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Fungal responses to reactive oxygen species

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Reactive oxygen species (ROS) such as hydrogen peroxide, produced externally or during normal metabolism, can damage different cell components and usually trigger a counteracting antioxidant response. The fact that animals and humans utilize ROS and related nitrogen reactive species to prevent fungal infection has generated great interest in defining the components of the antioxidant response and studying their role as virulence determinants in fungi. Here we review the role of specific enzyme and non-enzyme mediated antioxidant mechanisms in virulence, as well as the signal transduction mechanisms that fungal cells use to perceive high ROS levels and induce gene expression. We focus on *Schizosaccharomyces pombe* antioxidant responses, which involve a prokaryotic-type multistep phosphorelay coupled to a stress-response MAP kinase pathway and an AP-1 type transcription factor, in relation to homologous mechanisms in *Aspergillus nidulans* and the human pathogen *A. fumigatus*. Compared to *S. pombe* and other unicellular fungi, filamentous fungi have additional mechanisms to handle ROS, such as the presence of a larger number of phosphorelay sensor kinases, antioxidant enzymes and secondary metabolites with antioxidant functions. In addition, filamentous fungi have enzymes like the NADPH oxidases, which regulate multicellular development through ROS production and therefore, offer a unique opportunity to study the interplay between ROS production, perception and detoxification, and the role of these processes in cell differentiation and pathogenesis.

Keywords antioxidant, oxidative stress, *Aspergillus*, SakA, MAPK, AP-1, ROS signaling, signal transduction

Introduction

Reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are byproducts of normal aerobic metabolism, produced mainly by partial reduction of oxygen during respiration. In the presence of traces of metal ions, $O_2^{\bullet -}$ can react with H_2O_2 and generate the more reactive singlet oxygen ($^1O_2$). H_2O_2 can oxidize iron sulfur centers and cysteines in certain proteins or react with transition metals and produce the hydroxyl radical ($HO^{\bullet}$), which can oxidize virtually any cell molecule, causing DNA damage, protein inactivation, protein cross-linking and fragmentation, and lipid peroxidation. Cells have a number of mechanisms to maintain low intracellular ROS levels, which collectively constitute the antioxidant response. The enzymes superoxide dismutases (dismutate $O_2^{\bullet -}$ to H_2O_2), catalases (decompose H_2O_2 to H_2O), peroxidases (decompose H_2O_2 through other substrate oxidation), glutathione peroxidases (use glutathione to decompose H_2O_2 to H_2O) and peroxiredoxins (use reducing power to decompose H_2O_2 to H_2O), are the most ubiquitous effectors of the enzyme-mediated antioxidant response [1–3].

Some ROS are so damaging that humans and other organisms evolved ways to produce them in a regulated fashion, as part of a defense mechanism against all kinds of pathogens, including fungi like *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* [4,5]. Indeed, phagocytic cells utilize a

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NADPH enzyme complex to produce ROS, which combined with nitric oxide ($NO^{\bullet}$) produce the nitrogen reactive species peroxynitrite. All these oxidants produced as an oxidative burst have a powerful microbial killing activity. Chronic granulomatous disease (CGD), a human illness caused by a defective NADPH oxidase, is characterized by recurrent bacterial and fungal infections and a mice CGD model shows high susceptibility to *Staphylococcus aureus* and *A. fumigatus* infections [4]. A less direct role for ROS in microbial killing has been proposed from results showing that mice deficient in neutrophil serine proteases, but normal in ROS production, are susceptible to fungal infection [6]. Additional evidence has led to propose that the major role of neutrophil NADPH oxidase and associated ion fluxes is to activate digestive enzymes within the phagosome [7]. However, as discussed here, there is good evidence to support both oxidative and non-oxidative mechanisms in the microbicidal activity of phagocytic cells.

Here we briefly review two important aspects of ROS elimination as they relate to fungal pathogen virulence: the role of specific enzymes and non-enzyme-mediated antioxidant mechanisms in virulence, as well as the signal transduction mechanisms that fungal cells use to perceive high ROS levels and induce gene expression. First, we focus on clear cases in which mutation of an antioxidant mechanism has affected fungal virulence. In the second part, we review *Schizosaccharomyces pombe* responses to ROS and highlight the similarities and differences with what is known in the filamentous fungi *Aspergillus nidulans* and opportunistic human pathogen *A. fumigatus*.

