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The *Plasmodium* serine-type SERA proteases display distinct expression patterns and non-essential *in vivo* roles during life cycle progression of the malaria parasite

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Summary

Parasite proteases play key roles in several fundamental steps of the *Plasmodium* life cycle, including haemoglobin degradation, host cell invasion and parasite egress. *Plasmodium* exit from infected host cells appears to be mediated by a class of papain-like cysteine proteases called ‘serine repeat antigens’ (SERAs). A SERA subfamily, represented by *Plasmodium falciparum* SERA5, contains an atypical active site serine residue instead of a catalytic cysteine. Members of this SERAser subfamily are abundantly expressed in asexual blood stages, rendering them attractive drug and vaccine targets. In this study, we show by antibody localization and *in vivo* fluorescent tagging with the red fluorescent protein mCherry that the two *P. berghei* serine-type family members, *PbSERA1* and *PbSERA2*, display differential expression towards the final stages of merozoite formation. Via targeted gene replacement, we generated single and double gene knockouts of the *P. berghei* SERAser genes. These loss-of-function lines progressed normally through the parasite life cycle, suggesting a specialized, non-vital role for serine-type SERAs *in vivo*. Parasites lacking *PbSERAser* showed increased expression of the cysteine-type *PbSERA3*. Compensatory mechanisms between distinct SERA subfamilies may thus explain the absence of phenotypical defect in SERAser disruptants, and challenge the suitability to develop potent antimalarial drugs based on specific inhibitors of *Plasmodium* serine-type SERAs.

Introduction

Intracellular pathogens have evolved numerous strategies to exit their host cells after completion of replication and growth and depletion of host cell nutrients (Hybiske and Stephens, 2008). Cellular exit is often an active biological process triggered by the pathogen and accompanied by consecutive breaching of the membrane of the parasitophorous vacuole (PV) that harbours the pathogen and the host cell plasma membrane.

*Plasmodium* and other apicomplexan parasites are obligate intracellular pathogens that need to efficiently enter and exit their respective host cells in order to propagate and progress along the life cycle. Studies with broad-spectrum cysteine inhibitors have indicated central roles for proteolytic events during egress of merozoites, the invasive stage of the malarial parasite in the pathogenic red blood cell cycle, out of the PV and the erythrocyte plasma membrane (Salmon et al., 2001; Wickham et al., 2003). *Plasmodium* appears to compartmentalize proteins that function specifically in parasite egress in specialized electron-dense secretory organelles termed ‘exonemes’ (Yeoh et al., 2007). Exonemes contain the subtilisin-like serine protease subtilase 1 (SUB1) that is essential for parasite growth and can proteolytically activate a family of papain-like proteases termed ‘serine-repeat antigens’ (SERAs), which in turn may mediate parasite egress through subsequent processing of cellular substrates. Therefore, exoneme discharge may trigger a proteolytic cascade that ultimately leads to cytolysis and parasite exit (Yeoh et al., 2007). Understanding the cellular roles of SERAs, which constitute major substrates of
SUB1, may ultimately lead to the identification of parasite and/or host cell substrates and the underlying molecular mechanisms of proteolysis. Direct support for the proposed roles for SERAs in parasite egress comes from experimental genetics. Loss of PbSERA5/ECP1 function results in viable and motile sporozoites that are defective in exiting the midgut oocyst in the insect vector (Aly and Matuschewski, 2005). Remarkably, members of the SERA multigene family appear to have arisen from multiple gene duplication events. In P. falciparum eight out of nine SERAs are located in tandem on chromosome 2 (Aoki et al., 2002; Miller et al., 2002). Similarly, in the rodent malaria model parasite P. berghei the five SERAs are tandemly arranged in a head-to-tail fashion on chromosome 3 (Kooij et al., 2005).

This gene organization is evolutionary conserved and is a hallmark of the SERA multigene family (Arisue et al., 2007; McCoubrie et al., 2007).

All Plasmodium SERAs contain a central, papain-like protease domain and numerous cysteine residues. Intriguingly, this class of proteins appears to be absent in a number of related apicomplexan parasites, such as Toxoplasma gondii or Cryptosporidium parvum, suggesting that their respective roles are restricted to malaria parasites. Despite their overall sequence similarity in their central protease domain, Plasmodium SERA proteins can be classified into four major groups that form two distinct phylogenetic clusters (Hodder et al., 2003; Kooij et al., 2005; Arisue et al., 2007; McCoubrie et al., 2007). The active site cysteine SERAs (SERAcyss) form three separate groups within one cluster, whereas those with an active site serine (SERAser) form a fourth monophyletic group.

The three orthologous SERAcyss groups appear to be well conserved across the genus Plasmodium. Two groups, represented by P. falciparum PISERA6 and PISERA7, respectively, are expressed in asexual parasites, whereas the third and most ancestral group, represented by PISERA8, is not (Aoki et al., 2002; Miller et al., 2002). Targeted gene deletion of the P. berghei orthologue of PISERA8, termed 'egress cysteine protease 1' (ECP1), confirmed a dispensable role in the mammalian host and instead revealed an essential function for sporozoite egress from oocysts in the mosquito vector (Aly and Matuschewski, 2005). By analogy, members of the PISERA6 and PISERA7 groups may function in parasite egress out of mammalian host cells.

