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The antioxidant systems in *Toxoplasma gondii* and the role of cytosolic catalase in defence against oxidative injury

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Summary

Superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxins form an antioxidant network protecting cells against reactive oxygen species (ROS). Catalase is a potent H_2O_2-detoxifying enzyme, which is unexpectedly absent in some members of the Kinetoplastida and Apicomplexa, but present in *Toxoplasma gondii*. In *T. gondii*, catalase appears to be cytosolic. In addition, *T. gondii* also possesses genes coding for other types of peroxidases, including glutathione/thioredoxin-like peroxidases and peroxiredoxins. This study presents a detailed analysis of the role of catalase in the parasite and reports the existence of antioxidant enzymes localized in the cytosol and the mitochondrion of *T. gondii*. The catalase gene was disrupted and, in addition, *T. gondii* cell lines overexpressing either catalase or a cytosolic 1-cys peroxiredoxin, TgPrx2, under the control of a strong promoter were created. Analysis of these mutants confirmed that the catalase activity is cytosolic and is encoded by a unique gene in *T. gondii*. Furthermore, the catalase confers protection against H_2O_2 exposure and contributes to virulence in mice. The overexpression of Prx2 also increases protection against H_2O_2 treatment, suggesting that catalase and other peroxidases function as a defence mechanism against endogenously produced reactive oxygen intermediates and the oxidative stress imposed by the host.

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Introduction

Reactive oxygen species (ROS) including hydrogen peroxide, the superoxide radical and the hydroxyl radical, are produced as byproducts of oxygen metabolism in all cells. In addition, immune effector cells including neutrophils, eosinophils and macrophages release superoxide anion radicals as part of the oxidative burst during a microbial infection (Callahan *et al*., 1988; McGonigle and Dalton, 1998; Nathan and Shiloh, 2000). The resulting microenvironmental oxidative stress is toxic to various parasites and, in order to counteract this, pathogens are equipped with specific ROS-detoxifying mechanisms (Miller and Britigan, 1997). All protozoan and helminth parasites (Henkle-Duhrsen and Kampkotter, 2001) examined to date contain at least some of the main antioxidant enzymes. Catalase is acting downstream of superoxide dismutase (SOD) and is a central component of the enzymatic detoxification pathways that prevent the formation of the hydroxyl radical by detoxifying H_2O_2. In addition to catalase, most aerobic cells are equipped with glutathione peroxidases (GPx) and the novel family of peroxiredoxins (Prx), which are also capable of decomposing H_2O_2 (McGonigle and Dalton, 1998; Rhee *et al*., 2001; Hofmann *et al*., 2002). Prxs are peroxidases with a molecular size of 20–30 kDa, which distribute differently in organelles, provide defence against oxidative damage and also appear to participate in signalling by controlling H_2O_2 concentration (Wood *et al*., 2003). There are two groups of Prx, depending on one or two redox active cysteine residues being present. All of them have a cysteine residue at position 47 or nearby (Cys-47), and the 2-cys-Prxs contain a second cysteine residue in the C-terminal region, named Cys-170 (McGonigle *et al*., 1998; Henkle-Duhrsen and Kampkotter, 2001). In 1-cys-PRXs, the sequence surrounding the catalytic cysteine is PVCT, whereas in 2-cys-Prxs, the active site sequence is FVCP. The 2-cys-Prxs exist as homodimers, orientated in a head-to-tail-manner (Kang *et al*., 1998a).

Toxoplasma gondii is an obligate intracellular parasite replicating inside a parasitophorous vacuole in a broad range of host cells including macrophages. This parasite expresses a superoxide dismutase (Odberg-Ferragut *et al*., 2000), a catalase (Ding *et al*., 2000; Kaasch and Joiner, 2000) and at least one peroxiredoxin (Son *et al*.,

2001). Traditionally, catalase is considered as a peroxisome marker, but the existence of peroxisomes in *T. gondii* is still a matter of debate as catalase was reported to localize in the cytosol as well as in discrete structures in the parasite (Ding *et al*., 2000; Kaasch and Joiner, 2000). The presence of a cytosolic catalase has been described in several organisms including yeast (Wieser *et al*., 1991), plants (Guan and Scandalios, 1995), *Caenorhabditis elegans* (Taub *et al*., 2003) and mammalian cells (Yamamoto *et al*., 1988). In a wide range of pathogenic bacteria, fungi and helminths, catalase is a significant virulence factor, combating the oxidative burst generated by the host (Rocha *et al*., 1996; Wysong *et al*., 1998; Ramarao *et al*., 2000; Kotze and McClure, 2001). In contrast, many protozoan parasites appear to lack a gene coding for catalase. This enzyme is absent in *Trypanosoma* (Halliwell, 1999), *Leishmania*, *Plasmodium falciparum* (http://PlasmoDB.org/) and *Theileria parva* genomes (Nene *et al*., 2000), whereas the gene is present in *T. gondii*, *Eimeria tenella* and *Neospora caninum.*

In at least some of these parasites, the presence of other ROS-detoxifying enzymes, GPxs and Prxs, is likely to contribute critically to the antioxidant defence. Some Prxs are able to decompose not only hydrogen peroxide but also alkyl hydroperoxides. In Kinetoplastida, the Prxs reduce peroxides using tryparedoxin instead of thioredoxin as the intermediate electron donor; trypanothione reductase and Prxs have been proved to be critical for the survival of *Leishmania* in macrophages (Dumas *et al*., 1997; Tovar *et al*., 1998; Castro *et al*., 2002). In the present study, we have demonstrated that *T. gondii* possesses complete thioredoxin- and glutathione-based antioxidant systems. A 1-cys-Prx (TgPrx2) and a 2-cys-Prx (TgPrx1) localize in the cytosol, and a second 2-cys-Prx (TgPrx3) is targeted to the single mitochondrion. The last enzyme is probably assisted by two mitochondrial superoxide dismutases, TgSOD2 and TgSOD3, to eliminate the ROS produced by oxidative phosphorylation. Preliminary data on Prx2 suggest that this peroxidase can protect against H_2O_2. The ablation of the catalase gene by double homologous recombination and the generation of mutants overexpressing the gene confirmed that this cytosolic enzyme confers protection against oxidative stress both *in vitro* and *in vivo*.

Results

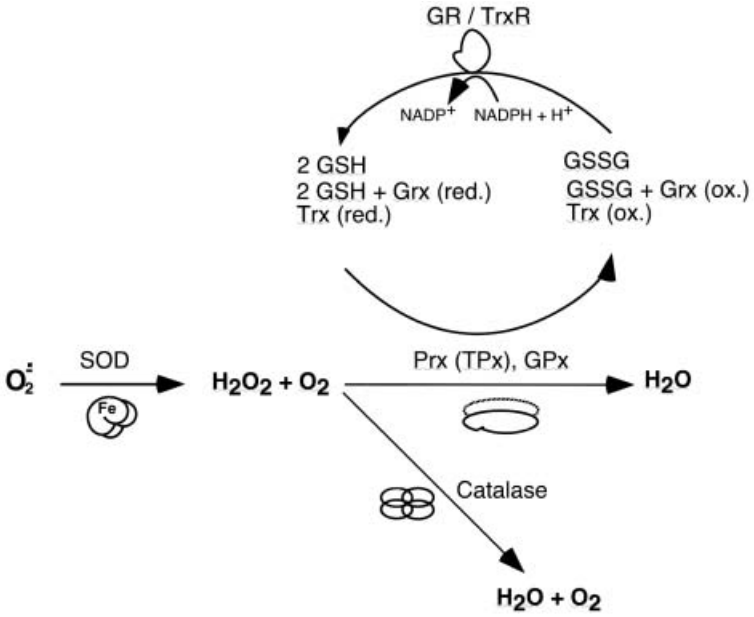
The antioxidant system of T. gondii

The progress in *Toxoplasma* genome sequencing and the generation of more stage-specific ESTs have provided a considerable source of information. We undertook a database mining approach to define the network of antioxidant enzymes present in *T. gondii*. The parasite possesses superoxide dismutases, which catalyse the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. Apart from catalase, peroxiredoxin-type peroxidases are present and also potentially involved in the degradation of hydrogen peroxide (Fig. 1A). The *T. gondii* genes identified so far are listed in Fig. 1B. The subcellular distribution of the gene products is indicated when it has been determined experimentally, and the stage specificity of expression is based on the abundance of stage-specific ESTs (http://www.ebi.ac.uk/blast2/parasites.html).

