MINIREVIEW

Two-component signal transduction in human fungal pathogens

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Abstract

Signal transduction pathways provide mechanisms for adaptation to stress conditions. One of the most studied of these pathways is the HOG1 MAP kinase pathway that in Saccharomyces cerevisiae is used to adapt cells to osmostress. The HOG1 MAPK has also been studied in Candida albicans, and more recently observations on the Hog1p functions have been described in two other human pathogens, Aspergillus fumigatus and Cryptococcus neoformans. The important, but not surprising, concept is that this pathway is used for different yet similar functions in each of these fungi, given their need to adapt to different environmental signals. Current studies of C. albicans focus upon the identification of two-component signal proteins that, in both C. albicans and S. cerevisiae, regulate the HOG1 MAPK. In C. albicans, these proteins regulate cell wall biosynthesis (and, therefore, adherence to host cells), osmotic and oxidant adaptation, white-opaque switching, morphogenesis, and virulence of the organism.

Introduction

Among the challenges facing clinicians treating immunocompromised and immunodeficient patients are secondary infections from bacteria and fungi. Of the fungal infections, those caused by Candida species are among the most common. Nosocomial candidiasis is now the third most common fungal disease in countries where this information is provided. Candidiasis is associated primarily with individuals suffering from human immunodeficiency virus (HIV) infection, the premature newborn and post-surgery patients, as well as transplant and cancer patients or those with genetic immunodeficiencies (Wenzel, 1995; Blumberg et al., 2001; Husain et al., 2003, 2005; Singh et al., 2003, 2005). The type of candidiasis, superficial, mucosal or invasive, depends upon the underlying host defect, such that HIV+ patients develop oral or esophageal disease, while deficiencies in numbers or functions of neutrophils contribute to invasive disease. Much has been done to develop new antifungal drugs, but many of these compounds are either toxic to the host (amphotericin B) as fungi are eukaryotic and share many metabolic features of the human host, or the drugs are fungistatic, resulting in the selection of Candida microbiota that are either intrinsically resistant to the drugs (triazoles) or develop resistance (Perfect, 2004). One of the goals of developing an effective antifungal treatment is to target those metabolic processes and other virulence attributes that appear to be unique to fungi. One area of target discovery that has been understudied is signal transduction in a variety of important clinical species of fungi. While there are a number of mitogen-activated protein kinase pathways (MAPK) found in fungi that are common to mammalian cells, the proteins of these pathways may be sufficiently different in sequence among fungi and mammalian cells to create specificity for target exploitation and development of drugs that target these proteins. Other signal proteins use different amino-acid residues for phosphotransfer, including aspartic acid and histidine. The latter type of signaling input regulates at least one fungal MAPK (HOG1), and includes a series of proteins that are fairly unique to bacteria, fungi, and higher plants, the two-component signal transduction proteins (Posas et al., 1996; Schaller, 1997; Chang et al., 1998; Ketela et al., 1998; Charizanis et al., 1999; Inoue et al., 2001; Grefen & Harter, 2004). The purpose of this review is to provide current knowledge of two-component signal transduction in C. albicans, Aspergillus fumigatus, and Cryptococcus neoformans, the only human fungal pathogens where these
proteins have been described. The relevance of this information is to gauge the importance of these pathways in fungal virulence, as well as the importance of using these proteins as targets for antifungal drug development.

**Two-component signal transduction in human pathogenic fungi**

Two-component signal transduction pathways in bacteria regulate a number of distinct processes, including virulence and drug resistance (Roychoudhury et al., 1993; Sola-Landa et al., 1998; Hayashi et al., 2000; Moskowitz et al., 2004). In the last several years since the discovery of a hybrid two-component system in *Saccharomyces cerevisiae*, it has been shown that a number of important fungal pathogens also have these pathways. The majority of the two-component pathways have remained unexplored in pathogenic fungi in comparison to the HOG1 MAPK pathway of *S. cerevisiae* (Hohmann, 2002). The availability of genome sequences of pathogens in addition to *Candida albicans* has established that *A. fumigatus*, for example, has as many as 13–15 histidine kinases (http://www.tigr.org/tdb/e2k1/afu1/), following the general theme that filamentous *Ascomycetes* have considerably more of these proteins than yeasts such as *C. albicans*, which has only three histidine kinases (Alex et al., 1998; Calera et al., 1998; Nagahashi et al., 1998; Srikantha et al., 1998; Selitrennikoff et al., 2001). On the other hand, little information is available on the functions of the *A. fumigatus* proteins, other than Fos1p, which is described below.

