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Identification, cloning, and functional expression of three glutathione transferase genes from *Aspergillus fumigatus*

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Abstract

Analysis of the genome of the human pathogen, *Aspergillus fumigatus*, revealed the presence of several putative glutathione transferase (GST) open reading frames. Three *A. fumigatus* GST genes, termed *gstA*, *B*, and *C*, were cloned and recombinant proteins expressed in *Escherichia coli*. Functional analysis of recombinant gstA–C confirms that the enzymes exhibit GST activity and glutathione peroxidase activity. RT-PCR confirmed low basal expression of *gstA* and *gstC* which was markedly up-regulated (at least 4×–10×) in the presence of either H_2O_2 or 1-chloro-2,4-dinitrobenzene (CDNB). *GstB* expression was only observed in the presence of CDNB. These results demonstrate for the first time the existence of three functional GSTs in *A. fumigatus* and strongly suggest a role for these enzymes in the response of the organism to both oxidative stress and xenobiotic presence.

Keywords: GST; MALDI-TOF; Xenobiotic resistance; Oxidative stress; Fungal

1. Introduction

Aspergillus fumigatus is a human pathogenic fungus capable of inducing a range of disease states in patients with pre-existing lung damage or immunosuppression following organ transplantation (Daly and Kavanagh, 2001). Three forms of aspergillosis are recognised clinically: saprophytic, allergic, and invasive, with the latter form having a mortality rate of >90% in some patient groups (Denning, 1998). Conventional therapy relies upon the use of amphotericin B and, more recently, on novel azole derivatives and the echinocandin class of anti-fungal agents, but mortality rates remain high. *A. fumigatus* displays the ability to withstand attack by macrophages and neutrophils and develop in a potentially hostile environment. Toxin-mediated inhibition of oxidative burst in alveolar macrophages and polymorphonuclear leukocytes by conidia and hyphae is well characterised (Bertout et al., 2002; Mitchell et al., 1997; Muryama et al., 1996). In addition, the physical size of developing hyphae prevent phagocytosis by alveolar macrophages and there is emerging evidence that *A. fumigatus* may be able to tolerate entry of xenobiotics as a result of amphotericin B treatment creating apertures in the fungal cell membrane (Ellis, 2002).

Glutathione transferases (GST; EC 2.5.1.18) are dimeric phase II detoxification enzymes with the ability to conjugate a broad range of potentially harmful xenobiotics to glutathione (GSH), thereby rendering them more susceptible to removal from the cell. GSTs have also been shown to exhibit GSH-dependent peroxidase activity and thus may be involved in resistance to oxidative stress. Cytosolic GSTs have been identified in almost all organisms, with mammalian GSTs the most clearly characterised. These enzymes have been implicated in pesticide resistance in plants and insects (Sheehan et al., 2001), and

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some GST polymorphisms are thought to alter cancer susceptibility in mammals (Hayes and Pulford, 1995). GSTs are divided into several classes based upon substrate specificity, sequence similarity (particularly in the N-terminal region which is involved in GSH binding), immunological cross-reactivity and, where available, structure similarity. GST classes include α, μ, π, θ, σ, ζ, ω, and κ classes, with insect specific (δ and ϵ), plant specific (φ and τ) and bacterial (β) classes also described (Sheehan et al., 2001). In addition, it is likely that many more classes have been already characterised exist in the broad ranging GST category; for example, new protozoan and fungal GST classes have been proposed (Cha et al., 2001; Takada et al., 2004).

Until recently, relatively little was known about the presence and role of GST in fungi, however it is now clear that GST isoforms exist in a number of fungal species including *Schizosaccharomyces pombe*, *Aspergillus nidulans*, *Saccharomyces cerevisiae*, *Issatchenkia orientalis*, *Yarrowia lipolytica*, *Cunninghamella elegans*, *Mucor circinelloides*, and *Phanerochaete chrysosporium* (Cha et al., 2001; Choi et al., 1998, 2002; Dowd et al., 1997; Dowd and Sheehan, 1999; Foley and Sheehan, 1998; Fraser et al., 2002; Kim et al., 2001; Tamaki et al., 1999; Shin et al., 2002; Veal et al., 2002). Fungal GSTs exhibit differential expression patterns, with some isoforms shown to be expressed inducibly in the presence of xenobiotics or oxidative stress. For example, of two GSTs identified in *I. orientalis*, only one was constitutively expressed, and both were induced in the presence of *o*-dinitrobenzene (*o*-DNB) (Choi et al., 1998). Three GSTs in *S. pombe* were induced by oxidative stress, and mutants lacking $gst1^+$ and $gst2^+$ or $gst3^+$ were more sensitive to the presence of the anti-fungal drug fluconazole, thereby indicating a role for GST in mediating anti-fungal drug tolerance (Cho et al., 2002; Kim et al., 2001; Shin et al., 2001; Veal et al., 2002).

