Replication of *Plasmodium* in reticulocytes can occur without hemozoin formation, resulting in chloroquine resistance

Jing-wen Lin,¹,⁹ Roberta Spaccapelo,⁴ Evelin Schwarzer,⁵ Mohammed Sajid,¹ Takeshi Annoura,¹ Katrien Deroost,⁶ Raimond B.G. Ravelli,² Elena Aime,⁴ Barbara Capucci,⁴,⁹ Anna M. Mommaas-Kienhuis,² Tom O’Toole,⁷ Frans Prins,³ Blandine M.D. Franke-Fayard,¹ Jai Ramesar,¹ Séverine Chevalley-Maurel,¹ Hans Kroeze,¹ Abraham J. Koster,² Hans J. Tanke,² Andrea Crisanti,⁴,⁸ Jean Langhorne,⁹ Paolo Arese,⁵ Philippe E. Van den Steen,⁶ Chris J. Janse,¹ and Shahid M. Khan¹

Most studies on malaria-parasite digestion of hemoglobin (Hb) have been performed using *P. falciparum* maintained in mature erythrocytes, in vitro. In this study, we examine *Plasmodium* Hb degradation in vivo in mice, using the parasite *P. berghei*, and show that it is possible to create mutant parasites lacking enzymes involved in the initial steps of Hb proteolysis. These mutants only complete development in reticulocytes and mature into both schizonts and gametocytes. Hb degradation is severely impaired and large amounts of undigested Hb remains in the reticulocyte cytoplasm and in vesicles in the parasite. The mutants produce little or no hemozoin (Hz), the detoxification by-product of Hb degradation. Further, they are resistant to chloroquine, an antimalarial drug that interferes with Hz formation, but their sensitivity to artesunate, also thought to be dependent on Hb degradation, is retained. Survival in reticulocytes with reduced or absent Hb digestion may imply a novel mechanism of drug resistance. These findings have implications for drug development against human-malaria parasites, such as *P. vivax* and *P. ovale*, which develop inside reticulocytes.
by aspartic and papain-like cysteine endoproteases, Hb unfolds and becomes accessible to further proteolysis by downstream proteases. In the *P. falciparum* DV, there are four aspartic proteases (plasmepsins) and two papain-like cysteine proteases (falcipains) capable of hydrolyzing native Hb (Goldberg, 2005; Subramanian et al., 2009). Gene disruption studies of hemoglobinases demonstrated that *P. falciparum* has developed redundant and overlapping Hb degradation pathways, demonstrating the importance of Hb digestion for the parasite (Liu et al., 2006; Bonilla et al., 2007). However, Hb is a poor source of methionine, cysteine, glutamine, and glutamate; in addition, human Hb contains no isoleucine and *P. falciparum* blood-stage parasite growth is most effective in culture medium supplemented with these amino acids, especially isoleucine (Liu et al., 2006). These data indicate that *P. falciparum* parasites are not only dependent on Hb digestion, but also import exogenous amino acids (Liu et al., 2006; Elliott et al., 2008).

Most studies on Hb degradation have been performed using *P. falciparum* maintained with mature RBCs (normocytes) in vitro. It is unknown whether observations on *P. falciparum* Hb digestion made in vitro can be directly translated to parasites replicating in vivo or for parasites developing in reticulocytes such as the human parasite *P. vivax* and *P. ovale*. For example, mechanisms of resistance to some drugs that interfere with Hb digestion and heme detoxification (e.g., chloroquine) differ between *P. vivax* and *P. falciparum* (Baird et al., 2004; Baird et al., 2012) indicating that there may be differences in their Hb digestion pathways.

To obtain a better insight into Hb digestion in parasites developing in vivo we used a rodent malaria parasite, *P. berghei*, which preferentially invades reticulocytes. We show a high level of functional redundancy among the predicted hemoglobinases, as 6 of the 8 are dispensable in vivo. Unexpectedly, we were able to create parasite mutants lacking the enzymes known to initiate Hb digestion. These parasites were able to multiply in reticulocytes without Hz formation and were resistant to chloroquine.

**RESULTS AND DISCUSSION**

**High degree of functional redundancy among Plasmodium hemoglobinases**

To determine the essential nature of individual enzymes involved in *P. berghei* Hb digestion, we performed a loss-of-function analysis on eight predicted *P. berghei* hemoglobinases. The selection was based on the corresponding *P. falciparum* orthologous proteases with a characterized role in Hb digestion and/or located in the digestive vacuole (DV; Table S1 and Fig. 1 A). These include the aspartic protease, plasmeplisin 4 (PM4), a single enzyme equivalent to the 4 *P. falciparum* plasmeplins (PM1–4); berghepean-2 (BP2), equivalent to the 2 *P. falciparum* DV falcipains FP-2 and FP-3; bergheylisin (BLN), the ortholog of *P. falciparum* falcilysin; dipetidyl peptidase 1 (DPAP1); and 4 aminopeptidases (aminopeptidase P [APP], M1-family alanyl aminopeptidase [AAP], M17-family leucyl aminopeptidase [LAP], and M18-family aspartyl aminopeptidase [DAP]). In addition, we included heme detoxification protein (HDP) and 3 enzymes related to DV proteases with undefined roles in Hb digestion (bergheplain 1, the ortholog of *P. falciparum* falcipain 1 and 2 dipetidyl peptidases, DPAP2 and DPAP3). We successfully generated gene-deletion mutants for pm4, bp1, bp2, dpap1, dpap2, dpap3, app, lap, and dap; however, multiple attempts to disrupt hbd, app, and hdp were unsuccessful (Table S2). We previously reported that disruption of pm4 in *P. berghei* results in the lack of all aspartic protease activity in the DV (Spaccapelo et al., 2010). Similarly, *P. falciparum* has been shown to survive without DV PM activity (Bonilla et al., 2007). We were able to generate mutants lacking BP2, whereas *P. falciparum* blood stages survive without FP2 but not FP3 (Sijwali et al., 2006). We also generated mutants that lack genes encoding DPAP1, APP, and LAP, whereas *P. falciparum* orthologs have been reported to be refractory to disruption (Table S1; Klemba et al., 2004; Dalal and Klemba, 2007). We were unable to select parasites lacking expression of AAP and BLN, and the *P. falciparum* orthologous genes aap and hbd have also been reported to be resistant to disruption and shown to play additional roles outside DV (Dalal and Klemba, 2007; Ponpuak et al., 2007). We were also unable to select mutants lacking HDP expression, suggesting an essential role for *P. berghei* blood stages, as has been proposed for *P. falciparum* (Jani et al., 2008). The successful deletion of 6 of the 8 genes encoding hemoglobinase indicates a high level of redundancy in vivo among these enzymes.

