The HOG MAPK pathway in *Candida albicans*: more than a osmosensing pathway

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Abstract

In 1993, Brewster and Gustin described the existence of a kinase whose activity was essential for *Saccharomyces cerevisiae* to grow in environments with high osmolarity. This led to the discovery of the HOG pathway, a MAP kinase (MAPK) pathway that has revealed crucial to respond to wide range of stress conditions frequently encountered by fungi in their common habitats. MAPK signaling is initiated at the plasma membrane, where triggering stimuli lead to a phosphorylation cascade that ultimately activates transcription factors to ensure an appropriate adaptive response. In pathogenic fungi, the HOG pathway gains special significance as it is involved in traits related to pathogenicity; these include biofilm formation, adhesion to surfaces and morphogenetic and epigenetic transitions. It also plays a role in controlling both the pathogen and the commensal state program. Understanding the signals leading to its activation, the elements of the pathways and the targets of the pathway is therefore of primary importance in the design of novel antifungals.

Although fungal infections are not as frequent as those derived from bacteria, they remain as an important cause of human diseases. *Candida albicans* is a pathogenic yeast that normally inhabits the human body and frequently causes life-threatening invasive infections (called candidiasis) that account for ~19% of the infections in intensive care units. This microorganism behaves as a harmless commensal of the gastrointestinal and vaginal tract of humans in a significant (albeit not precisely determined) percentage of individuals but is able to cause disease once the host defense mechanisms are compromised (Gow et al. 2012; Iliev and Leonardi 2017; Leonardi et al. 2018; Netea and Brown 2012; Romani 2011). This ability to proliferate within the human host is the most probable cause for the high incidence of its infections. Factors such as neutropenia, immune disorders, cancer, diabetes, long-term broad-spectrum antibacterial treatment and catheters are predisposing factors to candidiasis. *Cryptococcus neoformans* is a basidiomycete fungus acquired through inhalation from pigeons' excreta. This yeast may proliferate once inhaled under some conditions; the capsule is an important virulence factor that facilitates blood dissemination and can lead to fatal meningitis. *Aspergillus* spp. infections are frequently acquired from spores present in the environment; proliferation within immunocompromised individuals may develop in a systemic and fatal disease and are very difficult to control even in specialized hospital units. These three examples illustrate the fact that while most fungi normally cause mild superficial infections, their diseases can be very serious and are far from being controlled in every clinical situation. Effective therapy is much more limited for these infections given the similarity of fungal cells (eukaryotic) with human cells and the development of resistance against certain antifungals that may limit their utility in a near future. New therapeutic options are still necessary and research has therefore focused in the identification of virulence traits as novel therapeutic targets.

MAPK routes are essential sensor mechanisms that transduce environmental inputs to biochemical events that ensure adaptation to the new physiological situation. These routes are conserved among all eukaryotic organisms and control proliferation and stress responses in mammalian cells (Widmann et al. 1999). The central module is composed of three kinases that become sequentially activated by phosphorylation (Kultz 1998). Sensor mechanisms exist at the plasma membrane that connect the stimuli to the MAPK central module, which include G-protein coupled receptors, two-component systems based on histidine-aspartic phosphorylation sensors, integral membrane proteins or tyrosine kinase receptors (Herskowitz, 1995). The signal is eventually transferred to a MAP kinase kinase kinase (MAPKKKs) that phosphorylates a MAP kinase kinase (MAPKKs) which in turn phosphorylates a MAP kinase (MAPKs). The MAPK translocates to the nucleus where it activates different factors that lead to an adaptive transcriptional response. Activation of these pathways is transient as permanent is frequently prejudicial to the cell; therefore, there are negative and positive feedbacks mechanisms to ensure a timely and regulated response as well as ways to ensure the specificity of the response via scaffold proteins (Saito 2010).

