Expression and Activity of Catalases Is Differentially Affected by GpaA (Ga) and FlbA (Regulator of G Protein Signaling) in Aspergillus fumigatus

Kwang-Soo Shin¹,* and Jae-Hyuk Yu²

¹Department of Microbiology and Biotechnology, Daejeon University, Daejeon 300-716, Korea
²Departments of Bacteriology and Genetics, University of Wisconsin, Madison, WI 53706, USA

Abstract  Vegetative growth signaling of the opportunistic human pathogenic fungus Aspergillus fumigatus is mediated by GpaA (Gα). FlbA is a regulator of G protein signaling, which attenuates GpaA-mediated growth signaling in this fungus. The flbA deletion (∆flbA) and the constitutively active GpaA (GpaAQ204L) mutants exhibit enhanced proliferation, precocious autolysis, and reduced asexual sporulation. In this study, we demonstrate that both mutants also show enhanced tolerance against H₂O₂ and their radial growth was approximately 1.6 fold higher than that of wild type (WT) in medium with 10 mM H₂O₂. We performed quantitative PCR (qRT-PCR) for examination of mRNA levels of three catalase encoding genes (catA, cat1, and cat2) in WT and the two mutants. According to the results, while levels of spore-specific catA mRNA were comparable among the three strains, cat1 and cat2 mRNA levels were significantly higher in the two mutants than in WT. In particular, the ∆flbA mutant showed significantly enhanced and prolonged expression of cat1 and precocious expression of cat2. In accordance with this result, activity of the Cat1 protein in the ∆flbA mutant was higher than that of gpaAQ204L and WT strains. For activity of the Cat2 protein, both mutants began to show enhanced activity at 48 and 72 hr of growth compared to WT. These results lead to the conclusion that GpaA activates expression and activity of cat1 and cat2, whereas FlbA plays an antagonistic role in control of catalases, leading to balanced responses to neutralizing the toxicity of reactive oxygen species.

Keywords  Aspergillus fumigatus, Catalases, FlbA, GpaA, H₂O₂, Regulator of G protein signaling

During biological oxidation processes the toxic reactive oxygen species (ROS) are generated by incomplete reduction of oxygen with reduced electron carriers in all aerobic pathogenic fungi [1, 2]. ROS derived from host defense mechanisms can destroy major cellular components and effectively kill the spore and hyphae of pathogens. To protect these lethal effects, the fungi boosted various antioxidant enzyme activities of superoxide dismutases (SODs), which convert superoxide to hydrogen peroxide (H₂O₂), and catalases, which detoxify H₂O₂ to water and molecular oxygen. FlbA, regulator of G protein signaling protein, regulates vegetative growth signaling negatively, mediated by a heterotrimeric G protein and ∆flbA mutant shows autolysis in Aspergillus nidulans [3-7]. Our genetic studies in the opportunistic human pathogen Aspergillus fumigatus have demonstrated that the FadA homolog GpaA mediates vegetative proliferation, which is attenuated by FlbA, which in turn enables asexual development (conidiation) to occur [7, 8]. Our recent comparative proteomic studies have suggested that the absence of flbA may lead to increased catalase activity [9]. In this study, we further investigate the roles of FlbA and GpaA in differently governing the expression and activity of catalases in A. fumigatus.

MATERIALS AND METHODS

Fungal strain and culture conditions. A. fumigatus AF293 (WT) [10], ∆flbA4 (pyrG1, ∆flbA4::pyrG), and
tJH4.04 (pyrG1, gpaA<sup>Q204L</sup>, pyrG<sup>+</sup>) strains were used [8]. Glucose minimal medium with 0.1% (w/v) yeast extract (MMY) with supplements was used for general culture of fungal strains [11, 12]. For liquid submerged culture, approximately 5 × 10<sup>4</sup> conidia/mL were inoculated into liquid MMY and incubated at 37°C, 250 rpm. The susceptibility of WT and mutant strains to varying concentrations of H<sub>2</sub>O<sub>2</sub> was tested by performance of plate assays. Drop dilution assay was performed in a series of 10-fold dilutions derived from a starting inoculum of 1.0 × 10<sup>6</sup> conidia per spot. For the hyphal sensitivity test, mycelial plugs cut from the growing edge of five-day old colonies from MMY agar medium were transferred to plates containing 10 mM H<sub>2</sub>O<sub>2</sub> and incubated at 37°C for four days.

