Activity of the Calcium Channel Pore Cch1 Is Dependent on a Modulatory Region of the Subunit Mid1 in Cryptococcus neoformans

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Cryptococcus neoformans and other fungi express the Cch1-Mid1 channel (CMC channel) in their plasma membrane (1–7). C. neoformans is a fungal pathogen that causes life-threatening disease primarily in patients with a compromised immune system (8, 9). The survival of C. neoformans in low Ca\(^{2+}\) environments requires the activity of CMC (3–5). The notion of the role of CMC as the only high-affinity Ca\(^{2+}\) channel in the plasma membrane is supported by genetic analysis demonstrating that strains of C. neoformans lacking CCH1 or MID1 display growth sensitivity under conditions of limiting extracellular, free [Ca\(^{2+}\)] (3, 4). Store-operated Ca\(^{2+}\) (SOC) entry in eukaryotes involves the influx of Ca\(^{2+}\) across the plasma membrane in response to the depletion of Ca\(^{2+}\) from endoplasmic reticulum (ER) stores (10–14). A prolonged depletion of Ca\(^{2+}\) due to stresses inflicted on the ER can result in cell death unless the Ca\(^{2+}\) is restored to homeostatic levels (11, 13, 14).

The recent electrophysiological characterization of CMC revealed that the channel is gated by the depletion of intracellular ER Ca\(^{2+}\) stores, indicating that CMC functions primarily to replenish these stores and reestablish Ca\(^{2+}\) homeostasis (2). This notion was supported by evidence from recordings of inward Ca\(^{2+}\) currents that were specifically activated upon the depletion of intracellular ER Ca\(^{2+}\) stores with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, cesium salt (BAPTA-AM; a Ca\(^{2+}\) chelator), or thapsigargin (inhibitor of the SERCA Ca\(^{2+}\)-ATPase) (2). Cch1 was also found to be highly permeable with respect to to both Ca\(^{2+}\) and Ba\(^{2+}\) ions, while La\(^{3+}\) specifically blocked Cch1-mediated Ca\(^{2+}\) currents (2). Ca\(^{2+}\) movement through Cch1 was not voltage dependent, suggesting that CMC is not gated by voltage. This was consistent with the lack of the highly conserved voltage sensor, a hallmark of voltage-gated channels, within the Cch1 protein sequence (3, 15–17). Collectively, these studies indicated that CMC displayed some features intrinsic to SOC channels (10–12, 14, 18).

The survival of C. neoformans under conditions of ER stress was dependent on the expression of CMC, further indicating that Cch1 and its partner Mid1 play a critical role in the restoration of Ca\(^{2+}\) homeostasis (2). Similarly, yeast (Saccharomyces cerevisiae) survival under conditions of ER stress is supported by an active CMC that likely promotes Ca\(^{2+}\) influx during secretory Ca\(^{2+}\) depletion, consistent with the electrophysiological evidence (5, 19, 20). The activation of Cch1-mediated Ca\(^{2+}\) currents is dependent on the coexpression of Mid1, indicating that Mid1 is an essential component of CMC (2). This is consistent with earlier genetic data that demonstrated identical phenotypic defects of strains lacking either MID1 or CCH1 (10, 11, 21, 22). Unlike other accessory proteins of channel pores, Mid1 does not mediate the trafficking of Cch1 to the plasma membrane, but Mid1 expression in yeast appears to be stabilized by Cch1 (4, 23). Although it has been shown that Mid1 in S. cerevisiae (ScMid1) can operate as a stretch-activated nonselective cation channel under certain conditions, we found that CnMid1 does not have any independent channel activity under conditions of Ca\(^{2+}\) depletion, suggesting that its primary role, although elusive, is to activate Cch1 (2, 24).

In this study, we sought to resolve the contribution of Mid1 to the role of CMC in C. neoformans through the analysis of Mid1-truncated mutants and site-directed mutagenesis of Mid1. Microscopy, biochemical, and electrophysiological analysis revealed that Mid1 contains a modulatory region in its C-terminal tail that

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strains were constructed as previously described (2–4, 25). Strains were 
recovered from 15% glycerol stocks at 
serum and antibiotics (penicillin-streptomycin [PEN-STREP]) in a 5%
cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf
medium. Cells of 
serotype A) and 
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is required for establishing Ca^{2+} homeostasis. This region consists
of the last 24 amino acids of Mid1 and functions as a modulatory
region that affects Cch1 channel activity directly and mediates the
trafficking of Mid1 to the plasma membrane. The modulatory
region may serve as a likely point of contact between Cch1 and
Mid1. This report demonstrates that restoring Ca^{2+} homeostasis
via CMC in C. neoformans is dependent on the fundamental role of
a modulatory region of Mid1.