Before discussing these proteins in human pathogens, we first briefly mention those of bacteria, and, as a model for the studies of human pathogens, the HOG1 MAPK pathway of *S. cerevisiae* (Table 1). Advances in molecular biology of *C. albicans* have enabled improvements in genetic manipulation of this species. Other than the two-component proteins of *C. albicans*, the only other fungal pathogens thus far studied are *A. fumigatus*, which has a histidine kinase (Fos1p) that shares homology to the hybrid-histidine kinases by their structural arrangement. The database has also revealed a number of two-component signaling proteins as well as MAPK pathways including HOG1 (Fernandes et al., 2005).

**Three types of hybrid histidine kinases**

Given the lack of functional data for many of these histidine kinases, and for the purposes of this review, we have arranged the hybrid-histidine kinases by their structural organization (Fig. 3). From our observations, it appears that there are three types of histidine kinases (HKs) among the human pathogens, although in a recent whole-genome study primarily of the filamentous *Ascomycetes*, as many as 11 classes of histidine kinases have been developed, all of which participate to regulate signal transfer. In non-stressed cells, signaling is initiated by an autophosphorylation of a membrane-bound sensor kinase, Shn1p, which is a hybrid-histidine kinase (Ota & Varshavsky, 1993; Posas et al., 1996) (Fig. 1b). A hybrid histidine kinase contains both a conserved histidine residue (which is autophosphorylated under non-stressed conditions) and a conserved aspartate residue (which receives the phosphate from the histidine residue). The signal then is transferred from the aspartate on the sensor kinase to an intermediate histidine kinase, Ypd1p, which contains a single conserved histidine residue; phosphorylated Ypd1p then interacts with the response regulator protein, Skn1p that becomes phosphorylated on an aspartate residue within its receiver domain. Phosphotransfer can also proceed via another response regulator, Skn7p from Ypd1p, but apparently for different purposes including oxidant adaptation and cell wall biosynthesis (Li et al., 1998, 2002b). Both Skn1p and Skn7p contain a single conserved aspartate receiver domain. The difference in stimulus determines which response regulator is activated/inactivated. Because phosphotransfer occurs in unstressed cells, the effect of phosphorylated Skn1p is turning off the downstream HOG1 MAPK pathway, as it is unable to bind to the Skk2/Ssk2 MAPKKK. On the other hand, when cells are stressed by high osmolarity, phosphotransfer does not occur as described above. Skn1p (unphosphorylated) binds to Ssk2/Ssk22, causing phosphorylation of the latter protein and subsequent phosphotransfer to Pbs2p (MAPKK) and, subsequently, Hog1p (MAPK) (Fig. 2, and see below). Upon phosphorylation of Hog1p, is translocated to the nucleus and transcriptional events occur that result in adaptation to osmotic stress. Readers are referred to the study of Hohmann for details of osmoadaptation in *S. cerevisiae* (Hohmann, 2002).

Most of what is known about two-component signal transduction in pathogenic fungi has centered on two-component signaling in *C. albicans* (Table 1). Advances in molecular biology of *C. albicans* have facilitated improvements in genetic manipulation of this species. Other than the two-component proteins of *C. albicans*, the only other fungal pathogens thus far studied are *A. fumigatus*, which has a histidine kinase (Fos1p) that shares homology to the *C. albicans* Nik1p histidine kinase, and *Cryptococcus neoformans* response regulator (Skn7p) (both are discussed below). Additionally, an in silico search of the *Paracoccidioides brasiliensis* database has also revealed a number of two-component signal transduction proteins as well as MAPK pathways including HOG1 (Fernandes et al., 2005).
which are based upon gene annotation (Catlett et al., 2003). For our purposes, the first (Type I) is that of the canonical membrane-bound histidine kinase, which contains two transmembrane regions, as well as the respective histidine kinase and response regulator signaling domains (Fig. 3). The best example of this type of protein is Sln1p (Ota & Varshavsky, 1993). Other than in \textit{Saccharomyces cerevisiae}, only the \textit{C. albicans SLN1} gene has been partially characterized (Nagahashi et al., 1998; Yamada-Okabe et al., 1999). Sln1p is the sensor hybrid histidine kinase for the HOG1 MAPK signal transduction pathway (Posas et al., 1996). In \textit{S. cerevisiae} deletion of the gene results in a lethal phenotype, and this lethality is due to activation of Ssk1p, Sln1p’s cognate response regulator (Ota & Varshavsky, 1993; Tao et al., 1999). The constitutive activation of Ssk1p in the \textit{shl} deletion mutant causes the HOG pathway to be activated without osmostress, which results in pathway overproduction of glycerol (Ostrander & Gorman, 1999; Tao et al.,

