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The actin-related protein Sac1 is required for morphogenesis and cell wall integrity in *Candida albicans*

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ABSTRACT

Candida albicans is a common pathogenic fungus and has aroused widespread attention recently. Actin cytoskeleton, an important player in polarized growth, protein secretion and organization of cell shape, displays irreplaceable role in hyphal development and cell integrity. In this study, we demonstrated a homologue of *Saccharomyces cerevisiae* Sac1, in *C. albicans*. It is a potential PIP phosphatase with Sac domain which is related to actin organization, hyphal development, biofilm formation and cell wall integrity. Deletion of *SAC1* did not lead to insitiol-auxotroph phenotype in *C. albicans*, but this gene rescued the growth defect of *S. cerevisiae sac1*Δ in the insitiol-free medium. Hyphal induction further revealed the deficiency of *sac1*Δ/Δ in hyphal development and biofilm formation. Fluorescence observation and real time PCR (RT-PCR) analysis suggested both actin and the hyphal cell wall protein Hwp1 were overexpressed and mislocated in this mutant. Furthermore, cell wall integrity (CWI) was largely affected by deletion of *SAC1*, due to the hypersensitivity to cell wall stress, changed content and distribution of chitin in the mutant. As a result, the virulence of *sac1*Δ/Δ was seriously attenuated. Taken together, this study provides evidence that Sac1, as a potential PIP phosphatase, is essential for actin organization, hyphal development, CWI and pathogenicity in *C. albicans*.

1. Introduction

Candida albicans is a common commensal of our mucosal surfaces and intestinal tracts, usually without pathogenicity. But this pathogenic fungus may lead to serious infections in vulnerable patients (David, 2012; Kadosh and Lopez-Ribot, 2013). Several factors determine the virulence of *C. albicans*, including morphogenesis, adhesion, biofilm formation and adaptation to host niches (Calderone and Fonzi, 2001). Morphogenesis, also termed as dimorphic switching, in particular, has been noted as the most crucial for infection and pathogenesis in this pathogen (Gow et al., 2012).

In general, there are three main forms of *C. albicans* cells, yeast, pseudohypha and hypha. Although the yeast form is considered to be associated with fungal dissemination in host tissues, the hyphal form has been demonstrated to be most invasive. The defect in hyphal formation would impair infection ability of *C. albicans*, even causing avirulence of this pathogen. It is generally accepted that morphogenetic switching from yeast to hypha is a confusing and complicated program, which is affected by environmental conditions such as temperature, pH and CO_2, and is related to specific expression of hypha-related genes and polarized transport of morphogenesis factors (Gow et al., 2012; Inglis et al., 2013; Saville et al., 2003). Especially, many hypha-related proteins would be expressed and localized at specific subcellular positions to coordinate this asymmetric growth. In this process, the actin cytoskeleton, consisted of actin patches and actin cables, displays asymmetric distribution and is responsible for this polarized growth (Akashi et al., 1994; Berman, 2006). Due to the essential role of the actin cytoskeleton in polarized growth, the fungus evolves an elaborate mechanism to maintain the polarity of this

Abbreviations: PI, phosphatidylinositol; PIP phosphatase, phosphatidylinositol phosphate phosphatase; PI(3)P, phosphatidylinositol-3-phosphate; PI(4)P, phosphatidylinositol-4-phosphate; PI(3,5)P, phosphatidylinositol-3,5-phosphate; PI(4,5)P, phosphatidylinositol-4,5-phosphate; CWI, cell wall integrity; CFW, calcofluor white; 5-FOA, 5-fluoroorotic acid; FBS, fetal bovine serum; SC, synthetic complete; SD, synthetic drop-out; cAMP, cyclic adenosine monophosphate; GPI, glycosylphoshatidylinositol.