MATERIALS AND METHODS

Cell culture and reagents. All C. neoformans var. grubii strains (H99
MA1a serotype A) and S. cerevisiae strains (W303 MA1a, mid1Δ)
were recovered from 15% glycerol stocks at −80°C prior to use in these
experiments. The mid1Δ, cch1Δ, and mid1Δ cch1Δ mutant C. neoformans
strains were constructed as previously described (2–4, 25). Strains
were maintained on YPD (1% yeast extract, 2% peptone, and 2% dextrose)
medium. Cells of C. neoformans were cultured in YPD medium at 30°C for
24 h. Cells from the HEK293 human embryonic kidney cell line were
cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf
serum and antibiotics (penicillin-streptomycin [PEN-STREP]) in a 5%
CO_2 incubator at 37°C. HEK293 cells were purchased from ATCC (CRL-
1573) (2). Where indicated, a cell-impermeant form of 1,2-bis(2-
aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, cesium salt (BAPTA; Invitrogen/Molecular Probes, Carlsbad, CA), was added to YPD medium
(26).

Plasmids and transfection. The cDNA of C. neoformans MID1 (CnMID1) was cloned into the pcDNA3.1/CT-GFP-TOPO expression plasmid, under the control of the cytomegalovirus (CMV) promoter, using conventional molecular biological techniques as previously described (2). Briefly, the MID1 amplicon was generated from cDNA amplified by conventional molecular biological techniques as previously described (2). HEK293 cells that had been transfected with MID1-cDNA-GFP or the Mid1-GFP truncated or point mutants were biotinylated according to the manufacturer’s instructions (Invitrogen) (2). The cDNA of CCH1 was also expressed in HEK293 cells as previously described (2). Approximately 1 μg plasmid DNA was added to 100 μl of Opti-MEM I reduced-serum media and mixed with 1.75 ml of Lipofectamine 2000, and the mixture was incubated for 30 min at room temperature. The mixture was mixed with 1.75 ml of Lipofectamine 2000, and the mixture was incubated for 30 min at room temperature. The mixture was mixed with 1.75 ml of Lipofectamine 2000, and the mixture was incubated for 30 min at room temperature. The mixture was mixed with 1.75 ml of Lipofectamine 2000, and the mixture was incubated for 30 min at room temperature. The mixture was mixed with 1.75 ml of Lipofectamine 2000, and the mixture was incubated for 30 min at room temperature. The mixture was mixed with 1.75 ml of Lipofectamine 2000, and the mixture was incubated for 30 min at room temperature. The mixture was mixed with 1.75 ml of Lipofectamine 2000, and the mixture was incubated for 30 min at room temperature. The mixture was mixed with 1.75 ml of Lipofectamine 2000, and the mixture was incubated for 30 min at room temperature.

Sensitivity spot assays. For spot assays, C. neoformans strains and S.
cerevisiae strains were cultured in YPD medium overnight at 30°C. Cul-
tures were pelleted and resuspended in sterile H_2O. Serially diluted cells
(10^6, 10^5, 10^4, 10^3) were added to YPD agar plates supplemented with
1 mM BAPTA (2).

TABLE 1 Primers used in this study

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Primer designation</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>MID1-cDNA-F</td>
<td>ATGCCAGCGAGAGAGTGTA</td>
</tr>
<tr>
<td>2</td>
<td>MID1-cDNA-R</td>
<td>CTATCCGTTCACCATCTAT</td>
</tr>
<tr>
<td>3</td>
<td>MID1-F-point mutations</td>
<td>ATGCCAGCGAGAGAGGTGATTTCCA</td>
</tr>
<tr>
<td>4</td>
<td>MID1-R-R619A</td>
<td>TTCCGTTCACCATGCTTTTCGGTG</td>
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<td>5</td>
<td>MID1-R-C621A</td>
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<tr>
<td>6</td>
<td>MID1-R-S605A</td>
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<tr>
<td>7</td>
<td>MID1-R-CA-124</td>
<td>GACGGCTCTCGTTCGTTGTTGAACCCG</td>
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<tr>
<td>8</td>
<td>MID1-R-CC-124</td>
<td>GTTTATGGGCGCATATAAGTTGGTCCCAA</td>
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<tr>
<td>9</td>
<td>MID1-R-CC-24</td>
<td>GCGCGCAACACACCTGTCGTAAGCAGA</td>
</tr>
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Primer no. Primer designation Primer sequence
1 MID1-cDNA-F ATGCCAGCGAGAGAGTGTA
2 MID1-cDNA-R CTATCCGTTCACCATCTAT
3 MID1-F-point mutations ATGCCAGCGAGAGAGGTGATTTCCA
4 MID1-R-R619A TTCCGTTCACCATGCTTTTCGGTG
5 MID1-R-C621A TTCCGTTACCCCATCTTATCCCAGCGATCTG
6 MID1-R-S605A TTCCGTTACCCCATCTTATCCCAGCGATCTG
7 MID1-R-CA-124 GACGGCTCTCGTTCGTTGTTGAACCCG
8 MID1-R-CC-124 GTTTATGGGCGCATATAAGTTGGTCCCAA
9 MID1-R-CC-24 GCGCGCAACACACCTGTCGTAAGCAGA

a R indicates a reverse primer (5’ to 3’ direction), and F indicates a forward primer (5’ to 3’ direction).

For spot assays, C. neoformans strains and S. cerevisiae strains were cultured in YPD medium overnight at 30°C. Cultures were pelleted and resuspended in sterile H_2O. Serially diluted cells (10^6, 10^5, 10^4, 10^3) were added to YPD agar plates supplemented with 1 mM BAPTA (2).

