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Ecm7, a regulator of HACS, functions in calcium homeostasis maintenance, oxidative stress response and hyphal development in *Candida albicans*

Xiaohui Ding [a,1], Qilin Yu [a,1], Ning Xu [a], Yuzhou Wang [a], Xinxin Cheng [a], Kefan Qian [a], Qiang Zhao [a], Biao Zhang [b], Laijun Xing [a], Mingchun Li [a,*]

[a] Key Laboratory of Molecular Microbiology and Technology, Ministry of Education, College of Life Science, Nankai University, Tianjin, PR China
[b] Tianjin Traditional Chinese Medicine University, Tianjin, PR China

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ABSTRACT

Calcium is a universal messenger that translates diverse environmental stresses and developmental cues into specific cellular and developmental responses. In yeast, Cch1 and Mid1 function as part of a high affinity Ca^{2+} influx system (HACS) that becomes activated rapidly in response to sudden stimuli. Here, we report that Ecm7, a regulator of HACS, plays important roles in calcium homeostasis maintenance, oxidative stress response and hyphal development in *Candida albicans*. Disruption of *ECM7* led to increased sensitivity to calcium-depleted conditions. Flow cytometry analysis revealed that Ecm7 mediated Ca^{2+} influx under high pH shock. Cycloheximide chase experiments further showed that *MID1* deletion significantly decreased the stability of Ecm7. We also provided evidences that *ecm7*Δ/Δ cells were hypersensitive to oxidative stress. *ECM7* deletion induced the degradation of Cap1 when exposed to H_2O_2 treatment. Besides, the *ecm7*Δ/Δ mutant showed a defect in hyphal development, which was accompanied with the decreased expression of hyphal related gene *HWP1*. Though subsequent experiments revealed that the *ecm7*Δ/Δ mutant showed similar virulence to the wild-type strain, the ability of invasion and diffusion of the mutant in mouse kidneys decreased. Taken together, Ecm7 plays important roles in the adaptation and pathogenicity of *C. albicans*.

1. Introduction

Cells are frequently exposed to environmental stresses. Under these non optimal conditions, generating proper adaptive responses is necessary for their survival. Ca^{2+}-mediated signaling of stress conditions is used by virtually every eukaryotic cell to regulate a wide variety of cellular processes through a transient increase of cytosolic Ca^{2+}. The increase in cytosolic Ca^{2+} can be a consequence of external Ca^{2+} influx via the plasma membrane high-affinity Ca^{2+} influx system (HACS) (Batiza et al., 1996; Catterall, 2000; Matsumoto et al., 2002) or the release of vacuolar Ca^{2+} into the cytosol through the vacuole-located Ca^{2+} channel Yvc1 (Denis and Cyert, 2002; Palmer et al., 2001). After acting as a second messenger, the cytosolic free Ca^{2+} level declines rapidly to the normal level through the action of Ca^{2+} pumps and exchangers.

In *Saccharomyces cerevisiae*, HACS becomes activated in response to high pH (Viladevall et al., 2004), oxidative stress (Popa et al., 2010), mating pheromones (Iida et al., 1990; Iida et al., 1994; Muller et al., 2001) or compounds such as azole-class antifungal agents that inhibit essential enzymes in the endoplasmic reticulum (Hong et al., 2010; Kaur et al., 2004; Locke et al., 2000), indicating that HACS serves as an important defense system used by fungi to resist certain kinds of toxins and some common antifungal agents. It is well-known that HACS consists of two subunits, Cch1 and Mid1, which are homologous and analogous to the catalytic α-subunits and regulatory α2δ-subunits of mammalian voltage-gated calcium channels (VGCC), respectively (Locke et al., 2000; Viladevall et al., 2004). However, recent studies have revealed that Ecm7, a member of the PMP-22/EMP/MP20/Claudin superfamily of transmembrane proteins, is required for HACS functions in most conditions. Besides, Ecm7, as a new regulator of HACS, directly or indirectly interacts with subunits of HACS and regulates HACS through unknown mechanisms in *S. cerevisiae* (Martin et al., 2011; Van Itallie and Anderson, 2006).

Abbreviations: HACS, high affinity Ca^{2+} influx system; CHX, cycloheximide; SC, synthetic complete; SD, synthetic drop-out; HBSS, Hank's balanced salt solution; VGCC, voltage-gated calcium channels; EGTA, ethylene glycol bis(2-aminoethyl) tetraacetic acid; 5-FOA, 5-fluoroorotic acid; FBS, fetal bovine serum; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate.

* Corresponding author. Present address: Department of Microbiology, College of Life Science, Nankai University, Tianjin 300071, PR China. Fax: +86 22 23508800.
E-mail address: nklimingchun@163.com (M. Li).

[1] These authors contributed equally to this work.

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Candida albicans is the most common fungal agent of invasive disease in humans (Pappas et al., 2003; Sandven, 2000). It can grow in distinct morphological states, including yeast, pseudohyphae and true hyphae (Sudbery et al., 2004). *C. albicans* yeast cells switch to hyphal growth when phagocytosed or exposed to serum, which is thought to assist its invasion of host tissues and escape from phagocytotic killing (Lo et al., 1997). A major mechanism of the host defense system against fungal infection is via the production of reactive oxygen species (ROS) by macrophage cells (Murphy, 1991). Oxidative stress therefore is a frequent challenge for *C. albicans* to survive. During the long-term evolution, *C. albicans* has developed a variety of ways to sense the environmental changes. Ca^{2+}, as a major second messenger, mediates a series of intracellular processes in response to many stimuli. Two subunits of HACS have so far been identified in this organism: Cch1 and Mid1, homologous to Cch1 and Mid1 from *S. cerevisiae*, respectively. Cch1 and Mid1 have been implicated in many functions in *C. albicans*, such as high pH response (Wang et al., 2011), oxidative stress tolerance, morphogenesis and virulence (Yu et al., 2012). However, the identification and function of Ecm7 have not been explored in *C. albicans*.

