

# Glutathione Reductase-null Malaria Parasites Have Normal Blood Stage Growth but Arrest during Development in the Mosquito<sup>\*[5]</sup>

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Malaria parasites contain a complete glutathione (GSH) redox system, and several enzymes of this system are considered potential targets for antimalarial drugs. Through generation of a  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS)-null mutant of the rodent parasite *Plasmodium berghei*, we previously showed that *de novo* GSH synthesis is not critical for blood stage multiplication but is essential for oocyst development. In this study, phenotype analyses of mutant parasites lacking expression of glutathione reductase (GR) confirmed that GSH metabolism is critical for the mosquito oocyst stage. Similar to what was found for  $\gamma$ -GCS, GR is not essential for blood stage growth. GR-null parasites showed the same sensitivity to methylene blue and eosin B as wild type parasites, demonstrating that these compounds target molecules other than GR in *Plasmodium*. Attempts to generate parasites lacking both GR and  $\gamma$ -GCS by simultaneous disruption of *gr* and *\gamma-gcs* were unsuccessful. This demonstrates that the maintenance of total GSH levels required for blood stage survival is dependent on either *de novo* GSH synthesis or glutathione disulfide (GSSG) reduction by *Plasmodium* GR. Our studies provide new insights into the role of the GSH system in malaria parasites with implications for the development of drugs targeting GSH metabolism.

The glutathione (GSH) and thioredoxin redox systems of *Plasmodium* constitute the primary lines of defense against

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reactive oxygen damage and other forms of chemical stress (1–3). Enzymes involved in antioxidant defense in *Plasmodium* species, such as glutathione reductase (GR),<sup>6</sup> have been proposed as promising targets for the development of novel antimalarials. GR has attracted a lot of attention as a potential therapeutic target based on biochemical studies that have shown *in vitro* inhibition of the enzyme and *in vivo* parasite death after treatment with methylene blue (MB) and eosin B among others (4–10).

*Plasmodium* spp. have a fully functional GSH redox system. The low molecular weight GSH is not only an important intracellular thiol redox buffer but also a cofactor for several redox enzymes, such as glutathione *S*-transferase and glutaredoxin (3, 11, 12). A number of studies focused on the GSH metabolic pathway in malaria parasites because altered GSH levels have been linked to resistance to antimalarial drugs (13–17). In *Plasmodium*, *de novo* synthesis of GSH contributes to the maintenance of intracellular GSH levels (18–20). GSH is synthesized by the sequential action of  $\gamma$ -glutamylcysteine synthase ( $\gamma$ -GCS) and glutathione synthase; both of the genes encoding these enzymes are present in the *Plasmodium* genome (21, 22). In addition, GSH is also generated by recycling glutathione disulfide (GSSG) back to GSH via GR. GSSG is produced during the degradation of ferriprotoporphyrin IX, by the reduction of glutaredoxin, or during the reductive reactions catalyzed by glutathione *S*-transferase (3, 11, 12). The ratio between GSH and its oxidized form, GSSG, is maintained far on the side of the reduced form of GSH mainly by the action of GR (18) and the export of excess GSSG (23–25).

We have recently shown that *de novo* synthesis of GSH is not essential for survival of blood stages in the rodent malaria parasite, *Plasmodium berghei* (20). This was an unexpected finding in view of the expression of enzymes for *de novo* synthesis of GSH in blood stages (21, 22) and the anticipated important role that GSH plays both as a thiol redox buffer and in detoxification of free ferriprotoporphyrin IX produced during hemoglobin digestion (26–28). Blood stages of parasites lacking the enzyme

<sup>6</sup> The abbreviations used are: GR, glutathione reductase (EC 1.8.1.7); MB, methylene blue;  $\gamma$ -GCS,  $\gamma$ -glutamylcysteine synthetase; 5-FC, 5-fluorocytosine; p.i., postinfection; Trx, thioredoxin; TrxR, thioredoxin reductase; ROS, reactive oxygen species; contig, group of overlapping clones.

## Glutathione Reductase in Malaria Parasite Development

$\gamma$ -GCS ( $\Delta\gamma$ -gcs) showed only a minor reduction in growth rate compared with wild type (WT) parasites (20). In contrast, analysis of  $\Delta\gamma$ -gcs parasites demonstrated the pivotal role of *de novo* synthesis of GSH for development in the mosquito. Parasites lacking this enzyme show a complete block in oocyst development and were unable to develop sporozoites. Interestingly, the disruption of  $\gamma$ -gcs caused a significant reduction but not complete depletion of GSH in blood stage parasites. A likely explanation for the residual GSH in blood stages is that it is derived from the host erythrocyte. This may be a result of the uptake and transport of host GSH (or host GSSG) to the parasite food vacuole via hemoglobin-containing endocytic vesicles as proposed by Platel *et al.* (15, 20). The import and use of host enzymes by *Plasmodium* has recently been demonstrated for another enzyme involved in antioxidant defense. *Plasmodium falciparum* blood stages were shown to import the human redox-active protein peroxiredoxin 2 into its cytosol for peroxide detoxification (29). These observations indicate that the uptake and use of host enzymes in the erythrocyte is not an uncommon phenomenon/event in malaria parasites.

To further investigate the importance of the GSH metabolism in *Plasmodium* and the essential role of the enzymes involved in GSH formation, we have generated *P. berghei* parasites lacking GR expression. Similar to what was reported for  $\gamma$ -gcs, GR is not essential for parasite blood stage development, but it does play a critical role during oocyst development in the mosquito. These results emphasize the key role of GSH metabolism during *Plasmodium* development in the mosquito. In addition, we attempted to simultaneously disrupt *de novo* GSH synthesis and GR-mediated reduction of GSSG to GSH by knocking out both the  $\gamma$ -gcs and *gr* genes. We were unable to generate the double knock-out parasites, which indicate that blood stages cannot completely rely on the use of host GSH (or enzymes) or any other *Plasmodium*-derived alternative pathway for GSH metabolism. These results demonstrate that *de novo* synthesis of GSH or its formation through reduction of GSSG by a *Plasmodium*-derived GR are required in order to maintain intracellular GSH levels necessary for blood stage parasite survival. The analysis of  $\gamma$ -GCS-null and GR-null mutant parasites provide novel insights on the role of the GSH metabolism in the malaria parasite life cycle with implications for the development of drugs targeting enzymes involved in this pathway.

### EXPERIMENTAL PROCEDURES

*P. berghei* Parasites and Mice—*P. berghei*, ANKA strain, reference lines that stably express fluorescent reporter proteins were used: line 507c11 (WT GFP), which expresses GFP by the constitutive *eef1a* promoter (30) (RMgm-7; see the Leiden University Medical Center Web site) and line 820c11m1c11 (WT Fluo-frmg), which expresses GFP by a male gametocyte-specific promoter and red fluorescent protein by a female gametocyte-specific promoter (31) (RMgm-164; see the Leiden University Medical Center Web site).

Experiments were carried out using Swiss-CD1 female mice (4–6 weeks old; Charles River Laboratories, Wilmington, MA) or Swiss-OF1 female mice (OF1-ico, Construct 242; age 6 weeks old; Charles River Laboratories). Animal work at the University

of Puerto Rico was conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, the Institutional Animal Care and Use Committee, and regulations of the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Animal experiments in The Netherlands were performed after a positive recommendation of the Animal Experiments Committee of the Leiden University Medical Center was issued to the licensee. The Animal Experiment Committees are governed by section 18 of the Experiments on Animals Act and are registered by the Dutch Inspectorate for Health, Protection, and Veterinary Public Health, which is part of the Ministry of Health, Welfare, and Sport. The Dutch Experiments on Animals Act was established under European guidelines (EU directive 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes).

*Generation of P. berghei Mutants Lacking Expression of GR ( $\Delta$ gr Parasites)*—Four different replacement constructs were made to disrupt the *gr* gene. The *P. berghei* glutathione reductase DNA sequence (gene identifier in PlasmoDB PB001317.02.0, PB300369.00.0 and in GeneDB PBANKA\_102340) was retrieved from PlasmoDB, and the complete sequence of the gene was obtained from contig PB\_RP2849. For the first construct, DNA fragments from the 5' region (exon 2 and part of exon 3) and the 3' region of the *gr* gene were cloned flanking the *tdhfr-ts* (*Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase) selection cassette of plasmid pL0001 (MR4). An 885-bp DNA fragment from exon 2 to exon 3 of the *gr* locus was PCR-amplified with primers 3087 (5'-GGGGGTACCCAGGAATAAGGCAAAAAGTTGCTTTGGTTCG-3'; KpnI site is underlined) and 3088 (5'-GGGAAGCTTCATAGTCAAGATGCTCAAACCTCCGTCCATC-3'; HindIII site is underlined) and cloned into KpnI/HindIII-digested pL0001 vector to obtain pL0001-5'*gr*. To generate the 3' targeting region, a fragment of 754 bp was PCR-amplified with primers 3089 (5'-GGGGGATCCGTTGCTATAAATGCGGGGCGATTATTAGCTG-3'; BamHI site is underlined) and 3090 (5'-GGGTCTAGACCTTCTTTGATCATATCCCCTTATTTTGTTCATC-3'; XbaI site is underlined) and cloned into BamHI/XbaI pL0001-5'*gr*-digested plasmid to create the final disruption vector, pL1282. For transfection, pL1282 was linearized with KpnI/SacII and transfected into purified schizonts of *P. berghei* line WT GFP.