Mutants lacking PM4, DPAP1, BP1, LAP or APP exhibited a significant reduction in asexual multiplication rates (growth rates) compared with WT parasites, whereas growth rates of the other 4 mutants were not significantly reduced (Table 1). In addition, Δpm4 and Δapp mutants showed a significant reduction in Hz production quantified in mature schizonts (Sz; 8–24 nuclei) using reflection contrast polarized light microscopy (Fig. 1 A and B). Trophozoites (Tz) of Δapp and Δpm4 have an aberrant morphology as visible on Giemsa-stained smears, exhibiting an accumulation of translucent vesicles inside their cytoplasm (Fig. 1 C). The reduced Hz production in Δpm4 and Δapp mutants indicate that *P. berghei* blood stages can develop into mature Sz despite significantly reduced Hb digestion. The lower growth rate of mutants lacking downstream hemoglobinases DPAP1 and LAP (but with WT Hz levels) indicates that these enzymes are either important in effectively releasing amino acids from Hb peptides or that they have additional functions. Parasites lacking APP unexpectedly also had reduced levels of Hz. However, both APP and LAP are shown to have additional roles, e.g., in cytosolic peptide turnover (Dalal and Klemba, 2007). Although it remains to be investigated, APP may have an indirect effect on the initial steps of Hb digestion, either resulting from its involvement in establishment of the DV or due to feedback mechanisms that inhibit Hb digestion in the absence of APP.

Parasites lacking both PM4 and BP2 are restricted to reticulocytes and produce smaller Szs with less merozoites

Because in *P. berghei* PM4 is the only DV aspartic protease and BP2 is the single syntenic ortholog of the two *P. falciparum* falcipains.
and reticulocytes, mature Tz and Sz were exclusively found in reticulocytes (unpublished data), indicating that pm4bp2 parasites, while retaining their ability to invade all RBCs, are unable to develop in normocytes. This may be related to greater abundance/diversity of amino acids and proteins present in reticulocytes (Allen, 1960), or due to other physical characteristics of reticulocytes, for example, their larger size and reduced Hb content may provide more space for growth in the absence of Hb digestion.

Analysis of Giemsa-stained images revealed Δpm4Δbp2-Sz to be small, occupying only 25–65% of the RBCs, compared with 60–90% of WT Sz (n > 30; Fig. 2 A). This size reduction was confirmed by ImageStream flow cytometry on

Figure 1. Δpm4 and Δapp parasites show reduced Hz levels and an aberrant morphology. (A) Reflection contrast polarized light microscopy was used to quantify Hz production inside an iRBC; representative images of Hz in mature SzS are shown. Hz crystals are scattered in SzS and were used for Hz quantification; a single Hz cluster is only observed in fully segmented SzS (blue box); BF, bright field; bars, 5 µm. (B) Relative light intensity (RLI) of polarized light was measured for individual SzS (n > 30; Student’s t test; ***, P < 0.0001). (C) Aberrant morphology of Δpm4 and Δapp Tzs exhibiting reduced Hz production and an accumulation of translucent vesicles (indicated by arrows) in their cytoplasm. Bars, 5 µm. The phenotype was confirmed with two independent mutants.
live WT and Δpm4Δbp2 Szs expressing GFP under the control of the Szs-specific ama-1 promoter (Fig. 2 B). Analysis of Giemsa-stained parasites indicated that Δpm4Δbp2 Szs had fewer merozoites (Fig. 2 A). Measuring GFP and Hoechst fluorescence intensity by ImageStream and standard flow cytometry confirmed that mature Δpm4Δbp2-Sz have 40% reduction in both total DNA and (ama-1-based) GFP expression levels compared with WT Sz, indicating a significant reduction in the total number of merozoites per Sz (Fig. 2, C and D). Combined, these observations demonstrate that Δpm4Δbp parasites produce smaller Sz with less daughter merozoites than WT Sz.

Parasites lacking both PM4 and BP2 can form Szs in the absence of detectable Hz

Most Δpm4Δbp2 Tzs have an amoeboid-like appearance with translucent vesicles inside their cytoplasm, and their Tzs and Szs have little or no visible Hz (Fig. 2 A). Ultrastructural analyses confirmed this, showing that Δpm4Δbp2 Tzs contained a higher number of cytostomes or endocytic vesicles filled with material that had a structural appearance similar to RBC cytoplasm (Fig. 3 A). This indicates that the absence of PM4 and BP2 does not affect the uptake of Hb from the RBC cytoplasm. Electron microscopic images revealed that 37% of the Tzs contained dark-stained (electron-dense) vesicles that are completely absent from WT parasites (Fig. 3 A).

