The Cch1-Mid1 High-Affinity Calcium Channel Contributes to the Virulence of Cryptococcus neoformans by Mitigating Oxidative Stress

Kiem Vu,a Jennifer M. Bautos,b and Angie Gelli a

Department of Pharmacology, School of Medicine, University of California, Genome and Biomedical Sciences Facility, Davis, California; Veterinary Genetic Laboratory, School of Veterinary Medicine, University of California, Davis, California

Pathogenic fungi have developed mechanisms to cope with stresses imposed by hosts. For Cryptococcus spp., this implies active defense mechanisms that attenuate and ultimately overcome the onslaught of oxidative stresses in macrophages. Among cellular pathways within Cryptococcus neoformans’ arsenal is the plasma membrane high-affinity Cch1-Mid1 calcium (Ca2+) channel (CMC). Here we show that CMC has an unexpectedly complex and disparate role in mitigating oxidative stress. Upon inhibiting the Ccp1-mediated oxidative response pathway with antimycin, strains of C. neoformans expressing only Mid1 displayed enhanced growth, but this was significantly attenuated upon H2O2 exposure in the absence of Mid1, suggesting a regulatory role for Mid1 acting through the Ccp1-mediated oxidative stress response. This notion is further supported by the interaction detected between Mid1 and Ccp1 (cytochrome c peroxidase). In contrast, Cch1 appears to have a more general role in promoting cryptococci survival during oxidative stress. A strain lacking Cch1 displayed a growth defect in the presence of H2O2 without BAPTA [(1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid, cesium salt] or additional stressors such as antimycin. Consistent with a greater contribution of Cch1 to oxidative stress tolerance, an intracellular growth defect was observed for the cch1Δ strain in the macrophage cell line J774A.1. Interestingly, while the absence of either Mid1 or Cch1 significantly compromises the ability of C. neoformans to tolerate oxidative stress, the absence of both Mid1 and Cch1 has a negligible effect on C. neoformans growth during H2O2 stress, suggesting the existence of a compensatory mechanism that becomes active in the absence of CMC.

It is well established that Ca2+ is a critical secondary messenger that initiates and regulates a plethora of signaling events. For this reason, cytosolic Ca2+ levels are exquisitely controlled by regulating the movement of calcium ions into and out of cells via ion channels and transporters (1–3). Fluctuations of Ca2+ in the cytosol are transduced via calcium sensors like calmodulin, which, upon calcium binding, activates calcineurin and CaMK (Ca2+/calmodulin-dependent protein kinases). Calcineurin is a Ca2+/calmodulin-activated serine/threonine protein phosphatase highly conserved among eukaryotes. In fungi such as Cryptococcus neoformans, Candida albicans, and Saccharomyces cerevisiae, calcineurin regulates the transcription of genes involved in mating, cell viability, and response to cell stress (4–6). The improper regulation of Ca2+ can produce significant cell damage and ultimately lead to cell death (7).

In fungal cells, the Cch1-Mid1 channel complex (CMC) represents the only high-affinity Ca2+ channel in the plasma membrane that mediates the specific influx of Ca2+ (2). While Cch1 functions as the pore of the channel, Mid1 associates with Cch1 and, in a manner that is not completely understood, facilitates the movement of Ca2+ from the extracellular milieu to the cytosol (8, 9). Under conditions of endoplasmic reticulum (ER) stress or low ER Ca2+, the Cch1-Mid1 channel becomes activated and functions to replenish Ca2+ stores (1, 2, 10, 11).

It is not known if CMC is required for survival of cryptococci within the macrophage environment. Cryptococcus neoformans is a facultative intracellular pathogen capable of replicating within the phagolysosome and promoting host cell lysis (12, 13). Given the oxidative stresses that can promote fluctuating levels of cytosolic Ca2+ within active macrophages (14) and the specific role of CMC as the only high-affinity Ca2+ channel in the plasma membrane of C. neoformans, we sought to resolve whether CMC played a role in promoting survival of C. neoformans within an intracellular environment like the macrophage (15). Here we show that both Mid1 and Cch1 promote C. neoformans survival upon exposure to oxidative stress in vitro, with Cch1 making an additional contribution to cryptococci survival in macrophages.

A yeast two-hybrid screen using the C-terminal region of Mid1 spanning two cysteine-proline (CP) dipeptides and predicted heme-binding sites revealed an interaction with cytochrome C peroxidase (Ccp1), a known heme-binding protein (16). Ccp1 is a mitochondrial antioxidant protein that reduces hydrogen peroxide to water and is released into the cytosol during stress (16). Upon inhibition of the Ccp1-mediated oxidative response pathway with antimycin, we found that strains expressing Mid1 in the absence of Cch1 shows significant resistance to antimycin toxicity, consistent with the interaction observed for Mid1 and Ccp1. Surprisingly, a strain lacking both Mid1 and Cch1 showed significant resistance to oxidative stress in vitro, suggesting a compensatory mechanism that is active in the absence of CMC. Together, our work revealed that CMC plays a complex role in the mitigation of oxidative stress in C. neoformans with Mid1 and Cch1 having disparate contributions to oxidative stress resistance.

