages at an MOI of 2:1, in the presence of 1 μg/ml monoclonal antibody 18B7 (provided by Arturo Casadevall [14]), 50 U/ml of recombinant mouse gamma interferon (Roche Molecular Biochemicals, Mannheim, Germany), and 0.3 μg/ml lipopolysaccharide (Sigma-Aldrich, St. Louis, MO). After 1 h of incubation at 37°C in the presence of 10% CO_2, unattached *C. neoformans* cells were washed off by aspiration with fresh medium and the macrophage-yeast coculture was incubated for a further 24 h, at which time the macrophages were lysed with ice-cold H_2O containing 0.05% sodium dodecyl sulfate and serial dilutions of the lysate were plated on YPD. CFU served as the measure of *C. neoformans* virulence.

Three *C. elegans* system endpoints were used to examine the virulence prop-

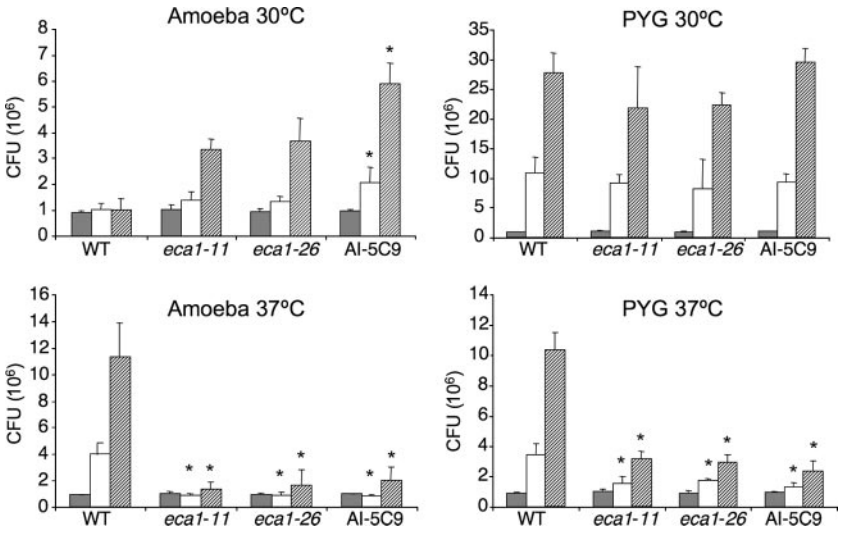

FIG. 1. *eca1* mutation enhances virulence in the amoeba *A. castellanii*. Cryptococcal yeast cells of the wild type (WT), *eca1* insertion mutant AI-5C9, and two independent *eca1*Δ deletion mutants (*eca1-11* and *eca1-26*) were inoculated into amoeba cell culture at an MOI of 2:1 (left panels) or into amoeba-free medium (PYG; right panels), and were cultured at either 30°C (top panels) or 37°C. A total of 1×10^6 cryptococcal cells were inoculated into amoebae or medium (gray bars), and fungal cells were isolated 24 h (white bars) or 48 h (hatched bars) after incubation, with growth measured by plating on YPD medium and counting CFU after culture at 30°C for 2 days. *, $P < 0.05$.

erties of the *C. neoformans* strains: nematode survival, nematode progeny production, and fungal burden within nematodes. *C. elegans* worms were monitored for survival on *C. neoformans* lawns as described previously (50, 51). Fungal strains were grown in 2 ml of YPD medium with kanamycin (45 μg/ml), ampicillin (100 μg/ml), and streptomycin (100 μg/ml) with shaking at 30°C for 24 to 48 h. Lawns were prepared by spreading 10 μl of each culture on 35-mm tissue culture plates containing BHI agar with the same antibiotics. The plates were incubated at 30°C for 24 h and then at 25°C for 24 h. Approximately 60 nematodes at the L4 stage were transferred from NGM seeded with *E. coli* strain OP50 to each of four BHI plates per *C. neoformans* strain. The plates were incubated at 25°C, and the worms were examined for survival at 24-h intervals with a Nikon SMZ645 dissecting microscope. At each interval, inviable worms were counted and removed.

For *C. elegans* progeny quantification, *C. neoformans* lawns were prepared as described above. One nematode at the L4 stage was moved from NGM plates seeded with *E. coli* OP50 to each of 24 lawns per *C. neoformans* strain. At 24-h intervals, living worms were transferred to new lawns for each strain, and at 72 h the living worms were removed. Each BHI agar pad was inverted onto a 100-mm tissue culture plate containing NGM agar with streptomycin (100 μg/ml) and seeded with *E. coli*. The plates were incubated at 25°C for 48 h, and the progeny were counted. Only worms that survived all 3 days were included in calculating the total progeny laid over the course of 72 h.

The *C. neoformans* burdens within infected *C. elegans* were quantified as described previously (34), but with 10 animals at the L4 stage in each group and only one 48-h time point of exposure to *Cryptococcus* lawns prepared on BHI plates as described above. After exposure, animals were washed twice in 8-μl drops of M9 medium on a BHI agar plate containing antibiotics as described above, in order to remove surface cryptococcal cells. Each group of 10 worms was then placed in 40 μl of M9 medium with 1% Triton X-100 and ground with a pestle. The volume was adjusted to 600 μl with M9 medium containing 1% Triton X-100, and the final suspension was serially diluted and plated on YPD agar containing the same antibiotics. The plates were incubated for 48 h at 30°C, and CFU were counted.

For virulence in the wax moth assay, each *G. mellonella* larva was injected in the terminal pseudopod with *C. neoformans* cells (1×10^5 in 5 μl PBS). Larvae were incubated at 30 or 37°C, and virulence was measured by scoring the survival of the larvae every 24 h.

RESULTS

Isolation of a hypervirulent, temperature-sensitive insertion mutant. Nonmammalian models have been developed to test virulence properties of *C. neoformans* and other human pathogens, leading to their use in forward genetic screening to identify attenuated mutants and thereby genes required for virulence. During a screen for *C. neoformans* strains with altered virulence in amoeba, one hypervirulent strain (AI-5C9) was identified out of 590 insertional mutants examined (Fig. 1), and its in vitro phenotypes were analyzed for known virulence attributes. Capsule and melanin were produced at wild-type levels. Strain AI-5C9 displayed normal growth at 30°C on YPD medium or the PYG medium used in amoeba interactions. However, when cultured at 37°C, growth of mutant AI-5C9 was significantly reduced compared with that of the wild-type strain, H99 (Fig. 1 and 2A). Although strain AI-5C9 exhibits reduced growth at 37°C, the cells display normal cellular morphology even after being cultured at 37°C for 2 days (data not shown). Because of its paradoxical increase in virulence in amoeba yet reduced growth rate at mammalian body temperature, the mutant strain was further characterized.