These vesicles are very similar to those that have been described in P. falciparum Tz when Hb trafficking or digestion is blocked by inhibitors, and it has been proposed that these vesicles are cytostome derived and contain concentrated undigested and/or denatured Hb (Fitch et al., 2003; Vaid et al., 2010). In addition, although WT Tz contained large numbers of Hz crystals, >40% of Δpm4Δbp2 Tz had no visible Hz crystals (Fig. 3 A), reflecting our observations with Giemsa-stained images of Tz and Sz (Fig. 2 A). Next, we used reflectance contrast polarized light-microscopy to determine the amount of Hz in maturing Δpm4Δbp2-Sz and WT-Sz. The relative light intensity (RLI) values of Δpm4Δbp2-Sz at all stages of maturation were strongly reduced (78–87% reduction compared with WT Sz; Fig. 3 B and Table 1), and whereas all WT Sz had Hz, a large percentage (35–48%) of Δpm4Δbp2-Sz had no detectable Hz (Fig. 3 B), having RLI values the same as uninfected RBCs. The severe reduction in Hz production was also reflected in vastly reduced Hz deposition in organs of Δpm4Δbp2-infected mice compared with WT- and even Δpm4-infected mice (Fig. 3 C).

Table 1. Growth and virulence characteristics of blood stages of gene deletion mutants

| Gene deletion mutant | Day to 0.5–2% parasitemiaa | Multiplication rateb | Hz productionc |
|----------------------|-----------------------------|---------------------|----------------|
| WT                  | 8 (0.2); n = 40             | 10.0 (0.7)          | 198.8 (69.8)   |
| Δpm4-a               | 9–11; n > 10               | 5.8 (0.5–7.0 (1.0)f | 129.5 (41.7)f  |
| Δpm4-b               | 9 (0); n = 2               | 7.7 (0)f           | 134.5 (47.6)f  |
| Δbp2-a               | 8 (0); n = 5               | 10.0 (0)f          | 177.5 (45.1)   |
| Δbp2-b               | 8 (0); n = 6               | 10.0 (0)           | 188.4 (71.5)   |
| Δdpap1-a             | 9.5 (0.7); n = 2           | 7.0 (1.0)f         | 174.6 (34.0)   |
| Δdpap1-b             | 9 (0); n = 4               | 7.7 (0)f           | 189.2 (62.7)   |
| Δopp-a               | 12 (0); n = 1              | 4.6 (0)f           | 131.8 (50.5)f  |
| Δopp-b               | 12 (0); n = 4              | 4.6 (0)f           | 111.4 (49.7)f  |
| Δdap                | 8 (0); n = 3               | 10.0 (0)           | 223.8 (65.7)   |
| Δlap                | 15.5 (0.7); n = 2          | 3.3 (0.2)f         | 213.6 (78.7)   |
| Δbp1-a              | 9.7 (0.6); n = 3           | 6.8 (0.8)f         | 186.2 (49.2)   |
| Δbp1-b              | 9 (0); n = 1               | 7.7 (0)f           | n.d.           |
| Δdpap2              | 8.3 (0.4); n = 4           | 9.4 (1.0)          | 187.8 (64.6)   |
| Δdpap3-a             | 8.3 (0.6); n = 3           | 9.2 (1.3)          | 184.5 (86.3)   |
| Δdpap3-b             | 8 (0); n = 5               | 10.0 (0)           | 193.3 (46.8)   |
| Δpm4Δbp2-a          | 12, 16, 20; n = 3          | 3.4 (1.1)f         | 27.2 (36.5)f   |
| Δpm4Δbp2-b          | 21, 24; n = 2              | 2.3 (0.1)f         | 46.1 (51.2)f   |

n.d., not determined.

aThe day on which the parasitemia reaches 2–5% in mice infected with a single parasite in cloning assays. The mean of one cloning experiment and standard deviation are shown (n = the number of mice). For the Δpm4Δbp2 mutants, the days for individual clones are shown.

bThe multiplication rate (mean and SD) of asexual blood stages per 24 h, calculated based on the "day to 0.5–2% parasitemia" in the cloning assay.

cRelative light intensity (mean and SD) of Hz crystals in individual Szs, determined by polarized light microscopy. See Fig. 1.

WT, wild type. P. berghei ANKA reference lines (cl15cy1, 676m1cl1, and 1037cl1). Data from >10 independent experiments.

*p < 0.0001; Student’s t test.
aspartyl and cysteine endopeptidases overlap in their ability to hydrolyze Hb. Interestingly, the \( \Delta bp2 \) mutant has a normal growth rate and produces WT levels of Hz, whereas \( \Delta pm4 \) parasites have a reduced growth and Hz production. These observations demonstrate that although PM4 is able to fully compensate for the function of BP2, BP2 can only partly compensate for the loss of PM4.

Gametocytes of parasite lacking both PM4 and BP2 are fertile despite their smaller size and reduced Hz levels

In mice infected with \( \Delta pm4\Delta bp2 \) parasites, male and female gametocytes were readily detected. Similar to Sz, they were 23% smaller than WT gametocytes, and their cytoplasm had strongly reduced or no Hz crystals (Fig. 4 A). Most \( \Delta pm4\Delta bp2 \) male gametocytes produced motile gametes (79.3 ± 4.6%), which are able to fertilize female gametes and produce ookinetes; these undergo meiosis and become tetraploid (Fig. 4, B and C). The conversion rates of \( \Delta pm4\Delta bp2 \) female gametes into ookinetes were comparable to those of WT parasites (60.0 ± 6.1%; Fig. 4 C). Analysis of Hz levels in WT and \( \Delta pm4\Delta bp2 \) ookinetes revealed that they also had reduced