A second construct (pL1309) was generated for attempts to disrupt *gr* in parasites lacking the enzyme  $\gamma$ -GCS ( $\Delta\gamma$ -gcs) (20). The  $\Delta\gamma$ -gcs parasites contain the *tdhfr-ts*-selectable marker, and therefore we generated a disruption construct with the human *hdhfr* (dihydrofolate reductase)-selectable marker to be able to select with the drug WR99210. The 5' and 3' *gr*-targeting regions were PCR-amplified with primer set 3085/3086 (5'-GGGGGATCCACACCCACCTATAGGAACAATAGTTTATCTG-3' and 5'-GGGTCTAGACTAAGCATAAATAATGCACATTCGGATTTCCCG-3') and 3087/3088 (5'-GGGGGTACCCAGGAATAAGGCAAAAAGTTGCTTTGGTTCG-3' and 5'-GGGAAGCTTCATAGTCAAGATGCTCAAACCTCCGTCCATC-3') and cloned BamHI/XbaI and KpnI/HindIII sites (restriction site underlined) into pL0006 (MR4), which contains the *hdhfr*-selectable marker under the control of the *eef1a* promoter. For transfection, pL1309 was linearized with

Scal and transfected into purified schizonts of  $\Delta\gamma$ -gcs parasites. For generation of the third construct, pL1377, the 5' and 3' *gr*-targeting regions were PCR-amplified with primer sets L3742/L3743 (5'-gggAAGCTTCGCTAGTTTATATACAGTGG-3' and 5'-tccCCGCGGCATGAACTTTTCTATTTC-TTCTAC-3') and L3680/L3681 (5'-cggGGTACCGTTGCTA-TAAATGCGGGGCGATTATTAGCTG and 5'-ccgGATAT-CCCTTCTTTGATCATATCCCTTATTTTGTGTC) and cloned HindIII/SacII and EcoRV/KpnI (restriction sites underlined) into pL0035 (MR4), which contains the *yfcu-hdhfr*-selectable marker under the control of the *efl1a* promoter (32). For transfection, pL1377 was linearized with HindIII, EcoRI, and Scal and transfected into purified schizonts of *P. berghei* line WT GFP. To obtain complete replacement of the ORF of *gr*, we generated a fourth construct, pL1538. This construct is similar to pL1377 except that the 5' region of pL1377 is exchanged (HindIII/SacII) with a 5'-UTR of *gr*, which was PCR-amplified with the primer set L4530/L4049 (5'-gccAAGCTTGTGTGT-TATAAGGCCTGTAAGTGTC-3' and 5'-TCCCCGCGGCG-ATTAATCATAAACCATATTTG-3'; restriction site underlined). For transfection, pL1538 was linearized with HindIII, EcoRI, and PvuI and transfected into purified schizonts of *P. berghei* line WT Fluo-frmg.

Transfection, selection, and cloning of mutant parasite lines were performed as described (33). Correct integration of the construct into the genome of mutant parasites was analyzed by diagnostic PCR and Southern blot analysis of digested genomic DNA or field inverted gel electrophoresis-separated chromosomes (33). Correct integration of pL1282 into the *gr* locus was confirmed by Southern blot analysis of digested genomic DNA using *tgdhfr-ts*- and *gr*-specific probes. The 921-bp *tgdhfr-ts* probe was obtained by digesting pL0001 with SalI, and the 768-bp *gr*-specific probe was obtained by PCR amplification of the entire *gr* coding sequence (*gr* ORF probe) using primers 97 F (5'-GGGGGATCCATGGTTTATGATTTAATCGTTATT-GGAGGAGGAAGTGG-3') and 99 R (5'-GGGGGTACCGA-TATCACATAAACAATTTGTGTAGTCATGCCATTGC-3') and digesting the PCR product with restriction enzymes SpeI and PacI. The 768-bp fragment was gel-extracted and purified using the Wizard SV gel and the PCR Clean-up system (Promega). The 3'-UTR *pbdhfr-ts* probe (33) and the EcoRV/KpnI fragment of pL1358 (3' *gr* probe) were used to analyze the correct integration of pL1377 and pL1358, respectively. The 3' *gr* probe was also used to detect the chromosomal location of the *gr* locus.

The positive-negative selectable marker system was used to generate a mutant lacking expression of GR without a drug-selectable marker in its genome. Parasites of the WT GFP line were transfected with pL1377, which contains the *hdhfr-yfcu*-selectable marker, and mutant parasites were selected with pyrimethamine (33), resulting in parasite line 1195. Before applying negative selection, parasites were cloned by limiting dilution, and one clone, 1195c1, was selected for further analysis. Correct integration of construct pL1377 in 1195c1 was shown by diagnostic PCR (data not shown) and Southern analysis of separated chromosomes (supplemental Fig. S2). Negative selection with 5-fluorocytosine (5-FC) was performed as described (32); three mice infected with 1195c1 were treated

with 5-FC starting at a parasitemia of 0.1–0.5% with a daily single dose of 0.5 ml of 20 mg/ml drug/day for a period of 4 days. Resistant parasites were collected between days 5 and 7 after initiation of the 5-FC treatment, and the genotype was analyzed by diagnostic Southern analysis to confirm removal of the drug-selectable marker *hdhfr-yfcu* by a recombination event between the two 3'-UTR *dhfr* sequences (supplemental Fig. S2). Parasites from one of the four mice (mouse 1) that had been treated with 5-FC were cloned by limiting dilution, resulting in line 1195c1m1c1 ( $\Delta gr4$ ). This line was used in attempts to disrupt  $\gamma$ -GCS with constructs pL1217 and pL1223 (20) using the positive selection as described above.

**Analysis of *gr* Transcription/Expression by Northern and Western Analysis**—Total RNA was isolated from blood stage parasites according to standard methods. To determine *gr* transcription, Northern blots containing RNA from blood stages were hybridized with a probe of the 5'-UTR *gr* region (5' *gr* probe) which was PCR-amplified with primer set L3087/L3088 (5'-GGGGGTACCCAGGAATAAGGCAAAAGTTGCTTT-GGTTCG-3' and 5'-GGGAAGCTTCATAGTCAAGATGCTC-AACTTCCGTCCATC-3'). As a loading control, Northern blots were hybridized with primer L644R, which hybridizes to the blood stage large subunit ribosomal RNA (34). To detect expression of the GR protein, we used a polyclonal pGR antibody produced in rabbits against the full-length recombinant GR protein, kindly provided by Prof. Heiner Schirmer (Heidelberg University). As a loading control, we used a mouse monoclonal antibody (B5) against a 58-kDa *P. berghei* co-chaperone protein (35, 36).

**Determination of Total GSH Levels in Blood Stage Parasites**—Total GSH levels in blood stage parasites were determined according to published methods (20, 37, 38). Blood (0.8–1.0 ml) was harvested by heart puncture from mice infected with  $\Delta gr$  mutants and WT parasites (10–20% parasitemia). White blood cells were removed using a Plasmodipur filter (Euro-Diagnostica), and the infected erythrocytes were washed (6 mM EDTA saline solution) and differentially lysed with saponin (0.15%). Parasites were resuspended at a final concentration of  $50 \times 10^6$  parasites/100  $\mu$ l in buffer (3.5 mM MgCl<sub>2</sub>, 110 mM KCl, 40 mM NaCl, 20 mM Hepes, 6 mM EDTA, pH 7.4) containing protease inhibitors (0.01 mg of leupeptin A, 0.001 mg of pepstatin A, 0.35 mg of PMSF). Subsequently, parasites were lysed by three freeze/thaw cycles. Parasite extracts were treated with 100  $\mu$ l of 12.5 mM dithioerythritol to reduce total GSH. The derivatization was carried out using monobromobimane. Total GSH in the effluent was measured using a Hewlett Packard 1046A programmable fluorescence detector (excitation 388 nm; emission 491 nm). GSH calibration curves were performed every time a set of samples was analyzed. In order to analyze the GSH data, a dot plot was generated. A one-tailed unpaired *t* test with the Welch correction for differences in variance was employed to test for differences in the GSH levels measured. This correction was used because a significant difference between the variances in the WT versus  $\Delta gr$  data was observed. A standard outlier detection test was used to analyze the GSH values (39). Extreme outliers at the upper level of the data were detected exceeding 3 times the interquartile range.