The *ECA1* gene encodes a SERCA-type calcium pump. Segregation analysis of the progeny produced by mating the AI-5C9 mutant with the congenic *MAT***a** strain KN99**a** confirmed that the NAT resistance marker is linked with the temperature-sensitive phenotype. Strain AI-5C9 mated normally with KN99**a**, producing wild-type amounts of viable basidiospores. Of those progeny analyzed, 6/13 were nourseothricin resistant and temperature sensitive and 7/13 were nourseothricin sensitive and temperature resistant, indicating 100% genetic linkage. Likewise, in crosses between **a** *eca1* and α *eca1* mutants, mating occurred to produce filaments and basidiospores (data not shown). The site of insertion of the plasmid into the *C. neoformans* genome was identified by inverse PCR and DNA sequencing. The AI-5C9 mutant carries an insertion in a gene encoding a predicted protein of 1,006 amino acids that belongs to the P-type calcium ATPase family. The corresponding gene in the serotype D strain JEC21 genome, which has been se-

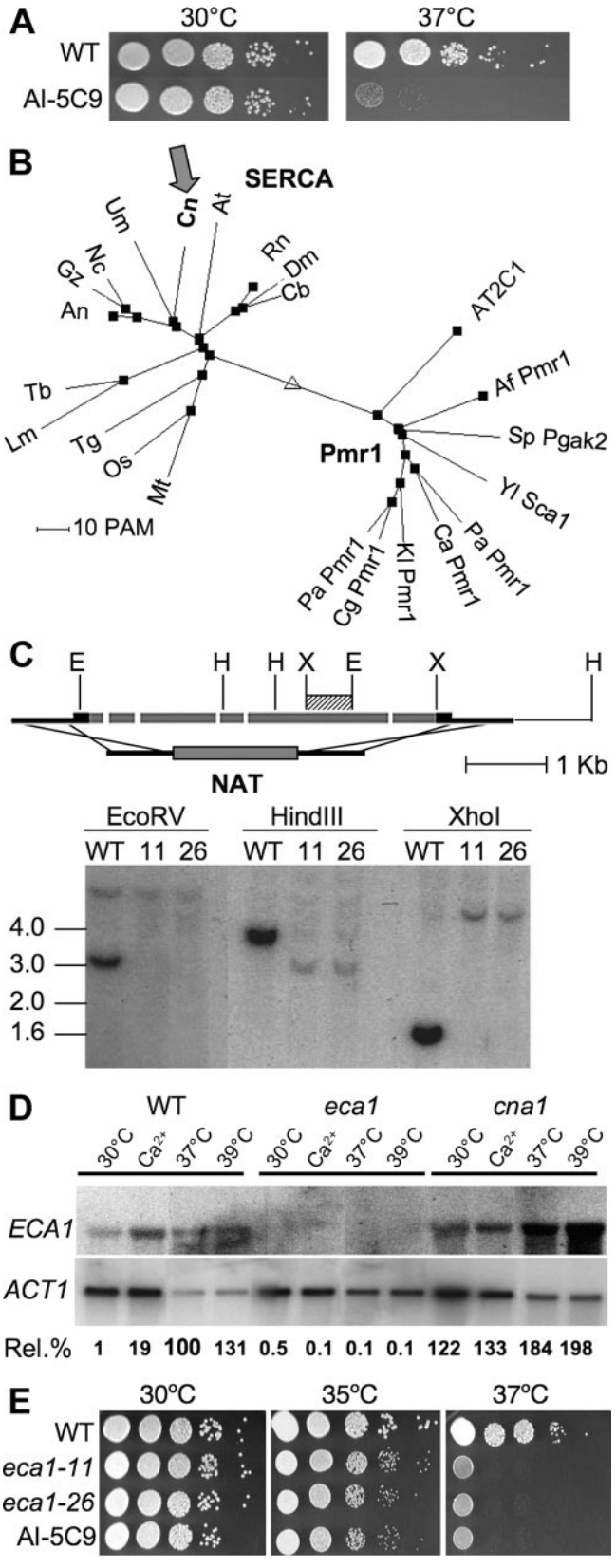

FIG. 2. Characterization of *C. neoformans* Eca1. (A). Temperature-sensitive growth of *eca1* insertion mutant AI-5C9. Ten-fold dilutions of AI-5C9 and the wild-type (WT) H99 cells were plated on YPD agar and cultured at 25°C or 37°C for 2 days. (B) Phylogenetic analysis of Eca1 and its homologs. Amino acid sequences of *C. neoformans* Eca1 (Cn) and P-type Ca^{2+} pumps from other organisms were aligned using the MultAlin program (17). A phylogenetic tree was deduced to depict the evolutionary relationships of the homologs. All of the SERCA-type pumps cluster at the left node (labeled SERCA), and the fungal Pmr1 or mammalian AT2C1 pumps cluster at the right node (labeled Pmr1). (C). Schematic diagram of the targeted deletion of the *ECA1* gene. The genomic DNA structure of *ECA1* is illustrated as gray boxes for the exons, and the entire coding region was replaced with a nourseothricin resistance cassette (NAT). Shown are the results of Southern analysis of genomic DNA from two independent isolates, the *eca1-11* (lanes 11) and *eca1-26* (lanes 26) mutants, and parental strain H99 (WT). Genomic DNA was digested with EcoRV, HindIII, or XhoI. The probe used is indicated by the hatched bar. (D) Northern blot analysis of *ECA1* expression. Total RNAs were isolated from wild-type, *eca1*, and *cna1* cells cultured at 30°C in YPD medium (30°C) or YPD medium supplemented with 20 mM of $CaCl_2$ (Ca20) and cells cultured in YPD medium at 37°C (37°C), and the Northern blot was hybridized with ^{32}P-labeled probes for actin (*ACT1*) and *ECA1*. The autoradiography images were analyzed with NIH *Image* software to quantify the expression levels. Transcription of *ECA1* in each sample was normalized by the *ACT1* gene and compared with the 37°C-treated wild-type sample, the value of which was set to 100%. The relative levels of *ECA1* expression are listed below the images (Rel. %). (E). Deletion of *ECA1* resulted in temperature-sensitive phenotypes identical to that of AI-5C9. Ten-fold serial dilutions of *eca1-11*, *eca1-26*, and wild-type strains were plated on YPD agar and cultured at 30°C, 35°C, or 37°C for 2 days.

quenced and annotated (45), is CNH02370. Although its closest homolog in *Saccharomyces cerevisiae* is Pmr1, which is a Ca^{2+} pump localized to the Golgi apparatus, the *C. neoformans*-encoded protein is more closely related to SERCA pumps in basidiomycetous fungi and other organisms, including plants, worms, fruit flies, mice, and humans. This is illustrated by the phylogenetic analysis of the deduced amino acid sequences of the *C. neoformans* gene and related P-type calcium pumps (Fig. 2B). The closest homolog of CNH02370 in the GenBank database is the *ECA1* gene from *U. maydis* (E = 0.0) (1), and the *C. neoformans* gene was therefore named *ECA1*. Alignment of the Eca1 amino acid sequence with that of rabbit SERCA1, whose structure has been solved by X-ray crystallography and well studied, showed that not only do they share a high degree of similarity in protein primary structure (54% identity, 68% similarity), but the overall domain structure and critical amino acid residues, such as the nucleotide binding sites and a phosphorylation site (DKTGT), are all conserved.