Figure 2. Szs of mutants lacking PM4 and BP2 expression are smaller in size and produce fewer merozoites. (A) Representative Giemsa-stained images showing the difference in size and merozoites production of \( \Delta pm4\Delta bp2 \) iRBCs relative to WT. Bars, 5 µm. (B) Images of Hoechst stained mature WT and \( \Delta pm4\Delta bp2 \) Szs expressing GFP under Sz-specific ama1 promoter in their cytoplasm (right) and their size measurement by ImageStream flow cytometry (left). Bars, 5 µm. The size of individual iRBC was determined from bright field images (B) and the size of Sz was measured from the combined GFP (G) and Hoechst (H) images (i.e., G+H). M, merged images (\( n > 250; ***; \ P < 0.0001; \) Student’s \( t \) test). (C) The dot plot (left) generated from ImageStream flow cytometry shows the GFP- and Hoechst-fluorescence of individual WT- (green) and \( \Delta pm4\Delta bp2 \)- (red) Szs, and the GFP expression and DNA content were quantified (right). (D) The GFP and DNA content of mature Szs were measured by standard flow cytometry; mature Szs were selected in Gate 1 (left) and the quantification shown as bar graphs (right). All data are representative of two independent experiments (bar graphs show mean fluorescence intensity with SEM; ***; \ P < 0.0001; Student’s \( t \) test).
Hz-levels (57%; Fig. 4 C). These observations demonstrate that both asexual and sexual blood-stages can complete development despite the severely impaired Hb digestion.

Parasites lacking both PM4 and BP2 are resistant to chloroquine but retain their sensitivity to artesunate

We tested the sensitivity of the Δpm4Δbp2 parasites to artesunate (AS) and chloroquine (CQ); their mode of action is believed to depend on Hb digestion/Hz formation (Egan et al., 2004; Klonis et al., 2011). As a control we used sulfadiazine (SD), an inhibitor of folic acid synthesis (Kinnamon et al., 1976). AS and SD treatment of WT- and Δpm4Δbp2-infected BALB/c mice resulted in rapid clearance of parasites from bloodstream in 3–4 and 4–5 d after start of AS (50 mg/kg body weight, i.p.) and SD (35 mg/liter in drinking water) treatment, respectively (Fig. 5 A). Similarly, BALB/c mice infected with WT parasites rapidly cleared their infection after CQ (288 mg/liter in drinking water) treatment (3–4 d). In contrast, mice infected with pm4bp2 parasites, first exhibited an increase in parasitemia for 3 d, followed by a slow decline (Fig. 5 A). Parasites with normal morphology were present 6 d after start of CQ treatment (Fig. 5 B). Untreated
Restricted to development in reticulocytes and produced less Hz (Platel et al., 1999). It has been proposed that CQ-resistance of parasites with reduced Hz is due to detoxification of hemin by elevated levels of glutathione in parasites inside reticulocytes, thus precluding heme-polymerization and preventing CQ activity (Platel et al., 1999; Fidock and DeSilva, 2012). However, our observations may provide a more direct explanation for CQ-resistance and reduced Hz production in these parasites, namely that these parasites, like the \( \Delta pm4\Delta bp2 \), digest less Hb in reticulocytes. Our observations may have particular relevance for the human malaria parasite, \textit{Plasmodium vivax}, which is also reticulocyte-restricted. \textit{P. vivax} CQ resistance appears to be different from \textit{P. falciparum} (Suwanarusk et al., 2007; Baird et al., 2012), and no clear association has been found with mutations in the same genes that typify \textit{P. falciparum} CQ resistance (\textit{pfcrt} or \textit{pfmdr1}). In addition, DV formation in \textit{P. vivax} appears to resemble more DV formation in \textit{P. berghei} than in \textit{P. falciparum}. Tzs of \textit{P. falciparum} have a large central DV, as Hb-containing cytostomes merge rapidly and Hz formation occurs principally in this single DV. In contrast, \textit{P. berghei} has many small Hb-containing vesicles, in which Hb digestion/Hz formation occurs, resulting in scattered Hz granules throughout BALB/c mice also resolved a \( \Delta pm4\Delta bp2 \) infection 3–4 wk after infection, indicating that host immunity may contribute to the decline of parasitemia in CQ-treated mice. We therefore performed infections and CQ treatment in Rag2\(^{-/-}\)\(\gamma c\)^{-/-} mice (deficient in B, T, and NK cells). Whereas CQ-treated, WT-infected mice cleared a 2–5% WT infection within 3 d, in \( \Delta pm4\Delta bp2 \)-infected mice parasites persisted for >20 d of CQ-treatment (Fig. 5 C), during which only Hz-negative \( \Delta pm4\Delta bp2 \) parasites were observed in circulation (Fig. 5 D). To examine whether \( \Delta pm4\Delta bp2 \) parasite resistance to CQ is lost at higher concentrations of CQ and conversely if resistance to AS is observed at lower concentrations of AS, we performed drug sensitivity assays using 5 and 10 mg/kg AS (in BALB/c mice) or with 600 mg/liter CQ (in Rag2\(^{-/-}\)\(\gamma c\)^{-/-} mice). These experiments revealed that, at lower AS doses, drug sensitivity does not significantly differ between \( \Delta pm4\Delta bp2 \) and WT parasites, and that \( \Delta pm4\Delta bp2 \) parasites are resistant to CQ even at high doses (Fig. 5 E).

