Possible Involvement of Surface Antigen Protein 2 in the Morphological Transition and Biofilm Formation of Candida albicans

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ABSTRACT

Surface antigen protein 2 (Csa2) is a member of the Candida albicans Common in Fungal Extracellular Membranes (CFEM) protein superfamily. We previously established its role in iron acquisition in C. albicans. However, the other roles of Csa2 remain unknown. Here, we compared growth, morphological transition, and biofilm formation among wild-type, Csa2-mutant, and complemented strains of C. albicans. Deletion of the Csa2 gene resulted in smaller and reduced colony growth, significant attenuation of the dimorphic transition under serum-inducing conditions, and reduced biofilm formation; complementation restored these levels to those of the wild-type. Our findings demonstrated that Csa2 participated in yeast-to-hyphae morphological switching under serum-inducing conditions and contributed to the biofilm formation of C. albicans. This work, therefore, provides novel insights into the potential roles of Csa2 in virulence of C. albicans.

Key words: biofilm formation, Candida albicans, common in fungal extracellular membranes (CFEM) protein, dimorphism, morphological transition

Introduction

Candida albicans, a major fungal pathogen, causes mucosal and systemic infections in immunocompromised hosts. The pathogenicity of Candida is attributed to certain virulence factors, such as those contributing to host defense evasion, dimorphism, and biofilm formation on host tissue. C. albicans possesses the ability to undergo a morphological transition from a single-yeast form to pseudohyphal and hyphal forms while inside a host in response to favorable host conditions, such as the presence of serum and optimum body temperature (37°C). C. albicans is capable of taking up iron from hemoglobin and hemeproteins through erythrocyte lysis, hemoglobin binding, heme extraction, and endocytosis. Members of the C. albicans heme-receptor protein family that possess the common in several fungal extracellular membranes (CFEM) domain (i.e., Csa1, Csa2, Pga7 [Rbt6], Rbt5, and Rbt51 [Pga10]) are thought to be involved in this system of iron uptake. We previously demonstrated that surface antigen protein 2 (Csa2) is involved in the binding and scavenging of iron from human hemoglobin during C. albicans hyphal growth. Nasser et al. recently reported the structure-function analysis of Csa2 proteins to characterize the CFEM relay network of heme-iron acquisition in C. albicans. However, despite great interest in the function of Csa2, its roles are still poorly understood.

In this study, the role of Csa2 was examined under serum-induced conditions, and biofilm formation was evaluated for a csa2 Δ/Δ mutant, a parental C. albicans strain (BWP17 + pClp30) as the wild-type control, and a csa2 Δ/Δ::CSA2 complemented strain. Our findings contribute to further understanding the function of Csa2 and its
role in the pathogenesis of *C. albicans* infections.

**Materials and methods**

**Strains**

The wild-type *C. albicans* strain (BWP17 + pClp30) and the csa2 Δ/Δ mutant were kindly provided by Professor B. Hube of Friedrich Schiller University, Germany. The csa2 Δ/Δ::CSA2 complemented strain was established as described previously.

**Growth medium and hyphal growth conditions**

To observe *C. albicans* hyphal development in liquid medium, we inoculated late-exponential-phase cultures containing 1 × 10⁵ cells grown in YPD (1% yeast extract, 2% peptone, and 2% glucose per liter) at 30°C into fresh hyphae-inducing YPD + 10% serum medium and incubated them at 37°C for 1, 3, or 5 h. After incubation, the hyphal portion was assessed microscopically, and the number of hyphal cells was counted as previously described. Briefly, *C. albicans* yeast cell suspensions were spread on hyphae-inducing solid medium and incubated at 37°C for 6 days. To induce hyphal development on solid medium, we cultured *C. albicans* yeast cells overnight at 30°C in YPD liquid medium and washed the cells twice with sterile water before plating. The *Candida* suspensions were then spread on hyphae-inducing solid medium and incubated at 37°C for 6 days. Photomicrographs of colonies were taken with an OLYMPUS microscope and photo adaptors.

**Biofilm formation**

Biofilm formation was evaluated with the optimized *Candida* biofilm microtiter assay reported by Krom et al. Briefly, *C. albicans* strains grown overnight at 30°C in yeast nitrogen base medium (YNB, pH 7; BD Difco, Sparks, MD, USA) containing 50 mM glucose were harvested, washed in sterile phosphate-buffered saline (pH 7.2), and suspended to a density of 1 × 10⁸ CFU/mL. Wells of a 96-well microtiter plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were coated for 30 min at 37°C with 100 μL of 50% fetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA). The wells were washed once with 200 μL phosphate-buffered saline and incubated with 100 μL of the cell suspension for 90 min at 37°C. Nonadherent cells were removed by washing twice with 200 μL phosphate-buffered saline. Subsequently, biofilm formation was stimulated by incubating the wells in 200 μL YNB (pH 7) containing 50 mM glucose for 24 h.

**XTT reduction assay**

After incubation, biofilm formation was assessed using a sodium3’-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) reduction assay according to the manufacturer’s protocol (Cell Proliferation Kit II; Roche Diagnostics, Mannheim, Germany) by measuring the colorimetric change at 490 nm. Briefly, 50 μL of the XTT labelling mixture was added to each well, and the plates were then incubated in the dark at 37°C for 1 h. A colorimetric change was measured in a microtiter plate reader (model 3550; Bio-Rad, Hercules, CA, USA).