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**Fig. 1.** Two-component signal transduction. (a) A bacterial two-component signal transfer is illustrated. A sensor histidine kinase is autophosphorylated on a histidine residue and the signal is subsequently transferred to a response regulator on an aspartate residue. The phosphorylated response regulator acts as a transcriptional regulator adapting cells to stress or other functions related to virulence. (b) In \textit{Saccharomyces cerevisiae}, two-component signaling includes three proteins, a sensor hybrid histidine kinase, a phosphohistidine intermediate and a response regulator. The response regulator can activate a downstream MAPK pathway or in the case of a different response regulator protein, directly activates gene transcription. Thus, in this system a series of four phosphorylations involving histidine-aspartate-histidine-aspartate occur prior to downstream events. (c) As in (b). The proteins of the HOG MAP kinase pathway are shown. In unstressed conditions, phosphorylation of Ssk1p inhibits activation of the HOG MAPK pathway, but in osmostressed cells, unphosphorylated Ssk1p activates the Ssk2/22 MAPKK and subsequent phosphorylation of Pbs2p and Hog1p. Phosphorylated Hog1p then translocates to the nucleus for transcription of genes associated with osmoadaptation.

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**Fig. 2.** The HOG MAPK signal pathway. Individual proteins are shown, Ssk2p, Pbs2p, and Hog1p, and functions assigned to each protein of \textit{Candida albicans}. The role of Ssk1p in activation is shown. Stress factors do not activate Ssk1p; instead, Ssk1p is not phosphorylated during stress, enabling it to bind to Ssk2p.
Table 1. Two-component signal transduction proteins in Candida albicans

| Required for virulence | Sln1 | Nik1 | Chk1 | Ypd1 | Ssk1 | Skn7 |
|-----------------------|------|------|------|------|------|------|
| Yes                   | Yes  | Yes  | Yes  | Unknown | Yes | No |
| Yes                   | Yes  | No   | Yes  | Unknown | Yes | No |
| No                    | No   | No   | Yes  | Unknown | Yes | Yes |

Fig. 3. The three types of histidine kinases identified in Candida albicans showing domains of each protein. Each is a hybrid histidine kinase containing both an H-box for histidine phosphorylation, and a receiver domain (RR) with its aspartate residue that participates in phosphotransfer. Only the Sln1p has sequences indicating a transmembrane location. The Nik1p has N-terminal repeat sequences (Rep) of unknown function and a second H-box while the Chk1p contains a partial Ser/Thr MAP kinase domain and a GAF signaling domain.

1999). Functions of the Ssk1p of C. albicans are described below. The C. albicans SLN1 gene was identified by its ability to complement a conditional shn1 mutant in S. cerevisiae (Nagahashi et al., 1998). Though the CaSLN1 gene can functionally replace ScSLN1, a strain lacking CaSLN1 is viable. This at least suggests that the CaSln1 protein is functionally similar to its S. cerevisiae counterpart, but its importance as a single protein is not as critical for viability in Candida as it is in Saccharomyces. However, a strain of C. albicans which lacks Sln1p has reduced virulence in a mouse model (Yamada-Okabe et al., 1999). Additionally, Cash1 cells grown under high osmotic conditions are viable, yet have a lower growth rate (Nagahashi et al., 1998). This may indicate that a secondary pathway is also involved in responding to osmotic stress. Additionally, other effects have been observed in the C. albicans shn1 mutant that relate to a cell wall assembly and maintenance function of Shn1p. For example, shn1 mutant cells have altered transcript levels of certain N- and O-mannosyltransferases (Kruppa et al., 2004a).

The second type of HK was first identified in Neurospora crassa. The canonical protein from this organism is OS-1 (Schumacher et al., 1997). One of the three hybrid histidine kinases of C. albicans falls into this type and was identified in two independent studies (Alex et al., 1998; Selitrennikoff et al., 2001) (Fig. 3). This protein is referred to in the literature as Cos1 or Nik1 (Alex et al., 1998; Selitrennikoff et al., 2001). This protein is believed to be cytoplasmic, as it lacks any distinguishing transmembrane domains, but contains a series of 90 amino-acid repeat sequences in addition to both of the canonical histidine kinase and response regulator domains as well as an apparent second histidine kinase domain. The role of CaCos1/Nik1p in the cell is still unclear; in N. crassa the Os-1 protein is needed for responding to osmoadaptation and S. cerevisiae NIK1 transcript levels are low in hyphae than in budding yeast cells. Deletion of NIK1 in the WO-1 strain background results in white and opaque cells that have a reduced frequency of phenotypic switching, indicating a probable if not minor role in regulating white/opaque phenotypic switching (Srikantha et al., 1998).

