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The Sko1 protein represses the yeast-to-hypha transition and regulates the oxidative stress response in *Candida albicans*

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ABSTRACT

Cells respond to environmental changes triggering adaptive responses which are, in part, mediated by a transcriptional response. These responses are complex and are dependent on different transcription factors. The present work reports the implication of the Sko1 protein in several processes relevant to the physiology of *Candida albicans*. First, Sko1 acts as transcriptional repressor of genes involved in pathogenesis and hyphal formation, which results in increased expression of the hyphal related genes *ECE1* and *HWP1* without significant changes in the virulence using a mouse model of systemic infection. Second Sko1 is involved in the response to oxidative stress and *sko1* mutants increase the sensitivity of *hog1* to the myelomonocytic cell line HL-60. Genome-wide transcriptional analysis after hydrogen peroxide treatment revealed that *sko1* mutants were able to generate an adaptive response similar to wild type strains, although important differences were detected in the magnitude of the transcriptional response. Collectively, these results implicate Sko1 as an important mediator of the oxidative stress response in *C. albicans*.

1. Introduction

Cells are frequently exposed to environmental stress. Under these non optimal conditions, the generation of a proper adaptive response is necessary to guarantee survival. When yeast cells are exposed to mild osmotic stress, they undergo a response which is accomplished, at least it part, by the HOG (high osmolarity glycerol) MAP kinase cascade (Brewster et al., 1993). In *Saccharomyces cerevisiae*, this route is activated by two upstream branches (Banuett, 1998; Gustin et al., 1998; Posas et al., 1998a). The first one depends on the transmembrane protein Sln1, whose basal autophosphorylation activates Ypd1 and Ssk1 (Posas et al., 1996; Tao et al., 2002) and prevents activation of the Hog1 MAP kinase. Under osmotic stress, unphosphorylated Ssk1 leads to phosphorylated Ssk2/Ssk22 that activates the Pbs2 MAPK kinase (MAP kinase kinase) (Boguslawski, 1992) and the Hog1 protein (Brewster et al., 1993). A second input comes from the Sho1, Cdc42, Ste20/Ste50 and Ste11 (the MAP kinase kinase kinase) proteins that also lead to activation of Pbs2 (O'Rourke and Herskowitz, 1998; Posas et al., 1998a,b; Raitt et al., 2000). The Sho1 transmembrane protein is responsible for attaching this complex to regions more vulnerable to osmotic stress.

It has become recently clear that the HOG pathway is also important for oxidative stress resistance in *S. cerevisiae* (see Ikner and Shiozaki (2005) for a review of the role of MAPK pathways in oxidative defense mechanisms). Although initial studies reported that Hog1 was not responsive to hydrogen peroxide (Schüller et al., 1994). It was later shown that Hog1 is indeed activated in response to certain oxidants (Singh, 2000; Haghnazari and Heyer, 2004; Bilsland et al., 2004). Reinforcing these data, mutants altered in the HOG pathway are more sensitive to oxidants (Singh, 2000; Rep et al., 2001). The yeast response to oxidative stress is, at least in part, developed at the transcription level. Some genes like *YAP1* (Harshman et al., 1988), *YAP2* (Bossier et al., 1993; Stephen et al., 1995), *MSN2/MSN4* (Kobayashi and McEntee, 1993; Estruch and Carlson, 1993), *SKN7* (Krems et al., 1996; Morgan et al., 1997; Lee et al., 1999) or *SKO1* (Rep et al., 2001) among others (see Moye-Rowley (2002) for a review) participate in the induction of oxidative defense mechanisms. Sko1 has been shown to mediate the recruitment of the Tup1–Ssn6/Cyc8 complex to the promoter regions of certain *HOG1*-dependent genes (Proft and Serrano, 1999; Proft et al., 2001). Phosphorylation of Sko1 occurs in response to osmotic stress at multiple sites within the N-terminal region (Proft et al., 2001); this converts the Sko1–Cyc8–Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF allowing the expression of targets genes (Proft and Struhl, 2002). Deletion of *SKO1* also modifies the induction of certain oxidative stress responsive genes. therefore establishing a link between Sko1 and

Abbreviations: MAP kinase, mitogen activated protein kinase.