The *ECA1* gene was deleted by homologous recombination to generate two independent *eca1*Δ deletion mutants (Fig. 2C). Southern blot analysis confirmed that the *ECA1* coding region was deleted, resulting in a null mutant (Fig. 2C). *ECA1* expression was undetectable in the *eca1*Δ mutants, as shown by Northern blotting (Fig. 2D), as expected for deletion mutants. The *ECA1* transcript was induced after treatment of wild-type cells at 37°C for 1 h, and its expression was similar to that of the wild type in another temperature-sensitive *C. neoformans* strain background (calcineurin *cna1* mutant). When two independent *eca1*Δ strains (*eca1-11* and *eca1-26*) were cultured at the permissive growth temperature (30°C) or elevated temperatures (35°C and 37°C), both *eca1*Δ isolates displayed the same enhanced virulence in amoebae and temperature sensitivity as

the original AI-5C9 insertion mutant (Fig. 1 and 2E). The *eca1*Δ mutants grew at a rate similar to that of the wild type at 30°C. At 35°C, the mutants grew more slowly than the wild type, while at 37°C their growth was dramatically inhibited (Fig. 2E). An *eca1*Δ deletion mutant was crossed to the congenic strain KN99**a**, and 24 progeny were analyzed. The 8 nourseothricin resistance mutants were all temperature sensitive, while the 16 nourseothricin-sensitive strains grew like the wild type at 37°C, indicative of genetic linkage between the *eca1* mutation and the temperature-sensitive growth phenotype. A wild-type copy of *ECA1* was reintroduced into the *eca1*Δ strain, and it complemented the temperature-sensitive phenotype (data not shown). Together, these results confirm that Eca1 regulates virulence in amoebae and is important for *C. neoformans* growth at high temperatures.

The *eca1*Δ mutant is hypersensitive to calcineurin inhibitors. Previous research on *C. neoformans* has identified a set of temperature-sensitive mutants affected in the Ca^{2+}-calmodulin-calcineuin A and B pathway; thus, it seemed probable that Eca1 might govern calcineurin signaling. The temperature-sensitive phenotype of *eca1* mutants is shared with *cna1* or *cnb1* calcineurin mutants. The *eca1* phenotype may result from either increased cytosolic Ca^{2+} causing overactivation of calcineurin or even reduced Ca^{2+} availability in response to stresses if Ca^{2+} derived from the ER is used in signaling. However, *eca1* mutants are also hypersensitive to calcineurin inhibitors, indicating that any interaction with Eca1 and calcineurin is not within a simple linear pathway (Fig. 3A). At the semipermissive temperature of 35°C, both CsA and FK506 inhibited the growth of *eca1*Δ mutants similarly. At 37°C, the growth of wild-type and *eca1*Δ cells was completely inhibited by CsA (Fig. 3B) or FK506 (not shown), displaying the temperature-sensitive phenotype associated with impaired calcineurin signaling caused by these drugs. However, when the cells were returned to 30°C and allowed to grow for 2 days, *eca1*Δ mutant cells were not able to recover, while both H99 and *cna1*Δ cells recovered and resumed growth. Interestingly, when *eca1*Δ mutant cells were treated at 37°C for 2 days in the absence of CsA, they were able to recover after being shifted back to 30°C (Fig. 3B). Therefore, at 37°C, while CsA is fungistatic to H99, it becomes fungicidal to *eca1*Δ mutant cells. This indicates that *eca1* mutant cells are viable but fail to proliferate at 37°C, calcineurin is required for *eca1* mutant cell viability, and Eca1 and calcineurin function at least in parallel pathways in *C. neoformans*.

***eca1*Δ is hypersensitive to ER and osmotic stresses.** Because SERCA pumps such as Eca1 are responsible for transporting Ca^{2+} into the ER and Ca^{2+} ions are required for correct folding of newly synthesized proteins and for protein secretion, we tested whether the *eca1* mutant is sensitive to ER stress. First we examined whether the *eca1*Δ mutation might affect tolerance to calcium stress. Indeed, elevated levels of calcium (100 mM or 250 mM) in YPD medium inhibited the growth of the *eca1*Δ mutant strains at either 30°C or 35°C (Fig. 4A). On the other hand, depletion of Ca^{2+} with 10 mM of the calcium chelator EGTA also inhibited growth of the *eca1*Δ strain. Although either high concentrations of Ca^{2+} or Ca^{2+} depletion inhibited the growth of the wild-type strain H99, the inhibition of *eca1*Δ mutant cells was much more severe. This indicates