In contrast, the cellular roles of SERAser proteins, which together form the most diverse group, remain largely unsolved. The founding member PISERA5 localizes to the PV of mature schizonts (Delplace et al., 1987; Miller et al., 2002). Purified recombinant PISERA5 protein exhibits only limited chymotrypsin-like autoproteolytic activity and cleavage of polypeptide substrates is negligible (Hodder et al., 2003). However, this group stands apart, because (i) expression analysis revealed that SERAser genes, particularly SERA5, are very abundantly expressed in P. falciparum late trophozoites and schizonts, the parasite stages preceding parasite egress from their host erythrocytes (Aoki et al., 2002; Lasonder et al., 2002; Miller et al., 2002), (ii) antibodies against PISERA5 inhibit parasite erythrocytic growth in vitro through agglutination of merozoites and ruptured schizonts (Pang et al., 1999), (iii) infected individuals in malaria-endemic areas exhibit high antibody titres against SERAser proteins, and most prominently SERA5 (Okech et al., 2001; Aoki et al., 2002; Okech et al., 2006), and (iv) high anti-PISERA5 antibody titres correlate with protection against severe disease (Okech et al., 2006). In a monkey model immunization with a purified recombinant PISERA5 fragment induces protection against challenge infection (Inselburg et al., 1991). Therefore, SERAser proteins represent the most promising group of all SERAs for potential therapeutic and vaccine targets.

In this study, we investigated the cellular roles of SERAser by experimental genetics in the model rodent malaria parasite P. berghei. Unexpectedly, we could exclude an essential role for all SERAser during the P. berghei life cycle. Our data suggest that this monophyletic SERA group evolved in the absence of vital roles for the parasite.

### Results

Plasmodium berghei serine-type SERA proteases

The model rodent malaria parasite P. berghei encodes two members of the SERAser subfamily, PbSERA1 and PbSERA2 (Aly and Matuschewski, 2005; Kooij et al., 2005).

Direct sequencing of cDNA from asynchronous blood stages permitted identification of the complete coding sequences (GenBank accession numbers: EU917224 and EU917225 for PbSERA1 and PbSERA2 respectively). Comparison of the P. berghei orthologues with the founding member PISERA5 (PFB0340c) illustrates the overall amino acid sequence similarity (Fig. 1A) of ~35% to the human malaria protein. A hallmark of this subfamily is the replacement of the catalytically active cysteine residue by a corresponding serine residue (Fig. 1B). The other amino acids of the catalytic centre, i.e. an amineterminal glutamine and a carboxyterminal asparagine, are well conserved, apart from a histidine residue, which is changed to a methionine residue in the homologues of rodent malaria parasites (Hodder et al., 2003; Arisue et al., 2007; McCoubrie et al., 2007).

In order to initiate a genetic characterization of the SERAser subfamily, we profiled their expression by
RT-PCR analysis (Fig. 2A). In good agreement with *P. falciparum* expression data (Aoki et al., 2002; Miller et al., 2002), both transcripts are readily detectable in blood stage merozoites. Both transcripts are absent during sporozoite maturation and expression commences again during liver stage development. Both *PbSERA1* and *PbSERA2* appear to be abundantly expressed late in liver stage development (Fig. 2A), as observed previously (Schmidt-Christensen et al., 2008). Together, these data suggest that the two SERAser genes are expressed during formation of liver stage and blood stage merozoites.

We further quantified the relative transcript abundance for the five *P. berghei* SERA genes using real-time RT-PCR (Fig. 2B). The two SERAser genes, *PbSERA1* and *PbSERA2*, were the most abundantly expressed in blood stages. In late liver stages, *PbSERA1*, *PbSERA2* and *PbSERA3* were expressed at a similar level, whereas *PbSERA4* transcripts were less abundant. As expected (Aly and Matuschewski, 2005), *PbSERA5* was not expressed in liver or blood stages (Fig. 2B). These data confirm that the SERAser genes (and particularly *PbSERA2*) are the SERAs most prominently expressed in blood stages, consistent with *P. falciparum* expression data (McCoubrie et al., 2007).

**Cellular localization of PbSERA1 and PbSERA2**

We next investigated the localization and the expression timing of *PbSERA1* and *PbSERA2*. For this purpose we generated parasite lines expressing the endogenous SERA1 and SERA2 proteins fused to the mCherry red fluorescent protein (Shaner et al., 2004). This was achieved by transfection of targeting vectors that contain an amino-terminally truncated *PbSERA1* or *PbSERA2* copy and in-frame fusion of the mCherry coding region, followed by the DHFR/TS 3′ untranslated region (Fig. 3A). Upon a single cross-over event, integration of these constructs is predicted to result in an allelic duplication, resulting in a mCherry-tagged full-length copy and a non-transcribed 5′ truncated version of the *PbSERA1* or *PbSERA2* gene.

Transfection was performed in *P. berghei* ANKA parasites expressing GFP (Janse et al., 2006), leading to green fluorescent parasites that express a red fluorescent SERA1 or SERA2 protein. Genotyping by PCR using specific primer combinations confirmed the desired integration events (Fig. S1).

We first performed live cell imaging of blood stages of the transgenic *PbSERA1/mCherry* and *PbSERA2/mCherry* parasites. Similarly to SERA5 in *P. falciparum* (Delplace et al., 1987), both *PbSERA1/mCherry* and *PbSERA2/mCherry* were detected in late schizonts, but not in early blood stages (Fig. 3B). *PbSERA1/mCherry* and *PbSERA2/mCherry* parasites developed normal asexual and sexual blood stages, and could be transmitted to *Anopheles* mosquitoes, resulting in the formation of sporozoites (data not shown). In good agreement with our transcription analysis, *PbSERA1/mCherry* and *PbSERA2/mCherry* were barely detectable during the mosquito stages (data not shown).
We next investigated expression of mCherry-tagged SERAs during liver stage development in vitro. As observed with blood stages, the fusion proteins were not detected in early liver stages, but were abundantly expressed in late liver stages (Fig. 3C and D). Remarkably, PbSERA1/mCherry and PbSERA2/mCherry showed clearly distinct expression patterns in liver stages. PbSERA1/mCherry was detected in mid and late liver stages, and localized predominantly to the PV, which constitutes the parasite/host interface (Fig. 3C). In contrast, PbSERA2/mCherry became detectable only at the end of liver stage development, with an intracellular distribution in the parasite (Fig. 3D). Interestingly, PbSERA1/mCherry was not detected in merosomes, in contrast to PbSERA2/mCherry, which gave a strong signal associated with individual merozoites inside merosomes (Fig. 3E). Collectively, these data indicate that both PbSERA1 and PbSERA2 are expressed in late blood and liver stages. Importantly, the two proteins apparently distribute to distinct compartments, and only PbSERA2 remains associated with merozoites after PV membrane (PVM) rupture.