Protein analysis. Biotinylation of HEK293 cells expressing Mid1-GFP or the Mid1 truncated GFP mutants or the Mid1 point GFP mutants was performed according to the protocol described in the manual for a Pierce cell surface biotin protein isolation kit (Thermo Scientific) and as described previously (2). HEK293 cells that had been transfected with Mid1-GFP or the Mid1-GFP truncated or point mutants were biotinylated according to the specifications outlined in the kit. Biotinylated protein samples were separated on a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes using semidry transfer (BioRad). Western blotting for Mid1 was performed using a rabbit polyclonal antibody to GFP (Abcam, Cambridge, MA) (1:5,000) as the primary an-

**Mid1 Modulatory Region Is Required for Cch1 Activity**

[Note: The rest of the text is not visible in the provided snippet, but it likely continues with the experimental details and results discussed in the context of Mid1 and its role in C. neoformans.]
tibody followed by detection with a goat polyclonal antibody to rabbit immunoglobulin G (IgG) (H&L horseradish peroxidase [HRP]; Abcam, Cambridge, MA) (1:5,000).

Confocal microscopy. Cells of C. neoformans were grown to mid-log phase in YPD overnight at 30°C. Cells were fixed in 3% formaldehyde for 1 h at 30°C and washed twice, and spheroplasts were obtained by digesting cell walls with 40 mg/ml lyzing enzyme from Trichoderma harzianum (Amersham) in 1 M sorbitol–10 mM sodium citrate (pH 5.8) for 3 h at 30°C. Cells were then washed in 1 M sorbitol–10 mM sodium citrate buffer, diluted in phosphate-buffered saline (PBS), and dried on microscope slides. Cells were then incubated with a peptide antibody raised against Cch1 (Antibodies Incorporated, Davis, CA) (1:500 dilution) plus 1 mg/ml bovine serum albumin (Sigma) at 4°C overnight. The specificity of the primary peptide antibody for Cch1 was confirmed in previous publications (2, 4). Cells were subsequently washed extensively in PBS and then incubated for 1 h with fluorescein isothiocyanate (FITC)–conjugated secondary antibody (Abcam, Cambridge, MA) (1:1,000). To examine the localization of Mid1 and Mid1 truncated mutants in HEK293 cells, cells expressing plasmids containing Mid1-GFP mutant fusion proteins were fixed in 4% paraformaldehyde for 20 min at room temperature, washed in PBS, and subsequently visualized with a confocal microscope. Immunofluorescence analysis of Cch1 was performed in HEK293 cells that had been fixed in 4% paraformaldehyde and permeabilized. A primary peptide antibody against the C terminus of Cch1 (Antibodies Incorporated, Davis, CA) was added to HEK cells at a dilution of 1:500 as previously described (1–6). Cells were then incubated with a peptide antibody raised against Cch1 (Antibodies Incorporated, Davis, CA) (1:500 dilution) plus 1 mg/ml bovine serum albumin (Sigma) at 4°C overnight. The specificity of the primary peptide antibody for Cch1 was confirmed in previous publications (2, 4). Cells were subsequently washed extensively in PBS and then incubated for 1 h with fluorescein isothiocyanate (FITC)–conjugated secondary antibody (Abcam, Cambridge, MA) (1:1,000). To examine the localization of Mid1 and Mid1 truncated mutants in HEK293 cells, cells expressing plasmids containing Mid1-GFP mutant fusion proteins were fixed in 4% paraformaldehyde for 20 min at room temperature, washed in PBS, and subsequently visualized with a confocal microscope. Immunofluorescence analysis of Cch1 was performed in HEK293 cells that had been fixed in 4% paraformaldehyde and permeabilized. A primary peptide antibody against the C terminus of Cch1 (Antibodies Incorporated, Davis, CA) was added to HEK cells at a dilution of 1:500 as previously described (2, 4, 25). Cch1 was visualized with a secondary antibody (1:1,000) conjugated with Texas Red (Abcam) (2, 4, 25). Fluorescence was examined using a Carl Zeiss LSM-5 inverted confocal microscope. Mid1 and Cch1 were examined with a laser line (488 nm and a 568 nm) and a 40× objective. The images were scanned and captured at a resolution of 1,024 by 1,024 pixels.

