Molecular Genetics Evidence for the in Vivo Roles of the Two Major NADPH-dependent Disulfide Reductases in the Malaria Parasite*4,5

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Malaria-associated pathology is caused by the continuous expansion of Plasmodium parasites inside host erythrocytes. To maintain a reducing intracellular milieu in an oxygen-rich environment, malaria parasites have evolved a complex antioxidative network based on two central electron donors, glutathione and thioredoxin. Here, we dissected the in vivo roles of both redox pathways by gene targeting of the respective NADPH-dependent disulfide reductases. We show that Plasmodium berghei glutathione reductase and thioredoxin reductase are dispensable for proliferation of the pathogenic blood stages. Intriguingly, glutathione reductase is vital for extracellular parasite development inside the insect vector, whereas thioredoxin reductase is dispensable during the entire parasite life cycle. Our findings suggest that glutathione reductase is the central player of the parasite redox network, whereas thioredoxin reductase fulfils a specialized and dispensable role for P. berghei. These results also indicate redundant roles of the Plasmodium redox pathways during the pathogenic blood phase and query their suitability as promising drug targets for antimalarial intervention strategies.

Antioxidant enzymes play a decisive role in rapidly growing organisms, including tumor cells and pathogens. Upon a malaria infection, the protozoan parasite Plasmodium continuously multiplies inside host erythrocytes. This specialized lifestyle necessitates the safe management of high oxygen tension. Moreover, Plasmodium is under constant exposure to oxidative and nitrosative stress, either generated endogenously by the high metabolic rate or produced exogenously by immune effector cells of the host in response to parasite infection (1, 2).

Reactive oxygen species (ROS)4 include the superoxide anion (O2•−) formed by univalent reduction of oxygen and the hydroxyl radical (OH•), which can be generated from hydrogen peroxide and the superoxide anion in the presence of iron ions. An imbalance between production and detoxification of these reactive intermediates can lead to local oxidative stress and ultimately damage vital molecules, such as proteins, lipids, or DNA.

To maintain and defend their reducing intracellular milieu, numerous organisms including malaria parasites have evolved a dual antioxidative system based on the cysteine-containing redox-active peptides glutathione (GSH) and thioredoxin (Trx) (1–3). It should be noted that this dual system also provides the reducing equivalents for converting nucleotides to deoxyribonucleotides, an essential process for DNA synthesis and rapid cell proliferation. Glutathione is the major low molecular weight antioxidant in Plasmodium parasites (4), and it is kept at a high level in the reduced state by the antioxidant enzyme glutathione reductase (GR) (5), which uses NADPH regenerates in the pentose phosphate pathway as an electron donor. In addition, a complete Trx system, consisting of NADPH, thioredoxin reductase (TrxR), Trx, and a number of Trx-dependent peroxidases, has been characterized in Plasmodium (6–8). Curiously, the malarial and related apicomplexan parasites lack catalase and a classical, selenium-containing glutathione peroxidase, two central antioxidant enzymes present in a wide range of organisms to protect themselves against hydrogen peroxide. In kinetoplastid parasites, the thioredoxin and glutathione systems are replaced by a unique redox system based on trypanothione (9, 10).

The apparent deficiency of such a unique antioxidant defense system in Plasmodium further underscores the central importance of the two antioxidant pathways, represented by GR and TrxR, in the parasite. Therefore, the redox metabolism in Plasmodium significantly differs from that of the host cells, a fundamental requirement for potential drug development (11). Specific drugs targeting these enzymes and other proteins of the Plasmodium redox network impair the impressive anti-ox-

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4 The abbreviations used are: ROS, reactive oxygen species; GR, glutathione reductase; GSH, glutathione; MB, methylene blue; Trx, thioredoxin; TrxR, thioredoxin reductase; RiboR, ribonucleotide reductase; TRAP, thrombospondin-related anonymous protein.
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Similarly, a gene replacement strategy was chosen to disrupt the rodent Plasmodium GR gene. The two flanking regions (1,000 and 560 bp) were amplified using the following primers: PbGR_Rep1for and PbGR_Rep1rev for the 1,000-bp fragment, and PbGR_Rep2for and PbGR_Rep2rev for the 560-bp fragment using Pb genomic DNA as template (supplemental Table S2). Cloning into the modified transfection vector resulted in pPbGRRep+. P. berghei transfections and positive selection were done by Nucleofector technology (13). Clonal parasites were obtained by limiting dilution of single parasites into recipient NMRI mice.