In *C. albicans*, four MAPK pathways have been described (Monge et al. 2006; Román et al. 2007). The cell integrity (or PKC) pathway regulates the biogenesis of the cell wall, morphogenesis, biofilm formation and virulence. The Cek1-mediated pathway, also involved in cell wall formation, was discovered as regulating mating and has been shown to regulate vegetative and invasive growth. Cek2, homologue to ScFus3, is essential for an efficient mating process. We will here focus on the role of the HOG pathway in *C. albicans*, mediated by the MAPK Hog1 and in particular, in its involvement in important traits which have been grouped in stress, morphogenesis and virulence/commensalism. It must be indicated that, obviously, this is done for clarity in the review. We will, where appropriate, discuss its relation with the cell integrity and *CEK1* pathways as they coordinately regulate some traits. We will also highlight its functionality in other fungal pathogens without extensive analyzing their role in other models such as *S. cerevisiae* or *Schizosaccharomyces pombe* or plant pathogens which have been the subject of excellent reviews (Hohmann 2015; Ikner and Shiozaki 2005; Perez and Cansado 2010). A short section collecting the role of HOG pathway in *C. neoformans* and certain *Aspergillus* species is included at the end of the review.

Stress

In *C. albicans*, the HOG (High Osmolarity Glycerol) pathway was first described to mediate adaptation to high osmotic environments. *HOG1* was cloned by complementation of *S. cerevisiae hog1* mutants and its deletion resulted in an increased susceptibility to osmotic stress and a failure to accumulate glycerol, indicating that this metabolite is a compatible solute in *C. albicans* as in *S. cerevisiae* (San José et al. 1996). This phenotype was not only observed in *hog1* cells but also in *pbs2* and *ssk2* mutants, being Pbs2 and Ssk2 the MAPKK and the only (in contrast with *S.cerevisiae*) MAPKKK respectively of the route (Arana et al. 2005; Cheetham et al. 2007). Interestingly, in *C. albicans* Hog1 is not only activated by osmotic but also by oxidative stress (Alonso-Monge et al. 2003), a somehow unexpected result when discovered although previously suggested from the phenotype observed in *S. cerevisiae* (Singh 2000). In *C. albicans*, *hog1* mutants are susceptible to a range of oxidants (hydrogen peroxide, *t*-BOOH and diamide), heavy metals (Cd^{2+} and As^{3+}) and UV light, a phenotype also observed in *pbs2* and *ssk2* mutants (Alonso-Monge et al. 2003; Cheetham et al. 2007). It has been shown that H_2O_2 arrests the *C. albicans* cell cycle and Hog1 is required to resume the growth after the stress (Correia et al. 2016),

How is the oxidative signal transduced to Hog1 is presently unknown. In *S. cerevisiae*, the HOG pathway is activated by two different upstream branches (Figure 1). The *SLN1*-branch relies on a two-component system formed by *SLN1*, *YPD1* and *SSK1* genes that activate the redundant Ssk2/Ssk22 MAPKKKs (Posas et al. 1996). The *SHO1*-branch (Posas and Saito 1997) connects Sho1, a transmembrane protein that interacts with Msb2 and Hkr1 (Cullen et al. 2004; O'Rourke and Herskowitz 2002), Opy2 and Ste50 with Ste20, a PAK-like kinase. Both branches converge at Pbs2 (Posas *et al.*, 2000;de Nadal *et al.*, 2002;Hohmann, 2002). In *C. albicans*, although both branches exist, only the *SLN1*-branch is responsible for the activation of Hog1 by oxidative stress, as *ssk1* and *ssk2* mutants fail to activate Hog1 in response to hydrogen peroxide (Chauhan et al. 2003). This was also confirmed later using *ssk2* mutants (Cheetham et al. 2007), indicating that there is no redundancy at the Ssk2 level in *C. albicans*. The *C. albicans* *SHO1*-branch is not involved in the activation of Hog1 but rather in the activation of Cek1 (Román et al. 2005), the homolog of the *S. cerevisiae KSS1*. In fact, Msb2, a mucin-like protein, does not mediate activation of Hog1 but Cek1 and rather controls the formation of the cell wall (Román et al. 2009). Interestingly, it has been shown that deletion of upstream components of this branch in combination with *SSK1* does not impair activation of Hog1 or glycerol accumulation in response to osmotic stress, although the cells remain sensitive to high osmolarity (Román *et al.*, 2005;2009b;Herrero de *et al.*, 2013). This suggests the existence of a third signaling branch that mediates Hog1 activation upon osmotic stress (Cheetham et al. 2011), as occurs with the response to arsenite.