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**Determination of GR Activity in Blood Stage Parasites**—Parasite extracts were prepared as described above for the GSH determination. Protein content in parasite extracts was determined by a DC assay (Bio-Rad). GR activity was assayed at 25 °C in *P. berghei* protein extracts by monitoring the decrease of NADPH absorbance at 340 nm using a 96-well plate in a Spectra Max 190 spectrophotometer (Molecular Devices). Parasite extract (0.33 mg/ml protein) and 0.35 mM NADPH was added to a buffer containing 50 mM potassium phosphate (pH 7.0), 200 mM KCl, 1 mM EDTA (40, 41). The reaction was started by adding 0.5 mM GSSG. In order to detect any residual redox activity associated with the parasite extract, blank reactions (no GSSG) were carried out. Enzyme activity is reported as units (micromol of NADPH/min)/mg of protein. GraphPad Prism 5.02 software was used for statistical analyses. A one-way analysis of variance was used to compare the GR specific activity in WT and  $\Delta gr$  using a 0.05 confidence level for significance. A *t* test with Bonferroni's correction for multiple comparisons was used to test for differences between the  $\Delta gr$  and WT values.

**Determination of Growth and Multiplication of Asexual Blood Stages**—The multiplication rate of asexual blood stages *in vivo*, determined during the cloning procedure (42), was calculated as follows. The percentage of infected erythrocytes in Swiss (CD1 or OF1) mice injected with a single parasite was determined at days 8–11 by counting Giemsa-stained blood films. The mean asexual multiplication rate per 24 h was then calculated assuming a total of  $1.2 \times 10^{10}$  erythrocytes/mouse (2 ml of blood). The percentage of infected erythrocytes in mice infected with reference lines of the *P. berghei* ANKA strain consistently ranged between 0.5 and 2% at day 8 after infection, resulting in a mean multiplication rate of 10 per 24 h (36, 42). In addition, the asexual growth and multiplication was determined in groups of five Swiss-CD1 mice that were infected intraperitoneally with  $1\text{--}2 \times 10^2$  parasites. The parasitemia (equal to the percentage of infected erythrocytes) was determined by counting daily Diff-Quick-stained slides of tail blood during a period of 11 days. Mice were sacrificed when they showed signs of cerebral complications, such as ruffled fur, hunching, wobbly gait, limb paralysis, convulsion, and coma, usually on day 10 or 11 after infection.

**Determination of the Susceptibility of Parasite Blood Stages to MB and Eosin B**—The susceptibility of blood stage parasites to MB and eosin B was determined *in vitro*. In addition, the susceptibility to MB was determined *in vivo* in Swiss CD-1 mice (see below). *In vitro* susceptibility was determined by standard drug susceptibility assays in which schizont maturation (DNA synthesis) is measured by fluorescence-activated cell sorting analysis (FC assay) (43, 44). MB (Sigma) and eosin B (Sigma) were dissolved in water to final stocks of 0.2 mM/liter and 500 nM/liter, respectively. Serial dilutions with complete culture medium (RPMI1640 with 20% fetal calf serum) were prepared, ranging from 0.2 to 200 nM/liter for MB and from 2.5 to 250 nM/liter for eosin B. In the FC assays, ring forms/young trophozoites were incubated for a period of 24 h at 37 °C in 24-well plates in complete culture medium containing serial dilutions of the drugs, allowing the ring forms/young trophozoites to develop into mature schizonts. After the culture period, the cells were stained with 2  $\mu$ M Hoechst 33258 dye for 1 h at 37 °C.

Stained cells were analyzed using a FACScan (LSR II, BD Biosciences). UV excitation of Hoechst 33258 dye was performed with an argon ion laser (450/50 nm), and the (infected) erythrocyte population was selected by gating on forward/side light scatter. The fluorescence intensity of a total of 100,000 cells/sample was measured, and data analysis was performed using CellQuest software (BD Biosciences) and FlowJo software (Tree Star, Inc.). The mean fluorescence intensity of the infected erythrocyte population, which is proportional to the mean DNA content of the parasites (45), was calculated for each drug concentration in triplicate. For calculation of the growth inhibitory curves, the mean fluorescence intensity value of samples with the highest drug concentration (*i.e.* with maximum inhibition of growth) was subtracted from the mean fluorescence intensity value of the samples with the other drug concentrations used and the control samples without drug as described (43). Growth-inhibitory curves and statistical analysis of the data were performed using GraphPad Prism software (GraphPad Software, Inc.). The non-linear regression function for sigmoidal dose-response (variable slope) of the GraphPad Prism software is used to calculate the (best fit)  $EC_{50}$  values.

Susceptibility of blood stage parasites to MB (Sigma) was tested *in vivo* as described previously by Arora and Srivastava (46) with some modifications. Briefly, three groups of mice (five mice each) were infected intravenously with  $10 \times 10^6$  parasites/mouse ( $\Delta gr$  or WT). MB administration (*i.p.*, 2.5 mg/kg) started 1 day after infection, twice a day, for a period of 6 days. Parasitemia was analyzed by light microscopy of Diff-Quick-stained thin blood smears, starting 1 day postinfection (*p.i.*) and every other day until completion of the experiment (day 6).

**Determination of Production of Gametocytes, Ookinets, and Oocysts**—Gametocyte production was determined in synchronized *in vivo* infections as described (47). The gametocyte conversion rate is the percentage of ring forms developing into gametocytes under standardized conditions. Ookinete production is determined by standard *in vitro* fertilization and ookinete maturation assays (48, 49). The ookinete conversion rate is defined as the percentage of female gametes that develop into mature ookinets determined by counting female gametes and mature ookinets in Giemsa-stained blood smears 16–18 h after *in vitro* induction of gamete formation. Oocyst development, oocyst production, and sporozoite production were monitored in *Anopheles stephensi* mosquitoes as described (50). Oocyst and sporozoite numbers were counted in infected mosquitoes at 10–12 days and 21–22 days *p.i.* The size of oocysts was determined in parallel. Salivary glands from 10 mosquitoes were dissected and homogenized in a homemade glass grinder in 1 ml of PBS, pH 7.2, and sporozoites were counted in a Bürker-Türk counting chamber using phase-contrast microscopy (51).

**Analysis of Oocyst Development by Immunofluorescence Microscopy and Transmission Electron Microscopy**—Oocyst development was assessed using specific antibodies. Mosquito midguts were dissected at 2 and 12 days *p.i.*, fixed in 4% paraformaldehyde, and blocked with 3% bovine albumin serum. The blood bolus was removed from midguts dissected at day 2 *p.i.* prior to incubation with an antibody specific for the surface protein, Pbs21 (mAb 13.1) (52) of ookinets and young

oocysts. Detection of stained oocysts was done by fluorescent microscopy (400 $\times$  magnification) after incubation with a Rhodamine Red<sup>TM</sup>-X goat anti-mouse IgG (H + L) (Invitrogen). Midguts dissected at day 12 p.i. were stained with antibodies against PbCap380, an oocyst capsule protein (anti-PbCap380) (53), and PbCSP, recognizing the circumsporozoite protein (anti-PbCSP mAb 3D11) (54). Oocysts were detected by immunofluorescence assay after incubation with Rhodamine Red<sup>TM</sup>-X goat anti-mouse IgG (H + L) or Texas Red<sup>®</sup>-X goat anti-rabbit IgG (H + L) (Invitrogen), respectively, in fluorescence microscopy (400 $\times$  magnification). To analyze infectivity of sporozoites, infected mosquitoes ( $n = 50$ ) at day 21 were fed on naive mice (2 mice/experiment) for a period of 15–20 min. The presence of parasites in the mouse blood stream was monitored daily (from day 6 to day 12 after feeding of the mosquitoes) by Giemsa-stained thin smears of tail blood.

The transmission electron microscopy analysis of oocysts was carried out in midguts of *A. stephensi* infected mosquitoes dissected at day 6. Midguts were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature and processed as described by Quittnat *et al.* (55) using a Philips 410 Electron Microscope (Eindhoven, The Netherlands) under 80 kV.

## RESULTS

**Generation of *P. berghei* Mutants Lacking Expression of GR ( $\Delta gr$ )**—GR of *P. berghei* is encoded by a single copy gene (*gr*; GeneDB PBANKA\_102340) located on chromosome 10 (Fig. 1). This gene is composed of three exons, 1494 bp in length, and encodes a 497-amino acid-long polypeptide with a predicted molecular mass of  $\sim 56.5$  kDa. Northern analysis showed the presence of a  $\sim 2.8$ -kb transcript (Fig. 1E). Similar to its homolog in *P. falciparum*, Western blot analysis of blood stage protein extracts using a polyclonal antibody against recombinant pfGR detected a protein of  $\sim 56$  kDa. The GR of *P. berghei* shows 80% identity with the *P. falciparum* GR homolog and differs only by the absence of three amino acids (position 133–135) not affecting functional domains (supplemental Fig. S1). Both proteins contain the pyridine nucleotide-disulfide oxidoreductase, dimerization, and the FAD/NAD-linked reductase domains, which are important for their enzymatic activity (see GeneDB). The *P. berghei* GR also contains the 34-residue insertion in the central domain (from 315 to 348; supplemental Fig. S1) found in *P. falciparum* (56).

