Distinct properties of the egress-related osmiophilic bodies in male and female gametocytes of the rodent malaria parasite Plasmodium berghei

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Summary

Gametogenesis is the earliest event after uptake of malaria parasites by the mosquito vector, with a decisive impact on colonization of the mosquito midgut. This process is triggered by a drop in temperature and contact with mosquito molecules. In a few minutes, male and female gametocytes escape from the host erythrocyte by rupturing the parasitophorous vacuole and the erythrocyte membranes. Electron-dense, oval-shaped organelles, the osmiophilic bodies (OB), have been implicated in the egress of female gametocytes. By comparative electron microscopy and electron tomography analyses combined with immunolocalization experiments, we here define the morphological features distinctive of male secretory organelles, hereafter named MOB (male osmiophilic bodies). These organelles appear as club-shaped, electron-dense vesicles, smaller than female OB. We found that a drop in temperature triggers MOB clustering, independently of exposure to other stimuli. MDV1/PEG3, a protein associated with OB in Plasmodium berghei females, localizes to both non-clustered and clustered MOB, suggesting that clustering precedes vesicle discharge. A P. berghei mutant lacking the OB-resident female-specific protein Pbg377 displays a dramatic reduction in size of the OB, accompanied by a delay in female gamete egress efficiency, while female gamete fertility is not affected. Immunolocalization experiments indicated that MDV1/PEG3 is still recruited to OB-remnant structures.

Introduction

Plasmodium, the agent of malaria disease, causes 207 million new cases and more than half a million deaths annually (WHO World Malaria Report, 2013). During its complex life cycle, malaria parasites alternate between a vertebrate host and a mosquito vector of the genus Anopheles. Parasites injected by an infected mosquito undergo an asymptomatic multiplication inside host hepatocytes. Subsequently, thousands of merozoites are released into the circulation, where they invade red blood cells (RBC). With the exception of invasive, short-lived merozoites, liver and blood stages are obligate intracellular parasites. They develop inside a parasitophorous vacuole (PV), the membrane of which (PVM) represents an interface between parasite and host cell cytoplasm (Zuccala and Baum, 2011). Asexual blood stages are responsible for symptoms associated with malaria disease, while circulating non-replicative gamete precursors (gametocytes) mediate transmission. When male and female gametocytes are ingested by a female mosquito during a blood meal, gamete differentiation (gametogenesis) is triggered by a drop in temperature and contact with molecules of the mosquito midgut microenvironment, such as xanthurenic acid (XA; Billker et al., 1998). A signalling cascade is activated, which involves a...
rapid rise of cytosolic calcium (Billker et al., 2004) and protein kinase G activation (McRobert et al., 2008).

Molecular events leading to gamete maturation and egress from host erythrocyte occur over a short time (15–20 min; Sinden, 1983). The female gametocyte escapes from the host erythrocyte by forming a single macrogamete, competent for fertilization. In contrast, prior to male gametocyte egress from the host cell, three rounds of DNA replication take place in these cells, followed by nuclear division. Eight axonemes are assembled, finally resulting in the release of eight flagellated microgametes. With rapid swimming movements, they reach and then fertilize female macrogametes, therewith initiating the mosquito stages (reviewed in Pradel, 2007).

Egress from the host cell is central both for proliferation of asexual stages and transmission to the mosquito vector. Asexual merozoite escape from the host erythrocyte has been investigated in detail in the human malaria parasite Plasmodium falciparum (reviewed in Blackman and Carruthers, 2013). An inside-out model of merozoite egress was experimentally confirmed. Parasitophorous vacuolar membrane disruption was found to be preceded by swelling of the PV compartment and shrinkage of the erythrocyte cytoplasm (Glushakova et al., 2005; Chandramohanadas et al., 2011). Gametocyte activation and egress was investigated both in the human malaria parasite P. falciparum and in the rodent P. berghei. As in the case of asexual merozoites, activated gametocytes emerge from the host cell by an inside-out process, which involves PV rupture at multiple sites (Sologub et al., 2011; Deligianni et al., 2013) followed by disruption of erythrocyte membrane (EM) at a single breakage point (Sologub et al., 2011). The essential role of a perforin-like protein (PPLP2) in EM disruption during microgamete egress was shown in P. berghei (Deligianni et al., 2013).

Growing evidence implicated electron-dense membrane-bound organelles, referred to as osmiophilic bodies (OB) in egress of activated gametocytes. Ultrastructural studies showed that OB migrate to the gametocyte plasma membrane upon activation (Sinden et al., 1976; Aikawa et al., 1984). OB disappear after discharging their content into the PV, concurrently with PV membrane disruption (Sologub et al., 2011).

Three OB residents, gametocyte-specific molecules, have been implicated in the egress process. The Pfg377 protein (PF3D7_1250100), expressed in female gametocytes of P. falciparum, has a role in OB biogenesis (Alano et al., 1995; Severini et al., 1999). Deletion of the encoding gene causes a dramatic reduction in OB number and a significant decrease in female gametocyte egress efficiency (de Koning-Ward et al., 2008). This reduced efficiency in escaping from the RBC resulted in a significant reduction of oocysts and sporozoite number when the mutant parasites were transmitted to Anopheles stephensi mosquitoes (de Koning-Ward et al., 2008). MDV-1/PEG3 has been characterized both in human and rodent malaria parasites. In P. falciparum (PF3D7_1216500), it localizes primarily to gametocyte PV (Furuya et al., 2005; Silvestrini et al., 2005; Lanfrancotti et al., 2007), while in P. berghei (PBANKA_143220), it resides exclusively in female OB and in vesicle-like structures of male gametocytes (Ponzi et al., 2009). The GEST protein (PBANKA_131270), characterized only in P. berghei, co-localizes with MDV1/PEG3 in both male and female gametocytes (Talman et al., 2011). P. berghei parasites lacking either MDV1/PEG3 (Lal et al., 2009; Ponzi et al., 2009) or PbsGEST (Talman et al., 2011) are defective in gametocyte egress. In these parasites, gametocytes of both genders fully develop inside RBC but remain entrapped within the PV after activation of the gametogenesis process.