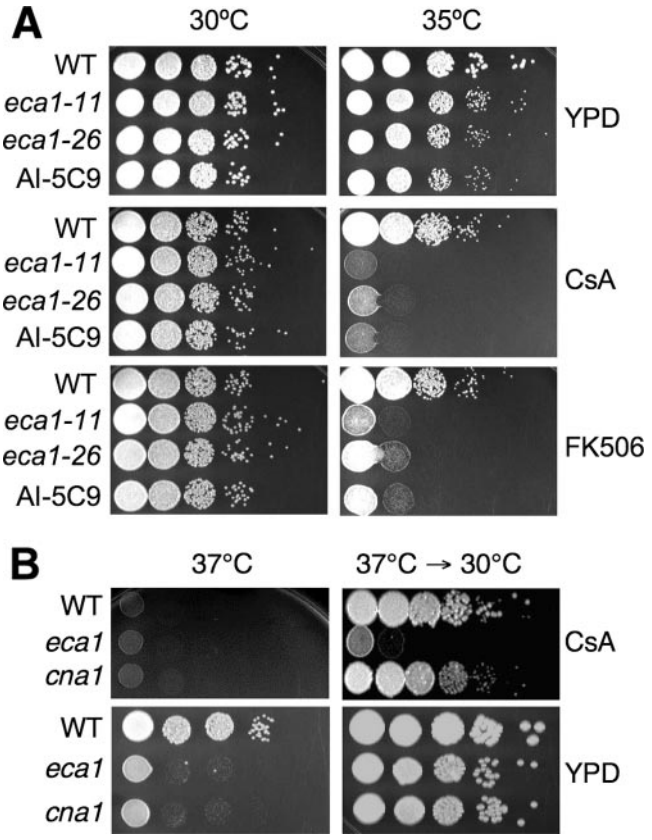

FIG. 3. Growth and viability of the *eca1* mutants are reduced by calcineurin inhibitors. (A). Ten-fold serial dilutions of *eca1-11*, *eca1-26*, the insertion mutant AI-5C9, and wild-type (WT) cells were plated on YPD agar or YPD medium supplemented with CsA or FK506 and cultured at 30°C or 35°C for 2 days. (B). Wild-type, *eca1*, or *cna1* cells were plated on YPD medium or YPD medium supplemented with CsA and cultured at 37°C for 2 days before being transferred to 30°C and cultured for 2 more days.

that Eca1 is involved in Ca^{2+} homeostasis and protection against Ca^{2+} stresses.

We also tested ER stress by adding tunicamycin to the growth medium to disrupt protein N glycosylation. Treatment with 100 nM tunicamycin moderately inhibited the *eca1*Δ mutant at 30°C and severely inhibited its growth at 35°C (Fig. 4A). This hypersensitivity indicates that Eca1 is involved in tolerance to ER stress.

To test whether the temperature sensitivity of the *eca1* mutants was due to overactive CaMKs, the *eca1* strain was treated with the CaMK-specific inhibitor KN-93 and the noninhibitory analog KN-92 (68). YPD solid medium was inoculated with a lawn of cells, a 6-mm filter paper disk soaked in either analog (10 μl at 5 mM) or the dimethyl sulfoxide solvent was placed on the lawn, and the strains were grown at 30°C and 37°C. Treatment with the drug was unable to rescue the temperature sensitivity of the *eca1*Δ mutant strain (data not shown), suggesting that the temperature sensitivity is more likely due to ER stress rather than enhanced Ca^{2+}-calmodulin-dependent kinase activity. However, one caveat in this experiment is the assumption that these molecules are efficiently taken up by *C. neoformans* as in other fungi.

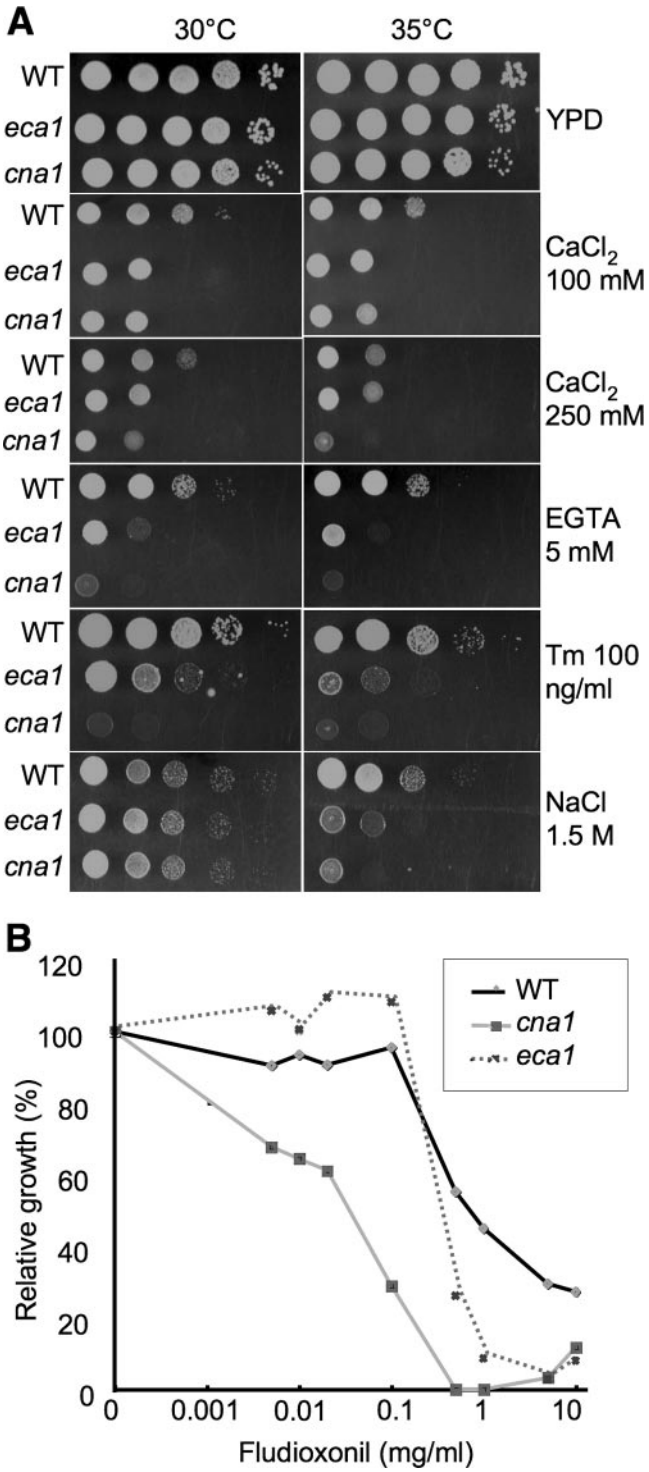

FIG. 4. Deletion of *ECA1* results in hypersensitivity to multiple stresses. (A) Ten-fold dilutions of wild-type (WT), *eca1*, or *cna1* cells were plated on YPD medium or YPD medium supplemented with 100 mM or 250 mM $CaCl_2$, 10 mM EGTA, 0.1 μg/ml tunicamycin (Tm), or 1.5 M NaCl and cultured at 30°C or 35°C for 2 days. (B) Wild-type, *eca1*, or *cna1* cells were cultured in RPMI medium (pH 7.0) containing 10-fold serial dilutions of fludioxonil and cultured at 30°C for 2 days. Growth was determined by measuring the optical density at 595 nm. The graph shows the percentage of growth relative to cells cultured in medium containing no fludioxonil.