To get further insights into the distribution of PbSERAser proteins, we performed immunofluorescence analysis of late liver stages, using antibodies generated against the C-terminus of PbSERA1 (anti-SERA1C) or the central domain of PbSERA2 (anti-SERA2M) respectively (Fig. 1A). In late liver stages,

Fig. 2. Expression profiling of P. berghei SERAser.
A. RT-PCR analysis of SERA1 and SERA2 expression in mosquito midgut (mg) and salivary gland (sg) sporozoites, mid (24 h) and late (48 h, 72 h) liver stages, liver stage merosomes (mer) and blood stage merozoites (BS mer). The merozoite and sporozoite-specific transcripts, MSP1 and TRAP, and the constitutive GAPDH transcript were added as controls. cDNAs were synthesized from mRNA in the presence (+) or absence (-) of reverse transcriptase. Genomic DNA (gDNA) was added as an amplification control.
B. Quantitative RT-PCR analysis of P. berghei SERA gene expression in WT mixed blood stages (BS), purified schizonts (late BS) or infected HuH7 cell cultures 65 h post-infection (late LS). Relative gene expression was normalized to MSP1 expression level, and is shown as the mean of two independent experiments (±SD).
staining with anti-SERA1C antibodies was mostly restricted to the PVM, as shown by colocalization with the PVM marker exported protein 1 (EXP1) (Fig. 4A, upper panels). This pattern was also observed at more advanced stages of development, in cytomeres and fully differentiated merozoite-containing parasites (Fig. 4A, middle and lower panels). This distribution is reminiscent of the fluorescence pattern observed in parasites harbouring a mCherry tag at the C-terminus of \( \textbf{PbSER} \text{A} \) (Fig. 3C). In contrast, anti-SERA2M antibodies showed a more complex distribution in late liver schizonts and cytomeres, staining both the PVM and more internal structures (Fig. 4B, upper and middle panels).

Interestingly, in terminal liver stages, just prior to the release of merozoites, SERA2M was detected at the periphery of the parasites, in the PV and/or PVM compartment, as well as the host cell cytoplasm (Fig. 4B, lower panels). This distribution differs from that of the C-terminal fragment of SERA2, based on the fluorescence pattern in \( \textbf{PbSER} \text{A}2 \) parasites (Fig. 3D). Together, these results strongly suggest that SERA2 is processed towards the end of liver stage development, with the central putative papain-like domain being released in the vacuolar space, while the C-terminal domain (visualized by the mCherry tag) remains associated with the merozoites. In good agreement with this hypothesis, Western blot analysis of purified blood stage schizonts demonstrated that SERA2 processed forms were enriched in saponin extracts, as compared with Triton X-100 (Fig. S2), consistent with localization to the PV compartment of cleaved products that contain the central domain recognized by the anti-PbSER2M antibodies.

**Generation of \( \textbf{PbSER} \text{A}1 \) and \( \textbf{PbSER} \text{A}2 \) knockout parasites**

We next wanted to study the cellular functions of \( \textbf{PbSER} \text{A}1 \) and \( \textbf{PbSER} \text{A}2 \) in the \textit{Plasmodium} life cycle. Based on the refractoriness to gene knockout of \( \textbf{PfSER} \text{A}5 \) (McCoubrie \textit{et al.}, 2007), we expected that at least one member of the \( \textit{P. berghei} \) \( \textbf{SER} \text{Aser} \) family plays a vital role during asexual growth and therefore cannot be targeted by classical reverse genetics. We generated replacement vectors that were designed to disrupt the corresponding gene.
sequent cloning of single parasites resulted in multiple clonal sera1(−) and sera2(−) parasite lines. Genotyping by PCR using specific primer combinations confirmed the expected recombination events in sera1(−) and sera2(−) parasite lines (Fig. 5C), and RT-PCR demonstrated the complete absence of SERA1 transcripts and SERA2 transcripts in sera1(−) and sera2(−) blood stage parasites respectively (Fig. 5D). Furthermore, Western blot analysis of purified blood schizonts demonstrated the absence of PbSERA1 and PbSERA2 proteins in sera1(−) and sera2(−) blood stage parasites respectively (Fig. 6). Collectively, these results confirm the successful disruption of the corresponding gene.

PbSERA1 and PbSERA2 are dispensable during the parasite life cycle

We then analysed the phenotypes of sera1(−) and sera2(−) parasites. The successful disruption of SERA1 and SERA2 genes in P. berghei blood stages indicated that both are dispensable during blood stage multiplication of the parasite. Both sera1(−) and sera2(−) parasites produced gametocytes and exflagellation of male gametocytes was similar to WT parasites (data not shown). Transmission to Anopheles stephensi mosquitoes and oocyst development were also normal when compared with WT parasites (Table 1). Both sera1(−) and sera2(−) oocysts produced sporozoites, which invaded mosquito salivary glands as efficiently as WT parasites (Table 1). These findings demonstrate that SERA1 and SERA2 are dispensable for the P. berghei life cycle in the mosquito vector, in good agreement with the absence of gene expression in the mosquito stages (Fig. 2A). Sporozoites from sera1(−) and sera2(−) parasites displayed normal gliding motility (Table 1), and were infective to rats (Table 2). Importantly, after intravenous injection of sporozoites or administration through mosquito bites, the natural transmission route, we observed no delay in latency as compared with WT parasites (Table 2). This clearly shows that hepatic merozoites are formed and released normally in sera1(−) and sera2(−) P. berghei parasites. This was confirmed by in vitro experiments.