These observations provide strong evidence that secretory organelles are central in both male and female gametocyte egress. By combining ultrastructural, immunolocalization and genetic approaches, we defined morphological features characteristic of male-specific secretory organelles and refer to them as MOB. Comparative electron microscopy and electron tomography revealed significant differences in size and morphology between female OB and MOB. We showed that clustering of MOB is an early event in male gametogenesis induced in vitro. It requires a drop in temperature (from 37°C to 21°C) but is not dependent on an increase in pH or the presence of XA as activation factors. While the MDV1/PEG3 is associated with both OB and MOB, we found that the P. berghei g377, like the P. falciparum orthologue, localizes to female OB. In P. berghei mutants lacking Pbg377, female gametocytes develop normally but display a dramatic reduction in size of OB that lack their typical oval-shaped morphology. This structural change is accompanied by a delay in egress efficiency but not in female gamete fertility.

Results

Pbg377 localizes to OB of P. berghei female gametocytes morphologically distinct from secretory vesicles of males

To characterize expression and localization of the P. berghei g377 (Pbg377; PBANKA_146300), we raised mouse antibodies against the C-terminal portion of the protein. The immune serum specificity was confirmed by western blot analysis on purified wild-type (WT) gametocytes and on mixed blood stages of isogenic P. berghei parasites (HPE) that are unable to produce gametocytes (Fig. 1A). Two main protein bands were detected exclusively in the gametocyte preparation,
indicating that Pbg377 expression is confined to sexual stages. The presence of multiple bands suggests that Pbg377, as in the case of *P. falciparum* Pf377, is subjected to post-translational cleavage. The size of the two detected polypeptides does not account for the entire protein (expected size of 309.2 kDa), suggesting the presence of additional fragments not recognized by the antibodies.

In immunofluorescence assay (IFA), Pbg377-specific antibodies stained dot-like structures only in female gametocytes (Fig. 1B). We used antibodies against the nuclear protein SET, which strongly labels the nucleus of male gametocytes (Pace et al., 2006), to distinguish between males and females. Double IFA using α-Pbg377 and α-MDV1/PEG3 antibodies showed that these proteins localize to the same vesicular structures (Fig. 1C) in female gametocytes.

Immune electron microscopy (IEM), revealed that both Pbg377 and MDV1/PEG3 reside in the characteristic oval-shaped OB of female gametocytes, while only MDV1/PEG3 was detected in OB-like, electron-dense organelles of male gametocytes (Fig. 2). In transmission electron microscopy (TEM), these appear as elongated, club-shaped structures, smaller than female OB (Fig. 2). Here we define these OB-like organelles ‘male osmiophilic bodies’ (MOB).

Electron tomography of gametocytes, followed by three-dimensional reconstruction, was employed to compare the size and morphology of OB and MOB. As shown in Fig. 3, the two organelles differ significantly both in morphology and size. Male osmiophilic bodies were found to be more slender than OB, and their volume was only ~1/3 of that of OB.

**MOB cluster after activation of male gametocytes**

Discharge of the OB content into the PV is believed to be essential for gametocyte egress from the infected RBC. In activated female gametocytes, OB accumulate at the gametocyte plasma membrane in multiple foci, which may be associated with PVM rupture sites (Wirth and Pradel, 2012). Conversely, in activated male gametocytes, MOB seem to coalesce in only a few focal points (Lal et al., 2009). To achieve a better understanding of these male-specific organelles, we followed MOB behaviour after gametocyte activation and simultaneously monitored OB for comparison. Gametocytes were activated by a drop in temperature (from 37°C to 21°C) and an increase in pH (from 7.2 to 8.2). After activation, gametocytes were collected at different time points and stained with α-MDV1/PEG3. As shown in Fig. 4A, within the first minute, female OB move to the cell periphery. In contrast, MOB appear to coalesce in a few spots, which display a bright fluorescence (Fig. 4B). Interestingly, we observed that a temperature drop was sufficient to trigger MOB clustering (Fig. 4C and Supporting Information Fig. S1), while discharge of their content, monitored by IFA, occurred within 5–8 min only when the pH was increased (Supporting Information Fig. S2). Parasitophorous vacuolar membrane disruption was verified in parallel by TEM (not shown). It appears that MOB are routed to the parasite plasma membrane as clusters of separated vesicles (Supporting Information Fig. S2). This indicates that the peculiar coalescence of MOB in a few foci is not accompanied by membrane fusion events leading to larger organelles. IEM images confirmed that these clusters are stained by α-MDV1/PEG3 (Fig. 4D).
Conversely, early after activation, OB migrate to the cell periphery as separate vesicles (Supporting Information Fig. S2). We never observed OB clustering in activated P. berghei females.

In order to determine whether mobilization of Ca\(^{2+}\) stores in gametocytes is implicated in OB and MOB dynamics, we treated mature gametocytes with the Ca\(^{2+}\) chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM). It has been shown that treatment of gametocytes with BAPTA-AM blocks Ca\(^{2+}\) mobilization in the cytosol, thereby inhibiting early events associated with gametogenesis (Billker et al., 2004). Gametocytes were incubated with 100 \(\mu\)M BAPTA-AM and then activated. After 20 min of incubation at 37°C, chelator treatment resulted in a block of exflagellation (data not shown). We then stained OB and MOB in activated gametocytes with \(\alpha\)-Pbg377 or \(\alpha\)-MDV1/PEG3 antibodies. This analysis revealed that the block of Ca\(^{2+}\) release inhibits the dynamics of both OB and MOB, i.e. the migration to the cell periphery or clustering (Supporting Information Fig. S3). Interestingly, this treatment also affects recruitment of the protein SET into the nucleus.