Integration-specific PCR amplification of the GR(−) parasites was done using the following primers: B3D+ for and GRtest rev, as well as B3D+rev and GRtest for. To validate the purity of the clonal GR(−) parasites, a GR-specific amplification using the primer PbGRstart and PbGRend was used for genomic DNA templates, and GRfor and GRrev, as well as MSP1for and MSP1rev for cDNA preparations (supplemental Table S2).

Immunoblotting—Polyclonal rabbit antibodies against P. falciparum glutathione reductase and thioredoxin reductase were obtained from Eurogentec (Seraing, Belgium) using highly purified recombinant proteins. The specificity of the antibodies was proven by Western blot on P. falciparum whole cell lysates. Rodents were infected with WT, TrxR(−), or GR(−) blood stage parasites, respectively, and cytosolic parasite proteins were extracted using 2 M urea buffer. For immunoblot analysis, proteins were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad) by electroblotting. TrxR (59.7 kDa) and GR (56.5 kDa) were detected by incubation of membranes with polyclonal anti-P. falciparum TrxR or GR antisera (dilution 1:1,500). Bound antibodies were detected using peroxidase-coupled anti-rabbit and anti-mouse antibodies, to detect PbTrxR, PbGR, actin, and PbHSP70 (14), respectively. Immunostained proteins were visualized with enhanced chemiluminescence detection ( Pierce). Recombinant P. falciparum TrxR was used as a positive control. The anti-Dicyostelium discoideum actin antiserum, which cross-reacts with apicomplexan actins, was kindly provided by Dr. Markus Meissner (Heidelberg).

Plasmodium Life Cycle and Phenotypic Analysis of Mutant Parasites—Anopheles stephensi mosquitoes were kept at 21 °C, 80% humidity, and daily feeding on 10% sucrose. Asynchronous blood-stages of P. berghei ANKA-GFP (WT) (14), TrxR(−), and GR(−) parasites were maintained in NMRI mice and checked for gametocyte formation and exflagellation of microgametocytes prior to mosquito feeding. For mosquito infection, A. stephensi mosquitoes were allowed to blood-feed on anesthetized mice for 15 min. Dissection of mosquitoes was conducted at days 10, 14, and 17 to determine infectivity and sporozoite numbers in midguts and salivary glands, respectively. To analyze liver stage development, sporozoites were deposited onto a semi confluent monolayer of hepatoma cells (HuH7) and incubated for 2 h, followed by washing and incubation in cell culture medium. Liver stages were detected after 48 h with a primary antibody directed against the P. berghei heat shock protein 70 (PbHSP70) (14), followed by an Alexa Fluor 488-conjugated anti-mouse antibody. To analyze sporozoite infectivity in vivo, Sprague-
Dawley rats were injected intravenously with 10,000 WT or TrxR(−) sporozoites. Parasitemia was monitored by daily blood smears. The occurrence of a single parasite marked the first day of patency.

Determination of Total Glutathione and Free Thiols in Parasite Extracts—Three days after infection of mice with either WT, GR(−), or TrxR(−) parasites, parasitemia was determined, mice were sacrificed, and parasites were cultured for 16–18 h to synchronize parasites. The parasites were then purified by gradient centrifugation (55% Nycodenz in PBS). After washing and final centrifugation the total parasite volume was determined taking into account cell volume and residual buffer. The suspension was mixed with 3 volumes of 5% sulfosalicylic acid, centrifuged, and the supernatant was stored at −80 °C and used for the determination of total glutathione and free thiols. The glutathione content was measured by the GR-coupled 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)-GSH-recycling assay (15). A standard curve was prepared using appropriate concentrations of GSH and sulfosalicylic acid. The concentration of free thiols was determined spectrophotometrically on the basis of their reaction with DTNB (ε_{412 \text{ nm}} = 13.6 \text{ mm}^{-1} \text{ cm}^{-1}) (16).