Fig. 4. Immunofluorescence analysis of PbSERA1 and PbSERA2 in late liver stages. P. berghei infected HepG2 cells were fixed at different time points (48–58 h) post infection and analysed on single cell level using IFA. Developmental stages are indicated on the left. Localization of the C-terminal region of PbSERA1 (A) and the central domain of PbSERA2 (B) were determined using specific rat antibodies (red). As a marker for the PVM, we used chicken anti-EXP1 antibodies (green), and DNA was labelled with DAPI (blue). Scale bar: 5 μm. Note the distribution of SERA1C to the PV compartment in all stages, whereas SERA2M localizes both to the PV and internal parasite structures in schizonts and cytomeres, but only to the PV compartment after merozoite differentiation.

Fig. 5. Targeted gene disruption of the P. berghei SERAser genes.

A and B. Replacement strategy to generate the sera1(−) and sera2(−) parasites (A), and the sera1(−)/2(−) parasites (B). The wild-type (WT) SERAser genomic loci are targeted with KpnI/SacII-linearized replacement plasmids (pREP) containing 5′ and 3′ untranslated regions adjacent to the SERAser open reading frames and the dhfr/ts-positive selectable marker. Upon a double cross-over event the open reading frame is replaced by the selectable marker. Replacement-specific test and WT primer combinations are indicated by arrows and expected fragments as lines.

C. Replacement-specific PCR analysis. The successful replacement event is verified by a primer combination (test) that can only amplify a signal from the REP locus. Absence of the WT signal from sera1(−), sera2(−) and sera1(−)/2(−) parasites confirms the purity of the clonal populations.

D. RT-PCR analysis of SERA1, SERA2 and MSP1 transcripts in WT, sera1(−), sera2(−) and sera1(−)/2(−) blood stage parasites. RT-PCR was performed in the presence (+) or absence (−) of reverse transcriptase (RT). Parasite genomic DNA (gDNA) was included as an amplification control.
which demonstrated that sera1(−) and sera2(−) parasites form exoerythrocytic forms (EEFs) in cultures, in numbers comparable with WT parasites (Table 1). Together, these findings demonstrate that individual SERAser are dispensable during the *P. berghei* life cycle.

P. berghei lacking both SERAser progress normally through the parasite life cycle.

We hypothesized that compensatory mechanisms within the SERAser subfamily may explain the absence of phe-
notypical defect in single gene disruptants. Therefore, we generated parasite lines with a double PbSERA1 and PbSERA2 gene deletion. To this end, we used a replacement vector containing the 5′ region of PbSERA1, the selection cassette and the 3′ region of PbSERA2 (Fig. 5B). Remarkably, after transfection of P. berghei parasites with this targeting construct, we could select and isolate sera1(-)/2(-) parasite populations. Genotyping by PCR using specific primer combinations confirmed the expected recombination events (Fig. 5C). Furthermore, RT-PCR and Western blot analysis demonstrated the complete absence of SERA1 and SERA2 transcripts (Fig. 5D) and proteins (Fig. 6), respectively, in sera1(-)/2(-) blood stage parasites, confirming the simultaneous disruption of both SERA1 and SERA2 genes. As expected, immunofluorescence analysis confirmed the absence of staining of sera1(-)/2(-) late liver stages with anti-SERA1 and anti-SERA2 antibodies, which also demonstrates the specificity of the staining pattern observed in WT parasites with these antibodies (Fig. S3 and Fig. 4).

Table 1. Phenotypic analysis of SERAser(-) mutants.

| Experiment | Parasite population | Infectivity | Mean no. of sporozoites/infected mosquito | Gliding motility | EEF 24 h | EEF 48 h |
|------------|---------------------|-------------|------------------------------------------|-----------------|----------|----------|
|            |                     |             | Midgut | Salivary glands |                         |          |          |
| I          | WT                  | 95%         | 47 000 | 23 500 | ++ | 222 (±16) | 170 (±24) |
|            | sera1(-)            | 90%         | 33 300 | 30 550 | ++ | 200 (±04) | 161 (±17) |
| II         | WT                  | 85%         | 17 500 | 11 200 | ++ | 235 (±19) | 131 (±12) |
|            | sera2(-)            | 65%         | 38 100 | 8 050  | ++ | 113 (±12) | 96 (±15)  |
|            | sera1(-)/2(-)       | 90%         | 34 550 | 16 600 | ++ | 242 (±05) | 112 (±22) |
| III        | WT                  | 85%         | 31 500 | 14 000 | ++ | 237 (±11) | 102 (±08) |
|            | sera1(-)/2(-)       | 75%         | 36 750 | 11 400 | ++ | 229 (±28) | 102 (±19) |

a. Gliding motility was visualized by CSP labelling of salivary gland sporozoites on glass slides, and motile sporozoites were counted using a fluorescence microscope.

++, continuous, multiple trails in more than 30% of sporozoites.
Table 2. Infectivity of SERAser(−) mutants to rats.

| Parasite population | Sporozoite dosea | No. infected animals | No. injected animals | Prepatency (days)b |
|---------------------|------------------|----------------------|----------------------|-------------------|
| WT                  | 10 000           | 8/8                  | 3.3                  |
| Mosquito bite       |                  |                      |                      |
| sera1(−)            | 10 000           | 5/5                  | 3.4                  |
| sera2(−)            | 20 000           | 3/3                  | 3.3                  |
| sera1(−)/2(−)       | 50 000           | 1/1                  | 3.0                  |
| Mosquito bite       | 10 000           | 5/5                  | 3.4                  |

a. Sporozoites were either injected intravenously at the doses indicated or delivered by natural mosquito bite via exposure of anaesthetized SD rats to 5 infected Anopheles mosquitoes.

b. Prepatency is the time until the first detection of an erythrocytic stage parasite in Giemsa-stained thin blood smears after sporozoite infection.