**OB number and morphology is affected in female gametocytes lacking Pbg377**

In order to define the role of the OB-resident protein Pbg377 in P. berghei gametocytes, we used standard genetic modification technologies to delete the Pbg377 gene. In two independent transfection experiments, we successfully generated and selected gene deletion

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mutants (Supporting Information Fig. S4A). The cloned lines $\Delta Pbg377\text{-}a$ (600cl1) and $\Delta Pbg377\text{-}b$ (622cl1) were used for genotyping. Genotype and Southern analyses of digested DNA and/or of chromosomes separated by pulse field gel electrophoresis, confirmed correct deletion of the $Pbg377$ gene (Supporting Information Fig. S4B). Northern analysis showed the absence of $Pbg377$ transcripts (Supporting Information Fig. S4C). The lack of the encoded protein was demonstrated by IFA and western blot analysis on mature gametocytes of the mutant lines (Fig. 5).

TEM analysis of $\Delta Pbg377$ female gametocytes did not detect typical OB but only tiny osmiophilic vesicles, which were reduced in size compared with OB of WT females (Fig. 6). The altered morphology of OB in mutant parasites was confirmed by determining minimum and maximum size values of more than a hundred OB sections present in TEM micrographs of WT and $\Delta Pbg377$ gametocytes (Supporting Information Fig. S5). These observations indicate that $Pbg377$ may have a structural role in defining the shape of female OB. Absence of this protein does not result in a near complete lack of OB, as described for $P. falciparum$ mutant lacking Pfg377, but instead results in a dramatic change in OB morphology. Notably, in $\Delta MDV/PEG3$, a mutant lacking expression of MDV1/PEG3 (Ponzi et al., 2009), OB size is comparable to that of WT female gametocytes (Supporting Information Fig. S6A).

**MDV1/PEG3 localizes to OB-like structures in $\Delta Pbg377$**

We asked whether subcellular localization of MDV/PEG3, detected both in OB and MOB of WT gametocytes, is
affected in \( \Delta Pbg377 \) gametocytes. In immunolocalization experiments, MDV1/PEG3-specific antibodies mark dot-like structures of both female and male gametocytes of \( \Delta Pbg377 \) (Supporting Information Fig. S6B). This suggests that secretory organelles are still present in both genders of the \( \Delta Pbg377 \) mutant parasites. We next followed the dynamics of MDV/PEG3-positive OB and MOB in \( \Delta Pbg377 \) gametocytes. One minute after activation, OB stained by MDV/PEG3 antibodies relocate to the gametocyte periphery, while MOB tend to form clusters (Supporting Information Fig. S7). These observations support the notion that \( \Delta Pbg377 \) female gametocytes still contain OB-like structures, which are able to respond to drop in temperature and pH increase similarly to OB of WT female gametocytes.

We were unable to decorate OB-like structures in \( \Delta Pbg377 \) females by IEM using MDV1/PEG3-specific antibodies, whereas these antibodies readily detected OB of the WT gametocytes in IEM and both WT and \( \Delta Pbg377 \) OB in IFA. This lack of staining of \( \Delta Pbg377 \) OB in IEM might be due to the small size of OB in \( \Delta Pbg377 \) females, which prevents efficient labelling.

**Female gametocytes lacking Pbg377 are fertile although egress is delayed**

As expected, \( \Delta Pbg377 \) parasites showed WT asexual multiplication rates *in vivo* (Supporting Information Table S1). Production of gametocytes was slightly reduced in clone \( \Delta Pbg377-b \) (622cl1) compared with WT,
Fig. 5. Characterization of ∆Pbg377 parasites.
A. Male and female gametocytes of ∆Pbg377 mutant were subjected to double IFA using mouse α-Pbg377 and rabbit α-SET antibodies. Representative images show that Pbg377 is absent in ∆Pbg377 sexual stages.
B. Mature gametocytes purified from WT and ∆Pbg377 mutant (KO) were probed with α-Pbg377 immune serum and successively with α-MDV1/PEG3 as a loading control. Scale bar, 5 μm.

Fig. 6. Ultrastructural analysis of ∆Pbg377 and WT gametocytes. Female gametocytes of ∆Pbg377 (bottom panels) are characterized by OB smaller in size than WT female gametocyte OB (top panels). OB are arrowed in enlarged sections. RER, rough endoplasmic reticulum; Nu, nucleus.
while the other clone examined was similar to WT (Supporting Information Table S1). Standard in vitro ookinete production assays were performed to determine the percentage of female gametes that transform into ookinetes. Ookinete production of \( \Delta Pbg377 \) was similar to WT parasites (Supporting Information Table S1). Analysis of oocyst and sporozoites production in \textit{Anopheles stephensi} confirmed the normal fertility of \( \Delta Pbg377 \) (Table S1).

\textit{P. falciparum} female gametocytes lacking Pfg377 are delayed in egress from the host erythrocyte. We therefore analysed in detail egress of \textit{P. berghei} males and females. Gametocytes of the WT and \( \Delta Pbg377 \) parasites were collected from infected mice and activated by dilution in RPMI supplemented with XA. 15 and 20 min after activation, parasites were fixed and immunolabelled with TER-119, an antibody against a RBC surface protein. To distinguish between male and female gametocytes, we made use of the SET antibody and DNA staining. 15 min post-activation a reduction of approximately 60% in female egress was observed in mutant parasites compared with the WT, while males emerged normally, as expected (Fig. 7). At 20 min, egress of mutant female gametocytes was comparable to the WT. These observations indicate that in the absence of normal OB, female gametocytes are able to egress from the host erythrocyte, although less efficiently than the WT.