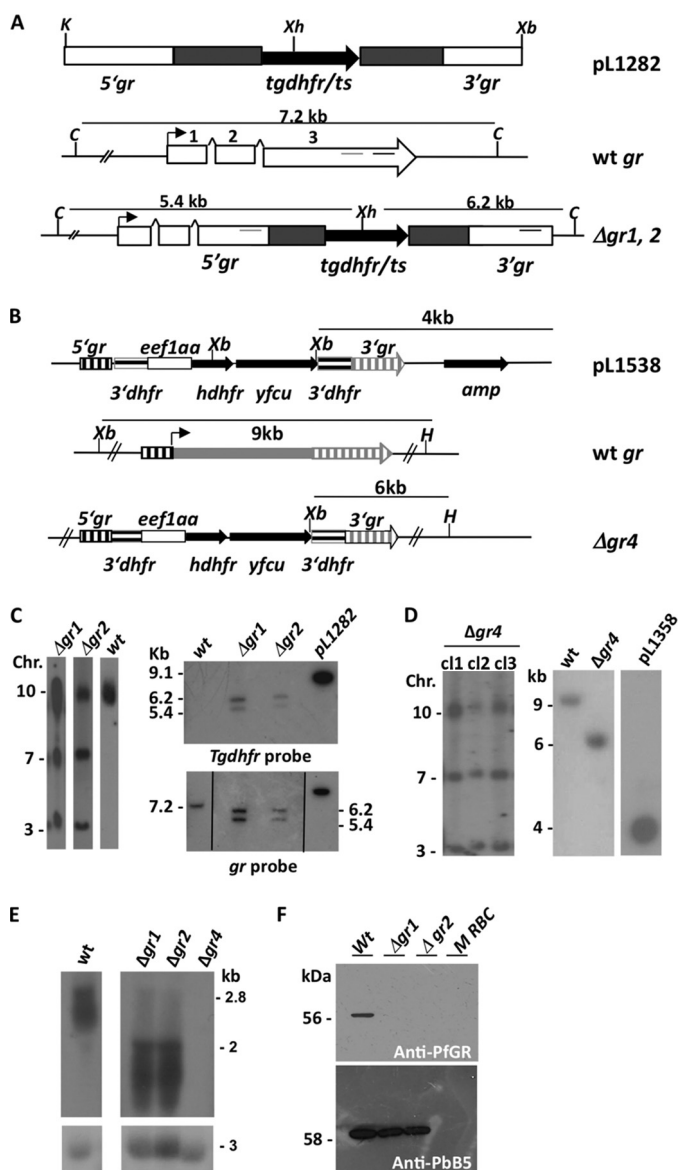
To examine the role of GR in *P. berghei* growth and development, the *gr* gene was disrupted using standard genetic modification technologies (33). In four independent experiments (experiments 1000, 1028, 1195, and 1513) *P. berghei* ANKA parasites of the reference line WT GFP or reference line WT Fluo-frmg were transfected with DNA constructs aimed at the disruption of the gene via double cross-over integration. In transfection experiments 1000 and 1028, integration of the construct pL1282 resulted in the replacement of a  $\sim 250$ -bp region of the *gr* gene of WT GFP by the *tdhfr-ts*-selectable marker cassette (Fig. 1A). Two independent clones ( $\Delta gr1$  and  $\Delta gr2$ ) were further analyzed. Similarly, in experiment 1195, the  $\sim 700$ -bp region of the *gr* gene of WT GFP was replaced with the

*hdhfr-yfcu*-selectable marker cassette using construct pL1377 (supplemental Fig. S2A). This mutant parasite line was generated in order to remove the selectable marker from the genome by negative selection using the drug 5-FC (32). After removal of the selectable marker cassette (see below), one clone ( $\Delta gr3$ ) was used for further analysis. In experiment 1513, a fourth mutant ( $\Delta gr4$ ) was generated using pL1538 construct. Integration of this construct resulted in the replacement of the complete 1.8-kb *gr* ORF of WT Fluo-frmg with the *hdhfr-yfcu*-selectable marker cassette (Fig. 1B). Correct integration of constructs into the parasite genome was confirmed by Southern analysis of digested DNA and/or field inverted gel electrophoresis separated chromosomes (Fig. 1, C and D, and supplemental Fig. S2B). The absence of the WT *gr* mRNA transcripts in the different mutant lines was demonstrated by Northern analysis of total mRNA from blood stages (Fig. 1E). Smaller sized transcripts ( $\sim 2$  kb) were detected in the mutant  $\Delta gr1$  and  $\Delta gr2$  lines. These correspond to the predicted size of transcripts, which include the first two and part of the third exon, which remain after disrupting the *gr* locus using pL1282. These small transcripts are absent in  $\Delta gr4$  in which the complete ORF has been replaced (Fig. 1E). Western analysis using a polyclonal antibody against recombinant *P. falciparum* GR showed the absence of the  $\sim 56$ -kDa fragment of *P. berghei* GR in the  $\Delta gr$  mutants (Fig. 1F and supplemental Fig. S2C). In addition, the predicted protein fragment of 21 kDa corresponding to the remaining 213 amino acids in the  $\Delta gr1$  and  $\Delta gr2$  was not detected, indicating the absence of a functional GR protein.

GR activity was determined in blood stage protein extracts of both WT and mutant parasites in the presence or absence of GSSG by monitoring the disappearance of NADPH in real time. The mean GR activity of WT parasites in the presence of GSSG was 0.016 units of enzyme activity/mg of total protein, as determined by the rate of NADPH disappearance (Fig. 2A). These values are comparable with those reported by Sarma *et al.* (56) in crude extracts of blood stages of several *P. falciparum* strains (range of 9–35 milliunits/mg total protein). In the absence of GSSG, the mean activity in WT parasites (0.001) was  $>15$  times lower than in the presence of GSSG. As expected, disruption of the *gr* gene resulted in a drastic reduction of the NADPH-dependent activity in  $\Delta gr$  blood stages (Fig. 2A). This result shows that GR is the major enzyme in the parasites for reduction of GSSG. However, the low residual activity in  $\Delta gr$  (0.0014 and 0.0030), only observed in the presence of GSSG, suggests that reduction of GSSG might be carried out by other enzymes (see “Discussion”).

**Intracellular GSH Levels in the Absence of Endogenous GR Activity**—The main function of GR enzymes is to convert GSSG into GSH and thereby contribute to the maintenance of GSH homeostasis. Therefore, the absence of endogenous GR activity in the  $\Delta gr$  mutants could potentially lead to altered intracellular GSH levels. Total GSH levels in blood stages from WT,  $\Delta gr1$ , and  $\Delta gr2$  parasites were quantified by HPLC as described previously (20, 37, 38). We initially determined the optimal concentration of DTE (6.25 mM) required to completely reduce known amounts of GSSG (supplemental Fig. S3). Highly reproducible values of GSH in blood stage parasites from multiple *P. berghei* wild type lines were obtained with mean values rang-

## Glutathione Reductase in Malaria Parasite Development



**FIGURE 1. Generation of parasites lacking expression of GR ( $\Delta gr$  parasites).** *A*, schematic representation of the disruption vector pL1282, the WT *gr* locus (numbers indicate the exons) and the locus after disruption. Lines within exon 3 represent the conserved domains of GR. Restriction sites (K, KpnI; X, XbaI) and the size of restriction fragments used for diagnostic Southern blots (see *C*) and the *tgdhfr*-selectable marker are indicated. This construct was used for generation of  $\Delta gr1$  and  $\Delta gr2$  and deletes part of exon 3. *B*, schematic representation of the disruption vector pL1538, the WT *gr* locus, and the locus after disruption. Restriction sites (H, HindIII; Xb, XbaI) and size of restriction fragments used for diagnostic Southern blots (see *D*) and the *yfcu-hdhfr*-selectable marker are indicated. This construct was used for generation of  $\Delta gr4$  and deletes the complete *gr* ORF. *C*, Southern analysis of separated chromosomes (left) and digested genomic DNA (right), confirming correct disruption of *gr* in  $\Delta gr1$  and  $\Delta gr2$  parasites. Hybridization of  $\Delta gr$  chromosomes with the 3' *dhfr* probe recognizes the construct integrated into *gr* on chromosome 10, the endogenous *dhfr-ts* gene on chromosome 7, and the integrated GFP construct on chromosome 3. The 3' *gr* probe hybridizes to the *gr* gene on chromosome 10 (right lane). Hybridization of digested DNA with the *tgdhfr-ts* and the *gr* ORF probes recognized the expected DNA fragments indicated in *A*. Genomic DNA from WT and  $\Delta gr$  parasites were digested with ClaI (C)/XhoI (X). *D*, Southern analysis of separated chromosomes (left) and HindIII/XbaI-digested genomic DNA (right), confirming correct disruption of *gr* in  $\Delta gr4$  parasites. Hybridization of  $\Delta gr$  chromosomes with the 3' *dhfr* probe recognizes the construct integrated into *gr* on chromosome 10, the endogenous *dhfr-ts* gene on chromosome 7, and the integrated GFP construct on chromosome 3. Hybridization of digested DNA with the 3' *gr* probe recognizes the expected DNA fragments indicated in *B*. *E*, Northern analysis of *gr* transcription. Blood stages RNA was hybridized with the 3' *gr* probe and as a loading

