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The Stress-Activated Signaling (SAS) Pathways of a Human Fungal Pathogen, *Cryptococcus neoformans*

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***Cryptococcus neoformans* is a basidiomycete human fungal pathogen that causes meningoencephalitis in both immunocompromised and immunocompetent individuals. The ability to sense and respond to diverse extracellular signals is essential for the pathogen to infect and cause disease in the host. Four major stress-activated signaling (SAS) pathways have been characterized in *C. neoformans*, including the HOG (high osmolarity glycerol response), PKC/Mpk1 MAPK (mitogen-activated protein kinase), calcium-dependent calcineurin, and RAS signaling pathways. The HOG pathway in *C. neoformans* not only controls responses to diverse environmental stresses, including osmotic shock, UV irradiation, oxidative stress, heavy metal stress, antifungal drugs, toxic metabolites, and high temperature, but also regulates ergosterol biosynthesis. The PKC (Protein kinase C)/Mpk1 pathway in *C. neoformans* is involved in a variety of stress responses, including osmotic, oxidative, and nitrosative stresses and breaches of cell wall integrity. The Ca^{2+}/calmodulin- and Ras-signaling pathways also play critical roles in adaptation to certain environmental stresses, such as high temperature and sexual differentiation. Perturbation of the SAS pathways not only impairs the ability of *C. neoformans* to resist a variety of environmental stresses during host infection, but also affects production of virulence factors, such as capsule and melanin. A drug(s) capable of targeting signaling components of the SAS pathway will be effective for treatment of cryptococcosis.**

KEYWORDS : *Cryptococcus neoformans*, Human fungal pathogens, SAS pathways, Signaling, Stress-response

Cryptococcus neoformans is a basidiomycete human fungal pathogen that causes meningitis in patients immunocompromised by AIDS, organ-transplantation, or anticancer-chemotherapy and immunocompetent individuals alike. *C. neoformans* is generally classified into four serotypes (A to D) with different pathogenic characteristics during host infection (Hoang *et al*., 2004). Serotype A (*C. neoformans* var. *grubii*) is the most commonly isolated clade worldwide, and along with serotype D (*C. neoformans* var. *neoformans*) causes life-threatening meningitis mainly in immunocompromised populations. In contrast, the serotype B and C clades (*C. neoformans var. gattii*), recently renamed *Cryptococcus gattii*, can cause the fatal disease in both healthy individuals and immunocompromised patients.

C. neoformans has both heterothallic sexual and homothallic unisexual life cycles. Supporting the existence of the heterothallic life cycle, *C. neoformans* is isolated as either the **a** (*MAT* **a**) or α (*MAT* α) mating type. In nature, the *C. neoformans MAT* α strain is more prevalent and more virulent (in serotype D but not serotype A) than the *MAT* **a** strain (Kwon-Chung *et al.*, 1992; Lin and Heitman, 2006). Even though no difference in virulence was observed between serotype A *MAT* α (H99) and *MAT* **a** (KN99) strains, the *MAT* α strain was found to be more efficient in disseminating into the central nervous system (CNS) and passing through the blood-brain-barrier (BBB) than the *MAT* **a** strain during co-infection of both mating types (Nielsen *et al.*, 2005). When *MAT* α and *MAT* **a** strains co-exist under certain environmental conditions, such as nutrient limitation, each mating-type cell secretes a small peptide, called a pheromone, to trigger cell-cell fusion and filamentous growth (Kwon-Chung, 1976), after which a dikaryon is formed. Subsequently, a basidium is formed at the end of the filamentous structure where karyogamy (nuclear fusion) occurs. As a result of the mating event, four chains of basidiospores are produced from the surface of basidium (Fraser *et al.*, 2003). However, the ratio difference of α and **a** mating type cells is so big that the sexual differentiation by mating is not common in the environment (Kwon-Chung and Bennett, 1978). *C. neoformans* also undergoes a homothalic unisexual life-cycle, named monokatyotic fruiting (Lin *et al.*, 2005). This type of differentiation is regarded as a form of self-sex, an occurrence which could increase the ability to survive under harsh conditions. During monkaryotic fruiting, two cells of the same mating type (mostly α type) fuse together and undergo hyphae-like sexual mating. During the process, ploidy changes occur, leading to the production of diploid blastospores (Tscharke *et al.*, 2003; Lin *et al.*, 2005). Generally, *MAT* α and serotype D strains are more efficient in monokaryotic fruiting than *MAT* **a** and serotype A strains, respectively (Wickes *et al.*, 1996).

