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The type II Ca^{2+}/calmodulin-dependent protein kinases are involved in the regulation of cell wall integrity and oxidative stress response in *Candida albicans*

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ABSTRACT

The type II Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) are thought to play a vital role in cellular regulation in mammalian cells. Two genes *CMK1* and *CMK2* in the *Candida albicans* genome encode homologues of mammalian CaMKs. In this work, we constructed the *cmk1*Δ/Δ, the *cmk2*Δ/Δ and the *cmk1*Δ/Δ*cmk2*Δ/Δ mutants and found that CaMKs function in cell wall integrity (CWI) and cellular redox regulation. Loss of either *CMK1* or *CMK2*, or both resulted in increased expression of CWI-related genes under Calcofluor white (CFW) treatment. Besides, CaMKs are essential for the maintenance of cellular redox balance. Disruption of either *CMK1* or *CMK2*, or both not only led to a significant increase of intracellular ROS levels, but also led to a decrease of the mitochondrial membrane potential (MMP), suggesting the important roles that CaMKs play in the maintenance of the mitochondrial function.

1. Introduction

Fluctuations in intra cellular Ca^{2+} levels are known to initiate responses to environmental stimuli in a wide variety of cell types. One of the principal mediators of this Ca^{2+} signal in eukaryotic cells is calmodulin, a small Ca^{2+}-binding protein. Upon binding Ca^{2+}, calmodulin then changes its conformation, forming the Ca^{2+}–calmodulin complex that controls the activity of several key regulatory enzymes. In mammalian cells, this Ca^{2+}–calmodulin complex provides the essential ability to decode Ca^{2+} signals, acting to modulate the activities of a large number of protein kinases, the protein phosphatase calcineurin, nucleotide cyclases and phosphodiesterases, Ca^{2+} transporters and nitric oxide synthases [1,2].

In *Saccharomyces cerevisiae*, calmodulin is an essential protein, yet this essential function can still be performed by mutant proteins that do not bind Ca^{2+} [3]. The yeast Ca^{2+}–calmodulin complex is therefore dispensable for viability, even though it normally functions as an activator of a number of regulatory proteins. Notable Ca^{2+}–calmodulin targets are calcineurin and the type II Ca^{2+}–calmodulin dependent protein kinases (CaMKs) [4–6]. Calcineurin is important in cellular regulation in yeast. Its loss causes defects in the adaptation to endoplasmic reticulum stresses and osmostress [7–9].

At least two genes in *S. cerevisiae* encode homologues of mammalian CaMKs, which are responsible for decoding intracellular Ca^{2+} ion fluctuation in terms of a Ca^{2+}-mediated physiological response. They are *CMKI* and *CMK2* [4,10]. The deduced amino-acid sequences of Cmk1 and Cmk2 are 60% identical and 90% similar. Though gene-disruption analysis has revealed that single null mutants (*cmk1*Δ and *cmk2*Δ) and the double mutant (*cmk1*Δ*cmk2*Δ) grow normally at 17, 23, 30, and 37 °C and show no defects in meiosis or sporulation, Cmk2 plays an important role in suppressing tunicamycin-caused ROS accumulation [7]. Besides, as a putative substrate of Ste11, Cmk2 is essential for the maintenance of cell wall integrity (CWI). In fission yeast, Cmk2 is essential for oxidative stress response, and is identified as a new factor involved in oxidative stress-activated Sty1 MAP kinase response [11].

Candida albicans, the major human fungal pathogen, causes a range of disorders from mild infections to life-threatening diseases

Abbreviations: CaMKs, the type II Ca^{2+}/calmodulin-dependent protein kinases High affinity Ca^{2+} influx system; CWI, cell wall integrity; CFW, Calcofluor white; SC, synthetic complete; SD, synthetic drop-out; MMP, the mitochondrial membrane potential; 5-FOA, 5-fluoroorotic acid; DCFH-DA, 2′,7′-dichlorodihydro-fluorescein diacetate.

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[12,13]. Like other living cells, *C. albicans* cannot avoid the frequent challenge of oxidative stress by phagocytes when it survives and causes diseases in host [14]. Besides, *C. albicans* itself also generates various oxidative agents, such as reactive oxygen species (ROS), from the mitochondrial respiratory chain in the normal aerobic metabolism process. ROS, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, can damage many of the cellular components [15–17]. Therefore, regulation of the redox homeostasis is important for cellular functions [18,19]. To maintain intracellular redox homeostasis in *C. albicans*, a series of regulation mechanisms are involved, such as Ca^{2+}-mediated signaling pathways [20,21] and Hog1-mediated MAPK pathways [22,23]. However, the function of CaMKs in oxidative stress response or other aspects has not been explored in *C. albicans*.

Recently, we identified two genes encoding CaMKs in *C. albicans*, named *CMK1* and *CMK2*. In this work, we studied the roles of CaMKs in CWI and cellular redox regulation by constructing the *cmk1*Δ/Δ, the *cmk2*Δ/Δ and the *cmk1*Δ/Δ*cmk2*Δ/Δ mutants. We found that CaMKs play an important role in CWI. Loss of either *CMK1* or *CMK2*, or both resulted in the expression of CWI-related genes under CFW treatment. Besides, they are essential for the maintenance of the cellular redox balance. Disruption of either *CMK1* or *CMK2*, or both not only led to a significant increase of intracellular ROS levels, but also led to a decrease of the mitochondrial membrane potential (MMP), suggesting an important role that CaMKs play in the maintenance of the mitochondrial function.

