Roles of calcineurin and Crz1 in antifungal susceptibility and virulence of *Candida glabrata*

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Running title: Role of the calcineurin pathway in *C. glabrata*

ABSTRACT

A *Candida glabrata* calcineurin mutant exhibited increased susceptibility to both azole antifungal and cell-wall damaging agents, and was also attenuated in virulence. Although a mutant lacking the downstream transcription factor Crz1 displayed a cell wall-associated phenotype intermediate to that of the calcineurin mutant and was modestly attenuated in virulence, it did not show increased azole susceptibility. These results suggest that calcineurin regulates both Crz1-dependent and -independent pathways depending on the type of stress.

Infections caused by the opportunistic fungal pathogen *Candida glabrata* are often difficult to treat due in part to its intrinsic or rapidly acquired resistance to azole antifungals (25). Calcineurin, a serine-threonine-specific protein phosphatase (1), has attracted attention as a new target of antifungal therapy based on the studies in several pathogenic fungi including *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (reviewed in reference 31). To date, very little is known about the calcineurin pathway in *C. glabrata*, although it has been reported that azole antifungals and calcineurin inhibitors have mild synergistic effects against *C. glabrata* wild-type strains (8, 15, 22). The transcription factor Crz1 is a downstream effector of calcineurin and is involved in azole tolerance in *C. albicans* (14, 23, 28); however, a Crz1 homolog in *C. glabrata* has yet to be characterized. Therefore, our objective was to evaluate the potential roles of calcineurin and its downstream target Crz1 in antifungal tolerance and virulence of *C. glabrata* through the characterization of mutant phenotypes.

Calcineurin is a heterodimer consisting of a catalytic A subunit and a Ca^{2+}-binding regulatory B subunit, and the association between the two subunits is necessary for phosphatase activity (19). To genetically disrupt calcineurin, we completely deleted the *CNB1* open reading frame (ORF) encoding the regulatory B subunit. *C. glabrata* orthologs of *CNB1* and *CRZ1* were identified in the genome database Genolevures (http://www.genolevures.org/). The primers and strains used in this study are listed in Tables 1 and 2, respectively. *C. glabrata* cells were propagated in minimal medium (0.7% yeast nitrogen base without amino acids, 2% dextrose) at 30°C, unless otherwise noted. Gene deletion was performed using the one-step PCR–based technique as described previously (13). Briefly, a 1-kb *Xho*I fragment containing *C. glabrata HIS3* was excised from pCgACH (17) and inserted into pBluescript II

SK+ (Stratagene, La Jolla, CA) to yield pBSK-HIS. A deletion construct was amplified from pBSK-HIS with primers tagged with the 100-bp sequences homologous to the flanking regions of the target ORF. Transformation of *C. glabrata* was performed using the lithium acetate (LiAc) protocol (6). Both PCR and Southern blotting were performed to verify that the desired homologous recombination occurred at the target locus without ectopic integration. To construct a centromere-based plasmid containing a *C. glabrata TRP1* marker, a 1,025-bp *Sac*I-*Kpn*I fragment containing the *Saccharomyces cerevisiae PGK1* promoter, a polylinker, and the *C. glabrata HIS3* 3' flanking region was excised from pGRB2.2 (12) and inserted into the corresponding site of pCgACT (17) to yield pCgACT-P. The entire ORFs of *C. glabrata CNB1* and *CRZ1* were amplified from the genomic DNA of CBS138 (10) and inserted into pCgACT-P to generate pCgACT-PNB and pCgACT-PRZ, respectively. The constructed plasmids were verified by sequencing before use. Complemented strains were made by transforming mutant strains with a plasmid construct containing the corresponding wild-type gene.

To examine the susceptibility of the generated mutants to antifungal agents, MIC assays were performed with a commercially prepared colorimetric microdilution panel (ASTY; Kyokuto Pharmaceutical Industrial Co., Ltd.) (Table 3) (24). Although increased azole susceptibility was observed in the Δ*cnb1* strain, the Δ*crz1* strain displayed susceptibility levels similar to, or in some instances lower than, those of wild-type cells. The *CNB1*-complemented strain displayed recovered azole tolerance. Neither the Δ*cnb1* nor Δ*crz1* strain had an effect on amphotericin B susceptibility. Next, we monitored the percent viability of each strain in the presence and absence of fluconazole as described previously (15). Although the antifungal activity of fluconazole is generally fungistatic, the drug was

fungicidal for the Δ*cnb1* strain (Fig. 1). In contrast, the deletion of *CRZ1* did not affect the antifungal activity of fluconazole. These results suggest that calcineurin is involved in azole tolerance via a Crz1-independent pathway in *C. glabrata*.