Patch clamp measurements. Experiments were performed using conventional whole-cell and single-channel patch clamp techniques (29, 30). In order to measure currents through Cch1, HEK cells expressing Cch1 and Mid1 were visually selected based on bright GFP fluorescence. Currents were stimulated by passive Ca$^{2+}$ store depletion with a pipette solution containing 10 μM BAPTA-AM or by activation with thapsigargin (100 μM). Recording electrodes were pulled with a vertical puller (HEKA Instruments Inc., Bellmore, NY) from borosilicate glass capillaries, coated with Sylgard, fire polished, and filled with a solution containing either BAPTA-AM or thapsigargin plus 130 mM Cs$^+$-positive (Cs$^+$) methane sulfonate, 5 mM MgCl$_2$, 500 μM Mg-ATP, and 20 mM HEPES (pH 7.2) with CaOH. Recording electrodes had a tip resistance of ∼5 MΩ when placed in an external solution containing 2, 5, or 10 mM CaCl$_2$ or BaCl$_2$ plus 140 mM NMDG (N-methyl-D-glucamine) (or 140 mM Na$^+$ methanate sulfonate in divalent-free solution and free of NMDG), 10 mM glucose, and 10 mM HEPES (pH 7.4) with NaOH. Whole-cell currents were measured by voltage ramps (−120 to +60 mV, lasting 200 ms) from a holding potential of −60 mV. All voltages have been corrected for liquid junction potentials. Currents were filtered at 2 kHz with a four-pole Bessel filter (Dagan) contained in the Dagan amplifier and sampled at 5 kHz. The amplifier was interfaced with a Digidata 1322A digitizer (Axon Instruments, Foster City, CA) in order to digitize data. All data were corrected for leak currents. For single-channel measurements, the outside-out patch configuration was used. Both the extracellular and pipette solutions used for single-channel recordings were the same as those used in whole-cell experiments. Single-channel records were obtained at voltages of −60 mV and −80 mV and sampled at 25 kHz. Currents were analyzed with pCLAMP 9.0 software (Axon) and graphed with SigmaPlot 8.0 software on an IBM 3-GHz computer.

**RESULTS**

A low, free Ca$^{2+}$ assay identified the C terminus of Mid1 as a regulatory region required for CMC activity. In order to determine the functional activity of Mid1 in C. neoformans, truncated mutants of Mid1 that lacked regions of the C terminus and N terminus were created. We reasoned that since Mid1 is an integral membrane protein, both of the predicted cytosolic ends of the Mid1 protein represented viable regions for association with Cch1 (2, 19, 20). Three C-terminal-truncated mutants were created: (i) a Mid1-CA-124 mutant (lacking 124 amino acids); (ii) a Mid1-CB-91 mutant (lacking 91 amino acids); and (iii) a Mid1-CC-24 mutant (lacking 24 amino acids) (Fig. 1A). Three N-terminal-truncated mutants of Mid1 were also created, but despite several attempts, expression of these mutants in HEK293 cells or in C. neoformans was not detected (data not shown). These results indicated that the N-terminal region of the Mid1 protein might be required for stability of the Mid1 polypeptide.

It is known that Mid1 is an essential partner and coregulator of Cch1 in C. neoformans, yeast, and other fungi (1–6). Accordingly, a strain of C. neoformans lacking Mid1 displays growth sensitivity under conditions of limited extracellular Ca$^{2+}$ (∼100 nM)—a similar phenotype was reported for the cch1Δ deletion strain (2, 3, 5). Thus, we used a simple functional assay based on this phenotype to resolve whether the channel activity of Cch1 was dependent on the C-terminal and/or N-terminal regions of the Mid1 protein. To do this, we monitored whether the expression of the Mid1 truncated mutants in the mid1Δ deletion background could rescue the mid1Δ deletion strain sensitivity in the presence of low, free Ca$^{2+}$ medium. For this assay, growth of C. neoformans and yeast strains on agar plates with YPD media (where the free [Ca$^{2+}$] was ∼0.140 mM) and growth on YPD medium plus BAPTA, a Ca$^{2+}$ chelator (where the free [Ca$^{2+}$] was ∼100 nM), were compared (Fig. 1B and C) (26).

The assays revealed that a mid1Δ deletion strain of C. neoformans expressing a Mid1-CC-24 truncated mutant (lacking the last 24 amino acids) displayed a significant sensitivity to an extracellular environment that was low in free Ca$^{2+}$, suggesting that CMC activity was compromised (Fig. 1B). A similar result was observed for the mid1Δ deletion strain expressing the Mid1-CA-124 mutant or the Mid1-CB-91 mutant (data not shown). However, the expression of full-length Mid1 in the mid1Δ strain did indeed rescue the growth sensitivity of the mid1Δ deletion strain in low, free Ca$^{2+}$, suggesting that the assay worked as predicted (Fig. 1B). These results suggested that CMC activity was dependent on the C-terminal tail (the last 24 residues) of Mid1. Interestingly, the expression of CnMid1 full-length cDNA in yeast (S. cerevisiae) did not rescue the mid1Δ deletion strain sensitivity of yeast, suggesting significant differences in the amino acid sequences of CnMid1 and ScMid1 and/or a lack of accessory proteins in yeast that are required for Cch1 channel activity in C. neoformans (Fig. 1C).