Another member of this type of HK is the Fos1 protein from A. fumigatus (Pott et al., 2000). The Fos1 protein shares 28% identity with N. crassa Os-1 protein and is 27%
identical to *C. albicans* Nik1p/Cos1p. Fos1p contains an H-box, ATP-binding domain and D box present in Nik1p of *C. albicans*, but lacks the 90 amino-acid repeat sequences found in CaNik1p. Similar to CaNik1p, Fos1p lacks any predicted transmembrane domains and is likely cytoplasmic. Deletion of *FOS1* in *A. fumigatus* results in a viable phenotype, however aside from normal morphology, the cells have a decreased ability to generate conidia, but germination appears to be unaffected. In addition, a *fos1* null strain has increased resistance to novozym 234 as well as to the dicarbamides Vinclozolin, Iprodione, and Procymidone (Pott et al., 2000). The *fos1* mutant is not affected by osmolarity, nor is there any increase in oxidant sensitivity. Given this, Pott et al. suggested that Fos1p may play a role in cell wall assembly (Pott et al., 2000). Furthermore, in a mouse model, *fos1* cells are markedly reduced in their virulence, but are not avirulent, suggesting an important role in the pathogenicity of *A. fumigatus* (Clemons et al., 2002).

The third type of the hybrid HK belongs to a group of proteins containing the canonical protein Chk1p (also known as HK1p, or CaHK1p) (Calera et al., 1998; Calera & Calderone, 1999a) (Fig. 3). In pathogenic fungi, Chk1p thus far has only been identified in *C. albicans*. Cells that lack Chk1p are avirulent in a disseminated model of candidiasis, but this can be partially attenuated if a chk1 mutation is accompanied by deletion of either *sln1* or *nik1* (Calera et al., 1999; Yamada-Okabe et al., 1999). The *chk1* null strain is also inhibited for growth and killed at a higher percentage when cultured in the presence of human polymorphonuclear leukocytes (PMNs) (Torosantucci et al., 2002).

There are two orthologous proteins in *Schizosaccharomyces pombe* (Mak2 and Mak3), that function as sensors for oxidative stress in place of Sln1p (Buck et al., 2001). Functionally, the Chk1 and Mak proteins are different, as the Mak2 and 3 proteins form a heterodimer that senses changes in oxidant levels in the *Sch. pombe* environment. Though Chk1p and Mak2 and 3 share homology in regard to domain organization, the Chk1p lacks one domain (PAS), which is present on both Mak proteins. PAS domains have been suggested to be sensors of oxygen, redox potential, light and other stimuli (the reader is referred to recent reviews on PAS function by Gu et al., and Taylor et al. Taylor & Zhulin, 1999; Gu et al., 2000). While Chk1p clearly lacks PAS domains, it is still possible that Chk1p senses oxidants or has some other role in response to oxidative stress. A *chk1* null strain does have some sensitivity to hydrogen peroxide but does not appear to be directly involved in regulating oxidative stress through the HOG pathway. However, the *CHK1* transcript is elevated in response to oxidative stress, and the levels of Chk1p appear to be regulated through the Sln1p-Ssk1p-Hog1p pathway (Li et al., 2004).

The *chk1* cells have many changes in cell wall architecture; these include changes in glucan structure, resulting in shorter β-1,3 chains with an increase in β-1,6-glucon branching (Kruppa et al., 2003) (Fig. 4). Also, cell wall mannan is altered such that the acid-labile fraction of N-linked mannan is truncated significantly (Kruppa et al., 2003). This is further supported by transcript analysis of a large group of mannosyltransferases, which show that a number of them have altered transcripts, very similar to those seen in both the *shl1* and *nik1* mutants (Kruppa et al., 2004a). These changes in cell wall structure may also play a role in the reduced ability for a *chk1* null cell to adhere to a reconstituted esophageal cell line (Li et al., 2002a). Therefore it appears that each of the *C. albicans* HKs plays a role in cell wall assembly, maintenance and integrity, but thus far only the changes affecting a *chk1* null cell have been closely examined.