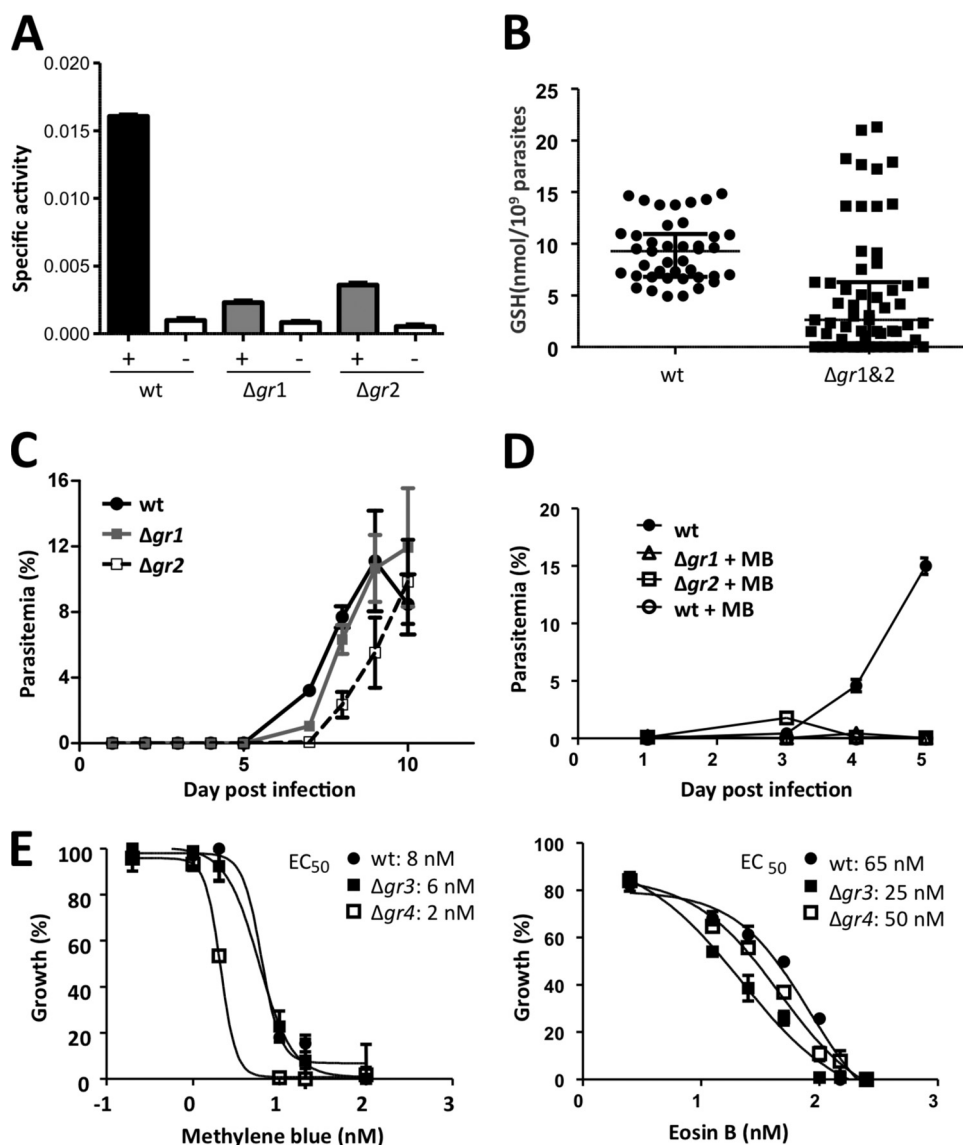
control with the L644R probe recognizing the large subunit ribosomal RNA. In WT parasites, a transcript of  $\sim 2.8$  kb was detected. In the  $\Delta gr1$  and  $\Delta gr2$  parasites, a smaller transcript of  $\sim 2.0$  kb was detected, corresponding to exons 1 and 2 and part of exon 3, which remain in the genome after integration of pL1282 (see *A*). In  $\Delta gr4$ , no transcripts were detected. *F*, Western analysis of GR expression. Protein extracts of blood stages were reacted with a rabbit polyclonal antiserum against *P. falciparum* GR. In WT, the GR protein of 56 kDa is detected, which is absent in the  $\Delta gr$  parasites (see also supplemental Fig. S2). Antibody PbB5 is used as a loading control. Uninfected mouse red blood cells (MRBC) were run in each experiment.

ing between 7 and 15 nm/10<sup>9</sup> cells (Fig. 2B, supplemental Fig. S3) (20), which are comparable with values reported for *P. falciparum* (14, 57). The total GSH level was significantly lower in the  $\Delta gr1$  and  $\Delta gr2$  parasites as compared with WT (Fig. 2B). High variability between samples was observed in the total GSH levels of the mutant parasite lines (mean = 5.04 nmol/10<sup>9</sup> parasites (range 0–21.33; *n* = 59)), whereas GSH values in WT were less variable (mean = 9.24 nmol/10<sup>9</sup> parasites (range 4.92–14.84; *n* = 41)). In a number of  $\Delta gr$  experiments, GSH levels of <1 were detected, in agreement with the levels we reported in mutants lacking *de novo* synthesis of GSH (20). These results show that the lack of GR affects GSH levels in *Plasmodium*, resulting in lower levels of total GSH. The large variation of GSH levels in the  $\Delta gr$  parasite samples examined, some showing higher GSH levels than those observed in WT parasites, suggests that the absence of GR deregulates GSH homeostasis. Because we measured only GSH levels after reduction with DTE, we have no data on the relative contribution of oxidized and reduced GSH to the variable GSH levels in the  $\Delta gr$  parasites. Notwithstanding the absence of the ratios between GSSG and GSH, it appears that the proposed loss of regulatory control of GSH homeostasis cannot be restored by other enzymes with a putative role in GSH reduction, such as thioredoxin or plasmoredoxin.

**Growth Rate of Parasite Blood Stages in the Absence of Endogenous GR Activity**—The generation of mutants lacking GR expression demonstrates that the activity of a *Plasmodium* GR is not essential for survival of the blood stages. To determine if the absence of GR has a possible negative effect on the parasite growth rate during blood stages, we analyzed *in vivo* growth in more detail. The growth rate was first determined by analyzing parasitemias in the experiments in which the mutant parasites were cloned by limiting dilution. Such an analysis is a sensitive method to quantify differences in growth during the early phase of *in vivo* infections when the availability of suitable host cells (reticulocytes) is not a limiting factor (20, 42). In these experiments, we observed no effect on the growth rate of the mutant parasites. All mice infected with a single  $\Delta gr$  parasite showed a parasitemia level of 0.5–2% at 8 days p.i., and therefore  $\Delta gr$  parasites have an asexual multiplication rate of 10 per 24 h, similar to WT parasites (Table 1). To further examine the potential effect of the absence of GR on blood stage growth rate, the course of parasitemia was followed for a period of 11 days in groups of mice infected with 1–2  $\times 10^2$  mutant or WT parasites (Fig. 2C). In parasites harboring up to 10–15% parasitemia, no significant differences were observed in growth rate. Differences in parasitemias were observed between WT and mutant infected mice when parasitemias were higher than 15% and mice suffered from cerebral complications (Fig. 2C). In conclu-

control with the L644R probe recognizing the large subunit ribosomal RNA. In WT parasites, a transcript of  $\sim 2.8$  kb was detected. In the  $\Delta gr1$  and  $\Delta gr2$  parasites, a smaller transcript of  $\sim 2.0$  kb was detected, corresponding to exons 1 and 2 and part of exon 3, which remain in the genome after integration of pL1282 (see *A*). In  $\Delta gr4$ , no transcripts were detected. *F*, Western analysis of GR expression. Protein extracts of blood stages were reacted with a rabbit polyclonal antiserum against *P. falciparum* GR. In WT, the GR protein of 56 kDa is detected, which is absent in the  $\Delta gr$  parasites (see also supplemental Fig. S2). Antibody PbB5 is used as a loading control. Uninfected mouse red blood cells (MRBC) were run in each experiment.





**FIGURE 2. Phenotype of  $\Delta gr$  parasites; GR activity, GSH production, *in vivo* blood stage growth, and sensitivity to methylene blue and eosin B.** *A*, GR activity in blood stage parasites as determined by monitoring the disappearance of NADPH in real time by a decrease in the absorbance at 340 nm in the absence (–) or presence of GSSG (+). Measurements were significantly different between WT,  $\Delta gr1$ , and  $\Delta gr2$  parasites ( $p < 0.0001$ , *t* test with Bonferroni's correction for multiple comparisons). Bars, S.D. *B*, GSH levels in blood stages of WT,  $\Delta gr1$ , and  $\Delta gr2$  parasites. One-way analysis of variance showed significant differences between WT and  $\Delta gr$  parasites ( $p < 0.0001$ ; unpaired *t* test with the Welch correction for differences in variance). The median and 10th and 90th percentiles are shown. *C*, mice infected with  $\Delta gr$  and WT parasites show a comparable course of infection (parasitemia) in Swiss-CD1 mice infected with  $1\text{--}2 \times 10^2$  parasites. The average parasitemia of groups of five mice is shown. Bars, S.D. *D*, the course of parasitemia in groups of five mice infected with  $\Delta gr$  or WT parasites and treated with MB for a period of 6 days is shown. Parasitemia in untreated mice infected with WT is shown as a control. No differences were found between the growth pattern of MB-treated WT and  $\Delta gr$  mutants. *E*, inhibition of the *in vitro* growth of blood stages MB (left) and eosin B (right). Inhibition of growth was determined by measuring the rate of DNA synthesis using flow cytometry (FC assay).  $EC_{50}$  values are shown in the graphs. Error bars, S.D.

sion, our results show that the lack of GR expression has no effect or only a very minor effect on growth and multiplication of the asexual blood stage parasites.