To examine cell wall-associated phenotypes in the Δ*cnb1* and Δ*crz1* strains, we examined their susceptibilities to different types of cell wall-damaging agents, including micafungin (inhibitor of β1,3-glucan synthesis), Congo red (inhibitor of chitin and β-glucan fiber formation), and calcofluor white (inhibitor of chitin polymer assembly), using a previously described method (15, 20, 26). Micafungin was kindly provided by Astellas (Tokyo, Japan) and dissolved in distilled water. Decreased micafungin tolerance was observed in the Δ*cnb1* and Δ*crz1* strains compared to that in the wild-type control and this was reversed in the reconstituted strains (Fig. 2). While the Δ*cnb1* strain showed decreased tolerance to both Congo red and calcofluor white, the Δ*crz1* strain exhibited only moderately decreased tolerance to Congo red and was unaffected by calcofluor white exposure (Fig. 2B). These results suggest that the calcineurin-Crz1 pathway plays a role in the response to β1,3-glucan defects and that calcineurin also regulates a Crz1-independent pathway(s) in response to impaired chitin construction in *C. glabrata*.

To date, the involvement of calcineurin and Crz1 in virulence has not been reported in *C. glabrata*. In contrast to the *C. neoformans* calcineurin mutant (21), deletion of either *CNB1* or *CRZ1* did not affect cell growth at 37°C in *C. glabrata* (data not shown), which is a necessary prerequisite for comparing virulence levels. We therefore performed a virulence assay using a murine model of disseminated candidiasis as described previously (5). Briefly, groups of 10 female, 8-week-old, BALB/c mice (Charles River Laboratories Japan, Inc., Japan) were infected via the lateral tail vein. The mice were euthanized 7 days after injection

to determine the number of organ CFU. In this study, no mice died before euthanasia. Statistical analyses were performed using the Kruskal-Wallis test with Dunn's posttest for multiple comparisons. A *P* value of <0.05 was considered statistically significant. Mice infected with the Δ*cnb1* strain showed significantly reduced fungal burden in all examined organs compared to those infected with the wild-type control and *CNB1*-complemented strains (Fig. 3). Decreased numbers of CFU of the Δ*crz1* strain were statistically significant in the kidney but not in the liver and spleen. The results from this assay indicate that the loss of calcineurin results in attenuated virulence while a deletion of *CRZ1* causes only a partial reduction.

This is the first report characterizing the phenotypes of *C. glabrata CNB1* and *CRZ1* mutants, and it has identified both similarities and differences with findings for other fungi. For example, the observed *C. glabrata* Δ*cnb1* strain phenotype, which is characterized by an increased susceptibility to azoles and cell wall-damaging agents as well as decreased virulence, is consistent with previous findings for other pathogenic fungi, such as *C. albicans* (2-4, 27), *C. neoformans* (11, 18, 21), and *A. fumigatus* (9, 30). To date, an ortholog of Crz1 in *C. neoformans* has not been identified and a mutant phenotype associated with azole susceptibility in *A. fumigatus* has yet to be reported; thus, the full importance of this transcriptional factor is not clear for these fungi. Although the virulence of a Δ*crz1* mutant is highly attenuated in *A. fumigatus* (7, 29), this mutation has little effect on virulence in both *C. albicans* (14, 23) and *C. glabrata* (Fig. 3). In contrast to that in *C. albicans* (14, 23, 28), the loss of Crz1 did not result in increased azole susceptibility in *C. glabrata*. In addition, the *C. glabrata* Δ*crz1* strain exhibited increased susceptibility to micafungin and Congo red but not to calcofluor white. Taken together, these results indicate that calcineurin-mediated Crz1

regulation is dependent upon the type of stress and that the regulatory mechanisms vary among fungal species. Further characterization of these mutant phenotypes will help to discover a novel and conserved calcineurin target in pathogenic fungi.