**Results and discussion**

Table 1 and Fig. 1 illustrate the effects of CSA2 gene deletion on the *C. albicans* dimorphic transition and colony morphology. CSA2 gene deletion induced colony morphological changes in *C. albicans* (Fig. 1). Although cells of the *C. albicans* wild-type, csa2 Δ/Δ mutant, and complemented strains formed wrinkled colonies on hyphae-inducing medium agar plates containing 10% fetal calf serum at 37°C, the colonies of the csa2 Δ/Δ mutant were smaller and had relatively rough wrinkles (Fig. 1b) compared with the *C. albicans* wild-type and complemented strains (Fig. 1a, c). Complementation of the CSA2 gene restored the level of hyphal development to that of the wild-type strain (Table 1).

Moreover, the size of the colonies in the mutant strain was smaller than that of the wild-type and csa2 Δ/Δ::CSA2 complemented strains (Fig. 1). Specifically, the average maximum diameters of 10 selected colonies were 1.27 ± 0.07 cm for the wild-type, 0.75 ± 0.09 cm for the csa2 Δ/Δ mutant, and 1.18 ± 0.05 cm for the csa2 Δ/Δ::CSA2 complemented strain. The yeast-form colonies, however, exhibited no differences among these three strains.

The ratio of *C. albicans* cells undergoing the blastospore-to-filament transition was significantly attenuated in the csa2 Δ/Δ mutant compared with that in the wild-type strain (Table 1). The ratio of the csa2 Δ/Δ mutant undergoing dimorphic transition under serum-inducing conditions was reduced by approximately 40% at 1 h and by 30% at 3 h and 5 h compared with that of the wild-type.
strain. Complementation of the CSA2 gene restored the level of dimorphic transition to that of the wild-type strain (Table 1).

Taken together, these results demonstrated that CSA2 gene deletion resulted in a colony morphological change and significantly decreased the ratio of C. albicans cells undergoing the blastospore-to-filament transition under filament-inducing conditions in serum. Therefore, the CSA2 gene may play a role in the normal colony morphogenesis and dimorphic transition of C. albicans under serum-inducing conditions.

Fig. 2 shows the effects of CSA2 gene deletion on C. albicans biofilm formation. The biofilm formation of the csa2 Δ/Δ mutant was reduced to approximately 40% that of the wild-type strain and was fully restored to the wild-type level in the csa2 Δ/Δ:CSA2 complemented strain. Péretz et al. previously reported the abnormal biofilm-forming ability of several C. albicans mutants in genes encoding other CFEM superfamily proteins using XTT reduction assay. The mutant strains, e.g., pga10 Δ, rbt5 Δ, and csa1 Δ, also exhibited approximately 20-40% reduction in 24-h biofilm formation compared with the parental strain⁶. These results suggested that CFEM superfamily proteins were critical for biofilm formation in C. albicans.

An important feature related to the pathogenesis of C. albicans is its ability to switch between different morphological forms. C. albicans can grow in a single-celled, budding yeast form (blastospore) or in a filamentous form (pseudohyphae and true hyphae). Mutants defective in
filamentous growth are known to be less virulent in systemic infections, indicating that the filamentous form exhibits more invasive properties, which promote tissue penetration and enable escape from host immune cells. The C. albicans biofilm is composed of a mixed structure of yeast cells and hyphal elements; thus, biofilm development is also suggested to play important roles in the dimorphic switch. Multiple signal transduction pathways are known to be involved in the regulation of hyphal morphogenesis and virulence in C. albicans. The Cph1-mediated mitogen-activated protein kinase and cAMP-dependent protein kinase A pathways are well-characterized signaling pathways that participate in dimorphic regulation. Therefore, it will be important to investigate the role of Csa2 during signal transduction in the serum-induced yeast-to-hyphae transition.

In conclusion, CSA2 gene deletion affected the normal colony morphogenesis of C. albicans on serum-containing solid medium and impaired the ability to undergo yeast-to-hyphae switching under serum-induced conditions. In addition, reduced biofilm formation was observed in the csa2 Δ/Δ mutant compared with that in the wild-type and csa2 Δ/Δ::CSA2 complemented strains. To the best of our knowledge, this is the first work describing the potential participation of Csa2 in yeast-to-hyphae switching under serum-inducing conditions and in biofilm formation in C. albicans. These findings suggested that Csa2 acted as a possible regulator of biofilm formation in C. albicans, thereby contributing to its infective and pathogenic mechanisms. Based on our previous report, Csa2 may be involved in both the utilization of iron from human hemoglobin during hyphal growth and regulation of switching to the hyphal form in C. albicans, suggesting the potential importance of Csa2 in its ability to switch from the yeast to hyphal form and continue to grow while inside the host.

Additional genetic approaches are currently being used to elucidate the molecular basis underlying the contribution of Csa2 to filamentation and biofilm formation in C. albicans and to further characterize this important protein. Thus, Csa2 also has the potential to serve as basis for the development of new antifungal treatments.

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Conflict of Interest
None declared.

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