**Susceptibility of Blood Stages to MB and Eosin B in the Absence of Endogenous GR Activity**—Blood stages of various *Plasmodium* species are highly susceptible to MB and eosin B, which are reported to target *Plasmodium* GR. Eosin B (4',5'-dibromo-2',7'-dinitrofluorescein), is a red dye, which effectively inhibits the growth of apicomplexan parasites, such as

*T. gondii* and *P. falciparum* (7, 58). The relative safety of eosin has been established previously, and it is approved by the Food and Drug Administration for use in drugs and cosmetics. For *Plasmodium*, it has been shown that eosin B is a highly selective, potent inhibitor with no indication of cross-resistance with other clinically utilized compounds, suggesting that eosin B is acting via a novel mechanism. Its anti-malarial mode of action appears to be multifaceted and is reported to include the enzymatic inhibition of glutathione reductase and thioredoxin reductase (7).

Using *in vitro* growth inhibition assays, low  $IC_{50}$  values in the range of 5–10 nM/liter for MB and 125 nM/liter for eosin B have been effective for *P. falciparum* (7, 9, 59, 60). In addition, treatment of *Plasmodium vinckei petteri*- and *Plasmodium yoelii nigeriensis*-infected mice with 1–50 mg/kg MB efficiently cures the mice (10, 46). Hereby, it is predicted that parasites lacking GR should exhibit less sensitivity to these drugs. Surprisingly, the normal growth and multiplication of the blood stages of  $\Delta gr$  parasites and their susceptibility to these inhibitors brings into question whether GR is the major target of these compounds. We compared the sensitivity of WT and  $\Delta gr$  blood stages to eosin B (*in vitro*) and MB (*in vitro* and *in vivo*) using standardized assays for determination of drug susceptibility in *Plasmodium*. No major differences in sensitivity to these drugs were detected between WT and  $\Delta gr$  parasites, with  $IC_{50}$  ranging between 2 and 8 nM/liter and between 25 and 65 nM/liter for MB and eosin B, respectively (Fig. 2E). Comparable with these results, treatment of  $\Delta gr$ -infected

mice with a dose previously reported to cure WT-infected mice was also efficient in controlling the parasitemias of  $\Delta gr$ -infected mice (Fig. 2D). In conclusion, our results provide strong evidence that GR is not the major target for MB and eosin B in *P. berghei* blood stages, and the very similar  $IC_{50}$  values of these compounds for WT and  $\Delta gr$  parasites indicate that these compounds target *Plasmodium* molecules other than GR.

**Failure to Generate Mutants Lacking Both GR and  $\gamma$ -GCS**—We have previously shown that *P. berghei* blood stage parasites

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**TABLE 1**  
Growth characteristics of blood and mosquito stages of  $\Delta gr$  and wild type parasites

| Mutant                      | <i>In vivo</i> multiplication rate <sup>a</sup> | Gametocyte production <sup>b</sup> | Ookinete production <sup>c</sup> | Oocyst production <sup>d</sup> | Sporozoite production <sup>e</sup>  |
|-----------------------------|---|------------------------------------|----------------------------------|--------------------------------|-------------------------------------|
|                             |   | %                                  | %                                |                                |                                     |
| $\Delta gr1$ (1000cl3)      | 10 (0; n = 4)                                   | ND <sup>f</sup>                    | ND                               | 165 (0–395)                    | 0                                   |
| $\Delta gr2$ (1028cl1)      | 10 (0; n = 7)                                   | ND                                 | ND                               | ND                             | 0                                   |
| $\Delta gr3$ (1195cl1)      | 10 (0; n = 4)                                   | ND                                 | ND                               | ND                             | ND                                  |
| $\Delta gr3$ (1195cl1m1cl1) | 10 (0; n = 5)                                   | 18.7 (2.1)                         | 78.0 (3.6)                       | ND                             | ND                                  |
| $\Delta gr4$ (1513)         | 10 (0; n = 3)                                   | 19.3 (2.5)                         | 84.3 (4.0)                       | 108 (0–240)                    | 0                                   |
| Wild type                   | 10 (0; n = 10)                                  | 15–25                              | 50–90                            | 215 (0–821)                    | $7 \times 10^4$ to $12 \times 10^4$ |

<sup>a</sup> The multiplication rate of asexual blood stages per 24 h was determined in mice infected with a single parasite.

<sup>b</sup> Gametocyte production (gametocyte conversion rate) is the percentage of blood stage parasites that develop into gametocytes under standardized *in vivo* conditions.

<sup>c</sup> Ookinete production (ookinete conversion rate) is the percentage of female gametocytes that develop into mature ookinete in standard *in vitro* assays for fertilization and ookinete development.

<sup>d</sup> Oocyst production: mean number and range of oocyst numbers in *A. stephensi* counted in 20–40 mosquitoes at days 10–12 after the infectious blood meal.

<sup>e</sup> Sporozoite production: number (range) of sporozoites per salivary gland of *A. stephensi* mosquitoes counted at day 21 after the infectious blood meal.

<sup>f</sup> ND, not determined.

are not dependent on *de novo* synthesis of GSH for their survival, as was demonstrated by generation of mutants lacking the enzyme  $\gamma$ -GCS (20). In order to examine whether blood stage parasites can survive without *de novo* synthesis of GSH and without a functional GR, we attempted to disrupt the  $\gamma$ -gcs in a mutant lacking expression of GR. For these experiments, we generated the  $\Delta gr3$  mutant, which lacks a drug-selectable marker, to enable us to take advantage of the  $\gamma$ -gcs disruption construct, which contains the *tgdhfr*-selectable marker (20). We removed the drug-selectable marker, a fusion of the *hdhfr* and *yfcu* genes, from the genome of mutant  $\Delta pbgr3$  by negative selection with 5-FC (supplemental Fig. S2) as described by Braks *et al.* (32). Three attempts to disrupt the  $\gamma$ -gcs gene using two different constructs (pL1217 and pL1223) in the  $\Delta gr3$  mutant were unsuccessful, whereas WT parasites were readily transfected with the same constructs (20) (data not shown). In addition, we attempted to disrupt the *gr* gene in  $\gamma$ -gcs mutants ( $\Delta \gamma$ -gcs1) (2, 20), with construct pL1309 containing the *hdhfr* as a selectable marker. This selectable marker allows selection of mutants using treatment with the drug WR99210 (61). In five attempts, we were unsuccessful at selecting parasites with both the  $\gamma$ -gcs and the *gr* genes disrupted. In conclusion, our results provide strong evidence that blood stage parasites cannot survive without GR and  $\gamma$ -GCS and indicate that the maintenance of cytosolic GSH levels needed for blood stage survival requires either *de novo* GSH synthesis or the formation of GSH through reduction of GSSG by a *Plasmodium* GR. These results also strongly suggest and further support the notion that blood stage parasites cannot depend solely on host-derived GSH and GR for their GSH metabolism.

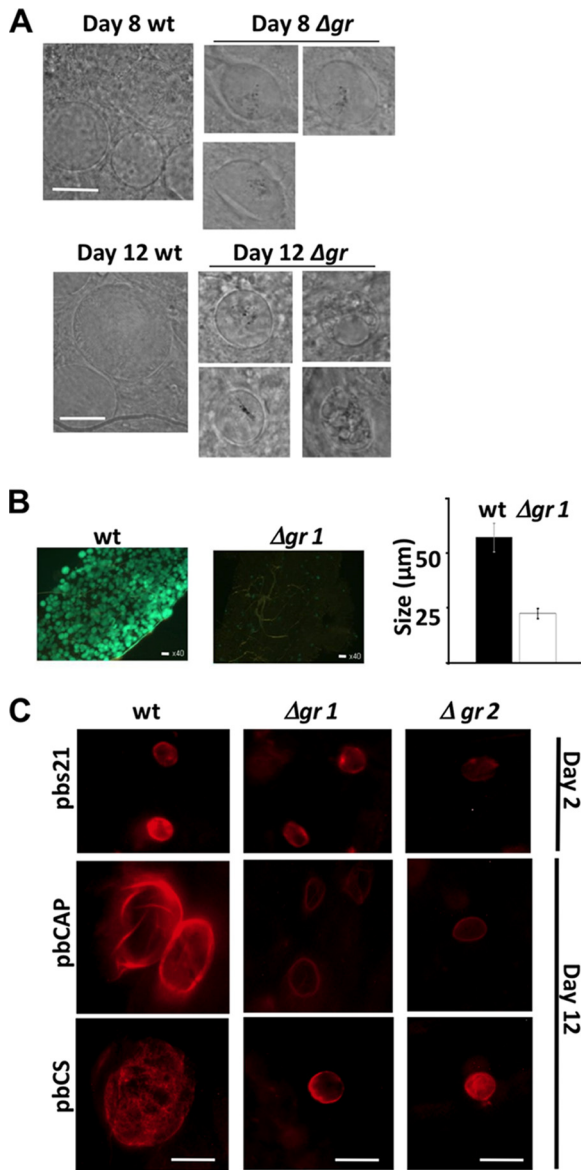
**Arrested Oocyst Development in Mutants Lacking Expression of GR**—In contrast to the independence of blood stages on *de novo* synthesis of GSH (and the likely uptake and use of host-derived GSH by these stages), we have demonstrated the critical role of *de novo* GSH synthesis in the development of the oocyst stage of *P. berghei*. Parasites lacking expression of  $\gamma$ -GCS were unable to produce sporozoites as a result of complete blockage of early oocyst development, demonstrating that survival of the parasites within the mosquito is critically dependent on parasite-derived GSH (20). In this study, the essential role of GR during both sexual development and development in the mosquito was analyzed. Mutants lacking GR expression produced normal numbers of gametocytes that produced fertile gametes and ookinetes comparable with WT parasites (Table 1). In

