

Two glutathione peroxidases in the fungal pathogen *Cryptococcus neoformans* are expressed in the presence of specific substrates

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Glutathione peroxidases catalyse the reduction of peroxides by reduced glutathione. To determine if these enzymes are important for resistance to oxidative stress and evasion of the innate immune system by the fungal pathogen *Cryptococcus neoformans*, two glutathione peroxidase homologues, which share 38% identity, were identified and investigated. In this study, these peroxidases, Gpx1 and Gpx2, their localization, their contribution to total glutathione peroxidase activity, and their importance to the oxidative and nitrosative stress resistance of *C. neoformans* are described. It is shown that the two glutathione peroxidase genes are differentially expressed in response to stress. While both *GPX1* and *GPX2* are induced during *t*-butylhydroperoxide or cumene hydroperoxide stress and repressed during nitric oxide stress, only *GPX2* is induced in response to hydrogen peroxide stress. Deletion mutants of each and both of the glutathione peroxidases were generated, and it was found that they are sensitive to various peroxide stresses while showing wild-type resistance to other oxidant stresses, such as superoxide and nitric oxide. While the glutathione peroxidase mutants are slightly sensitive to oxidant killing by macrophages, they exhibit wild-type virulence in a mouse model of cryptococcosis.

INTRODUCTION

The tripeptide glutathione is important in antioxidant defence, xenobiotic and eicosanoid metabolism, and regulation of the cell cycle and gene expression (Dickinson & Forman, 2002). Glutathione peroxidases (Gpx) are a part of the glutathione system, which is known to be one of the main thiol antioxidant systems in the cell in addition to the thioredoxin system. It has been suggested that the thioredoxin and glutathione systems are maintained independently (Trotter & Grant, 2003), though compensation between these two systems has been observed (Inoue *et al.*, 1999). Glutathione peroxidases are known to reduce peroxides in the presence of two molecules of reduced glutathione (GSH), forming one molecule of glutathione disulfide (GSSG) as a by-product.

Glutathione peroxidases have been shown to exhibit a broad substrate range, including hydrogen peroxide, organic peroxides and peroxyxynitrite (Arteel *et al.*, 1999; Arthur, 2000). All three glutathione peroxidases from *Saccharomyces cerevisiae* express phospholipid hydroperoxidase activity as well (Avery & Avery, 2001). Glutathione peroxidase gene expression has been studied in *S. cerevisiae* and induction of

these genes is observed in response to oxidative stress or glucose repression (Inoue *et al.*, 1999).

The glutathione system has not been studied in any fungal pathogen to date, but enzymes important for resistance to oxidative and nitrosative stress have been linked to both virulence and viability of *Cryptococcus neoformans* (de Jesus-Berrios *et al.*, 2003; Cox *et al.*, 2003; Missall *et al.*, 2004b, 2005; Missall & Lodge, 2005). For example, flavohaemoglobin denitrosylase, Fhb1, which is necessary for nitric oxide resistance (de Jesus-Berrios *et al.*, 2003), and the thiol peroxidase, Tsa1, which is important for both oxidative and nitrosative stress resistance (Missall *et al.*, 2004b), contribute significantly to virulence in *C. neoformans*. In addition, thioredoxin reductase, upon which Tsa1 is thought ultimately to be dependent for reduction, has been shown to be induced during oxidative and nitrosative stress and to be necessary for viability in *C. neoformans* (Missall & Lodge, 2005). While some enzymes important for resistance to stress, including a superoxide dismutase, are dispensable for virulence (Giles *et al.*, 2005), other enzymes have been shown to be compensatory, their action only necessary for resistance in the absence of another stress-related enzyme. For example, only in the absence of Tsa1 is the laccase Lac2 induced in response to nitrosative stress and therefore important for resistance (Missall *et al.*, 2005).

Abbreviation: GFP, green fluorescent protein.

The importance of glutathione peroxidases to the virulence of a pathogen has only been studied in the bacterium *Streptococcus pyogenes*, in which its GpoA has been shown to be necessary for pathogenicity (Brenot *et al.*, 2004). Glutathione peroxidases have not been studied in any fungal pathogen (Missall *et al.*, 2004a), though a glutathione peroxidase homologue has been shown to be transcriptionally abundant during experimental cryptococcosis (Steen *et al.*, 2003). Here, we determine the expression patterns and localization of the two glutathione peroxidases and test their importance to stress resistance, macrophage survival and mammalian virulence of the fungal pathogen *C. neoformans*.

METHODS

Fungal strains and media. H99, a well-characterized virulent clinical isolate of *C. neoformans* serotype A, was used as the wild-type strain. *C. neoformans* was grown on rich medium, YPD (1% yeast extract, 2% bacto-peptone and 2% glucose) or minimal medium, YNB, pH 4.0 (6.7 g l⁻¹ yeast nitrogen base without amino acids, 20 g l⁻¹ glucose and 25 mM sodium succinate at pH 4.0). Solid media contained 2% bacto-agar. Selective YPD media contained either 200 units ml⁻¹ hygromycin (Calbiochem), 100 µg ml⁻¹ nourseothricin (Werner BioAgents, Jena-Cospeda, Germany), or 200 µg ml⁻¹ geneticin (Gibco).

Localization using green fluorescent protein (GFP). The plasmid containing the coding sequence for GFP (accession U73901) was generously provided by John Perfect. Primers used to amplify this coding region annealed to bases 5–24 and 710–730. Overlap PCR technology (Davidson *et al.*, 2002) was used to generate the 3' GFP fusion constructs, which included the entire genomic sequence of the desired gene and ~1 kb of the upstream sequence, as well as the G418 resistance marker.