Discussion

Escape from the host cell is essential for survival of intracellular parasites. In \textit{Plasmodium}, this key process is tightly regulated and leads to the eventual rupture of PVM and host cell membrane with an inside-out mechanism (reviewed in Wirth and Pradel, 2012; Blackman and Carruthers, 2013). In sexual stages of both \textit{P. falciparum} and \textit{P. berghei}, PVM ruptures at multiple sites soon after activation, concomitant with the discharge of OB, specialized electron-dense secretory organelles, specific for female gametocytes (Sologub \textit{et al}., 2011; Deligianni \textit{et al}., 2013; Andreadaki \textit{et al}., unpublished).

Functional studies on these key organelles are difficult because of the limited number of OB-associated molecules characterized so far. In \textit{P. berghei}, MDV1/PEG3 (Ponzi \textit{et al}., 2009) and GEST (Talman \textit{et al}., 2011) were detected in vesicle-like structures of both genders. IEM or co-immunolocalization studies confirmed that both MDV1/PEG3 (Ponzi \textit{et al}., 2009) and \( PbGEST \) (Talman \textit{et al}., 2011) mark female gametocyte OB. The female-specific Pfg377 is the only protein associated to OB of \textit{P. falciparum} gametocytes (Alano \textit{et al}., 1995). The lack of this molecule causes a significant delay in female gamete egress (de Koning-Ward \textit{et al}., 2008), likely because of a dramatic reduction in OB number.

We show here that the \textit{P. berghei} orthologue, Pbg377, is a female-specific protein and localizes to OB. Co-immunolocalization experiments indicated that Pbg377 and MDV1/PEG3 mark the same vesicular structures in female gametocytes, suggesting that OB are homogeneous in protein content.

In parasites deficient for Pbg377 (\( \Delta Pbg377 \)), we observed a delay in female gametocyte egress, while males escape normally from RBC. The percentage of gametes that transform into ookinetes in vitro was not
affected, indicating no defect in fertility of macro-
gametes. This was also confirmed in vivo through the
analysis of oocysts and sporozoites produced in Anopheles stephensi mosquitoes. Taken together,
these observations suggest that, although a delay in
egress is detectable, fertility of the females is not
compromised.

TEM analysis of female gametocytes lacking Pbg377
showed electron-dense, OB-like structures that were sig-
nificantly reduced in size, compared with OB of WT
female gametocytes. In contrast, OB morphology is not
affected in female gametocytes, In contrast, OB morphology is not
affected in MDV1/PEG3, a mutant lacking expression of
MDV1/PEG3 (Ponzi et al., 2009). These observations
strongly support a structural role of Pbg377 in the
biogenesis of these organelles. However, lack of Pbg377
does not prevent recruitment of egress-related proteins
into OB-remnant structures. We, in fact, detected MDV1/
PEG3 in ΔPbg377 female gametocytes. MDV1/PEG3-
containing vesicular structures respond to a drop in
temperature and increase of pH, similarly to OB of WT
gametocytes, and may support, although less efficiently,
female gametocyte egress.

In contrast to Pbg377, the OB-resident proteins MDV1/
PEG3 (Ponzi et al., 2009) and GEST (Talman et al., 2011)
have a clear role in egress of both male and female
P. berghei gametocytes. In the absence of these proteins,
both sexes are severely affected in emergence from the
RBC, although no alteration in OB shape or in the ability
to discharge their content was observed in ΔMDV/PEG3
(this study and Ponzi et al., 2009). Recruitment of egress-
related proteins into OB and MOB strongly supports a key
role of these organelles in gametocyte escape from the
host RBC. Moreover, the active involvement of proteins in
the egress process indicates that mechanical forces
caused by formation of the highly motile male gametes
are not sufficient to rupture the PVM/EM membranes in
WT parasites. Consistent with these observations is the
fact that P. berghei parasites lacking a member of plant-
like calcium-dependent protein kinases, CDPK4 are still
capable to egress from the host cell, despite a severe
defect in axoneme formation and lack of exflagellation
(Billker et al., 2004).

Immune electron microscopy studies detected
MDV1/PEG3 in both OB and MOB, while electron
tomography revealed distinctive morphological features
of these gender-specific electron-dense vesicles.
Female OB are oval-shaped and their volume is approxi-
mately three times greater than the one of club-shaped
MOB.

In non-activated gametocytes, both kinds of vesicles
are dispersed throughout the cytoplasm. In activated
females, OB are uniformly distributed underneath parasite
surface before fusing to the plasma membrane. In acti-
vated males, MOB progressively coalesce in few focal
points as previously observed (Lal et al., 2009). Ultrastructural analysis revealed that MOB become
organized in large clusters that, in some cases, come into
contact with the parasite membrane. MOB clustering
occurs in response to a drop in temperature, but increase
of pH or addition of XA is required for efficient vesicle
discharge. Under optimal conditions, MDV1/PEG3-
specific fluorescence is greatly reduced 5–8 min after
activation, suggesting that the majority of MOB released
their content over this time.

It is conceivable that release of MOB content into PV
and the subsequent PVM disruption occurs in one or few
delivery sites and not at multiple points as in the case of
females (Sologub et al., 2011). Egress of motile males
might be similar to egress of asexual merozoites from the
host RBC. Breakdown of the host cell membrane by
merozoites initiates, in fact, with the formation of a single
pore followed by curling of the membrane and a physical
ejection of merozoites in the blood (Abkarian et al.,
2011).

The peculiar spatial organization of secretory vesicles,
observed only in activated males, likely reflects a
specific mechanism of vesicle discharge. The best
described case of vesicle clustering has been reported
for small synaptic vesicles (SVs), which fuse with the
plasma membrane and release neurotransmitters in
response to a wide variety of stimuli (reviewed in
Shupliakov et al., 2011). Electron tomographic studies
showed that SVs are held together by filaments and
elongated proteins. The nature of these inter-vesicular
tethers has not been completely elucidated. The dynam-
ics of SV clustering is regulated by the phosphorylation
state, while destabilization of the intra-vesicular matrix
and membrane fusion events are likely triggered by Ca2+
influx and de-phosphorylation.