Compared to unicellular fungi, filamentous fungi have supplementary mechanisms to handle ROS, such as the presence of a larger number of antioxidant enzymes [8,9], and the ability to produce secondary metabolites with antioxidant function [10]. Furthermore, filamentous fungi with NADPH oxidases can produce and handle ROS in a regulated fashion to regulate multicellular development [2,11].

Virulence related to mechanisms that cope with reactive oxygen and nitrogen species

As indicated, ROS and nitrosative species have been shown to be important in the defense against pathogens [4]. Likewise, several antioxidant enzymes have been implicated in the defense of fungal pathogens against the ROS produced by macrophages and polymorphonuclear neutrophils. $O_2^{\bullet -}$ is eliminated by cytosolic, mitochondrial, and extracellular superoxide dismutases. A whole battery of enzymes decomposes H_2O_2: catalases, catalase-peroxidases, peroxidases, glutathione peroxidases and the glutathione system, peroxiredoxins and the thioredoxin system.

In *C. neoformans* the expression of the SOD1 gene increases at the host temperature (37°C) and a *sod1* mutant lacking the cytosolic Cu,Zn SOD is killed by ROS in a cell free system, and is significantly less virulent than the wild type strain in a murine inhalation infection model [12]. Mitochondrial SOD2p is crucial for survival of *C. neoformans* var. *gatti* at the host temperature, to cope with different stress conditions and is important for virulence in a mouse model of cryptococcosis. Strains lacking both, SOD1p and SOD2p, were even more susceptible to oxidative and other types of stress, and incapable to produce the experimental disease [13].

The high level of redundancy in H_2O_2 elimination mechanisms makes it difficult to assign a definitive role in pathogenesis for a given antioxidant enzyme. Thus, although more sensitive to H_2O_2 than wild type, *A. fumigatus* null mutant strains in the spore specific catalase (CatAp) or the mycelial catalases (Cat1p and Cat2p), were as sensitive to macrophage (conidia) and polymorphonuclear neutrophil (mycelium) killing as the wild type strain. However, the double *Δcat1 Δcat2* mutant showed delayed infection in a rat model of aspergillosis [9]. On the other hand, peroxiredoxins, which are slow H_2O_2 consuming but highly abundant proteins in all cells, proved to be important not only for resistance to H_2O_2 stress but also for virulence in *C. neoformans*. From four peroxiredoxins present in *C. neoformans*, only one (*tsa1*), a two cysteine-type (2-Cys) peroxiredoxin, is important for growth, H_2O_2 resistance, and is necessary for virulence [14]. The 2-Cys peroxiredoxins are reduced by thioredoxins. *C. neoformans* has two small dithiol thioredoxin proteins (Trx1 and Trx2), which have overlapping functions but are differentially expressed [15]. A *trx1Δ* mutant strain shows reduced growth and is sensitive to oxidative and nitrosative stress, whereas the *trx2Δ* strain only shows low sensitivity to nitrosative stress. However, mutation of both genes is required to reduce virulence in an inhalation mouse model of infection [15]. Thioredoxin reductase uses NADPH to reduce the oxidized form of thioredoxin. There is a single, probably essential, thioredoxin reductase gene in *C. neoformans*, which is inducible by nitrosative stress [16].

As $NO^{\bullet}$ and its derivatives are important reactive species in the macrophage response to fungal infection, it is also expected that enzymes that detoxify reactive nitrogen species might be relevant to pathogenicity. In contrast to the redundancy in the systems for H_2O_2 elimination, there are few mechanisms for the disposal

of $NO^{\bullet}$. *C. neoformans* has a single gene for $NO^{\bullet}$ oxygenase (*fhb1*), also called flavohemoglobin denitrogenase, and a single gene for 5-nitrosoglutathione reductase (*gno1*) [17]. In fact, $NO^{\bullet}$ generated by the inducible nitric oxide synthase (iNOS) in mammal hosts, exerts a fungistatic effect against *C. neoformans* and $NO^{\bullet}$ oxigenase Fhb1 protects the fungus during infection. The *gno1Δ* null mutant was not affected in virulence, the *fhb1Δ* null mutant showed attenuated virulence, and the double *gno1Δ fhb1Δ* mutant strain showed further attenuation in its virulence [17].