Our observations on the ability of \textit{Plasmodium} parasites to develop without detectable Hz formation that are resistant to CQ indicate a novel mechanism of resistance against drugs that target Hb proteolysis. Interestingly, previous studies on CQ-resistant \textit{P. berghei} lines revealed that parasites are more restricted to development in reticulocytes and produced less Hz (Platel et al., 1999). It has been proposed that CQ-resistance of parasites with reduced Hz is due to detoxification of hemin by elevated levels of glutathione in parasites inside reticulocytes, thus precluding heme-polymerization and preventing CQ activity (Platel et al., 1999; Fidock and DeSilva, 2012). However, our observations may provide a more direct explanation for CQ-resistance and reduced Hz production in these parasites, namely that these parasites, like the \( \Delta pm4\Delta bp2 \), digest less Hb in reticulocytes.

Our observations may have particular relevance for the human malaria parasite, \textit{Plasmodium vivax}, which is also reticulocyte-restricted. \textit{P. vivax} CQ resistance appears to be different from \textit{P. falciparum} (Suwanarusk et al., 2007; Baird et al., 2012), and no clear association has been found with mutations in the same genes that typify \textit{P. falciparum} CQ resistance (\textit{pfcrt} or \textit{pfmdr1}). In addition, DV formation in \textit{P. vivax} appears to resemble more DV formation in \textit{P. berghei} than in \textit{P. falciparum}. Tzs of \textit{P. falciparum} have a large central DV, as Hb-containing cytostomes merge rapidly and Hz formation occurs principally in this single DV. In contrast, \textit{P. berghei} has many small Hb-containing vesicles, in which Hb digestion/Hz formation occurs, resulting in scattered Hz granules throughout
Moreover, Plasmodium vivax, like P. berghei, has only 1 DV plasmepsin (PM4) in contrast to P. falciparum which encodes 4 DV plasmsins (Table S1). While P. vivax has 3 DV vivapains, 2 of them, VX2 and 3, are syntenic orthologs of FP-2 and 3 the other, VX4, is nonsyntenic with the FPs but shows greater

the cytoplasm of the parasite, these clusters coalesce when schizogony starts (Slomianny et al., 1985). It has been suggested that the uptake of Hb from reticulocytes by the process of micropinocytosis results in multiple small vesicles containing Hb and Hz in both P. berghei and P. vivax (Jeffers, 2010). Moreover, P. vivax, like P. berghei, has only 1 DV plasmepsin (PM4) in contrast to P. falciparum which encodes 4 DV plasmepsins (Table S1). While P. vivax has 3 DV vivapains, 2 of them, VX2 and 3, are syntenic orthologs of FP-2 and 3 the other, VX4, is nonsyntenic with the FPs but shows greater
sequence similarity to BP2 (Na et al., 2010; Table S1). These observations indicate that mechanisms of Hb uptake and digestion in *P. vivax* more closely resembles *P. berghei* than *P. falciparum* and future research is required to see if this also translates into similar mechanisms to resistance to drugs that exclusively target Hb digestion pathways.

Although Δpm4Δhp2 parasites are resistance to CQ, they retain sensitivity to AS. Although the precise mode of action of artemisinin and related derivatives remains contentious, it is believed that their activity results from activation by reduced heme iron in the DV (Eastman and Fidock, 2009). Our results suggest AS activity is not affected by reduced Hb digestion or absence of Hz formation. Alternatively AS activation in Δpm4Δhp2 parasites in reticulocytes may derive from a labile pool of heme that exists in reticulocytes for Hb synthesis. *P. vivax* resistance to artemisinins has not been reported; however, most of the artemisinin-based combination therapies have proven efficacy against chloroquine-resistant strains of *P. vivax* (Price et al., 2014).

We show that, very much counter to expectation, Hb digestion and Hz formation appears not to be essential to parasite survival when parasites develop in reticulocytes. Indeed, our findings support the notion that *Plasmodium* parasites retain multiple modes of development and survival during blood stage development, which has important implications for antimalarial drug design, in particular drugs that target Hb digestion and Hz formation.

**MATERIALS AND METHODS**

**Experimental animals and parasites.** Female C57BL/6, BALB/c, Swiss OF1 mice (6–8 wk old; Charles River) and female Rag2<−/−>ge<−/−> mice (6–8 wk old; bred in MRC NIMR) were used. All animal experiments performed at the Leiden University Medical Center (LUMC) were approved by the Animal Experiments Committee of the LUMC (DEC 10099; 12042; 12120). All animal experiments performed at the University of Perugia were approved by Ministry of Health under the guidelines D.L. 116/92). All animal experiments performed in MRC NIMR, were performed after review and approval by the MRC National Institute for Medical Research Ethical Review Panel in strict accordance to current UK Home Office regulations, and conducted under the authority of UK Home Office Project License PPL 80/2358. The Dutch, Italian, and British Experiments on Animal Act were established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes).

Two reference *P. berghei* ANKA parasite lines were used: line c115cy1 (WT) and reporter line 1037cl1 (WT-GFP-Lucschiz; mutant RMgm-32). This reporter line contains the fusion gene gfp-luc under control of the Sz-specific ama1 promoter integrated into the silent 250 bp gene locus (PBANKA_030600) and does not contain a drug-selectable marker (Spaccapelo et al., 2010).

**Generation of *P. berghei* mutants.** DNA constructs used to disrupt genes were based on the standard plasmids pL0001 [MRA-770], pLTg3DHFR, and pL0035 [MRA-850] and by modified PCR methods based on pL0040 and pL0048 (Lin et al., 2011). Targeting sequences for homologous recombination were PCR amplified from *P. berghei* ANKA (c115cy1) genomic DNA using primers specific for the 5' or 3' end of each gene using primer sets of P1/P2, P3/P4, respectively (see Table S3 for the primer sequences).

