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The transmembrane protein Opy2 mediates activation of the Cek1 MAP kinase in *Candida albicans*

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ABSTRACT

MAPK pathways are conserved and complex mechanisms of signaling in eukaryotic cells. These pathways mediate adaptation to different stress conditions by a core kinase cascade that perceives changes in the environment by different upstream elements and mediates adaptation through transcription factors. In the present work, the transmembrane protein Opy2 has been identified and functionally characterized in *Candida albicans*. This protein is required to trigger Cek1 phosphorylation by different stimuli such as the resumption of growth from stationary phase or the addition of the cell wall disturbing compounds zymolyase and tunicamycin. *opy2* mutants display susceptibility to cell wall disturbing compounds like Congo red. However, it does not play a role in the adaptation to high osmolarity or oxidative stress, in close contrast with the situation for the homologous protein in *Saccharomyces cerevisiae*. The overexpression of Opy2 in a *S. cerevisiae opy2 ssk1* mutant partially complemented the osmosensitivity on solid medium by a Hog1-independent mechanism as well as the abnormal morphology observed in this mutant under high osmolarity. The electrophoretic pattern of CaOpy2 tagged version in *S. cerevisiae* suggested similar post-translational modification in both microorganisms. This protein is also involved in pathogenesis as revealed by the fact that *opy2* mutants displayed a significantly reduced virulence in the *Galleria mellonella* model.

1. Introduction

Cells respond and adapt to external changes through signal transduction pathways. Among the different mechanisms of cell signaling described in eukaryotic cells the MAPK (Mitogen Activated Protein Kinase) mediated pathways are essential. These signaling routes are conserved in all eukaryotic cells and are implicated in several relevant physiological functions for the cell (Kultz, 1998). MAPK pathways consist of a module of three kinases (a MAP kinase kinase kinase, a MAP kinase kinase and a MAP kinase) that are activated by successive phosphorylation in response to different signals through others kinases, two-component systems and/or heterotrimeric G proteins.

Candida albicans is a human commensal that is able to cause infection under certain circumstances and therefore, it is considered as an opportunistic pathogen (Odds, 1988). These infections range from superficial to systemic which are frequently difficult to treat. Although others species of *Candida* have increased their prevalence as nosocomial systemic infections, *C. albicans* still remains as the major cause of fungemia in hospitals of developed countries. In *C. albicans* four MAP kinases have been currently identified: Mkc1, the homolog to *Saccharomyces cerevisiae* Slt2 (Navarro-García et al., 1995); Cek1, homolog to Kss1 (Csank et al., 1998); Cek2, homolog to Fus3 (Chen et al., 2002) and Hog1, homolog to the Hog1 MAP kinase (San José et al., 1996). Mkc1 participates in the cell wall integrity pathway and is implicated in cell wall biogenesis, the response to cell wall stress and others like biofilm formation and virulence (Diez-Orejas et al., 1997; Kumamoto, 2005; Navarro-García et al., 1998, 2005, 1995). Cek2 participates in mating as *cek1 cek2* double mutants are unable to mate. Cek1is involved in invasive hyphal growth, mating efficiency, cell wall construction, quorum sensing and virulence in a systemic murine model (Chen et al., 2002; Csank et al., 1998; Román et al., 2009a, 2005, 2009b). Finally, the Hog1 kinase responds to different stresses such as oxidative, osmotic and the presence of metals, etc. (Alonso-Monge et al., 2003; Smith et al., 2004). This kinase is also implicated in the yeast-to-hypha transition, cell wall biogenesis, oxidative metabolism and virulence (Alonso-Monge et al., 2009, 1999; San José et al., 1996).

In *S. cerevisiae*, Hog1 perceives the osmotic stress signal via two branches: the Msb2, Sho1, Ste50, Ste11 branch and the Sln1–Ypd1–Ssk1, Ssk2/Ssk22 branch (O'Rourke and Herskowitz, 2002; Posas et al., 1996). In addition, another transmembrane mucin Hkr1

Abbreviations: MAPK, Mitogen Activated Protein Kinase; SVG, Sterile Vegetative Growth.