**Nucleic acid isolation and manipulation.** Isolation of total RNA was performed as described previously [13]. Five micrograms (5 μg) of total RNA was reverse-transcribed to cDNA using EcoDryTM Premix (Clontech, Mountain View, CA, USA). Quantitative PCR (qRT-PCR) assays were performed according to the manufacturer’s instructions (Qiagen, Valencia, CA, USA) using 96-well optical plates and a Rotor-Gene Q (Qiagen, Valencia, CA, USA). Primers used for qRT-PCR are shown in Table 1. PCR conditions were: 95°C, 15 min followed by 95°C, 30 sec and 55°C, 30 sec for 40 cycles. Amplification of one single specific target DNA was checked by melting curve analysis (+0.5°C ramping for 10 sec, from 55°C to 95°C). The expression ratios were normalized to EF1α expression, and calculated according to the ΔΔC<sub>t</sub> method [14]. To verify the absence of genomic DNA contamination, negative controls, in which reverse transcriptase was omitted, were used for each gene set. Three independent biological replicates were performed.

**Protein extraction and analysis.** For protein extraction, 250 mL flasks containing 100 mL liquid MMY were inoculated with WT and mutant strain conidia and grown at 37°C for two days. After washing with phosphate buffered saline (pH 7.4), mycelia were suspended in lysis buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) and homogenized using a Mini Bead-Beater (BioSpec Products, Bartlesville, OK, USA). The homogenate was centrifuged in a microcentrifuge for 5 min at 15,000 rpm at 4°C, and the supernatant was used for further analyses. For detection of catalase activity on gels, the mycelial extracts were subjected to non-denaturing polyacrylamide gel electrophoresis, and the ferricyanide-negative stain was used to locate bands containing catalase activity [15]. Peroxidase activity on gels was detected according to the method of Wayne and Diaz [15] with a modification of using o-dianisidine instead of diaminobezidine as the substrate.

**RESULTS AND DISCUSSION**

Catalase is regarded as a putative virulence factor with the capability of scavenging H<sub>2</sub>O<sub>2</sub> produced by host phagocytes. Phylogenetic analyses place the catalases into four distinct clades: clade P (peroxisomal catalases), clade C (cytoplasmic catalases), clade A (conidia-specific catalases), and clade B (secreted catalases) [16]. There are three types of catalases

| Table 1. Primers used for qRT-PCR |
|-----------------------------------|
| Primer  | Sequence (5' → 3') | Purpose |
| Oligo275 | AGCCACGGCTCTTCTACAAT | cat1 forward |
| Oligo276 | TTTCATTCCACCGGGAGATT | cat1 reverse |
| Oligo277 | ATCCGACGAGGAGACGAGA | catA forward |
| Oligo278 | GAGCCTTTCCAATCCACGCIA | catA reverse |
| Oligo281 | GGTGCTCAACACACACACGAG | cat2 forward |
| Oligo282 | ATGTCGCAAGGTTGAACAAA | cat2 reverse |
| Oligo346 | CCAAGTGCTGTCGAGGCTCCTCT | EF-1α forward |
| Oligo347 | GAACGTACAGCAACAGTCTGG | EF-1α reverse |

