A Subset of \textit{Plasmodium falciparum} SERA Genes Are Expressed and Appear to Play an Important Role in the Erythrocytic Cycle*

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The \textit{Plasmodium falciparum} serine repeat antigen (SERA) has shown considerable promise as a blood stage vaccine for the control of malaria. A related protein, SERPH, has also been described in \textit{P. falciparum}. Whereas their biological role remains unknown, both proteins posses papain-like protease domains that may provide attractive targets for therapeutic intervention. Genomic sequencing has recently shown that SERA and SERPH are the fifth and sixth genes, respectively, in a cluster of eight \textit{SERA} homologues present on chromosome 2. In this paper, the expression and functional relevance of these eight genes and of a ninth \textit{SERA} homologue found on chromosome 9 were examined in blood stage parasites. Using reverse transcriptase-PCR and microarray approaches, we demonstrate that whereas mRNA to all nine \textit{SERA} genes is synthesized late in the erythrocytic cycle, it is those genes in the central region of the chromosome 2 cluster that are substantially up-regulated at this time. Using antibodies specific to each SERA, it was apparent that SERA4 to -6, and possibly also SERA9, are synthesized in blood stage parasites. The reactivity of antibodies from malaria-immune individuals with the \textit{SERA} recombinant proteins suggested that SERA2 and SERA3 are also expressed at least in some parasite populations. To examine whether \textit{SERA} genes are essential to blood stage growth, each of the eight chromosome 2 \textit{SERA} genes was targeted for disruption. Whereas genes at the periphery of the cluster were mostly dispensable (SERA2 and -3 and SERA7 and -8), those in the central region (SERA4 to -6) could not be disrupted. The inability to disrupt SERA4, -5, and -6 is consistent with their apparent dominant expression and implies an important role for these genes in maintenance of the erythrocytic cycle.

Malaria remains one of the most devastating diseases of mankind. It has been estimated that there are as many as 500 million clinical cases of malaria each year including 1–2 million deaths. The emergence of widespread drug-resistant parasites and insecticide-resistant vectors has intensified the need for novel drug target strategies and an effective vaccine. One molecule that has potential to serve both as a vaccine and a drug target to control \textit{Plasmodium falciparum}, the most important cause of human malaria, is the serine repeat antigen (SERA).1 also known as serine stretch protein (SERP) or P126 (1–3). SERA, which is produced in large amounts late in the blood stage cycle, is the target of protective immune responses and possesses a domain that may function as a protease (4–10).

\textit{P. falciparum} SERA/SERP is synthesized as a 113–126-kDa precursor protein and is localized to the parasitophorous vacuole (1, 2, 11). Synchronous with merozoite release, the precursor is processed into N-terminal 47-kDa, centrally located 56-kDa, and C-terminal 18-kDa fragments. The 47- and 18-kDa fragments remain linked by a disulfide bond(s), whereas the 56-kDa domain undergoes further processing to a 50-kDa species in a manner that is inhibited by leupeptin and E64 (1, 12, 13). The covalently associated N- and C-terminal fragments appear to be associated with the merozoite surface, whereas the central 50- and 6-kDa species are shed upon schizont rupture (14). This central fragment of SERA shows a high degree of homology to the homologous family of cysteine proteases (6, 7); however, it is not yet known if SERA possesses proteolytic activity.

SERA is well recognized as a vaccine candidate antigen (see Ref. 15 for a review). Recombinant proteins derived from the N-terminal 47-kDa domain are highly immunogenic and elicit antibodies that inhibit erythrocyte invasion and parasite replication \textit{in vitro} (14, 16–18). In \textit{vivo} studies using rodent and primate models demonstrated that the 47-kDa domain conferred significant protection from parasite challenge (19–22).