Toxoplasma gondii *possesses two mitochondrial superoxide dismutases*

Toxoplasma gondii tachyzoites express a cytosolic superoxide dismutase (TgSODB1) that was characterized previously (Odberg-Ferragut *et al*., 2000). Two additional SOD genes are present in the parasite genome and EST databases. The amino acid sequence alignment of these SODs with *P. falciparum*-derived PfSOD1 (Baert *et al*., 1999) revealed a very high level of conservation (Fig. 2A). In contrast to mammals, which possess Cu/ZnSOD and MnSOD but lack FeSOD, these apicomplexan enzymes contain the conserved residues characteristic for the utilization of iron as metal cofactor. Interestingly, TgSOD2, TgSOD3 and a second SOD present in the annotated *Plasmodium* genome exhibit large N-terminal extensions compared with TgSOD1 and PfSOD1 (Fig. 2A). Although TgSOD3 possesses a classical mitochondrial targeting signal (according to the programs http://www.cbs.dtu.dk/services/TargetP/and MitoProt), TgSOD2 presents a rather unusual extension preceded by a putative signal peptide targeting the enzyme to the endoplasmic reticulum. This configuration is reminiscent of the bipartite targeting signal for the apicoplast (Waller *et al*., 1998). The subcellular localization of these proteins was determined by the generation of stable parasite lines expressing TgSOD2 and TgSOD3 carrying a Ty-1 epitope tag inserted at their C-terminus. The cDNA for SOD2 was amplified by reverse transcription polymerase chain reaction (RT-PCR) using tachyzoite total RNAs as template. In contrast, the cDNA corresponding to TgSOD3 could not be amplified from tachyzoites, and the genomic sequence was used for vector construction. Both transgene products were targeted into the single tubular mitochondrion of *T. gondii* as demonstrated by indirect immunofluorescence assay (IFA) (Fig. 2B). *TgSOD1* and *TgSOD2* are transcribed in tachyzoites based on the abundance of stage-specific ESTs and successful RT-PCR amplifications. In contrast, all ESTs specific to *TgSOD3* were restricted to the partially sporulated oocysts, and no RT-PCR product was obtained from tachyzoite total RNAs.

A



B

Antioxidant systems in *Toxoplasma gondii*

Protein	Name	Accession	References	Stage	Localization
Superoxide- dismutase	SODB1	AF029915	Odberg-Ferragut et al 2000	tachyzoite/bradyzoite	cytosolic
	SOD2	AY176062	this study	constitutive	mitochondrial
	SOD3	AY254045	this study	sporulated oocyst	mitochondrial
Catalase	CAT	AF161267	Kaasch and Joiner, 2000 Ding et al 2001, this study	constitutive	peroxisomal? cytosolic
Glutathione reductase	GR	AF041450			
Thioredoxin reductase	TrxR?	AA519618			
Thioredoxin II	Trx	BG657266	Rahlfs & Beckers, 2001	constitutive	cytosolic?
Glutaredoxin	Grx1	BM131493			
	Grx2	BM040167		sporulated oocyst	
Gluthathione/thioredoxin peroxidase	GPx1?	AY043228		tachyzoite/bradyzoite	
	GPx2?	BM039715		sporulated oocyst	
Peroxiredoxins 2-cys	Prx1	AF305718	Son et al., 2001	constitutive	cytosolic
	Prx3	AY251021	this study	constiutive	mitochondrial
Peroxiredoxin 1-cys	Prx2	AF397213	this study	tachyzoite/bradyzoite	cytosolic

Fig. 1. *T. gondii* antioxidant systems.
A. Pathways for the detoxification of reactive oxygen intermediates (superoxide anion radical, O_2^- and hydrogen peroxide, H_2O_2) by superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), thioredoxin peroxidase (TPx) and peroxiredoxins (Prx). When peroxidases are coupled to sulphydryl reducing systems such as thioredoxin (Trx) or glutathione (GSH) with or without glutaredoxin (Grx), glutathione disulphide (GSSG) and oxidized thioredoxin are reduced by glutathione reductase (GR) and thioredoxin reductase (TrxR), respectively, at the expense of NADPH.
B. Genes encoding glutathione- and thioredoxin-based antioxidant proteins present in *T. gondii.* The accession numbers; reference articles; life stage specificity of expression based on the abundance of ESTs and the subcellular localization (when determined experimentally) are included.