RESULTS

Expression Profiling of Plasmodium berghei Glutathione and Thioredoxin Reductases—We first studied mRNA levels of the two major Plasmodium NADPH-dependent reductases by quantitative real-time RT-PCR (Table 1). In comparison, the highest transcript levels for glutathione reductase (GR) and thioredoxin reductase (TrxR) were determined in merozoites as it is also the case for ribonucleotide reductase (RiboR). Abundant expression of redox enzymes is consistent with the high metabolic and multiplication rates of Plasmodium during blood stage development. In liver stages about 1/10 of these transcript levels were reached for GR and TrxR, i.e. 11.4 and 16%, respectively (Table 1). In the insect vector, in salivary glands the transcript levels of redox enzymes is consistent with the high metabolic and multiplication rates of Plasmodium during blood stage development. Toward parasite growth under in vitro culture conditions (17). Toward drug target validation in the mammalian host, we targeted the P. berghei TrxR locus, employing a replacement strategy (Fig. 1A). Unexpectedly, by a single transfection attempt, we successfully integrated the PbTrxR disruption plasmid. This parental population was genotyped to ensure the correct replacement of the TrxR locus with the positive selection marker Tgdhfr/its. After double crossover homologous recombination, the TrxR open reading frame is substituted by the selection marker, resulting in the loss-of-function TrxR(−) allele. Replacement- and WT-specific test primer combinations and expected fragments are shown as arrows and lines, respectively. B, confirmation of the TrxR gene disruption by replacement-specific PCR analysis with primer combinations that amplify a signal in the recombinant locus (test) only. The absence of a WT-specific signal in the clonal TrxR(−) population confirms the purity of the mutant parasite line. C, depletion of TrxR transcripts in TrxR(−) parasites. cDNAs from WT and TrxR(−) blood stages were used as templates for TrxR-specific PCR reactions. Amplification of GR transcripts was used as a positive control. D, Western blot analysis of WT and TrxR(−) blood stages. Extracts from WT or TrxR(−) (16 μg of total protein each) were separated on a 10% SDS gel and probed with the polyonal anti-PfTrxR serum (upper panel) or a polyclonal anti-actin serum (lower panel). As a positive control 80 ng of recombinantly expressed P. falciparum thioredoxin reductase (rec. TrxR) was added.

FIGURE 1. Targeted deletion of the P. berghei TrxR. A, replacement strategy for targeted gene disruption of PbTrxR. The wild-type TrxR locus (WT) is targeted with a KpnI (K)/SacI (S) linearized replacement plasmid (pTrxR Rep) containing the 5′- and 3′-UTRs of PbTrxR and the positive selection marker Tgdhfr/its. After double crossover homologous recombination, the TrxR open reading frame is substituted by the selection marker, resulting in the loss-of-function TrxR(−) allele. Replacement- and WT-specific test primer combina-

Determination of Total Glutathione and Free Thiols in Parasite Extracts—Three days after infection of mice with either WT, GR(−), or TrxR(−) parasites, parasitemia was determined, mice were sacrificed, and parasites were cultured for 16–18 h to synchronize parasites. The parasites were then purified by gradient centrifugation (55% Nycodenz in PBS). After washing and final centrifugation the total parasite volume was determined taking into account cell volume and residual buffer. The suspension was mixed with 3 volumes of 5% sulfosalicylic acid, centrifuged, and the supernatant was stored at −80 °C and used for the determination of total glutathione and free thiols. The glutathione content was measured by the GR-coupled 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)-GSH-recycling assay (15). A standard curve was prepared using appropriate concentrations of GSH and sulfosalicylic acid. The concentration of free thiols was determined spectrophotometrically on the basis of their reaction with DTNB (ε_{412 \text{ nm}} = 13.6 \text{ mm}^{-1} \text{ cm}^{-1}) (16).