A gene showing strong homology to the \textit{SERA} gene has been identified in \textit{P. falciparum} (23). This gene, termed \textit{SERPH}, encodes a protein that is also localized to the parasitophorous vacuole and that has very similar characteristics to \textit{SERA}, including the presence of a centrally located papain-like protease domain. The likely presence of at least one other \textit{SERA} gene homologue has also been reported (10). Recently, nucleotide sequencing of \textit{P. falciparum} chromosome 2 identified the presence of eight \textit{SERA}-like genes located in a cluster in a central region of the chromosome (24). These \textit{SERA} genes are arranged in a head-to-tail array with \textit{SERA} and \textit{SERPH} the

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1 The abbreviations used are: SERA, serine repeat antigen; SERP, serine stretch protein; RT-PCR, reverse transcriptase-PCR; GST, glutathione S-transferase; KAHRP, knob-associated histidine-rich protein.

2 W. B. Li and T. Horii, submitted for publication.
fifth and sixth genes in the cluster, respectively. A similar cluster of five SERA-like genes has been identified in *Plasmodium vivax* (25), and further homologues have been discovered in the rodent malaria parasite *Plasmodium vinckei* (26). The presence of relatively large SERA multigene families in divergent *Plasmodium* species suggests an important biological role for the proteins encoded by these genes.

Whereas all the SERA proteins are predicted to possess a central domain with strong homology to the papain family of cysteine proteases, some, including SERA itself, were found to contain a highly unusual cysteine to serine substitution in the active site position (6, 7, 24). Furthermore, in certain *P. vivax* and *P. vinckei* SERA homologues, substitutions were observed in other important catalytic site residues, raising the possibility that at least some of these molecules may no longer function as proteases (25). Importantly, however, the protease domain of some SERA homologues, including SERPH, have no such substitutions.

Very little is known about the function of the SERA proteins, and the fundamental reason for the requirement of so many homologues is unknown. In this paper, we analyze the expression of the eight chromosome 2 SERA genes using a combination of RT-PCR, microarray, antigenicity studies and gene “knockout” approaches. We also identify and similarly characterize a ninth SERA homologue present on *P. falciparum* chromosome 9. These studies reveal that genes in the central region of the chromosome 2 cluster, but not those toward the periphery, are expressed in mature blood stage forms and appear to play an important role in maintenance of the erythrocytic cycle.

**EXPERIMENTAL PROCEDURES**

**Parasite Culture and Transfection**—*P. falciparum* parasites were cultured and synchronized as per standard procedures (27, 28). Enriched trophozoite and schizont preparations used in Western blot analysis were obtained by Percoll purification (29). Ring stage parasites (~5% parasitemia) were transfected with 80 μg of purified plasmid DNA (Plasmid Maxi Kit; Qiagen) as previously described (30) except using modified electroporation conditions (31). Parasites were cultured in a 10-cm culture dish for 48 h prior to selection with 2.5 μM WR99210. DNA (Plasmid Maxi Kit; Qiagen) as previously described (30) except using RNeasy minicolumns (Qiagen) according to the manufacturer’s instructions. Total RNA (20 μg) was reverse transcribed overnight using SuperScript II (Invitrogen) according to the manufacturer’s instructions with a modification of the dNTP stock that resulted in final concentrations of 500 μM dATP, dCTP, and dGTP and 250 μM dTTP and 5- (3-aminomethyl)-dUTP (Sigma; catalog no. A0410). The reactive amino group of 5- (3-aminomethyl)-dUTP was used to conjugate 2 μg of the purified cDNA preparations with 100 μg of the fluorescent dye esters, NHS-Cy5 (Amersham Biosciences; catalog no. NAP-0565) and NHS-Cy3 (Amersham Biosciences; catalog no. PA25001). Microarrays were hybridized overnight at 42 °C with 500 ng of each fluorescently labeled probe in 25% formamide, 5 × SSC, 0.1% SDS. Arrays were washed twice at room temperature with 1× SSC, 0.2% SDS, followed by two stringent washes (0.1× SSC) prior to scanning on a ScanArray 4000 (GSI Lumonics). Data was extracted from the raw images using Spot software (4, 5) and filtered in GeneSpring (Silicon Genetics).