A

```
                    10         20         30         40         50         60
                    |          |          |          |          |          |
PfSOD       ------------------------------------------------------------
TgSOD1      ------------------------------------------------------------
TgSOD2      MSITAVLVPALRLSAALVAAPIGLALETAALTTSHGPRQVTSRFLHLCPPSASLSSCNKT
TgSOD3      ---------------------------------MFAAVARRTATGTLPGAVTLFGRKS

                    70         80         90         100        110        120
                    |          |          |          |          |          |
PfSOD       ------------------------MVITLPKLKYALNALSPHISEETLNFHYNKHHAGYV
TgSOD1      ------------------------MVFTLPPLPYAHDALAPHISSETLQFHHGKHHAGYV
TgSOD2      VQGRIAHVRSPYLQRDAHCFSTNACAFTLPPLPYAEDALAPHISAETLRFHHGKHHAAYV
TgSOD3      FEGYYRDYPLHPRNFGVMASLFSTGKFELPALPYDYNALEPYISAKTLKFHHDKHHATYV

                    130        140        150        160        170        180
                    |          |          |          |          |          |
PfSOD       NKLNTLIKDTPFAEKSLLDIVKESSGAIFNNAAQIWNHTFYWDSMGP---DCGGEPHGEI
TgSOD1      AKLNGFIEGTAFAGKTLEEVIRTSTGAIFNNAAQVWNHTFYFSSMKPPMSGGGGEPTGRL
TgSOD2      NKLNGFIQGTPFADKTLEDIVKNSSGAIFNNAAQAWNHEFYFKSMKPASAGGGGEPDGLL
TgSOD3      KNLNELIKGTDYENQKLEDIIRNADGPTFNNAAQAWNHTFFWNSMKP---NGGGEPTGLV

                    190        200        210        220        230        240
                    |          |          |          |          |          |
PfSOD       KEKIQEDFGSFNNFKEQFSNILCGHFGSGWGWLAL-NNNNKLVILQTHDAGNPIKDNTGI
TgSOD1      LDEIKKEFTSVENFKDEFSKVAAGHFGSGWAWLVWDKQGKKVGIEQTHDAGTPITPMKV
TgSOD2      KKEIEAAFSSFAQFKDEFSKLAAGHFGSGWAWIVWDKQQKKIVLEQTHDANTPITDTMKV
TgSOD3      KEQIDRCFSSFSKYKEEFSEKATKHFGSGWAWLVWCNEKRRLEVIETHDAGNPIT-MKKW

                    250        260        270        280        290
                    |          |          |          |          |
PfSOD       PILTCDIWEHAYYIDYRNDRASYVKAWWNLVNWNFANENLKKAMQK-----------
TgSOD1      PLLCCDVWEHAYYLDRKNDRPAYIKAWWNVVNWDFASKNLENALK------------
TgSOD2      PLLCCDVWEHAYYLDKKNDRPAYIEGWWSVVNWGFAAENLVKALESN----------
TgSOD3      PIITCDLWEHGYYLDRQNDRGAYVKSWWSVVNWDFANEKLEKAMRGDPIDETVGGGQ
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B

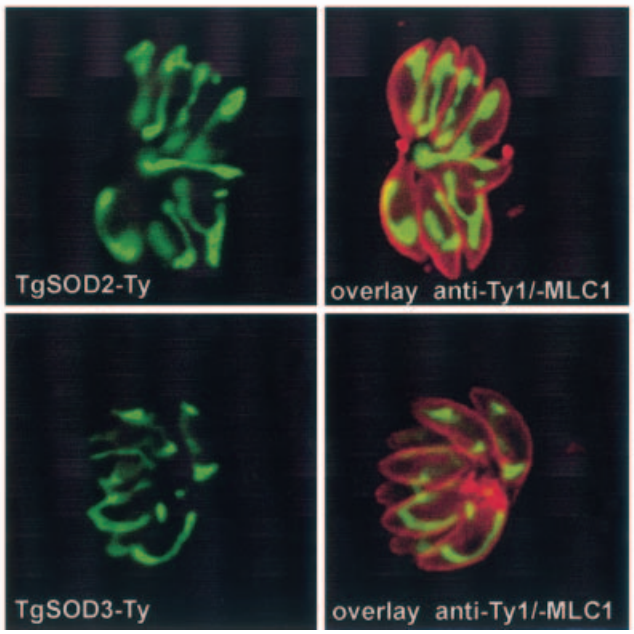

Fig. 2. *T. gondii* superoxide dismutases.
A. Amino acid sequence alignment of the *T. gondii* and *P. falciparum* superoxide dismutases. *TgSOD2* and *TgSOD3* genes were identified in the *T. gondii* genome database, and their predicted amino acid sequence revealed an amino-terminal extension compared with SOD1. The iPSORT WWW Service predicted a mitochondrial targeting signal for TgSOD3 but not for TgSOD2, which appears to be composed of a hydrophobic N-terminal sequence resembling a putative ER signal peptide (sequence underlined) followed by an extension. TgSOD1, AC AF029915; TgSOD2, AC AY176062; TgSOD3, AC AY254045; PfSOD Z49819. Line boxes represent the residues crucial for the distinction between FeSODs and MnSODs (Baert *et al.*, 1999).
B. HFF cells were infected with tachyzoites expressing TgSOD2Ty and TgSOD3Ty. The localization of the SODs was documented by double IFA and confocal microscopy, using anti-Ty1 and anti-MLC1 (*T. gondii* myosin light chain 1) antibodies. MLC1 serves as marker of the pellicle of the parasites (Herm-Gotz *et al.*, 2002).

Identification of other H_2O_2-removing enzymes in T. gondii

Three Prx genes are present in the *T. gondii* genome sequence database, including a 2-cys-Prx, named here TgPrx1 and described previously (Son *et al.*, 2001). A second 2-cys-Prx gene, *TgPrx3*, codes for a 283-amino-acid polypeptide containing a putative mitochondrial targeting signal. The predicted cleavage site generates an N-terminal presequence of 82 amino acids. The two *T. gondii* 2-cys-Prx genes were aligned with the cytosolic and mitochondrial *P. falciparum* genes PfTPx1 and PfTPx2 respectively (Rahlfs and Becker, 2001) (Fig. 3A). As in *P. falciparum*, a 1-cys-Prx gene (TgPrx2) is also present in the *T. gondii* genome, and its amino acid sequence was aligned with the Pf1-cys-Prx (Kawazu *et al.*, 2000) (Fig. 3A). The deduced amino acid sequence of TgPrx2 suggested a cytosolic localization. The subcellular localization of TgPrx2 and TgPrx3 was verified experimentally. The cDNAs corresponding to Prx2 and Prx3 were used to express the protein with a C-terminal Ty-1 epitope tag. Parasite cell lines expressing these transgenes were analysed by IFA, which confirmed that TgPrx3

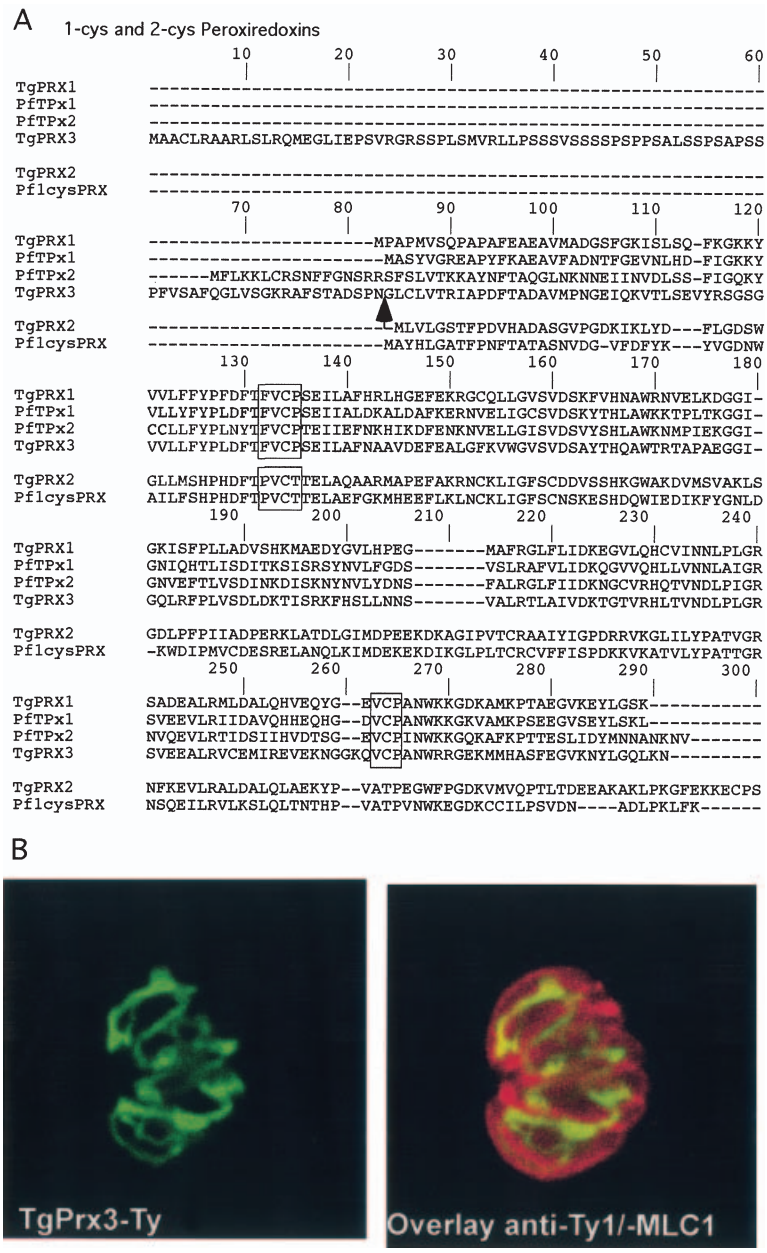

Fig. 3. *T. gondii* peroxiredoxins.
A. Amino acid sequence alignment of *T. gondii* 1-cys and 2-cys peroxiredoxins with the thioredoxin peroxidases (TPx) and 1-cys peroxiredoxin of *P. falciparum*. The residues conserved around the catalytic site are boxed. TgPrx1 and TgPrx3 are 2-cys peroxiredoxins. TgPrx2 is a 1-cys peroxiredoxin. TgPrx3 was identified in the *T. gondii* genome database. The predicted amino acid sequence of the *Prx3* gene exhibits an amino-terminal extension, corresponding to a mitochondrial targeting signal. The predicted cleavage site for the targeting signal on TgPrx3 is indicated by an arrow. PfTPx1, AC AF225977; PfTPx2, AC AF225978; TgPrx1, AC AF305718; TgPrx3, AC AY251021; TgPrx2, AC AF397213; Pf1-cysPrx, AC Q9XXW9).
B. Targeting of TgPrx3 to the mitochondrion was determined by double IFA using anti-Ty1 and anti-MLC1 on intracellular tachyzoites stably transformed with the expression plasmid pT8Prx3Ty-HX.

is targeted exclusively to the mitochondrion (Fig. 3B) and TgPrx2 is cytosolic (Fig. 4B) with a staining signal reinforced at the periphery of the parasite.

Cloning and characterization of T. gondii *Prx2*

Rabbit polyclonal antibodies raised against a peptide corresponding to the predicted C-terminal amino acid sequence of Prx2 (amino acids 193 to 209) enabled us to characterize the expression of Prx2 in *T. gondii*. A 25 kDa protein was detected by Western blot analysis of *T. gondii* lysate (Fig. 4A). No cross-reaction was visible with the host cell lysates (data not shown). The abundance of Prx2 was identical in the parental and mutant strains lacking or overexpressing catalase, which indicated that the absence of catalase was not compensated by an enhanced level of Prx2. The cytosolic localization of endogenous Prx2 was confirmed by IFA (Fig. 4B). At least one additional copy of *TgPrx2* controlled by the tubulin 1 promoter was stably introduced in *RHhxgprt*$^-$ to create *RHPrx2ov*. The overexpression of Prx2Ty protein was confirmed by Western blot analysis (Fig. 4A). A higher molecular weight product of ≈50 kDa was detectable in all *RHPrx2ov* and *RHhxgprt*$^-$ strains. Analysis of cell lysates by Western blot after separation on SDS-PAGE under non-reducing conditions revealed that Prx2 now

