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

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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 enhanced 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.
and 2.

DNA ligase, and transformed into


cysteine

Mid1 harboring the CP1 mutation (C648AP649A [CP1x]), the CP2 mutation

with alanine or left unchanged, yielding three possible mutations: (i)

C656AP657A [CP2x]) or both CP 1 and CP 2 mutations (C 648AP649A

and proline residues in the CP dipeptides were either replaced

expression plasmid Jmm180 under the control of a

constitutive actin promoter was designated Mid1 CPwt.

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 (Thermo Sci-

entific Phusion Hot Start II; ThermoScientific) was used to generate

mutations in Mid1 cDNA using the primer pairs listed in Table 1. The

cysteine and proline residues in the CP dipeptides were either replaced

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Mid1 harboring the CP1 mutation (C648AP649A [CP1x]), the CP2 mutation

(C656AP657A [CP2x]) or both CP1 and CP2 mutations (C648AP649A

C656AP657A [CP2x2]). The Mid1 site-directed mutagenesis constructs and the

construct harboring the wild-type Mid1 cDNA clone (CPwt) were then

introduced into the strain of C. neoformans using biolistic transforma-

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-2a 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% heat-inactivated fetal bovine serum, 1% nones-

sential amino acids, 50 μg/ml penicillin-streptomycin (P/S), and 1

μg/ml lipopolysaccharide (LPS), and 1 μg/ml monocolonal antibody (MAB) 18B7 (a gift from A. Casadevall) for 1 h.

Cells were washed three times with 1X PBS and either incubated over-

night 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.

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, 5

μM 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 approxi-

mately 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 mea-

sured with the Bio-Rad QuickStart bovine serum albumin (BSA) standard

set. Protein lysates were separated on a 6.5% SDS-PAGE gel and semidry

blotted onto a polyvinylidene difluoride (PVDF) membrane. Purified mouse anti-Sos1 (BD Biosciences) at 1:500 and secondary horseradish

TABLE 1 Primers used to generate Mid1 CP mutant expression constructs

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>Mid1 Cp1x2x</td>
<td>Mid1-CP1x-R</td>
<td>CCATTGATACCGCGGCTGAGCGAT</td>
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<td></td>
<td>Mid1-CP2x-F</td>
<td>GCATCAGGGGCGCGAGAACCC</td>
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<td></td>
<td>Mid1-CP2x-F</td>
<td>GCATCAGGGGCGCGAGAACCC</td>
</tr>
<tr>
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<td>Mid1-CP1-R</td>
<td>CCATTGATACCGCGGCTGAGCGAT</td>
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<tr>
<td></td>
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<tr>
<td>Mid1 Cpwt</td>
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<td>ATAGCGCGCGTACGGCGGAGAGAGGGTATTTCAAAAAAGG</td>
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<tr>
<td></td>
<td>Midi_MfeI_R</td>
<td>GCTCAATTGCTACGGTTAACCATCTATTTCCCCAGCGAT</td>
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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 [Ca²⁺] in the presence of the calcium-specific chelator BAPTA (2), suggesting a requirement for Mid1 during oxidative stress (arrows). Serially diluted cells (10⁴, 10³, 10², and 10¹) from wild-type H99, the mid1Δ mutant, and the Mid1-reconstituted (mid1Δ::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'-ACCGGATCCATGACAACTCTATCGCCCGCTCCCTATATGCGCCATAATAATGCTGC-3'), and the reverse primer was Y2H_Mid1R4_NotIR (5'-ACCCGGGCGCCACTATGTCGCTTCCCCAAGGC-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-pSos bait and prey plasmids were cotransformed into a temperature-sensitive 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 leu- tial positives were selected by growth at the restrictive temperature of 37°C into a temperature-sensitive membrane. Both Mid1C-pSos bait and prey plasmids were cotransformed into the Cch1-Mid1C plasmid to create a Mid1C-hSos fusion "bait" plasmid. The forward primer was Y2H_Mid1Cterm_BamHF2 (5'-ACCGGATCCATGACAACTCTATCGCCCGCTCCCTATATGCGCCATAATAATGCTGC-3'), and the reverse primer was Y2H_Mid1R4_NotIR (5'-ACCCGGGCGCCACTATGTCGCTTCCCCAAGGC-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-pSos bait and prey plasmids were cotransformed into a temperature-sensitive 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 mid1Δ strain, transformed into Invitrogen ElectroMAX DH5α E. coli electrocompe- tent E. coli, prepared using a midikit (Qiagen), and sequenced. Potential positives were identified by a BLAST search in the Broad Institute (http://www.ebi.ac.uk/Tools/maa/tcoffee/).

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 Ca²⁺ 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 [Ca²⁺] (~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-[Ca²⁺] 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 mid1Δ strain displayed a growth defect in the presence of 2 mM and 4 mM BAPTA (a Ca²⁺-selective chelator) in contrast to the wild-type strain (Fig. 1). Interestingly, the growth defect of the mid1Δ strain was exacerbated upon exposure to the oxidative stress-inducing agent H₂O₂ under conditions of limited extracellular [Ca²⁺] (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 cch1Δ 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 oxidative stress response, we sought to identify the protein-binding partners of Mid1 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
We chose to use only a specific region of the C-terminal cytosolic portion of Mid1 in order to construct the hSos-Mid1 fusion protein. Specifically, (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.