As observed with the single gene mutants, sera1(−)/2(−) displayed no obvious defect during asexual blood stage growth (Fig. 7) and sexual stage differentiation (data not shown). The double disruptants could be transmitted to mosquitoes, where parasite development was not affected (Table 1). sera1(−)/2(−) parasites produced normal numbers of sporozoites (Table 1), which were motile and as infective to rodents as WT parasites (Table 2). As observed with single gene disruptants, sera1(−)/2(−) parasites formed EEFs in vitro, in similar numbers as WT (Table 1). Furthermore, the number of detached infected cells released in culture supernatants was similar between WT (473 ± 200 merozoites per well) and sera1(−)/2(−) parasites (433 ± 250 merozoites per well), and mice injected with 500 WT merozoites (n=3) or sera1(−)/2(−) merozoites (n=3) all developed a patent blood stage infection at day 2 post injection. Together, these data establish that in the absence of SERAser, P. berghei parasites progress normally through their life cycle. In particular, parasites lacking SERA1 and SERA2 display no detectable defect in egress from infected host cells.

P. berghei lacking SERAser display increased expression of the cysteine-type SERA3

Finally, we analysed the impact of SERAser gene disruption on the expression of the cysteine-type SERAs PbSERA3 and PbSERA4. Quantitative PCR on cDNA prepared from purified blood schizonts showed a modest reduction of SERA1 transcript levels in sera2(−) parasites, whereas SERA2 transcript levels were not significantly modified in sera1(−) parasites. In sharp contrast, in both sera1(−) and sera2(−) parasites there was an upregulation of PbSERA3, but not SERA4, expression (Fig. 8A). The increase in PbSERA3 transcript levels was even more pronounced in parasites lacking both SERAser (Fig. 8A). To confirm a compensatory upregulation of PbSERA3 in the SERAser loss-of-function parasite lines we analysed the protein levels in merozoites (Fig. 8B). PbSERA3 was detected mainly in saponin extracts, confirming its localization to the PV compartment in blood stages, as reported previously (Schmidt-Christensen et al., 2008). In good agreement with the transcript profiling we detected an increase in PbSERA3 protein by 2- and 2.5-fold in sera1(−) and sera1(−)/2(−) parasites respectively. These results indicate a potential functional link between distinct SERA subfamilies, and provide a possible explanation for the absence of phenotypical defect in PbSERAser-deficient parasites.

Discussion

The most important finding from our study is the non-essential role of the SERAser subfamily in vivo in the model rodent malaria parasite P. berghei. We successfully deleted the single genes of SERA1, SERA2 and generated a SERA1/2 double knockout. Both single mutants and the double mutant showed no apparent defect at any phase of the *Plasmodium* life cycle under standard conditions. Most importantly, the successful generation of the double mutant demonstrates that redundancy is not an essential feature of the SERAser family in vivo. This finding was unexpected, because SERAser members are abundantly expressed in asexual blood stages (Aoki et al., 2002; Miller et al., 2002) and at least one member

![Fig. 7. Blood stage growth of sera1(−)/2(−) double knockout P. berghei parasites.](image)

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As a loading control an anti-HSP70 antibody was used. Analysed by Western blot using antibodies specific for good agreement, we now show that the rodent malaria parasites do not rely on SERAser genes for parasite life cycle progression. We propose that members of this monophyletic group evolved gradually and independent of strong selection pressure for parasite growth. However, although SERAser do not play any vital function in rodent malaria parasites, we cannot exclude a role of immunological pressure in evolution of this family, including its expansion in the human parasites.

Notably, one important feature distinguishes the *P. falciparum* SERAser proteins from their other relatives (Hodder et al., 2003). In their central domain they retain a histidine residue that is thought to be part of the catalytic triad. This residue is generally substituted by leucine in *P. vivax* and *P. knowlesi* SERAser proteins, except for *P. vivax* SERA2/ *P. knowlesi* SERA5, and by methionine in the rodent *Plasmodium* species. Hence, we currently cannot formally exclude distinct roles of the histidine-containing *P. falciparum* proteins, although their in vitro proteolytic activity is weak (Hodder et al., 2003). This possibility can now be addressed experimentally by generating a trans-species complementation of the *PfSERA5* deletion that consistently appears to be required for *P. falciparum* growth under in vitro culture conditions (Miller et al., 2002; McCoubrie et al., 2007). If complementation with *PbSERA1* or *PbSERA2* rescues the growth defect, an essential role for the histidine residue, and perhaps the *PfSERA5* gene as a whole, can be excluded in vivo, and vice versa. Because of the absence of an apparent phenotype in the *PbSERA1(-)/2(-) double mutant, the reverse experiment, i.e. complementation with *PfSERA5*, would not be informative. Although indirect, such an approach will eventually substantiate an important in vivo role for *PfSERA5*.

Our expression and localization studies with specific antibodies and fluorescently tagged parasite lines show that *PbSERA1* and *PbSERA2* have distinct expression patterns, both in terms of expression timing and subcellular localization. After proteolytic processing, N-terminal and C-terminal parts of SERA proteins remain covalently linked, while the central domain is released (Li et al., 2002). The functions of the SERA N- and C-terminal domains remain unknown. It should be noted that the N- and C-terminal regions of *PbSERA1* and *PbSERA2* show only 34% and 29% amino acid sequence identity, respectively, whereas the central protease-like domain is more conserved (67% identity). Because in our mCherry constructs the fluorescent moiety is fused to the C-terminal end of SERA1 and SERA2, the fusion proteins only allow tracking of the C-terminal moieties of the corresponding SERA, and not the papain-like central domain after proteolytic processing.

Interestingly, by combining C-terminal tagging of *PbSERA2* and antibodies specific for the central domain of *PbSERA2*, we found that the two domains localize to
distinct compartments after formation of liver stage merozoites. While the C-terminal region of SERA2 remains associated with merozoites, the central putative papain-like domain localizes predominantly to the vacuolar compartment. This was also evidenced by differential Western blot analysis of vacuolar versus parasite protein fractions, which showed preferential accumulation of SERA2 processed forms (containing the central domain) in the vacuolar compartment of asexual blood stage parasites. This suggests that PbSERA2 is proteolytically cleaved at the end of *Plasmodium* schizont, the central domain being released in the vacuolar space while the C-terminus remains associated with merozoites.