C. neoformans is a ubiquitous fungus found in diverse

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environmental niches, including soil, trees and bird guano (Idnurm *et al.*, 2005). Infections propagules have form of either spore or dried yeast cells. The main infection route is the respiratory tract of the human host, which leads to the pulmonary infection. Subsequently, the pathogen disseminates into diverse organs (but mainly to the CNS), resulting in life-threatening meningoencephalitis (Sukroongreung *et al.*, 1998; Liu *et al.*, 2002).

C. neoformans has several virulence factors. Two well known virulence factors are antiphagocytic polysaccharide capsule (Chang *et al.*, 1996) and antioxidant melanin (Nosanchuk *et al.*, 1999). The capsule prevents *C. neoformans* from being phagocytosed by macrophages and from being dehydrated (Aksenov *et al.*, 1973). Capsule formation is influenced by iron limitation and physiological CO_2 levels (Zaragoza *et al.*, 2003). Four genes (*CAP10*, *CAP59*, *CAP60* and *CAP64*) are known to be required for capsule formation, but their biochemical properties have not been reported (Chang and Kwon-Chung, 1994, 1998, 1999; Chang *et al.*, 1995, 1996, 1997). Mutants with these genes deleted lack capsule formation and are avirulent (Chang and Kwon-Chung, 1994). The *C. neoformans* capsule is composed of several carbohydrate components. The major component is glucuronoxylomannan, accounting for more than 80% of capsule components, and the minor components are galactoxylomannan and mannoprotein (Vartivarian *et al.*, 1993). Like capsule, the virulence factor melanin plays an important role in debilitating the host defense mechanism during infection (Kwon-Chung *et al.*, 1982). Melanin is converted from catecholamine by Lac1 or Lac2. Originally, it was thought that Lac1 was the only enzyme capable of producing melanin, but microarray data demonstrated that Lac2 is not only homologous to Lac1, but also contributes to melanin production (Zhu and Williamson 2004; Pukkila-Worley *et al.*, 2005). Cells with the ability to synthesize melanin are more resistant to oxidation, ultraviolet irradiation, and high temperature, than non-melanized cells (Jacobson and Emery, 1991; Wang *et al.*, 1995; Wang and Casadevall, 1994; Rosas and Casadevall, 1997).

The ability to sense and respond to diverse extracellular signals is indispensable for all living organism to survive under a plethora of environmental stresses. Fungal pathogens maintain cellular homeostasis by developing their unique signaling pathways to counteract these environmental cues. Therefore, the stress response has been one of major virulence determinants for *C. neoformans*. For example, the ability to survive at host physiological temperature, 37~39°C, is considered to be one of the major virulence factors for human fungal pathogens including *C. neoformans* and *C. albicans* (Kwon-Chung and Rhodes, 1986; Ernst, 2000). The ability to sense and respond to diverse environmental stresses is mediated by four major signaling pathways in *C. neoformans*. These pathways are the: i) HOG (high osmolarity glycerol response), ii) PKC/Mpk1 MAPK (mitogen-activated protein kinase), iii) calcium-dependent calcineurin, and iv) RAS signaling pathways. In this review, we focus on the major stress-activated signaling (SAS) pathways controlling stress response and virulence of *C. neoformans*.

The HOG Pathway

From fungi to mammals, the stress-activated MAPK pathway is evolutionarily conserved and plays a crucial role in responding to a plethora of environmental cues (Bahn, 2008). Depending on the species, however, the MAPK pathway has some unique characteristics. For example, a two-component-like phosphorelay system is found to be a major signaling cascade upstream of the stress-activated MAPK pathway in fungi, but not in mammals (Bahn, 2008). *S. cerevisiae* contains a stress-activated MAPK, named Hog1, a protein involved in sensing, responding, and adapting to diverse environmental cues including pheromone sensing and initiation of sporulation during mating, filamentous growth under nitrogen starvation, and the response to osmotic and cell wall stresses (Chen and Thorner, 2007).

The HOG pathway is one of the key SAS pathways in *C. neoformans* and consists of multiple signaling components (Fig. 1). The signaling cascade upstream of the Hog1 MAPK module is the multi-component phosphorelay system, comprised of a hybrid sensor histidine kinase (HK), including Tco1 and Tco2 (Bahn *et al.*, 2006), a histidine-containing phosphotransfer protein (HPt) Ypd1, and response regulators (RR), Ssk1 and Skn7. The Hog1 MAPK module consists of Ssk2 MAPK kinase kinase (MAPKKK), Pbs2 MAPK kinase (MAPKK), and Hog1 MAPK. Downstream of the Hog1 MAPK, multiple stress defense and protein kinase genes have been identified recently (Ko *et al.*, 2009).

