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Research Article

A Ferroxidase, Cfo1, Regulates Diverse Environmental Stress Responses of *Cryptococcus neoformans* through the HOG Pathway

Kyung-Tae Lee[1], Jang-Won Lee[1], Dohyun Lee[2], Won-Hee Jung[3] and Yong-Sun Bahn[1,*]

[1]*Department of Biotechnology, Center for Fungal Pathogenesis, Yonsei University, Seoul 120-749, Korea*
[2]*Nutrex Technology Co., Ltd., Seongnam 463-400, Korea*
[3]*Department of Systems Biotechnology, Chung-Ang University, Anseong 456-756, Korea*

Abstract The iron uptake and utilization pathways play a critical role in allowing human pathogens, including *Cryptococcus neoformans,* the causative agent of fatal meningoencephalitis, to survive within the mammalian body by competing with the host for iron. Here we show that the iron regulon is also required for diverse environmental stress responses and that in *C. neoformans,* it is regulated by the high-osmolarity glycerol response (HOG) pathway. Between *CFO1* and *CFO2,* two ferroxidase genes in the iron regulon, *CFO1* but not *CFO2* was induced during oxidative and osmotic stress. Interestingly, we found that the HOG pathway repressed basal expression of both *CFO1* and *CFO2*. Furthermore, when the HOG pathway was blocked, *CFO2* also responded to oxidative and osmotic stress and the response of *CFO1* was increased. We also established that *CFO1* plays a major role in responding and adapting to diverse environmental stresses, including oxidative and genotoxic damage, osmotic fluctuations, heavy metal stress, and stress induced by cell membrane destabilizers. Therefore, our findings indicate that in *C. neoformans*, the iron uptake and utilization pathways are not only required for iron acquisition and survival, but also play a significant role in the environmental stress response through crosstalk with the HOG pathway.

Keywords Cfo1, Cfo2, *Cryptococcus neoformans*, Hog1, Iron

Iron acquisition is considered a critical aspect of microbial survival within a mammalian host [1]. *Cryptococcus neoformans*, a human fungal pathogen causing fatal meningoencephalitis [2], similar to other pathogens, competes with the host to acquire iron for its survival and virulence. Available iron sources within the mammalian host include transferrin and hemoglobin [3]. *C. neoformans* is able to utilize these iron sources, and the pathways and regulatory mechanisms associated with iron utilization in this pathogen have been at least partly identified [4-8]. Among the pathways required for iron utilization, the high-affinity reductive iron uptake pathway, which is composed of cell surface reductases; an iron permease; and a ferroxidase, was shown to play a major role in virulence. The iron permease is encoded by *CFT1* and the mutants lacking this gene are deficient in growth in the presence of the inorganic iron source $FeCl_3$ as well as when transferrin is the sole iron source. Moreover, *cft1Δ* mutants display attenuated virulence in the murine model of cryptococcosis [5]. *CFO1* encodes ferroxidase in the high-affinity reductive iron uptake pathway and this protein is coupled with Cft1 at the cell membrane. Mutants lacking the *CFO1* gene show similar phenotypes to the *cft1* mutants, suggesting this protein cooperates with Cft1 and contributes to pathogenesis. Intriguingly, deletion of *CFO1* influences azole antifungal sensitivity, suggesting a role in ergosterol synthesis [4]. Other iron sources available in the mammalian body include heme and siderophores. However, the deletion of *CFT1* or *CFO1* does not affect the utilization of heme and siderophores, suggesting only a minor contribution of these iron sources to the virulence of *C. neoformans*. Indeed, mutants lacking the siderophore transporter *SIT1* are as virulent as wild type [8]. Much attention has been paid to defining the regulatory mechanisms of the genes involved in iron utilization. So far, Cir1, a GATA type transcription factor, has been identified as a major regulatory protein for the

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***Corresponding author**
E-mail: ysbahn@yonsei.ac.kr

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control of the iron regulon in *C. neoformans* [6]. Contributions of other pathways and proteins such as the cAMP pathway and Tup1 have also been suggested [5, 9, 10].