RNA extraction and cDNA synthesis. Following the appropriate treatments, 50 ml *C. neoformans* cells was collected by centrifugation at 1800 g for 5 min, washed once with distilled water, and lyophilized overnight. The lyophilized pellet was then vortexed with 3 ml glass beads (1 mm, Biospec) and resuspended in 4 ml TRIzol Reagent (Invitrogen). After sitting at room temperature for 5 min, 800 µl chloroform was added and the mixture was shaken for 30 s. This cell lysate was then centrifuged at 4000 r.p.m. for 10 min, and the supernatant was transferred to a new tube. 2-Propanol (2 ml) was added, followed by incubation for 10 min at room temperature and centrifugation at 4000 r.p.m. for 10 min. After washing with 75% ethanol, the pellet was resuspended in water and incubated with DNase I at 37 °C for 1 h. The RNA was extracted again with TRIzol and chloroform and precipitated with 2-propanol, as above. The dried pellet was resuspended in 300 µl RNase-free water (Gibco) and stored at -80 °C. First strand cDNA was made using the First Strand cDNA synthesis kit for RT-PCR (Roche).

Real-time PCR. *C. neoformans* H99 was grown in minimal media at 30 °C overnight with shaking. Exponentially growing cells were treated with *t*-butylhydroperoxide, cumene hydroperoxide, hydrogen peroxide or sodium nitrite and allowed to grow at 30 °C with shaking for 2 h. RNA was extracted and first strand cDNA made as described above. This cDNA was used as template in a real-time PCR reaction using SYBR Green PCR reagents (Sigma) according to the manufacturer's recommendations. The *GPX1* primers annealed to bases 210–231 and 857–877 of the 838 bp gene, and the *GPX2* primers annealed to bases 102–122 and 847–867 of the 859 bp gene. Base numbering relates to the genomic sequence starting with the start codon ATG. The DNA Engine Opticon (MJ Research) was

used as the fluorescence detector with the following protocol for the PCR reaction: 35 s at 94 °C, 50 s at 53 °C, 50 s at 72 °C, and a plate reading was repeated for a total of 40 cycles after a hot start of 4 min at 94 °C. A melting curve was performed at the end of the reaction to confirm a single product. A series of 10-fold dilutions of the cDNA was used in both the control and the experimental reactions. The induction data were taken from dilutions that came up 3.3 cycles apart, indicating that the reaction was in the linear range. The data were normalized to actin cDNA expression amplified in the set of PCR reactions.

Generation of deletion constructs. An overlap PCR gene deletion technology (Davidson *et al.*, 2002) was used to generate gene-specific deletion cassettes of *GPX1* (GenBank accession no. XM 570772) and *GPX2* (GenBank accession no. XM 568531) that included a nourseothricin (McDade & Cox, 2001) or hygromycin (Hua *et al.*, 2000) cassette and resulted in the deletion of the entire coding regions of the appropriate genes. The double *gpx1Δgpx2Δ* mutant was generated by deleting the *GPX2* coding sequence from the *gpx1Δ* mutant. Mutant strains were reconstituted by fusing a ~3 kb fragment that contained the *GPX1* or *GPX2* coding sequence and ~1 kb of the putative promoter sequence to a G418 (Hua *et al.*, 2000) or nourseothricin (McDade & Cox, 2001) selectable marker, and the entire construct was transformed into the appropriate mutant strains.

Transformation of *C. neoformans*. H99 and mutant strains were transformed using biolistic techniques (Toffaletti *et al.*, 1993; Hua *et al.*, 2000). Cells were grown in YPD to late exponential phase, concentrated, and plated onto YPD agar for transformation. The cells were bombarded with 0.6 µm gold beads (Bio-Rad) which were coated with DNA of the target construct according to the manufacturer's recommendations. Following the transformation, the cells were incubated at 30 °C for 4 h on non-selective media to allow for recovery, and then transferred with 0.8 ml sterile PBS to the appropriate selective media. Transformants were observed in 3–5 days.

Analysis of transformants. To isolate stable transformants, all transformants were passaged five times on non-selective YPD medium and then tested for resistance to the appropriate selective marker. Only those transformants that grew as well on the selective media as on non-selective media were used as stable transformants. A three-primer PCR screen was used to prove homologous integration on both the 5' and 3' ends of the deletion construct (Nelson *et al.*, 2003). In this manner, homologous recombinants can be distinguished from wild-type. A PCR screen using primers outside the deletion construct will amplify the entire gene region, demonstrating that a single copy of the transforming DNA has been inserted at the desired locus. Southern blots were performed to screen for single integration in the genome. Single bands were observed on all Southern blots when probed with a selectable marker-specific probe. All deletion strains generated for this work had a single deletion construct homologously integrated at the appropriate locus, and no other insertions in the genome (data not shown).

Genomic DNA preparation. Genomic DNA was prepared by a modification of the glass-bead DNA extraction protocol described by Fujimura & Sakuma (1993). *C. neoformans* cells were suspended in a microfuge tube in 500 µl lysis buffer (50 mM Tris, pH 7.5, 20 mM EDTA, 1% SDS), with 400 mg glass beads (425–600 µm, Sigma G-9268). Cells were disrupted by vortexing for 5 min, followed by a 10 min incubation at 70 °C. After brief vortexing, 200 µl 5 M potassium acetate and 150 µl 5 M sodium chloride were added. The tubes were placed on ice for 20 min and centrifuged at 14 000 r.p.m. for 20 min. The supernatant was mixed with 500 µl phenol/chloroform and spun for 2 min at 14 000 r.p.m. The aqueous phase was then mixed with 450 µl chloroform and spun for 2 min

at 14 000 r.p.m. The DNA was then precipitated by the addition of 200 μ l ethanol, washed with 70% ethanol, dried, and resuspended in 50 μ l deionized water.