**Nucleic Acid Analysis**—Genomic DNA was isolated from mixed trophozoite/schizont stage parasites as described (34). Manipulation of recombinant DNA and analysis of nucleic acids by Southern blot hybridization were carried out using standard procedures (35). DNA was labeled with [α-32P]dATP using the Bresalet DNA labeling kit as per the manufacturer’s instructions. Unincorporated nucleotides were removed using ProbeQuant™ G-50 Micro Columns (Amersham Biosciences). For RT-PCR, *P. falciparum* D10 total DNA was isolated from saponin-lysed (0.15% saponin) infected erythrocytes using the RNasey minikit as per the manufacturer’s instructions (Qiagen). RT-PCR was performed on the extracted RNA using the Superscript First Strand Synthesis System (Invitrogen). SERA genes were amplified from the cDNA with Tq polymerase (Invitrogen) using the following oligonucleotides (restriction sites are underlined): SERA1/for (5′-AACCTCA-GTGTAGATTATTATGTCATCC-3′) and SERA1/rev (5′-CTAACTCAGAAATATTCTCTCC-3′), SERA2/for (5′-GTAATTCGTAT-ACCATTGGTG-3′) and SERA2/rev (5′-CCTCGAGCTATTTCTGCG-3′), SERA3/for (5′-CTCTCGAAGCTTAATCAGATCC-3′) and SERA3/rev (5′-CTCTCGAGCTATTTCTGCG-3′), SERA4/for (5′-ACCTCGAGTATTCATCACTGATAATG-3′) and SERA4/rev (5′-CTCTCGAGCTATTTCTGCG-3′), SERA5/for (5′-GGGCTCGACGTTTGTATATTTTCTCTCC-3′), SERA6/for (5′-GGGCTCGACGTTTGTATATTTTCTCTCC-3′) and SERA6/rev (5′-GTAATTCGTAT-ACCATTGGTG-3′), SERA7/for (5′-TAATGAGATTTTGCTCGG-3′) and SERA7/rev (5′-GGTGAGCAAGGAATTTTCTCTCC-3′), and SERA8/for (5′-GAGTATCCATAGATCTACATTC-3′) and SERA8/rev (5′-CTGC) and SERA9/rev (5′-CTGC).

**DNA Expression and Purification**—The DNA sequences corresponding to the N-terminal regions of SERA1–9 genes were amplified from *P. falciparum* (D10) genomic DNA using the following oligonucleotides (restriction sites are underlined): SERA1N/for (5′-GAGGACGGATCCATGATTTTCTTCTCCGACCTG-3′) and SERA1N/rev (5′-CCTCGAGCTATTTCTGCG-3′) and SERA1N/rev (5′-CCTCGAGCTATTTCTGCG-3′), SERA2N/for (5′-GGTATACATTTCTCTCCGACCTG-3′) and SERA2N/rev (5′-GTAATTCGTAT-ACCATTGGTG-3′), and SERA2N/rev (5′-GGTATACATTTCTCTCCGACCTG-3′). SERA2N/rev (5′-GGTATACATTTCTCTCCGACCTG-3′) and SERA3N/rev (5′-CCTCGAGCTATTTCTGCG-3′), SERA3N/rev (5′-CCTCGAGCTATTTCTGCG-3′) and SERA4N/rev (5′-GAGGACGGATCCATGATTTTCTTCTCCGACCTG-3′), SERA4N/rev (5′-GAGGACGGATCCATGATTTTCTTCTCCGACCTG-3′) and SERA5N/rev (5′-GGGCTCGACGTTTGTATATTTTCTCTCC-3′), SERA5N/rev (5′-GGGCTCGACGTTTGTATATTTTCTCTCC-3′) and SERA6N/rev (5′-GGTGAGCAAGGAATTTTCTCTCC-3′), SERA6N/rev (5′-GGTGAGCAAGGAATTTTCTCTCC-3′) and SERA7N/rev (5′-CCACAATCTGACGTTTATTTTCTCTCCGACCTG-3′), SERA7N/rev (5′-CCACAATCTGACGTTTATTTTCTCTCCGACCTG-3′) and SERA8N/rev (5′-GGTGAGCAAGGAATTTTCTCTCCGACCTG-3′), SERA8N/rev (5′-GGTGAGCAAGGAATTTTCTCTCCGACCTG-3′) and SERA9N/rev (5′-CCACAATCTGACGTTTATTTTCTCTCCGACCTG-3′), and SERA9N/rev (5′-CCACAATCTGACGTTTATTTTCTCTCCGACCTG-3′).