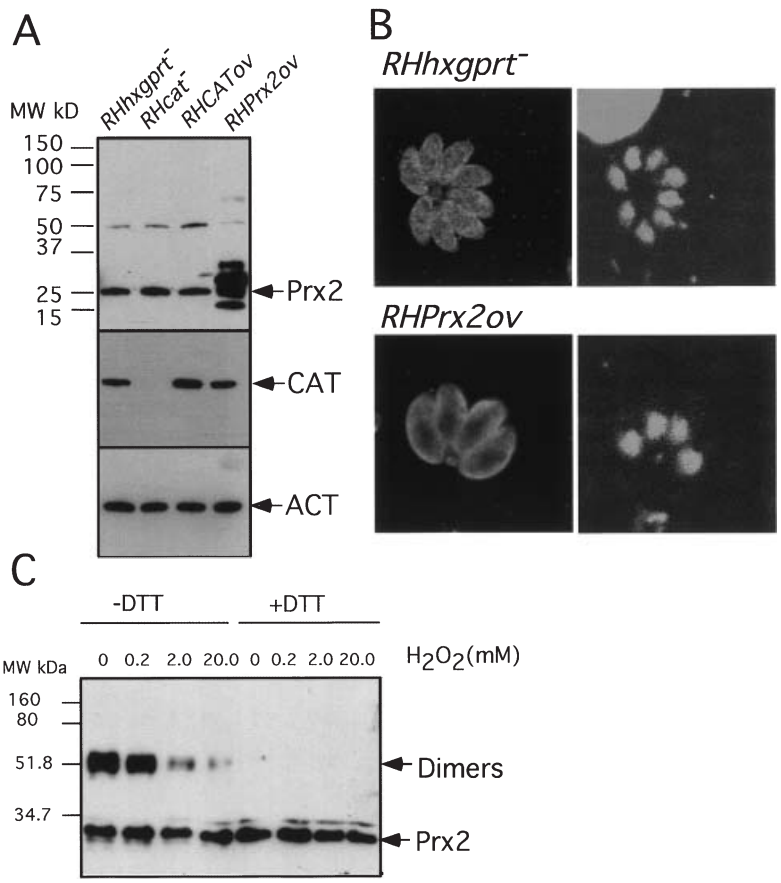

Fig. 4. Characterization of Prx2 expression and localization in *T. gondii*.
A. Immunoblot analysis of parental and mutant strains using antibodies raised against a specific peptide in the C-terminal region of TgPrx2. The same blot was reprobed with antibodies against *T. gondii* catalase and actin. In the RHPrx2ov, Prx2Ty migrated slightly higher than the endogenous protein because of the addition of the Ty-1 tag, but the additional higher and lower bands detected are not explained.
B. Expression and cytosolic distribution of Prx2 was confirmed by IFA on intracellular tachyzoites by confocal microscopy. Overexpression of Prx2 in *RHPrx2ov* revealed a more intense as well as a more peripheral distribution of the protein (left). DAPI staining showed the localization of the parasite nucleus (right).
C. Further analysis of Prx2 was performed by treating extracellular parasites with increasing concentrations of H_2O_2 for 1 h. Total extracts were analysed on SDS-PAGE with or without DTT present in the loading buffer.

migrated as a diffuse band at twice the predicted size (Fig. 4C). When extracellular parasites were treated with increasing doses of H_2O_2 for 1 h, the 50 kDa product disappeared. Similar results were obtained with bacterial recombinant protein (data not shown). These results suggest that the 50 kDa product is generated by the dimerization of Prx2.

Overexpression of Prx2 increased resistance to H_2O_2

To examine the functional role of Prx2 in *T. gondii*, the ability of *RHPrx2ov* to resist H_2O_2 treatment was measured. To avoid any interference by the host cell detoxifying systems and damage of the host cells, freshly released extracellular parasites were treated with H_2O_2, washed and then allowed to infect human foreskin fibroblast (HFF) cells in normal media. In these experiments, the total number of parasites that established an infection was expressed as a percentage of the number of vacuoles in untreated parasites (Fig. 5A). Subsequently, replication of parasites was assessed by counting the number of parasites per vacuole in cells infected with *RHPrx2ov* or *RHhxgprt⁻* with or without H_2O_2 pretreatment (Fig. 5B). These experiments revealed that the percentage of vacuoles containing only one parasite increased significantly after treatment of *RHhxgprt⁻*, whereas the *RHPrx2ov* was unaffected by the treatment (Fig. 5B).

Generation and characterization of parasite mutants lacking the catalase gene

The disruption of the catalase gene was achieved by homologous recombination of a vector containing an HXGPRT-expressing cassette flanked by 1.7 kbp and 2.7 kbp of the 5′ and 3′ flanking sequence of the catalase gene respectively. Western blotting of parasite lysates confirmed that the 57 kDa protein corresponding to catalase was detectable in *RHhxpgrt⁻* but was absent in *RHcat⁻* mutants (Fig. 6A). The *RHcat⁻* strain was complemented by co-transfection of a vector expressing catalase under the control of tubulin 1 and using the chloramphenicol acetyltransferase gene as a marker of selection to generate *RHcat⁻com*. The strain expressed more catalase than wild-type parasites possibly because of the integration of more than one copy of the transgene or because the tubulin promoter is stronger than the catalase gene in controlling sequences. The vector was also integrated in *RHhxpgrt⁻* and gave rise to the *RHCATov* mutant, which contained more than one copy of the *CAT* gene. Western blot analysis confirmed that *RHcat⁻com* and *RHCATov*

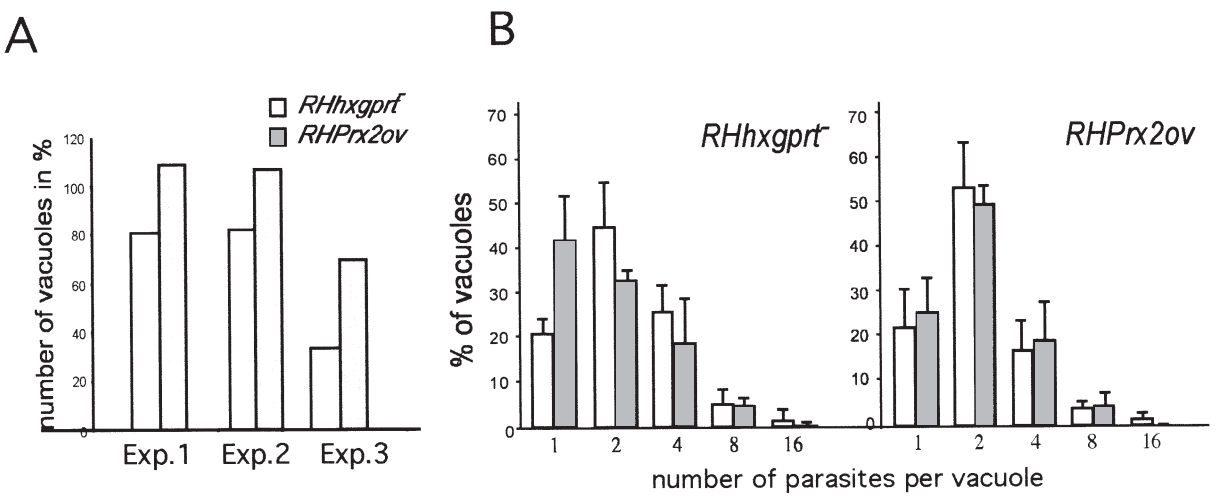

Fig. 5. Effect of H_2O_2 on parental *RHhxgprt⁻* and *RHPrx2ov* mutant strains.
A. Freshly released tachyzoites were incubated for 3 h in the presence of 200 μM H_2O_2, inoculated onto monolayers of HFF cells and incubated for 24 h in normal medium. Twenty-four hours after infection, the numbers of vacuoles were counted by scoring multiple fields under the microscope. Three independent experiments are presented, and the data are expressed as a percentage of the number of vacuoles formed by H_2O_2-treated parasites compared with untreated control parasites (extracellular parasites were incubated for 3 h in the presence of 2×10^{-4} M H_2O_2, washed and added to host cells).
B. The rate of parasite replication was compared between H_2O_2-treated and untreated parasites by counting the numbers of parasites per vacuole (more than 200 vacuoles were analysed for each strain and condition). The data are expressed as percentage of vacuoles containing one, two, four, eight and 16 parasites. Mean values of the three independent experiments are shown. Error bars indicate the standard deviations. The grey and white bars correspond to the treated and untreated parasites respectively.

expressed at up to threefold excess of catalase protein compared with *RHhxpgrt⁻* parasites (Fig. 6A). IFA performed on *RHhxpgrt⁻* and *RHcat⁻* strains using several anti-catalase antibodies demonstrated consistently that the diffuse cytosolic staining was the only specific labelling that corresponded to the catalase. In contrast, the various punctuated staining patterns observed previously (Ding *et al*., 2000; Kaasch and Joiner, 2000) persisted in the *RHcat⁻* mutant and therefore were unspecific (Fig. 6B). The *RHcat⁻com* and *RHCATov* mutants also showed an unambiguous cytosolic staining, considerably more intense than in the parental strain (Fig. 6C). The replacement of the *CAT* gene with *HXGPRT* was also obtained in the Prugniaud (*PRU*) strain. PRU is a type II cyst-forming avirulent strain of *T. gondii* that is more appropriate than RH for *in vivo* studies. Initially, the parental *PRUhxgprt⁻* mutant used to generate *PRUcat⁻* was obtained by homologous recombination (M. Soete *et al*., unpubl.) as described previously (Donald *et al*., 1996). The absence of catalase in *PRUcat⁻* was confirmed by Western blot and IFA (data not shown).