Local increase of Ca2+ concentration and activation of
calcium-dependent protein kinases are required for
egress of Apicomplexan parasites Toxoplasma gondii and
P. falciparum (reviewed in Blackman and Carruthers,
2013). It has been shown that treatment of gametocytes
with the Ca2+ chelator BAPTA-AM blocks Ca2+ mobilization
in the gametocyte cytosol and thereby inhibiting early
events associated with gametogenesis (Billker et al.,
2004). We observed that both OB repositioning to the
female gametocyte periphery and MOB clustering are
affected in gametocytes treated with BAPTA-AM. These
observations indicate that OB dynamics is dependent on
Ca2+ release.

Evidence presented in this study indicates that molecu-
lar events leading to gametocyte egress differ between
female and male gametocytes. A further dissection of
this process, essential for fertilization and development within
the mosquito, may provide knowledge for designing strat-
egies that can effectively block transmission.
Experimental procedures

Experimental animals and P. berghei ANKA reference lines

Female Swiss OF1 mice (6–8 weeks) from Charles River were used. All animal experiments performed at the LUMC were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 07171; DEC 10099). The Dutch Experiments on Animal Act is established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes).

P. berghei ANKA parasite lines used in this study were the WT reference line c15cy1 (Janse et al., 2006) and the non-gametocyte producer line HPE.

Generation and genotyping of mutants lacking Pbg377 (ΔPbg377)

To disrupt the Pbg377 gene (PBANKA_146300), the standard plasmid pL0001 (MRA-770, http://www.mr4.org) was used, which contains the pyrimethamine resistant T gondii (Tg) dihydrofolate reductase-thymidylate synthase (dhfr/ts) as a selectable marker (SM) under the control of the P. berghei dhfr promoter. Targeting sequences for homologous recombination were polymerase chain reaction (PCR) amplified from P. berghei ANKA (c15cy1) genomic DNA using primers specific for the 5′ or 3′ end of each gene:

- 2307/2308 (5′-CGGGGTACCacacagcaatgtgcaagac; 5′-CCGAGTCCTtagtttctgtgatataactagg) and 2309/2310 (5′-CGCGATTCgccaccoccgacatcaacatalag; 5′-CGCGGATCCgggtcggtgatcgtttaatg). The PCR-amplified target sequences were cloned either upstream or downstream of the SM to allow for integration of the construct into the targeting regions by double crossover homologous recombination. The final DNA construct was linearized with KpnI/BamHI before transfection. Transfection and selection of transformed parasites was performed using standard genetic modification technologies for P. berghei (Janse et al., 2006) using P. berghei ANKA (c15cy1) as the parent parasite line. Cloned parasite lines were obtained from two independent transfections (exp. 600 and 622) by the method of limiting dilution. Correct integration of DNA constructs and disruption of target sequences as described in (Spaccapelo et al., 2001) was determined in Giemsa-stained blood smears made 16–18 h post-activation.

Oocyst and sporozoite production in Anopheles stephensi mosquitoes. For mosquito transmission experiments, female A. stephensi mosquitoes were fed on mice infected with WT parasites or mutants. Oocyst development, oocyst production and sporozoite production was monitored in infected mosquitoes as described in (Sinden, 1997). Oocyst and sporozoite numbers were counted in infected mosquitoes at 11–14 days and 19–22 days after infection respectively. Salivary gland sporozoites were isolated and counted as described (Annoura et al., 2012).
Sporozoite infectivity. To determine in vivo infectivity of sporozoites, Swiss OF1 mice were infected with $1 \times 10^4$ salivary gland sporozoites by intravenous injection, as previously described (Sinden, 1997). Blood stage infections were monitored by analysis of Giemsa-stained thin smears of tail blood collected on days 4–8 after inoculation of sporozoites. The prepatent period (measured in days post-sporozoite infection) is defined as the day when a blood stage infection with a parasitemia of 0.5–2% is observed.

TEM

Nycomeden-purified gametocytes were fixed in 2% glutaraldehyde, 2% paraformaldehyde, 2 mM CaCl$_2$ in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C, and processed according to (Perry and Gilbert, 1979). Parasites were washed in cacodylate buffer and post-fixed with 1% OsO$_4$ in 0.1 M sodium cacodylate buffer for 1 h at RT, treated with 1% tannic acid in 0.05 M cacodylate buffer for 30 min and rinsed in 1% sodium sulphate in 0.05 M cacodylate buffer for 10 min. Post-fixed specimens were washed, dehydrated through a graded series of ethanol solutions (30–100% ethanol) and embedded in Agar 100 (Agar Scientific Ltd, UK). Ultrathin sections, obtained by an UC6 ultramicrotome (Leica), were stained with uranyl acetate and Reynolds’ lead citrate and examined by an EM208 Philips electron microscope.

Immunoelectron microscopy

Nycomeden-purified gametocytes were processed for immuno-electron microscopy according to published protocols (Newman, 1989). Briefly, samples were fixed overnight at 4°C with 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer. Next, the suspension was gently washed in sodium cacodylate buffer, dehydrated in ethanol serial dilutions and embedded in LR White, medium-grade acrylic resin (London Resin Company, UK). The samples were polymerized in a 50°C oven for 72 h. Ultrathin sections, collected on gold grids, were stained with uranyl acetate and Reynolds’ lead citrate and examined by an EM208 Philips electron microscope.