In *C. albicans*, the transcription factor Sko1 and not Cap1 (Zhang et al. 2000) (the homolog of *S. cerevisiae* Yap1 (Moye-Rowley et al. 1989)) is responsible, at least partially, for this oxidant specific response (Alonso-Monge et al. 2010). This was inferred from the different oxidant phenotype of *hog1* and *cap1* mutants, indicating that each gene controls different subsets of genes and later confirmed upon completion of the *C. albicans* genome by transcriptomic analysis (Enjalbert et al. 2006) where a core of Hog1-stress responsive genes was identified. Sko1 regulates the response to osmotic stress in *S. cerevisiae* (Proft et al. 2001) but also controls a set of oxidative responsive genes (Rep et al. 2001). In *C. albicans*, *sko1* mutants increase the sensitivity of *hog1* mutants to oxidants (Alonso-Monge et al. 2010) and also to the mielomonocytic cell line HL-60, whose killing mechanisms occur via generation of oxidative stress. In addition, a genome wide transcriptional analysis revealed different expression of specific oxidative stress response genes, indicating that Sko1 is responsible, at least partially, of this behavior. Interestingly, Hog1 also becomes phosphorylated in response to arsenate (Urrialde et al. 2015) in an Ssk1-dependent process, similarly to what occurs in *S. pombe* (Rodriguez-Gabriel and Russell 2005). Both arsenate and arsenite increase reactive oxidant species in *C. albicans*, linking arsenic ion metabolism with the HOG pathway (Urrialde et al. 2017). Recently, it has been reported that the oxidation of Hog1 in a specific Cys residue mediates the response to nitrosative stress (Herrero-de-Dios et al. 2018). This suggests that alternative posttranslational modification (not only phosphorylation) regulate the stress-specific outputs of MAPK pathways.

Morphogenesis and cell wall biogenesis

Morphogenesis is an essential trait related to virulence in fungi (Rooney and Klein 2002). *C. albicans* is a dimorphic fungus, being able to switch from a unicellular yeast-like form to a hyphal form in response to stimuli frequently encountered in the human host, where this conversion is favored. Nutritional signals are important for filamentation, as nitrogen deprivation, oxygen availability, serum or pH strongly influence this response. Filamentation is therefore a very complex and environmentally triggered program where several transcription factors and stimuli have been involved (Liu 2001).

The HOG pathway plays a repressive role on filamentation in *C. albicans*. *hog1*, *pbs2* or *ssk2* display enhanced hyphal formation as shown by their increased ability to form filaments under not fully inducing conditions such as reduced concentrations of serum (Alonso-Monge et al. 1999; Arana et al. 2005; Cheetham et al. 2007) or their appearance on solid media with limiting nitrogen concentration (Eisman et al. 2006). These mutants show a crosstalk between the HOG and the Cek1 mediated pathways rendering in a hyperphosphorylation of Cek1 MAPK which regulates filamentation. In contrast, *ssk1* mutants show a reduced ability to filament even in the presence of serum, a phenotype not suppressed by the overexpression of *HOG1* (Calera et al. 2000). Interestingly, *hog1* mutants (as well as *pbs2* and *ssk2*) express the GATA transcription factor Brg1, a promoter of hyphal formation (Su et al. 2013). As Brg1 is a target of the nutrient sensing Tor1-mediated pathway, this supports the idea that diminishing Tor1 activity via an altered metabolic state nutrition leads to a decrease in Hog1 signaling (by activation of the Ptp2 and Ptp3 phosphatases) that ultimately lead to Brg1 expression.