Based on our recent transcriptome analysis identifying environmental stress response genes in *C. neoformans*, the high-osmolarity glycerol response (HOG) pathway also plays some role in the ion transport and metabolic pathway. Basal expression levels of the *SIT1*, *CFO1, CFO2*, and *CFT1* genes are all highly induced in the HOG pathway mutants compared to the wild-type strain [11]. The *Cryptococcus* HOG pathway consists of the two-component phosphorelay system and the mitogen-activated protein kinase (MAPK) system. The Hog1 MAPK module is composed of the Ssk2 MAPK kinase kinase, the Pbs2 MAPK kinase, and the Hog1 MAPK itself. Upstream of the Ssk2-Pbs2-Hog1 module the multicomponent phosphorelay system, which comprises hybrid sensor histidine kinases including Tco1 and Tco2; a histidine-containing phosphotransfer protein Ypd1; and response regulators Ssk1 and Skn7, relays signals to Ssk2 [12-16]. The HOG pathway is not only involved in a plethora of environmental stress responses in *C. neoformans*, but also controls the virulence and sexual differentiation of *C. neoformans* [12-16]. Downstream of the Hog1 MAPK multiple stress defense genes, transcription factors, and protein kinase genes have been identified by our microarray analysis, including Ena1 (cation transporter), Hrk1 (Hog1-regulated kinase 1) and Ubc6 (ubiquitin-conjugating enzyme) [11, 17].

In this study, we provide evidence showing that Cfo1 plays significant roles in response and adaptation to diverse environmental stresses through the HOG pathway in *C. neoformans*. This provides further information about the regulatory mechanisms for and roles of Cfo1/2 in the stress response of *C. neoformans*.

MATERIALS AND METHODS

Strains and media. *C. neoformans* strains listed in Table 1 [4, 14, 18-20] were cultured and maintained in yeast extract-peptone-dextrose (YPD) medium or yeast extract-peptone (YP) medium for inducing glucose starvation.

Expression analysis by northern blotting. Each strain was cultured in YPD medium at 30°C for 16 hr and subcultured in fresh YPD medium at 30°C until optical density at 600 nm (OD_{600}) reached 1.0. The zero time sample was collected and the remaining culture was mixed with an equal volume of liquid YPD containing 3 M NaCl (for final 1.5 M NaCl concentration) or was treated with the indicated concentration of H_2O_2. After treatment, the culture was further incubated at 30°C, and samples from each indicated time point were lyophilized overnight. Total RNA was isolated by the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as previously described [11]. Ten μg of total RNA from each strain was used for the northern blot. Electrophoresis, membrane transfer, hybridization, and membrane washing were performed by following the protocols previously described [21]. Gene-specific probes were amplified by PCR with primer pairs: B1921 (5'-CGGGGTAAGTCGTTCTTTC-3') and B1922 (5'-TCCTC-ATCCAACTCTCCAG-3') for *CFO1* and B1923 (5'-TTAC-CTATGCCACAGCCAACCC-3') and B1924 (5'-TTCGTC-CAAGTGTTCGGCAC-3') for *CFO2*.

Phenotypic analysis. Each strain was grown in YPD medium at 30°C for 16 hr, serially diluted at ten-fold (1 to 10^4), and spotted on 2% agar-based YPD medium containing the indicated concentration of oxidizing agents (hydrogen peroxide and diamide), a cell membrane destabilizer (sodium dodecyl sulfate [SDS]), a cell wall stress agent (Congo red [CR]), a genotoxic agent (hydroxyurea [HU]), osmotic shock agents (NaCl and KCl), or a heavy metal stress agent ($CdSO_4$).

RESULTS

***CFO1* expression was induced by oxidative and osmotic stresses and both *CFO1* and *CFO2* expression was repressed by the HOG pathway.** Our prior comparative transcriptome analysis of the HOG pathway in response to oxidative and osmotic stress response revealed that several genes involved in iron transport and metabolism are negatively regulated by the HOG pathway [11]. *CFO1* and *CFO2* ferroxidases are genes regulated by the HOG pathway. Basal expression levels of *CFO1* and *CFO2* are induced by 4- to 7-fold respectively in the *hog1Δ* and *ssk1Δ* mutants [11] indicating that *CFO1* and *CFO2* are regulated by the HOG pathway in *C. neoformans*.