TABLE 1

| Gene    | Merozoites | Midgut | Salivary gland | Liver stages |
|---------|------------|--------|----------------|--------------|
| GR      | 100        | 4.0    | 10.7           | 11.4         |
| TrxR    | 100        | 3.6    | 0.35           | 16.0         |
| RiboR   | 100        | 2.7    | 1.43           | 2.72         |
| TRAP    | 0.63       | 100    | 97.7           | 0.47         |

Transcript levels of P. berghei GR, TrxR, and selected control genes during Plasmodium life cycle progression. Shown are mean values of triplicate measurements. The highest expression levels were set to 100%.

Generation of Thioredoxin Reductase Knockout Parasites—We first investigated the in vivo role of the thioredoxin arm of the parasite redox network, represented by TrxR (gi: 68076031). Thioredoxin is the standard electron donor for ribonucleotide reductase and is therefore upstream of the de novo synthesis of deoxyribonucleotides (1–3). Previous work in the P. falciparum system had suggested that PfTrxR is vital for parasite growth under in vitro culture conditions (17). Toward drug target validation in the mammalian host, we targeted the P. berghei TrxR locus, employing a replacement strategy (Fig. 1A). Unexpectedly, by a single transfection attempt, we successfully integrated the PbTrxR disruption plasmid. This parental population was genotyped to ensure the correct replacement of the TrxR locus with the positive selection marker (data not shown). The population was subsequently used for the selection of clonal, TrxR-deficient parasite populations. We obtained three TrxR knockout clones, named TrxR(−), which exclusively showed the predicted mutant locus as verified by replacement-specific genotypic analysis (Fig. 1B). To confirm the absence of TrxR transcripts in TrxR(−) parasites, RT-PCR and subsequent cDNA synthesis were performed with poly(A)+ RNA from mixed blood stages (Fig. 1C). As predicted, no TrxR transcripts were detected in the knockout parasite lines, verifying the purity of the clonal populations. Moreover, Western blot analysis of TrxR(−) blood stages with a PfTrxR-specific, anti-peptide antiserum confirmed the complete absence of the protein in TrxR(−) parasites (Fig. 1D). This finding already indicated that TrxR is dispensable for the growth of asexual blood stage parasites.

Targeted Gene Deletion of P. berghei Glutathione Reductase—This finding prompted us to investigate the role of the other
arm of the parasite redox network, represented by GR (gi: 56499726). We first targeted the PbGR gene locus with an integration vector that disrupts the gene locus via a single-crossover event (data not shown). Several attempts to disrupt the gene were not successful, while an integration control that recovered the WT GR copy yielded recombinant parasites (data not shown). To distinguish between an essential function and difficulties in targeting the gene, we constructed a replacement vector containing the PbGR 5'- and 3'-untranslated regions that flank the positive selection marker cassette (Fig. 2A). Upon a double-crossover event, this vector is predicted to delete the entire PbGR locus. After transfection and continuous selection with the antifolate pyrimethamine, we obtained a parental population that was used for single parasite cloning. Genotyping of the PbGR gene locus with an inte- rmediate redundant gene. The successful generation of GR- and/or TrxR-deficient parasites already demonstrates that this gene is not essential during the pathogenic blood-stage cycle in vivo. 

**FIGURE 2. Targeted deletion of P. berghei GR.** A, replacement strategy for targeted gene disruption of PbGR. The wild-type GR locus is targeted with a SacII/KpnI(K)-linearized replacement plasmid containing the 5'- and 3'-UTRs of PbGR and the positive selection marker Tgdhfr-ts. After double crossover homologous recombination, the GR open reading frame is substituted by the selection marker, resulting in GR knockout parasites. Replacement, -locus-, and WT-specific test primer combinations and expected fragments are shown as arrows and lines, respectively. B, genotyping of the GR(-) disruption by PCR analysis with primer combinations that amplify signals in the recombinant locus (test 1 and 2), the GR locus (5'- and 3'-UTR), and the WT locus (WT). The absence of a WT-specific signal in the clonal GR(-) population confirms the purity of the mutant parasite line. C, depletion of GR transcripts in GR(-) parasites. cDNAs from WT and GR(-) late stage schizonts were used as templates for GR-specific PCR reactions. Amplification of merozoite surface protein 1 (MSP1) transcripts was used as a positive control. D, Western blot analysis of WT, GR(-), and TrxR(-) blood stages. Parasite extracts were obtained after saponin lysis (left) or Triton X-100 lysis (right) of infected erythrocytes, separated on a 10% SDS gel and probed with the polyclonal anti-PbGR serum (upper panel) or a monoclonal anti-PbHSP70 antibody (lower panel). Note the absence of the GR-specific signal in the GR(-) line and comparable GR steady state levels in WT and TrxR(-) parasites.

parasites is not due to compensatory up-regulation of a potential redundant gene. The successful generation of GR-deficient parasites already demonstrates that this gene is not essential during the pathogenic blood-stage cycle in vivo. 