In addition to antioxidant enzymes, other factors related to ROS detoxification are important for fungal pathogen virulence. Melanin, capsule polysaccharides and mannitol are known virulence factors in different pathogens. Melanin is known to react with most ROS, acting as a buffer against external ROS. The ability to produce melanin has been long recognized as a bacterial and fungal virulence factor, after it was found that mutations that eliminate melanin production resulted in strains with significantly attenuated virulence (reviewed in 18). Two laccases in *C. neoformans* are necessary for melanin production, using L-DOPA as substrate, and both are important to resist macrophage killing [19]. Also in *C. neoformans*, there are four CAP genes required for capsule formation [20], and six CAS genes required for polysaccharide synthesis, all of which are necessary for virulence [21]. In addition, *C. neoformans* produces large amounts of mannitol, a known scavenger of hydroxyl radical, during infection. Mutants that produce less mannitol are less virulent and more sensitive to neutrophil-mediated oxidative killing [22].

Signal transduction mechanisms in fungal responses to oxidative stress

Studies, in which fungal models *Saccharomyces cerevisiae* and *S. pombe* have been challenged by external ROS, show that eukaryotes evolved novel mechanisms to perceive and eliminate ROS. These mechanisms involve a prokaryotic-type multistep phosphorelay coupled to a stress-response MAP kinase pathway, as well as an AP-1 type transcription factor [2,23].

Specific MAPKs mediate multiple stress signaling

To respond to environmental changes, eukaryotes utilize mitogen-activated protein kinase (MAPK) phosphorylation cascades. A MAPK cascade is composed by a kinase MAPKKK (also called MEKK) that phosphorylates and activates a second kinase MAPKK (or MEK), which in turn phosphorylates and activates a MAPK. Once active, this MAPK phosphorylates substrates such as transcription factors or other proteins, which results in expression of genes related with the response to the initial stimulus. A specific group of homologous MAPKs or SAPKs (stress activated protein kinases) mediate responses to different types of stress: Hog1 (high osmotic glycerol) principally mediates responses to high osmolarity in *S. cerevisiae*, whereas mammalian JNK and p38, and *S. pombe* Spc1 (also called Sty1) are activated by multiple forms of stress such as high osmolarity, oxidative stress, nutrient starvation, heat shock and UV light.

Fungal MAPKs involved in oxidative stress sensing

Among fungi, only *S. pombe* Spc1/Sty1 [23–26], *C. albicans* CaHog1 [27,28] and *A. nidulans* SakA [2,29] have been shown to be activated by ROS and actually mediate oxidative stress responses. Spc1/Sty1 becomes phosphorylated in response to exogenous H_2O_2, is required for induction of more than 200 antioxidant response genes such as *ctt1*$^{+}$, the only catalase gene present in this organism, and to survive high H_2O_2 treatments. As shown in Table 1, Spc1 is activated by MAPKK Wis1 (also called Sty2), which in turn gets activated by redundant MAPKKKs Wis4 and Win1. Once phosphorylated, Spc1 becomes localized to the nucleus where it phosphorylates and activates Atf1, a transcription factor homologous to human ATF2. Atf1

Table 1 *Aspergillus* proteins related to components of the MAPK module involved in oxidative stress sensing in *Schizosaccharomyces pombe*. *Saccharomyces cerevisiae* proteins are shown for comparison; their role in mediating oxidative stress responses is not yet clear. *Aspergillus nidulans* and *Aspergillus fumigatus* proteins are indicated by locus tag ID with GenBank accession number indicated in parenthesis. [1]Wis4/Win1 orthologue appears miss-annotated as a C-domain of protein AN1180.2. bZIP TF indicates transcription factors of the bZIP family.

MAPK Component	*S. pombe*	*A. nidulans*	*A. fumigatus*	*S. cerevisiae*
MAPKKK	Wis4, Win1	SskB AN1180.2[1] (XP_658784)	Afu1g10940 (XP_752459)	Ssk2, Ssk22
MAPKK	Wis1/Sty2	PbsB AN0931.2 (XP_658535)	Afu1g15950 (XP_752961)	Pbs2
MAPK	Spc1/Sty1	SakA/HogA, MpkC	SakA, MpkC	Hog1p
bZIP TF	Atf1	AtfA	Afu3g11330 (XP_754486)	Sko1

appears to mediate most of the transcriptional responses regulated by Spc1/Sty1 [25,30,31]. Recently, *csx1*$^+$ was identified as a gene essential to survive oxidative stress treatment. The Csx1 protein binds to and stabilizes *atf1*$^+$ mRNA under oxidative stress to maintain normal levels of Atf1 under these conditions. However, microarray analysis of cells under oxidative stress identified a number of genes whose expression is dependent on *csx1*$^+$ but not *atf1*$^+$. This and the fact that null *csx1*$^-$ mutants are more sensitive to oxidative stress than null *atf1*$^-$ mutants, indicates additional functions of Csx1 in *S. pombe* oxidative stress response [32].