The DNA construct targeting hp1 was provided by P. Sinnis (Johns Hopkins University, Baltimore, MD). Transfection, selection, cloning, and genotyping of transformed parasites was performed using standard genetic modification technologies for *P. berghei* (Janse et al., 2006). To generate Δpm4Δhp2 mutant, haldfr<yfu> selectable marker was removed by negative selection (Braks et al., 2006) from Δhp2-b (based on pL0035) and pm4 gene was subsequently targeted in Δhp2-b<Δhp2> line. Table S2 provides details of all gene deletion experiments, such as experiment number, targeting construct, parental line for transfection, and RMgmDB IDs (Rodent Malaria Genetically Modified Parasites Database). Details for targeting construct generation (maps and sequences) and genotyping results can be found in RMgmDB and Table S4 includes primers used for genotyping including diagnostic PCR, Southern analyses of chromosomes separated by pulsed-field gel electrophoresis, Northern PCR, and RT-PCR.

**In vivo asexual multiplication (growth) rates.** The multiplication (growth) rate of asexual blood-stages in mice was determined during cloning of the gene-deletion mutants as described before (Spaccapelo et al., 2010) and was calculated as follows: the percentage of infected erythrocytes (parasitemia) in Swiss OF1 mice injected with a single parasite was determined by counting Giemsa-stained blood films when parasitemas reach 0.5–2%. The mean asexual multiplication rate per 24 h was then calculated assuming a total of 1.2 × 10^{10} erythrocytes per 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 0.5–2% at day 8 after infection, resulting in a mean multiplication rate of 10 per 24 h.

**Gametocyte and ookinete production.** Gametocyte production is defined as the percentage of ring forms developing into mature gametocytes during synchronized infections (Janse and Waters, 1995). Male gamete production (exflagellation) and ookinete production was determined in standard in vitro fertilization and ookinete maturation assays (Janse and Waters, 1995); ookinete production is defined as the percentage of female gametes that develop into mature ookinetes under standardized in vitro culture conditions. Female gamete and mature ookinete numbers were determined on Giemsa-stained blood smears made 16–18 h after activation. Histo level of ookinetes were quantified.

**Sz and gametocyte size measurements.** For the Giemsa-stained smears, pictures were taken using a Leica microscope from randomly chosen fields of 300–400 RBCs, and all iRBCs were measured in these fields. The sizes of iRBCs and the parasites were measured by ImageJ by getting on the areas of parasites and iRBC. For ImageStream flow cytometry analysis, iRBCs containing Szs of WT-GFP-Lucschiz and Δpm4Δhp2 parasites were collected from infected BALB/c mice with a high parasitemia (10–30%) and enriched by Nycodenz density centrifugation (Janse et al., 2006). Purified parasites were then collected in complete RPMI-1640 culture medium and stained with Hoechst-33258 (2 µmol/liter; Sigma-Aldrich) for 1 h at room temperature. Cultured, mature Szs of WT *P. berghei* ANKA (c115cy1) were used as unstained control; Hoechst stained c115cy1 (Hoechst only) and nonstained WT-GFP-Lucschiz (GFP only) were used as single-color controls. The analyses were performed using an Anims ImageStream X imaging cytometer (Anims Corp.) and images were analyzed using the IDEAS image analysis software.

**Hz and Hb quantification.** Hz was quantified in SzS using different methods. Hz was quantified by measuring the relative light intensity (RLI) of Hz crystals in Szs and ookinetes by reflection contrast polarized light microscopy. Szs were either collected from overnight in vitro blood stage cultures or directly from tail blood when Szs were present in the peripheral circulation. Blood smears were made from cultured parasites or from tail blood and stained with Hoechst-33342 (2 µmol/liter; Sigma-Aldrich) for 20 min. Szs (8–24 nuclei) with scattered Hz were selected and pictures were taken with a LeicaDM/IRB microscope (Leica) which was adapted for reflection contrast polarized light microscopy (Cornelise-ten Velde et al., 1988). The RLI of Hz crystals in the Szs was measured using ImageJ software (NIH).

In addition, Hb and Hz from WT and Δpm4Δhp2 Sz-iRBC were quantified by measuring the hemoglobin luminol-enhanced luminescence (Schwarzar et al., 1994). For these experiments, 10^{7} purified SzS were collected by flow sorting from infected blood of BALB/c mice with a high parasitemia.
To quantify Hz deposition in organs of infected mice, groups of 8 BALB/c mice were i.p. infected with $10^5$ WT, Δpm4, or Δpm4Δhbp2 parasites. At different parasitemia stages, mice were sacrificed and subjected to hematoxylin and eosin (H&E) staining, destained, dehydrated in an ethanol series, and embedded in paraffin. Sections were cut at 3-5 µm thickness and counterstained with hematoxylin and eosin. The area of heme deposition was measured using ImageJ software.

For organ H&E slides, similar methods were applied, and the area of heme deposition was calculated. The area of Hz deposition was represented as the percentage of the total area of the organ.

Graphical and statistical analyses. All graphs and statistical analyses were made in GraphPad Prism. All error bars indicate SEM and all p-values were derived from unpaired Student’s t-tests.

Supplemental material. Table S1 shows genes targeted in this study. Table S2 shows details of transfection experiments aiming at deletion of P. berghei genes encoding hemoglobinases. Table S3 shows gene deletion constructs and primers. Table S4 shows primers for genotyping gene-deletion mutants. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20141731/DC1.

We would like to thank Dr. Photini Sinnis for providing us with a P. berghei bp1 gene deletion construct, J.J. Underwater for electron microscopy sample preparation, and Guido de Roo and Sabrina Veld for assistance with flow cytometry experiments.

J.-W. Lin was supported by the China Scholarship Council-Leiden University Joint Program and C.J. Janse by a grant of the European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreement no. 242095.