qRT-PCR, quantitative PCR.
FlbA of *A. fumigatus* Regulates Catalases in *A. fumigatus*: CatA (Afu6g03890), produced in conidia; and Cat 1 (Afu3g02270) and Cat2 (Afu8g01670), produced in hyphae [17, 18]. Both the catA deletion and cat1 disrupted mutants showed increased sensitivity to H$_2$O$_2$ [17, 18]. The susceptibilities of the ∆flbA and gpaA$_{Q204L}$ mutants against H$_2$O$_2$ were determined in comparison with WT. Drop dilution assay showed that the two mutants were more resistant than WT, where the ∆flbA mutant exhibited higher tolerance than the gpaA$_{Q204L}$ mutant (Fig. 1A). The radial growth of WT was approximately 62% of that of ∆flbA strain, suggesting approximately 1.6 fold enhanced growth by the absence of flbA (Fig. 1B). These results led us to hypothesize that expression and activities of catalases are elevated in the ∆flbA and gpaA$_{Q204L}$ mutants. We performed qRT-PCR for examination of mRNA levels of the three catalases. According to one report, CatAp is the only catalase present in the resting conidia required for conidial resistance to heat, denaturing agents, and metal ions [18]. Accordingly, catA mRNA accumulation at low levels was observed in hyphae of all tested strains (Fig. 2). On the contrary, levels of cat1 mRNA in ∆flbA and gpaA$_{Q204L}$ strains appeared to be low at 24 hr, highly increased at 48 hr, and then slightly decreased at 72 hr. The highest level of accumulation of cat1 mRNA was observed in ∆flbA strain at 48 hr of growth (Fig. 2). However, the expression pattern of cat2 mRNA was somewhat different from that of cat1. Accumulation of the cat2 mRNA began to occur at 24 hr and remained at high levels until 48 hr in both mutants and then became indistinguishable among the three strains, including WT (Fig. 2). These results suggest that the absence of FlbA and the activation of GpaA result in enhanced expression of the cat1 and cat2 genes. We then performed the zymogram assay for determination of catalase activity; according to our findings, two catalases (Cat1p and Cat2p), while at different levels, were present in all tested strains (Fig. 3A). The Cat1p activity of ∆flbA strain was higher than those of gpaA$_{Q204L}$ mutant and WT, and the highest activity was observed in ∆flbA strain at 72 hr (Fig. 3A). Distinct from CatAp and Cat1p, the Cat2p protein is a bifunctional catalase-peroxidase, conferring resistance to heat, heavy metals, and sodium dodecyl sulfate [18]. In all tested strains, Cat2p activities were not detected at 24 hr, but began to be detectible at 48 hr, and reached the highest level at 72 hr. The patterns of peroxidase activities were similar to those of Cat2p activities.

![Fig. 2. mRNA levels of the three catalase genes in WT, ∆flbA, and gpaA$_{Q204L}$ strains.](image)

| Gene | Culture Time (hr) | WT | ∆flbA | gpaA$_{Q204L}$ |
|------|------------------|-----|-------|---------------|
| catA | 24               | 1   | 0.7   | 0.8           |
|      | 48               | 0.9 | 0.7   | 0.8           |
|      | 72               | 1   | 0.7   | 0.8           |

*Fig. 2.* mRNA levels of the three catalase genes in WT, ∆flbA, and gpaA$_{Q204L}$ strains. mRNA levels of catA, cat1, and cat2 in growing cells of WT, ∆flbA, and gpaA$_{Q204L}$ strains were determined by quantitative PCR (qRT-PCR). Cultures were incubated in liquid MMY and mRNA levels were normalized using the EF1α gene, according to the ∆∆Ct method. Data are expressed as the mean ± standard deviation from three independent experiments. Student’s *t*-test: *p* < 0.005, **p** < 0.001.

![Fig. 3. Native polyacrylamide gel electrophoresis of the mycelial protein extracts of WT, ∆flbA, and gpaA$_{Q204L}$ strains stained for catalase (A) and peroxidase (B). Equal amount of protein (50 µg) was loaded.](image)

*Fig. 3.* Native polyacrylamide gel electrophoresis of the mycelial protein extracts of WT, ∆flbA, and gpaA$_{Q204L}$ strains stained for catalase (A) and peroxidase (B). Equal amount of protein (50 µg) was loaded. Note that the patterns of peroxidase activities were similar to those of Cat2p activities.
generated by respiration leading to increased production of various ROS; thus, the fungus has evolved to couple the enhanced production of defensive cellular enzymes with proliferation.