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structure. Many factors, including actin binding proteins (ABPs), signal molecules and ion homeostasis, have been demonstrated to be responsible for the accurate actin organization (Cantiello, 1997; Li et al., 2005). For example, deletion of *CDC42* results in disturbance of polarized organization of actin, indicating the actin cytoskeleton is a downstream part of signal transduction in regulating polarized morphogenesis (Brand et al., 2014; Ushinsky et al., 2002).

In *Saccharomyces cerevisiae*, Sac1, an important actin-related protein, was originally discovered as a 'suppressor of actin' (Novick et al., 1989). Further studies demonstrated that this protein participates in many cellular processes, such as organization of the actin cytoskeleton, secretory pathway, vacuolar function, ER function and sphingolipid metabolism (Blagoveshchenskaya and Mayinger, 2009; Foti et al., 2001). Deletion of *ScSAC1* also results in inositol auxotrophy (Whitters et al., 1993), implying an essential role in inositol metabolism. Furthermore, this protein has the activity of phospholipase and could dephosphorylate PI(3)P, PI(4)P, PI(3,5)P_2 *in vitro*. But its activity of dephosphorylating PI(4)P is likely to be the origin of its functions (Guo et al., 1999; Hsu and Mao, 2013). Coincidently, there are a variety of proteins containing Sac domain, such as *Sc*Sac1, *Sc*Fig4, *Sc*Inp51, *At*Sac1, *h*Sac1 and *r*Sac1, possessing phospholipase activity (Guo et al., 1999; Rohde et al., 2003). In *Arabidopsis*, *At*Sac1 plays crucial roles in cell elongation and cell wall biosynthesis by the regulation of actin cytoskeleton (Zhong et al., 2005). However, in *C. albicans*, the homolog of *Sc*Sac1 and other proteins containing Sac domain remains to be investigated.

In this study, we identified a protein containing the Sac domain in *C. albicans*, also termed as Sac1, and investigated its role in inositol metabolism, morphogenesis and cell wall integrity. This protein was proposed to have potential phospholipase activity similar to *Sc*Sac1 and other proteins containing this domain. Although there is no growth differences of *sac1*Δ/Δ on inositol-free medium and synthetic complete medium, *SAC1* could rescue inositol auxotrophy of *Scsac1*Δ completely, suggesting the functional similarity between Sac1 and *Sc*Sac1, at least in inositol metabolism. We further demonstrated Sac1 functions in hyphal development, Hwp1 localization, cell wall integrity (CWI), chitin distribution and consequently attenuated virulence of this pathogen. We speculate that the phenotypes of *sac1*Δ/Δ may be attributed to abnormal localization of actin cytoskeleton, which is associated with changed PI levels.

2. Materials and methods

2.1. Strains and growth conditions

All strains used in this study are listed in Table 1. BWP17 was the parental strain and used as the wild-type strain in subsequent experiments. Strains were routinely cultivated in YPD medium (1% yeast extract, 2% peptone, 2% glucose) supplemented with 80 μg/ml uridine, synthetic complete medium (SC, 0.67% yeast nitrogen base without amino acids, 2% glucose, 0.2% complete amino acid mixture) or synthetic drop-out medium (SD, SC medium without specific ingredients). To obtain the homozygous strain without *URA3* gene, the SD medium supplemented with 0.1% 5-fluoroorotic acid (5-FOA) was used for counter-selecting. The media M199, RPMI-1640 (Gibco) and YPD containing 10% (v/v) FBS (fetal bovine serum) were used for morphogenesis analysis. For dot assay experiments, different concentrations of calcofluor white (CFW), Hygromycin B and Congo Red were added into YPD medium, respectively.

2.2. Strains and plasmids construction

Primers used in our study are all listed in Table 2.