The general regulatory mechanism of the phosphorelay system in fungi is as follows. After exposure to certain external cues, a hybrid sensor HK in the phosphorelay system autophosphorylates a His residue in its HK domain and transfers the phosphate group to an Asp residue in the RR domain in the same hybrid sensor HK. Subsequently, the phosphate in the RR domain is delivered to a His residue in the HPt and then transferred to an Asp residue in a response regulator (Bahn, 2008). Next, the activated response regulator interacts with the autoinhibitory domain of the Ssk2-like MAPKKK and induces the autophosphorylation of a Thr residue in the Ser/Thr kinase domain. In turn, the activated MAPKKK phosphorylates the Ser and Thr residues of the Pbs2-like MAPKK, which then dually phosphorylates the Thr and Tyr residues of the Hog1-like MAPK (Bahn, 2008). In *C. neoformans*, Tco1 and Tco2 HKs act redundantly and independently

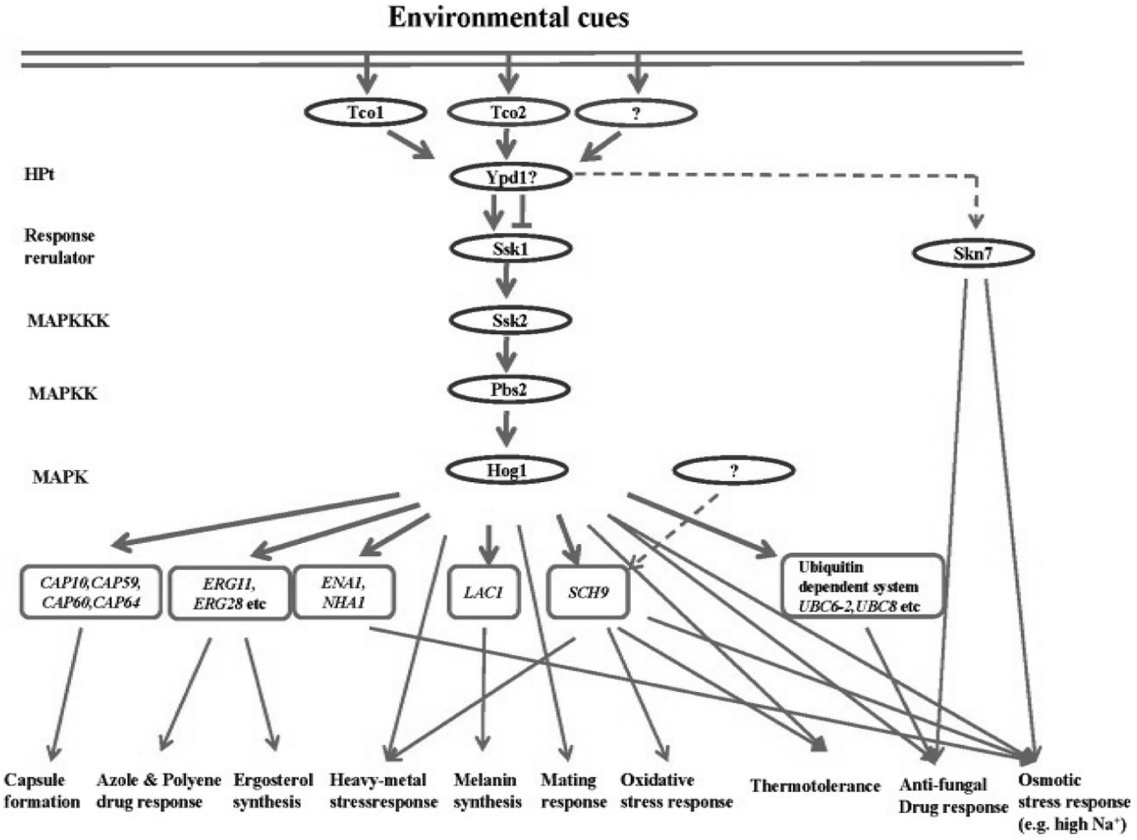

Fig. 1. The *C. neoformans* HOG pathway. The HOG pathway consists of multiple signaling components and is involved in a variety of stress responses, including osmotic, oxidative, and heavy metal stresses, breaches of cell wall integrity, and antifungal drug responses. Following exposure to an environmental cue, signals are transmitted to downstream genes of the HOG pathway via a two-component-like phosphorelay system and a HOG MAPK module composed of Ssk2 MAPK kinase kinase, Pbs2 MAPK kinase, and Hog1 MAPK.

for either positive or negative regulation of the Hog1 MAPK. However, the Ssk1 response regulator appears to positively regulate the Hog1 MAPK module since phenotypes of the *ssk1Δ* mutant are mostly identical to those of the *hog1Δ* mutant (Bahn *et al*., 2006). Another *C. neoformans* response regulator, Skn7, is also involved in stress responses to high salt conditions, oxidative damages (e.g. *tert*-butyl hydroperoxide), and the antifungal drug fludioxonil. Skn7, however, appears to act independently from the Hog1 MAPK module (Fig. 1) (Bahn *et al*., 2006).