**Protein Expression**—The recombinant proteins were expressed in E. coli and purified as described (36). GST fusion proteins were harvested by suspending bacteria expressing 4 M. J. Buckley (2000) CSIRO Mathematical and Information Services, New South Wales, Australia (available on the World Wide Web at www.cmis.csiro.au/iap/spot.htm).
SERA proteins in 30 ml of Bug Buster™ (Novagen) in the presence of Complete™ protease inhibitor mixture tablets (Roche Molecular Biochemicals) for 30 min followed by sonication. The SERA proteins were purified from the supernatant using glutathione-Sepharose as described by the manufacturer (Amersham Biosciences). GST alone was produced using the pGEX-4T-1 plasmid. The GST fusion proteins and GST alone were dialyzed overnight in PBS before further use.

Generation of SERA Antibodies—To generate SERA antisera, 6-week-old female BALB/c mice and 3-month-old New Zealand White rabbits were immunized with 40 and 150 μg of SERA GST fusion proteins, respectively, in Freund's complete adjuvant. Animals were boosted three times with 35 and 120 μg of protein in Freund's incomplete adjuvant 5 weeks post injection, after which the animals were bled for serum collection. Anti-GST antibodies were removed from rabbit serum using a GST-bound CNBr-activated Sepharose 4B column (Amersham Biosciences) according to the manufacturer's instructions. Anti-SERA IgG fractions were further isolated by applying the rabbit serum to a Protein G-Sepharose column (Amersham Biosciences). Total IgG was eluted from the column with 0.1 M glycine, 0.15 M NaCl (pH 2.6). Eluted fractions (2 ml) were neutralized using 200 μl of 2 M Tris, pH 8.0.

SDS-PAGE and Western Blotting—Parasite proteins obtained from enriched trophozoite and schizont preparations were separated on 6, 12, and 15% SDS-PAGE reducing gels and transferred to nitrocellulose membranes. Membranes were probed with either rabbit anti-SERA1–9 polyclonal antibodies or pooled human sera derived from adults living in Papua New Guinea (denoted PNG-P, PNG-M, or PNG-B) (37). Melbourne serum was collected from nonmalaria exposed individuals currently living in Melbourne, Australia. Horseradish peroxidase-conjugated sheep anti-rabbit or sheep anti-human IgG (Silenus Laboratories) were used for detection, and bands were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences).

RESULTS

Centrally Located SERA Genes Are Transcribed More Strongly than Peripheral SERA Genes in Mature Blood Stages—A search of the P. falciparum data base (available on the World Wide Web at www.PlasmodDB.org) revealed the presence of nine SERA gene homologues, eight in a cluster on chromosome 2 (24) and one on chromosome 9 (Fig. 1). In this figure, these genes are referred to as SERA1–SERA9, with SERA5 and SERA6 being the previously described SERA/SERP and SERPH genes, respectively (3, 38). Ironically, only SERA5 encodes an N-terminal serine stretch from which the family derives its name. All genes are predicted to possess the four-exon structure characteristic of SERA genes in this and other Plasmodium species with the exception of SERA8, which is predicted to possess an additional exon and a premature stop codon. All SERA homologues encode a centrally located “papa-like” protease domain, although in SERA1–SERA5 and in SERA9 the active site cysteine residue is present as a serine. Phylogenetic analysis performed on all known Plasmodium SERA genes reveals that SERA9 is most closely related to the other P. falciparum SERA genes with an “active site” serine 5. The origins and function of this “rouge” SERA gene remain to be determined; however, at present, there are no obvious features that distinguish this gene from SERA1–SERA5.

RT-PCR analysis was used to determine which members of the SERA multigene family are expressed in parasitized erythrocytes. Initially, cDNA was synthesized by reverse transcription of parasite RNA isolated from an asynchronous culture of P. falciparum parasites (D10 line) using a poly(dT) primer. This material was subjected to PCR using primers specific for each SERA gene amplifying across two introns as shown in Fig. 1B. The same primers were also used to amplify the genes from parasite gDNA. Appropriately sized cDNA and gDNA fragments were detected on an ethidium bromide-stained agarose gel (Fig. 1C). Sequencing of these PCR products confirmed the identification of the amplicons and of the intron/exon boundaries. It should be noted that although these fragments were sequenced for correct identification of each gene, rather than to identify polymorphisms, the sequence obtained from the D10 parasite line was almost identical to that published for 3D7 for each gene (24). These results indicate that each of the nine SERA genes is transcribed in blood stage parasites.