Multiple alignment of the primary structures of Mid1 reveals a high degree of similarity in the latter half of Mid1 but not within the modulatory region. A protein structure for Mid1 has not yet been resolved; however, previous results have determined that Mid1 forms an oligomeric structure that exists primarily as an integral plasma membrane protein and specifically associates with Cch1 (2, 5). In silico analysis suggested that Mid1 may have 2 to 4 predicted transmembrane regions and that both the C-terminal and N-terminal ends likely face the cytoplasm. Interestingly, similar analyses revealed that the N-terminal region of Mid1 lacks a predicted signal sequence that is often present in plasma membrane-bound proteins. This is consistent with previous observations of ScMid1 characteristics (20, 31).
Analysis of the Mid1 amino acid sequence alignments revealed some degree of similarity in the region closest to the C terminus; however, very little similarity was observed within the modulatory region (last 24 residues) (see Fig. S1 in the supplemental material) (32, 33). The conservation of 11 of the 12 cysteine residues within the latter half of Mid1 was striking, and it indicated a potential role for this group of cysteines in complex formation (see Fig. S1 in the supplemental material [black filled triangles]). Another noteworthy feature of the Mid1 alignment was the presence of two conserved heme-regulatory cysteine-proline (CP) motifs (CP dipeptide) at position 924 and position 932 (see Fig. S1 in the supplemental material [black filled ovals]). This motif has been implicated in mediating cell signaling events that in some cases involved a Ca\(^{2+}\)-dependent Slo1 potassium (BK) channel that operated via the binding of heme to the CP motifs (21, 34–38).

Mid1 trafficking to the plasma membrane is dependent on a modulatory region within the C-terminal end. To further explore the mechanism of Mid1, confocal microscopy was used to examine the Mid1-CC-24 truncated mutant protein in vivo. To do this, a Mid1-CC-24–DsRed fusion protein was constructed and expressed in C. neoformans. As a control, we examined the mid1\(\Delta\) deletion strain of C. neoformans expressing the Mid1-CC-24–DsRed fusion protein by light microscopy and found that these cells appeared physically unaltered, as expected (Fig. 2A, differential interference contrast [DIC]). Confocal microscopy was then used to examine the expression of the Mid1-CC-24–DsRed fusion protein. Unexpectedly, the Mid1-CC-24 truncated mutant protein was not observed on the cell surface of cryptococci as is typically observed for both Mid1 and Cch1 full-length proteins (Fig. 2B, C, and D). Indeed, it has been previously shown that Cch1 and Mid1 colocalize and reside in the plasma membrane as a complex (2, 4, 5). Instead, the Mid1-CC-24 –DsRed fusion protein appeared to be largely cytosolic, indicating that Mid1 could not localize to the plasma membrane of C. neoformans independently of the 24 amino acids in its C-terminal region (Fig. 2B).

We further examined this region of Mid1 more closely in HEK293 (human embryonic kidney) cells. The HEK293 cell line was chosen because it represents an excellent expression system for characterizing channel proteins and because we previously successfully expressed the Cch1-Mid1 channel complex in HEK293 cells and demonstrated by patch clamp analysis that Cch1 is activated by the depletion of intracellular Ca\(^{2+}\) stores (2). Our aim in this study was to determine whether the C-terminal region of Mid1 directly affected Cch1 channel activity. Thus, prior

![FIG 1 Activity of the Cch1 channel in C. neoformans is dependent on a C-terminal modulatory region of the subunit, Mid1. (A) Three truncated mutants of Mid1, each lacking a portion of the C terminus, were constructed. Mid1-CA-124 lacked 124 amino acids (aa), Mid1-CB-91 lacked 91 amino acids, and Mid1-CC-24 lacked 24 amino acids. Each mutant was constitutively expressed in the mid1\(\Delta\) deletion background of C. neoformans under the control of the actin promoter (27). (B) Sensitivity spot assays were used to identify regions of the Mid1 protein critical for Cch1 activity. The Mid1-CC-24 truncated mutant did not rescue the sensitivity of the mid1\(\Delta\) deletion strain on low, free [Ca\(^{2+}\)] medium, suggesting that this region is critical for Cch1 activity. (C) A wild-type yeast (Saccharomyces cerevisiae) strain was transformed with full-length CnMid1. Two representative transformants expressing CnMid1 did not restore growth of the Scmid1\(\Delta\) deletion strain on low, free [Ca\(^{2+}\)] medium.](http://ec.asm.org)
with this analysis, Ca\textsuperscript{2+} out an intact C terminus as observed in
that Mid1 could not localize to the surface of HEK293 cells with-
not expressed on the surface of HEK293 cells, further indicating
mutants (Mid1-CA-124, Mid1-CB-91, and Mid1-CC-24) were
terminal-truncated mutants (Fig. 3A). This suggested that these
were not detected in HEK293 cells expressing the three Mid1 C-
terminus of Cch1 and a Texas Red-conjugated secondary antibody were used to visualize Cch1 as reported
previously (2, 4). The specificity of the primary peptide antibody to Cch1 was
confirmed in previous publications (2, 4). Scale bars represent 10 \(\mu\)M.