In *C. albicans*, the HOG pathway is also involved in the formation of chlamydospores. These structures are frequently considered (but not yet proven) to be forms of resistance, able to survive under harsh environmental conditions (Staib and Morschhauser 2007). As chlamydospores are not usually observed *in vivo* (Cole et al. 1991) and their proportion is scarce (chlamydospores are usually formed at the tip of the growing hypha), their study has been hampered. Hog1 is essential for chlamydospore formation (Alonso-Monge et al. 2003) by a mechanism that is independent of the Efg1 regulator, a key regulatory gene involved in this developmental process (Sonneborn et al. 1999). Both *efg1* and *hog1* are unable to form chlamydospores but overexpression of *EFG1* gene in a *hog1* mutant -and vice versa- did not restore the phenotype. The reasons for the involvement of Hog1 in this morphogenetic process are presently unclear. One possibility could be the involvement of Nrg1 (a repressor of hyphal formation (Murad et al. 2001)) that could be regulated by Hog1, as Nrg1 is also involved in chlamydospore formation (Staib and Morschhauser 2005). Another explanation could invoke the fact that these structures are formed under low oxygen concentrations and absence of light, suggesting a role for oxidative stress in this process. Recently, a role for mitochondrial complex I has been demonstrated in gastrointestinal commensalism (Huang et al. 2017). The authors also demonstrate that mannitol-dependent morphogenesis is controlled by an oxidative stress signaling pathway that requires Hog1 and Brg1 repression.

The HOG pathway is also involved in cell wall biogenesis. Deletion of *hog1* confers a resistance to certain compounds such as nikkomycin Z (an inhibitor of chitin synthesis) other drugs such as Congo red or calcofluor white that interfere with the correct assembly of the cell wall (Alonso-Monge et al. 1999; Román et al. 2005), phenotypes that are not completely shared by *pbs2* mutants (Arana et al. 2005). It has been proposed, based on transcriptional studies, that the HOG pathway regulates chitin synthesis coordinately with other pathways (Munro *et al.*, 2007). This could involve other MAP kinases, such as Cek1, which has been also shown to be important in cell wall formation (Eisman et al. 2006; Navarro-García et al. 2005). Osmotic stress can be triggered by an increase in external osmolites but also through the action of killer toxins; interestingly, the *C. albicans* Hog1 has been implicated in the survival to killer toxins from *Debaryomycess hansenii* (Morales-Menchen et al. 2018), pointing towards a role for this pathway in the construction of the cell wall.

Commensalism and Pathogenesis

Since the HOG pathway plays so many crucial roles in the biology of *C. albicans*, it is not surprising the relevance of this pathway in fungal pathogenesis. In fact, although *hog1* mutants are hyperfilamentous (therefore, expecting to cause increased tissue damage), they show an attenuated virulence in a mouse model of systemic infection (Alonso-Monge *et al.*, 1999). This can be partially explained by their sensitivity to oxidative and nitrosative stress, even more pronounced in *pbs2* strains (Arana et al. 2005), which could result in sensitivity to the phagocytic attack by macrophages and neutrophils that generate reactive oxygen species (ROS) or reactive nitrogen species (RNS) to allow fungal clearance (Arana et al. 2007). A similar behavior is observed with other mutants of the pathway, indicating that the HOG route is important as a therapeutic target.