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Hog1-dependent oxidative stress response in *S. cerevisiae* (Rep et al., 2001). Sko1 is also regulated by the cAMP/protein kinase A (PKA) signaling pathway in response to osmotic stress: PKA phosphorylates Sko1 near the bZIP domain (Proft et al., 2001). Sko1p is localized in the nucleus of unstressed cells but redistributes to the cytosol upon severe salt stress by a mechanism that is independent of Hog1 and Bcy1, the regulatory subunit of PKA (Pascual-Ahuir et al., 2001).

In *C. albicans* the HOG pathway is an important mediator of virulence and *hog1* mutants display reduced virulence in a mouse model of experimental infection (San José et al., 1996; Roman et al., 2007). This route plays a repressive role over the yeast-to-hypha transition (Alonso-Monge et al., 1999; Arana et al., 2005; Eisman et al., 2006) and *hog1* mutants are, therefore, hyperfilamentous. Avirulence of *hog1* mutants can be explained, at least in part, by their enhanced susceptibility to oxidative stress and killing mediated by phagocytes (Alonso-Monge et al., 2003a; Arana et al., 2007). In agreement with this, oxidative stress (Alonso-Monge et al., 2003b) – as well as osmotic (San José et al., 1996) and heavy metal stress (Smith et al., 2004) – activate the pathway. Some downstream mediators of the HOG pathway have been identified in the last years. Although *hog1* mutants share some phenotypes with *cap1* mutant (homolog to yeast *yap1*), localization, phenotypic characterization and biochemical evidence indicate that Cap1 is not a downstream target of Hog1 (Alonso-Monge et al., 2003a). a conclusion also inferred from genome wide studies (Enjalbert et al., 2006). Recently, the Sko1 protein has been shown to be phosphorylated in a Hog1-dependent manner in response to osmotic stress (Rauceo et al., 2008). This study also concluded that Sko1 is involved in the response to caspofungin and participates in cell wall construction. We have characterized here the role of Sko1 in the oxidative stress response of this fungus. We demonstrate by genome wide analysis that Sko1 acts mainly as a repressor of gene expression. We also demonstrate a role for this protein in yeast-to-hypha transition and its implication in the regulation of the MAP kinase network.

2. Materials and methods

2.1. Strains and growth conditions

Yeast strains are listed in Table 1. For clarity and unless otherwise stated, *geneX* will always indicate the homozygous *genX*/*geneX* Ura$^+$ strain. Reint indicates that an *SKO1* fused to GFP gene was reintroduced at the *LEU2* locus of the *C. albicans* mutant strains genome. The results showed in the present work were obtained using the RM100 strain and the mutants derived from it, except the genome-wide transcription analyses which were performed using CAF2 as parental strain and the *sko1* mutant in this background. Phenotypic results shown in this manuscript were similar for both backgrounds. Yeast strains were grown at 37 °C (unless otherwise stated) in YEPD medium (1% yeast extract, 2% peptone and 2% glucose) or SD minimal medium (2% glucose, 0.67% yeast nitrogen base without aminoacids) with the appropriate auxotrophic requirements at 50 μg/ml (final concentration). pH was adjusted to 4.3 or 6.7. The morphology of cells under different growth conditions was tested using YEPD supplemented with bovine fetal serum (BFS) at different concentrations or SD liquid medium at different pHs supplemented or not with 5% BFS. Morphology of the colonies was analyzed plating 50–100 colony forming units (C.F.U.) on YEPD. Spider medium (1% mannitol, 1% nutrient broth, 0.2% $K_2{\cdot}HPO_4$ and 1.35% agar) and SLADH medium (Gimeno et al., 1992) plates that were incubated for 7 days at 37 °C. Growth in liquid medium was estimated as the absorbance at 600 nm (A_{600}). *C. albicans* was transformed using the lithium acetate method (Köhler et al., 1997).