**FIGURE 3. P. berghei glutathione reductase and thioredoxin reductase are dispensable for asexual parasite growth.** A, GR(-) (red line) and TrxR(-) (green line) parasites cause high-level parasitemia in vivo. Displayed are in vivo growth curves of WT (blue lines) and knockout parasites. Five naive animals each were injected intravenously with 1,000 asexual parasites of the respective parasite populations. Parasitemia was determined every 24 h after infection by microscopic examination of Giemsa-stained blood smears. B, in vivo growth curves of WT, GR(-) and TrxR(-) parasites under constant exposure to methylene blue (25 mg/kg/day). Treatment started immediately after infection with 1,000 WT or mutant parasite lines, respectively.

parasites and parasitemia follow-up every 24 h after infection with 1,000 WT or mutant parasite lines, respectively. To test whether GR and/or TrxR serve auxiliary role(s) during blood stage infection, an in vivo growth assay was conducted by intravenous infection of 1,000 asexual parasites and parasitemia follow-up every 24 h after infection (Fig. 3A). Interestingly, after an initial delay in proliferation of GR(-) parasites, parasitemias of these and TrxR(-) and WT parasite lines reached comparable peak levels at day 7 after infection. This finding shows that depletion of GR, but not TrxR, may affect onset of parasite proliferation under in vivo growth conditions, but clearly excludes an essential function of both NADP-dependent disulfide reductases for establishment of blood stage infections.

We next wanted to determine whether loss of GR and/or TrxR function affects parasite growth in vivo under oxidative stress. To this end, we monitored parasitemia under enhanced stress conditions. We used methylene blue (MB) because this antimalarial had been shown to challenge the parasite’s intracellular reducing milieu through the generation of pro-oxidant H2O2 (18). WT-, GR(-)-, and TrxR(-)-infected mice were
treated orally with 25 mg MB/kg/day. We selected this sublethal concentration, because dose finding experiments revealed rapid elimination of WT parasites with 100 mg MB/kg/day (data not shown). *In vivo* growth of the GR-deficient parasites, even under these enhanced oxidative stress conditions, was comparable to WT parasites (Fig. 3B). Notably, *TrxR* (-) parasites were delayed by 2 days under MB treatment, but eventually reached substantial parasitemia levels. These findings exclude central roles of *P. berghei* GR or *TrxR* in antioxidant defense.

**Total Glutathione and Non-protein-bound Free Thiols Are Unaffected in Mutant Parasites**—To test whether glutathione levels are altered in the parasite lines that lack one functional NADPH-dependent disulfide reductase we measured total glutathione concentrations in purified asexual blood stage parasites (Table 2). Total glutathione concentrations were found to be slightly, but non-significantly, lower in GR- and *TrxR*-deficient parasites than in the WT parasites (*p* > 0.05; unpaired *t* test). Under the experimental conditions chosen, the concentrations of free thiols in the parasite extracts were determined to be 60, 79, and 62% of the total glutathione values, in WT, GR-, and *TrxR*-deficient cells, respectively (*p* > 0.05; unpaired *t* test). Together, these data do not indicate alterations of parasite glutathione levels in the absence of either NADPH-dependent disulfide reductase.