To address how *CFO1* and *CFO2* are regulated by

Table 1. Strains used in this study

Name	Genotype	Parent	Reference
H99	*MATα*		[18]
YSB64	*MATα hog1Δ::NAT-STM#177*	H99	[14]
YSB51	*MATα ras1Δ::NAT-STM#150*	H99	[19]
YSB42	*MATα cac1Δ::NAT-STM#159*	H99	[20]
	MATα cfo1Δ::NAT	H99	[4]
	MATα cfo2Δ::NAT	H99	[4]
	MATα cfo1Δ::NAT CFO1-NEO	*MATα cfo1Δ::NAT*	[4]
	MATα cfo2Δ::NATCFO2-NEO	*MATα cfo2Δ::NAT*	[4]

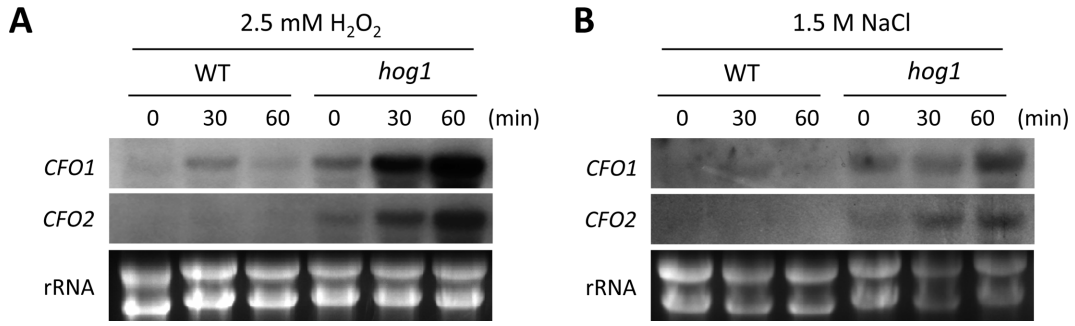

Fig. 1. High-osmolarity glycerol response (HOG)-dependent regulation of the expression of *CFO1* and *CFO2* under oxidative and osmotic stress conditions. WT (H99) and *hog1Δ* strains were cultured and synchronized to 1.0 of OD_{600nm}. Then each strain was treated with 2.5 mM H_2O_2 (A) or 1.5 M NaCl (B) for each indicated incubation time and sampled for total RNA extraction. Northern blot analysis was performed using *CFO1*- or *CFO2*-specific probe. Ethidium bromide staining results of rRNA were used as loading controls.

environmental stresses, first we monitored expression patterns of *CFO1* and *CFO2* under oxidative and osmotic stress (Fig. 1). When *C. neoformans* was exposed to 2.5 mM H_2O_2, *CFO1* expression was induced after 30 min, while *CFO2* expression was not detected (Fig. 1A). In agreement with our previous microarray data, basal expression levels of both *CFO1* and *CFO2* were much higher in the *hog1Δ* mutant than the wild-type strain (Fig. 1A). Interestingly, we found that both *CFO1* and *CFO2* were both strongly induced by H_2O_2 in the *hog1Δ* mutant background. This suggests that the HOG pathways control basal and induced expression levels of ferroxidase genes *CFO1* and *CFO2* during adaptation to oxidative stress in *C. neoformans*.

In contrast to oxidative stress, *CFO1* was only very weakly induced and *CFO2* was not detected in response to osmotic stress (1 M NaCl) (Fig. 1B). Nevertheless, both *CFO1* and *CFO2* showed robust induction in the *hog1Δ* mutant after 30 min of 1 M NaCl treatment (Fig. 1B). Taken together, these data suggest that expression of two ferroxidase genes, *CFO1* and *CFO2*, are regulated by both environmental stresses and the HOG pathway