Southern hybridizations. Approximately 10 μ g of genomic DNA from each strain was digested with various restriction endonucleases according to the manufacturer's recommendations. Restriction fragments were separated on a 1% agarose gel and transferred to nylon membranes using a Turbo-Blot apparatus (Schleicher & Schuell) and $10 \times$ SSC as transfer buffer. Probes for Southern analysis were prepared by random priming (random priming kit; Roche) using 50 μ Ci (1.9 MBq) [α - 32 P]dCTP (Amersham AA0005) according to the manufacturer's instructions. The blots were incubated in 10 ml of a $6 \times$ SSC, 0.1% SDS and 5% non-fat dry milk (Carnation) solution for 1 h at 65 °C, then probe was added to this solution, and the blots were hybridized at 65 °C overnight. The blots were washed twice in $2 \times$ SSC, 0.1% SDS at room temperature for 10 min and once for 10 min in $0.2 \times$ SSC, 0.1% SDS that had been prewarmed to 65 °C.

Oxidative and nitrosative stress plates. Solid minimal media were made with designated amounts of hydrogen peroxide, *t*-butylhydroperoxide, cumene hydroperoxide or sodium nitrite. *C. neoformans* strains were grown to mid-exponential phase in YNB, pH 4.0, and 10-fold dilutions were made. Aliquots (5 μ l) of the undiluted and diluted cultures for each strain were spotted onto the solid minimal media and grown at 30 °C for two nights.

Protein lysate preparation. *C. neoformans* cells were grown to mid-exponential phase in YPD at 30 °C. The cells were collected by centrifugation, washed three times in sterile PBS and resuspended in chilled lysis buffer [40 mM Tris/HCl, pH 9.0, 4% CHAPS, $1 \times$ complete protease inhibitor cocktail (Roche)] at a concentration of 2×10^9 cells ml $^{-1}$. One millilitre of cells and 2.2 g of 0.5 mm zirconium/silica beads were added together in a 2 ml tube, and the cells were disrupted on a Biospec BeadBeater for 30 s at 50 000 r.p.m., repeated seven times, alternated with 2 min on ice. The cell debris was removed by centrifugation (10 000 r.p.m. for 20 min) and syringe filtered (0.45 μ m). The supernatant was assayed using a Bio-Rad RC-DC protein assay. Typical lysates resulted in 5–7 mg protein ml $^{-1}$.

Glutathione peroxidase activity. Total protein lysates were assayed for glutathione peroxidase activity using the BIOXYTECH GPx-340 colorimetric assay (OxisResearch, Portland, OR). Briefly, the lysate is added to a solution containing glutathione, glutathione reductase and NADPH. The enzymic reaction is initiated by adding *t*-butylhydroperoxide as a substrate and the A_{340} is recorded for 5 min. The oxidation of NADPH to NADP $^{+}$, which is directly proportional to the Gpx activity, results in a decrease in A_{340} . Controls lacking lysate or glutathione solution were performed to ensure specificity of glutathione-dependent Gpx activity. Twofold dilutions of total-protein lysates were used to ensure that the activity measurements observed were in the linear range.

Macrophage assay. RAW 264.7 macrophages were diluted to 10^5 cells ml $^{-1}$ in Dulbecco's Modified Eagle Medium (DMEM). A 100 μ l volume (10^4 cells) of macrophages was plated into each well of a pre-treated microtitre dish. *C. neoformans* cells grown in YNB, pH 4.0, overnight were diluted in DMEM to 10^5 cells ml $^{-1}$. The cryptococcal cells were added to the macrophages at an m.o.i. of 1 and incubated at 37 °C and 5% CO $_2$ for 24 h. One hundred microlitres of 5% SDS was added to each well and the mixture was incubated at room temperature for 5 min to lyse the macrophages. Serial dilutions were plated on YPD agar and incubated at 30 °C for 2 days. Control wells without macrophages were done for each strain to control for growth of cryptococcal cells in DMEM media. Data presented are representative of three independent experiments.

Inhalation mouse model. *C. neoformans* strains were grown at 30 °C with shaking for two nights in YPD. The cells were centrifuged, washed in endotoxin-free PBS and resuspended in endotoxin-free PBS. The cells were counted on a haemocytometer and diluted to 1×10^7 cells ml $^{-1}$. CBA/J female mice (Jackson Laboratories) were anaesthetized and allowed to inhale 5×10^5 (50 μ l) cells, which were dripped into the nares (Cox *et al.*, 2000). Mice were weighed before and during the course of infection. Mice were sacrificed by CO $_2$ asphyxiation once they reached 80% of their original body weight. At this point, the mice showed signs of being morbidly ill, including a ruffled coat, lethargy, a hunched posture, unstable gait and loss of appetite.

RESULTS

C. neoformans encodes two glutathione peroxidases

Since the glutathione system has not been investigated in any fungal pathogen to date, we looked for the presence of glutathione peroxidases in the *C. neoformans* genome (Loftus *et al.*, 2005). By using the glutathione peroxidase conserved protein domain (NCBI GSHPx domain, pfam00255), we identified two putative GPX genes, which we have designated GPX1 and GPX2. These two genes encode glutathione peroxidases which share 49% similarity between their amino acid sequences. They also share homology with glutathione peroxidases from other organisms, including *S. cerevisiae*, *Strep. pyogenes* and human (Fig. 1). Gpx1 and Gpx2 contain the highly conserved glutathione peroxidase residues as well as many of the predicted amino acid residues that have been shown to be conserved among phospholipid hydroperoxide glutathione peroxidases (Avery & Avery, 2001). In addition, the GPX transcripts from *C. neoformans*, like those of *S. cerevisiae* and *Strep. pyogenes*, do not have the mammalian selenocysteine codon (UGA), but have a traditional cysteine codon (UGU) in its place.