Differently from *PbSERA1/mCherry*, which was no longer detected after PVM rupture, *PbSERA2/mCherry* was clearly found associated with liver and blood stage merozoites. This is reminiscent of *PfSERA5* localization on the surface of free merozoites after processing (Pang et al., 1999; Okitsu et al., 2007). Although the different expression patterns of *PbSERA1* and *PbSERA2* might be explained by differential stability of their N- and C-terminal domains after proteolytic processing, our observations raise the possibility that different SERAs fulfill different functions. *PbSERA1* and *PbSERA2* are particularly abundant in the final stages of liver and erythrocytic schizont maturation, which is compatible with a potential role in merozoite egress. Based on their differential localization, the two *P. berghei* SERAser may act at different steps of egress. In such a scenario, *PbSERA1*, which localizes predominantly in the PV, could be involved in the rupture of the PVM. The *PbSERA2* C-terminal domain, which remains associated with merozoites after the PVM rupture, may rather play a role during subsequent steps, such as rupture of the host cell membrane or preparation of merozoites for invasion. Nevertheless, such roles, if any, can only be auxiliary, because parasites that lack SERA1 and SERA2 display no defects in egress of liver and blood stage merozoites, or natural malaria transmission in general.

So far, only *PbSERA5/ECP1* (the orthologue of *PfSERA8*) was demonstrated to play a role during parasite egress. Interestingly, *PbSERA5/PfSERA8* stands apart in the SERA family as it is expressed only in the mosquito stages and is the only SERA that apparently lacks a SUB1 cleavage site (Yeoh et al., 2007). In this regard, it is noteworthy that inhibitors of *P. falciparum* SUB1, which cleaves (and presumably activates) SERAs, are more potent inhibitors of merozoite invasion than of merozoite egress (Yeoh et al., 2007). While this observation could be due to different pharmacodynamic properties of the inhibitors, it is also compatible with a predominant role of SERAs during merozoite invasion of erythrocytes rather than parasite egress. The potential use of several invasion pathways may result in compensatory mechanisms explaining why single (and double) knockout parasites display no obvious defect during their progression through the life cycle. Because of the absence of detectable phenotype in parasites lacking SERAser genes, the loss-of-function approach cannot discriminate between a role of SERAser proteins during egress versus merozoite invasion. Gain-of-function approaches may constitute an alternative strategy, although it is probably difficult to overexpress SERAser, which are already highly expressed in normal parasites. Other strategies such as using blocking antibodies may help unraveling the function of the SERAser proteins.

Interestingly, we found that *P. berghei* parasites lacking both SERAser had increased levels of the cysteine-type SERA3, both at transcript and protein levels. Although we cannot formally exclude an effect of the modification in *cis* of the SERA gene locus in the knockout parasite lines, this observation suggests a potential functional link between SERAs belonging to distinct subfamilies. It has been shown before that *P. falciparum* parasites with a deletion of *PfSERA4* gene display increased RNA levels of *PfSERA5*, which is refractory to gene deletion (McCoubrie et al., 2007). Similarly, attempts to knockout *PfSERA3* gene in *P. berghei* remained unsuccessful so far (E.D. Putrianti, F.F. Masduki, and K. Matuschewski, unpubl. data), suggesting that *PbSERA3* may play an essential role during *P. berghei* blood stage infection.

The crystal structure of the central protease-like domain of *PfSERA5* was recently solved, revealing several anomalies in the active site, in addition to the serine substitution (Hodder et al., 2009). These structural features question the role of SERA5 as an actual protease, and may explain the limited proteolytic activity of recombinant *PfSERA5 in vitro* (Hodder et al., 2003). In the absence of a clear proteolytic activity of SERAser proteins under physiological conditions, multiple non-catalytic cellular roles, including regulatory functions, have to be considered. An attractive hypothesis is a potential function in substrate recognition. In analogy to ubiquitin E2-like variants of the ubiquitin/proteasome pathways that lack active site cysteine residues and form heterodimers with E2 enzymes (VanDemark et al., 2001), SERAser, while catalytically inactive on their own, could act in concert with canonical SERAcs proteases and provide crucial substrate binding sites. In such a scenario, the diverse group of abundant and degenerate SERAser proteins would bind and recruit substrates to the proteases, thereby enhancing their overall proteolytic activity in the cell. While not essential, the presence of multiple substrate recognition proteins may greatly enhance the adjustment of the parasite to a changing environment prior to parasite exit of the respective host cell.

Importantly, a non-vital role *in vivo* does not exclude the potential of SERAser members for antimalaria subunit
vaccine development. There is precedence for a non-essential target antigen, namely MSP3 (Mills et al., 2002), which shows similar promising characteristics in a monkey challenge trial and functional assays with sera from immunized individuals (Hisaeda et al., 2002; Druilhe et al., 2005). The choice of candidate antigens for incorporation into a subunit vaccine does not necessitate an essential role in the parasite but should rather be based on functional assays (Matuschewski, 2006). Antibodies against PISERa5 inhibit parasite erythrocytic growth in vitro through agglutination of merozoites and ruptured schizonts (Pang et al., 1999). Similarly to PISERa5, the C-terminal region of PbSERA2 associates with merozoites, and therefore constitutes a potential target for inhibitory antibodies. In this regard, P. berghei mouse infection may thus represent a valuable in vivo model to evaluate antimalarial vaccines targeting SERA antigens. In contrast, target validation by reverse genetics is a prerequisite for preclinical development of tailor-made inhibitors against a target protein. Our results show that parasites grow normally in the absence of any SERAser members and cast profound doubt on the suitability to translate potential PISERa5 or other SERAser inhibitors into potent antimalarial drugs.