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Address correspondence to Angie Gelli, aggelli@ucdavis.edu.
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The Table 1 Mutations in the Mid1 CP motifs 1 and 2 (CP1 and CP2) were suggested with NotI and MfeI, ligated into the Jmm180 plasmid using T4 DNA ligase. C. neoformans into the constitutive actin promoter using standard methods (C2920; Sigma), and paraquat (36541; Sigma) were pre pared according to the manufacturer’s instructions. 

Mid1 harboring the CP1 mutation (C648AP649A [CP1x]), the CP2 mutation (C656AP657A [CP2x]), or both CP 1 and CP 2 mutations (C648AP649A C656AP657A [CP1x2x]) were streaked on yeast-peptone-dextrose (YPD) plates and incubated overnight at 30°C. Colonies were then grown in liquid YPD overnight at 30°C until log phase (approximately 16 h). Cultures were regrown a second time (to log phase) by adding 100 μl of liquid culture to fresh YPD to ensure that yeast colonies were healthy and robust. Ten milliliters of each cultured strain was washed with sterile 1X phosphate-buffered saline (PBS) (4,000 rpm, 4 min, 25°C) and resuspended in YPD. The concentration of the original culture was determined by hemacytometer count (Brite-Line) and diluted in 1X PBS to appropriate concentrations. Where indicated, a cell-impermeant calcium chelator [BAPTA: 1,2-bis(2-aminophenoxy)ethane-N,N,N,N’-tetraacetic acid, cesium salt] ([Invitrogen/Molecular Probes, Carlsbad, CA) was added to YPD medium (2). In the presence of BAPTA, the final free [Ca2+]i was approximately 100 nm (2). Antimycin A (A8674; Sigma), FCCP [carbonyl cyanide 4-( trifluoromethoxy)phenylhydrazine- and paraquat] (C9290; Sigma), and paraquat (36541; Sigma) were prepared according to the manufacturer’s instructions.

Generation of Mid1 expression constructs. Mid1 cDNA was cloned into the C. neoformans expression plasmid Jmm180 under the control of a constitutive actin promoter using standard methods (17). Briefly, Mid1 cDNA was amplified via PCR from C. neoformans cDNA using the primers Mid1_NotI_F and Mid1_MfeI_R (Table 1). The cDNA was then digested with NotI and MfeI, ligated into the Jmm180 plasmid using T4 DNA ligase, and transformed into Escherichia coli. Positive bacterial transformants were selected on LB plates and incubated overnight at 37°C with 10% CO 2 in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% nontoxic amino acids, 50 μg/ml penicillin-streptomycin (P/S), and 10% NCTC-109 medium. J774A.1 cells were grown in 25-cm 2 or 75-cm 2 flasks and used between passages 4 and 15. For the assay, 2.5 × 107 J774A.1 cells were counted using a hemacytometer and grown in 96-well culture plates overnight. An H99 strain of C. neoformans was cultured overnight in YPD at 30°C with agitation. Cultures were then synchronized/normalized based on the optical density at 600 nm (OD600) and allowed to grow overnight. By using a hemacytometer, 1 × 107 cells of C. neoformans were added to the macrophages supplemented with 100 U/ml mouse gamma interferon (IFN-γ), 0.3 μg/ml lipopolysaccharide (LPS), and 1 μg/ml monoclonal antibody (MAB) 18B7 (a gift from A. Casadevall) for 1 h. Cells were washed three times with 1X PBS and either incubated overnight with fresh DMEM plus supplemental medium or lysed with 100 μl of ice cold 0.05% SDS for CFU analysis. The following day, the medium was removed from wells and set aside. Approximately 100 μl of ice-cold 0.05% SDS was added to lyse macrophages. The mixture was combined with medium for serial dilutions and plated on plates containing YPD plus P/S. The plates were incubated for 48 h at 30°C, and the number of CFU was determined.

Sensitivity spot assay. Frozen strains of C. neoformans were maintained in 15% glycerol stocks at −80°C were plated on YPD agar plates and incubated at 30°C for 2 days. A single colony was placed in a 10-ml glass test tube with fresh liquid YPD and grown overnight in a roller drum apparatus (14 to 16 h) until cells reached mid-log phase. Cells were diluted to 10 4 to 10 5, and 7 × 10 4 cells was spotted on freshly made YPD with or without H 2O 2. H 2O 2 was added to YPD agar once the agar had cooled to at least 60°C. Plates were incubated at 30°C for 2 days.