K. Deroost was supported by a PhD grant of the Agency for Innovation by Science and Technology (IWT), and P.E. Van den Steen was supported by a grant of the Research Fund of the KU Leuven, and by the Fund for Scientific Research (FW.O.-VLanderen).

J. Langhorne is supported by the MRC, UK (U117584248).

The authors declare no competing financial interests.

Submitted: 7 September 2014
Accepted: 8 April 2015

REFERENCES

Allen, D.W. 1960. Amino acid accumulation by human reticulocytes. Blood. 16:1564–1571.
Baird, J.K. 2004. Chloroquine resistance in Plasmodium vivax. Antimicrob. Agents Chemother. 48:4075–4083. http://dx.doi.org/10.1128/AAC.48.11.4075-4083.2004
Baird, K.J., J.D. Maguire, and R.N. Price. 2012. Diagnosis and treatment of Plasmodium vivax malaria. Adv. Parasitol. 80:203–270. http://dx.doi.org/10.1016/B978-0-12-307900-1.00004-9
Bonilla, J.A., T.D. Bonilla, C.A. Yowell, H. Fujioka, and J.B. Dame. 2007. Critical roles for the digestive vacuole plasminogen of Plasmodium falciparum in vacuolar function. Mol. Microbiol. 65:64–75. http://dx.doi.org/10.1111/j.1365-2958.2007.05768.x
Braks, J.A., B. Franke-Fayard, H. Kroese, C.J. Janse, and A.P. Waters. 2006. Development and application of a positive-negative selectable marker system for use in reverse genetics in Plasmodium. Nucleic Acids Res. 34:69. http://dx.doi.org/10.1093/nar/gnj033
Cornelee–ten Velde, I., J. Bonnet, H.J. Tanke, and J.S. Ploem. 1988. Reflection contrast microscopy. Visualization of [peroxidasie-generated] diamino benzoic acid polymer products and its underlying optical phenomena. Histochemistry. 89:141–150.
Dalal, S., and M. Klenka. 2007. Roles for two aminopeptidases in vacuolar hemoglobin catabolism in Plasmodium falciparum. J. Biol. Chem. 282:35978–35987. http://dx.doi.org/10.1074/jbc.M703643200
Deroost, K., N. Lays, S. Noppen, E. Martens, G. Opdenakker, and P.E. Van den Steen. 2012. Improved methods for haemoglobin quantification in tissues yield organ- and parasite-specific information in malaria-infected mice. Malar. J. 11:166. http://dx.doi.org/10.1186/1475-2875-11-166
Eastman, R.T., and D.A. Fidock. 2009. Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. Nat. Rev. Microbiol. 7:864–874. http://dx.doi.org/10.1038/nrrmicro.2009.239
Egan, T.J., K.R. Koch, P.L. Swan, C. Clarkson, D.A. Van Schalkwyk, and P.J. Smith. 2004. In vitro antimalarial activity of a series of cationic 2,2′-bipyridyl- and 1,10-phenanthrolineplatinum(II) benzoylthiourea complexes. J. Med. Chem. 47:2926–2934. http://dx.doi.org/10.1021/jm031312g
Elliott, D.A., M.T. McIntosh, H.D. Hosgood III, S. Chen, G. Zhang, P. Baeova, and K.A. Joiner. 2008. Four distinct pathways of hemoglobin uptake in the malaria parasite Plasmodium falciparum. Proc. Natl. Acad. Sci. USA. 105:2463–2468. http://dx.doi.org/10.1073/pnas.0711067105
Faas, F.G., M.C. Avramut, B.M. van den Berg, A.M. Mommaas, A.J. Koster, and R.B. Ravelli. 2012. Virtual nanoscopy: generation of ultra-large high resolution electron microscopy maps. J. Cell Biol. 198:457–469. http://dx.doi.org/10.1083/jcb.201201140
Fidock, M., and B. DeSilva. 2012. Bioanalysis of biomarkers for drug development. Bioanalysis. 4:2425–2426. http://dx.doi.org/10.4155/bio.12.253
Fitch, C.D., G.Z. Cai, Y.F. Chen, and J.S. Ryerse. 2003. Relationship of chloroquine-induced redistribution of a neutral aminopeptidase to hemoglobin accumulation in malaria parasites. Arch. Biochem. Biophys. 410:296–306. http://dx.doi.org/10.1016/S0003-9861(02)00688-4
Goldberg, D.E. 2005. Hemoglobin degradation. Curr. Top. Microbiol. Immunol. 295:275–291.
Jani, D., R. Nagarkatti, W. Beatty, R. Angel, C. Sleighbordick, J. Andersen, S. Kumar, and D. Rathore. 2008. HDP-a novel heme detoxification protein
from the malaria parasite. PLoS Pathog. 4:e1000053. http://dx.doi.org/10.1371/journal.ppat.1000053
Janse, C.J., and A.P. Waters. 1995. Plasmodium berghei: the application of cultivation and purification techniques to molecular studies of malaria parasites. Parasitol. Today (Regul. Ed.). 11:138–143. http://dx.doi.org/10.1016/0169-4758(95)80133-2
Janse, C.J., J. Ramesar, and A.P. Waters. 2006. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei. Nat. Protoc. 1:346–356. http://dx.doi.org/10.1038/nprot.2006.53
Jeffers, V. 2010. Shedding light on Plasmodium knowlesi food vacuoles. A thesis submitted to Comelled Facultes for the Natural Sciences and for Mathematics of the Ruperto-Carola University of Heidelberg for the degree of Doctor of Natural Sciences.