It is believed that Chk1p is present in the cytoplasm, because neither transmembrane sequences nor any localization signal to the membrane is found in the protein. Whether or not Chk1p is an environmental sensor is uncertain. Recently it has been shown that a *chk1* null strain does not respond to *E. E*-farnesol (a quorum sensing molecule) that normally prevents yeast from hyphal transition (Hornby et al., 2001; Kruppa et al., 2004b). Inhibition of the yeast-to-hyphae shift normally requires a very small amount of farnesol (i.e. ~25 μM), whereas the *chk1* null strain is not responsive to farnesol at concentrations up to 250 μM (Kruppa et al., 2004b). This is the first genetic evidence that a hybrid HK is involved in eukaryotic quorum sensing. While a cell harboring Chk1p responds to farnesol, there does not appear to be any recognition site present for farnesol in the amino-acid sequence. However, it is also possible that Chk1p is downstream of a receptor for farnesol. Given the paradigm for two-component signaling through a sensor, if Chk1p is the sensor then there should be...
a downstream response regulator protein that provides transcriptional activation of genes associated with growth in the presence of farnesol. It is also possible that Chk1p is acting as a response regulator (downstream of either Sln1p or Nik1p); however this is not likely, due to the fact that a strain lacking either sln1 or nik1 responds normally to farnesol (Kruppa et al., 2004b). A third model is that Chk1p acts alone as the sensor and response regulator, as it does contain both sets of domains (HK and RR) and an additional MAPK-like domain, that in turn could function as a MAPKKK feeding into a MAPK signaling pathway.

The Ypd1p phosphohistidine intermediate

Another protein that is involved in eukaryotic two-component systems and serves as a phosphotransfer intermediate in the signaling process is Ypd1p (Posas et al., 1996) (Fig. 1c). Ypd1p has been identified in Saccharomyces cerevisiae as a member of the Hog1 pathway just downstream of Sln1p (Posas et al., 1996; Ketela et al., 1998). In pathogenic fungi, the YPD1 orthologue has been cloned in C. albicans, but not characterized in any other pathogenic fungus (Calera et al., 2000a). Calera et al. have demonstrated that the C. albicans Ypd1p protein can complement a S. cerevisiae ypd1 null strain (Calera et al., 2000a). This indicates that C. albicans Ypd1p plays a similar role in the phosphotransfer mechanism as seen in two-hybrid signal transduction in S. cerevisiae. The role of Ypd1p is to act as a shuttle between the hybrid histidine kinase and to transfer its phosphate to a response regulator (Janiak-Spens et al., 2000). In S. cerevisiae and C. albicans two response regulators are downstream of Sln1p-Ypd1p (Ketela et al., 1998; Li et al., 1998; Calera et al., 2000b; Singh et al., 2004). How Ypd1p can distinguish which response regulator to interact with when Sln1p is stimulated is not fully understood, as both response regulators are known to be activated in response to different stimuli.

The response regulators of Candida albicans

There are two possible routes for signal transfer in S. cerevisiae, each of which requires a response regulator protein. In S. cerevisiae and C. albicans there are two response regulators, one of which regulates gene activity by its action as a transcription factor (Skn7p), while the second mediates gene activity by interacting with the MAPKKK Ssk2/Ssk22p of the HOG MAPK pathway (Ssk1p). Below we describe the functions of both response regulators identified thus far in C. albicans.

The Skn7p response regulator, initially identified in S. cerevisiae, acts as a transcription factor when activated by heat or oxidant stress following Sln1p-Ypd1p phosphotransfer (Ketela et al., 1998; Charizanis et al., 1999; Lee et al., 1999). Additionally, Skn7p has functional roles in the cell cycle and cell wall regulation (Bouquin et al., 1999; Hohmann, 2002). In C. albicans, the SKN7 gene was identified from the Candida genome sequence (Singh et al., 2004). CaSkn7p shares similarities with S. cerevisiae and Schizosaccharomyces pombe homologues with regard to a heat shock factor DNA-binding domain and its response regulator receiving domain (Singh et al., 2004). The Caskn7 null strain has some impairment of filamentation on 10% serum or M199 pH 7.5 agar plates. The null mutant also has increased sensitivity to hydrogen peroxide and t-butyl hydroperoxide, indicating a role in responding to oxidant stress (Singh et al., 2004).