In order to investigate the transcriptional regulation of the chromosome 2 SERA genes, parasite RNA isolated from the same number of synchronized ring, trophozoite, and schizont stage parasites (D10 line) was subjected to RT-PCR as described above. Appropriately sized cDNA fragments representing these eight SERA genes were detected in mature parasites but not in early stage parasites (Fig. 2). The presence of SERA5 and SERA6 mRNA in mature blood stage forms of the parasite has previously been described (3, 23, 10), consistent with the above results. Although not strictly quantitative, it appears from this experiment that maximal expression for each of the eight chromosome 2 SERA genes occurs in mature parasites. This is in contrast to the expression pattern for the KAHRP control that is transcribed earlier in the parasite life cycle as expected. Note that since the SERA9 gene was not available.

5 M. Delorenzi and B. S. Crabb, unpublished data.
until relatively recently, it was not included in this experiment. However, in a separate time course experiment, mRNA from SERA9 appeared to be regulated similarly to the other SERAs (data not shown).

To further examine SERA transcription, we used microarray analysis to measure the extent of increase in transcription of SERA mRNA from young blood stage forms (rings) to mature forms (trophozoites/schizonts). By this analysis, only the centrally located SERA genes in chromosome 2 cluster, as well as SERA9, showed a significant increase in expression in mature forms (Fig. 3A). In addition, SERA3 appeared to be substantially more strongly transcribed than other SERA genes. A repeat set of microarray experiments in which the two mRNA samples were labeled with the alternate fluorescent dye revealed almost identical results (Fig. 3B). It should be noted that the microarray datum included here was part of a genome-wide expression analysis that revealed several “hot spots” of mRNA expression increase from young to mature forms.6 On chromosome 2, the SERA and MSP2/4/5 (39) loci were the most obvious in this regard.

Only Proteins Encoded by Centrally Located SERA Genes Are Detected in Blood Stage Parasites—We aimed to generate antibodies specific for each of the SERA proteins. To identify variable regions suitable for recombinant protein expression, we aligned the amino acid sequences of the nine P. falciparum SERAs together with full-length SERA homologues identified in P. vivax (25), P. knowlesi, and several rodent malaria species that we identified by interrogation of Plasmodium sequence data bases (available on the World Wide Web at www. Plasmodb.org). Six highly conserved blocks were identified, five of which shared ~45% amino acid identity across the family (Fig. 4). The remaining region (block 5) encompasses the protease-like domain and is even more conserved showing ~60% identity. In contrast, a number of highly variable regions were identified, the most divergent of which is at the N terminus (Fig. 4, solid line). This region shares less than 10% identity between any two SERA proteins. Moreover, it is universally hydrophilic and, in the case of SERA5, has been shown to be immunogenic (14, 17, 40). Hence, the N-terminal 50–100 residues of each P. falciparum SERA were expressed in E. coli as a GST fusion protein, and antiserum to each was raised in both rabbits and mice.

To investigate the specificity of the rabbit antisera, each was tested for reactivity with a panel of SERA1–SERA9 GST fusion proteins by Western blotting following depletion of GST-reactive antibodies. Antiserum to each immunogen recognized only the respective fusion protein (Fig. 5). Since the expressed regions are mostly overlapping, this result suggests that specific antibodies had been raised to the SERA region of each fusion protein.