The Hog1 MAPK is found in both *C. neoformans* serotype A and serotype D. The role of Hog1 in *C. neoformans*, and its regulatory mechanism, however, varies between different clinical and environmental *C. neoformans* strains (Hoang *et al*., 2004). Most notably, the Hog1 MAPKs in a majority of *C. neoformans* are uniquely regulated in response to environmental stresses. In most other fungi and in mammals, the Hog1 MAPK is unphosphorylated and localizes to the cytosol under unstressed condition, but is rapidly phosphorylated for translocation into the nucleus and activation of target genes. By contrast, in a number of *C. neoformans* strains, including the serotype A H99 strain, Hog1 is constitutively phosphorylated, found in both the cytosol and nucleus even under unstressed conditions, and rapidly dephosphorylated for activation by stress (Bahn, 2008). This type of Hog1 regulation is not observed in all *C. neoformans* strains. In a minority of *C. neoformans* strains, such as the serotype D JEC21 stain, Hog1 is regulated in a conventional manner similar to other fungi (Bahn, 2008). This difference appears to account for the different roles of the HOG pathway between *C. neoformans* strains. For instance, the *hog1Δ* mutant generated in the H99 strain background, but not the *hog1Δ* mutant generated in the JEC21 background, shows an increased sensitivity to high temperature (39~40°C) and hydrogen peroxide. In addition, the mating capability is enhanced in the *hog1Δ* mutant of the H99 strain, but not of the JEC21 strain. In the H99 strain background, mutation of genes in the HOG pathway induces pheromone production and cell-cell fusion in the early stage of mating (Bahn *et al*., 2005). The major benefit of having constitutively phosphorylated Hog1 appears to be increased stress-resistance. Notably, the *C. neoformans* strains with constitutively phosphorylated Hog1 are more

stress-resistant than those having unphosphorylated Hog1 under unstressed condition.

The HOG pathway in *C. neoformans* not only responds to osmotic stress, but also controls diverse environmental stress responses against UV irradiation, oxidative stress, heavy metal stress, antifungal drugs, toxic metabolites, and high temperature (Bahn *et al.*, 2005, 2006, 2007; Kojima *et al.*, 2006; Bahn, 2008). Moreover, the HOG pathway is involved in controlling production of two major *C. neoformans* virulence factors, capsule and melanin, which are also regulated by the cAMP signaling pathway (Alspaugh *et al.*, 1997, 2002; D'Souza *et al.*, 2001; Bahn *et al.*, 2004). Supporting this finding, DNA microarray analyses have shown that genes required for production of capsule, such as *CAP10*, *CAP59*, *CAP60* and *CAP64*, and melanin, such as *LAC1*, were found to be induced following inactivation of the HOG pathway (Ko *et al.*, 2009).

Recently, a genome-wide transcriptome analysis of the HOG pathway has led to the discovery of a number of HOG downstream target genes. In response to osmotic stress, the *ENA1* gene, encoding a putative P-type ATPase sodium pump, was found to be mainly regulated by Ssk1 and Hog1 (Ko *et al.*, 2009). In *S. cerevisiae*, Ena1 is required for long-term adaptation to osmotic shock and is also activated by Hog1 (Proft and Struhl, 2004). The *C. neoformans ena1Δ* mutant is hypersensitive to osmotic shock under glucose starvation and low pH conditions (Ko *et al.*, 2009; Idnurm *et al.*, 2009). Interestingly, Idnurm and co-workers reported that Ena1 is required for full virulence of *C. neoformans* (Idnurm *et al.*, 2009). Following exposure to oxidative shock, a number of genes are regulated in a Hog1-dependent manner (Ko *et al.*, 2009). These include *SCH9*, encoding a protein kinase, and *UBC6-2* and *UBC8*, two genes that appear to be involved in the ubiquitin proteasome system. In *S. cerevisiae*, Sch9 kinase regulates transcription factors, such as Sko1, that respond to osmotic shock (Pascual-Ahuir and Proft, 2007). The *C. neoformans sch9Δ* mutant shows hypersensitivity to osmotic shock and fludioxonil, phenotypes that are distinguished from those of the *hog1Δ* mutant, indicating that Sch9 appears to be governed by both Hog and other pathways (Ko *et al.*, 2009). The protein degradation pathway, including the ubiquitin proteasome system, is also commonly activated by oxidative stress in other eukaryotic organisms (Vandenbroucke *et al.*, 2008).