Catalase activity was measured in whole-cell extracts from extracellular tachyzoites of the parental *RHhxgprt⁻*, *PRUhxgprt⁻* strains and the *RHcat⁻*, *RHCATov* and *PRUcat⁻* mutant strains. Both *RHcat⁻* and *PRUcat⁻* mutants were essentially deficient in catalase activity, whereas *RHCATov* and *RHcat⁻com* showed 1.5- to twofold increased catalase activities compared with *RHhxgprt⁻* (Ding, 2002) and consistent with the increased level of protein (Fig. 6A). These results confirmed that the *CAT* gene expresses a classical and functional enzyme and is the only source of catalase activity in *T. gondii.* This was expected, as the fivefold coverage of the *T. gondii* genome sequence contains only a single catalase gene. We also tested extracts from all the parasite lines for glutathione peroxidase (GPx) activity. Despite the fact that several genes potentially coding for glutathione peroxidase are present in the database (Fig. 1B), no activity was detectable over background level in tachyzoites, using cumene hydroperoxide as substrate (data not shown).

RHcat⁻ *mutants are more susceptible to treatment with* H_2O_2 *than wild-type parasites*

The cytosolic localization of catalase suggests a potential role in protecting the parasites against oxidative stress produced by the host cell. *In vitro* assays were performed to investigate the phenotypic consequences of either the absence or the overexpression of catalase in parasites treated with H_2O_2. Parasite invasion and multiplication were determined 48 h later by counting the number of vacuoles containing more than four parasites (Fig. 7A). All strains were killed at concentrations of 10^{-2} M H_2O_2 but were differently susceptible at lower concentrations. The *RHcat⁻* mutants were sensitive to 50 μM H_2O_2, whereas

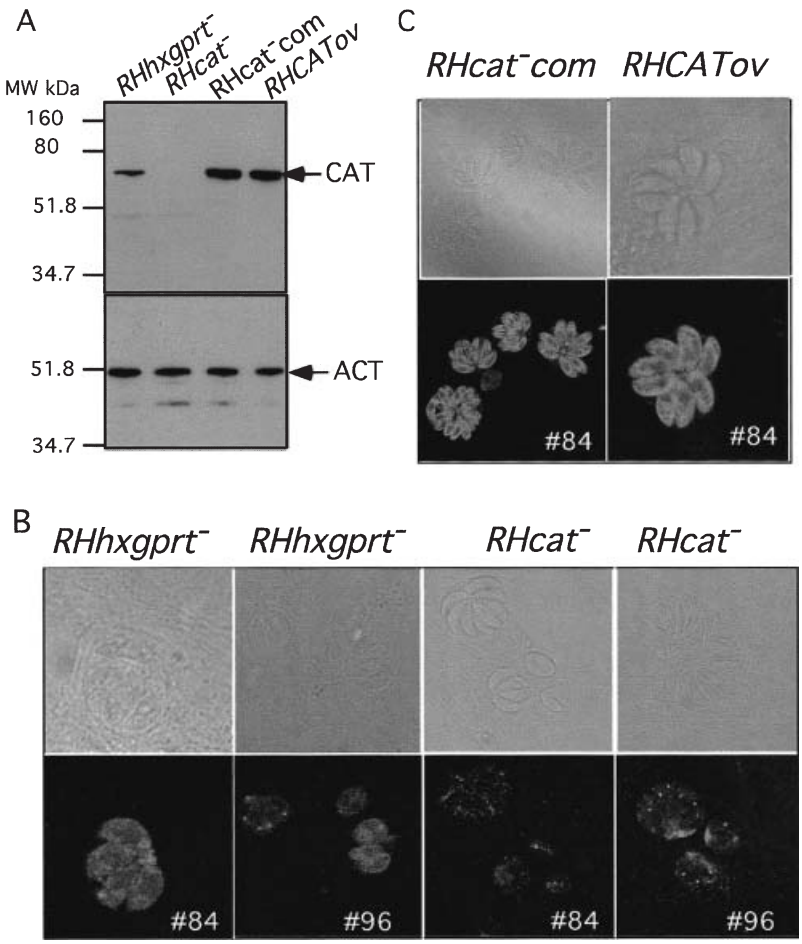

Fig. 6. Characterization of *T. gondii* catalase mutant strains.
A. Total lysates of freshly released 5×10^6 tachyzoites from control (*RHhxgprt*⁻) and catalase knock-out (*RHcat*⁻), catalase knock-out complemented (*RHcat*⁻*com*) and catalase overexpressor (*RHCATov*) were analysed by immunoblot using rabbit antisera raised against *T. gondii* catalase. The blot was stripped and probed again with anti-actin or anti-MIC4 antibodies as a control for equal loading. CAT and ACT correspond to catalase and actin respectively.
B. Subcellular localization of catalase in *RHhxgprt*⁻ and *RHcat*⁻ documented by confocal microscopy including phase contrast. Tachyzoites were inoculated into host cell monolayers and grown for 24 h followed by IFAs using rabbit polyclonal antibodies 84 and 96 against catalase.
C. Subcellular localization of catalase in *RHcat*⁻*com* and *RHCATov* detected with antisera 84.

the other strains were not significantly affected at this concentration (Fig. 7A). The parental *RHhxgprt*⁻ strain was susceptible to 100 μM H_2O_2 but, in comparison, both *RHCATov* and *RHcat*⁻*com* were notably more resistant. These results were confirmed by plaque assays, which monitored parasite survival up to 1 week after treatment (data not shown). The increased susceptibility of *RHcat*⁻ to H_2O_2 treatment compared with the *RHhxgprt*⁻ strain illustrates that CAT is required for an optimal infectious cycle of *T. gondii*. To clarify further whether CAT is important for the immediate survival of the parasite and its invasion of host cells or multiplication in the parasitophorous vacuole, complementary experiments were undertaken. Extracellular parasites treated for 3 h with 200 μM H_2O_2 were again allowed to infect host cells. Twenty-four hours later, the total number of vacuoles and the number of parasites per vacuole were determined in treated and untreated parasites. Both *RHhxgprt*⁻ and *RHcat*⁻ exhibited a severe reduction in immediate survival with only 20–40% of invasion after H_2O_2 treatment, compared with *RHCATov* and *RHcat* (Fig. 7B). The distribution of the number of parasites per vacuole was scored in order to compare the rate of replication between treated and untreated parasites (Fig. 7C). In *RHcat*⁻, 70% of the vacuoles contained only one parasite, and no vacuole contained more than eight parasites compared with untreated parasites. In conclusion, parasites lacking catalase were mostly unable to replicate even if they were not immediately killed by H_2O_2 and still able to invade host cells. In contrast, parasites expressing normal amounts of catalase replicated efficiently intracellularly, although their invasiveness was clearly affected by H_2O_2 treatment. The overexpression of catalase conferred to the parasites an enhanced resistance to H_2O_2 leading to an increased fitness and invasiveness.

Parasites lacking catalase have reduced virulence in mice

The phenotypic consequences for the parasites lacking the *CAT* gene were examined in the mouse model of lethal toxoplasmosis. RH is a type I virulent strain of *T. gondii* that typically kills mice with a LD_{100} of a single infectious

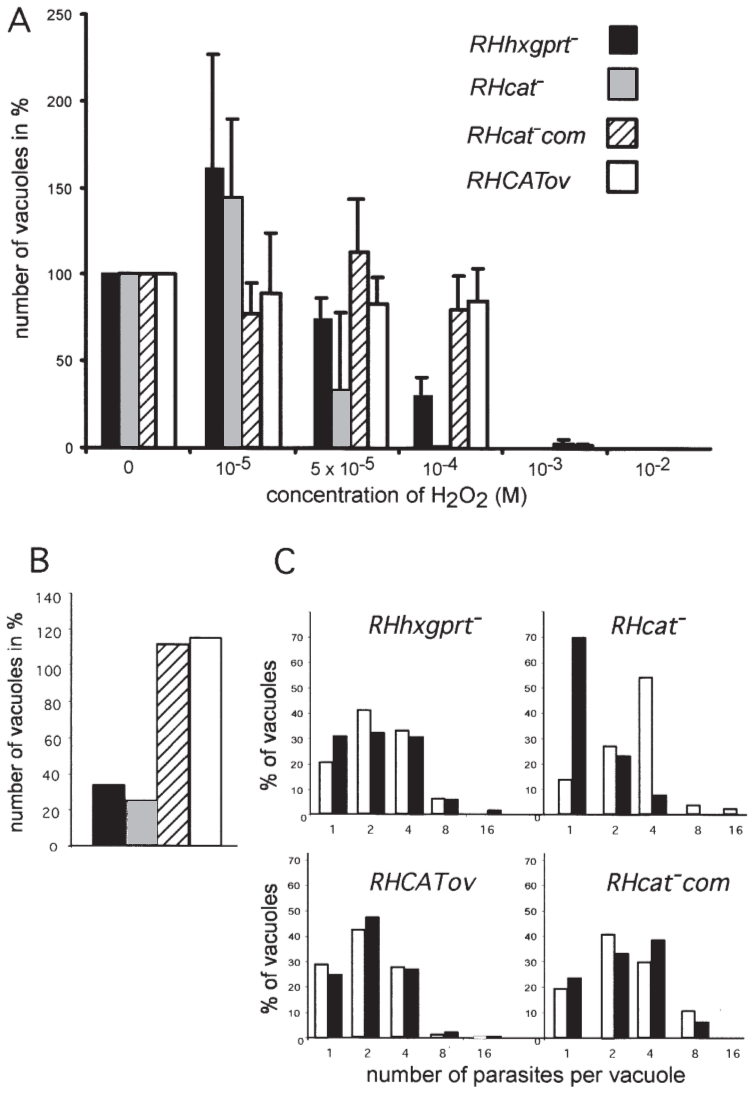

Fig. 7. Effect of hydrogen peroxide (H_2O_2) on parental and mutant strains of *T. gondii*.
A. Freshly released tachyzoites of the different strains were incubated for 1.5 h in the presence of increasing concentrations of H_2O_2 as indicated on the *x*-axis. The percentage of vacuoles containing more than four parasites in treated compared with untreated parasites was scored after 48 h.
B. Freshly released tachyzoites were incubated for 3 h in the presence of 200 μM H_2O_2. The inoculate was transferred onto monolayers of HFF cells and incubated in normal medium. To determine the parasite survival after treatment, the number of vacuoles was counted by scoring multiple fields under the microscope 24 h after infection. The data are expressed as a percentage of the number of vacuoles in H_2O_2-treated parasites compared with untreated control parasites (the same code was used for each strain as in A).
C. The rate of replication of parasites that successfully invaded the host cells was then determined. The number of parasites per vacuole with and without H_2O_2 treatment was counted for all four mutants (more than 200 vacuoles were analysed for each condition and strain). The data are expressed as a percentage of vacuoles containing one, two, four, eight and 16 parasites. Graphs show one representative experiment, which was reproduced three times with similar results. The grey and white bars correspond to the treated and untreated parasites respectively.