Kinnamon, K.E., A.L. Ager, and R.W. Orchard. 1976. Plasmodium berghei: combining folic acid antagonists for potentiation against malaria infections in mice. Exp. Parasitol. 40:95–102. http://dx.doi.org/10.1016/0014-4894(76)90070-9
Klemba, M., I. Gluzman, and D.E. Goldberg. 2004. A Plasmodium falciparum dipeptidyl aminopeptidase I participates in vacuolar hemoglobin degradation. J. Biol. Chem. 279:43000–43007. http://dx.doi.org/10.1074/jbc.M408123200
Klonis, N., M.P. Crespo-Ortiz, I. Bottova, N. Abu-Bakar, S. Kenny, P.J. Rosenthal, and L. Tilley. 2011. Artemisinin activity against Plasmodium falciparum requires hemoglobin uptake and digestion. Proc. Natl. Acad. Sci. USA. 108:11405–11410. http://dx.doi.org/10.1073/pnas.1104063108
Lin, J.W., T. Annoura, M. Sajid, S. Chevalley-Maurel, J. Ramesar, O. Klop, B.M. Franke-Fayard, C.J. Janse, and S.M. Khan. 2011. A novel ‘gene insertion/marker out’ (GIMO) method for transgene expression and gene complementation in rodent malaria parasites. PLoS ONE. 6:e29289. http://dx.doi.org/10.1371/journal.pone.0029289
Liu, J., E.S. Istvan, I.Y. Gluzman, J Gross, and D.E. Goldberg. 2006. Plasmodium falciparum ensures its amino acid supply with multiple acquisition pathways and redundant proteolytic enzyme systems. Proc. Natl. Acad. Sci. USA. 103:8840–8845. http://dx.doi.org/10.1073/pnas.0601876103
Na, B.K., Y.A. Bae, Y.G. Zo, Y. Choe, S.H. Kim, P.V. Desai, M.A. Avery, C.S. Craik, T.S. Kim, P.J. Rosenthal, and Y. Kong. 2010. Biochemical properties of a novel cysteine protease of Plasmodium vivax, vivapain-4. PLoS Negl. Trop. Dis. 4:e849. http://dx.doi.org/10.1371/journal.pntd.0000849
Platel, D.F, F Mangou, and J. Tribouley-Duret. 1999. Role of glutathione in the detoxification of ferriprotoporphyrin IX in chloroquine resistant Plasmodium berghei. Mol. Biochem. Parasitol. 98:215–223. http://dx.doi.org/10.1016/S0166-6851(98)00170-4
Pouliquen, M., M. Klemba, M. Park, I.Y. Gluzman, G.K. Lamappa, and D.E. Goldberg. 2007. A role for falcilysin in transit peptide degradation in the Plasmodium falciparum apicoplast. Mol. Microbiol. 63:314–334. http://dx.doi.org/10.1111/j.1365-2958.2006.04443.x
Price, R.N., L. von Sieglin, N. Valecha, F. Nosten, J.K. Baird, and N.J. White. 2014. Global extent of chloroquine-resistant Plasmodium vivax: a systematic review and meta-analysis. Lancet Infect. Dis. 14:982–991. http://dx.doi.org/10.1016/S1473-3099(14)70853-2
Schwarrer, E., F. Turrini, and P. Aree. 1994. A luminescence method for the quantitative determination of phagocytosis of erythrocytes, of malaria-parasitized erythrocytes and of malarial pigment. Br. J. Haematol. 88:740–745. http://dx.doi.org/10.1111/j.1365-2141.1994.tb05112.x
Siwali, P.S., J. Koo, N. Singh, and P.J. Rosenthal. 2006. Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of Plasmodium falciparum. Mol. Biochem. Parasitol. 150:96–106. http://dx.doi.org/10.1016/j.molbiopara.2006.06.013
Slomiany, C., G. Pruesser, and P. Charet. 1985. Comparative ultrastructural study of the process of hemoglobin degradation by P.berghei (Vincke and Lips, 1948) as a function of the state of maturity of the host cell. J. Protozool. 32:1–5. http://dx.doi.org/10.1111/j.1550-7488.1985.tb00303.x
Spaccapelo, R., C.J. Janse, S. Caterbi, B. Franke-Fayard, J.A. Bonilla, L.M. Syphard, M. Di Cristina, T. Dottorni, A. Savarno, A. Cassone, et al. 2010. Plasmepsin 4-deficient Plasmodium berghei are virulence attenuated and induce protective immunity against experimental malaria. Am. J. Pathol. 176:205–217. http://dx.doi.org/10.1016/j.ajpath.2010.09.050
Subramaniam, S., M. Harth, Y. Choe, R.K. Niles, E.B. Johansen, J. Legac, J. Gut, I.D. Kerr, C.S. Craik, and P.J. Rosenthal. 2009. Hemoglobin cleavage site-specificity of the Plasmodium falciparum cysteine proteases falcipain-2 and falcipain-3. PLoS ONE. 4:e5156. http://dx.doi.org/10.1371/journal.pone.0005156
Suwanarusk, R., B. Russell, M. Chavchich, F. Sulfetin, E. Kengsangalee, V. Kosaisavee, B. Prasetyorini, K.A. Piera, M. Barends, A. Brockman, et al. 2007. Chloroquine resistant Plasmodium vivax: in vitro characterisation and association with molecular polymorphisms. PLoS ONE. 2:e1089. http://dx.doi.org/10.1371/journal.pone.0001089
Vaid, A., R. Ranjan, W.A. Smythe, H.C. Hoppe, and P. Sharma. 2010. PIP3K, a phosphatidylinositol-3 kinase from Plasmodium falciparum, is exported to the host erythrocyte and is involved in hemoglobin trafficking. Blood. 115:2500–2507. http://dx.doi.org/10.1182/blood-2009-08-238972