For generating the reconstituted plasmid pDDB78-SAC1, the *SAC1* complementary fragment which was composed of 898 bp promoter region, 1857 bp ORF and 333 bp terminator region was amplified by SAC1-5con and SAC1-3con, digested with *Spe* I and *Eco*R I and recombined into the plasmid pDDB78. To construct the *sac1::LEU1* cassette for *ScSAC1* disruption, a 1036 bp fragment was amplified by Sc.SAC1-5DR and Sc.SAC1-3DR from the wild-type genome of *S. cerevisiae* (Invitrogen, USA), and then cloned into pGEM-T easy vector (Promega, USA), generating the plasmid T-ScSAC1. This plasmid was digested with *Xba* I and *Hind*III and the fragment containing the *LEU1* screening marker from plasmid T-LEU was inserted, obtaining the plasmid T-*sac1::LEU1*. For homology analysis of *SAC1* in *S. cerevisiae* and *C. albicans*, the *C. albicans SAC1* fragment was amplified with the primers CtoS-Sac1-5com and CtoS-Sac1-3com from BWP17 genome, and then cloned into the YE-P_{PGK1}, generating the final plasmid YE-P_{PGK1}-SAC1.

To obtain the *SAC1*-disrupted mutant *sac1*Δ/Δ(NKF301), the *sac1::ARG4* cassette was amplified from pRS-ARG4Δ*Spe*I with the deletion primers SAC1-5DR and SAC1-3DR and transformed into BWP17. The obtained heterozygous mutant was transformed with the *sac1::URA3-dpl200* cassette amplified from pDDB57, generating NKF301. The recombinant strains were confirmed with the detection primers SAC1-5det and SAC1-3det. To obtain the *sac1*Δ/Δ strain without *URA3* selectable marker (NKF302), NKF301 was streaked on the SD medium containing 5-FOA. To construct the *SAC1* reconstituted strain *sac1*Δ/Δ + *SAC1* (NKF303), NKF302 was transformed with the *Nru*I-digested pDDB78-SAC1. To investigate *ACT1* expression, BWP17, NKF302 and NKF303 were transformed with *Bgl*II-digested pAU34M-GFP, generating the strains NKF306, NKF307 and NKF308, respectively. For expression analysis of *HWP1*, the fragment amplified from pHWP1-GFP with primers HG-5′ and HG-3′ was transformed into BWP17, NKF302 and NKF303, obtaining the strains NKF309, NKF310 and NKF311, respectively. To avoid the effect of *URA3* on virulence analysis, *Bgl*II-*Pst*I-digested pLUBP (containing *URA3* and *IRO1*) was transformed into BWP17, NKF302 and NKF303 to generate the corresponding strains BWP17*, NKF304 and NKF305, with the *URA3* gene at its normal genetic loci.

To obtain *Scsac1*Δ (NKF315), the *sac1::LEU1* cassette was amplified from T-*sac1::LEU1* with the deletion primers Sc.SAC1-5DR and Sc.SAC1-3DR and transformed into INVSc1. Then the plasmid YE-P_{PGK1}-SAC1 was transformed into NKF315 to generate *Scsac1*Δ + *CaSAC1* (NKF316).

2.3. Filamentous growth assays

RPMI-1640 and YPD medium with 10% (v/v) FBS were used for liquid induction of filamentous growth. RPMI-1640 and M199 solid media were used for observation of colony morphology, and YPD containing 10% (v/v) FBS with 1.5% agar was used for invasive growth. For colony morphology observation, all strains were diluted in sterile water to an OD_{600} of 0.8, then dotted on the corresponding plates and cultured 4–5 days at 30 °C and 37 °C. In invasive growth experiments, the colonies incubated in solid hyphal inducing conditions were washed and vertically sliced, obtaining 0.1 mm slices for microscopic observation. In embedded growth experiments, cells were cultured to exponential phase, washed, mixed in molten YPD semi-solid medium (1% agar) and incubated at 25 °C for 8 days.

2.4. Adhesion and biofilm formation assays

The ability of adhesion and biofilm formation on polystyrene surface was performed as follows. Cells were overnight cultured in YPD medium, washed with PBS and resuspended in RPMI-1640 medium. The cells were then incubated on 24-well polystyrene microtiter plates at 37 °C for 4 h (adhesion analysis)