organism within 7 days (Pfefferkorn and Pfefferkorn, 1976). We therefore decided to evaluate the impact of catalase knock-out in the Prugniaud strain. This strain has a much higher but also more variable LD_{100} than RH. In addition to *PRUcat*⁻, a clone of *PRUhxgprt*⁻ transfected with the plasmid encoding the *HXGPRT* gene but with an intact *CAT* locus was used as a control (*PRU*). Mice (C57BL/6) were infected with three doses of each strain, and the results of two independent experiments are presented (Fig. 8). Both experiments showed comparable results. The rate of death of infected mice was dependent on the infection dose, and mice infected with *PRU* reproducibly began to succumb to the infection significantly earlier than mice infected with *PRUcat*⁻. Furthermore, notably more mice survived in the groups infected with *PRUcat*⁻ compared with those infected with *PRU* parasites beyond 17 days after infection. These results indicated that the disruption of the catalase gene led to an attenuated virulence of the parasites in mice.

Discussion

ROS are generated by the incomplete reduction of oxygen during respiration in mitochondria and as side-products of a variety of metabolic reactions in cytoplasm, peroxisomes, endoplasmic reticulum and plasma membrane. All aerobic organisms require mechanisms that limit molecular damage caused by ROS and, additionally, intracellular pathogens must protect themselves against the oxidative burst imposed by the host. *T. gondii* is able to infect virtually all cell types including immune cells such as macrophages, eosinophils and neutrophils. *In vitro* assays have implicated the role of ROS in the toxoplasmacidal activity of human monocytes and interferon gamma-activated

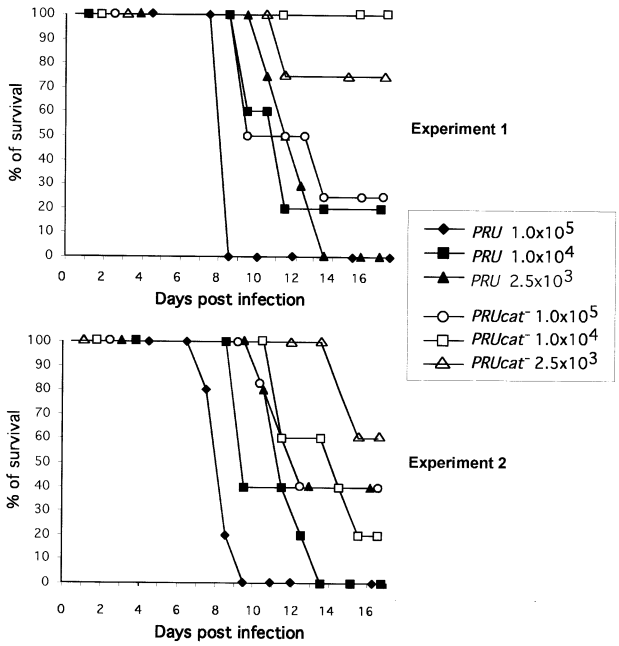

Fig. 8. Virulence of parental strain *PRU* and catalase knock-out mutant *PRUcat*⁻ in mice. Groups of six C57BL/6 mice were infected intraperitoneally with 2.5×10^3, 10^4 and 10^5 tachyzoites of *PRU* or *PRUcat*⁻. The survival of mice after infection was monitored over 17 days. The seroconversion of all the surviving mice was confirmed by Western blot analysis 3 weeks after infection. The results of two independent experiments are presented.

human macrophages (Wilson and Remington, 1979; Murray *et al*., 1985). Therefore, the establishment of an infection and the continued survival of *T. gondii* is likely to be particularly reliant on an effective and networked antioxidative system.

To protect itself against ROS produced during active oxidative respiration (Vercesi *et al*., 1998), *T. gondii* relies on two superoxide dismutases, TgSOD2 and TgSOD3, and the 2-cys-Prx, TgPrx3. We demonstrated here that all three enzymes are targeted to the *Toxoplasma* single tubular mitochondrion (Melo *et al*., 2000) (Fig. 1B). It is unclear yet whether the parasite possesses a mitochondrial thioredoxin/thioredoxin reductase (Trx/TrxR) system to supply TgPrx3 with reducing equivalents. *P. falciparum* contains a mitochondrial 2-cys-Prx, PfTPx2, and the second PfSOD might potentially be a mitochondrial enzyme. The unusual bimodal targeting signal of TgSOD2 might resemble the novel class of chimeric signals with dual targeting properties that were recently reported to modulate the rate of mitochondrial targeting (Robin *et al*., 2002) or a signal able to target to the apicoplast; nevertheless, SOD2Ty appears to localize only to the mitochondrion (Fig. 2B).

The cytosol of *T. gondii* is very well equipped with antioxidant enzymes including catalase and two Prxs. Despite the fact that we were unable to detect any glutathione peroxidase enzyme activity in total-cell lysates of tachyzoites, the genome sequence database clearly contains potential Gpxs, which await characterization (Fig. 1B). Some of these putative peroxidases might actually use thioredoxin rather than glutathione as reducing agent, and the *T. gondii* Trx was not included in the assay to test such a possibility. Additionally, the examination of the EST database suggested that these genes could be more abundantly expressed in the bradyzoite and sporulated oocyst stages than in tachyzoites. *P. falciparum* possesses a Gpx-like TPx (Sztajer *et al*., 2001) and three Prxs very similar to the ones present in *T. gondii*, but lacks a catalase. A previous report suggested that the host catalase can be taken up by the parasite and accumulate in the food vacuole (Clarebout *et al*., 1998). The two 2-cys-Prx, PfTPx1 and the mitochondrial PfTPx2, have been shown to use electrons provided by thioredoxin, whereas the specific thiol dependency of the 1-cys-Prx (Pf1-cys-Prx) and its mode of action are not entirely resolved (for a review, see Rahlfs *et al*., 2002). Whether the biochemical properties of the *Toxoplasma* Prxs are similar to those of their counterpart in *Plasmodium* remains to be determined. In both parasites, genes coding for thioredoxins and thioredoxin reductases as well as glutaredoxin and glutathione reductases are present, and their products are most probably assisting these Prxs (Fig. 1B) (Rahlfs *et al*., 2002). Both glutathione- and thioredoxin-based antioxidant systems have been documented to be of vital importance for *Plasmodium* survival in erythrocytes (Luersen *et al*., 2000; Krnajski *et al*., 2002), but their precise role in and contribution to antioxidant defence remain to be elucidated. Overexpression of Prx2 confers protection against H_2O_2, but this does not necessarily imply that this is the primary function of this enzyme. Our data suggest that Prx2 might exist as a dimer. This characteristic has been described for the 2-cys-prx, which are known to form H_2O_2-sensitive head-to-tail dimers via a disulphide bridge between the two conserved cysteine residues (Koo *et al*., 2002). The crystal structure of a 1-cys-prx (PrxVI) has been resolved and revealed the peroxidatic cysteine as a stable sulfenic acid and that the protein exists as a dimer (Choi *et al*., 1998). Further investigations will be necessary to resolve how the dimer is formed and if it is relevant for peroxidase activity.

In enzymatic terms, the peroxiredoxins have moderate efficacy compared with catalase or even compared with the seleno-peroxidases (Hofmann *et al*., 2002). In organisms possessing a catalase, the peroxiredoxins are unlikely to play a pivotal role in antioxidant defence, and recent studies suggest that these enzymes might fulfil quite distinct functions.

A mammalian 1-cys-prx has been described as a bifunctional enzyme, exhibiting phospholipase A2 activity in addition to the non-selenium glutathione peroxidase