The most notable observation of the HOG pathway transcriptome analysis is that it regulates genes involved in ergosterol biosynthesis. Inhibition of the HOG pathway, such as mutation of the *SSK1* and *HOG1* genes, increases the expression levels of a number of genes, such as *ERG11*, a gene involved in ergosterol biosynthesis. Supporting this finding, it was observed that cellular ergosterol contents increase by inhibiting the HOG pathway. Confirming this, the *hog1Δ* mutant was found to be resistant to azole drugs, such as fluconazole, but is hypersusceptible to amphotericin B (Ko *et al.*, 2009). This provides an important combination antifungal therapeutic method, one which will increase efficacy but decrease toxicity of amphotericin B, a drug that is currently the most widely used for treatment of cryptococcosis.

The PKC/Mpk1 Pathway

In *C. neoformans*, the PKC (Protein kinase C)/Mpk1 signaling pathway is involved in responding to a variety of environmental stresses, including osmotic stress, oxidative stress, nitrosative stress, and breaches of cell wall integrity. Moreover, deletion of the *PKC1* gene induces an increase in capsule size and reduces the ability to synthesize melanin when compared to the wild-type. This pathway comprises several signaling components, including the small GTP-binding protein Rho1, PKC, Bck1 MAPKKK, Mkk1/Mkk2 MAPKK, and Mpk1 MAPK, a gene which is homologous to Slt2 in *S. cerevisiae* (Levin, 2005). It has been reported that DAG (diacylglycerol) functions as a second messenger activating mammalian protein kinase C. The production of DAG is regulated by Ipc1 (inositolphosphorylceramide synthase-1) (Heung *et al.*, 2004). Specifically, DAG influences melanin synthesis in *C. neoformans* by regulating Pkc1 (Fig. 2).

The PKC/Mpk1 pathway has been well characterized in *S. cerevisiae*. The PKC/Mpk1 MAPK pathway in *S. cerevisiae* regulates the organization of the actin cytoskeleton and cell wall integrity (Delley and Hall, 1999). Pkc1 plays a more crucial role in sensing and responding to environmental cues (Levin *et al.*, 1990). In response to an oxidative stress, such as hydrogen peroxide and diamide, Pkc1 is activated by phosphorylation. This activation results in the formation of disulfide bonds in cytoskeletal proteins and induces disruption of the cell wall.

In *C. neoformans*, the PKC/Mpk1 pathway is also involved in responding to a diversity of stresses, including oxidative stress, and breaches of cell wall integrity (Fig. 2) (Gerik *et al.*, 2008). Following exposure to reactive oxygen or nitrogen species (ROS and RNS, respectively), the Mpk1 MAPK is activated by phosphorylation downstream of the PKC pathway in *C. neoformans*. Activation of the Mpk1 MAPK is not observed in the *pkc1Δ* mutant following exposure to nitrosative stress (e.g. $NaNO_2$), oxidative stress (e.g. hydrogen peroxide), or a cell wall damaging agent (e.g. calcoflour white), clearly indicating that phosphorylation of Mpk1 is governed by Pkc1 (Gerik *et al.*, 2008). Recently, it has been shown that the *bck1Δ*, *mkk1Δ* and *mpk1Δ* mutants exhibited hypersensitivity to fludioxonil, methylglyoxal, hydrogen peroxide, and high temperature, but not osmotic stress (Bahn *et al.*, 2007). Furthermore, the *pkc1Δ* mutant is hypersensitive to cell wall destabilizers such as SDS, calcoflour and