Recently Hog1 (and also Pbs2) have been also shown to play a critical role in the establishment of *C. albicans* in the mouse gut (colonization). *hog1* mutants are unable to colonize the mouse gut even in animals which have a strong reduction of bacterial microbiota by means of antibiotic treatment, indicating that the HOG pathway controls traits essential for the commensalism program (Prieto et al. 2014). The reasons for this behavior are presently unknown, especially as *hog1* mutants are sensitive to bile salts and also show a decreased adhesion to mucosa. However, it has been recently described that *C. albicans* cells are able to undergo a morphogenetic transition (called GUT) that makes cells especially prepared to grow in the mammalian tract (Pande et al. 2013). This transition is morphologically and genetically different from the already described white opaque transition and results in cells with enhanced metabolic abilities (Ene et al. 2016; Pande et al. 2013). The conversion from white to GUT cells is determined by Wor1, a master regulator of the white opaque transition, the mating competent phase of this pathogen (Huang et al. 2006; Srikantha et al. 2006) with Wor1-overproducing cells showing increased fitness in the mouse gut. It has been described that *HOG1* represses mating in *C. albicans* (Liang et al. 2014) as *hog1* a/a and α/α white cells are efficiently converted to opaque cells. Given the role of the a1/α2 heterodimer as repressor of Wor1 production, it is tempting to speculate that the HOG pathway could control GUT production via Wor1 or a yet unidentified element. Recently, in *C. glabrata* Hog1 has been shown to be responsible for lactic acid resistance and revealed essential for fungal cells to compete with *Lactobacillus*, a common member of endogenous microbiota (Beyer et al. 2018). Whether this could be occurring in the gastrointestinal tract for *C. albicans* is presently unknown.

Other fungi

The role of the HOG pathway in other yeasts shows similarities as well as distinctive features. In *Cryptococcus neoformans*, four serotypes are observed based on capsule composition: A (var. *grubii*), B and C (var. *gattii*) and D (var. *neoformans*). Most of the clinical isolates are of type A, the most pathogenic which are found to be more resistant to osmotic stress implying that the HOG pathway is involved (Cruz 2000). *HOG1* plays different roles depending on the serotype of the clinical strain. In H99 strain (a type A reference strain), *HOG1* represses capsule production, the most important virulence factor that enable colonization of the central nervous system CNS (Bahn et al. 2005). In addition, it also represses the production of melanin, a pigment accumulated within cells in response to specific environmental conditions which is also involved in oxidative stress resistance. By contrast, this is not observed in the less virulent laboratory type D strain JEC21, indicating how evolution has shaped this pathway for host adaptation. Not surprisingly, *HOG1* controls filament formation and *hog1* and *pbs2* mutants show reduced virulence in animal models of cryptococcosis (Bahn et al. 2005). Interestingly, *hog1* mutants show also enhanced ability to mate, a phenotype that resembles the situation already indicated for *C. albicans*. It has been recently hypothesized that the opposite role that the PKA and HOG signaling pathways play in response to glucose availability is crucial for the adaptation within the host in either glucose deficient (lungs) or not (CNS) environments (Banerjee et al. 2016).

In *Aspergillus nidulans*, the HOG pathway is activated in response to high osmolarity exclusively by the *SLN1*-branch, and the existence of a proline-rich motif in the Pbs2 homolog allows receiving the input from the Sho1-branch (Furukawa et al. 2005). *A. fumigatus* share many of the elements of the HOG pathway. *sakA* and *pbs2* mutants (lacking the MAPK and MAPKKs respectively) show enhanced sensitivity to osmotic and oxidative stress, as well as to

amphotericin B and itraconazole antifungals which induce oxidative stress. However, it seems that *sakA* plays a key role in the physiology of *A. fumigatus* since it also regulates growth and conidia germination (reviewed in (Ma and Li 2013)). Deletion of the group III histidine kinase NikA showed morphological defects (low conidia and abnormal hyphae), sensitivity to high osmolarity and resistance to fungicides such as fluodioxonil, which have been described to activate the HOG pathway (Hagiwara et al. 2013).

Conclusions

While the HOG pathway was initially considered to be an osmotic responsive route responsible for allowing environmental yeasts (such as those on grapes' surfaces) to survive in water limitations periods, it is evident that this is not the case. Rather, the HOG is involved (and evolved) in human pathogenic fungi (not only *C. albicans*) to coordinately regulate traits such as cell wall biogenesis, morphogenesis and resistance to stress which have profound implications in their adaptation to the host. The existence of drugs which target this pathway combined with the availability to develop efficient and robust chemical and genetic screenings will enable to identify new drugs that may contribute to the control of human fungal diseases.

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