Ca^{2+} is an important second messenger regulating numerous cellular functions, including osmotic stress tolerance. We examined whether the *eca1*Δ mutant has any defect in osmostress tolerance by treatment with a high concentration of NaCl. We found that at 30°C, the *eca1*Δ mutant is as sensitive to 1.5 M NaCl as the wild-type strain, H99 (Fig. 4A). However, at 35°C, while the growth of H99 was also further inhibited by NaCl, the *eca1*Δ mutant showed an even higher level of sensitivity. We also treated the *eca1*Δ mutant with fludioxonil, an antifungal drug that causes osmotic stress by activating the Hog1 mitogen-activated protein kinase and inducing the intracellular accumulation of glycerol (32, 40, 54, 76). While more than 10 mg/ml fludioxonil is required to inhibit growth by 80% in the wild type, less than 1 mg/ml was required to achieve 80% growth inhibition of the *eca1*Δ mutant at 30°C (Fig. 4B).

Mutation of calcineurin (*cna1*Δ) aggravates the phenotypes conferred by *eca1*Δ mutation. To examine further the relationship between Eca1 and calcineurin, we constructed an *eca1*Δ *cna1*Δ double mutant strain by crossing the nourseothricin-resistant *eca1*::*NAT* mutant in the H99 (*MAT*α) background with the neomycin-resistant *cna1*::*NEO* strain in the KN99**a** background (*MAT***a**). The *eca1*Δ *cna1*Δ strain was tested for temperature sensitivity, as well as sensitivity to ER stresses and hyperosmolarity. When cultured on YPD medium or YPD medium containing 100 mM $CaCl_2$, 5 mM EGTA, 0.1 mM tunicamycin, or 1.5 M NaCl, the double mutant displayed a more severe sensitivity than the *eca1*Δ mutant alone. The *eca1*Δ *cna1*Δ mutant was also more sensitive to high temperature than the *eca1*Δ mutant (Fig. 5B), which is consistent with the increased sensitivity of a single *eca1*Δ strain when treated with calcineurin inhibitors (see Fig. 3). These findings provide further evidence that Eca1 functions in parallel with calcineurin, and Eca1 may also function upstream or with other Ca^{2+}-dependent proteins or processes to control calcium activation by calmodulin.

Temperature-dependent roles of Eca1 in *C. neoformans* virulence. Using the amoeba *A. castellanii* as described previously (65), the *ECA1* gene was identified in *C. neoformans* in a screen for mutants with altered virulence (Fig. 1), and the data presented thus far show that the Eca1 Ca^{2+} pump is involved in stress tolerance. We further investigated a role for Eca1 in virulence and its interaction with calcineurin. First we examined the virulence phenotype of *eca1* mutants in the murine macrophage infection model. Cells of two independent *eca1*Δ mutant strains, the insertion mutant AI-5C9, and the wild-type strain H99 were inoculated into macrophage J774A.1 cultured cells and incubated at 37°C in the presence of 5% CO_2. Virulence was measured by the growth of *C. neoformans* cells as defined by CFU at the 24-h time point. As shown in Fig. 6A, growth of the *eca1* mutants was significantly lower than that of the wild type. This demonstrates that the *eca1* mutants exhibit reduced virulence in the murine macrophage model. However, the reduced virulence of *eca1* mutants may simply reflect their reduced proliferation or viability at elevated temperature, as indicated by the growth of the *eca1* mutants in DMEM at 37°C (Fig. 6A) and as observed in previous experiments.

Because experiments with murine models of cryptococcosis are performed at 37°C, to analyze the contribution of Eca1 to *C. neoformans* virulence, we took advantage of wax moth (*G. mellonella*) larvae (52), an alternative host model that can grow

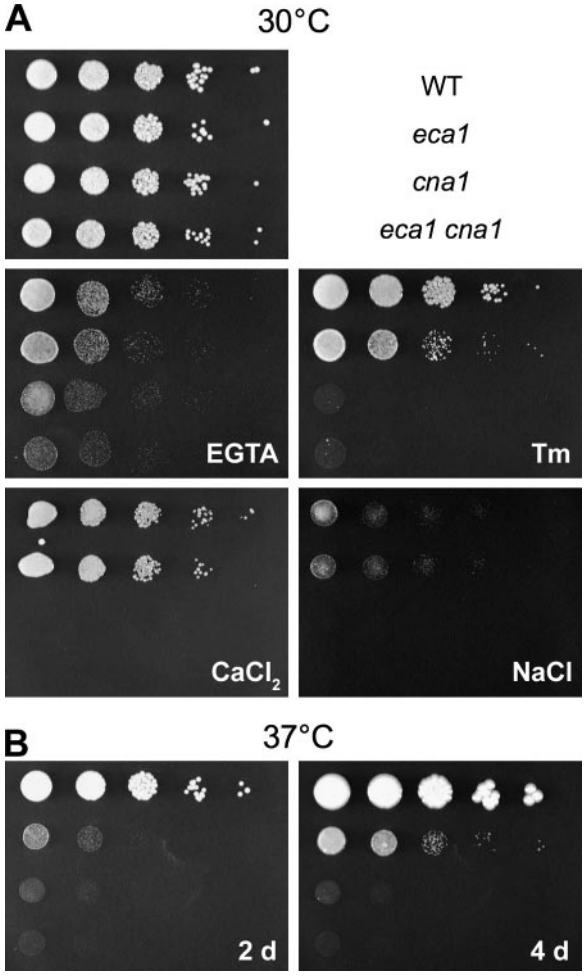

FIG. 5. Calcineurin mutation exacerbates *eca1* mutant phenotypes. (A) Ten-fold serial dilutions of the wild-type (WT), *eca1*, *cna1*, and *eca1 cna1* mutant cells were plated on YPD medium or YPD medium supplemented with EGTA (5 mM), tunicamycin (Tm; 0.1 μg/ml), $CaCl_2$ (250 mM), or NaCl (1.5 M) and cultured at 30°C for 2 days. (B). Ten-fold serial dilutions of the wild-type or *eca1*, *cna1*, and *eca1*Δ *cna1*Δ mutant cells were plated on YPD medium and cultured at 37°C for 2 or 4 days.

at temperatures ranging from 22 to 37°C, and the nematode *C. elegans* (growth at 25°C). For the wax moth larva infection model, 1×10^5 *C. neoformans* cells were injected into the wax moth larvae and the larvae were incubated at 30°C or 37°C. The virulence of each strain was measured by the death/survival of the larvae injected with it. At 30°C, 80 to 90% of the larvae infected with wild-type or *eca1* mutant cells died by day 13, whereas 70 to 90% of the larvae infected with the *cna1* or *eca1 cna1* mutant cells survived (Fig. 7; $P < 0.05$, Mann-Whitney test), showing that while calcineurin does contribute to virulence in this model at 30°C, Eca1 does not. However, when incubated at 37°C, the wild-type strain killed all moth larvae within 6 days, whereas the *eca1* strain showed reduced virulence, and the *cna1* or *eca1 cna1* mutants exhibited an even greater attenuation in virulence compared to the wild type ($P < 0.05$, Mann-Whitney test), in accord with the temperature-sensitive phenotype of the *eca1*, *cna1*, and *eca1 cna1* mutant strains.