Though the skn7 null has an increased sensitivity to oxidant stress, its ability to persist in a murine model of disseminated candidiasis is similar to a wild-type strain with little or no attenuation of virulence (Singh et al., 2004). Therefore, the role of Skn7p in virulence of the cell is not of major importance, but clearly it has a role in survival from oxidant stress. It is possible that Skn7p acts as a secondary line of defense to oxidant stress when present in the host, as another protein Cap1p acts as a major regulator in C. albicans in responding to oxidant stress (Bahn & Sundstrom, 2001).

The second response regulator, Ssk1p, mediates its function through the HOG pathway in S. cerevisiae, regulating Hog1p activity in response to osmotic and oxidative stress through a MAP kinase cascade (Maeda et al., 1994, 1995; Singh, 2000) (Fig. 1c). Ssk1p is negatively regulated by Sln1p, in such a way that when Ssk1p is phosphorylated it is inactivated. When cells are stressed osmotically, the phosphorylation signal from Sln1p is interrupted and the now dephosphorylated Ssk1p can activate the MAPKKK Ssk2p, initiating the cascade of signals necessary to activate the HOG pathway (Posas & Saito, 1998).

The C. albicans SSK1 gene was first identified by Calera et al. (Calera & Calderone, 1999b). CaSsk1p shares 61.3% and 72.8% identity to the C-terminus of Ssk1p of S. cerevisiae and Mcs4p of Sch. pombe response regulators, respectively. The ScSsk1p functions downstream of Sln1p-Ypd1p in response to osmystress and oxidant stress (Maeda et al., 1995; Singh, 2000), while the Mcs4p functions in the adaptation of cells to a number of stress conditions, including heat and oxidant challenge (Cottarel, 1997; Shieh et al., 1997; Shiozaki et al., 1997; Buck et al., 2001; Nakamichi et al., 2002). Attempts were made to complement both a mcs4Δ and a conditionally lethal sln1Δsks2Δ double mutant that over expressed the PTP2 gene under P_GAL1 control. Overexpression of Ptp2Δ allows for rapid inactivation of Hog1p, thereby preventing cell death. However, the CaSSK1 could not complement the mutants in either gene (Calera & Calderone, 1999b). It was suggested that this could be due to CaSsk1p performing a function different from that of its
orthologues in *S. cerevisiae* and *Sch. pombe*, or that the inability to complement might actually be due to the inability of either yeast species to efficiently translate four CUG codons in such a way that a functional CaSsk1p would be expressed (Calera & Calderone, 1999b).

Deletion of the CaSSK1 gene results in a viable phenotype; however, it does have an impaired ability to form filaments on solid media such as Spider, 10% serum and M199 (pH 7.5), but can form hyphae in liquid media just as wild-type cells (Calera et al., 2000b). However, under nitrogen-limiting conditions on solid medium the ssk1 strain does form filaments and hyperinvaldes the medium, indicating a possible role for SSK1 in regulating a response to nutritional stress (Calera et al., 2000b).

The ssk1 null strain also is avirulent in a murine model of disseminated candidiasis, indicating that SSK1 plays an important role in the virulence of the pathogen (Calera et al., 2000b). Further in vitro studies using human polymorphonuclear leukocytes (PMNs) showed that cells that lack the SSK1 gene have an increased level of morbidity when compared to wild-type cells (Du et al., 2005), suggesting that Ssk1p has an important function regarding the ability to survive when phagocytized by neutrophils.

Initially, experimental challenges with 1 M sorbitol or 2 mM hydrogen peroxide suggested that the strains were not affected in either osmotic or oxidant stress response (Calera et al., 2000b). However, a recent study has demonstrated that growth of ssk1 is sensitive to oxidative stress from t-butyl hydroperoxide, hydrogen peroxide, menadione, or potassium superoxide (Chauhan et al., 2003). Activation of the HOG1 pathway upon challenge by hydrogen peroxide stress or osmotic stress was analyzed in the ssk1 null strain. It was shown that when the ssk1 null mutant was grown in the presence of 1.5 M sodium chloride, the Hog1p was phosphorylated as effectively as the wild type, suggesting that another pathway was responsible for Hog1p activation. Whereas, when the ssk1 strain was grown in the presence of 10 mM hydrogen peroxide, Hog1p was not phosphorylated in contrast to a wild-type strain. This result further demonstrates that Ssk1p is essential for activation of Hog1p in response to oxidant challenge (Chauhan et al., 2003). In addition, a number of genes are upregulated in the ssk1 null mutants, including the CHK1 hybrid histidine kinase gene, suggesting that there may be some transcriptional regulation occurring between the two-component proteins (Chauhan et al., 2003; Li et al., 2004).