In *A. nidulans*, studies aimed at understanding the antioxidant response and its relation to development identified four different catalases in this fungus, which are expressed at different times, cell compartments or cell-types [8,33]. The *catA* and *catB* genes encode a spore-specific and a mycelial catalase, respectively. Oxidative and other stress conditions induce the accumulation of both *catA* and *catB* mRNAs but CatA activity can only be detected in asexual and sexual spores, where it confers protection against H_2O_2 and heat shock treatments [33,34]. In contrast, CatB activity [35] and expression of a *catB::lacZ* reporter gene is readily induced by oxidative stress caused by paraquat or H_2O_2 treatments [36]. The *A. nidulans sakA* gene (also called *hogA*), identified as an *spc1*$^+$ homologue, encodes a MAPK that plays functions that are similar to those reported for Spc1/Sty1: first, SakA is activated by osmotic and oxidative stress treatments and can replace Spc1 functions in *S. pombe*. Second, it is activated by osmotic and oxidative stress in *A. nidulans*. Third, it is required for normal conidiospore CatA activity [29] and for induction of the *catB* gene in response to H_2O_2 [2]. In addition, SakA is essential for the long-term survival of intact conidiospores, as well as for hydrogen peroxide and heat shock resistance in germinated conidiospores [29]. Interestingly, this last phenotype is also observed in *ΔcatA* null mutants [34].

As shown in Table 1, homologues of all components of the *S. pombe* stress MAPK cascade can be found in *A. nidulans* and *A. fumigatus* [37], as well as in virtually every fungi with a genome sequence available. In contrast to *S. cerevisiae*, S. *pombe* and *C. albicans*, filamentous fungi *A. nidulans*, *A. fumigatus* and *A. oryzae* contain a second SAPK, similar to SakA, called MpkC [29,38]. Deletion of *mpkC* gene in *A. nidulans* did not cause sensitivity to high osmolarity or any other visible phenotype (K. Jahng *et al*., unpublished) and it was not possible to obtain double *ΔsakA ΔmpkC* mutants from sexual crosses involving single MAPK mutants (O. Sánchez and J. Aguirre, unpublished; [39]). Although it is not possible to detect phosphorylated MpkC in wild type or *ΔsakA* strains [29], Furukawa *et al*. (2005) generated strains that overexpressed MpkC and detected its phosphorylation in response to osmotic stress [39]. These results suggest that SakA and MpkC might play partially redundant functions in *A. nidulans*.

MAPKKK and MAPK genes homologous to *S. cerevisiae ssk2/ssk22* and *pbs2* genes identified in the *A. nidulans* genome database [37] were deleted. Corresponding *ΔsskB* and *ΔpbsB* mutants were more sensitive than null *sakA* mutants to high osmolarity and failed to phosphorylate SakA in response to both, osmotic and oxidative stress, indicating that SskB and PbsB are part of the stress SakA MAPK cascade [39].

In *A. fumigatus*, *sakA* mRNA accumulates in response to increased salt concentrations and conidiospores from a *ΔsakA* null mutant show slow germination and growth rates under high osmolarity conditions. In addition, SakA regulates conidiospore germination in response to the nitrogen source in the

Table 2 *Aspergillus* proteins related to the phosphorelay components involved in oxidative stress sensing in *Schizosaccharomyces pombe*. *Saccharomyces cerevisiae* homologues are shown as reference; only Skn7 is known to mediate oxidative stress responses. *Aspergillus nidulans* and *Aspergillus fumigatus* proteins are indicated by locus tag ID with GenBank accession number indicated in parenthesis. Putative sensor kinases contain PAS or GAF domains as indicated. TF indicates transcription factor.