GPX1 and GPX2 are differentially expressed during stress

To understand if the glutathione peroxidases in *C. neoformans* are transcriptionally regulated in response to stress, we studied the expression patterns of these two genes during peroxide and nitric oxide stress. Using real-time PCR, we observed induction of both the glutathione peroxidase genes in response to *t*-butylhydroperoxide and cumene hydroperoxide, while we only observed induction in response to hydrogen peroxide in GPX2 (Fig. 2).

Since it has been suggested that glutathione peroxidases may also catalyse the reduction of some reactive nitrogen species, we looked at the expression of GPX1 and GPX2 during nitric oxide stress, and observed down-regulation of both genes (Fig. 2), suggesting a different mechanism independent of the glutathione peroxidases for nitrosative stress resistance in *C. neoformans*.

CnGpx1	MTFPDSIAKFGYESLPGDVANKSFYDLKAKLPGSKGDL	40
CnGpx2	MSDIYSYSVEFPKSTLP-----	17
ScGpx1	MQEFPYSFSPIDENGNFPF-----	18
SpGpoA	MPNLYDFTVKAQDGSDIS-----	18
HsGpx1	MCAARLAAAAAQSVYAFSARPLAGGEP-----	29
.		
CnGpx1	FSTLKGKVVLIIVNTASKCGFTP-QYTGLEELHKTYGDKGLVVLGFPSPNEF	89
CnGpx2	LSDLKGGKTLFVNVASKCGLTP-QYKDLQALHEKYGDKGLAIIGFPCNQF	66
ScGpx1	FNSLRNKVVLIVNVASHCAFTP-QYKELEYLYEKYKSHGLVIVAFPCGF	67
SpGpoA	LVDYKEKVVLVVNTATKCGLTP-QYQALQALYDTHDKGFVLDLDFPCNQF	67
HsGpx1	LGSLRGGKVVLLIENVASLGGTTVRDYTMNELQRRLGPRGLVVLGFPNQF	79
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.		
CnGpx1	GGQEPGSDDDIAQFCTLN-----HGVTFFPLMKKSEVNGKNMNEVFAWLKS	134
CnGpx2	KAQEPGTDDEVLQFCQVN-----YGVTFPIAKKGDVNGENTQPIWKYLKE	111
ScGpx1	GNQEFKDKKEINKFCQDK-----YGVTFPIIHKIRCNQKQDPVYKFLKN	112
SpGpoA	LNQAPGDABEINHFCSLT-----YHTTFPRFAKIKVNGKADPLFTWLKE	112
HsGpx1	GHQENAKNEEILNLSLKYVRPGGGFEPNFMLEFKCEVNGAGAHPLFAFLRE	129
	* . . . * . . . * . . . * . . . * . . . * . . . * . . . *	
.		
CnGpx1	QKGENVG-----GLAGTTAIKWNFTKFLINKEGKCVGRYGS	170
CnGpx2	N-----AEPVSDIDWNFSKFLVK-DGKITWFFPAR	140
ScGpx1	SVS-----GKSGIKMIKWNFEKVVVDRNGKVVKRFSC	144
SpGpoA	EK-----SGPLGKRIEWNFTKFLIDQNGQVIKRYSS	143
HsGpx1	ALPAPSDDATALMTDPKLIWSPVCRNDVAWVNFELVLPVDPVPLRRYSR	179
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.		
CnGpx1	STKPEKLKEEIEKLL	185
CnGpx2	STKVS---DVEAAL	151
ScGpx1	MTRPLELCPPIEELLNQPPEEQI	167
SpGpoA	KTDPKLIBEDLKALLG	159
HsGpx1	RFQTIDIEPDIEALLSQGPSCA	201
	. * .	

Fig. 1. Alignment of glutathione peroxidase amino acid sequences from *C. neoformans* (*CnGpx1* and *CnGpx2*), *S. cerevisiae* (*ScGpx1* NP012899), *Strep. pyogenes* (*SpGpoA* AAK33582.1) and human (*HsGpx1* P07203). Shaded boxes indicate the conserved glutathione peroxidase triad active site, traditionally including (seleno)cysteine, glutamine and tryptophan. Similarity and identity among these five glutathione peroxidases are indicated by '.' and '*', respectively.

Glutathione peroxidase mutants are sensitive to various peroxides

To understand the importance of the glutathione peroxidase enzymes to the stress resistance of *C. neoformans*, we generated deletion mutants of each and both of these enzymes. While the *gpx* mutants are both morphologically similar to wild-type and show normal melanin and capsule production, they respond differently in the presence of peroxide stress. Both the *gpx1Δ* and *gpx2Δ* mutants are hypersensitive to cumene hydroperoxide (an aromatic hydroperoxide), but at high concentrations, the *gpx2Δ* mutant is slightly more sensitive than the *gpx1Δ* mutant

(Fig. 3). Conversely, the *gpx1Δ* mutant, but not the *gpx2Δ* mutant, is hypersensitive to *t*-butylhydroperoxide (an alkyl hydroperoxide) (Fig. 3). All phenotypes observed were complemented by reintroduction of the appropriate *GPX* gene, as shown (Fig. 3). Since we show that both enzymes are induced during various peroxide stresses, these results may suggest a difference in the localization of the two Gpx enzymes, a difference in their activity, or an alternative regulation in the deletion mutants.