The response regulator (Skn7p) of *Cryptococcus neoformans*

Recent work by Wormly et al. has characterized an SKN7 gene in *Cryptococcus neoformans* (Wormley et al., 2005). CnSkn7p shares 69% similarity with the *Candida albicans* and *Saccharomyces cerevisae* Skn7 proteins, but most of the similarity is restricted to the heat shock factor domain and response regulator (signal receiving) domain. Deletion of the gene results in a strain with a flocculent phenotype when grown at 30 °C in YPD. Flocculation could only be disrupted when cells were grown in the presence of 10% mouse serum, and not in the presence of 1 M glucose or 1 M mannose (Wormley et al., 2005). The skn7 strain is not significantly susceptible to cell wall inhibitors, an indication that there is no dramatic alteration of cell wall in the mutant. The skn7 mutant is sensitive to challenge with 0.025 mM t-butyl hydroperoxide, thereby indicating a role in responding to oxidative stress. When tested for virulence in a mouse inhalation infection model it was shown that the skn7 null strain is attenuated, but if incubated over a prolonged period will eventually cause death (Wormley et al., 2005). These results indicate that kn7p is important for responding to oxidative stress, but its role in virulence is not clear.

The Hog1 MAP kinase pathway of *Candida albicans*

The HOG1 MAP kinase pathway is composed of three proteins, the MAPKKK Ssk2p, MAPKK Pbs2p, and MAPK Hog1p (Figs 1 and 2). Presumably these proteins operate in *Candida albicans* the same way as in *Saccharomyces cerevisiae*, although epistasis experiments have not been performed in the former. In *S. cerevisiae*, the Ssk2p and Ssk22p are redundant proteins, whereas an Ssk22p has not been identified in the *C. albicans* genome. Deletion mutants in the CaSSK2 have not been described, so that its function is only presumed to be similar to that of the ScSSK2. The Pbs2p MAPKK is critical to osmoadaptation in *S. cerevisiae* and for both osmo- and oxidant adaptation in *C. albicans* (Hohmann, 2002; Arana et al., 2005). Nuclear translocation of Hog1p was dependent upon the Pbs2 proteins. Interestingly, both Pbs2 and Hog1 proteins were important in regulating phosphorylation of other MAP kinases such as Mkc1 and Cek1, which regulate the cell wall integrity and invasive filamentous growth in vitro (Arana et al., 2005). Similar to the pbs2 deletion mutant, hog1 results in oxidant sensitivity along with avirulence, so these are at least two functions associated with the HOG MAPK pathway. Another underlying function that was mentioned briefly above in regard to the HOG pathway of *C. albicans* is its role in wall biosynthesis. Thus, deletion mutants hog1, ssk1, and pbs1 have cell wall defects and/or altered susceptibilities to anti-cell wall drugs (Alonso-Monge et al., 1999; Chauhan et al., 2003; Arana et al., 2005).

It is certain that at least two branches of the HOG pathway exist, which are initiated by two osmosensors, Sln1p (as described above) and Sho1p. The proof for two
branches in part is derived from mutant phenotypes in \textit{S. cerevisiae}. For example, the \textit{S. cerevisiae} ssk2/ssk22 double deletion mutant is sensitive to high osmolarity, but only in the \textit{sho1} mutant (Hohmann, 2002). Thus, the current picture of osmoregulation in \textit{S. cerevisiae} is that not only do two sensors exist, but also that there are different requirements for induction of either branch pathway. In this regard, it is speculated that the Sho1p branch primarily senses internal osmotic changes associated with cell growth and expansion, while the Sln1p branch regulates adaptation to external osmotic conditions. Further, the Sln1p branch monitors changes in lower concentrations of NaCl (100 mM), while Hog1p phosphorylation via the Sho1p sensor requires stimulation with at least 300 mM NaCl (Hohmann, 2002). Thus the Sln1 branch appears to be more sensitive to osmotic changes. In \textit{C. albicans}, the contribution of Sho1p to osmoadaptation, or even oxidant adaptation, is unknown.

While these details have not been established in \textit{C. albicans}, both \textit{pbs2} and \textit{hog1} mutants have been constructed and phenotypes assigned as described above (San Jose et al., 1996; Alonso-Monge et al., 1999, 2003; Arana et al., 2005).