Cfo1 played key roles in response and adaptation to oxidative, genotoxic, and heavy metal stresses. The Hog1-dependent differential expression of *CFO1* and *CFO2* in response to oxidative and osmotic stresses suggests that these ferroxidases could play some roles in response and adaptation to diverse environmental stresses other than the iron stress response. To address the role of Cfo1 and Cfo2 in environmental stress responses, we performed diverse stress susceptibility test with the *cfo1Δ* and *cfo2Δ* mutants. In agreement with the expression analysis data (Fig. 1A), the *cfo1Δ* mutant exhibited a greater susceptibility to H_2O_2 than wild type strain (Fig. 2A). However, the *cfo2Δ* mutant did not exhibit any increased sensitivity to H_2O_2 (Fig. 2A). In response to diamide, which is a thiol-specific oxidant but does not produce reactive oxygen species (H_2O_2 does), the *cfo1Δ* mutant, but not the *cfo2Δ* mutant, exhibited highly increased sensitivity. These data indicated that Cfo1, but not Cfo2, plays a major role in oxidative stress response in *C. neoformans*.

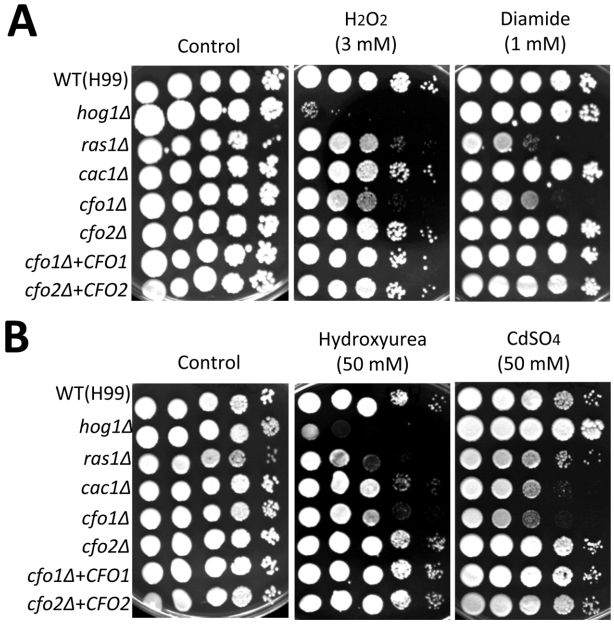

Fig. 2. Cfo1 played key roles in response and adaptation to oxidative, genotoxic, and heavy metal stresses. Each strain (WT [H99], *hog1Δ*, *ras1Δ*, *cac1Δ*, *cfo1Δ*, *cfo2Δ*, *cfo1Δ+CFO1*, and *cfo2Δ+CFO2*) was cultured for 16 hr in yeast extract-peptone-dextrose (YPD) medium, 10-fold serially diluted ($1\sim10^4$ dilutions), and spotted (3 μL of dilution) onto the YPD agar containing the indicated concentration of hydrogen peroxide, diamide for oxidative stress (A), hydroxyurea for genotoxic stress, and $CdSO_4$ for heavy metal stress (B). Spotted plates were incubated for 3 or 4 days and photographed.

Related to oxidative stress response, we have questioned whether Cfo1 and Cfo2 may play some roles in genotoxic and heavy metal stresses. The *cfo1Δ* mutant also exhibited increased sensitivity to HU and cadmium sulfate, which are known respectively to be a DNA damaging agent and a toxic heavy metal (Fig. 2B). In contrast, Cfo2 appeared to

