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Truncation of *Plasmodium berghei* merozoite surface protein 8 does not affect *in vivo* blood-stage development

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Running title: Full-length *P. berghei* MSP-8 is not required for blood-stage growth

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ABSTRACT
Merozoite surface protein 8 (MSP8) has shown promise as a vaccine candidate in the *Plasmodium yoelii* rodent malaria model and has a proposed role in merozoite invasion of erythrocytes. However, the temporal expression and localisation of MSP8 are unusual for a merozoite antigen. Moreover, in *P. falciparum* the MSP8 gene could be disrupted with no apparent effect on in vitro growth. To address the in vivo function of full-length MSP8, we truncated MSP8 in the rodent parasite *P. berghei*. PbΔMSP8 disruptant parasites displayed a normal blood-stage growth rate but no increase in reticulocyte preference, a phenomenon observed in *P. yoelii* MSP8 vaccinated mice. Expression levels of erythrocyte surface antigens were similar in *P. berghei* wild-type and PbΔMSP8-infected erythrocytes, suggesting that a parasitophorous vacuole function for MSP8 does not involve global trafficking of such antigens. These data demonstrate that a full-length membrane-associated form of PbMSP8 is not essential for blood-stage growth.
Merozoite surface protein 8 (MSP8) is a glycosylphosphatidyl inositol (GPI)-anchored membrane antigen originally identified in *Plasmodium yoelii* [1] and soon after in the human malaria parasite *P. falciparum* [2]. A distinctive feature of this protein is a C-terminal double epidermal growth factor (EGF)-like domain that resembles that seen in the dominantly expressed merozoite surface protein, MSP1. In MSP1, this double EGF module is considered an important target of protective antibodies and as a result has become a component of numerous strategies being trialled for efficacy as a blood-stage malaria vaccine [3,4]. The MSP8 EGF module is predicted to fold similarly to its MSP1 counterpart and, remarkably, using allelic replacement the MSP8 double EGF domains have been shown to complement the *in vitro* blood-stage function of the MSP1 EGFs [5]. Hence, MSP8 may also be a target of protective antibodies and indeed it has shown considerable promise as a protective immunogen in the *P. yoelii* rodent malaria model system, both alone and in combination with MSP1-based antigens [1,6,7]. One interesting facet of these rodent malaria vaccine studies was the observation that ‘breakthrough’ parasites appear to have an increased preference for reticulocytes [6]. This suggested that MSP8 plays a role in merozoite entry of mature erythrocytes and that vaccine escape parasites had an altered, MSP8-independent, invasion phenotype. Alternate invasion pathways that utilise distinct ligand-receptor combinations are well known, at least in *P. falciparum* [8]. Consistent with a role in merozoite invasion, recombinant *P. yoelii* MSP8 has been shown to bind murine erythrocytes although no difference was observed in the binding of reticulocytes or normocytes [9].
Despite these data, other observations are inconsistent with a generic role for MSP8 in erythrocyte attachment and/or invasion. While early observations demonstrated schizont/merozoite expression as expected for a merozoite antigen, in addition to expression in other stages of the life cycle [2], more recent studies have indicated that *P. falciparum* MSP8 is expressed predominantly in ring-stages where it resides in the parasitophorous vacuole and is probably attached to the parasite membrane [10,11]. In this latter study by Drew *et al* [10], no expression of *P. falciparum* MSP8 was observed in merozoites. Moreover, two independent gene knockout studies in *P. falciparum* have shown that the MSP8 gene is not required for seemingly normal blood-stage growth [10,12]. Similarly in *P. yoelii* peak expression appears to occur at an earlier time point than would be expected for a merozoite antigen, although here peak expression occurs within the trophozoite stages [9].