activity (Chen *et al*., 2000). Such an enzyme could potentially simultaneously regulate phospholipid turnover and protect against oxidative injury. Recent studies also suggest that 1-cys-prx enzymes are able to scavenge peroxides but can also reduce peroxidized membrane phospholipids, protecting cells against oxidant-induced plasma membrane damage (Manevich *et al*., 2002). Finally, because of their ability to metabolize lower concentrations of H_2O_2, the Prxs might participate in signalling cascades in pathways using hydrogen peroxide as a messenger (Kang *et al*., 1998b).

Artemisinin is an endoperoxide that is used as a drug to treat malaria. It also kills *T. gondii*, presumably via oxidative stress (Berens *et al*., 1998). In a previous report, the level of TgPrx1 was shown to increase rapidly in response to artemisinin treatment (Son *et al*., 2001). In this study, prolonged treatment of intracellular parasites with increasing doses of artemisinin was performed to assess the ability of the parental and mutant strains in coping with the drug pressure. The effects were analysed by plaque assays and revealed that the growth of all parasite clones generated in this study was strongly suppressed at a concentration of 10 μM artemisinin, and no significant difference in growth rate was observed between any of the clones suggesting an equal sensitivity to the drug (data not shown).

Previous studies describing the subcellular localization of catalase reached inconsistent conclusions (Ding *et al*., 2000; Kaasch and Joiner, 2000). The present study confirms that *T. gondii* possesses a unique cytosolic catalase, which is clearly in the cytosol and not in peroxisomes. The location is consistent with the complete absence of the PEX genes, which are obligatorily required for peroxisome biogenesis (Subramani *et al*., 2000), from both the *Plasmodium* genome and the *Toxoplasma* database. A catalase gene is also present in the closely related organisms *Neospora caninum* and *Eimeria tenella*, based on the presence of several ESTs in these parasite databases (http://www.ebi.ac.uk/blast2/parasites.html), and catalase enzymatic activity was reported previously in *Eimeria bovis* (Hughes *et al*., 1989), but the localization of the enzyme has not been determined in these parasites.

The intrinsic kinetic properties of catalase (extremely high k_{cat}) and its cytosolic localization suggest that this enzyme might be best suited to protect against host cell oxidative stress. The disruption of the catalase gene established that this gene is dispensable for *T. gondii* tachyzoites. The fact that the absence of catalase did not significantly alter parasite fitness when cultivated in HFF cells suggests that other peroxidases are able to confer protection against endogenously produced H_2O_2. Indeed, the presence of cytosolic and mitochondrial SODs and Prxs identified previously and in this study suggest that the parasite is well armed to neutralize endogenously generated ROS. In contrast, the parasites lacking catalase exhibited an increased susceptibility to externally applied H_2O_2 and reduced virulence in mice. The role of *T. gondii* catalase in contributing to parasite virulence is also further supported by Nischik *et al*. (2001). An attenuated *Toxoplasma* strain generated by continuous *in vitro* passage showed a lower level of catalase production compared with strains passaged in mice. The recombinant parasite lines overexpressing either catalase or Prx2 showed an increased resistance to oxidative stress imposed by H_2O_2. Together, these results establish that the *T. gondii* cytosolic catalase plays an important role against oxidant stress imposed by the host and thus contributes as a virulence factor *in vivo.* The presence of additional peroxidases such as TgPrx2 and the fact that *T. gondii* is able to infect a broad range of host cell types might explain why parasites lacking catalase did not completely lose their virulence.

Experimental procedures

Parasite strains and growth

Tachyzoites of RH and Prugniaud (PRU) strains of *T. gondii* were maintained by growth on monolayers of HFF or on African green monkey (Vero) cells, grown in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 5% or 10% fetal calf serum (Gibco). Clonal isolates of *RHhxgprt*$^-$ and *PRUhxgprt*$^-$ of *T. gondii* were used as recipient strains for the parasite transfection.

Generation of antisera specific against T. gondii *Prx2*

The synthesis and coupling of the specific peptide to keyhole limpet haemocyanin (KLH) as well as the generation of rabbit antisera to *T. gondii* Prx2 were performed by Eurogentec. Rabbits were immunized with a peptide, DEEAKAKLPKGFEKKEC, which corresponds to the amino acid position 193–209 of the predicted Prx2 protein sequence. The antisera were tested for specificity against *T. gondii* Prx2 protein by immunoblot and IFA.

DNA constructs

The vectors pT8SOD2Ty-HX and pT8Prx3Ty-HX were generated by exchanging the original fragment between the *Eco*RI and *Nsi*I sites of the vector pTUB8MycGFPPf.myotailTy-1-HX (Herm-Gotz *et al*., 2002) with the respective SOD2 and Prx3 cDNA fragments flanked by *Mfe*I and *Nsi*I/*Pst*I restriction enzyme sites. The SOD2 and Prx3 cDNAs were amplified by RT-PCR using total RNAs prepared from freshly released RH tachyzoites as template and the primers SOD2 sense (5′-CTTCAATTGCCTTTTTCGACAAAATGTCC ATCACAGCTGTCCTAGTG-3′), SOD2 antisense (5′-GG CATGCATTGTTGCTTTCAAGTGCTTTCACCAAGTTC-3′), Prx3 sense (5′-CTTCAATTGCCTTTTTCGACAAAATGGCG

GCTTGCCTTCGAG-3′) and Prx3 antisense (5′-GGCC TGCAGGGTTTTTCAGTTGTCCAAGGTAGTTCTTG-3′). The expression vector pT8SOD3Ty-HX was constructed by replacing the original fragment between the *Eco*RI and *Nsi*I sites of the vector pTUB8MycGFPPf.myotailTy-1-HX with the genomic sequence of TgSOD3 flanked with the same restriction enzyme sites. PCR amplification of the SOD3 gene was performed using genomic DNA isolated from freshly released tachyzoites with sense (5′-CGGGAATTCCCTT TTTCGACA AAATGTTTGCTGCAGTCGCACGGC-3′) and antisense 5′-GGCATGCATTCTGGCCTCCACCGACTGTC-3′) primers.

The cDNA that corresponded to the *T. gondii* Prx2 gene was generated by PCR using sense (5′-CCTTCAATTGC CTTTTTCGACAAAATGTTGGTCCTCGGCAGCACG-3′) and antisense (5′-GGCCATGCATGCGCCGACGGATCCGGAGC GTA-3′) primers and with *T. gondii* cDNA (Dr J. Mattsson) as template. The Prx2 cDNA fragment was flanked by the restriction enzyme sites *Mfe*I and *Nsi*I. The Prx2 expression vector pT8Prx2Ty-HXGPRT was constructed by replacing the original fragment between the *Eco*RI and *Nsi*I sites of the vector pT8MycGFPPf.myotailTy-1-HX with Prx2 cDNA.