2.2. Molecular biology procedures and plasmid constructions

Standard molecular biology procedures were used for all genetic constructions (Ausubel et al., 1993). For the disruption of the *SKO1* gene, the oligos RSKO1UP5′ and RSKO1LW5′ (all the oligonucleotides used as primers are listed in Table 2) were used to amplify a 452 bp 5′ region flanking the ORF and subcloned in the cloning vector pGEM-T (Promega). Similarly, oligonucleotides RSKO1UP3′) and RSKO1LW3′ were used to amplify a 436 bp 3′ flanking region of the ORF from *C. albicans* strain SC5314 and subcloned in the cloning vector pGEM-T. The 5′ and 3′ regions were excised from these constructions using the combination of enzymes *Sca*I–*Bgl*II and *Bam*HI–*Sph*I respectively and accommodated in the disruption plasmid pCUB6K1 generating the pIR-12 plasmid. pCUB6K1 comprises the *URA3* marker flanked by the *hisG* gene from *Salmonella typhimurium* resistance gene (Alonso-Monge et al., 1999). The pIR-12 plasmid was digested with *Sca*I and *Sph*I to force recombination at the *SKO1 locus* following the *URA3*-blaster deletion scheme (Fonzi and Irwin, 1993). Genomics DNAs were digested with *EcoR* I for the southern-blot assay. The probe used to confirm the deletion of the *SKO1* gene was generated by PCR amplification using the SKO1COMUP and SKO1COMLW. This strategy was used for the deletion of *SKO1* gene either in wild type or *hog1* backgrounds (strain CNC15, Table 1). For *SKO1* reintegration, a 1587 bp fragment which included *SKO1* ORF was amplified and subcloned in the cloning vector pGEM-T by using the primers o-SKO1cup and o-SKO1clw. A *Not*I–*Bam*HI fragment was then accommodated in the *Not*I–*Bam*HI restriction site of the plasmid pFA which carries the *ACT1* promoter and the *URA3* marker (Román et al., 2005) generating the pASKO1c plasmid. Then the gene encoding green fluorescent protein (GFP) was amplified by PCR using the primers o-GFPup and o-GFPlw. The GFP ORF fragment was inserted in the *Not*I site of the pASKO1c plasmid generating the pAGFPSKO1c plasmid. This plasmid was integrated in the *C. albicans* genome previous restriction with the enzyme *Kpn*I. The construction was integrated in the genome at the *LEU2 locus*.

2.3. Drug susceptibility assays

Drop tests were performed by spotting serial dilutions of cells onto YEPD plates supplemented with sodium chloride, sorbitol, H_2O_2, menadione, Congo red or Calcofluor White at the indicated concentrations. Plates were incubated at 37 °C overnight. Kinetic of susceptibility to oxidants was measured using logarithm phase cells. 10^7 cells were transferred to a 1.5 ml tube containing YEPD and the oxidative agents was added to the final concentration indicates. Tubes were incubated at 37 °C with shaking; then 5 μl samples were collected at different time points and spotted onto YEPD plates. The plates were incubated for 24 h at 37 °C and photographed.

2.4. Protein extracts and immunoblot analysis

Yeast strains were grown to an optical density of 1 at 37 °C in YEPD medium. To test the kinetic of the phosphorylation, hydrogen peroxide was added to the medium at a final concentration of 10 mM samples were taken at 5, 15 and 30 min after the challenge. Alternatively, different concentrations of hydrogen peroxide were added to cell cultures and samples were collected after 10 min of incubation. The exit from stationary phase assay was performed as follows: overnight cultures were refreshed in pre-warmed YEPD medium to A_{600} of 0.1; samples were collected after 15 and 30 min after the shift. Cell extracts were obtained as previously indicated (Martín et al., 1993, 2000). Even amounts of proteins were loaded onto gels as assessed by 280 nm measurement of the samples and Ponceau Red staining of the membranes prior to blocking and