Experimental procedures

Experimental animals

Animals were from Charles River Laboratories. All animal work was conducted in accordance with European regulations and approved by the state authorities (Regierungspräsidium Karlsruhe).

Reverse transcriptase PCR

Total RNA was purified from sporozoites, infected HuH7 cells or infected erythrocytes using the RNeasy kit (Qiagen). Reverse transcription was performed using the RETROscript kit (Ambion). cDNA was used as template for PCR amplification with primers specific for P. berghei SERA1 (forward, GTAATGCG ACAAGAAGAACACAG; reverse, CAGATTGATAGCATA AACTCTTGAC), SERA2 (forward, GGTTACCCGATATG GATCAAATATAATGG; reverse, GCCCTGTACGGAAGATG AACCTGGAAG), TRAP (forward, CCCCCGATCATGAACT CTAGAAAG), GAPDH (forward, ATGGCAATAACAAAAAGT GC GAATATTGG; reverse, TGTGGAATAGCATAATACAAAG AC CGG), MSP1 (forward, CTGGGAGGTGGAGGAGGCGCACT; reverse, AGCTACAGAATACACTACAAAT), or PbSERA3 (forward, GGTTAGCTCCGATATGG; reverse, GTGGTAGTTTGGGAG). Real-time qPCR was performed on cDNA preparations from mixed blood stages, purified blood stage schizonts or late liver stages in HuH7 cell cultures, using the ABI 7500 sequence detection system and Power SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer’s instructions. qPCR was performed in triplicates, with 1 cycle of 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s and 60°C for 45 s. Standard curves were generated for all primers using WT cDNA serial dilutions and gave amplification efficiencies of 90–100%. Data were analysed with the SDS 1.3.1 software (Applied Biosystems). Relative transcript abundance was normalized to MSP1 expression. The following primers were used for real-time PCR: SERA1 (forward, CAAATGCGGTAACAGCTAGATTAGTG; reverse, AATGGCATATACGTCCTGGCAGC), SERA2 (forward, ACAACAGGTCACTAGCCACAAAGG; reverse, CCC ACCATTTCTGGAGTTCTCAC), SERA3 (forward, GGTGAT GTTTTAGGGCTCCGATATGG; reverse, GTGTTGATTTTTT GAATTGTGGTGGG), SERA4 (forward, CAATTCCAGAAAA AATAGACATTACC; reverse, TTTAAACCAATCTTTGTACCT CGAAC), SERA5 (forward, CCAACTGGATTGACTTGA CATATAAG; reverse, ATAAAATCTGGTGTTGGAGGAG; reverse, TTTTGGGACAACACGAGG).

Generation of the mCherry-tagged SERA1 and SERA2 parasite lines

For targeted fluorescent tagging of SERA1 and SERA2, an integration vector was generated by amplification of a PCR fragment using P. berghei genomic DNA as template and primers mCherry-SERA1for (5′-ATAAGAATGCGGCGGCGCAGT AAGTTTGGCAGG-3′; NotI site is underlined) and mCherry-SERA1rev (5′-GGGACTAGTCATATAACCAAAGATAGCA ATCGCTCG-3′; Spel site is underlined), or mCherry-SERA2for (5′-ATAAGAATGCGGCGCAGTATGCTCTAGCTCAGCCGCAAG-3′; NotI site is underlined) and mCherry-SERA2rev (5′-TGC TCTAGATACGGCAAAAGTACTTATCCATACC-3′; XbaI site is underlined). Cloning into the P. berghei transfection vector that contained the mCherry sequence and PbDHFR/TS 3′ UTR resulted in plasmids pEDP05 and pEDP06, for tagging of PbSERA1 and PbSERA2 respectively. The targeting plasmids were linearized with HpaI and AarI respectively, and parasite transfections, positive selection, and parasite cloning was performed as described previously (Janse et al., 2006). Integration-specific PCR amplification of the mCherry-tagged SERA1 or SERA2 was generated using specific primer combinations. We obtained one parasite population each that was used for a systematic expression and localization analysis. Expression of the mCherry fusion proteins was analysed through direct detection of the red fluorescence of mCherry by confocal microscopy. Hoechst 33342 (Molecular Probes) was used to stain nuclei. Images were acquired on a Zeiss LSM510 confocal system using the red fluorescence of mCherry by confocal microscopy. Hoechst 33342 (Molecular Probes) was used to stain nuclei. Images were acquired on a Zeiss LSM510 confocal system (Zeiss, Germany) equipped with visible and UV laser lines, and processed with Adobe Photoshop software (Adobe Systems).

Generation of anti-PbSERA1 and anti-SERA2 antibodies

DNA fragments corresponding to the coding sequence of PbSERA1 C-terminal region (Lei et al., 2012) and PbSERA2 central (M) domain (Lys546-Pro714) were amplified from P. berghei cDNA by RT-PCR and cloned into pGEX6P-1 vector (Amersham, Buckinghamshire, England). Recombinant proteins were expressed in Escherichia coli BL21 cells (Stratagene) as glutathione S-transferase (GST) fusion proteins, and purified using
glutathione-agarose as described by the manufacturer (Amer- 
sham Biosciences). Purified proteins were used to immunize 
Lewis rats along with complete Freund adjuvant, followed by 
multiple boosting immunizations. A rat monoclonal antibody 
was generated against P. berghei SERA1-C. To this purpose, B cells 
were isolated from lymph nodes of one rat immunized against 
PbsERA1-C protein and fused to the mouse myeloma cell line 
P3X63.Ag8.653. The positive pools of hybridoma cells reacting 
with SERA1-C were screened by indirect ELISA. Single-cell 
clones were isolated by limited dilution, leading to the isolation 
of clone C65 (SERA1C), which was characterized further and found 
to bind an epitope (D892-EPASISTQ-E901) at the C-terminus of 
PbsERA1.