three independent experiments, we found that the oocyst numbers produced in *A. stephensi*, counted at day 10–12 p.i., were slightly lower than those produced by WT parasites (Table 1 and Fig. 3B), and the size of  $\Delta gr$  oocysts at day 10–12 was considerably smaller compared with the size of WT oocysts (Fig. 3, A and B) with a maximum size that was comparable with immature 6–8-day WT oocysts. Day 2 oocysts of the mutant parasites expressed Pbs21, a surface protein of ookinetes and early stage oocysts (52). Day 12 oocysts of mutant parasites expressed pbCAP380 (PBANKA\_121810; PB0000 71.00.0), a critical protein present in the oocyst capsule (Fig. 3C) (53). Although expression of the CS protein (PB001026.00.0; PBANKA\_040320) (54) could be detected in these oocysts (Fig. 3C), formation of sporozoites was absent in all  $\Delta gr$  oocysts at 10–12 days p.i. and also at later time points (13–21 days p.i.). In addition, no sporozoites were observed in *A. stephensi* salivary glands at day 21–22 p.i. (Table 1). Mosquitoes infected with  $\Delta gr$  parasites for 21 days did not transmit the infection to naive mice. Transmission electron microscopy analysis of 6-day-old oocysts showed clear signs of aberrant development of  $\Delta gr$  oocysts. These oocysts showed a vacuolar appearance of the cytoplasm, decreased number of nuclei, reduced endoplasmic reticulum in an electron-lucent cytoplasm, and signs of “damaged” mitochondria when compared with WT (Fig. 4).

## DISCUSSION

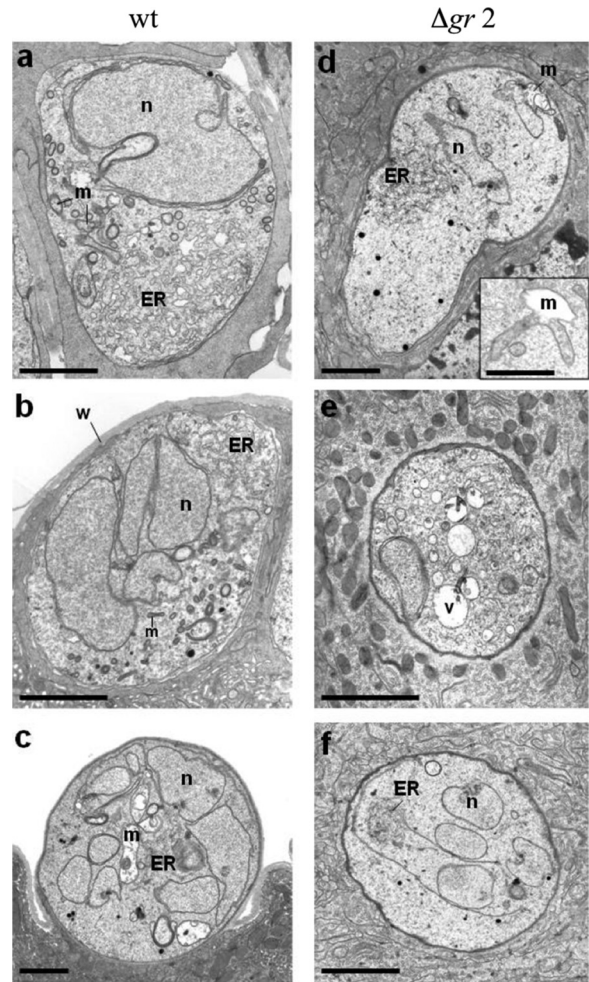
Recently, we demonstrated the critical role of *de novo* GSH synthesis in oocyst stage development in *P. berghei*. Oocysts of parasites lacking  $\gamma$ -GCS expression were not able to produce sporozoites as a result of the complete blockage in development, demonstrating that survival of the parasites within the mosquito is critically dependent on parasite-derived GSH (20). In this study, we demonstrate that GR, another enzyme involved in the GSH metabolic pathway, has an essential role in oocyst development (see Fig. 5 for an overview of the role of the different GSH enzymes at different points in the *Plasmodium* life cycle). Similar to  $\Delta \gamma$ -gcs parasites, the  $\Delta gr$  mutants produce normal numbers of ookinetes and produce oocysts; however, oocysts are blocked during their development and are not able to produce sporozoites. These observations demonstrate that the oocyst stage of *Plasmodium* is not dependent only on *de novo* GSH synthesis but also on a functional GSH redox cycle. Moreover, this suggests that GR is the major enzyme in the





**FIGURE 3. Development of oocysts of  $\Delta gr$  parasites.** A, light microscope pictures of oocysts of  $\Delta gr$  parasites in the midgut of *A. stephensi* mosquitoes at days 8 and 12 p.i., showing an aberrant morphology and reduced size. Scale bar, 25  $\mu m$ . B, GFP-expressing oocyst of WT and  $\Delta gr$  parasites, showing reduced size, reduced GFP expression, and lower numbers of oocysts (see Table 1). Scale bar, 50  $\mu m$ . C, day 2 and day 12 oocysts stained with antibodies against different oocyst proteins: Pbs21, a surface protein of ookinetes and young oocysts; PbCap380, a protein of the oocyst capsule; and PbCSP, a surface protein of sporozoites. Antibody-stained oocysts were detected by indirect fluorescence microscopy using Rhodamine Red<sup>TM</sup>-X goat anti-mouse IgG (H + L) or Texas Red<sup>®</sup>-X goat anti-rabbit IgG (H + L) secondary antibodies (Invitrogen). Scale bar, 25  $\mu m$ .

oocyst that is responsible for converting GSSG to GSH. These results provide evidence that the function of GR cannot be compensated by other reducing enzymes (e.g. plasmoredoxin (PB001241.02.0; PBANKA\_040220) or other enzymes of the thioredoxin (Trx) system (see below)) during mosquito stage development. In contrast to the essential role in the oocyst, *de novo* GSH synthesis and reduction of GSSG by a *Plasmodium*-derived GR appear not to be critical for the survival of the blood stages in the vertebrate host (Fig. 5). The absence of  $\gamma$ -GCS expression had only a minor effect on the growth rate of blood



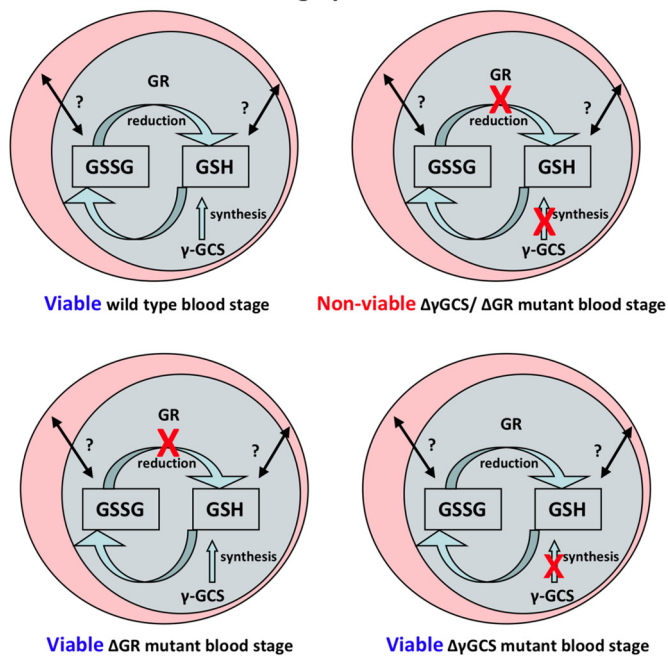
**FIGURE 4. Morphology of oocysts of  $\Delta gr$  parasites.** Transmission electron microscope analysis of WT (a–c) and  $\Delta gr 2$  (d–f) oocysts at day 6 postinfection of *A. stephensi* mosquitoes. WT oocysts show abundant ER, mitochondria (m), and large nuclei (n). Oocysts of  $\Delta gr$  parasites have a smaller size and show limited expansion of the ER, small nuclei with electron-lucent nucleoplasm, damaged mitochondria (m in a and inset), and large vacuoles (v). Scale bars, 2  $\mu m$ .

stage parasites (20), whereas in this study, no significant differences in intraerythrocytic growth were observed between parasites lacking GR and WT parasites. These observations are highly unexpected due to the importance of GSH as a thiol redox buffer, its anticipated role in *Plasmodium* as a cofactor for redox-active enzymes such as glutathione S-transferase and glutaredoxin (3, 62), and its participation in detoxification of free ferriprotoporphyrin IX that is produced during hemoglobin digestion (26–28).