*PbGR Is Vital for Sporogony Inside the Anopheles Vector*—Previous work established that endogenous glutathione biosynthesis is critical for mosquito stage development of the malaria parasite (19). We therefore wanted to test whether GR displays a comparable phenotype. We infected *A. stephensi* mosquitoes with WT and GR(-) parasites and monitored sporogony inside the mosquito vector (Fig. 4). Whereas WT infection resulted in high densities of mature oocysts, infections with GR(-) parasites showed smaller oocysts (Fig. 4A). Counting oocyst numbers per infected mosquito revealed a trend toward fewer GR(-) oocysts compared with WT, which was, however, not significant (Fig. 4B). Quantification of midgut-associated sporozoites, the extracellular stages that are formed upon successful sporogony, revealed a complete absence of GR(-) sporozoites (Fig. 4C). To corroborate our results, we exposed susceptible C57Bl/6 mice to 15 WT- and GR(-)-infected mosquitoes (WT, *n* = 3; GR(-), *n* = 6). As expected, none of mice exposed to GR(-)-infected mosquitoes developed malaria, whereas exposure to WT-infected *Anopheles* resulted in detectable parasitemia after 3 days. Together, these findings establish a vital role for GR during *Plasmodium* development inside the mosquito vector.

*TrxR Is Not Necessary for the Entire Life Cycle of P. berghei*—We finally followed the phenotype of *TrxR* (-) parasites during the entire *P. berghei* life cycle. No differences regarding gametocyte formation, transmission to *Anopheles* mosquitoes, and oocyst development could be observed between WT and *TrxR* (-) parasites (data not shown). We next investigated sporozoite development and salivary gland invasion by comparing sporozoite numbers in infected mosquito midgut oocysts and salivary glands (Table 3). Even though the infectivity of *TrxR* (-)-infected mosquitoes and the midgut sporozoite numbers were found to be significantly lower compared with WT-infected mosquitoes, the *TrxR*-deficient parasites could apparently compensate for lower overall infection rates, since the salivary gland-associated sporozoite numbers reached levels comparable to WT parasites. Testing mature salivary gland sporozoites for *in vivo* and *in vitro* infectivity in the mammalian host revealed no striking differences between the two parasite lines. Hepatocytes infected with *TrxR* (-) sporozoites were phenotypically indistinguishable from WT-infected cells, although consistently lower numbers of mature liver stage parasites were produced (Table 3). When testing *TrxR* (-) sporo-

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**TABLE 2**

| Parasite line | Total glutathione | Free thiols | n   |
|---------------|------------------|------------|-----|
| WT            | 2.3 (± 1.1)      | 1.4 (± 0.8) | 10  |
| GR(-)         | 2.2 (± 0.6)      | 1.7 (± 0.8) | 8   |
| *TrxR*(-)     | 1.9 (± 0.8)      | 1.2 (± 0.4) | 4   |

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**FIGURE 4. Glutathione reductase is vital for sporogony in the mosquito vector.** *A.* epifluorescence micrographs of infected *A. stephensi* midguts at day 14 after an infectious bloodmeal. WT parasites mature to sporozoite-containing oocysts (left, GR(-) parasites colonize the mosquito midguts but are arrested in sporogony (right). Bars, 40 μm. *Insets* show representative oocysts. Note the radial sporozoites emerging from the sporoblast in WT parasites. *Inset* bars, 16 μm. *B,* quantification of oocysts per infected mosquito for WT (blue) and GR(-) (red) parasites. C, midgut sporozoites per infected mosquito at day 14 after feeding. WT, *n* = 7; GR(-), *n* = 4. No sporozoites were detectable in GR(-)-infected mosquitoes even after extended monitoring up to day 31.
TABLE 3
Loss of TrxR function does not impair Plasmodium life cycle progression

| Parasite | Insectivitya | Midgutb | Sporozoites | Mice infected/mice inoculated (prepatent period)c |
|----------|--------------|---------|-------------|-----------------------------------------------|
| TrxR(−f) | 38% (±10%)   | 9,500 (±2,900) | 8,250 (±1,600) | 7/7 (d.4) 5/5 (d.4.6) |
| WT       | 77% (±18%)   | 27,800 (±5,400) | 12,100 (±2,200) | 4/4 (d.5) 6/6 (d.3.5) |

a Percentage of mosquitoes that contain oocysts at days 11–13 after the infectious bloodmeal.

b Midgut-associated sporozoites per infected mosquito at days 12–14 after the infectious bloodmeal.

c Salivary gland-associated sporozoites per infected mosquito at days 17–19 after the infectious bloodmeal.

d Liver stages are represented by total numbers of mature liver stages visualized 48 h after incubation of 10,000 salivary gland sporozoites with subconfluent cultured hepatocytes.