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Nycomeden-purified gametocytes were fixed in 2% glutaraldehyde, 2% paraformaldehyde, 2 mM CaCl$_2$ in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C, and processed according to (Perry and Gilbert, 1979). Parasites were washed in cacodylate buffer and post-fixed with 1% OsO$_4$ in 0.1 M sodium cacodylate buffer for 1 h at RT, treated with 1% tannic acid in 0.05 M cacodylate buffer for 30 min and rinsed in 1% sodium sulphate in 0.05 M cacodylate buffer for 10 min. Post-fixed specimens were washed, dehydrated through a graded series of ethanol solutions (30–100% ethanol) and embedded in Agar 100 (Agar Scientific Ltd, UK). Ultrathin sections, obtained by an UC6 ultramicrotome (Leica), were stained with uranyl acetate and Reynolds’ lead citrate and examined by an EM208 Philips electron microscope.

Antibodies

A Pbg377 fragment amplified using the primers gaccggtatgacacctttctct and gaccgagggctctcgcattctaggtcctagttgct was cloned in the BamHI-NotI sites of the pGEX-6P-1 vector. Pbg377 fragment was expressed and used to produce specific antibodies in BALB/c mice by intraperitoneal injection with 50 μg recombinant protein (first injection in Freund’s complete adjuvant; two further injections performed at 2-week intervals in Freund’s incomplete adjuvant). Mice were bled 1 week after the third immunization.

Working dilutions of antibodies used in this study were α-Pb377 mouse polyclonal, 1:300 in IFA, 1:2000 in western blot; α-MDV/PEG3 mouse polyclonal, 1:400 in IFA, 1:2000 in western blot; α-SET rabbit polyclonal (Pace et al., 2006) 1:200 in IFA, 1:5000 in western blot; Alexa Fluor 488 α-mouse TER-119 (BioLegend) 1:400 in IFA.

Indirect IFA

Immunofluorescence assay was performed at room temperature as follows: blood smears were fixed on glass slides for 1 h with 4% paraformaldehyde, washed in PBS and then treated with 0.1% Triton X-100 in PBS. After blocking overnight in PBS/3%BSA, slides were incubated 1 h in primary antibody, washed several times with PBS and then incubated 30 min in fluorescein- or rhodamin-conjugated goat α-mouse or α-rabbit secondary antibodies (1:400 dilution). Cell nuclei were labelled with DAPI. The specificity of the immune sera was checked in parallel using pre-immune sera. In double IFA experiments using mouse immune sera against Pbg377 and MDV/PEG3, slides were first incubated with α-Pbg377 immune serum as described above and fixed with 4% paraformaldehyde prior incubation with the second immune serum. α-MDV/PEG3 antibodies were labelled with Zenon kit Alexa Fluor 488 (Invitrogen), according with manufacturer’s protocol. Prior labelling IgG were purified from α-MDV/PEG3 immune serum using protein G agarose (Life Technologies). Ten micrograms of labelled IgG were used in IFA.

Western blot analysis

Western blot analysis was performed using MINI TRANS-BLOT® Bio-Rad apparatus at constant voltage (100 V) for 1 h, in transfer buffer (20% methanol, Tris 0.025 M, Glycine 0.192 M) onto Protran 0.22 microns membrane (Whatman). Incubation with antibodies was done. Primary and horseradish peroxidase-conjugated secondary antibody were incubated 1 h in PBS-Tween (0.05%) and membrane was developed using the ECL system (SuperSignalWest Pico, Thermo Scientific) according to manufacturer’s instructions.
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References

Abkarian, M., Massiera, G., Berry, L., Roques, M., and Braun-Breton, C. (2011) A novel mechanism for egress of malarial parasites from red blood cells. *Blood* **117**: 4118–4124.

Aikawa, M., Carter, R., Ito, Y., and Nijhout, M.M. (1984) New observations on gametogenesis, fertilization, and zygote transformation in *Plasmodium gallinaceum*. *J Protozool* **31**: 403–413.

Alano, P., Read, D., Bruce, M., Aikawa, M., Kaido, T., Tegoshi, T., et al. (1995) COS cell expression cloning of Pfg377, a *Plasmodium falciparum* gametocyte antigen associated with osmiophilic bodies. *Mol Biochem Parasitol* **74**: 143–156.

Annoura, T., Ploemen, I.H., van Schaijk, B.C., Sajid, M., Vos, M.W., van Gemert, G.J., et al. (2012) Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates. *Vaccine* **30**: 2662–2670.

Billker, O., Lindo, V., Panico, M., Etienne, A.E., Paxton, T., Dell, A., et al. (1998) Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. *Nature* **392**: 289–292.

Billker, O., Dechamps, S., Tewari, R., Wenig, G., Franke-Fayard, B., and Brinkmann, V. (2004) Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. *Cell* **117**: 503–514.

Blackman, M.J., and Carruthers, V.B. (2013) Recent insights into apicomplexan parasite egress provide new views to a kill. *Curr Opin Microbiol* **16**: 459–464.

Braks, J.A., Mair, G.R., Franke-Fayard, B., Janse, C.J., and Waters, A.P. (2008) A conserved U-rich RNA region implicated in regulation of translation in Plasmodium female gametocytes. *Nucleic Acids Res* **36**: 1176–1186.

Chandramohanadas, R., Park, Y., Lui, L., Li, A., Quinn, D., Blackman, M.J., and Carruthers, V.B. (2013) A perforin-like protein mediates disruption of the erythrocyte membrane during egress of *Plasmodium berghei* male gametocytes. *Cell Microbiol* **15**: 1438–1455.

van Dijk, M.R., Janse, C.J., Thompson, J., Waters, A.P., Braks, J.A., Dodemont, H.J., et al. (2001) A central role for P48/45 in malaria parasite male gamete fertility. *Cell* **104**: 153–164.

Furuya, T., Mu, J., Hayton, K., Liu, A., Duan, J., Nkrumah, L., et al. (2005) Disruption of a *Plasmodium falciparum* gene linked to male sexual development causes early arrest in gametocytogenesis. *Proc Natl Acad Sci USA* **102**: 16813–16818.

Glushakova, S., Yin, D., Li, T., and Zimmerberg, J. (2005) Membrane transformation during malaria parasite release from human red blood cells. *Curr Biol* **15**: 1645–1650.