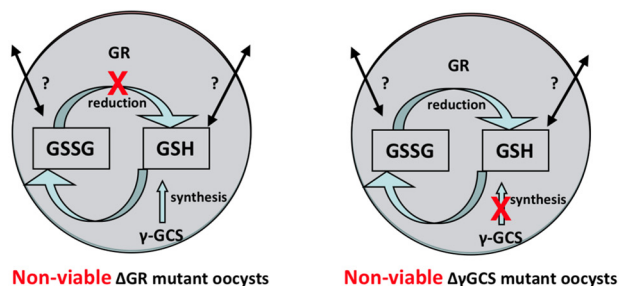
Blocking *de novo* GSH synthesis by disruption of  $\gamma$ -gcs caused a significant reduction but not complete depletion of GSH in blood stage parasites (20). Although it is assumed that *P. falciparum* does not utilize GSH from the host erythrocyte because the parasite membrane is permeable neither to host GSH nor to  $\gamma$ -glutamylcysteine synthetase (18, 63), it might be possible that the residual GSH levels detected in the  $\Delta \gamma$ -gcs blood stages were derived from the host erythrocyte (as indicated in Fig. 5) by uptake of host GSH via hemoglobin-containing endocytic vesicles, as suggested by Platel *et al.* (15). Blood stages of the malaria parasite endocytose large quantities of the surrounding

## Glutathione Reductase in Malaria Parasite Development

### A. Intracellular blood stage parasites



### B. Extracellular mosquito stage parasites (oocysts)



**FIGURE 5. Importance of GSH enzymes in the *Plasmodium* parasite life cycle.** A, analysis of mutants lacking expression of either GR or  $\gamma$ -GCS shows that neither the reduction of GSSG nor the *de novo* synthesis of GSH is essential for parasite blood stage multiplication (*lower panels*). It has been proposed that the intracellular blood stage parasites (*gray circles*) are able to take up and use GSH directly from the host erythrocyte (*pink circles*). Blood stage parasites lacking both  $\gamma$ -GCS and GR are not viable, indicating that blood stages cannot completely rely on host enzymes or alternative pathways of GSH reduction for their GSH metabolism (*upper right panel*). B, development of the extracellular oocyst stage in the mosquito is completely dependent on reduction and synthesis of GSH by its own enzymes. The *black arrows* represent the possible transport of GSH and GSSG in and/or out of the parasite.

erythrocyte cytoplasm and deliver it to a digestive food vacuole via those vesicles (64, 65). If uptake of GSH in endocytic vesicles is followed by transport into the parasite's cytoplasm, the possibility exists that the parasite might use host GSH for its own GSH metabolism. The same uptake might occur for host GSSG and GR because the cytoplasm of erythrocytes contains high levels of oxidized GSH and GR. Such an import and use of host molecules for its own GSH metabolism might provide an explanation for survival of blood stages in the absence of  $\gamma$ -gcs or gr expression. Blood stages of *P. falciparum* have been shown to import the human redox-active protein peroxiredoxin 2 into its cytosol for detoxification of peroxide (29), demonstrating that malaria parasites do use host-erythrocyte proteins in essential biochemical pathways. On the other hand, the absence of a phenotype in  $\Delta$ gr blood stages might also be explained by

redundancy in the process of GSSG reduction and the presence of alternative GSH-reducing pathways in *Plasmodium* or by a constant supply of GSH by an intact and active biosynthetic pathway. In both *Escherichia coli* and *Saccharomyces cerevisiae*, it was possible to generate mutants lacking GR expression (66, 67). Thioredoxin reductase was found to be essential for the growth of the *S. cerevisiae* GR-deficient cells (66). In *S. cerevisiae* GR-null mutants, the thioredoxin-thioredoxin reductase system (TrxR/Trx) functions *in vivo* as an alternative system to reduce GSSG (68). Similarly, *Arabidopsis* uses the NADPH-dependent thioredoxin system as a functional backup for cytosolic GR (69). Moreover, in *Drosophila melanogaster*, the thioredoxin reductase system fulfills the function of GR, which is absent in its genome (70). Interestingly, there is evidence that GSSG reduction can be performed by the TrxR/Trx system in *P. falciparum* (71), suggesting a functional link between the GSH and Trx pathway. Furthermore, *Plasmodium* expresses plasmoredoxin (PFC0166w; PB001241.02.0; PB ANKA\_040220), a redox-active protein belonging to the thioredoxin superfamily, which has been shown to reduce GSSG (72). These observations indicate that *Plasmodium* may indeed have alternative ways for reducing GSSG and therefore might not be dependent on either an active GR in the blood stages or on the use of a host-derived GR. Our observation of low but significant levels of NADPH utilization in the extracts of  $\Delta$ gr parasites in the presence of GSSG suggests the presence of other enzyme(s) that can catalyze the NADPH-dependent reduction of GSSG. It will be of importance to investigate whether these observations, a non-essential role of a *Plasmodium* GR and  $\gamma$ -GCS for *P. berghei* asexual blood stages, also hold true for the human parasites *P. falciparum* and *Plasmodium vivax*. As we have discussed before (20), it might be possible that the dependence on these enzymes may vary between *Plasmodium* species and that this may reflect differences in the ability of the parasites to take up and use GSH enzymes from the host cell.

The interesting question arises why such alternative or "back-up" systems for reduction of GSSG cannot compensate for the lack of GR during oocyst development in the mosquito? It might be possible that expression of enzymes of the TrxR/Trx system is lower in the oocyst stage compared with blood stages and/or increased oxidative pressure in the mosquito may require not only *de novo* synthesis of GSH but also a fully functional system of GR-dependent GSH reduction. Another explanation might be that the extracellular oocyst is now completely dependent on its own, endogenous enzymes of the redox systems and cannot use host enzymes, as has been suggested for the intracellular blood stages. Several studies have shown an increase of reactive oxygen species (ROS) in both the mosquito midgut and hemolymph resulting from an innate immune response against infection with *Plasmodium* (73–76). We had also previously suggested that *Plasmodium* might experience an increase in oxidative stress associated with the endogenous accumulation of ROS (20), resulting from a significant increase in mitochondrial activity during ookinete and oocyst development (77–79). Mitochondria are one of the main ROS producers in the cell (80, 81), and therefore the oocyst stage may have an increased dependence on GSH metabolism for maintaining



ROS reduction and redox homeostasis. Not unexpectedly, the oocysts of  $\Delta gr$  parasites showed highly comparable characteristics of degeneration during their development in comparison with degeneration of  $\Delta \gamma$ -gcs oocysts. Nuclear division was strongly reduced, no sporozoite formation was observed, and the endoplasmic reticulum, cytoplasm, and mitochondria showed clear signs of degeneration in 6-day-old oocysts.

The independence of blood stage growth on an active *Plasmodium* GR and  $\gamma$ -GCS has consequences for the development of drugs that specifically target these enzymes, and it might be expected that such drugs will not be able to efficiently inhibit blood stage growth and multiplication. Evidence has been presented that the antimalarial activity of MB and eosin B results from interactions with GR (5, 7, 46, 82). Our results demonstrate that GR is not the major target for these compounds, and the high susceptibility to MB of  $\Delta gr$  blood stages shows that interactions with targets other than *Plasmodium* GR must explain the antimalarial activity of this compound, as has been suggested previously (10, 46, 82, 83).

Notwithstanding the non-essential function of  $\gamma$ -GCS and GR for blood stage parasites, we have been unable to generate parasites that lack both enzymes. In an attempt to disrupt both genes in the same parasites, we specifically generated  $\Delta gr$  mutants lacking a drug-selectable marker. These parasites allowed us to perform disruption experiments using the same DNA constructs used to disrupt the  $\gamma$ -gcs locus in WT parasites (20). Multiple unsuccessful attempts to disrupt this locus in  $\Delta gr$  mutants indicate that blood stages cannot survive in the absence of both *de novo* synthesis of GSH and formation of GSH by GR-dependent reduction of GSSG. In addition, they demonstrate that blood stage parasites cannot completely rely on host GSH and enzymes for its GSH metabolism and also suggest that enzymes involved in the TrxR/Trx system cannot compensate completely for the loss of GR activity. Clearly, in order to maintain the cytosolic GSH levels needed for blood stage survival, either *de novo* synthesis of GSH is required or GSH has to be formed through reduction of GSSG by a *Plasmodium*-derived GR. In conclusion, our studies provide novel insights into the role of the GSH system in malaria parasites, both during development in the blood of the vertebrate host and during development in the mosquito. Moreover, the observations that  $\gamma$ -GCS and GR are non-essential for blood stage parasites call into question whether these enzymes are suitable targets for novel antimalarial drugs.

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