Depletion are supported by a recent report of PbTrxR(−) mutant throughout thePlasmodiumlife cycle.

**DISCUSSION**

To date, there is mainly biochemical and pharmacological evidence that the glutathione- and thioredoxin-based redox systems play important roles in blood stage malaria parasites (3, 20–22). As a complementation, experimental genetics in an in vivo model is an important tool for drug target validation. In the work described here we studied the essentiality of the two NADPH-dependent flavoenzymes glutathione reductase and thioredoxin reductase for the malaria parasite *P. berghei*. The two proteins are the principal electron donors for the glutathione and thioredoxin redox networks comprising, among others, peroxiredoxins, glyoxalases, glutathione S-transferase, plasmoperoxidase, thioredoxins, and glutaredoxins (1, 2, 23, 24).

Using double crossover replacement, we successfully deleted the GR locus in *P. berghei* blood stages. *P. berghei* TrxR was also efficiently targeted by gene disruption. Interestingly, the loss of GR function resulted in viable and infectious blood stage malaria parasites in vivo. However, for sporogony in the mosquito vector GR was found to be essential. In contrast to previous studies on *P. falciparum* (17), TrxR-deficient *P. berghei* parasites could be readily obtained. A systematic phenotypic analysis of the PbTrxR(−) mutant throughout the Plasmodium life cycle excluded an essential function for TrxR in any of the developmental stages.

The data on GR depletion are supported by a recent report of Vega-Rodriguez *et al.* (19). In their study, the authors disrupted the γ-glutamylcysteine synthetase (γ-GCS) gene of *P. berghei*. γ-GCS is a critical component of the GSH biosynthesis pathway. Gene disruption resulted in growth retardation and depletion of GSH levels in blood stage parasites. However, like in our study on GR, the gene was not found to be essential. In contrast, γ-GCS gene disruption had a dramatic effect on the development of mosquito stages resulting in reduced numbers of stunted oocysts that did not undergo full sporogony and, therefore, failed to produce sporozoites (19). Taken together, the two studies suggest that neither the synthesis nor the GR-based reduction of glutathione are essential for *P. berghei* blood stages in vivo. This observation is in contrast to biochemical studies which demonstrated lethal effects of a γ-GCS inhibition by l-buthionine sulfoximine on *P. falciparum* in vitro (21). The observed differences might be based on the different experimental approaches. However, they might also point to differences in glutathione metabolism and requirements in murine and human malaria parasites. Further studies, like knockouts of the GR and the γ-GCS genes in *P. falciparum* are required to answer this question.

As mentioned above, GR as well as γ-GCS (19) depletion lead to severe disturbance of the development of parasite mosquito stages. The discussed irreversible damage of oocyst mitochondria in γ-GCS depletion might point to enhanced oxidative stress based on limited availability of glutathione. Apart from different metabolic requirements, which might contribute to enhanced oxidative burden, the mosquito stages are extracellular parasite forms and thus potentially exposed to higher fluxes of reactive oxygen species. In addition, it should be kept in mind that dipiteric insects including Anopheles and Drosophila do not possess a genuine glutathione reductase (6, 25). In these organisms the non-enzymatic reduction of glutathione is, at least partially, effected by reaction with reduced thioredoxin. In the WT parasite, the available enzymatic reduction of glutathione by GR is likely to be much more efficient than in the GR-deficient vector. This constellation might explain why it is impossible for a GR-deficient parasite to develop within a GR-deficient host. Another important observation is the fact that in salivary glands of mosquitoes infected with WT parasites pronounced GR transcripts were determined whereas TrxR transcripts were hardly detectable (Table 1). This constellation points to a specific role of GR in mosquito stage parasites. This might also explain why the thioredoxin system cannot compensate GR-deficiency in these stages.