2. Materials and methods

2.1. Strains, culture, and growth of C. albicans

C. albicans strains used in this study are derivatives of the wild-type strain BWP17 and listed in Table 1. Except where noted, *C. albicans* cells were grown at 30 °C in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) supplemented with 80 μg/ml uridine, or in synthetic complete (SC) medium (adding 80 μg/ml uridine), or in synthetic drop-out (SD) medium. Synthetic drop-out medium was used for the selection of transformants. SC medium supplemented with 0.1% 5-fluoroorotic acid (5-FOA; Lancaster, USA) was used to counter-select for *URA3*. Solid media contained 2% agar.

2.2. C. albicans strain construction

All deletion strains were generated in the BWP17 background. For the deletion of *CMK2* gene, the BWP17 strain was transformed with PCR products amplified from the plasmid pRS-ARG4Δ*Spe*I with the deletion primers *CMK2*-5DR (5′-CCATCCATAGATACATCAATT AGTTATTACCCACTTCTTGTATTAATCCCTTTACTTAACTTTCCCAGTCA CGACGTT-3′) and *CMK2*-3DR (5′-TTTCCTTCTTCATCTTCAGTA TAATTGGCTACTTTTTCTTTATTTGCAGTTGCTGCATGTGTGGAATTGT GAGCGGATA-3′), and the heterozygous mutant was confirmed by PCR with the detection primers *CMK2*-5det (5′-CGGTCATCAAAC AGTTATCA-3′) and *CMK2*-3det (5′-TCAACCAACATTCAGAGAAG-3′). The obtained heterozygous mutant was then transformed with PCR products amplified from the plasmid pDDB57 with the deletion primers, to generate the *cmk2*Δ/Δ null mutant. The *ura3* auxotrophs were obtained on SC agar plates containing 0.1% 5-FOA and 80 μg/ml uridine. In order to get the *cmk1*Δ/Δ*cmk2*Δ/Δ double mutant, the *URA3* cassette amplified from the plasmid pDDB57 with the deletion primers *CMK1*-5DR (5′-ATACATATATAAATGTAGATTTTCCCCTAATT TTGGGTTTTCGCTTGTTCTCATCAACAATTTCCCAGTCACGACGTT-3′) and *CMK1*-3DR (5′-ATGTGATAAAGCTGGTGCTGACACCCCTCCTAC CTTTTGAAGAATATTTTTGATTTGATCGTGGAATTGTGAGCGGATA-3′) was used twice. First, the *cmk2*Δ/Δ strain was transformed with the *URA3* cassette, and the heterozygous mutant was confirmed by PCR with the detection primers *CMK1*-5det (5′-GTCATTATGGTACTCT-CAGG-3′) and *CMK1*-3det (5′-AGATCCATCTCTTGAAACTG-3′). After the heterozygous mutant was constructed, the *ura3* auxotrophs were obtained on SC agar plates containing 0.1% 5-FOA and 80 μg/ml uridine. Then the strain was transformed with the *URA3* cassette again, to generate the *cmk1*Δ/Δ*cmk2*Δ/Δ double mutant. As for the construction of the *cmk1*Δ/Δ mutant, it shares the same way used for the construction of the *cmk2*Δ/Δ mutant.

2.3. CFW sensitivity

CFW sensitivity tests were set up in 96-well polystyrene flat-bottom microtitre plates (Denmark). Cell suspension (100 μL of 1×10^6 cells ml^{-1}) in YPD medium, containing the CFW ranging from 0 to 80 μg/ml, was added to wells of a microtitre plate. The plate was covered with its lid, sealed with parafilm and incubated at 30 °C for 24 h. OD_{600} (optical density at 600 nm) of each well was determined by using a microplate reader and the growth as a percentage of control (% of control) was calculated. Cells were also grown in solid YPD medium with indicated CFW concentration.

2.4. Oxidative-stress assays

Overnight cultures were refreshed in YPD medium and grown to log phase at 30 °C. Series of 10-fold dilutions were prepared in YPD, and approximately 10^6, 10^5, 10^4, 10^3, and 10^2 cells were

Table 1
Strains and plasmids in this study.

	Genotype	Source
Strains		
BWP17	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	Wilson
NKH1	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk1::ARG4/CMK1*	This study
NKH2	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk1::ARG4/cmk1:: dp1200*	This study
NKH3	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk1::ARG4/cmk1:: dpl200,CMK1*	This study
NKH4	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk2::ARG4/CMK2*	This study
NKH5	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk2::ARG4/cmk2:: dp1200*	This study
NKH6	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk2::ARG4/cmk2:: dpl200,CMK2*	This study
NKH7	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk2::ARG4/cmk2:: dp1200 cmk1:: dpl200/CMK1*	This study
NKH8	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG cmk2::ARG4/cmk2::dp1200 cmk1:: dpl200/cmk1::URA3-dpl200*	This study
Plasmids		
pRS-ArgΔSpeI	Ap^R *ARG4*	Dana Davis
pDDB57	Ap^R *URA3*	Dana Davis
pDDB78	Ap^R *TRP1 HIS1*	Dana Davis