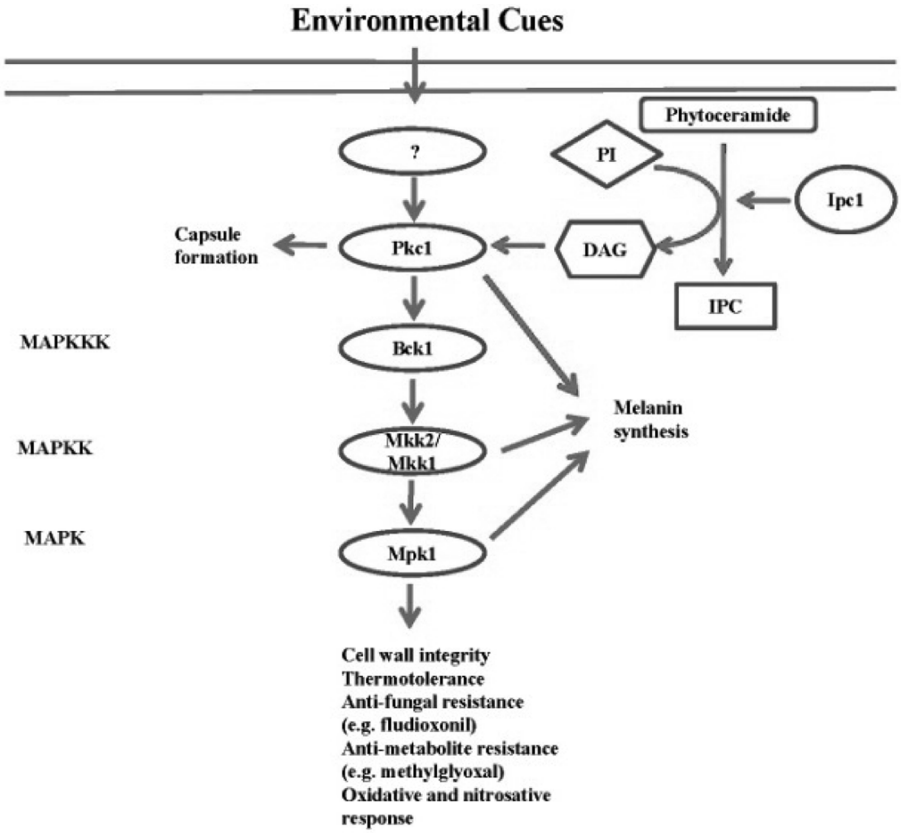

Fig. 2. The *C. neoformans* PKC/Mpk1 pathway. The PKC (Protein kinase C)/Mpk1 pathway governs cell wall integrity and stress responses against nitrosative and oxidative damaging agents and anti-fungal drugs. This pathway is composed of several components including the small GTP-binding protein Rho1, PKC, Ser/Thr MAPKKK Bck1, MAPKK Mkk1/Mkk2, MAPK Mpk1, and Ipc1 (inositol-phosphorylceramide synthase-1), an enzyme which produces DAG (diacylglycerol) for activating PKC.

congo red (Berridge *et al.*, 2003), indicating that Pkc1 plays an essential role in maintaining cell wall integrity. These characteristics may be related to the hyper-susceptibility of the *pkc1Δ* mutant to high temperature (Gerik *et al.*, 2008).

The PKC pathway influences capsule formation and melanin synthesis. Deletion of the *PKC1* gene induces an increase in capsule size, a phenotype not observed in the *bck1Δ*, *mkk2Δ* and *mpk1Δ* mutants (Gerik *et al.*, 2008). In addition, the *pkc1Δ* and *mkk2Δ* mutants show a reduction in melanin synthesis (Gerik *et al.*, 2005, 2008). For regulation of melanin formation, the PKC pathway is activated by the sphingolipid pathway. The role of the PKC pathway in capsule and melanin biosynthesis could be related to the ability to maintain cell wall integrity since the capsule is deposited into the cell wall and a laccase, an essential enzyme for the production of melanin, is located in the cell wall (Zhu *et al.*, 2001; Reese and Doering, 2003; Heung *et al.*, 2004).

The Ca^{2+}/Calcineurin Pathway

Ca^{2+} is an important secondary messenger governing a variety of stress responses in eukaryotic organisms. In *C. neoformans*, the Ca^{2+}-mediated signaling pathway also plays a critical role in adaptation to certain environmental stresses, such as high temperature and cell wall stress (Fig. 3) (Fox and Heitman, 2002; Kraus and Heitman, 2003). In response to certain external cues, the concentration of cytosolic Ca^{2+} is changed, an event which results in the transmission of signals from the membrane to the nucleus. For example, a rise in concentration of cytosolic Ca^{2+} leads to the activation of PKC, an enzyme which functions in a variety of cellular responses including cellular growth and activation of transcription factors (Berridge *et al.*, 2003).

The Ca^{2+} signaling pathway is well conserved from fungi to mammals (Kraus *et al.*, 2005). In this pathway, calmodulin and calcineurin play essential roles in the stress response of fungi (Kraus *et al.*, 2005). Calmodulin is a small cytosolic protein that binds four Ca^{2+} ions via the EF hand motifs forming a Ca^{2+}/calmodulin complex that acts as a Ca^{2+} sensor (Kraus and Heitman, 2003). The bound Ca^{2+} induces a conformational change of the calmodulin, resulting in the release of free energy (Ikura, 1996). In *S. cerevisiae*, the Ca^{2+} signaling pathway is also