FIG 2 Loss of the modulatory region of Mid1 alters the localization pattern of
Mid1 in \(C.\) neoformans. (A) Mid1-CC-24 –DsRed fusion protein was con-
structed by tagging the Mid1-CC-24 truncated mutant with DsRed (28) and
visualized by DIC and confocal microscopy. (A) A DIC image of cryptococci
expressing the Mid1-CC-24 –DsRed fusion protein revealed bright, spherical
cells of \(C.\) neoformans. (B and C) Interestingly, Mid1-CC-24 –DsRed appeared to be
mislocalized. This was in stark contrast to the full-length Mid1 (Mid1-
FL) dsRed fusion protein, which is predominately localized to the cell surface
of \(C.\) neoformans, consistent with its plasma membrane distribution as previ-
ously reported (2). (D) Immunofluorescence (Texas Red) of Cch1 revealed a
surface distribution in \(C.\) neoformans similar to that of Mid1, confirming that,
like Cch1, Mid1 is primarily localized to the plasma membrane in cells of \(C.\)
neoformans. A primary peptide antibody to the C terminus of Cch1 and a Texas
Red-conjugated secondary antibody were used to visualize Cch1 as reported
previously (2, 4). The specificity of the primary peptide antibody to Cch1 was
confirmed in previous publications (2, 4). Scale bars represent 10 \(\mu\)M.

FIG 3 The expression of Mid1 on the surface of HEK293 cells is dependent
on the C-terminal modulatory region. (A) Western blot analysis of surface
biotinylation of HEK293 cells expressing full-length Mid1-GFP fusion pro-
tein revealed two distinct bands corresponding to Mid1 (monomer, \(\sim 100\)
\(\text{kDa; complex, } \sim 200 \text{kDa (indicated by arrows)\}. In contrast, Mid1 poly-
peptides were not detected in Western blots of biotinylated HEK293 cells
expressing Mid1 truncation mutants (Mid1-CA-124, Mid1-CB-91, or
Mid1-CC-24). The Mid1-CA-124 mutant lacked 124 residues, Mid1-
CB-91 lacked 91 residues, and Mid1-CC-24 lacked 24 residues. Western
blotting for Mid1 was performed using a commercial rabbit polyclonal
antibody to GFP (1:5,000) as the primary antibody (Abcam, Cambridge,
MA) followed by detection with a goat polyclonal antibody to rabbit immu-
unoglobulin G (IgG) (1:5,000). (B and C) Surface expression of Cch1 is
independent of Mid1. Full-length Mid1 was tagged with a C-terminal GFP
expressed in HEK293 cells and visualized by confocal microscopy. Immuno-
fluorescence (Texas Red) of Cch1 (not tagged) in HEK293 cells showed
surface localization similar to that of Mid1. A primary peptide antibody to
the C terminus of Cch1 and a Texas Red-conjugated secondary antibody were
used to visualize Cch1 (2, 4). Scale bars represent 10 \(\mu\)M.

In striking contrast, the protein bands corresponding to Mid1 were not
detected in HEK293 cells expressing the three Mid1 C-
terminal-truncated mutants (Fig. 3A). This suggested that these
mutants (Mid1-CA-124, Mid1-CB-91, and Mid1-CC-24) were
not expressed on the surface of HEK293 cells, further indicating that
Mid1 could not localize to the surface of HEK293 cells with-
out an intact C terminus as observed in \(C.\) neoformans. Consistent
with this analysis, \(\text{Ca}^{\text{2+}}\) channel currents from HEK293 cells ex-
pressing Cch1-GFP and the Mid1-GFP truncated mutants were not
detected by conventional patch clamp techniques (data not
shown). Confocal microscopy revealed that Cch1 and Mid1 were
targeted to the surface of HEK293 cells independently of each
other; thus, the absence of CMC activity upon expression of the
Mid1 truncated mutants was not due to a defect in Cch1 trafficking
(Fig. 3B and C).

These results were further corroborated by confocal micros-
copy of HEK293 cells expressing the Mid1 truncated mutant-GFP
fusion proteins (Fig. 4). There is little doubt that polypeptides
referred to the Mid1 truncation mutants were indeed syn-
thesized and expressed in HEK293 cells, since strong fluorescence

FIG 4 C-terminal-truncated mutants of Mid1 are mislocalized in HEK293
cells. Confocal microscopy was used to examine the localization of three trun-
cated mutants of Mid1 in HEK293 cells. (A) Full-length Mid1-GFP under the
control of the CMV promoter was expressed in HEK293 and observed on the
cell surface. (B, C, and D) However, expression of the Mid1 truncated mutants
lacking specified regions of the C terminus (Mid1-CA-124, Mid1-CB-91, and
Mid1-CC-24) displayed a diffuse and predominately cytosolic localization
pattern unlike that of full-length Mid1. The Mid1-CA-124 mutant lacked 124
residues, Mid1-CB-91 lacked 91 residues, and Mid1-CC-24 lacked 24 residues.
signals corresponding to the Mid1 truncated mutant-GFP fusion proteins were clearly detected (Fig. 4B, C, and D). However, the Mid1 truncated mutant proteins appeared mislocalized in HEK293 cells, since the mutants were no longer observed on the surface of HEK293 cells (Fig. 4B, C, and D). The localization patterns of the Mid1 truncation mutants in HEK293 cells were strikingly different from those of the full-length Mid1 protein, which displayed a cell surface distribution in HEK293 cells (Fig. 4A).

These results supported the biotin labeling experiment data and further indicated that the last 24 amino acid residues in the C-terminal tail of Mid1 are critical for targeting Mid1 to the plasma membrane in HEK293 cells.