Site-directed mutagenesis of Mid1 cysteine dipeptide (CP) motif 1 and 2. Mutations in the Mid1 CP motifs 1 and 2 (CP1 and CP2) were generated via an inverse PCR method, using the Mid1 expression construct as a PCR template. A high-fidelity DNA polymerase (Thermoscientific Phusion Hot Start II; Thermoscientific) was used to generate mutagenic primers in Mid1 cDNA using the primer pairs listed in Table 1. The cysteine and proline residues in the CP dipeptides were either replaced with alanine or left unchanged, yielding three possible mutations: (i) Mid1 harboring the CP2 mutation (C650AP651A [CP2x]), the CP2 mutation (C650AP651A [CP2x]), or both CP1 and CP2 mutations (C648AP649A C650AP651A [CP1x2x]). The Mid1 site-directed mutagenesis constructs and the construct harboring the wild-type Mid1 cDNA allele (CPwt) were then introduced into the mid1A strain of C. neoformans using biolistic transformations (18). Positive transformants were then screened by colony PCR for genomic DNA integration. The expression of Mid1 in the biolistic transformants was then confirmed by RT-PCR and by growth sensitivity assay on YPD containing 2 mM BAPTA.

### Table 1: Primers used to generate Mid1 CP mutant expression constructs

| Target | Primer | Sequence |
|--------|--------|----------|
| Mid1 CP1x2x | Mid1-CP1x-R | CCATTGATACCGCGGCTGAGCATG |
| Mid1 CP2x | Mid1-CP2x-R | GCATCAGGGCCGACGAAAC |
| Mid1 CPwt | Mid1_NotI_R | ATAGCCGGCGGATCGCCAGCGAGAGGTGTATTTAAAAAG |

Macrophage infection assay. The J774A.1 (ATCC TIB-67; kindly provided by R. Tsolis) cell line is a lung macrophage-like cell line from a BALB/c, haplotype H-2 - reticulum sarcoma and commonly used as an intracellular survival assay for C. neoformans. Macrophages were grown at 37°C with 10% CO2 in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 50 μg/ml penicillin-streptomycin (P/S), and 10% NCTC-109 medium. J774A.1 cells were grown in 25-cm 2 or 75-cm 2 flasks and used between passages 4 and 15. For the assay, 2.5 × 107 J774A.1 cells were counted using a hemacytometer and grown in 96-well culture plates overnight. An H99 strain of C. neoformans was cultured overnight in YPD at 30°C with agitation. Cultures were then synchronized/normalized based on the optical density at 600 nm (OD600) and allowed to grow overnight. By using a hemacytometer, 1 × 107 cells of C. neoformans were added to the macrophages supplemented with 100 U/ml mouse gamma interferon (IFN-γ), 0.3 μg/ml lipopolysaccharide (LPS), and 1 μg/ml monoclonal antibody (MAB) 18B7 (a gift from A. Casadevall) for 1 h. Cells were washed three times with 1X PBS and either incubated overnight with fresh DMEM plus supplemental medium or lysed with 100 μl of ice cold 0.05% SDS for CFU analysis. The following day, the medium was removed from wells and set aside. Approximately 100 μl of ice-cold 0.05% SDS was added to lyse macrophages. The mixture was combined with medium for serial dilutions and plated on plates containing YPD plus P/S. The plates were incubated for 48 h at 30°C, and the number of CFU was determined.

Sensitivity spot assay. Frozen strains of C. neoformans were maintained in 15% glycerol stocks at −80°C were plated on YPD agar plates and incubated at 30°C for 2 days. A single colony was placed in a 10-ml glass test tube with fresh liquid YPD and grown overnight in a roller drum apparatus (14 to 16 h) until cells reached mid-log phase. Cells were diluted to 10 4 to 10 5, and 7 × 10 4 cells was spotted on freshly made YPD with or without H 2O 2. H 2O 2 was added to YPD agar once the agar had cooled to at least 60°C. Plates were incubated at 30°C for 2 days.

Protein analysis. A 50-ml culture of KN99a (congenic strain of H99) cells was grown overnight in YPD. Cells were pelleted and resuspended in 0.3 ml lysis buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 5 mM NaF, 0.3 mM EDTA [pH 8.0], 0.1% Nonidet-P40 detergent; stored at 4°C). Fresh dithiothreitol (DTT) (1 mM) and Sigma yeast protease inhibitor cocktail, which was used following the manufacturer’s instructions, were added. Samples were split into two 1.7-ml locking microcentrifuge tubes, and 425 to 600 μm acid-washed glass beads was added until there was approximately 1 to 2 mm between liquid and beads. Samples were vortexed at maximum speed for 1 min and placed on ice for 1 min for 5 cycles. Following cell lysis, the supernatant was collected and centrifuged at 4,000 rpm. The supernatant was collected, and total protein content was measured with the Bio-Rad QuickStart bovine serum albumin (BSA) standard set. Protein lysates were separated on a 6.5% SDS-PAGE gel and semidry transferred onto a polyvinylidene difluoride (PVDF) membrane. Purified mouse anti-Sos1 (BD Biosciences) at 1:500 and secondary horseradish
FIG 1 Mid1 plays a calcium-dependent role in mitigating oxidative stress induced by H2O2. Sensitivity spot assays were used to evaluate the requirement for Mid1 during oxidative stress. A strain of C. neoformans lacking Mid1 exhibited a significant growth defect upon exposure to H2O2 under conditions of limiting extracellular free [Ca\(^{2+}\)] in the presence of the calcium-specific chelator BAPTA (2), suggesting a requirement for Mid1 during oxidative stress (arrows). Serially diluted cells (10\(^5\), 10\(^4\), 10\(^3\), 10\(^2\), and 10\(^1\)) from wild-type H99, the mid1\(\Delta\) mutant, and the Mid1-reconstituted (mid1\(\Delta\)/Mid1) strain of C. neoformans were spotted onto YPD plates with either 1.5 mM H2O2 or 2 mM H2O2 in combination with either 2 mM or 4 mM BAPTA. Plates were incubated at 30°C overnight.

peroxidase (HRP)-conjugated goat anti-mouse Ig (BD Biosciences) at 1:1,000 were used for Western blotting.