In our study TrxR-deficient *P. berghei* parasites were viable throughout the Plasmodium life cycle, although infectivity and parasite counts in the midgut and the salivary glands of the insect vector, as well as in mouse liver were reduced (Table 3). These data exclude an essential function of TrxR in both mammalian host and vector. Notably, in a previous experimental approach TrxR(−) parasites could not be obtained in cultured erythrocytes infected with *P. falciparum* laboratory strain (17). Possible explanations for the strikingly different results include (i) frequently observed technical problems in the *P. falciparum* transfection system, (ii) an important role of TrxR for Plasmodium under in vitro culture conditions (which only partially reflect the physiological environment and lack feed-back regulation by the host), or (iii) a different role of TrxR in rodent and primate/human parasites, as also discussed above for GR.
Plasmodium NADPH-dependent Disulfide Reductases

The fact that neither GR nor TrxR are essential for blood stage *P. berghei* parasites might be explained by a functional overlap between the glutathione and the thioredoxin system. The major functions of the two systems include recycling of reduced glutathione and thioredoxin, antioxidant defense, redox regulation, and donation of reducing equivalents to other enzymes including ribonucleotide reductase, glutathione S-transferase, glyoxalases, and peroxidases (1–4). As mentioned above, numerous organisms including *Drosophila melanogaster* and *Anopheles* do completely lack a genuine GR. In these insects, glutathione reduction is maintained by the thioredoxin system (6, 25). As recently shown, also humans with inherited complete GR deficiency can live an almost normal life (26). Moreover, *P. falciparum* efficiently invades and develops inside GR-deficient erythrocytes (27). These observations further support the notion of a functional compensation by the thioredoxin system. Indeed, apart from thioredoxin, also plasmaredoxin, and in many organisms glutaredoxin, have been shown to reduce ribonucleotide reductase, which catalyzes the first step of DNA and RNA synthesis (1, 23). As recently shown by Sturm et al. (28) using interactome analyses, also the redox regulatory properties of thioredoxin, glutaredoxin, and plasmaredoxin are largely overlapping. Furthermore, the peroxiredoxins acting in *Plasmodium* are mainly thioredoxin but also glutaredoxin dependent (1). Concentrations of total glutathione and non-protein bound free thiols were found to not differ significantly between WT and GR (−) or TrxR (−) blood stage parasites. This result supports the finding that the parasites are viable in the absence of either of the two disulfide reductases, and that glutathione and thiol homeostasis are not largely disturbed. Taken together, the available data suggest that in *P. berghei* blood stage parasites, GR and TrxR might be able to largely compensate for each other. In addition, we cannot exclude that reduction by dihydrodipamide of glutaredoxins, and indirectly of glutathione disulfide and thioredoxin disulfide, can functionally replace the NADPH-dependent enzymes GR and TrxR (16, 29, 30).

Interfering with redox metabolism represents a promising approach to antiparasitic drug development (20) prominent examples being *Schistosoma mansoni* thioredoxin-glutathione reductase (31) and trypanothione reductase of *L. donovani* and *T. b. brucei* (32). Also *Plasmodium* GR was considered a leading drug target because (i) complete structural information, which permits computational inhibitor modeling, is available (33), (ii) detailed enzyme kinetic data provided the framework for high throughput inhibitor and mechanistic studies (34), and (iii) several antimalarial agents that presumably act against GR have been developed (18, 35, 36). TrxR was discussed as a promising enzymic drug target because (i) knockout data pointed toward essentiality of the enzyme for blood stage parasites (17), (ii) detailed mechanistic and kinetic properties of PfTrxR are known (Ref. 37, and references therein), (iii) structural differences between the selenoprotein hTrxR and PfTrxR occurring at the solvent exposed C-terminal end of the proteins were considered most attractive sites for directed inhibitor development (22, 37), and (iv) numerous PfTrxR inhibitors had been developed (38, 39).

According to our data in *P. berghei*, neither GR nor TrxR are essential for blood stage malaria parasites. In case the data should be transferable to *P. falciparum*, the potential of mere inhibitors of either disulfide reductase as drug candidate needs to be questioned. However, compounds inhibiting both, structurally highly related, disulfide reductases simultaneously and inhibitors acting on PfGR in mosquito stages should still be considered as antimalarial strategies. Furthermore, drugs like methylene blue that act as redox-cycling substrates of plasmoidal GR or TrxR (18, 20) are still very promising.

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