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has been reported as potential osmosensor of the Sho1 branch. Hkr1 and Msb2 act upstream Sho1 forming a complex which, upon high external osmolarity, triggers Sho1 mediated signaling (Tatebayashi et al., 2007). Opy2 is a transmembrane protein first postulated as a third osmosensor in the Sho1 branch that anchors Ste50 and Ste11 (Wu et al., 2006). The association between Ste50 and Opy2 is regulated positively by external nutritional conditions or negatively by activated MAP kinases. This association/dissociation controls signal transmission integrating different signals and MAPK network regulation (Yamamoto et al., 2010).

In *C. albicans* the analysis of MAPK pathways were performed originally with the MAP Kinases, actually, others elements have been included to draft the structure and organization of these pathways. Initially two branches were allocated in the HOG pathway leading to Pbs2 phosphorylation which is the Hog1 MAPKK (Arana et al., 2005). Nevertheless it seems that only the branch involving Sln1, Ypd1, Ssk1 and Skk2 plays an unequivocal role in the Pbs2–Hog1 signaling (Cheetham et al., 2007). The second via triggers Cek1 phosphorylation; these elements, Sho1, Msb2 and Ste11, belong to the so-called SVG (Sterile Vegetative Growth) pathway (Lee and Elion, 1999; Román et al., 2005). The Msb2 mucin mediates Cek1 activation upon tunicamycin addition (Román et al., 2009a), although the hierarchy among the transmembrane elements (Sho1 and Msb2) of the SVG pathway has not yet been analyzed.

Although Hog1 phosphorylation seems to be mediated by Ssk1 and Ssk2 in *C. albicans*, deletion of *SSK1* in addition to *MSB2* and *SHO1* genes rendered cells that are still able to activate Hog1 upon osmotic stress (Román et al., 2009b). The triple mutant *ssk1 sho1 msb2* displayed an aberrant cell morphology under hypertonic conditions in spite of accumulating intracellular glycerol and Hog1 translocation to the nucleus (Román et al., 2009b). These results suggest that there may be present others elements still unknown implicated in the phosphorylation of Hog1 under osmotic stress. In order to identify this/these elements, the *OPY2* gene was deleted in *C. albicans* and its function was analyzed both in the SVG and the HOG pathways. Our results demonstrate that the transmembrane protein Opy2 mediates Cek1 phosphorylation upon the presence of cell wall disturbing agents and during the resumption from stationary phase of growth. The *opy2* single mutant displayed cell wall defects while it plays no apparent role on the susceptibility to osmotic or oxidative stress. We also evidence of functional differences between *C. albicans* and *S. cerevisiae* regarding signaling evolution.

2. Material and methods

2.1. Strains and growth conditions

Yeast strains are listed in Table 1. For clarity, and unless otherwise stated, *opy2* will always indicate the homozygous *C. albicans* mutant *opy2/opy2* strain (strain CHO4-1), $opy2^{reint}$ designates the homozygous *opy2/opy2* where the *OPY2* gene was reintegrated at the *OPY2* region under the control of its own promoter (strain CHO39).

C. albicans strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 37 °C, unless indicated. Usually, overnight cultures were refreshed to an optical density of 0.1 (measured at 600 nm) and experiments were performed when cultures reached an optical density of 1 (at λ 600 nm) when exponential phase cells were required. In the case of stationary phase cells, cells from a 1 day overnight culture were routinely used. The cultures of strains carrying the *OPY2*–myc under the control of tetracycline inducible promoter were incubated in YPD supplemented with 10 μg/ml doxycycline for 1 day to ensure *OPY2–myc* full expression. *S. cerevisiae* strains were grown routinely in minimal medium (2% glucose, 0.67% yeast nitrogen base without aminoacids) supplemented with a synthetic complete URA-drop-out to impede loss of plasmid. Cultures were incubated at 30 °C, unless otherwise indicated.