ACKNOWLEDGEMENTS

This work was primarily supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0007836) to KSS. The work at UW was supported by the Intelligent Synthetic Biology Center of Global Frontier Project funded by the Ministry of Education, Science and Technology (2011-0031955) grants to JHY.

REFERENCES

1. Aguirre J, Ríos-Momberg M, Hewitt D, Hansberg W. Reactive oxygen species and development in microbial eukaryotes. Trends Microbiol 2005;13:111-8.
2. Torres MA, Jones JD, Dangl JL. Reactive oxygen species signaling in response to pathogens. Plant Physiol 2006;141:373-8.
3. Rosén S, Yu JH, Adams TH. The Aspergillus nidulans sfaD gene encodes a G protein beta subunit that is required for normal growth and repression of sporulation. EMBO J 1999;18:5592-600.
4. Seo JA, Han KH, Yu JH. Multiple roles of a heterotrimeric G-protein γ-subunit in governing growth and development of Aspergillus nidulans. Genetics 2005;171:81-9.
5. Seo JA, Yu JH. The phosducin-like protein PhnA is required for Gβγ-mediated signaling for vegetative growth, developmental control, and toxin biosynthesis in Aspergillus nidulans. Eukaryot Cell 2006;5:400-10.
6. Yu JH, Mah JH, Seo JA. Growth and developmental control in the model and pathogenic aspergilli. Eukaryot Cell 2006;5:1577-84.
7. Yu JH. Regulation of development in Aspergillus nidulans and Aspergillus fumigatus. Mycobiology 2010;38:229-37.
8. Mah JH, Yu JH. Upstream and downstream regulation of asexual development in Aspergillus fumigatus. Eukaryot Cell 2006;5:1585-95.
9. Shin KS, Park HS, Kim YH, Yu JH. Comparative proteomic analyses reveal that FlbA down-regulates gliT expression and SOD activity in Aspergillus fumigatus. J Proteomics 2013;87:40-52.
10. Brookman JL, Denning DW. Molecular genetics in Aspergillus fumigatus. Curr Opin Microbiol 2000;3:468-74.
11. Pontecorvo G, Roper JA, Hemmons LM, Macdonald KD, Bufton AW. The genetics of Aspergillus nidulans. Adv Genet 1953;5:141-238.
12. Käfer E. Meiotic and mitotic recombination in Aspergillus and its chromosomal aberrations. Adv Genet 1977;19:33-131.
13. Yu JH, Hamari Z, Han KH, Seo JA, Reyes-Dominguez Y, Scanzocchio C. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet Biol 2004;41:973-81.
14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−ΔΔC(T)} method. Methods 2001;25:402-8.
15. Wayne LG, Diaz GA. A double staining method for differentiating between two classes of mycobacterial catalase in polyacrylamide electrophoresis gels. Anal Biochem 1986;157:89-92.
16. Giles SS, Stajich JE, Nichols C, Gerrald QD, Alspaugh JA, Dietrich F, Perfect JR. The Cryptococcus neoformans catalase gene family and its role in antioxidant defense. Eukaryot Cell 2006;5:1447-59.
17. Calera JA, Paris S, Monod M, Hamilton AJ, Debeauvais JP, Diaquin M, López-Medrano R, Leal F, Latgé JP. Cloning and disruption of the antigenic catalase gene of Aspergillus fumigatus. Infect Immun 1997;65:4718-24.
18. Paris S, Wysong D, Debeauvais JP, Shibuya K, Philippe B, Diamond RD, Latgé JP. Catalases of Aspergillus fumigatus. Infect Immun 2003;71:3551-62.