Key amino acids in the C-terminal region of Mid1 promote CMC activity. Among the 24 amino acids within the C-terminal modulatory domain of Mid1, 3 amino acids were identified as potentially relevant residues. The charged amino acid (arginine; R619) and the only cysteine (C621) residue in this region mediating disulfide bond formation were selected for substitution experiments because of their key roles in bridging protein complexes. In addition, a predicted protein kinase C (PKC)-phosphorylated serine residue (S605) was selected because of its potential role in Ca\textsuperscript{2+} signaling. To establish the role of these residues in the molecular mechanism of Mid1, cysteine (C621A), arginine (R619A), and a serine residue (S605A) were replaced with alanine (Fig. 5A).

Single GFP fusion proteins of the Mid1 point mutations were constructed and examined in HEK293 cells. Interestingly, confocal microscopy revealed an altered localization pattern for all the Mid1 point mutations, with the exception of the Mid1 serine mutant (S605A) (Fig. 5B). In this case, HEK293 cells expressing the Mid1-S605A–GFP fusion revealed a localization pattern of the Mid1-S605A mutant that was similar to the expression of full-length, unaltered Mid1-GFP (Fig. 5B). In contrast, the most striking result occurred in HEK293 cells expressing the Mid1-R619A–GFP mutant protein, where a strongly diffuse localization pattern was observed (Fig. 5B). The Mid1-C621A–GFP mutant also appeared mislocalized, as determined by its presence throughout regions of the cytosol (Fig. 5B). This expression pattern was not consistent with the more uniform expression of Mid1-GFP along the surface of HEK293 cells (Fig. 5B).

To confirm the surface expression of the Mid1 serine mutant observed by confocal microscopy, HEK293 cells expressing Mid1-S605A–GFP, Mid1-R619A–GFP, Mid1-C621A–GFP, or Mid1-GFP were biotin labeled (Fig. 5C). Western blot analysis revealed that the polypeptides corresponding to full-length, unaltered Mid1 were detected in HEK293 expressing Mid1-S605A–GFP or Mid1-GFP (Fig. 5C), suggesting that the serine residue within the C-terminal modulatory region was not required for trafficking of Mid1 to the cell surface. However, Mid1 protein bands were not detected in biotin-labeled HEK293 cells expressing Mid1-R619A–GFP or Mid1-C621A–GFP mutants, indicating that both residues were required for the surface localization of Mid1 (Fig. 5C).

A predicted phosphorylation site in the modulatory domain of Mid1 alters the kinetics of CMC-mediated Ca\textsuperscript{2+} influx. Our results suggested that the serine residue in the C-terminal region of Mid1 was not involved in the trafficking of Mid1. However, since this residue is a predicted PKC-phosphorylation site and the only serine residue within this region, we questioned whether it might have a direct role in mediating Ca\textsuperscript{2+} influx via CMC. To examine this, the Mid1-S605A mutant was expressed in the mid1\textsuperscript{Δ} deletion strain of C. neoformans under the control of a constitutive, actin promoter (27). Reverse transcriptase PCR analysis revealed robust expression of the Mid1-S605A transcript in C. neoformans (Fig. 6A).

Interestingly, expression of the Mid1-S605A mutant in C. neoformans rescued the sensitivity associated with the mid1\textsuperscript{Δ} deletion strain on low, free Ca\textsuperscript{2+} media, suggesting that the lack of a S605 residue did not impair CMC-mediated Ca\textsuperscript{2+} influx (Fig. 6B). However, patch clamp analysis of Ca\textsuperscript{2+} channel currents from HEK293 cells expressing Cch1-GFP and Mid1-S605A–GFP revealed differences in the kinetics of CMC (Fig. 6C and D). Similar to previous electrophysiological characterization of CMC by ramp protocols, the depletion of ER Ca\textsuperscript{2+} stores by BAPTA-AM led to the development of robust, time-dependent Cch1-mediated Ca\textsuperscript{2+} currents (Fig. 6C) (2). We previously demonstrated that these inward Ca\textsuperscript{2+} currents activated specifically by Ca\textsuperscript{2+} store depletion were not detected in HEK293 cells expressing either Cch1 or Mid1 alone or an empty plasmid, indicating that Cch1 activity required Mid1 and, importantly, that the measured
Ca$^{2+}$ currents were not endogenous to HEK293 cells (2). The coexpression of Cch1 and Mid1-S605A in HEK293 cells resulted in Cch1-mediated Ca$^{2+}$/H$^{+}$ currents that displayed a more rapid activation and a significantly slower inactivation; however, current amplitude levels were largely unchanged (Fig. 6D). These differences in CMC kinetics suggest that Mid1 may have a direct role in regulating the activity of the Cch1 channel pore.

DISCUSSION

The first mechanistic study of Cch1 by electrophysiological characterization revealed that the Cch1-Mid1 channel complex is a Ca$^{2+}$-selective store-operated channel that is gated by the depletion of intracellular Ca$^{2+}$ (2). This conclusion is largely based on the direct measurement of Cch1-mediated inward Ca$^{2+}$ currents that displayed a more rapid activation and a significantly slower inactivation; however, current amplitude levels were largely unchanged (Fig. 6D). These differences in CMC kinetics suggest that Mid1 may have a direct role in regulating the activity of the Cch1 channel pore.