Sensitivity on solid media was tested on YPD supplemented with the indicated concentration of several compounds (hydrogen peroxide, menadione, NaCl, sorbitol, Congo red or calcofluor white). In the case of strains carrying the inducible version of tetracycline promoter (TET^P), deoxicycline was added to the plates at 20 μg/ml. 10^5, 10^4, 10^3 and 10^2 cells were spotted on the plates and incubated unless otherwise stated at 37 C to reveal differences of growth. In the case of *S. cerevisiae* strains, the temperature of incubation was 30 °C.

The assays in liquid medium to visualize cell morphology were performed as follow. Cells were grown overnight in YPD or YPD supplemented with 1 M NaCl and visualized with a Nikon TE2000 fluorescence inverted microscope equipped with CCD (Melville, NY). Digital images were acquired with an Orca C4742-95-12ER camera (Hamamatsu, Bridgewater, NJ) and processed with the HCImage software.

2.2. Genetic constructions

For the disruption of the *OPY2* gene, we constructed the pDOPY2 plasmid. A 0.72 kbp ORF flanking region was amplified by PCR using the primers OPY2KpnI and OPY2XhoI. This fragment was first subcloned in the commercial vector pGEM-T (Promega). Similarly, oligonucleotides OPY2NotI and OPY2SacI were employed to amplify a 0.529 kbp region flanking the 3′ of the *OPY2* gene of the CAF2 strain and cloned in pGEM-T vector. Both fragments were finally accommodated in the pSF2SA plasmid (Reuss et al., 2004) carrying the nourseothricin resistance marker (*CaSAT1*), thus obtaining pDOPY2. This plasmid was digested with *Kpn*I–*Sac*I to force the homologous recombination at the *OPY2* chromosomal region. Following the already described *SAT1* flipping method (Reuss et al., 2004), two rounds of integration/excision were necessary to generate homozygous mutants.

To construct the *OPY2*–myc fusion we previously generated the pNIM1_MoGFP_carboxi_ca_myc plasmid which derives from the pNIM1 vector (Park and Morschhauser, 2005). The primers: ETC.–myc-upper: 5′-CAGTTTGGTTCAGCACCTTGTCG-3′ and ETC–myc-lower: 5′-GCCAGATCTTTACAAGTCTTCTTCAGAAATCAATTTTTGTTCGCGGCCGCATTTGTATAGTTCATCCATGCCATGTG-3′) allowed to amplify the GFP fused to a myc tag from the pNIM1 vector. The PCR product was digested with *Sal*I and *Bgl*II, allowing the placement in the *Sal*I–*Bgl*II places again in the pNIM1 plasmid. We then amplified the *OPY2* ORF by PCR using the primers o-OPY2GFPmycUp (5′-GCGGTCGACAATCATGCCAATACCCAGATC) and o-OPY2GFPmycLow (5′-GCAGCGGCCGCCGCTCGAGAGTTTGTCAGGATCAGCAAAAG-3′) and genomic DNA form CAF2 strain as template. For the pNIM1–Opy2–myc construction, where the Opy2–myc fusion is under the control of the inducible tetracycline promoter, the PCR product (1615 bp) was cloned in the *Sal*I–*Not*I sites of pNIM1_MoGFP_carboxi_ca_myc after digestion with the same enzymes. The construction was integrated in the *ADH1 locus* after digestion with *Kpn*I–*Sac*II.

The *opy2* reintegrant strain was constructed using a vector that allowed the fusion of the gene to HA and the plasmid integration in the *ARD1 locus* (constructed and kindly provided by Daniel Prieto). The *OPY2* ORF plus 1.5 kpb upstream of the ATG was amplified using the primers: prOPY2opy2UP (CGGTCGACGAAAAGAAATCCCAATGACGTAGTGGTAGTG) and prOPY2opy2LOW (GCGGATCCTTTGTCAGGATCAGCAAAAGGGCTTC). After digestion with *Sal*I–*Bam*HI, the amplified sequence (3168 bp) was located in the mentioned plasmid. The final construction was integrated in the *ARD1 locus*