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TABLE 1. Primers used in this study

Primer [a]	Target gene	Sequence (5' – 3') [b]
For gene deletion		
CgCNB 100-F	*CNB1*	*GTATGTGATGCTTCTCACAGGGTTCAGACGGTTACAT ACCATCGCTTGAGAGTCATAGTAAATGTTCAGGTTCA CGATTAAATCATGCTTTCTCTTTGA***TAATACGACTC ACTATAGGGC**
CgCNB 100-R	*CNB1*	*GCGAACTCTGAAATGTAGATCAAGGATTATTCTGTCC TTGAAATGGGTGTTGATGTCCCTCACTAGGAAAGACA ACCACTTTACTATTGTAAGGGGTGA***CGCTCTAGAA CTAGTGGATCC**
CgCRZ 100-F	*CRZ1*	*GATAACGAGTTGGACGCCCTCTTTTGGAAGTCTGTTC TGGTTGCAGATGCTTATAGACCCTGGATCAAGCACTT CATTTCATTGGGATTACAGCTTTTC***TAATACGACTC ACTATAGGGC**
CgCRZ 100-R	*CRZ1*	*CACAATCTTGATTCTGAAGAAAAAAATTTATCATTAAA AATACTGGAGGTTTGTGTTAATTTATTCCAAAGTAACA CCCATCTCAGTTGCTTGAATATT***CGCTCTAGAACTA GTGGATCC**
For gene cloning		
CgCNB1-F2-5P	*CNB1*	ATCAAGGGAAATGGGAGC
CgCNB1-R1-5P	*CNB1*	CGCCCTAAGTTACATCTCTCCTCG
CgCRZ1-F1-E	*CRZ1*	CGGAATTCATGGGCGATAACGAAGAGGA
CgCRZ1-R1938-E	*CRZ1*	CGGAATTCTTATTCCAAAGTAACACCCATCTCA

[a] "F" and "R" indicate forward and reverse primers, respectively.

[b] Sequences homologous to flanking regions of the target ORF are shown in italics. Sequences shown in boldface are present in pBSK-HIS. Restriction sites are underlined.

TABLE 2. Strains used in this study.

Strain	Genotype or description	Reference or source
CBS138	Wild-type	(10)
2001T	Δ*trp1* (a derivative of CBS138)	(16)
2001HT	Δ*his3*, Δ*trp1* (made from 2001T)	(16)
TG11	2001T containing pCgACT-P	This study
TG161	Δ*cnb1*::*HIS3*, Δ*trp1* (made from 2001HT)	This study
TG162	TG161 containing pCgACT-P	This study
TG163	TG161 containing pCgACT-PNB	This study
TG171	Δ*crz1*::*HIS3*, Δ*trp1* (made from 2001HT)	This study
TG172	TG171 containing pCgACT-P	This study
TG173	TG171 containing pCgACT-PRZ	This study

TABLE 3. Antifungal susceptibilities of *C. glabrata* strains

Strain (genotype)	MIC (μg/ml) [a]				
	FLC	MCZ	ITC	VRC	AMB
TG11 (wild-type)	16	0.5	2	0.25	0.5
TG162 (Δ*cnb1*)	4	0.125	0.5	0.125	0.5
TG163 (Δ*cnb1* + *CNB1*)	16	0.5	2	0.25	0.5
TG172 (Δ*crz1*)	32	1	1	0.5	0.5
TG173 (Δ*crz1* + *CRZ1*)	16	0.5	1	0.25	0.5

[a] FLC, fluconazole; MCZ, miconazole; ITC, itraconazole; VRC, voriconazole; AMB, amphotericin B.

Figure legends

Fig. 1. Time-kill curves of *C. glabrata* wild-type and mutant strains exposed to fluconazole. Logarithmic-phase cells (5×10^5 CFU/ml) were incubated in minimal medium with agitation in the presence or absence of fluconazole at the indicated concentrations. The total number of cells was counted using a hemocytometer, and the number of viable cells was determined by plating the appropriate dilutions on yeast extract-peptone-dextrose (YPD) plates. The data are expressed as the percentages of viability and represent the means and standard deviations for three independent experiments.