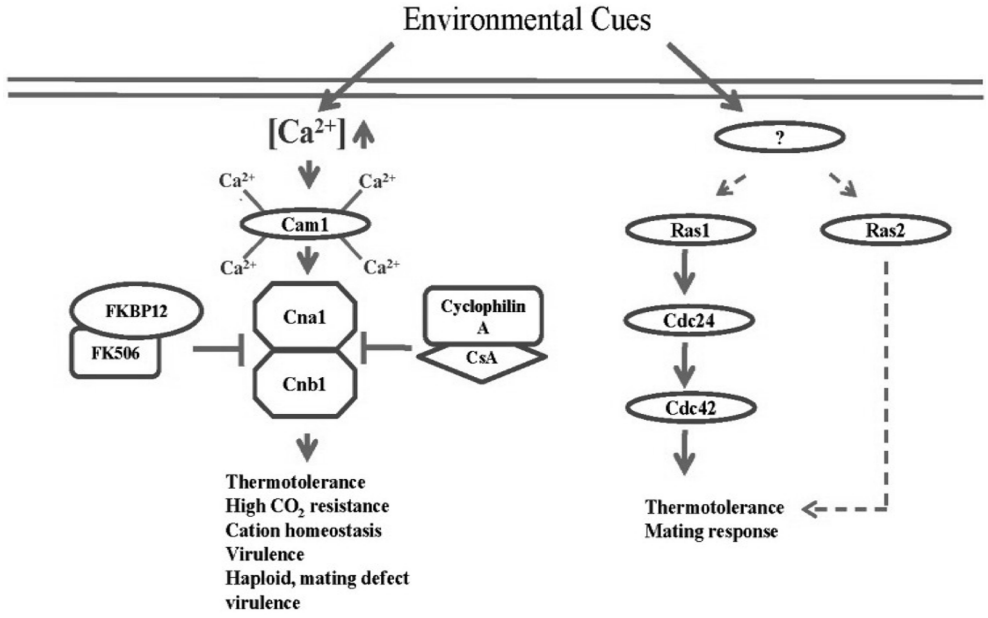

Fig. 3. The Ca^{2+}/calcineurin pathway and Ras-signaling pathway in *C. neoformans*. These two pathways are also involved in a variety of stress responses, including thermo- tolerance and pheromone-responsive mating. Calmodulin (Cam1) binds four Ca^{2+} molecules via four EF hand motifs. Subsequently, Ca^{2+}-bound Cam1 activates cal- cineurin, a complex composed of a catalytic subunit A and a regulatory subunit B. Cyclophilin A and FK506-binding protein (FKBP12) bound with CsA and FK506, respectively, inhibit the activation of calcineurin. The Ras-signaling pathway governs thermotolerance in a Cdc24 (a guanine nucleotide exchange factor) and Cdc42 (GTPase)-dependent manner. Ras1 and Ras2 share some functions in both thermotolerance and mating.

involved in cellular mechanisms, such as mitosis (by regulation of Nuflp/Spc110p, stress responses), and cytoskeleton rearrangement (Chin and Means, 2000; Cyert, 2001).

Calcineurin, a Ser/Thr specific phosphatase, is the target of Ca^{2+} bound calmodulin and its function is well conserved among eukaryotic cells (Aramburu *et al.*, 2000). The calmodulin-mediated activation of calcineurin is inhibited by chemical compounds such as cyclosporine A (CsA) and tacrolimus (FK506), two well known immunosuppressive drugs (Hemenway and Heitman, 1999). In the cell, CsA and FK506 form complexes with cyclophilin A and FK506-binding protein (FKBP12), respectively, and these complexes inhibit the action of calcineurin (Liu *et al.*, 1991). Calcineurin is a heterodimer composed of catalytic subunit A (Cna1), containing autoinhibitory and calmodulin binding domains, and Ca^{2+} binding regulatory B subunit (Cnb1) (Watanabe *et al.*, 1996).

The Ca^{2+}/calcineurin pathway is also involved in a variety of stress responses in *C. neoformans* (Fig. 3). The CsA does not affect the viability of *C. neoformans* at low temperature, but does at 37°C. This indicates that calcineurin is not essential at low temperature, but indispensable during infection of the host (Odom *et al.*, 1997). Furthermore, the *cna1Δ* mutant is hypersensitive to high CO_2 concentration and pH 7.3, conditions which are essential for the cells to infect the host. As a result, the *cna1Δ* mutant is avirulent (Odom *et al.*, 1997). Also, calcineurin A in *C. neoformans* is related to cation homeostasis. The mutant lacking calcineurin is sensitive to Li^{+} and provides resistance for Mn^{2+}, which is the opposite phenotype observed in *S. cerevisiae*. This indicates that the function of calcineurin diverged between the species.

Calcineurin B (subunit B) in *C. neoformans* is also indispensable for growth at 37°C. The *cnb1Δ* has defects in haploid fruiting and mating response, phenotypes not seen in *cna1Δ* mutants (Fox *et al.*, 2001). The *cnb1Δ* mutant is also avirulent like the *cna1Δ*.