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We identified a modulatory region that consisted of 24 amino acids in the C terminus of Mid1 that is critical for Mid1 trafficking and CMC activity. A strain of *C. neoformans* expressing a mutant of Mid1 lacking this region was not viable on low, free Ca$^{2+}$ medium, indicating that Cch1 could not mediate the influx of Ca$^{2+}$ from the extracellular environment in the absence of the Mid1 modulatory domain. This is consistent with the role of CMC as the only high-affinity Ca$^{2+}$ channel in the plasma membrane of *C. neoformans*. We found that the C-terminal tail mediated the trafficking of Mid1 to the cell surface; however, Mid1 did not affect the surface expression of the channel pore (Cch1), since we have shown that Cch1 is expressed in the plasma membrane independently of Mid1 (2). The inability of the Mid1 C-terminal tail mutant to activate Cch1 was likely due to its inability to associate with Cch1, since the Mid1 mutant was no longer targeted to the plasma membrane. Interestingly, a similar observation was made in *S. cerevisiae*, indicating some similarity in the functional mechanism of Mid1 (39). However, although the C-terminal modulatory region of Mid1 in *C. neoformans* appeared to be fundamentally important for Mid1 trafficking, as observed for yeast, it is surprising that very little similarity was observed in the amino acid sequence within this region among fungi. This finding argues against the presence of a conserved targeting sequence within the modulatory domain of Mid1. In the case of *C. neoformans*, Mid1 trafficking could be the result of palmitoylation—a posttranslational modification of the cysteine within the modulatory region (C621).
thioester linkage of a 16-carbon palmitate lipid to a cytosolic-exposed cysteine residue could promote anchoring of Mid1 to the plasma membrane (40). Indeed, many regulatory subunits of ion channels have been identified as being palmitoylated (41). The lipid modification of channel pore proteins and/or channel subunits can promote both the surface expression and regulation of ion channels (41). In C. neoformans, it has been shown that palmitoylation regulates different aspects of cytoplasmic proteins and G proteins (9, 22). In the case of Mid1, lipid modification of C621 could provide a viable mechanism for membrane association and this would support the essential function of C621 in the modulatory domain during Mid1 trafficking; however, more work is needed to confirm this notion. Biochemical analysis has revealed that Mid1 forms an oligomeric structure likely mediated by disulfide bonds. The 11 conserved cysteine residues within the C-terminal half of Mid1 likely play a role in Mid1 complex formation. Our results indicated that substitution of the only cysteine residue (C621) within the modulatory domain of Mid1 prevented the targeting of Mid1 to the plasma membrane and consequently precluded the functional activity of CMC. Nevertheless, it is not clear whether the C621 residue also mediates the formation of the Mid1 oligomeric complex.

Further analysis of Mid1 identified a key arginine residue within the modulatory domain that was crucial for Mid1 function. It is known that polar and charged amino acids primarily cover the surface of proteins and usually associate with each other. Positively charged amino acids form salt bridges with charged residues, and this noncovalent interaction is crucial because it provides stability to the folded protein conformation (42, 43). Thus, it is likely that the arginine residue is responsible for maintaining a stable conformation of the modulatory region of Mid1.

Although not part of the modulatory region, two CP dipeptides in the C-terminal region of Mid1 are highly conserved among fungi. It has been shown in eukaryotes that heme binds to proteins such as transcription factors and mitogen-activated protein (MAP) kinases through CP motifs (34, 35, 37). In addition to the role of heme in the transport and storage of oxygen, heme also provides stability to the folded protein conformation (42, 43). Thus, it is not surprising that Ca$^{2+}$ currents mediated by Cch1 were not detected upon the coexpression of Cch1 and the Mid1 C-terminal-tail-truncated mutants, since it has been shown that the channel pore of CMC cannot conduct Ca$^{2+}$ currents independently of Mid1 (2). It is intriguing, however, that the loss of a serine residue (S605) within the modulatory region of Mid1 altered the kinetics of the Cch1 channel pore but did not prevent Cch1-mediated Ca$^{2+}$ influx in C. neoformans or in HEK293 cells. Patch clamp analysis revealed a significantly faster activation of channel currents upon depletion of ER Ca$^{2+}$ with BAPTA-AM and a slower inactivation of the channel; however, Ca$^{2+}$ current amplitude levels remained the same. These results suggest that the modulatory region of Mid1 may directly control the activity of the Cch1 pore. This notion is consistent with the functional mechanisms of some ion channels found in mammalian cells. For example, the beta-subunit of voltage-gated calcium channels has been shown to regulate biophysical properties of high-voltage-activated Ca$^{2+}$ channels in addition to regulating their own functional expression (44).

In summary, Ca$^{2+}$ homeostasis in C. neoformans is established through the action of the Cch1-Mid1 channel complex whose activity is dependent on a crucial set of amino acids in the C-terminal tail of Mid1. This nonconserved modulatory region is essential for Mid1 trafficking and for CMC activity.

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