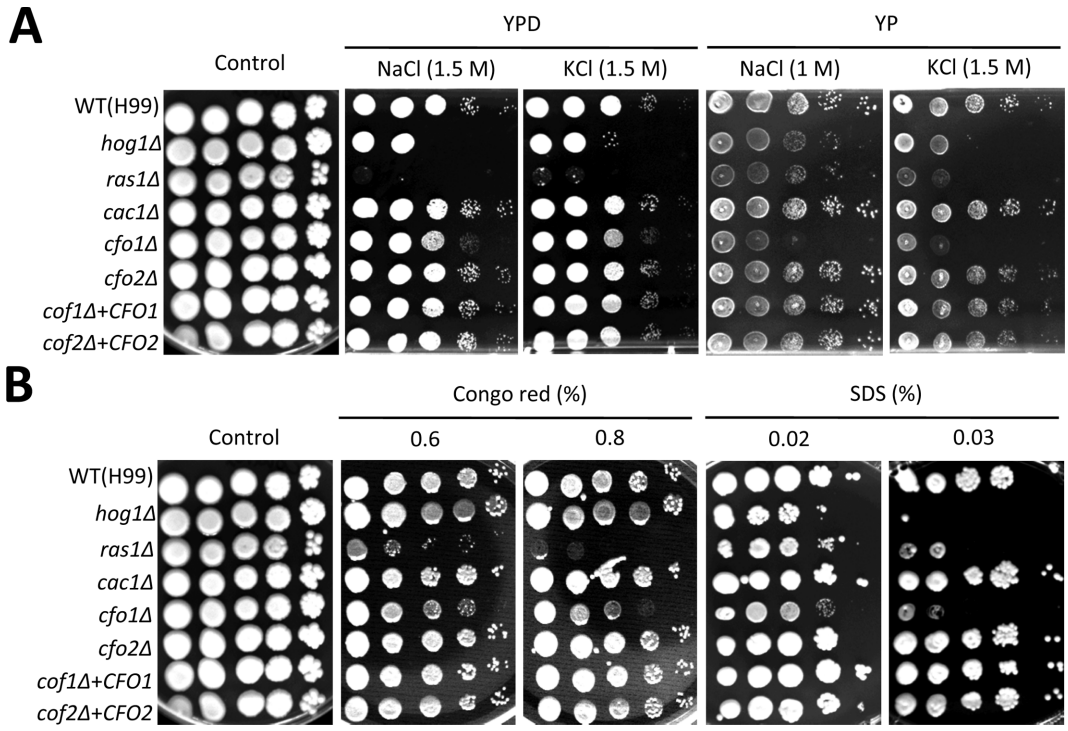

Fig. 3. Cfo1 was required for response and adaptation to osmotic stresses and cell wall and membrane integrity. Each strain (WT [H99], *hog1Δ*, *ras1Δ*, *cac1Δ*, *cfo1Δ*, *cfo2Δ*, *cfo1Δ*+*CFO1*, and *cfo2Δ*+*CFO2*) was cultured for 16 hr in yeast extract-peptone-dextrose (YPD) medium, 10-fold serially diluted ($1\sim10^4$ dilutions), and spotted (3 μL of dilution) onto YPD or east extract-peptone (YP) agar containing the indicated concentration of NaCl or KCl for oxidative stress (A). B, To test the cell membrane and cell wall integrity, YPD medium containing Congo red or sodium dodecyl sulfate (SDS) was used. Spotted plates were incubated for 3 or 4 days and photographed.

be dispensable for genotoxic and heavy metal stress responses (Fig. 2B).

Cfo1 played key roles in response and adaptation to osmotic stresses and was required for cell wall and membrane integrity. Although expression of *CFO1* and *CFO2* were not strongly induced in response to osmotic shock in the wild-type strain, both *CFO1* and *CFO2* were induced after 30 min upon 1 M NaCl treatment in the *hog1Δ* mutant (Fig. 1A), indicating that the ferroxidases may be required for response and adaptation to osmotic or salt stress response. Therefore, we tested sensitivity of the *cfo1Δ* and *cfo2Δ* mutants against osmotic stress (1 or 1.5 M NaCl or KCl). Under glucose-rich condition (YPD medium), the *cfo1Δ* mutant, but not the *cfo2Δ* mutant, exhibited slightly increased sensitivity to both 1.5 M NaCl and KCl (Fig. 3A). This osmosensitivity of the *cfo1Δ* mutant was even more evident under glucose-starved condition (YP medium), which was similar to the *hog1Δ* mutant (Fig. 3A). In contrast, the *cfo2Δ* mutant exhibited the wild-type level of osmosensitivity under the glucose-starved condition. In summary, Cfo1 was required for osmotic stress response while Cfo2 was dispensable.