These same rabbit antisera were tested for reactivity with D10 parasite proteins extracted from purified schizonts by Western blotting (Fig. 6A). Only anti-SERA4, anti-SERA5, and anti-SERA6 antibodies reacted strongly with proteins in the size range expected for full-length SERA proteins (100–130 kDa). This was also the case when parasite proteins from two other parasite lines, 3D7 and W2mef, were analyzed (data not shown). A similar panel of mouse antisera also reacted strongly to identically migrating parasite proteins in the appropriate lanes (data not shown). In contrast, the strongly reactive species observed in the rabbit anti-SERA7 lane (Fig. 6A) appeared to migrate too slowly for it to represent a SERA protein. More-

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6 R. Good and A. Cowman, unpublished data.
over, this protein was not detected by mouse anti-SERA7 antibodies, nor did it appear to localize to the parasitophorous vacuole by indirect immunofluorescence analysis (data not shown). Most importantly, this large molecular weight species was still detected in a parasite line in which the SERA7 gene was disrupted by gene targeting (see below; data not shown). A relatively weakly reactive species at \(110 \pm 10\) kDa was consistently observed with SERA9 antibodies (Fig. 6A).

It has previously been shown that full-length SERA5 migrates slightly more rapidly that SERA6 in SDS-PAGE gels under reducing conditions (14, 17, 40). This is consistent with the differences in the sizes observed here, although the actual molecular weights of these species appear a little smaller by our analysis. SERA4 protein has not been identified previously, and this protein appeared to migrate similarly to SERA5 at an apparent molecular mass of \(-100\) kDa under reducing conditions (Fig. 6A and B). Western blotting was also performed under nonreducing conditions in order to confirm that these species were indeed derived from different gene products. By this analysis, SERA4, SERA5, and SERA6 species are clearly separated (Fig. 6B). Also apparent under these conditions was the disappearance of more rapidly migrating species observed in the SERA5 and SERA6 lanes under reducing conditions. It is likely that these fragments represent disulfide-bonded processing products of these proteins that are known to accumulate in mature blood stage forms. To determine the subcellular localization of SERA4, indirect immunofluorescence was performed on schizont-stage parasites (D10 line). Both SERA5 and SERA6 are known to localize to the parasitophorous vacuole (1, 2, 23). Double labeling experiments revealed that SERA4 co-localizes with SERA5 (Fig. 7A). Consistent with parasitophorous vacuole staining, fluorescence was limited to the parasite but excluded from the merozoite, giving a “channeled” appearance in segmented schizonts. Furthermore, SERA4 also appeared to co-localize with the membrane-anchored merozoite surface protein 1 (MSP-1) in mature (segmented) schizonts, also consistent with a parasitophorous vacuole location for SERA4 (Fig. 7B). Double labeling performed in parallel except using a prebleed serum in place of the SERA4 antibodies confirmed the specificity of the SERA4 reagent (Fig. 7C). We caution that given that this is the first time the localization of this molecule has been examined, further studies using differ-

Fig. 4. Summary of a pairwise comparison of predicted SERA proteins. Data were derived from an alignment of all the full-length SERA protein sequences found in the malaria PlasmoDB database at the time of writing and included members from *P. falciparum* (eight sequences, with SERA8, a likely pseudogene, being excluded from the analysis), *P. vivax* (five sequences), *P. chabaudi* (one sequence), *P. knowlesi* (five sequences), and *P. yoelii* (four sequences)). The position of the active site cysteineserine is indicated (asterisk), as are the known processing sites in the SERA5 protein (arrows).
ent reagents and approaches (e.g. confocal microscopy and/or electron microscopy) are required to confirm the localization of SERA4.

Antibodies to SERA2–5 Are Elicited in Response to Natural Infection—In order to examine the possibility that other SERA proteins might be expressed in parasite populations in the field, the panel of recombinant SERA proteins were examined for reactivity to three different sets of pooled human antisera by Western blotting (Fig. 8). These sera were derived from adult Papua New Guineans resident in areas of intense *P. falciparum* transmission (37). These individuals are likely to have been infected on numerous occasions with *P. falciparum* and are considered clinically immune to malaria. Recombinant SERA4 was recognized by all three pools of human sera, whereas SERA2, SERA3, and SERA5 fusion proteins were recognized by one or two of the PNG pooled sera. The finding that not all PNG sera recognize the SERA5 recombinant antigen is consistent with a previous study using sera from Ugandan adults living in a holoendemic area (41). In contrast, other recombinant SERA proteins were not recognized by any of the PNG antibodies. As expected, the pooled sera from non-malaria-exposed individuals (Fig. 8, Melb) did not react with any of the recombinant SERA proteins when tested in parallel Western blots (note that PNG-B and non-malaria-exposed pools were tested at the same time under identical conditions). This result provides evidence that SERA2 and SERA3, in addition to SERA4–SERA6, may also be expressed in some *P. falciparum* parasites.