CytoTrap yeast two-hybrid assay. The cytosolic C terminus of Mid1 (270 amino acids [aa], corresponding to 813 bp) lacking the last 23 aa (corresponding to 72 bp) (required for localization to the plasmid membrane) was cloned into the multiple-cloning site (MCS) of a pSos plasmid to create a Mid1C-hSos fusion “bait” plasmid. The forward primer was Y2H_Mid1Cterm_BamHF2 (5'-ACCGGATCCATGACAACTCATATGGTCGTCT-3'), and the reverse primer was Y2H_Mid1R4_NotIR (5'-ACCGGGCCGCCACTATGTCGTCTCCACAAACGC-3'). A C. neoformans cDNA library was made using the CytoTrap XR library construction kit. The library "prey" was cloned into the pMyr plasmid containing a myristylation signal to the plasma membrane. Both Mid1C-hSos bait and prey plasmids were cotransformed into a temperature-sensitive cdc25 Saccharomyces cerevisiae strain. Potential positives were selected by growth at the restrictive temperature of 37°C on synthetic defined (SD) medium containing galactose but lacking leucine and no growth at 37°C on SD medium with glucose but without leucine. Potential positive pMyr plasmids were isolated from the cdc25 strain, transformed into Invitrogen ElectroMAX DH5α-E electrocompetent E. coli, prepared using a midikit (Qiagen), and sequenced. Potential positives were identified by a BLAST search in the Broad Institute E. coli database.

In silico bioinformatic analysis. The amino acid comparison of Mid1 was performed by ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalo/) and T-Coffee multiple-sequence alignment (http://www.ebi.ac.uk/Tools /msa/coffee/).

Statistical analysis. Statistical significance was determined by an unpaired t test with Welch’s correction (unequal variance) or by one-way analysis of variance (ANOVA) (GraphPad Prism 5.0).

RESULTS

Mid1 plays a role in mitigating oxidative stress in C. neoformans. As a facultative intracellular pathogen, C. neoformans can replicate within the phagolysosome and promote host cell lysis (12, 13). It is known that oxidative stress can lead to fluctuating levels of cytosolic Ca\(^{2+}\) within cells, including active macrophages (14); thus, we sought to resolve whether CMC played a role in promoting survival of C. neoformans during oxidative stress.

We have previously shown that strains of C. neoformans lacking the genes encoding the Cch1-Mid1 channel complex (CMC) display a significant growth defect in environments that are low in [Ca\(^{2+}\)] (~100 nM) (2, 8, 10, 19). This is expected, since CMC represents the only high-affinity calcium channel in the plasma membrane of C. neoformans. In low-[Ca\(^{2+}\)] environments, a strain lacking either Mid1 or Cch1 is incapable of mediating the influx of calcium from the extracellular milieu to the cytosol. This is consistent with the role of CMC in other fungal species (1, 5, 20, 21).

As predicted, the mid1\(\Delta\) strain displayed a growth defect in the presence of 2 mM and 4 mM BAPTA (a Ca\(^{2+}\)-selective chelator) in contrast to the wild-type strain (Fig. 1). Interestingly, the growth defect of the mid1\(\Delta\) strain was exacerbated upon exposure to the oxidative stress-inducing agent H2O2 under conditions of limited extracellular [Ca\(^{2+}\)] (Fig. 1). This suggested that Mid1 contributed to the survival of C. neoformans during oxidative stress likely in a calcium-dependent manner. In contrast, the cch1\(\Delta\) mutants exhibit a strong growth defect during H2O2 stress under high-calcium conditions (in the absence of BAPTA), suggesting a significant role for Cch1 in oxidative resistance (Fig. 2A).