Three virulence assay endpoints were used to assess the role of Eca1 and calcineurin in virulence in the model nematode *C. elegans*, to assess the role of temperature sensitivity in virulence, and to study whether the hypervirulent phenotype of *eca1* mutants was amoeba specific. The *C. elegans-C. neoformans* interaction experiments were performed at 25°C. For the strains tested, no major differences were observed in the assay that relied on the fungus' ability to kill the nematode (data not shown). However, in a second assay measuring the generation of nematode progeny after being infected with *C. neoformans*, differences were observed (Fig. 8). This assay relies on more virulent strains having a detrimental effect of nematode fertility, such that more progeny are produced from nematodes infected with attenuated strains compared to those infected with wild-type strains. Inoculation with the *eca1*Δ *cna1*Δ double mutant strain resulted in higher numbers of progeny compared to the wild type or the single *cna1*Δ mutant ($P < 0.005$, Mann-Whitney test) and also higher numbers of progeny from worms infected with the *eca1* mutant strain compared to the wild type ($P < 0.05$). Microscopic examination of the nematodes revealed that the *C. neoformans* yeast cells were present in the intestines of nematodes when exposed to the wild type or *eca1*Δ or *cna1*Δ mutants, but rarely for the *eca1*Δ *cna1*Δ double mutant (Fig. 8), although there was distension of the proximal intestine in this case, suggesting that the *eca1*Δ *cna1*Δ strain survived passage through the nematode grinder and was subsequently lysed in the intestine. CFU from the nematodes infected with these strains confirmed the microscopic observations, with fewer CFU being produced from the nematodes fed

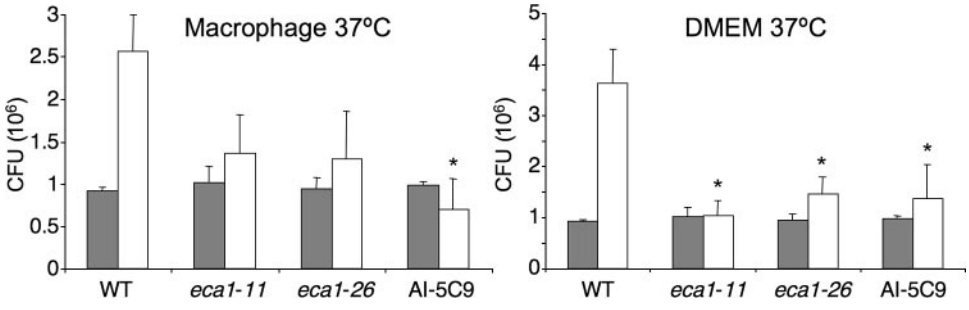

FIG. 6. The Eca1 SERCA pump is required for virulence in a murine macrophage model. Cryptococcal yeast cells of the wild type (WT), the *eca1* insertion mutant (AI-5C9), and two *eca1*Δ deletion mutants (*eca1-11* and *eca1-26*) were inoculated into macrophage J774A.1 cultured cells at an MOI of 2:1 (left panel) or into macrophage-free medium (DMEM; right panel). The cryptococcal strains were inoculated at 1×10^6 cells (gray bars), and after 24 h (white bars) of incubation, growth was measured by plating on YPD and counting CFU after culture at 30°C for 2 days. *, $P < 0.05$.

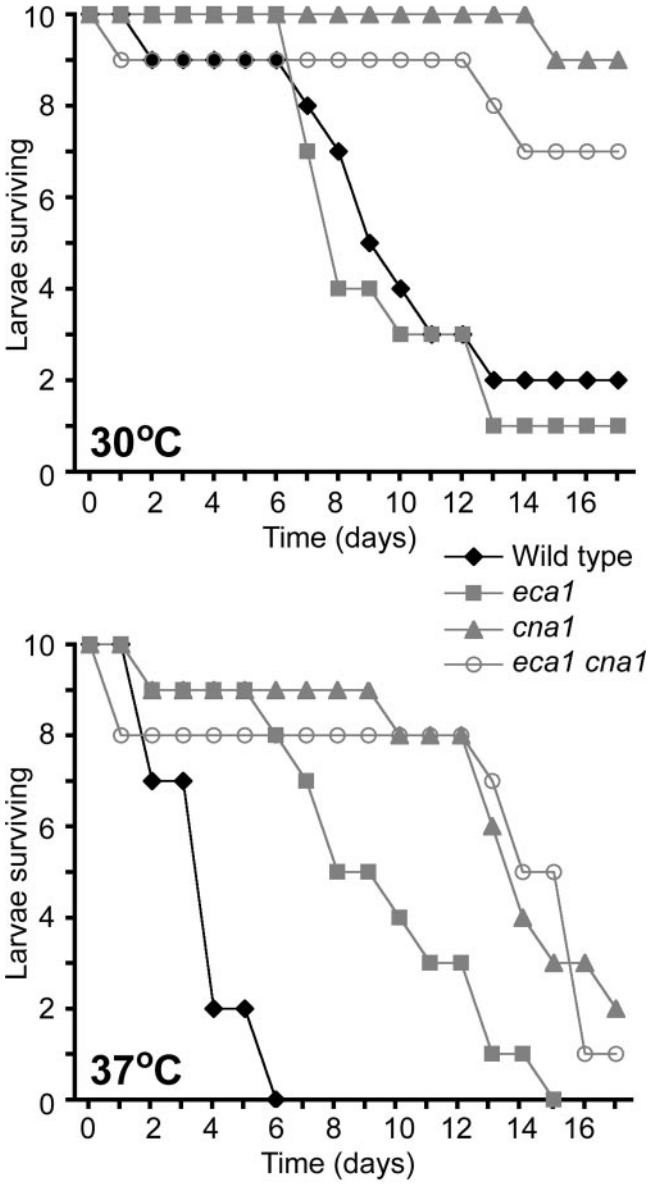

FIG. 7. Temperature influences the role of *C. neoformans* Eca1 SERCA pump and calcineurin in virulence in the wax moth *G. mellonella*. Ten wax moth larvae were injected with 1×10^5 cells of the wild-type or *eca1*Δ, *cna1*Δ, or *eca1*Δ *cna1*Δ strains and incubated at 30 or 37°C, and survival was monitored daily.

the *eca1*Δ *cna1*Δ mutant compared to other strains ($P < 0.05$). The intestines of the nematodes infected with the *eca1*Δ mutant also appeared to contain fewer *C. neoformans* cells, and infection with the *eca1*Δ mutant allowed more progeny to be produced than with the wild type. However, there was no statistically significant difference between CFU emanating from the wild type and *eca1* mutants. Thus, analysis of virulence in the nematode *C. elegans* reveals that only the *eca1*Δ *cna1*Δ double mutant shows a dramatic decrease in fungal viability within nematodes and the fertility of the infected nematodes, while the *eca1* or *cna1* single mutants have little or no effect compared to the wild type.