Increased osmosensitivity of the *cfo1Δ* mutant may result from defective cell membrane/wall integrity, which renders cells to be unable to resist osmotic pressure. To test this possibility, we have monitored sensitivity of the *cfo1Δ* mutant to SDS and CR, which are cell membrane and cell wall destabilizers, respectively (Fig. 3B). The *cfo1Δ* mutant exhibited increased sensitivity to both SDS and CR, while the *cfo2Δ* mutant was as resistant to the agents as the wild-type strain, indicating that Cfo1 was required for the maintenance of cell membrane and cell wall integrity.

DISCUSSION

In this study, we demonstrated that two ferroxidase genes in the iron uptake system, *CFO1* and *CFO2*, were differentially regulated during environmental stress response and adaptation via the HOG pathway, which is a key stress-activated signaling cascade in *C. neoformans*. When the HOG pathway was inhibited, expression of *CFO1* and *CFO2* was not only de-repressed at basal levels it was strongly induced upon oxidative and osmotic stresses. Between the two ferroxidases, we found that Cfo1 played major roles in responding and adapting to diverse environmental stresses, such as oxidative and genotoxic damage, osmotic fluctuations, heavy metal stress, and cell membrane destabilizers. Therefore, our study indicates that the iron uptake and metabolic systems are not only required for iron acquisition for survival, but also play a significant role in environmental stress response through a crosstalk with the stress-activated signaling

pathway.

The reason for the H_2O_2-mediated induction of *CFO1* is not clear at this point. However, it is conceivable that the ferroxidase, which catalyzes the oxidation of ferrous ion (Fe^{2+}) to ferric iron (Fe^{3+}), may be involved in the Fenton's reaction, where H_2O_2 reacts with Fe^{2+} to generate highly reactive, toxic hydroxyl radicals (•OH). Therefore, *C. neoformans* may induce the expression of *CFO1* to decrease ferrous iron, which may lead to reduction of the Fenton's reaction. This may explain why the *cfo1Δ* mutant, in which intracellular ferrous iron concentration should be high, is more susceptible to H_2O_2 than the wild-type strain because higher amounts of toxic hydroxyl radicals could be accumulated in the mutant.

Based on this proposal, the hypersensitivity of the *cfo1Δ* mutant to osmotic stress and cell membrane/wall destabilizers appeared to be related to the role of Cfo1 in maintaining proper ferric iron concentrations intracellularly. Even without the exogenously added H_2O_2, amount of hydroxyl radicals formed from Fenton's reaction between naturally occurring H_2O_2 (from superoxide anion) and ferrous ions should be higher in the *cfo1Δ* mutant than the wild-type strain. These reactive hydroxyl radicals may cause the peroxidation of membrane lipids, which lead to altered cell membrane permeability and decreased activity of membrane-bound enzymes. This assumption was supported by our data showing that the *cfo1Δ* mutant was indeed hypersensitive to high levels of NaCl or KCl, SDS, or CR.

One notable finding in this study is that the HOG pathway controlled both basal and induced levels of *CFO1* and *CFO2* in *C. neoformans,* respectively. Basal expression levels of both *CFO1* and *CFO2* were found to be highly enhanced in the *hog1Δ* mutant by northern blot analysis in this study and microarray analysis in a previous study [11]. The HOG pathway mutants exhibit increased sensitivity to oxidative (H_2O_2) and osmotic stress (NaCl or KCl) as well as cell membrane destabilizer (SDS), which is similar to the *cfo1Δ* mutant. It is possible that increased expression of *CFO1* in the *hog1Δ* mutant could be a compensatory mechanism that plays a role in maintaining cellular homeostasis.

Previously it has been shown that deletion of *CFO1*, but not *CFO2*, significantly reduces the virulence of *C. neoformans* [4]. Data presented in this study suggest that the attenuated virulence observed in *cfo1Δ* may result not only from perturbed iron homeostasis, but also from an abnormal response and adaptation to diverse environmental stresses that could occur during the progression of *C. neoformans* infection. In conclusion, the ferroxidase Cfo1, but not Cfo2, played significant roles in response and adaptation to diverse environmental stresses in *C. neoformans*.

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