An interaction between Mid1 and a novel binding partner, cytochrome c peroxidase (CcP1), was detected. To further resolve whether Mid1 played a direct role in an oxidative stress response, we sought to identify the protein-binding partners of M1 through the use of a modified version of the yeast two-hybrid system (Fig. 3A) (22, 23). Unlike traditional two-hybrid screens, where the interaction between the bait and the target (also

FIG 2 Cch1 contributes to oxidative stress tolerance and intracellular macrophage survival. (A) Tenfold serial dilutions (10\(^5\), 10\(^4\), 10\(^3\), and 10\(^2\)) of wild-type H99, the cch1\(\Delta\) mutant, the Cch1-reconstituted (cch1\(\Delta\)/Cch1) strain, and the cch1\(\Delta\) mid1\(\Delta\) mutant of C. neoformans were spotted on YPD alone or YPD with BAPTA (2 mM), H2O2 (3 mM), paraquat (0.313 mM), or FCCP (1.8 μg/ml). (B) The cch1\(\Delta\) strain displayed a modest but significant growth defect following 24 h of incubation within macrophages, suggesting that Cch1 played a role in promoting intracellular survival of C. neoformans. Statistical significance was determined by unpaired t test with Welch's correction (GraphPad Prism5).
FIG 3 A yeast-two hybrid screen revealed an interaction between Mid1 and Ccp1. (A) Schematic representation of a modified cytosolic version of the traditional yeast two-hybrid system that was used to identify binding partners of Mid1. During a physical interaction between bait (C-terminal region of Mid1) and target (cDNA library of C. neoformans with myristoylation signal), hSos is recruited to the plasma membrane, Ras signaling is activated, and growth of the cdc25-2 strain at the restrictive temperature (37°C) is restored. (B) Approximately 5 × 10^5 colonies were screened, and colonies that grew at the restrictive temperature (37°C) and in the presence of galactose were determined to be candidate colonies. Plasmids of candidate cDNAs were sequenced and cytochrome c peroxidase (Ccp1) was identified. In the presence of galactose at 37°C, the interaction between MAFB and pMyr5B served as a positive control and the lack of an interaction between pSos-MAFB and pl Lamin C served as a negative control for the genetic screen.

referred to as prey) occurs in the nucleus, the genetic interaction here takes places in the cytosol by exploiting the Sos–Ras recruitment system. Since Mid1 is a plasma membrane-resident protein, we reasoned that this two-hybrid method would increase the probability of identifying targets of Mid1 that were physiologically relevant.

Our two-hybrid approach takes advantage of the Ras pathway in S. cerevisiae in that when it is localized to the plasma membrane, the Ras guanyl nucleotides exchange factor Cdc25 stimulates GDP/GTP exchange on Ras (Fig. 3A) (24). The screen relies on a yeast mutant strain expressing a cdc25-2 allele that prevents growth at 37°C. Upon the physical interaction between the bait and target, hSos is recruited to the plasma membrane, Ras signaling gets activated and growth of the cdc25-2 strain at 37°C is restored (Fig. 3A).

In the case of Mid1, a cytosolic C-terminal portion of Mid1 (813 bp, 271 aa) lacking the last 23 amino acids was cloned into a pSos plasmid in order to construct the hSos-Mid1 fusion protein. We chose to use only a specific region of the C-terminal cytosolic portion of Mid1 from C. neoformans for several reasons. First, we found that the expression of the full-length Mid1 activated the Sos/Ras recruitment system on its own; therefore, this was not a viable choice for the screen. Second, we recently reported that the last 23 C-terminal amino acids are required for trafficking Mid1 to the plasma membrane; this would likely complicate the readout of the screen (8). Third, the predicted protein structure of Mid1 contains 3 or 4 putative transmembrane regions, indicating that these integral stretches of Mid1 protein would likely not bind to relevant cytosolic or membrane-bound targets. Lastly, as seen with other channel complex proteins, the C-terminal cytosolic regions are often involved in significant protein–protein interactions (19).

The hSos-Mid1 fusion protein was used as bait to screen a myristoylated, galactose-inducible cDNA library of C. neoformans. Expression of the hSos-Mid1 fusion protein was confirmed by Western analysis (data not shown). The temperature-sensitive cdc25-2 yeast strain expressing the cDNA library of C. neoformans and a C-terminal region of Mid1 (lacking the last 23 amino acids) predicted to be cytosolic was grown on glucose or galactose plates. Both a positive and negative control were included in the screen to ensure that the cdc25-2 strain had not reverted. Following the screening of approximately 5 × 10^5 colonies, only colonies that grew at the restrictive temperature (37°C) and in the presence of galactose were determined to be candidate colonies. Once the plasmids harboring the candidate cDNAs were sequenced, we found that one candidate encoded a cytochrome c peroxidase (Ccp1) (Fig. 3B).

Ccp1 and oxidative stress response in C. neoformans. Next we sought to resolve the physiological implication of the Mid1-Ccp1 genetic interaction. In C. neoformans, cytochrome c peroxidase (Ccp1) is known to protect against external oxidative stress-inducing agents (25, 26). It has been shown that treating cells with antimycin A inhibits the cytochrome c pathway and leads to the production of free radicals (26). We examined the effect of antimycin in strains of C. neoformans lacking either Mid1 or Cch1.