**The Hog1 (SakA) protein of \textit{Aspergillus fumigatus}**

The only other human pathogenic fungus of which the Hog1 (SakA) protein has been studied is \textit{Aspergillus fumigatus} (Xue et al., 2004). The SakA protein of this fungus is 82% identical to the \textit{Saccharomyces cerevisiae} Hog1p, and 79% identical to the \textit{Candida albicans} Hog1p. A gene-deleted strain has been constructed by disruption of the entire ORF of the SAKA gene. Compared to the sakA mutant, transcription of genes associated with osmostress adaptation was higher in the wild-type strain. Further, SAKA transcription increased in cells stressed with hydrogen peroxide. Osmotressed germings but not conidia of the sakA mutant underwent growth arrest; the latter germinated, albeit slower than wild-type conidia. A correlation between the presence of SakA and growth on complete (CM) and minimal (MM) media was observed in that the mutant appeared to germinate better on MM than wild-type cells, but both strains grew equally well on complete medium. This observation was taken to mean that the SakA protein is part of a pathway that senses nitrogen starvation, as the minimal medium contains nitrate as a nitrogen source, whereas the CM contains more complex nitrogen sources, such as yeast extract, peptone and tryptone. Thus, under nutritionally-poor growth conditions (poor nitrogen sources), SakA prevents, or at least delays, germination of conidia. Similar experiments were carried out with both good and poor carbon compounds, but differences among wild type and mutant were not observed. The conclusion is that the SakA protein in \textit{A. fumigatus}, in addition to its role in adaptation, also functions in nitrogen-sensing but not in carbon-sensing.

**The Hog1 protein of \textit{Cryptococcus neoformans}**

Recently, Bahn et al isolated and disrupted the \textit{HOG1} orthologue from \textit{Cryptococcus neoformans} (Bahn et al., 2005). \textit{HOG1} was disrupted in both the most virulent (serotype A) and least virulent (serotype D) clinical isolates. The Hog1 protein shares 73% with \textit{Candida albicans} and 65% identity with \textit{Saccharomyces cerevisiae}, and can complement the function of an \textit{S. cerevisiae} \textit{hog1} mutant. When the gene is deleted from the D serotype background, minimal effects on overall biology are observed, strains are sensitive to osmostress of 1.5 M KCl, and show resistance to 3 mM hydrogen peroxide stress (Bahn et al., 2005). Whereas with the Serotype A \textit{hog1} mutant, the strain is sensitive to 3 mM hydrogen peroxide stress. Curiously the activity of \textit{CnHOG1} in the serotype A background is opposite that of \textit{ScHOG1}, with Hog1p being constitutively phosphorylated until stressed by hyperosmotic conditions, whereas the serotype D strain behaves according to the phosphoactivation seen in \textit{S. cerevisiae}. Phosphorylation by Pbs2p is necessary for Hog1p activity, as Bahn et al. also demonstrated that a \textit{pbs2} mutant behaves similarly to the \textit{hog1} null mutant and is required for Hog1p activation (Bahn et al., 2005). This difference in phosphorylated Hog1p activity between the two serotypes is due to the activity of the phosphotyrosine-specific protein phosphatase, which regulates the catalytic activity of Hog1p. Despite the differences in phenotype, both serotypes are attenuated for virulence but will eventually confer a lethal phenotype over an extended period of incubation in a mouse infection model (Bahn et al., 2005).

**Outlook**

There are several conclusions that can be drawn from these data. First, two-component proteins of pathogenic fungi provide adaptation functions against oxidant and osmostress. Second, these proteins at least are required for virulence by regulating expression of downstream virulence factors, such as Als1p of \textit{Candida albicans}. Third, these proteins offer the advantage of selectivity in their exploitation as drug targets. Fourth, two-component proteins should be found in other pathogens, and as just mentioned, this could be used in developing broad-spectrum drugs. Fifth, the presence of apparent similar pathways in two different fungi does not preclude functional differences (Fig. 5). This conclusion is somewhat obvious given that each of the organisms illustrated in Fig. 5 is confronted with
different environmental stress conditions. The challenge at this time is to develop high throughput assays for synthetic or natural product screens. Emphasis should also be directed towards understanding the regulatory functions of these proteins; structure-function studies of these proteins will be quite useful in determining circuitry and interactions with other signal pathways. On the down side, functional assignments of histidine kinases in fungi such as Aspergillus fumigatus may be difficult to do given the redundancy in the genome. Thus a phenotype for a specific histidine kinase mutant may not be obvious.

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