The knock-out vector for catalase, pHXGPRTCATko, was constructed by inserting 5′ and 3′ flanking sequences of the *CAT* gene into the vector pminiHXGPRT (Donald *et al*., 1996). Fragments (1766 bp and a 2756 bp) were amplified by PCR from *T. gondii* genomic DNA using the primer pairs sense (5′-GGGGTACCTGGCTGTACGCTGGAGTTGACG-3′) and antisense (5′-CCCAAGCTTCCCTTCGGGGTCAA AAACAG-3′) for the 5′ flanking fragment and the pairs sense (5′-CAGGATCCGTTTCCCTGTTTGATGTTAGTC-3′) and antisense (5′-GGGCGGCCGCGCATTGGAACGTGAGGTA GGCGAGGTG-3′) for the 3′ flanking fragment respectively. These fragments were cloned into the vector pminiHXGPRT between *Kpn*I and *Hin*dIII and *Bam*HI and *Not*I sites respectively. The catalase expression vector, pTUBCAT-HX, was constructed by replacing the GFP coding sequence between *Eco*RI–*Pac*I from the plasmid pT8MycHisGFP-HX (unpublished) with the cDNA encoding for the *T. gondii CAT* gene. The catalase cDNA fragment flanked by the restriction enzyme sites *Mfe*I and *Pac*I was generated by amplifying from *T. gondii* cDNA by PCR with the sense (5′-GGCCC AATTGCCTTTTTCGACAAATGACTCAGGTTCCGCCCG-3′) and the antisense (5′-CGGTTAATTAACTACATCTTGGCTG GGTAGCACG-3′) primers.

Transfection and selection of stable transformants

To generate stable transformants, 5×10^7 extracellular parasites were transfected with appropriate plasmids and subjected to either mycophenolic acid/xanthine (MPA/X) or chloramphenicol selection accordingly (Kim, 1993; Donald *et al*., 1996). Parasites were transfected with 30, 40, 50 or 70 μg of linearized plasmids and cloned by limiting dilution in 96-well microtitre plates after selection. Positive stable transformants were assessed by IFA and immunoblotting and subcloned again. Disruption of the *CAT* gene was achieved by double homologous recombination in *RHhgprt*$^-$ and *PRUhxgprt*$^-$ mutant strains to generate *RHcat*$^-$ and *PRUcat*$^-$ respectively. *T. gondii* tachyzoites were transfected with the linearized pHXGPRTCATko and selected for resistance to MPA/X. The complementation of *RHcat*$^-$ from catalase was achieved by co-transfection of a plasmid expressing the catalase gene under the control of the tubulin 1 promoter pTUBCAT-HX together with a selectable plasmid expressing the chloramphenicol acetyltransferase gene, as described previously (Soldati and Boothroyd, 1993). Parasites overexpressing CAT, *RHCATov*, were obtained by stable transfection of *RHhxgprt*$^-$ parasites with pTUBCAT-HX using *TgHXGPRT* as the selectable gene. *T. gondii* mutants overexpressing Prx2, Prx3, SOD2 and SOD3 were created by stable integration of pT8Prx2Ty-HX, pT8Prx3Ty-HX, pT8SOD2Ty-HX and pT8SOD3Ty-HX into parasite *RHhxgprt*$^-$. In order to perform the *in vivo* analysis in the mouse model, a clone expressing HXGPRT and containing the intact locus for catalase (PRU) was used as a control.

Indirect immunofluorescence microscopy assay

All manipulations were carried out at room temperature. Tachyzoite-infected HFF cells on glass coverslips were fixed with 4% paraformaldehyde, 0.05% glutaraldehyde or 4% paraformaldehyde only, for 20 min, followed by 3 min incubation with 0.1 M glycine in PBS. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min and blocked in 2% bovine serum albumin in PBS for 20 min. The cells were then stained with the primary antibodies followed by Alexa 594 goat anti-rabbit or Alexa 488-conjugated goat anti-mouse antibodies (Molecular Probes, Cappel and Bio-Rad). For the detection of *T. gondii* catalase, sera 84 and 96 (Ding *et al*., 2000) were used. Anti-actin antibodies were kindly provided by Dr G. Gerisch, MPI Martinsried, Germany. The anti-Ty1 antibodies were kindly provided by Dr K. Gull (Manchester, UK).

Confocal images were collected with a Leica laser scanning confocal microscope (TCS-NT DM/IRB) using a 100× Plan-Apo objective with NA 1.30. Single optical sections were recorded with an optimal pinhole of 1.0 (according to Leica instructions) and 16 times averaging. Other micrographs were obtained with a Zeiss Axiophot equipped with a CCD camera (Photometrics type CH-250). Adobe PHOTOSHOP was used for image processing.

Immunoblots

Freshly released tachyzoites were harvested, washed in PBS, resuspended and boiled directly in SDS sample buffer. Polyacrylamide gels (12%) were run under reducing or non-reducing conditions in the presence or absence of 0.1 M dithiothreitol (DTT) in the sample buffer respectively. Proteins in the gel were transferred onto a nitrocellulose membrane using a semi-dry electroblotter and incubated with respective monoclonal antibodies (mouse ascites fluid) or rabbit polyclonal antisera in PBS, 0.05% Tween 20 and 5% non-fat milk powder. After washing, the nitrocellulose membrane was incubated for 1 h with a peroxidase-conjugated goat anti-mouse or anti-rabbit antibody (Bio-Rad), and bound antibodies were visualized using the ECL system, POD (Boehringer).

Hydrogen peroxide sensitivity assays

To analyse the growth inhibitory effect of H_2O_2, freshly lysed parasites were collected, washed twice with serum-free DMEM and treated with various concentrations of hydrogen peroxide in DMEM for 90 min or 3 h at 37°C, 5% CO_2. Treated and untreated parasites were then allowed to invade HFF monolayers after washing the parasites. The effect of H_2O_2 on the replication of intracellular parasites was investigated further by incubating freshly lysed parasites in serum-free DMEM in the presence of 2×10^{-4} M H_2O_2 for 3 h before allowing them to grow on HFF monolayers grown on glass coverslips. Non-invaded parasites were removed by washing the monolayers with PBS 2 h after incubation. After 24 or 48 h, cells were fixed and stained with anti-actin antibodies. The viability and intracellular replication of the parasites were determined by counting the number of vacuoles and the number of parasites per vacuole under the microscope. The effect of H_2O_2 treatment on Prx2 protein was assessed by incubation of freshly lysed parasites with various concentrations of H_2O_2 for 1 h.

Virulence assay in mice

C57BL/6 mice (6–8 weeks old; Harlan-Winkelmann) were infected intraperitoneally with different doses (2.5×10^3, 10^4 or 10^5 parasites per mouse) of control *PRU* and *PRUcat*$^-$ parasites freshly lysed from HFF cells. The survival of mice upon infection was monitored. Three weeks after infection, the seroconversion of surviving mice was examined by immunoblot analysis using complete RH tachyzoite lysate.

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