**Western blot analysis**

Parasite protein extracts were obtained from P. berghei 
Nycodenz-enriched blood stage schizont preparations, after 
lysis in saponin followed by Triton X-100, to differentiate the 
vacuolar compartment from the parasite fraction, as described (Schmidt-Christensen et al., 2008). Proteins were separated on 
10% SDS-PAGE reducing gels and transferred to PVDF mem-
branes (Amersham). Membranes were probed with anti-SERA1 
and anti-SERA2 rat antibodies, anti-SERA3 mouse antibodies 
(Schmidt-Christensen et al., 2008) or anti-HSP70 mouse anti-
bodies (Tsuij et al., 1994). Horseradish peroxidase-conjugated 
 goat anti-rat or anti-mouse antibodies (Sigma) were used for 
detection, and bands were visualized by enhanced chemilumi-
nescence (Amersham).

**Immunofluorescence assay**

For analysis of PbSERAser localization in late liver stages, 
infected HepG2 cells were fixed with 4% paraformaldehyde, per-
meabilized with ice-cold methanol and incubated with primary antibodies against PbsERA1C or PbsERA2M (rat). A chicken 
anti-EXP1 antibody was used to stain the PVM. Bound antibodies 
were detected using anti-rat Alexa Fluor 594- or anti-chicken 
Cy5- conjugated secondary antibodies (Molecular Probes, 
Leiden, the Netherlands). Nuclei were visualized with DAPI 
(Sigma-Aldrich, Germany). Immunofluorescence labelled cells 
were examined by confocal microscopy using the Olympus 
FV1000 (SIM scanner and spectral detection).

**Generation of the sera_{ser} knockout parasite lines**

For targeted replacement of PbSERAs1, a replacement vector 
was generated by amplification of two PCR fragments using P. 
berghei genomic DNA as template and primers SERA1_{forl} 
(5′-GGGTATTGACGAACAAAATAC-3′; KpnI site is underlined) and 
SERA2_{revl} (5′-GGACTAGTGTACCTCTCACC-GGAACCGTTTTG-3′; SacII site is underlined). The targeting vector to 
generate the SERA1/2 double mutant was cloned from the 
5′ flanking region of SERA1 and 3′ flanking region of SERA2 
fragments. Cloning into the P. berghei transfection vector (Thathy 
and Ménard, 2002) resulted in plasmids pEDP01, pEDP02 and 
pEDP03 for pSERA1(-), pSERA2(-) and pSERA1(-)/pSERA2(-) respectively. The targeting plasmids were linearized with 
KpnI/SacII, and parasite transfection, positive selection and para-
site cloning were performed as described previously (Janse 
et al., 2006). Transformations were performed in the P. berghei 
ANKA strain, except for sera2(-) parasites, which were gener-
gated in the P. berghei NK65 strain. Replacement-specific PCR 
amplifications of the corresponding SERA_{ser}(-) loci were generated 
using specific primer combinations. We obtained four, five 
and four independent sera1(-), sera2(-), and sera1(-)/ sera2(-) 
clonal parasite populations, respectively, that were phenotypically 
identical. Detailed analysis was performed with one representative 
clone each.

**Phenotypical analysis during the Plasmodium life cycle**

**in vivo**

Blood stage development was analysed in vivo in asynchronous 
infestations using NMRI mice. Gametocyte differentiation and 
exflagellation of microgametocytes were detected in mice before 
mosquito feedings. Anopheles stephensi mosquito rearing and 
maintenance was carried out under a 14 h light/10 h dark cycle, 
75% humidity and at 28°C or 20°C respectively. Sporozoite popu-
lations were separated and analysed as described previously 
(Vanderberg, 1975). For determination of sporozoite infectivity, 
and numbers of midgut- and salivary gland-associated sporozo-
ites, infected mosquitoes were dissected at days 10, 14 and 17 
after feeding respectively. For determination of the infectivity of 
sporozoites, infected mosquitoes were dissected at day 17 after 
feeding. Sporozoites were liberated from salivary glands and 
injected intravenously at the numbers indicated into young 
Sprague/Dawley (SD) rats. Patency was checked daily by 
Giemsa-stained blood smears.

**In vitro experiments**

For analysis of gliding motility, sporozoites isolated from infected 
mosquito salivary glands were deposited on glass slides coated 
with bovine serum albumin, and incubated at 37°C for 30 min. 
Trails left behind gliding parasites were then visualized using 
anti-CSP antibodies (Potocnjak et al., 1980). For analysis of EEF 
development, we used HuH7 cells cultured in DMEM supple-
mented with 10% FCS and antibiotics. P. berghei sporozoites 
were added in triplicate wells, incubated for 2 h at 37°C, and 
washed off. After 24 or 48 h, EEFs were revealed using primary antibodies against Plasmodium heat shock protein 70 (HSP70) 
(Tsuij et al., 1994).

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**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Genotyping of *PbSERA1/mCherry* (A) and *PbSERA2/mCherry* (B) clonal parasites. Confirmation of the predicted integration events by diagnostic PCR fragments, tests 1–4 (see Fig. 3A). Absence of the WT signal confirmed purity of the clonal parasite lines.

**Fig. S2.** Processed forms of *PtSERA2* localize preferentially to the PV compartment. Purified schizonts from WT *P. berghei* parasites were lysed in saponin (PV fraction), followed by Triton X-100 (parasite fraction). Protein extracts were analysed by Western blot using rat antibodies specific for the central domain of *PtSERA2*. Processed forms of SERA2 enriched in the PV fraction are indicated with an arrowhead, whereas other specific bands are shown with an arrow.

**Fig. S3.** Immunofluorescence analysis confirms the absence of SERA1 and SERA2 expression in *P. berghei sera1(−)/2(−)* parasites. HepG2 cells infected with *P. berghei* WT or *sera1(−)/2(−)* parasites at the cytomere stage were stained with antibodies against PbSERA1 (A) or PbSERA2 (B). PVM was stained with anti-EXP1 antibodies (cyan), and DNA was labelled with DAPI (blue). Scale bar: 5 µm.

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