Interestingly, whereas the H99 wild-type strain of C. neoformans displayed significant growth sensitivity to antimycin, strains that retained MID1 expression but lacked CCH1 (cch1Δ mutants) were significantly more resistant to antimycin stress (Fig. 4). This phenotype was observed in three separate isogenic cch1Δ strains (Fig. 4). Since the H99 wild-type (WT) strain (which has a func-
Cysteine-proline dipeptide residues in the C-terminal region of Mid1 are conserved among fungal species. A schematic diagram of the predicted protein structure of Mid1 illustrates the region of Mid1 that associated with Ccp1 in the genetic screen. TD, trafficking domain (8). TM, predicted transmembrane domains according to topology prediction software. It should be noted that the number of TM domains in CnMid1 has not been experimentally defined. The amino acid comparison of Mid1 was performed by ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalo/) and T-Coffee multiple-sequence alignment (http://www.ebi.ac.uk/Tools/msa/tcoffee/). Both alignment software programs produced similar results; therefore, only the alignment produced by ClustalW is shown. The two cysteine-proline (CP) dipeptide motifs highlighted in yellow are conserved in fungi and appear within the Ccp1-binding region.

FIG 5 Cysteine-proline dipeptide residues in the C-terminal region of Mid1 are conserved among fungal species. A graphical representation of the Mid1 amino acid multiple-sequence alignment for the region containing the two CP dipeptide motifs further demonstrated the sequence conservation at the CP dipeptide positions C648P649 (referred to here as CP1) and C656P657 (CP2) and the fairly high frequency of the CP amino acids at this position (Fig. 6A, arrows).

We sought to determine whether the two highly conserved CP dipeptides in Mid1 were involved in mitigating oxidative stress through its association with Ccp1. To accomplish this goal, site-directed mutagenesis was carried out on the full-length Mid1 cDNA, which was cloned into a C. neoformans expression plasmid (Jmm180) under the control of a constitutive actin promoter (Table 1). Three different mutant strains were constructed with the following amino acid substitutions: Mid1 CP mutant strain 1 has the Mid1 C648P649A dipeptide replaced with two alanines (Mid1 CP1x2x), and Mid1 CP mutant strain 2 has the Mid1 C656P657A dipeptide substituted with alanine residues (Mid1 C656A/P657A [CP2x]), and Mid1 CP mutant strain 3 has both CP dipeptides replaced with alanine residues (Mid1 C648A/P649A, C656A/P657A [CP1x2x]). As a control, the mid1Δ strain expressing the full-length wild-type Mid1 under the control of the constitutive actin promoter was also constructed (designated Mid1 CP+). We then used the BAPTA (low-[Ca2+] medium) assay to monitor whether the CP mutants were required for Mid1 functional activity and/or whether they mediated an oxidative stress response (Fig. 6B; also, see Fig. S1 in the supplemental material).

We found that in the presence of H2O2 or low extracellular [Ca2+], the mid1Δ strain expressing either the Mid1 C648A/P649A dipeptide mutant (CP1x) or the strain expressing the Mid1 C648A/P649A, C656A/P657A dipeptide mutant (CP1x2x) displayed a growth defect similar to that observed for the mid1Δ background strain (Fig. 1 and 5B). In addition, a similar phenotype was observed for these strains upon exposure to both H2O2 and limited extracellular [Ca2+] (Fig. 1 and 5B). Taken together, these results suggested that the C648A/P649A dipeptide (CP1) was likely crucial to the overall folding and structure of the Mid1 polypeptide, since its substitution produced a strain with a phenotype similar to that of...
the strain lacking Mid1 (mid1Δ null strain) in limited extracellular [Ca^{2+}]. This is consistent with a recent report that demonstrated that C^{48} is required for Mid1 function in Saccharomyces cerevisiae (27).

In contrast to the growth of the Mid1 Cρ^{1x} and Mid1 Cρ^{1x2x} mutant strains, the mid1Δ strain expressing the Mid1 C^{656A}P^{657A} dipeptide mutant (Cρ^{2x}) was viable on low- [Ca^{2+}] medium, similar to the H99 wild-type strain and to the Mid1 Cρ^{wt}-reconstituted control strain, suggesting that this second set of C^{656}P^{657} residues was not required for the functional activity of Mid1 in mediating Ca^{2+} uptake. In addition, the Mid1 C^{656A}P^{657A} dipeptide mutant (Cρ^{2x}) showed growth sensitivity similar to that of the wild-type strain when exposed to either H2O2, H2O2 stress with limited extracellular [Ca^{2+}], paraquat, or FCCP (Fig. 6B; also, see Fig. S1 in the supplemental material), suggesting that the role of Mid1 in attenuating oxidative stress was not dependent on the C^{656}P^{657} dipeptide residues.