To further address the *in vivo* function of a full-length, membrane associated form of MSP8, we targeted the *P. berghei* MSP8 gene (PlasmoDB identity number PB000173.03.0) for disruption and analysed blood-stage phenotypes. Gene targeting was performed by double-crossover recombination and was designed to result in truncation of the MSP8 gene such that the regions encoding the C-terminal EGF domains and GPI-anchor signal were deleted (Fig. 1A). *In vitro* cultured blood-stage parasites were transfected with the MSP8 knockout plasmid, pB3-MSP8-TR/3’, essentially as described elsewhere [13]. Following an initial PCR based screen for integration that detected the expected homologous integration-specific PCR products (data not shown), parasites were cloned and a
representative cloned line was analysed by Southern blotting using the 5’-
targeting sequence as a probe (Fig. 1B). This analysis showed the expected loss
of the endogenous hybridising band at 8 Kb and the presence of an integration
specific species at 5 Kb in the disrupted ΔMSP8 line. No other species were
observed, demonstrating that integration had proceeded by double crossover
recombination. Western blotting of parasite extracts with antibodies specific for
the MSP8 EGF domains (M8 Ab) confirmed the absence of MSP8 in the ΔMSP8
line, with detection of MSP119 used as a loading control for ΔMSP8 parasite
extracts (Fig. 1C). A time course analysis of protein expression also confirmed
that P. berghei MSP8, like its P. yoelii counterpart, is predominately expressed in
the trophozoite stage, substantially earlier than schizont-specific antigens such
as MSP1 (Fig. 1C). MSP8 was also detected in the ring stages and by
immunofluorescence assay (IFA) it was found to localise to the parasitophorous
vacuole (PV), where it is possibly anchored to the parasite membrane via its GPI-
anchor, especially given that it co-localises with MSP119 at this time. By schizont
stages, however, MSP8 was present in the food vacuole (Fig. 1D). The specificity
of this observed reactivity in Western blots and IFA was confirmed by testing the
reactivity of the same reagent against the ΔMSP8 disrupted line in parallel and
under identical conditions to wild-type P. berghei parasites. For merozoites,
however, weak apical reactivity was observed with the anti-MSP8 antibodies in
IFA but was indistinguishable in the ΔMSP8 and wild-type lines suggesting these
antibodies are weakly cross-reactive to another parasite protein (data not
shown). In P. yoelii, MSP8 appeared to have a surface localisation in the intact
although the specificity of this reactivity was not confirmed with a *P. yoelii* MSP8 knockout line [9]. In summary, our results with *P. berghei* confirm the two *P. falciparum* knockout studies, which show that MSP8 is expressed in the ring-stages [10,12] and localises to the PV [10]. While expression may progress to later stages in the cycle, our study and that by Drew *et al* [10] clearly show that the vast majority of full-length MSP8 is restricted to the food vacuole in mature forms.

Having confirmed that the *MSP8* gene could be disrupted in *P. berghei*, we examined the blood-stage phenotype of the ∆MSP8 parasite line. Wild-type *P. berghei* has a preference for invading reticulocytes [14,15], however, at low parasite burdens the majority of *P. berghei* parasites can be found inside mature RBCs, which is reflective of the high normocyte:reticulocyte ratio during the early stages of infection (see day 5 time point in Fig. 2A) [15]. As the levels of parasitemia increase from day 7 onwards, there is a marked increase in circulating reticulocytes in response to the decrease in erythrocyte levels, such that by day 11 post-infection reticulocytes account for up to 40% of circulating cells (Fig. 2B) [15]. With its preference for reticulocytes, therefore, the overwhelming majority of parasites can be found inside reticulocytes in the latter stages of infection. A similar finding to the wild-type parent was observed with the ∆MSP8 parasite line, in that there was no difference in the overall growth rates in Balb/c mice, nor was there a significant difference in overall reticulocyte preference at any time point, including on day 5 when only normocytes were observed (Figs. 2A). In addition, no significance differences in reticulocytosis or
haemoglobin levels were detected in mice infected with either parasite line over the course of the infection (Fig. 2B).

Another observation in *P. yoelii* MSP8 vaccination experiments was the detection of transcriptional differences in ‘breakthrough’ parasites in genes encoding proteins exported to the RBC cytosol and in some instances probably to the surface of infected RBCs [9]. In order to investigate if disruption of *MSP8* altered the expression of antigens on the surface of infected erythrocytes, we performed FACS analysis using antisera raised to wild-type *P. berghei* parasites on whole, unfixed parasite populations. Schizont-stage infected erythrocytes reacted most strongly with this reagent as expected since it is at this stage that the expression of surface antigens is greatest. However, under a range of conditions and in two separate experiments, no difference was seen in the reactivity of the ∆MSP8 and parental parasites, suggesting that the levels of surface antigen expression are similar (Fig. 2C). Hence, it appears unlikely, in this system at least, that MSP8 is a key component of a protein-export trafficking machinery predicted to be present in the parasitophorous vacuole of ring-stage parasites and which is responsible for the global export of antigens to the erythrocyte surface [16].

In summary, we have shown that a full-length membrane-associated form of *P. berghei* MSP8 is not required for merozoite invasion, blood-stage development or other aspects of blood-stage survival in Balb/c mice. It should be noted, given the design of the targeting vector used to create the ∆MSP8 disruptant line, that it is possible that a 35 kDa N-terminal fragment of MSP8 missing the C-terminal EGF domains and GPI-anchor is likely to be present in ∆MSP8 parasites, which
cannot be detected by the available antibodies raised against \textit{P. berghei} MSP8. We cannot rule out the possibility that this polypeptide performs the same function as full-length MSP8. However, such a role for this truncated form seems unlikely given the lack of the EGF domains and capacity for membrane association, features shared by all \textit{Plasmodium} MSP8 orthologs. Hence, the function of MSP8 remains a mystery. It is possible that its primary role is not in the blood stage and so was not revealed in this study, however, there is no evidence for strong \textit{MSP8} mRNA expression in other life stages in either \textit{P. falciparum} or \textit{P. berghei}. It is possible that in the rodent system MSP8 plays a redundant role in invasion [6,9] although this was not revealed in our study and also appears unlikely to be the case of \textit{P. falciparum} where it is completely absent from merozoites [10].