Interestingly, neither the *gpx* single mutants nor the double mutant is sensitive to superoxide (data not shown), hydrogen peroxide or nitric oxide stress (Fig. 3). Various

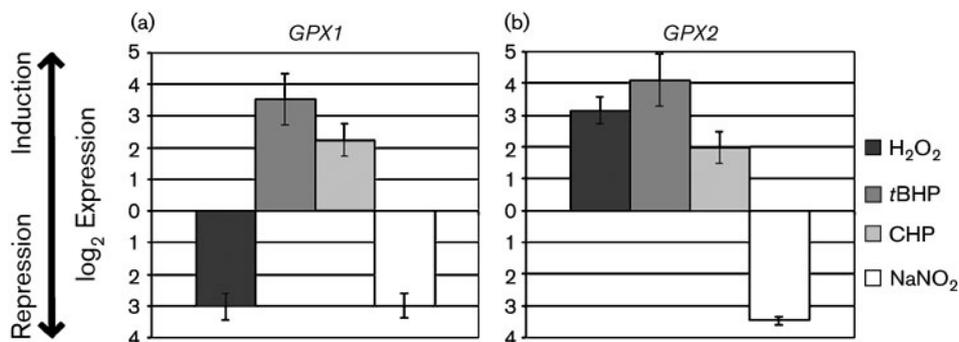


Fig. 2. GPX expression in H99 during oxidative and nitrosative stress. (a) *GPX1* and (b) *GPX2* expression in response to 250 μM hydrogen peroxide, 25 μM *t*-butylhydroperoxide (*t*BHP), 20 μM cumene hydroperoxide (CHP) or 250 μM sodium nitrite in H99.

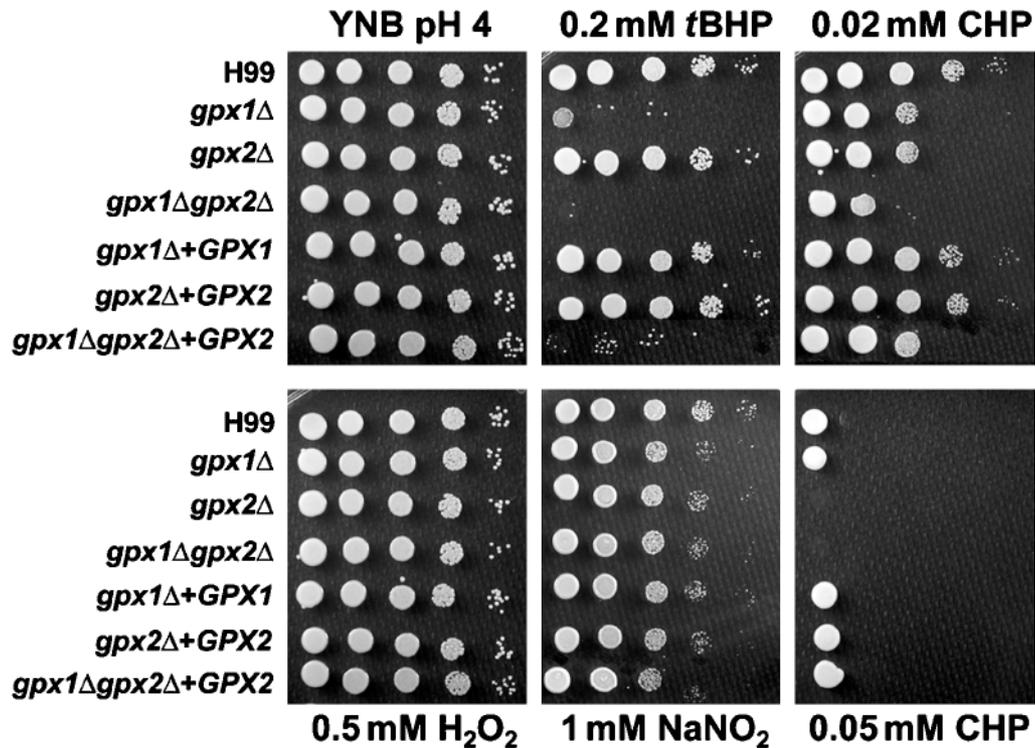


Fig. 3. *gpx* mutant phenotypes exposed to stress. Tenfold dilutions of 16 h YNB cultures were plated on YNB plates with the indicated stress. Plates were incubated at 30 °C for 48 h. Wild-type H99, glutathione peroxidase mutants and corresponding reconstituted strains are shown. *t*BHP, *t*-butylhydroperoxide; CHP, cumene hydroperoxide.

concentrations of these oxidants were used to test mutant sensitivities, with the highest concentration inhibiting wild-type growth (data not shown). The double *gpx1Δgpx2Δ* mutant shows increased sensitivities to both *t*-butylhydroperoxide and cumene hydroperoxide compared to the single mutants (Fig. 3), suggesting some functional redundancy between these peroxidases.

Gpx1 and Gpx2 are both localized to the cytoplasm

Since the *gpx* mutants show specific phenotypes to either *t*-butylhydroperoxide or cumene hydroperoxide, one hypothesis that would explain this difference is that the glutathione peroxidases are differentially localized. To determine the cellular localization of the glutathione peroxidases in *C. neoformans*, we fused the GFP coding sequence to the 3' end of the *GPX* genes and expressed these fusion constructs in the *gpx* mutants. By expressing Gpx–GFP in the *gpx* mutants, we were able to confirm the functionality of the resulting fusion proteins with *in vitro* peroxide sensitivity tests. Our localization studies show that both the functional Gpx1–GFP and Gpx2–GFP fusion proteins are localized in a similar manner in the cytoplasm throughout the cryptococcal cell (Fig. 4).