Hanssen, E., Dekiwadia, C., Riglar, D.T., Rug, M., Lengrubler, L., Cowman, A.F., et al. (2013) Electron tomography of *Plasmodium falciparum* merozoites reveals core cellular events that underpin erythrocyte invasion. *Cell Microbiol* **15**: 1457–1472.

Janse, C.J., and Waters, A.P. (1995) *Plasmodium berghei*: the application of cultivation and purification techniques to molecular studies of malaria parasites. *Parasitol Today* **11**: 138–143.

Janse, C.J., Haghparast, A., Speranca, M.A., Ramesar, J., Kroeze, H., del Portillo, H.A., and Waters, A.P. (2003) Malaria parasites lacking eef1a have a normal S/M phase yet grow more slowly due to a longer G1 phase. *Mol Microbiol* **50**: 1539–1551.

Janse, C.J., Ramesar, J., and Waters, A.P. (2006) High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. *Nat Protoc* **1**: 346–356.

de Koning-Ward, T.F., Olivier, A., Bertuccini, L., Hood, A., Silvestrini, F., Charvalias, K., et al. (2008) The role of osmiophilic bodies and Pfg377 expression in female gametocyte emergence and mosquito infectivity in the human malaria parasite *Plasmodium falciparum*. *Mol Microbiol* **67**: 278–290.

Kremer, J.R., Mastronarde, D.N., and McIntosh, J.R. (1996) Computer visualization of three-dimensional image data using IMOD. *J Struct Biol* **116**: 71–76.

Lai, K., Delves, M.J., Bromley, E., Wastling, J.M., Tomley, F.M., and Sinden, R.E. (2009) *Plasmodium* male development gene-1 (mdv-1) is important for female sexual development and identifies a polarised plasma membrane during zygote development. *Int J Parasitol* **39**: 755–761.

Manfrinacci, A., Bertuccini, L., Silvestrini, F., and Alano, P. (2007) *Plasmodium falciparum*: mRNA co-expression and protein co-localisation of two gene products upregulated in early gametocytes. *Exp Parasitol* **116**: 497–503.

McRobert, L., Taylor, C.J., Deng, W., Fifelman, Q.L., Cummings, R.M., Polley, S.D., et al. (2008) Gametogenesis in malaria parasites is mediated by the cGMP-dependent protein kinase. *PLoS Biol* **6**: e139.

Mastronarde, D.N. (1997) Dual-axis tomography: an approach with alignment methods that preserve resolution. *J Struct Biol* **120**: 343–352.

Mastronarde, D.N. (2005) Automated electron microscope tomography using robust prediction of specimen movements. *J Struct Biol* **152**: 36–51.

Newman, G.R. (1989) LR white embedding medium for
colloidal gold methods. In Colloidal Gold – Principles, Methods and Applications, Vol. 2. Hayat, M.A. (ed.). San Diego: Academic Press, pp. 47–73.

Pace, T., Olivieri, A., Sanchez, M., Albanesi, V., Picci, L., Siden Kiamos, I., et al. (2006) Set regulation in asexual and sexual Plasmodium parasites reveals a novel mechanism of stage-specific expression. Mol Microbiol 60: 870–882.

Perry, M.M., and Gilbert, A.B. (1979) Yolk transport in the ovarian follicle of the hen (Gallus domesticus): lipoprotein-like particles at the periphery of the oocyte in the rapid growth phase. J Cell Sci 39: 257–272.

Ponzi, M., Siden-Kiamos, I., Bertuccini, L., Curra, C., Kroeze, H., Camarda, G., et al. (2009) Egress of Plasmodium berghei gametes from their host erythrocyte is mediated by the MDV-1/PEG3 protein. Cell Microbiol 11: 1272–1288.

Pradel, G. (2007) Proteins of the malaria parasite sexual stages: expression, function and potential for transmission blocking strategies. Parasitology 134: 1911–1929.

Saito, N., Konishi, K., Takeda, H., Kato, M., Sugiyama, T., and Asaka, M. (2003) Antigen retrieval trial for post-embedding immunoelectron microscopy by heating with several unmasking solutions. J Histochem Cytochem 51: 989–994.

Severini, C., Silvestrini, F., Sennella, A., Barca, S., Gradoni, L., and Alano, P. (1999) The production of the osmophilic body protein Pg377 is associated with stage of maturation and sex in Plasmodium falciparum gametocytes. Mol Biochem Parasitol 100: 247–252.

Shupliakov, O., Haucke, V., and Pechstein, A. (2011) How synapsin I may cluster synaptic vesicles. Semin Cell Dev Biol 22: 393–399.

Silvestrini, F., Bozdech, Z., Lantfranchi, A., Di Giulio, E., Bultrini, E., Picci, L., et al. (2005) Genome-wide identification of genes upregulated at the onset of gametocyteogenesis in Plasmodium falciparum. Mol Biochem Parasitol 143: 100–110.

Sinden, R.E. (1983) Sexual development of malarial parasites. Adv Parasitol 22: 153–216.

Sinden, R.E. (1997) Infection of mosquitoes with rodent malaria. In Molecular Biology of Insect Disease Vectors: A Methods Manual. Crampton, J.M., Beard, C.B., and Louis, C. (eds). London.: Chapman and Hall, pp. 67–91.

Sinden, R.E., Canning, E.U., and Spain, B. (1976) Gametogenesis and fertilization in Plasmodium yoelii nigeriensis: a transmission electron microscope study. Proc R Soc Lond B Biol Sci 193: 55–76.

Sologub, L., Kuehn, A., Kern, S., Przyborski, J., Schillig, R., and Pradel, G. (2011) Malaria proteases mediate inside-out egress of gametocytes from red blood cells following parasite transmission to the mosquito. Cell Microbiol 13: 897–912.