Although clearly not essential, it is curious that MSP8 is expressed in the blood-stages in both human and rodent \textit{Plasmodium} species and that its localisation to the ring-stage parasitophorous vacuole is conserved across the species. In \textit{P. falciparum}, MSP8 also resides in detergent-resistant membranes where it appears to be present in a larger complex [10,17,18]. An as yet undetermined role for MSP8 or the MSP8-associated complex in the parasitophorous vacuole remains a possibility, however this study suggests that such a function is unlikely to involve a major role in protein trafficking.

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References


Figure legends

Fig. 1. Generation of a *P. berghei* ANKA line harbouring a truncated *MSP8* gene. (A) Schematic diagram of the *P. berghei* *MSP8* locus, the transfection vector pB3-MSP8-TR/3’ used to disrupt the endogenous MSP8 locus (linearised with *KpnI* and *BamHI*) and the predicted *MSP8* locus after integration that results in a truncation immediately prior to the start of the double EGF domains. The 5’- and 3’- *MSP8* targeting regions used to drive integration are indicated by heavy solid lines and were generated by PCR amplification of *P. berghei* genomic DNA using the oligonucleotides Pb8-5’F (5’-CGGGGTACCttttcttttattagcccactctctatatattatagg) and PB8-5’R (5’-CCGCTCGAGttaaattcgatttttttcaataaaatattctgaaatrrtttc) and PB-8 3’F (5’-GGGATATCgttaatatgcatatattatgttatttgaaactag) and PB-8 3’R (5’- CGCGGATccatcgttcgaaaaaaacagacaatgtac), respectively. The hsp86 3’ UTR is represented by a grey box and has been described previously [19]. The expected sizes of DNA fragments from wild-type and transgenic parasites resulting from digestion with *EcoRV* (Ev) are shown. The EGF region of *P. berghei* MSP8 to which the antibodies (M8 Ab) were derived is indicated [5]. Tg DHFR-TS, selectable marker cassette. (B) Southern blot analysis of digested genomic DNA showing homologous integration into the *MSP8* gene. Genomic DNA from wild-type (WT) *P. berghei* and the *MSP8* disrupted line that had been cloned (ΔMSP8) were digested with *EcoRV* and hybridised with the portion of *MSP8* that was used as the 5’-targeting sequence. (C) Western blot analysis of parasite extracts harvested from ring (R), early trophozoite (ET), trophozoite (T) and schizont (S) stages, using rabbit α-MSP8 or rabbit α-MSP119 antibodies (α-
PbM19 [19], used to confirm equal loading of parasite extracts in wild-type and 
ΔMSP8 disruptant lines and as a control for timing of expression of a schizont 
specific protein), both diluted 1/1000. (D) Localisation of MSP8 by IFA.
Acetone/methanol fixed smears of \textit{P. berghei} parasites from stages throughout 
the asexual life cycle were incubated with a mixture of rabbit \(\alpha\)-MSP8 (1:2000) or 
mouse \(\alpha\)-MSP1\textsubscript{19} (1:1000) antibodies, followed by incubation with a mixture of 
Alexa-Fluor 488-conjugated -rabbit IgG and Alexa-Fluor 568-conjugated goat 
anti-mouse IgG (both diluted 1:1000). Staining of parasite nuclei was performed 
using DAPI.

\textbf{Fig. 2.} Truncation of MSP8 in \textit{P. berghei} does not affect parasite growth, tropism 
or surface antigen expression. (A) Balb/c mice were infected intraperitoneally 
with erythrocytes parasitised with either \(10^6\) \textit{P. berghei} wild-type (WT) or ΔMSP8 
parasites. The total blood parasitemia (top) and the percentage of parasites 
invading reticulocytes (bottom) was determined over the course of the infection 
by thin tail blood smears stained with Giemsa. Shown in the mean ± SD 
observed in 8 mice. (B) The percentage of reticulocytes in the circulation, and the 
haemoglobin levels (measured using a HemoCue HB201 DM systems; 
HemoCue Australia) were monitored in the mice from day 3 post-infection. 
Shown in the mean ± SD observed in 8 mice. (C) Binding of \textit{P. berghei} wild-type 
(WT) and ΔMSP8 transgenic parasites to serum from mice unexposed to malaria 
parasites (pre) or from mice made hyperimmune to \textit{P. berghei} [??????]

]. The mouse serum was either diluted 1:20, with secondary goat anti-mouse 
Alexa IgG (488 nm) used at 1:500. Samples were analysed using a FACS
Calibur Flow cytometer and Flowjo Software. Data represents the average fluorescence intensity obtained from two independent experiments (labelled A and B), with all samples tested in duplicate.