Intracellular survival of C. neoformans is dependent on Cch1 but not Mid1. Interestingly, the mid1Δ strain was viable and did not display any growth sensitivity when tested in an intracellular survival assay using an immortalized murine lung macrophage cell line (J774A.1) (Fig. 7). In contrast, a strain lacking Cch1 (cch1Δ) exhibited a modest intracellular growth defect in the same in vitro assay (Fig. 2B). This was supported by spot sensitivity assays, which revealed that the cch1Δ strains had significant growth defects upon exposure to the oxidative stress-inducing agents H2O2 and paraquat and the mitochondrial inhibitor FCCP (Fig. 2A). The growth differences observed among the strains whose results are shown in Fig. 2A were due solely to the absence of Cch1 because the mid1Δ strain showed no defect when grown on H2O2,

FIG 6 The regulatory role of Mid1 in the Ccp1-mediated oxidative stress response is independent of the conserved CP dipeptides. (A) Graphical representation of the C-terminal region of the Mid1 protein containing the cysteine-proline (CP) dipeptides demonstrating the sequence conservation at the CP dipeptide positions CP1 (C^{648}P^{649}) and CP2 (C^{656}P^{657}) and the fairly high frequency of the CP amino acids at this position (arrows). (B) Site-directed mutagenesis was used to replace CP1, CP2, or CP1 and CP2 with alanine. The mid1Δ strains of C. neoformans expressing the Mid1 CP1 mutant, CP2 mutant, CP1-CP2 mutant, and wild-type CP were designated CP1x, CP2x, CP1x2x, and CPwt strains, respectively. CP2 was not required for mediating oxidative stress, but CP1 was required for the functional activity of Mid1, suggested by the significant growth defect of the CP1x mutant strain in limited-[Ca^{2+}] environments (2 mM and 4 mM BAPTA, a Ca^{2+}-specific chelator). This is consistent with the growth defect of the mid1Δ strain under similar conditions (Fig. 1).
paraquat, and FCCP under similar assay conditions (see Fig. S1 in the supplemental material). The resistance of the cchlΔ midlΔ double mutant to H2O2 and paraquat was unexpected, because our data showed that both Mid1 and Cch1 played a role in mitigating oxidative stress (Fig. 1, 3, and 7A), and we expected that the loss of both components of the Cch1-Mid1 channel complex would lead to a stronger growth defect phenotype during oxidative stress (Fig. 2A). This result was further supported by the intracellular macrophage assay, where the cchlΔ midlΔ mutant did not display a growth defect, in contrast to the growth defect observed for the cchlΔ strain (Fig. 2B).

To further confirm the contribution of CMC in oxidative stress resistance, we tested the growth of the cchlΔ midlΔ strain in the presence of FCCP, a protonophore known to disrupt mitochondrial functions in other fungi (28) (Fig. 2A and 8). As shown in Fig. 2A, the cchlΔ midlΔ double mutant was as defective as the cchlΔ strains in the presence of FCCP. This result was consistent with the protective role of Cch1 during oxidative stress, since mitochondrial functions are important for oxidative stress resistance in C. neoformans (26). A likely scenario for the resistance of the cchlΔ midlΔ strain to oxidative stress would be the existence of a compensatory mechanism that is activated in the absence of both Mid1 and Cch1 under H2O2 and paraquat stress but not with FCCP.

Since paraquat is a superoxide (O2−) generator and C. neoformans superoxide dismutase (SOD) can convert superoxide to H2O2 (25), it is likely that the compensatory response observed for the cchlΔ midlΔ mutant is activated in the presence of H2O2. To test this idea, the cchlΔ midlΔ strain was assayed in the presence of both FCCP and H2O2. We reasoned that the presence of H2O2 would activate this compensatory mechanism and alleviate the growth defect of the cchlΔ midlΔ mutant (Fig. 8). Consistent with the existence of a compensatory response pathway, we found that while the cchlΔ midlΔ strain is as sensitive to FCCP as the cchlΔ mutants, the addition of 2 mM H2O2 to the medium containing FCCP restored the growth of cchlΔ midlΔ to the level of the H99 WT strain, while the growth defect of the cchlΔ strains became more drastic (Fig. 8).

**DISCUSSION**

Oxidative stress implies that the cellular redox status has shifted to an increased oxidized state, and this shift may be a result of cells exposed to environmental oxidants or stresses like heavy metals, ionizing and UV irradiation, and others. Reactive oxygen or nitrogen species can also be endogenously produced under pathological conditions or the aging of cells (29, 30). Either way, once formed, oxidants can interact with cellular components and modify calcium-mediated signaling events (30).

It is well known that oxidative stress alters calcium signaling (31). In the presence of exogenous agents that produce acute oxidative stress, cells respond by altering and sustaining high cytosolic calcium levels via the influx of calcium across the plasma membrane and calcium release from intracellular stores like the ER (32). A rise in cytosolic calcium triggers a rise in mitochondrial calcium, and depending on the strength or type of oxidative stress, mitochondrial calcium accumulation can switch from a positive effect to a cell death signal (32, 33).

The mitochondrial antioxidant protein cytochrome c peroxidase (Ccpl) catalyzes the degradation of hydrogen peroxide (25, 34). Ccpl is a water-soluble heme-containing enzyme of the peroxidase family that takes reducing equivalents from cytochrome c and reduces hydrogen peroxide to water (34). In C. neoformans, Ccpl promotes resistance to oxidative stress-inducing agents in vitro, consistent with similar observations made with S. cerevisiae (25, 26, 35). In vivo, the cchlΔ strain of C. neoformans was as virulent as the wild-type strain, suggesting that Ccpl does not act alone and that other compensatory mechanisms are in place in order to mitigate oxidative stress (25).