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 nucleotide exchange factor Cdc25 stimulates GDP/GTP exchange on Ras (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 is activated, and growth of the cdc25-2 strain at the restrictive temperature (37°C) is restored.

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 × 10⁶ 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 (Ccpl) (Fig. 3B).

Ccpl and oxidative stress response in C. neoformans. Next we sought to resolve the physiological implication of the Mid1-Ccpl genetic interaction. In C. neoformans, cytochrome c peroxidase (Ccpl) 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 (cchlΔ) mutants were significantly more resistant to antimycin stress (Fig. 4).

FIG 3 A yeast-two hybrid screen revealed an interaction between Mid1 and Ccpl. (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 × 10⁶ 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 (Ccpl) was identified. In the presence of galactose at 37°C, the interaction between MAFB and pMyrSB 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.

FIG 4 Mid1 is protective and promotes cell survival under conditions that block the cytochrome c pathway. Spot sensitivity assays were used to further explore the connection between Mid1 and Ccpl. Strains of C. neoformans expressing Mid1 but lacking Cch1 were increasingly resistant to antimycin (Antim) (an inhibitor of the cytochrome c peroxidase [Ccpl] pathway), but the addition of H2O2 damped the resistance and increased the growth sensitivity. A strain lacking Mid1 and Cch1 was no longer viable under similar conditions suggesting that Mid1 is protective while the Ccpl pathway is blocked (arrow). Serially diluted cells (10⁵, 10⁴, 10³, 10², and 10¹) from wild-type H99, the cchlΔ mutant (three independent cchlΔ isogenic strains), a CCH1-reconstituted (cchlΔ/cchlΔ) strain, and a cchlΔ mid1Δ strain of C. neoformans were spotted onto YPD plates with 1 mM H2O2 either alone or in combination with increasing concentrations of antimycin. Plates were incubated at 30°C overnight.
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.

The protective role of Mid1 in oxidative stress conditions is independent of the conserved CP (cysteine and proline) dipeptide residues. It has been shown that Ccp1 can bind heme, and this association is mediated by CP dipeptide motifs (Fig. 5). The CP dipeptides are highly conserved among fungal species (Fig. 5, highlighting). We and others showed that Mid1 has 12 highly conserved cysteine residues near its C-terminal region, several of which are required for the functional activity of Mid1 (8). The two cysteine residues within the CP dipeptides belong to this group of 12 previously reported cysteines (27).

A graphical representation of the Mid1 amino acid multiple-sequence alignment for the region containing the two CP 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 C648P649 dipeptide replaced with two alanines (Mid1 C648AP649A [referred to here as CP1x]), Mid1 CP mutant strain 2 has the Mid1 C656P657 dipeptide substituted with alanine residues (Mid1 C656AP657A [CP2x]), and Mid1 CP mutant strain 3 has both CP dipeptides replaced with alanine residues (Mid1 C648AP657A [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 C648AP649A dipeptide mutant (CP1x) or the strain expressing the Mid1 C648AP649A-C656AP657A 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 C648P649 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^{548} is required for Mid1 function in *Saccharomyces cerevisiae* (27).

In contrast to the growth of the Mid1 CP1Δ and Mid1 CP1Δx2x mutant strains, the mid1Δ strain expressing the Mid1 C^{656P657A} dipeptide mutant (CP2Δx) was viable on low-[Ca^{2+}] medium, similar to the H99 wild-type strain and to the Mid1 CPwt-reconstituted control strain, suggesting that this second set of C^{656P657} residues was not required for the functional activity of Mid1 in mediating Ca^{2+} uptake. In addition, the Mid1 C^{656P657A} dipeptide mutant (CP2Δx) showed growth sensitivity similar to that of the wild-type strain when exposed to either H_{2}O_{2}, H_{2}O_{2} 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^{656P657} 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 H_{2}O_{2} 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 H_{2}O_{2},
FIG 7 Intracellular survival of C. neoformans is independent of Mid1. The J774A.1 macrophage cell line was used to examine whether Mid1 was required for intracellular survival. J774A.1 was infected with wild-type H99, the mid1Δ mutant, and the Mid1-reconstituted (mid1Δ::MID1) strain of C. neoformans for 4 h. Lysed macrophages were plated on YPD to determine intracellular growth of C. neoformans strains. CFU data were normalized by dividing the CFU counts from wells containing macrophages by the CFU counts from wells lacking macrophages (medium-alone control).

paraquat, and FCCP under similar assay conditions (see Fig. S1 in the supplemental material). The resistance of the cchlΔ mid1Δ 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 mid1Δ 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 mid1Δ 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 mid1Δ 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 mid1Δ 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 mid1Δ mutant is activated in the presence of H2O2. To test this idea, the cchlΔ mid1Δ 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 mid1Δ mutant (Fig. 8). Consistent with the existence of a compensatory response pathway, we found that while the cchlΔ mid1Δ 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 mid1Δ 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, Hap1 is a heme-activated tranскrip-
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 Qi 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 mitochondria to Pos9, a transcription factor that mediates the oxidative stress response (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 cryptococcal 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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