The RAS Pathway

The Ras-signaling pathway is another important SAS pathway that governs thermotolerance of *C. neoformans*. Ras is a monomeric GTPase activated by replacement of bound GDP with GTP, an event mediated by a guanine nucleotide-exchange factor (GEF), or inactivated by a GTPase-activating protein (GAP) (Bourne *et al.*, 1990; Bourne *et al.*, 1991). The Ras-signaling pathway has been implicated in growth, differentiation, and stress response of eukaryotic organisms. In mammals, Ras regulates cell proliferation via the Erk MAPK and a mutation in the *RAS* gene is regarded as one of the main causes of human cancers (Barbacid, 1987). In fungi, Ras is also involved in a variety of cellular responses, including cell cycle regulation and cAMP production (Thevelein, 1994; Jiang *et al.*,

1998). Although Ras is conserved from fungi to mammals, the role of Ras in cells varies among species. *S. cerevisiae*, for example, has two Ras proteins, Ras1 and Ras2 that control a variety of cellular responses, including the production of cAMP, regulation of MAPK signaling, diploid and haploid growth, and polarization of actin cytoskeleton (Toda *et al.*, 1985; Gimeno *et al.*, 1992; Stanhill *et al.*, 1999; Ho and Bretscher, 2001). In *Schizosaccharomyces pombe*, Ras also controls the MAPK pathway in the pheromone response during mating, but is not involved in the production of cAMP (Nielsen *et al.*, 1992; Hughes, 1995).

The Ras-signaling pathway is involved in cAMP signaling, environmental stress response, and pheromone response during mating in *C. neoformans* (Fig. 3) (D'Souza *et al.*, 2001; Waugh *et al.*, 2002, 2003). *C. neoformans* also expresses Ras1 and Ras2, and these two proteins not only have distinct roles in stress response, such as thermotolerance, but also share some functions (Waugh *et al.*, 2002). Although mutating both *RAS1* and *RAS2* in *S. cerevisiae* causes lethality, *C. neoformans ras1Δ ras2Δ* double mutants are viable but with growth defects under normal conditions (Kataoka *et al.*, 1984; Tatchell *et al.*, 1984; Waugh *et al.*, 2002). Ras1, but not Ras2, is required for growth at high temperature, mating response, and virulence (Alspaugh *et al.*, 2000; Waugh *et al.*, 2003). Overexpression of Ras2, however, partly restores the growth of the *ras1Δ* mutant at high temperature and completely suppresses its mating defect, indicating that these Ras proteins share some functions. Unlike other SAS pathways, however, the Ras-signaling pathway is not involved in production of virulence factors, such as melanin and capsule (Waugh *et al.*, 2002).

Recently, the downstream signaling network of the Ras pathway was partly elucidated. The guanine nucleotide exchange factor Cdc24 was found to be the downstream effector of Ras1 (Nichols *et al.*, 2007). The *cdc24Δ* mutant shows the same phenotype as the *ras1Δ* mutant in response to high temperature and actin polarization. The *cdc24Δ ras1Δ* double mutant shows no synergistic defect of growth at 37°C compared to the *cdc24Δ* and *ras1Δ*, clearly indicating that Ras1 and Cdc24 share a common signal transduction pathway. Recently, Cdc42 was found to be the target of the Ras1-Cdc24 signaling complex in *C. neoformans* (Nichols *et al.*, 2007).

Conclusion and Future Insights

C. neoformans is armed with various stress-activated signaling (SAS) pathways that promote survival of the pathogen in the harsh host environment. The SAS pathways of *C. neoformans* include the HOG, Ca^{2+}/calcineurin, PKC/Mpk1 MAPK, and Ras-signaling pathways. Perturbation of the SAS pathways not only impairs the capability of *C. neoformans* to resist a variety of environmental stresses during host infection, but also affects production of virulence factors, such as capsule and melanin. A drug(s) targeted to signaling components of the SAS pathway will be effective for treatment of cryptococcosis. This therapeutic strategy, however, has the following problems. First, most of the central signaling components of the SAS pathways are evolutionarily conserved and therefore their inhibitors could have side-toxicity. Exceptions include components of the two-component system, which are fungal-specific. Further identification and characterization of the downstream targets of the SAS pathways by DNA microarray or proteomics approaches will likely provide more ideal targets for development of antifungal therapies. Secondly, drugs targeting the SAS pathway will not be fungicidal. This problem could only be overcome by using them in combination with other drugs. For example, fluconazole and fludioxonil are known to have synergistic antifungal activity with the calcineurin inhibitor FK506 (Del Poeta *et al.*, 2000; Kojima *et al.*, 2006). Furthermore, it has been shown that inhibition of the HOG pathway renders cells hypersensitive to amphotericin B (Ko *et al.*, 2009). In conclusion, complete understanding of SAS pathways will provide unprecedented opportunities to develop novel antifungal therapy against *C. neoformans*.

Acknowledgements

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