Gpx1 and Gpx2 constitute a fraction of the total cellular glutathione peroxidase activity

We measured the glutathione peroxidase activity of wild-type and *gpx* mutants to determine the contribution of each Gpx enzyme to the total glutathione peroxidase activity of *C. neoformans* under non-stressed conditions. This glutathione peroxidase activity is dependent on the presence of reduced glutathione. We observed reduced activity in the *gpx1Δ* and *gpx2Δ* single mutants and an additive reduction in the double *gpx1Δgpx2Δ* mutant (Fig. 5). We still detected residual glutathione peroxidase activity in our double *gpx1Δgpx2Δ* mutant, suggesting that other enzymes in *C. neoformans* possess glutathione peroxidase activity.

GPX expression changes are observed in *gpx* mutants

To test whether deletion of one of the *GPX* genes results in induction of the other *GPX* gene as a compensation mechanism, we used real-time PCR to examine the transcriptional expression patterns of the *GPX1* or *GPX2* gene in response to the various peroxide stresses in the *gpx2Δ* or *gpx1Δ* mutant, respectively. While the baseline level of expression of the *GPX1* gene in the *gpx2Δ* mutant and of the *GPX2* gene in the *gpx1Δ* mutant was similar to wild-type (data not shown), we did observe differences in the

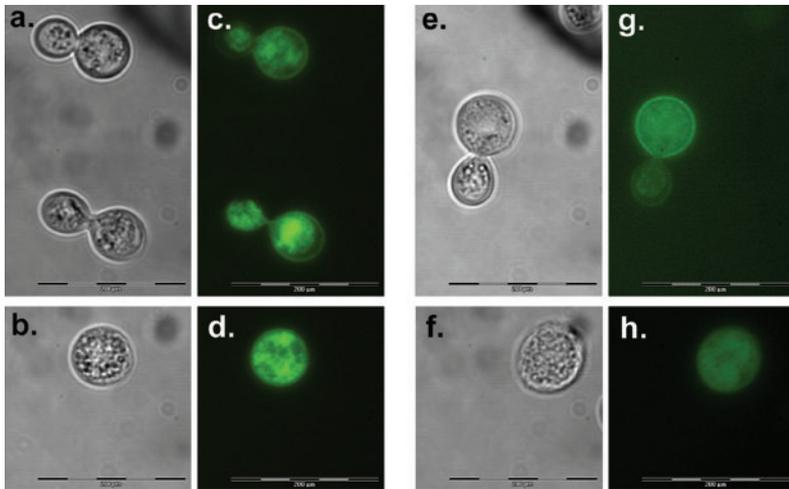


Fig. 4. GFP fusion protein localization of glutathione peroxidases. (a, b) Phase-contrast and (c, d) the corresponding GFP fluorescence of Gpx1-GFP; (e, f) phase-contrast and (g, h) the corresponding GFP fluorescence of Gpx2-GFP.

expression patterns in these mutants during stress. We found that in the *gpx1Δ* mutant, *GPX2* was induced in response to hydrogen peroxide or cumene hydroperoxide in a similar manner as in the wild-type (Fig. 6b, f), and in the *gpx2Δ* mutants, *GPX1* was highly induced in response to *t*-butylhydroperoxide or cumene hydroperoxide stress, similar to the induction observed in wild-type H99 (Fig. 6c, e). Interestingly, we observed an induction of *GPX1* in the *gpx2Δ* mutant in response to hydrogen peroxide stress (Fig. 6a), compared to the reduction in expression seen in the wild-type during this stress, suggesting a potential transcriptional compensation. In contrast, during *t*-butylhydroperoxide stress, *GPX2* was not induced in the *gpx1Δ* mutant as it was in the wild-type (Fig. 6d). This is likely to explain the specific sensitivity of the *gpx1Δ* mutant to *t*-butylhydroperoxide stress.

Glutathione peroxidase mutants are sensitive to oxidant killing by macrophages

To examine the importance of glutathione peroxidases in protection against the initial immune response of the host

during a cryptococcal infection, we infected RAW macrophages with the *gpx* mutants and with the reconstituted strains (Fig. 7). The *gpx1Δ*, *gpx2Δ* and *gpx1Δgpx2Δ* mutants were all slightly sensitive to macrophage killing compared to wild-type. Reconstituted strains partially, but significantly, complemented the sensitive phenotypes back to the appropriate parental phenotype.

Glutathione peroxidases are dispensable for virulence in *C. neoformans*

While the glutathione peroxidases appear to be important for peroxide stress and the complex oxidative environment of macrophages, we wanted to determine if they are critical to the virulence of *C. neoformans*, as this type of correlation has been shown before (Cox *et al.*, 2003; de Jesus-Berrios *et al.*, 2003). An inhalation mouse model of cryptococcosis was employed to determine the importance of glutathione peroxidases to the virulence of *C. neoformans*. We found that not only are the *gpx1Δ* and *gpx2Δ* mutants just as virulent as wild-type, but the *gpx1Δgpx2Δ* double mutant also exhibited wild-type

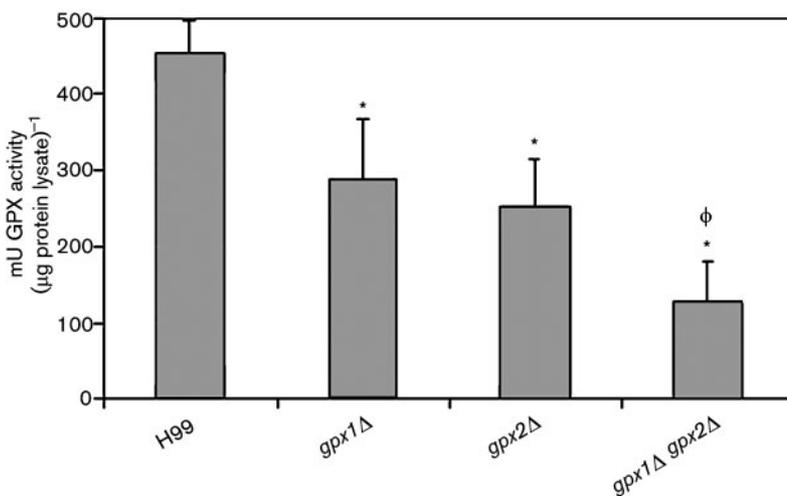


Fig. 5. Glutathione peroxidase activity in *C. neoformans*. Glutathione peroxidase activity in total protein lysates of wild-type and mutant cells. *, Significance compared to H99 ($P < 0.04$); φ, significance compared to either *gpx1Δ* or *gpx2Δ* ($P < 0.05$).