Fig. 2. Susceptibilities of *C. glabrata* wild-type and mutant strains to cell wall-damaging agents. (A) Logarithmic-phase cells (2.5×10^3 CFU/ml) were incubated in minimal medium in either the presence or absence of micafungin, and the optical density at 600 nm (OD_{600}) was measured after 24 h (left panel). The percentages of absorbance were calculated from the OD_{600} of each culture after 24 h of incubation in the presence of 0.03 μg/ml micafungin relative to those in the absence of micafungin (right panel). Data represent the means and standard deviations for three independent experiments. (B) Serial 10-fold dilutions of *C. glabrata* log-phase cells were spotted onto minimal medium plates containing micafungin, Congo red, or calcofluor white at the indicated concentrations. Plates were incubated at 30°C for 48 h. All sensitivity tests were repeated at least three times. *C. glabrata* strains were as follows: wild-type, 2001T containing an empty vector (strain TG11); Δ*cnb1*, a Δ*cnb1* strain containing an empty vector (strain TG162); Δ*cnb1* + *CNB1*, a *CNB1*-complemented strain made with pCgACT-PNB (strain TG163); Δ*crz1*, a

Δ*crz1* strain containing an empty vector (strain TG172); and Δ*crz1* + *CRZ1*, a *CRZ1*-complemented strain made with pCgACT-PRZ (strain TG173).

Fig. 3. Virulence assay using a mouse model of disseminated candidiasis. Groups of 10 mice were intravenously inoculated with 8×10^7 cells for each *C. glabrata* strain. Three target organs (liver, spleen, and bilateral kidneys) were excised 7 days after injection. Appropriate dilutions of organ homogenates were plated, and the numbers of CFU were counted after 3 days of incubation at 30°C. Numbers of recovered CFU from each organ are indicated for individual mice in the scatter plots. The geometric mean is shown as a bar. Representative data of two independent experiments are shown. *C. glabrata* strains are as follows: wild-type, TG11 (wild-type control); Δ*cnb1*, TG162 (Δ*cnb1* strain containing an empty vector); Δ*cnb1* + *CNB1*, TG163 (*CNB1*-complemented strain made with pCgACT-PNB); Δ*crz1*, TG172 (Δ*crz1* strain containing an empty vector); Δ*crz1* + *CRZ1*, TG173 (*CRZ1*-complemented strain made with pCgACT-PRZ). ¶ , $P < 0.05$ (Kruskal-Wallis test with Dunn's posttest).