We detected an interaction between Mid1 and Ccpl, but the regulatory role of Mid1 in the Ccpl-mediated oxidative stress response does not appear to depend on heme binding. Not only does heme serve as a prosthetic group in enzymes like Ccpl, it also functions as a signaling molecule that controls various cellular processes (36). For instance, HapI is a heme-activated transcrip-
tion factor that mediates oxygen sensing in *S. cerevisiae* (37), and cytochrome *c* peroxidase also functions as a heme-based H$_2$O$_2$ sensor that can mediate antioxidant stress (34). The association between Hap1 and heme may be dependent on a series of conserved cysteine-proline (CP) dipeptide residues that we identified in Mid1 as well. The CP dipeptides are known as the heme-regulatory motifs (34). Despite the high level of conservation of both CP dipeptides across fungal species, amino acid substitution analysis revealed that the loss of the second CP dipeptide had no effect on H$_2$O$_2$ stress response in *C. neoformans*. We could not assess the role of the first CP dipeptide, since this cysteine residue was critical for Mid1 functional activity, consistent with a previous finding (27).

Antimycin blocks the Ccp1 pathway by binding to Q site of cytochrome *c* reductase and thus inhibiting oxidation of ubiquinol in the electron transport chain of oxidative phosphorylation (38). Inhibition blocks formation of proton gradients across the inner membrane and inhibits production of ATP, as protons cannot flow through the ATP synthase complex. The depletion of ATP produces a rise in [Ca$^{2+}$] in both the cytosol and the mitochondria, which could lead to cytochrome *c* release and ultimately cell death (39). In certain cell types, blocking calcium influx by inhibiting L-type calcium channels in the plasma membrane is cytoprotective, likely due to prevention of the uncontrolled accumulation of calcium in mitochondria (33, 39). In this study, we found that uncoupling the Cch1-Mid1 channel complex so that Mid1 was expressed independently of Cch1 protected cryptococci from antimycin-induced stress. The underlying mechanism may reflect the inability of Mid1 to mediate the influx of extracellular calcium into the cytosol, which would prevent sustained accumulation of mitochondrial calcium and thus curtail cell death. Alternatively, the release of Ccp1 into the cytosol and its subsequent association with Mid1 may promote a complex cellular defense network, possibly one that transmits stress signals to the oxidative-stress response transcriptional (26). In yeast, Ccp1 functions as an antioxidant enzyme; however, it may also play a role as a sensor in conveying oxidative stress in the mitochondria to Pos9, a transcription factor that mediates the oxidative stress response (16).

The “individual” contributions of Mid1 and Cch1 to oxidative stress resistance in *C. neoformans* appear disparate and complex. Mid1 contribution to the mitigation of oxidative stress is likely less significant and more specific than that of Cch1. The growth differences between the mid1Δ mutants and the WT H99 strain can be discerned only in low-calcium medium, under antimycin stress in the absence of Cch1 (i.e., growth sensitivity of the cch1Δ mutants), and under antimycin in combination with H$_2$O$_2$ in the absence of both Mid1 and Cch1 (i.e., growth sensitivity of the cch1Δ mid1Δ mutant). In contrast, Cch1 appears to have a more general role in promoting cryptoccocal survival during oxidative stress. The growth defect of the cch1Δ mutants can be easily discerned by the addition of H$_2$O$_2$ in the absence of the calcium chelator BAPTA and without the need for additional stressors, such as antimycin. Consistent with a greater contribution of Cch1 to oxidative stress tolerance, an intracellular growth defect was observed for the cch1Δ strain, while no growth differences were observed for the mid1Δ mutant compared to the wild-type strain in the macrophage cell line J774A.1.

Interestingly, the cch1Δ mid1Δ double mutant was resistant to oxidative stress induced by both H$_2$O$_2$ and paraquat but remained susceptible to oxidative stress induced by FCCP. The resistance of the cch1Δ mid1Δ strain to H$_2$O$_2$ remained strong even in the presence of FCCP. Together, these data suggest that in the absence of both Mid1 and Cch1, a compensatory mechanism is active to promote *C. neoformans* survival in the presence of H$_2$O$_2$ and paraquat but not FCCP. This compensatory mechanism is likely to be robust, since it is able to restore *C. neoformans* growth to levels comparable to that of the WT strain in the presence of FCCP, a highly toxic drug that disrupts mitochondrial function. The ability of the cch1Δ mid1Δ strain to resist oxidative stress was further supported by the macrophage intracellular survival assays, where the cch1Δ mutant displayed an intracellular growth defect but the cch1Δ mid1Δ mutant did not, suggesting the presence of a complex web of factors that can alleviate oxidative stress independently of CMC.

Collectively, this study suggests that Cch1 and Mid1 participate in a multiprotein complex that mediates both Ca$^{2+}$ influx and intracellular Ca$^{2+}$ homeostasis. However, the observed differences in the phenotypic responses of the cch1Δ and mid1Δ mutant strains to oxidative stress suggest partially overlapping functions in mitigating oxidative stress.

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