Phosphorelay Component	*S. pombe*	*A. nidulans*	*A. fumigatus*	*S. cerevisiae*
Sensor Kinase (HK)	Mak1,	AN3101.2 (PAS) (EAA63672)	Afu3g12530(PAS) (XP_754368)	Sln1
	Mak2,	AN3102.2 (GAF) (EAA63673)	Afu3g12550 (GAF) (XP_754366)	
	Mak3			
Phosphotransfer Protein (HPt)	Mpr1	YpdA (EAA63906)	Afu4g10280 (XP_751798)	Ypd1
Response Regulator (MAPK-linked)	Mcs4	SskA (EAA61883)	Afu5g08390 (XP_753797)	Ssk1
Response Regulator (TF)	Prr1	SrrA (AAN75016)	Afu6g12520 (XP_751130)	Skn7

Table 3 *Aspergillus* proteins related to AP-1-like transcription factors involved in oxidative stress sensing. *Aspergillus nidulans* and *Aspergillus fumigatus* proteins are indicated as in Table 1.

AP1-like Component	*S. cerevisiae*	*S. pombe*	*A. nidulans*	*A. fumigatus*
bZip TF	Yap1	Pap1	AN7513.2 (EAA2846.2)	Afu6g09930 (XP_750882)

medium [40]. The role of *A. fumigatus* SakA in the oxidative stress response and pathogenicity has not yet been evaluated.

Fungal stress MAPKs are activated by a phosphorelay system

Two-component histidine kinase (HK) phosphorelay systems are prevalent in prokaryotes as a major mechanism to sense and adapt to the environment. In response to an environmental signal, the HK autophosphorylates in a conserved histidine residue and the phosphate is then relayed to a conserved aspartic acid residue in a response regulator (RR) protein, usually a transcription factor, resulting in changes in gene expression. In eukaryotic cells, phosphorelay systems have only been found in slime molds, fungi and plants, where they connect environmental signaling to stress MAPK cascades [reviewed in 23,41]. In *S. cerevisiae,* its only HK (Sln1) transmits osmotic stress signals to the SAPK Hog1, whereas the three HKs (Mak1-3) present in *S. pombe* transmit oxidative stress signals (Table 2). Mak1-3 kinases have PAS/PAC domains, which are often found in redox-regulated proteins. Mak2 and Mak3 [24], the phosphotransfer (Hpt) protein Mpr1 [26] and the response regulator Mcs4 [24,26] form a phosphorelay system needed to activate Spc1 in response to H_2O_2 and to induce Atf1-dependent gene expression [reviewed in 23]. Mak1 and Prr1, the second response regulator present in *S. pombe* seem to constitute a second Spc1-independent phosphorelay involved in oxidative stress sensing, as both are necessary for induction of the *ctt1*$^+$ catalase gene by H_2O_2 [24,42]. In *S. cerevisiae*, the Prr1 homologue Skn7 is also required for induction of a set of genes encoding anti-oxidant activities, such as catalase CTT1 and superoxide dismutase SOD1 [43].

The *A. nidulans* genome predicts 15 HKs, one Hpt and two response regulators. This seems true in *A. fumigatus* and other filamentous fungi (Table 2), but the number of putative HKs can range between 11 and 21 [41]. The HK TcsB, a Sln1 homologue, is dispensable to transmit osmotic and oxidative stress signals to SakA, whereas response regulator SskA protein is required for both functions [39]. The role of other phosphorelay components in the response to oxidative stress remains to be elucidated.

Fungal AP-1-like transcription factors involved in oxidative stress sensing

In mammalian cells, AP-1 (activating protein 1) describes a group of members of the Jun, Fos and ATF bZIP protein families, which form heterodimeric transcription factors to control genes that contain AP-1 sites. AP-1 activity regulates cell proliferation, apoptosis and differentiation in animal cells [44]. Yap1 (Yeast AP-1), first identified in *S. cerevisiae* by its ability to bind an heterologous AP-1 recognition element, has been thoroughly characterized as a transcription factor that plays an essential role in the induction of antioxidant genes [23,43,45,46]. In response to H_2O_2, Yap1 [45] and the homologous proteins *S. pombe* Pap1 [47], *C. albicans* Cap1 [48] and *Cochliobolus heterostrophus* CHAP1 [49] are accumulated in the nucleus, where they induce a set of genes needed to respond to this stress. Indeed, null mutants in the corresponding genes are hypersensitive to H_2O_2. All these fungal AP-1-like transcription factors have carboxy terminal (c-CRD) and amino terminal (n-CRD) cysteine-rich domains, which are critical for resistance to oxidative stress and for appropriate nuclear localization. As shown in Table 3, *A. nidulans* and *A. fumigatus* contain clear Yap1 homologues, whose role in the oxidative stress response and pathogenicity are yet to be evaluated.

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