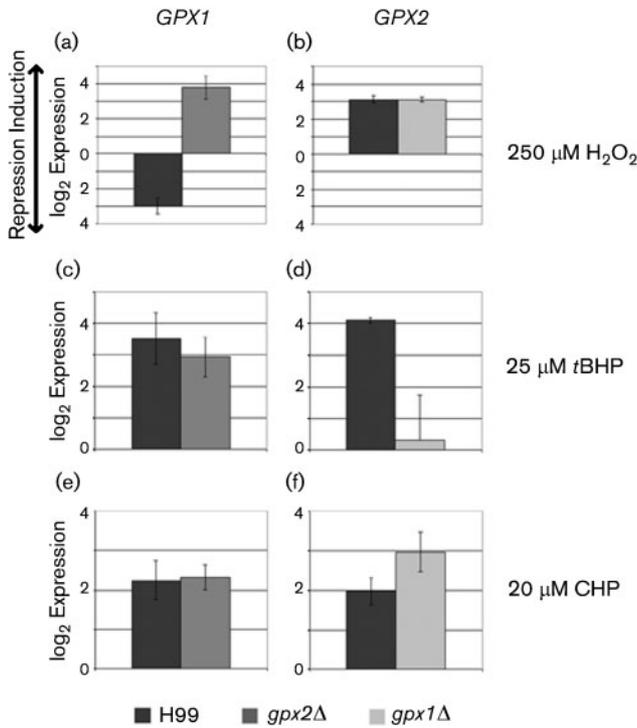


Fig. 6. GPX induction in *gpx* mutants. Left column, GPX1 expression during (a) hydrogen peroxide, (c) *t*-butylhydroperoxide (tBHP) and (e) cumene hydroperoxide (CHP) stress in H99 and *gpx2Δ* mutant. Right column, GPX2 expression during (b) hydrogen peroxide, (d) tBHP and (f) CHP stress in H99 and *gpx1Δ* mutant.

virulence in mice (Fig. 8), suggesting that the glutathione peroxidases are not necessarily compensating for each other *in vivo*. These results suggest that though the glutathione peroxidases are important for stress resistance, other antioxidant defence systems must be present *in vivo* during a

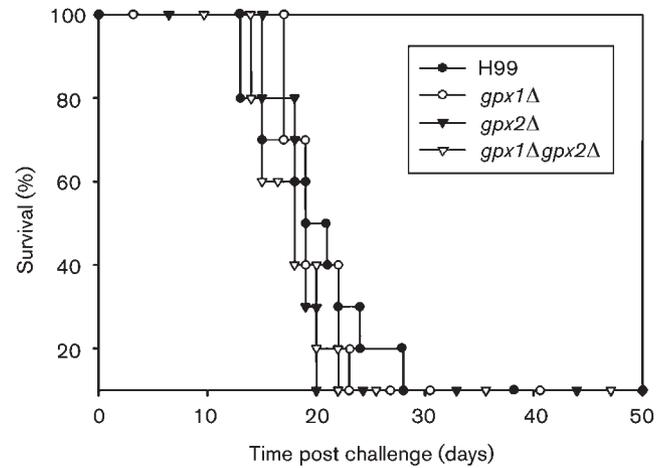


Fig. 8. Virulence of *gpx* mutants in mice. CBA/J mice were infected with 5×10^5 cryptococcal cells, as described in Methods. Ten mice were infected for each strain.

cryptococcal infection which are more important to its virulence in mice.

DISCUSSION

In this study, we have characterized two genes that encode glutathione peroxidases in *C. neoformans*, GPX1 and GPX2. We show specific expression-pattern differences between these two genes, while their protein products appear to share a similar pattern of localization within cells. Our expression analysis reveals that the glutathione peroxidases in *C. neoformans* are specifically induced during peroxide, but not nitric oxide stress. Interestingly, we do observe a difference in regulation of these two homologous genes during exposure to hydrogen peroxide. In *S. cerevisiae*, glutathione peroxidase expression is regulated by Yap1 and Skn7 (Tsuzi *et al.*, 2004), but these transcriptional activators

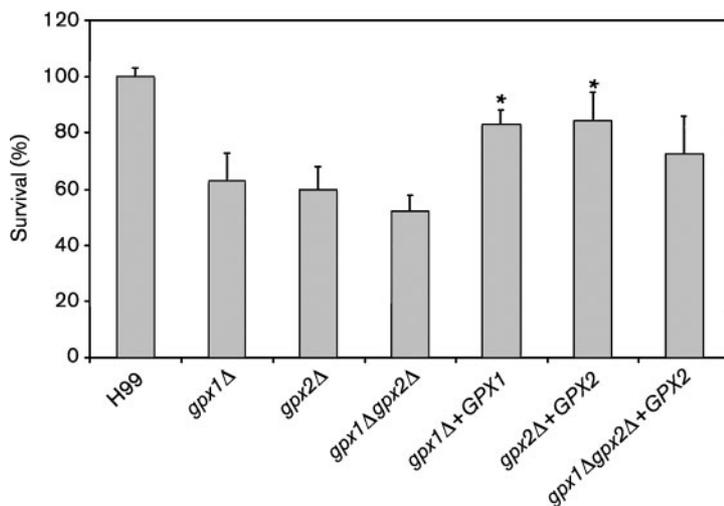


Fig. 7. Survival of *gpx* mutants in RAW macrophages. Strains were grown in YNB and incubated with macrophages at a m.o.i. of 1. The percentage of the original inoculum surviving after 24 h is shown. *, Significant difference ($P < 0.05$) compared to the respective parental mutant strain.