Fig. 1

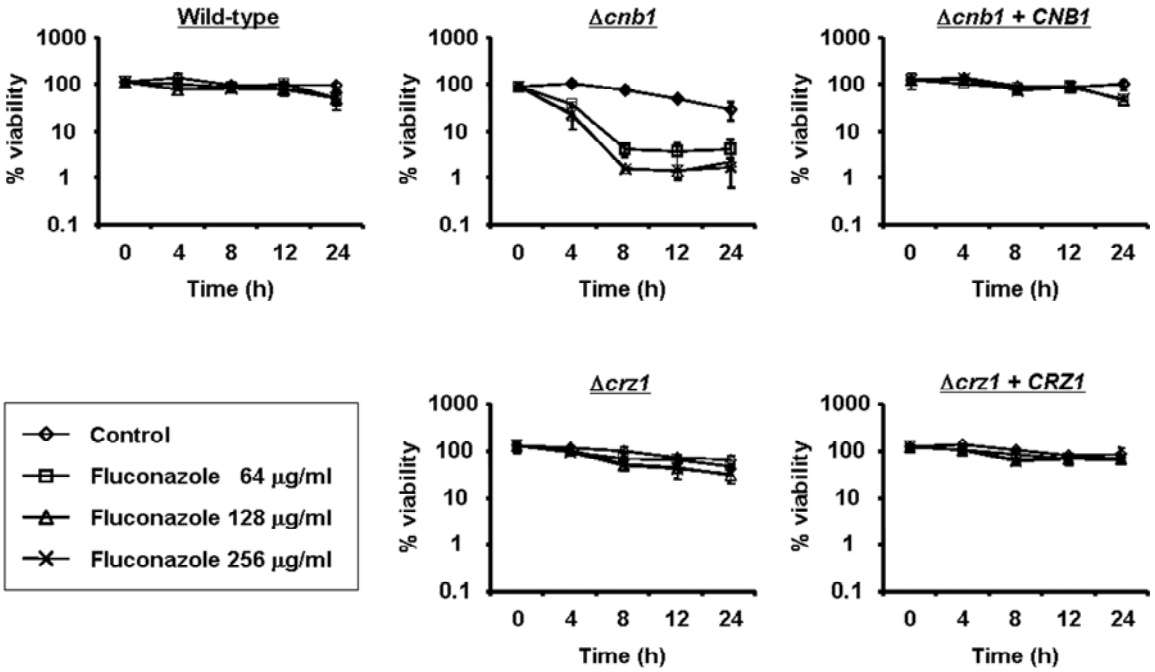

Wild-type
Δcnb1
Δcnb1 + CNB1
Δcrz1
Δcrz1 + CRZ1
% viability
1000
100
10
1
0.1
0
4
8
12
24
Time (h)
Control
Fluconazole 64 μg/ml
Fluconazole 128 μg/ml
Fluconazole 256 μg/ml

Fig. 2

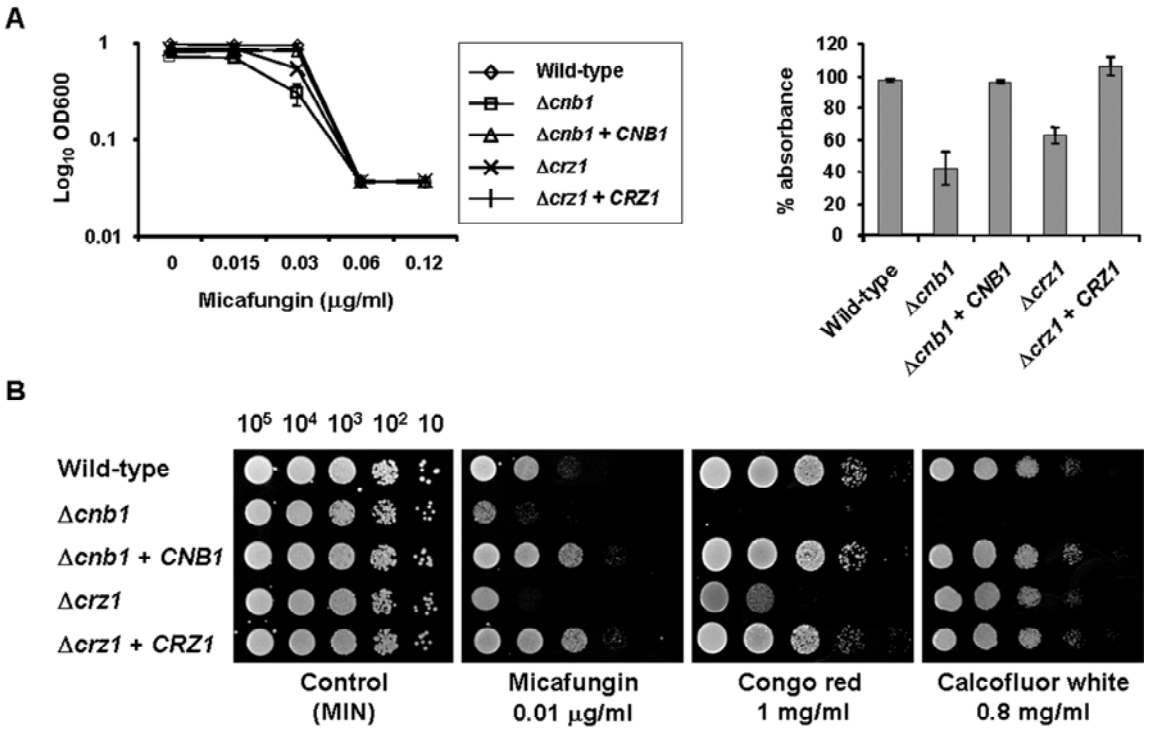
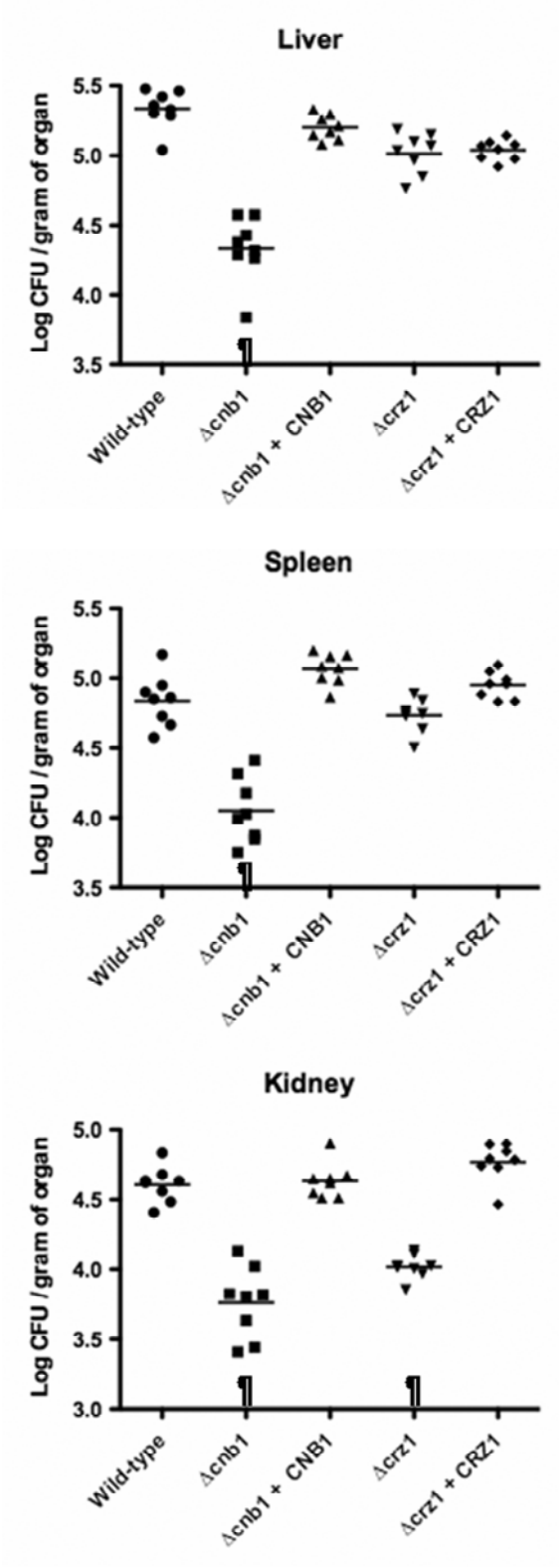