In this work, we described the identification and functional characterization of the homologue of *S. cerevisiae ECM7* in *C. albicans*. We constructed an *ecm7* null mutant strain and found that Ecm7 plays an important role in calcium homeostasis maintenance and is stabilized mainly by Mid1. We presented evidences that Ecm7 mediates oxidative stress response in a Cap1-dependent manner. Besides, the mutant exhibited a defect in hyphal development. Quantitative RT-PCR further revealed that disruption of *ECM7* down-regulated the expression of hyphal-specific gene *HWP1* under hyphal-inducing conditions. Subsequent experiments showed that *ECM7* deletion attenuated the ability of invasion and diffusion of yeast cells in mouse kidneys compared with the wild-type strain, though no significant changes were observed in the virulence between *ecm7*Δ/Δ and wild-type cells using a mouse model of systemic infection. These results indicated that Ecm7 is involved in the development of an adaptive response in this fungal pathogen.

2. Materials and methods

2.1. Strains and culture conditions

The strains used in this study are derivatives of the 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. Plasmid construction

The plasmids used in this study are also listed in Table 1. The reporter plasmid p$P_{IPF7817}$-*lacZ* was generated as follows. The upstream region of *IPF7817* enclosed from −1000 bp to ATG was amplified with primers *IPF7817*-Pdistal (5′-CTAAGTTGACAATATGAG-3′) and *IPF7817*-ATG (5′-CATATGTGTTGCAAATAGTTT-3′), and the resulting PCR product was ligated into pGEM-T easy vector (Promega, Madison, WI) to generate pGEM-T-$P_{IPF7817}$. pDDB211 (Baek et al., 2006) was digested with *Ase*I/*Mlu*I and ligated into *Nde*I/*Mlu*I-digested pGEM-T-$P_{IPF7817}$ to generate p$P_{IPF7817}$-*lacZ*. DAY414 strain was then transformed with the *Drd*I-digested p$P_{IPF7817}$-*lacZ* and the *Not*I/*Eco*RI-digested pDDB78 to generate p$P_{IPF7817}$-*lacZ* by in vivo recombination (Spreghini et al., 2003).

2.3. C. albicans strain construction

All deletion strains were generated in the BWP17 background. For the deletion of *ECM7* gene, the BWP17 strain was transformed with PCR products amplified from the plasmid pRS-ARG4Δ*Spe*I with the deletion primers *ECM7*-5DR (5′-GTTTTTTTTTTTTTCCGATTGGCTCTCACTCCTTTTTCTTCACACATACAAAACCTGCCTTTCCCAGTCACGACGTT-3′) and *ECM7*-3DR (5′-CGCATTTCTTCCTCGTGGTGATCGTCCTGATGAGCTGAATGACGAGGGTTATGGAATATGGTGGAATTGTGAGCGGATA-3′), and the heterozygous mutant was confirmed by PCR with the detection primers *ECM7*-5det (5′-TTGGGCTATTGTAGAGGGTT-3′) and *ECM7*-3det (5′-CTCGTCATCTTCTAGGTACG-3′). After the heterozygous mutant was constructed, it was then transformed with PCR products amplified from the plasmid pDDB57 with the deletion primers, to generate the *ecm7* null mutant. The *ura3* auxotrophs were obtained on SC agar plates containing 0.1% 5-FOA and 80 μg/ml uridine.

2.4. Sensitivity to calcium-depleted conditions

To test the sensitivity of strains to calcium-depleted conditions, overnight cultures were inoculated into YPD medium with the absorbance at 600 nm (A_{600}) = 0.1, supplemented with or without EGTA, a specific calcium chelator (Aghajanian et al., 1983; Popa et al., 2010; Wang et al., 2011). A_{600} was measured at the indicated time points. All samples were tested in duplicate.

2.5. Calcium measurement with flow cytometry

Evaluation of cytoplasmic calcium fluctuation was carried out as follows. Overnight cultures of strains were re-cultivated to mid-exponential phase at 30 °C in 30 ml YPD medium. The cells were then loaded with the calcium-sensitive indicator fluo-3-AM-ester (Sigma), which is a membrane-permeant form of fluo-3 (Gergely et al., 1997). Once passively loaded into cells, it is readily hydrolyzed to fluo-3 free acid by nonspecific esterases and becomes activated in the cytoplasm. Typically, 1 ml of yeast cells were loaded with fluo-3AM at a final concentration of 5 μM and incubated at 37 °C for 50 min. The cells were then washed twice in a Hank's balanced salt solution (HBSS) buffer (pH 7.2) and after counting, the cells were kept at room temperature until calcium flux measurements were performed. Calcium flux measurements were performed by flow cytometry, using a BD FACSCalibur flow cytometer (BD). The baseline ratio was acquired before the addition of the KOH solution. Data were analyzed off-line using the WINMDI 2.9.

2.6. Protein extraction and western blotting

The appropriate yeast strains were cultured at 30 °C in YPD medium to log phase, adjusted to an A_{600} of 0.5, and split equally into two aliquots. One aliquot was treated with 10 μg/ml cycloheximide (Sigma) at 30 °C for 1 h before processing, and the other was processed immediately. Processing involved centrifugation of 2.5 A_{600} units of cells at 4 °C, lysis of cells in the presence of protease inhibitors using glass beads, extraction of proteins with urea sample buffer, SDS–PAGE, and Western blotting as described previously (Locke et al., 2000). Western blotting was probed with anti-alpha tubulin antibody (Novus Biologicals, NB100-1639) as a loading control.

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