Spaccapelo, R., Janse, C.J., Caterbi, S., Franke-Fayard, B., Bonilla, J.A., Syphard, L.M., et al. (2010) Plasmepepin 4-deficient Plasmodium berghei are virulence attenuated and induce protective immunity against experimental malaria. Am J Pathol 176: 205–217.

van Spaendonk, R.M., Ramesar, J., van Wigchera, A., Eling, W., Beetsma, A.L., van Gemert, G.J., et al. (2001) Functional equivalence of structurally distinct ribosomes in the malaria parasite, Plasmodium berghei. J Biol Chem 276: 22638–22647.

Talman, A.M., Lacroix, C., Marques, S.R., Blagborough, A.M., Carzaniga, R., Menard, R., and Sinden, R.E. (2011) PbGEST mediates malaria transmission to both mosquito and vertebrate host. Mol Microbiol 82: 462–474.

Wirth, C.C., and Pradel, G. (2012) Molecular mechanisms of host cell egress by malaria parasites. Int J Med Microbiol 302: 172–178.

World Malaria Report (2013). World Health Organization.

Zuccala, E.S., and Baum, J. (2011) Cytoskeletal and membrane remodelling during malaria parasite invasion of the human erythrocyte. Br J Haematol 154: 680–689.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. MOB coalescence is triggered by a drop in temperature. Purified schizonts of the WT line were injected intravenously into a mouse to obtain synchronous blood stages. Tail blood was collected 30 h post-infection to recover mature gametocytes. Infected blood was immediately smeared and fixed in paraformaldehyde (A) or kept 15 min at 22°C and washed in PBS prior fixation (B). Blood smears were then and subjected to IFA.

Fig. S2. Transmission electron micrographs of activated WT gametocytes. (1) TEM images of an activated male (1–2 min after induction) with enlarged nucleus (Nu) and a cluster of MOB in the close vicinity of the plasma membrane (A), 5–8 min after activation axonemes (Ax) are being formed, while the large majority of MOB clusters disappear (B), suggesting that they fuse with parasite plasma membrane and discharge their content into the PV. At difference, OB moves to the periphery of an activated female gametocyte as separate vesicles (C) and discharge their content within 1–2 min (D). (2) IFA of male gametocytes, 5–8 min after induction. Male gamete flagella, stained with antibodies against tubulin-1, are being formed; MDV/PEG3-specific fluorescence is very faint, suggesting that the majority of protein has been discharged. Scale bar: 5 μm.

Fig. S3. Ca2+ chelator BAPTA_AM affects OB and MOB localization upon gametocyte activation. Mature gametocytes from a synchronous infection (30 h post-merozoite invasion) were pre-treated with the Ca2+ chelator BAPTA-AM (100 μM, 20 min at 37°C). The effectiveness of the treatment was monitored by inspecting exflagellations upon gametocyte induction. Exflagellations in treated sample were completely blocked, while normal progression of gametocytegenesis was observed in the control sample from the same mouse. Blood smears from BAPTA-treated and control parasites were stained with α-Pbg377 (A) or α-MDV/PEG3 (B). In both cases, OB and MOB relocation is hampered.

Fig. S4. Generation and genotyping of mutants lacking Pg377 (ΔPbg377). A. Schematic representation of gene-deletion construct targeting the open reading frame (ORF) of Pbg377 by double crossover homologous recombination, and the wild-type (wt) gene locus before and after disruption in two transfection experiments (exp 600 and 622). The construct contains a drug-selectable marker cassette (SM; white box) and gene target regions (black arrows). See the Materials and Methods section for primer sequences used to amplify the target regions.
Restriction sites for diagnostic Southern analysis (see B) are shown. B. Diagnostic Southern analysis of digested genomic DNA (right; BglII) or of pulsed field gel-separated chromosomes (left) confirm correct disruption of PbG377 in ΔPbG377 parasites. Separated chromosomes were hybridized with the 3′UTR of \( P. \) berghei \( dhfr/ts \) gene recognizing the endogeneous \( dhfr/ts \) locus on chromosome 7 and the integrated locus at chromosome 14. Digested DNA (BglII) was hybridized with a probe recognizing the \( Tgdhfr/ts \) SM (see A for the sizes of the DNA fragments). m: after mosquito passage of ΔPbG377 parasites. C. Northern analysis confirms the absence of Pbg377 transcripts in ΔPbG377 parasites. Northern blots were hybridized with a PCR probe specific for the 5′utr of PbG377. As a loading control, blots were hybridized with a Pbs25 specific probe (using a PCR-amplified PBANKA_051500.

Fig. S5. OB morphology and size is affected in ΔPbG377. Box-plot of maximum (A) and minimum (B) values of OB size in WT and ΔPbG377 (128 and 163 sections inspected, respectively). In WT female gametocytes, median of maximum and minimum values is, respectively, 1.7- and 1.5-fold higher than that observed in ΔPbG377 gametocytes. Data distribution around median show high variance in maximum values of both WT and KO parasites. This is expected for elongated structures and confirm tridimensional reconstruction of OB and MOB.

Fig. S6. Ultrastructural analysis of gametocytes of ΔPbg377 and ΔMDV1/PEG3 parasites. A. TEM images of ΔPbg377 female gametocytes show tiny OB, smaller than OB of ΔMDV/PEG3 females, which display WT size and morphology. B. In ΔPbg377 gametocytes, α-MDV/PEG3 stains OB-like structures and normal MOB; in ΔMDV/PEG3 gametocytes, α-Pbg377 stains OB morphologically similar to OB of the WT parasites.

Fig. S7. OB and MOB behaviour in activated ΔPbG377 gametocytes. After 1 min of induction, OB stained by MDV/PEG3 antibodies relocate to the gametocyte periphery, while MOB tend to form clusters similar to those observed in WT parasites.

Table S1. Phenotype analyses of ΔPbG377 parasites during blood and mosquito development.

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