A
Log10 OD600
1
0.1
0.01
0
0.015
0.03
0.06
0.12
Micafungin (μg/ml)
Wild-type
Δcnb1
Δcnb1 + CNB1
Δcrz1
Δcrz1 + CRZ1
% absorbance
120
100
80
60
40
20
0
Wild-type
Δcnb1
Δcnb1 + CNB1
Δcrz1
Δcrz1 + CRZ1
B
10^5 10^4 10^3 10^2 10
Wild-type
Δcnb1
Δcnb1 + CNB1
Δcrz1
Δcrz1 + CRZ1
Control (MIN)
Micafungin 0.01 μg/ml
Congo red 1 mg/ml
Calcofluor white 0.8 mg/ml

Fig. 3


Liver
Log CFU / gram of organ
5.5
5.0
4.5
4.0
3.5
Wild-type
Δcnb1
Δcnb1 + CNB1
Δcrz1
Δcrz1 + CRZ1
Spleen
Log CFU / gram of organ
5.5
5.0
4.5
4.0
3.5
Wild-type
Δcnb1
Δcnb1 + CNB1
Δcrz1
Δcrz1 + CRZ1
Kidney
Log CFU / gram of organ
5.0
4.5
4.0
3.5
3.0
Wild-type
Δcnb1
Δcnb1 + CNB1
Δcrz1
Δcrz1 + CRZ1