Taken together, the virulence phenotypes from multiple models at different temperatures suggest that both Eca1 and Cna1 contribute to virulence by supporting growth at mammalian body temperature; however, mutation of both genes has an additive effect and reduces virulence at room temperature.

DISCUSSION

Pathogenic fungi that normally exist outside the human host face challenges in transitioning from one environmental niche to another, particularly from the environment into the human host. Many rely on the ability to sense these changes via conserved signaling cascades to adapt successfully to new conditions. One such signaling pathway is that mediated by Ca^{2+}-calmodulin-calcineurin. Calcium signaling is important for *C. neoformans* and other human-pathogenic fungi to cope with stress and for their virulence (41).

In this study, we identified a new gene, *ECA1*, involved in Ca^{2+} homeostasis in *C. neoformans*. The gene is part of a conserved family of SERCAs that regulate Ca^{2+} levels in the ER lumen in many eukaryotes. In *C. neoformans*, this gene regulates resistance to various stresses and growth at 37°C and functions in parallel with and upstream of the calcineurin pathway. The closest homolog is the *ECA1* gene of the related basidiomycete plant-pathogenic fungus *U. maydis*, in which the *ECA1* gene was identified by analysis of a temperature-sensitive mutant (1). The growth phenotypes of the *U. maydis* and *C. neoformans eca1* mutants are very similar. One exception is that in *U. maydis* the altered cell morphology is clearly attributable to overactive CaMKs (1). However, in *C. neoformans eca1* mutants there is no defect in cellular morphology and a CaMK inhibitor did not rescue the temperature sensitivity of *eca1*. Rather, we hypothesize that many of the *C. neoformans eca1* phenotypes are due to impaired ER function resulting in misregulation of protein processing, suggesting a divergence in function between these two basidiomycetes. We performed a pilot microarray study to elucidate the effects of *eca1* deletion on gene transcription in *C. neoformans* (W. Fan, K. Kojima, and J. Heitman, unpublished data). While we were able to confirm the temperature regulation of transcription by subsequent Northern blot experiments for genes encoding proteins such as opsin, the cruciform DNA binding protein Hmp1, an oxidoreductase (GenBank accession no. CNC03730), ribosomal protein L35, and an Ero1-like protein (data not shown), no Eca1-dependent transcriptional response was identified, suggesting that other microarray experiments or approaches will be required to elucidate Eca1 functions.

We initially hypothesized that the temperature-sensitive phenotype of *eca1*Δ cells could be related to a role upstream in calmodulin-calcineurin signaling. Mutation of *ECA1* should impair Ca^{2+} import into the ER, increasing cytosolic Ca^{2+} levels and overactivating calcineurin. However, the *eca1 cna1* double mutant is more severely impaired than either *eca1* or *cna1* single mutants for a number of phenotypes, including temperature sensitivity and virulence, suggesting that Eca1 functions in parallel with calcineurin. We consider it likely that Eca1 also plays a role upstream of calcineurin by regulating cytosolic Ca^{2+} levels. Calcineurin is required for virulence in all human-pathogenic fungi in which it has been studied, but its roles in virulence differ between species. In *C. albicans*, calcineurin mutants grow like the wild type at 37°C but are killed

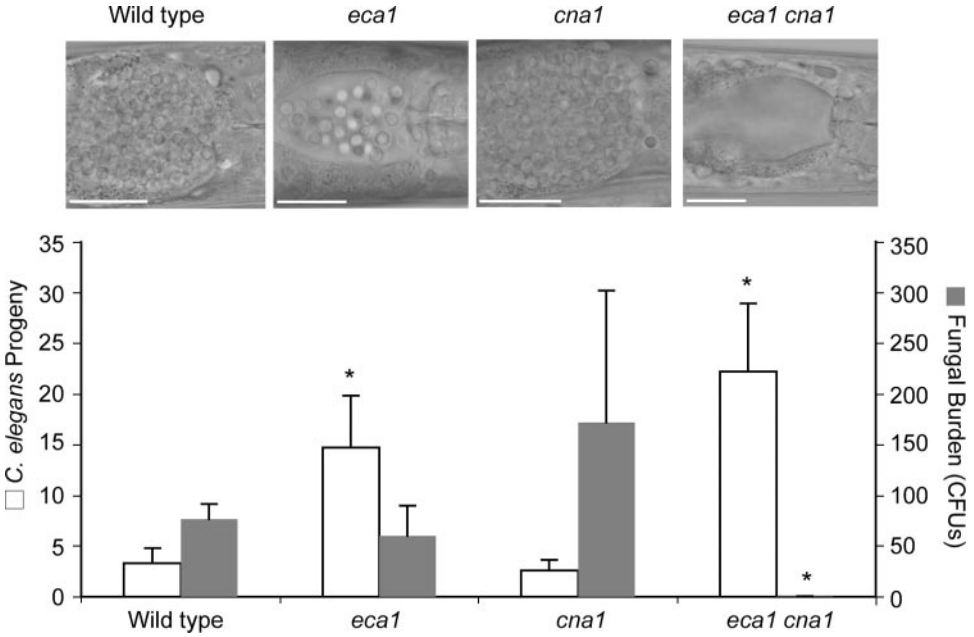

FIG. 8. Eca1 SERCA pump and calcineurin additively contribute to virulence in the nematode *C. elegans*. Micrographs of nematode intestines containing *C. neoformans* yeast cells. The grinder is to the right in each image. Bar = 20 μM. Shown is the generation of *C. elegans* progeny after exposure to *C. neoformans* strains (white bars). CFU are the average yeast colonies (± standard error) from individual *C. elegans* worms fed *C. neoformans*, with worms harvested from three plates (gray bars). The *eca1* single and *eca1 cna1* double mutants show statistically significant differences from the wild type. *, $P < 0.05$.

by calcium in serum (4, 8, 9, 61). In *A. fumigatus*, calcineurin mutants exhibit severe morphological defects (22, 66). In *C. neoformans*, calcineurin mutants cannot grow at 37°C (29, 55). Here we show that *C. neoformans* calcineurin mutants exhibit wild-type virulence at room temperature in *C. elegans* but reduced virulence in the wax moth at 30°C (a temperature at which there are no growth defects in vitro). Thus, based on the results in the wax moth, the virulence defect in mammalian models may not be attributed solely to temperature sensitivity in *C. neoformans*. This study highlights the use of nonmammalian models in understanding the genetic mechanisms governing virulence, especially when one phenotype of mutating a gene is temperature sensitivity. We note that because the *cna1* mutant was fully virulent in *C. elegans*, but not in the wax moth, this indicates that virulence determinants can differ between model heterologous hosts, providing unique vantage points from which to dissect branched signaling pathways controlling virulence.