The identification of a functional theta class GST (gene: *gstA*) in *A. nidulans* has further elucidated the role of GST in fungal metabolism. *GstA* appears to be up-regulated by the presence of either 1-chloro-2,4-dinitrobenzene (CDNB)[2] or H_2O_2 in the culture medium and may also play a role in mediating heavy metal resistance in *A. nidulans* (Fraser et al., 2002).

Given the significance of *A. fumigatus* as a human pathogen and the limited success of anti-fungal agents to treat aspergillosis, particularly in immunocompromised patients, it is surprising that the putative presence and role of GST has merited little attention. In addition, the potential role of fungal GST in allowing *A. fumigatus* to withstand neutrophil attack may represent a key element in the cell's ability to survive in the host and colonise pulmonary tissue. Here we describe the identification, cloning, heterologous expression and characterisation of three GST genes from *A. fumigatus*. We also investigate the response of GST gene expression following exposure of *A. fumigatus* to both CDNB and H_2O_2.

[2] *Abbreviations used:* CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; GST, glutathione transferase; GSH, glutathione; MALDI-TOF, matrix assisted laser desorption ionisation-time of flight; MEM, minimal essential medium Eagle; CALM, calmodulin.

2. Experimental

2.1. Genomic DNA isolation

Aspergillus fumigatus ATCC 26933 (obtained from the American Type Culture Collection, Maryland, USA) was used in this study. *Aspergillus* cultures were grown in 5%(v/v) fetal calf serum in minimal essential medium Eagle (MEM) (Sigma–Aldrich, Dorset, UK) for 2 days at 37 °C. Genomic DNA was isolated as described by Nicholson et al. (2001). Briefly, ca. 4 g *A. fumigatus* mycelia were crushed in liquid N_2 and suspended in 10 ml extraction buffer (10 mM Tris–HCl, 10 mM EDTA, 0.5% (w/v) SDS pH 8.0). Phenol:chloroform:isoamyl alcohol (25:24:1, 10 ml) was added to the mycelial suspension and mixed gently for 30 min. Phases were separated by centrifugation at 5000*g* at 4 °C. The aqueous layer was removed a fresh tube and phenol extraction repeated until the interface was clear. The final aqueous layer was treated with chloroform:isoamyl alcohol (24:1) and phases separated as before. The remaining aqueous layer was treated with ribonuclease A (20 μl; 10 mg ml^{-1}) at 37 °C for 30 min, followed by phenol extraction, then chloroform extraction. The DNA was precipitated from the aqueous layer with 2 volumes of 100% ethanol and 1/10 volume of LiCl (4 M) at −20 °C overnight. DNA was recovered by centrifugation at 13,000*g* for 10 min. The pellet was washed with 70%(v/v) ethanol, air-dried, and resuspended in 1 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

2.2. DNA sequence and bioinformatic analysis

All DNA sequence analysis was performed using a Perkin-Elmer ABI Prism 310 genetic analyser, commercially by MWG Biotech (Milton Keynes, UK) or Lark Technologies (Essex, UK) and sequence similarities were determined using the BLAST algorithm (www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). Sequence alignments and neighbor-joined phylogenetic trees were generated using ClustalW (Thompson et al., 1994; http://www.ebi.ac.uk/clustalw). A bootstrapping value of 1000 was used, with bootstrapping percentages noted at tree branch points. Trees were visualised in Treeview (Page, 1996; http://taxonomy.zoology.gla.ac.uk/rod/rod.html). Preliminary sequence data was also obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequencing of *A. fumigatus* genome is near completion with support from the Wellcome Trust and NIH.