do not appear to have significant homologues in *C. neoformans*. Understanding the different mechanism of regulation for these peroxidases will give an insight into the stress responses of *C. neoformans*.

Based on our deletion studies, Gpx1 appears to be necessary for defence against *t*-butylhydroperoxide, while Gpx2 appears to be more important for cumene hydroperoxide stress. Neither *gpx* mutant shows sensitivity towards hydrogen peroxide stress, though we show, based on gene expression, that there is a potential for compensation in the *gpx2Δ* mutant by induction of *GPX1* mRNA, possibly due to an increased effective concentration of peroxide seen by this mutant compared to wild-type. But since the double mutant is also not sensitive to hydrogen peroxide, this suggests that another peroxide defence system in *C. neoformans*, such as catalases, glutaredoxins or other peroxidases, is more efficient and specific for the stress induced by hydrogen peroxide compared to more complex hydroperoxides. In previous work, we have shown the importance of the thiol peroxidase Tsa1 to the hydrogen peroxide stress resistance of *C. neoformans* (Missall *et al.*, 2004b).

We observe *GPX* gene induction in the *gpx* mutants during cumene hydroperoxide stress similar to that of the *GPX* genes observed in wild-type, which is consistent with our functional analysis showing additive sensitivity to this peroxide in the double *gpx1Δgpx2Δ* mutant compared to the single mutants. Interestingly, while we see wild-type induction of *GPX1* in the *gpx2Δ* mutant during *t*-butylhydroperoxide stress, we observe no induction of expression of *GPX2* in the *gpx1Δ* mutant. These data suggest that there may not be any substrate specificity, but simply changes in expression that affect the sensitivities of the mutants to peroxides. It is possible that Gpx1 is important for the regulation of *GPX2* during this specific stress, as glutathione peroxidases have been implicated in the regulation of stress responses in *S. cerevisiae* by acting as a sensor and transducer of a hydroperoxide signal to a transcriptional activator (Delaunay *et al.*, 2002).

It is possible that in addition to these changes in expression in the glutathione peroxidase mutants, there is a difference in substrate specificity between the two enzymes. Substrate specificity has been observed among similar glutathione-type peroxidases of the parasite *Trypanosoma brucei* (Schlecker *et al.*, 2005). This would help explain the increased sensitivity to high concentrations of cumene hydroperoxide stress that we observe in the *gpx2Δ* mutant. Since we observe additional Gpx-independent glutathione peroxidase activity in *C. neoformans*, we are unable to determine a substrate specificity in the whole-cell-lysate activity assay. Future studies may identify this potential substrate specificity by purifying the two glutathione peroxidase enzymes.

Our functional analysis shows that both glutathione peroxidases are important for defence against the oxidants encountered in the macrophage environment of the host,

while they are dispensable for virulence in mice. This points out the complexity of a mammalian infection involving more than the macrophage stress. There are many factors that contribute to the ability of *C. neoformans* to evade the innate immune system and many more that contribute to the ability to cause disease. Our data may simply indicate that another antioxidant system is more important for defence against the oxidative and nitrosative stress to which *C. neoformans* is exposed during infection. Since mutation of the *GPX* genes does not affect virulence or resistance to hydrogen peroxide and nitric oxide, it is plausible that these two compounds, and not the more complex alkyl or aromatic peroxides to which the *gpx* mutants are sensitive, are more important to the host defence against *C. neoformans*. Both the thiol peroxidase Tsa1, which is important for hydrogen peroxide, *t*-butylhydroperoxide and nitric oxide resistance, and the flavohaemoglobin denitrosylase Fhb1, which is important specifically for nitric oxide resistance, are important to the virulence of *C. neoformans* (de Jesus-Berrios *et al.*, 2003; Missall *et al.*, 2004b). Another possibility is that in the absence of a *GPX*, other antioxidant enzyme(s) are upregulated *in vivo* to compensate for the lack of Gpx activity. For example, in *S. cerevisiae*, 1-Cys peroxiredoxins have been shown to possess glutathione peroxidase activity (Chen *et al.*, 2000). In addition, it has been reported that glutaredoxins, which are small glutathione-dependent oxidoreductases, can act as glutathione peroxidases and reduce peroxides in *S. cerevisiae* (Collinson *et al.*, 2002). It has also been observed that during oxidative stress, there is an overexpression of two of these glutaredoxins (Collinson *et al.*, 2002). We also show that *C. neoformans* has additional enzymes with glutathione peroxidase activity, which may function *in vivo*, compensating for the absence of the two glutathione peroxidases. After the source of the additional glutathione peroxidase activity is identified, the challenge will be to measure this potentially compensatory activity *in vivo*. These and other future studies may reveal that a similar mechanism of compensation within the glutathione system also exists in *C. neoformans*. In conclusion, while both glutathione peroxidases of *C. neoformans* are dispensable for virulence, these antioxidant enzymes are necessary for resistance to specific peroxides and survival of this fungal pathogen in macrophages.

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