Many of the phenotypes of *eca1* mutants are similar to those caused by calcineurin mutation, suggesting that conditions leading to both loss and gain of calcineurin activity might similarly perturb cellular physiology. Another component of calcium homeostasis that has been recently identified is the Cch1 voltage-gated Ca^{2+} channel that is required for calcium uptake in *C. neoformans* and predicted to be in the plasma membrane (44). Strains with *cch1* mutations show sensitivity to calcium chelation, like *eca1* mutants, but no temperature sensitivity. Thus, in the absence of Cch1 other mechanisms must exist to provide sufficient Ca^{2+} to activate calcineurin. Similarly, other components of calcium-calcineurin signaling show variation in phenotype when mutated, such as mutants of the calcineurin binding protein (*CBP1*), which exhibit wild-type growth at high temperature but a mating defect like calcineurin mutants (30), or a calmodulin mutant that has similar temperature sensitivity and mating defects to calcineurin mutation but is also hypersensitive to the calcineurin inhibitor FK506 (42). Taken together, these and other results suggest that variation exists between the phenotypes caused by perturbations to calcium homeostasis in *C. neoformans* and other fungi.

While SERCAs are crucial for maintaining Ca^{2+} homeostasis in many eukaryotes, including animals, plants, protists, and fungi like *C. neoformans* and *U. maydis*, organisms can function without this class of Ca^{2+} pump. For example, the model eukaryote *S. cerevisiae* and related hemiascomycete yeasts do not contain a SERCA protein, so intracellular calcium concentrations in these fungi are regulated by other Ca^{2+} transporters. One is Pmc1, which is localized to the vacuolar membrane, and the key role in *S. cerevisiae* of the vacuole, where most Ca^{2+} is stored, in maintaining Ca^{2+} levels has recently been demonstrated by screening the yeast deletion collection for strains that accumulate (or do not accumulate) ions; the majority of strains affected in Ca^{2+} levels were mutants that impair vacuole function (24). A second transporter, Pmr1, is localized to the Golgi apparatus, thereby controlling Ca^{2+} within these post-ER vesicles and also affecting Ca^{2+} levels within the ER itself (10). *S. cerevisiae* does contain a P-type ATPase (Cod1/Spf1) localized to the ER to regulate calcium levels (19, 69), but, to the best of our knowledge, there is no evidence that mutants of this gene exhibit interactions with the calcineurin pathway. Mutation of any of these transporters affects calcium homeostasis and reduces viability of *S. cerevi-*

siae under stress conditions. Despite different calcium transporter compositions, in both *C. neoformans* and *S. cerevisiae* (and the related hemiascomycete pathogens *C. albicans* and *Candida glabrata*) impairment of both calcineurin and ER functions leads to additive deleterious effects in these fungi (10), showing a commonality in overall cellular functions.

One hypothesis as to how *C. neoformans* maintains its ability to infect the human host is that it is under constant selection in the environment by predators, such as amoebae, nematodes, and insects (63). Passage of the fungus through the slime mold *Dictyostelium discoideum* enhanced virulence in a murine model of disease (64). Several genes required for virulence in the mammalian host are also required for virulence in heterologous hosts (31, 50). Furthermore, two recent screens for strains with attenuated virulence in *C. elegans* identified the *KIN1* and *ROM2* genes, which were subsequently shown to be required for virulence in murine models of cryptococcosis (51, 71). The original AI-5C9 *eca1* mutant strain was identified as hypervirulent in a screen for strains with altered virulence in an amoeba model of the disease. Subsequent studies show that the magnitude of virulence depends on the temperature of the experiment, as the *eca1* mutant has a growth and virulence defect at elevated temperatures but retains wild-type or enhanced virulence at room temperature. While mutation of *ECA1* would produce a *C. neoformans* strain that is better adapted to survive predation by certain environmental hosts like *Acanthamoeba castellanii* and still be virulent in nematodes and wax moth, it would not survive in the mammalian host or under other stressful conditions. Thus, selection for virulence in this amoeba species by an *eca1* mutation would decrease mammalian virulence. A reverse situation exists for the carbonic anhydrase Can2, whose deletion yields a strain that proliferates better than the wild type in a rabbit intracranial model of infection yet is unable to grow outside the animal host in the low CO_2 concentrations present in the atmosphere (6). Thus, mutations towards enhanced virulence must be balanced with other selective pressures acting on *C. neoformans*.

The proposal of Steenbergen et al. (65) for a role for environmental predators as enhancing *C. neoformans* virulence has stimulated considerable debate (see e.g., reference 43). While the differences in virulence for *eca1* and *cna1* mutants in diverse host systems could be interpreted as a consequence of these being nonnatural hosts, we argue in favor of alternative systems as virulence models for this fungus. As noted previously, the *C. neoformans* interaction with amoeba has been observed in nature (13, 16) and the fungus lives in soil, guano, and other debris coinhabited by other microbial predators, such as nematodes and insects. The *Cryptococcus* genus clusters with other basidiomycetes that are insect pathogens or insect associated (62). *C. neoformans* has been isolated from the intestines of beetles (7, 67). The wax moth is a common problem for beekeepers, and *C. neoformans* has been isolated from beehives in Turkey (25). Thus, *C. neoformans* could associate with these species in nature. Further support for studies with alternative hosts derives from analysis of Legionnaires' disease, in which it is well established that infectious *Legionella pneumophila* bacteria are maintained within amoebae in water sources (28, 60). Importantly, laboratory-isolated mutants of *L. pneumophila* exhibit reduced virulence in both amoebae and macrophages, in macrophages but not amoebae, or in amoebae but not macrophages (e.g., references 11 and 33 and reviewed in reference 70), representing a similar situation to that observed with *C. neoformans eca1* mutants. The differences in virulence of the *eca1* and *cna1* mutants in each model system may reflect underlying differences in host conditions (e.g., Ca^{2+} concentrations) in each model. This is analogous to recent discoveries showing differing virulence properties of *C. albicans* calcineurin mutants depending on host tissue, in that calcineurin is required for systemic but not vaginal infection (5, 56). These studies demonstrate the potential complexity in the host-pathogen-environment interactions that represent challenges for future research to understand and control infectious disease.

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