Most Genes Located at the Periphery of the SERA Cluster Are Not Essential to Blood Stage Growth—The expression data described provided evidence for the expression in blood stages of detectable levels of SERA4, SERA5, and SERA6 proteins, but not of other SERAs, in D10 and 3D7 parasite lines. In order to test whether this expression pattern corresponded to an essential role in blood stage growth, each gene in the chromosome 2 SERA locus (SERA1–SERA8) was targeted for genetic disruption by single crossover recombination (30, 42, 43). Note that since the SERA9 gene was identified only relatively recently, it has not yet been targeted for disruption. An internal region of each of the SERA genes 1–8 (~1 kb) was cloned into the transfection vector pH1 (Fig. 9) (44). Each plasmid was transfected into D10 parasites, and drug-resistant populations were derived following transfection. These eight transfected lines containing each of the different plasmids were selected to obtain parasites with integrated forms of the plasmid.

To test whether transfected plasmids had integrated into the corresponding SERA genes on chromosome 2, Southern blot analysis was used to probe restriction endonuclease-digested gDNA from both parental D10 and transfected parasite populations. Filters were probed with DNA fragments encompassing the unique targeting sequence in each plasmid (Figs. 9 and 10). The enzymes used were chosen to reveal a distinct size difference in fragments representing wild-type locus, integrated locus, and episomal plasmid when the blots are hybridized to a targeting sequence probe.

By this analysis, it was evident that all plasmids were transfected correctly and were initially maintained in an episomal form as expected (Fig. 10). Following selection for integration, it was evident that plasmids designed to disrupt SERA2, SERA3, SERA7, and SERA8 had integrated by single crossover homologous recombination into their respective genetic loci (Fig. 10). In these parasite populations, the complete disappearance of the fragment representing the endogenous locus, together with the appearance of fragments representing the targeted locus, confirmed that the vast majority of parasites in these populations possessed correctly integrated forms of the plasmid. It should be noted that plasmids that integrate by single crossover recombination often insert more than one plasmid copy into the locus. If this has occurred, a band corresponding to that expected for the episomal plasmid will be observed in populations that possess completely integrated forms of the plasmid.
The blood stages of these lines (summarized in Table I), is also in 3D7), together with their apparent strong expression in SERA4 have an important function that can be complemented by ex-
cycle or are differentially expressed and encode proteins that
stage growth of D10 parasites. It remains possible, however,
scribed event in this parasite (Fig. 10) (30).

The plasmid targeting elsewhere in the genome seemingly by
unsuccessful (data not shown).

Plasmids designed to disrupt SERA4, SERA5, and SERA6 did not integrate by homologous recombination but remained episomal throughout the selection procedure (Fig. 10). Repe-
tated attempts to disrupt these genes using single crossover
technology, including in the 3D7 parasite line, were similarly unsuccessful (data not shown). SERA1 was only targeted for integration on the one occasion, and this was unsuccessful with the plasmid targeting elsewhere in the genome seemingly by
homologous recombination, a rare although previously de-
scribed event in this parasite (Fig. 10) (30).

These data indicate that individually SERA2, SERA3, SERA7, and SERA8 are not absolutely necessary for blood stage growth of D10 parasites. It remains possible, however, that these genes are important for another stage of the life cycle or are differentially expressed and encode proteins that have an important function that can be complemented by expression of another SERA. In contrast, the inability to disrupt SERA4, SERA5, and SERA6 in the D10 line (and in some cases also in 3D7), together with their apparent strong expression in the blood stages of these lines (summarized in Table I), is consistent with an important role for some or all of these genes during the erythrocytic cycle.

DISCUSSION

Sequencing of the P. falciparum genome has unveiled the presence of numerous genes that show strong homology to well characterized parasite proteins. The related blood stage pro-
tiens SERA and SERPH (termed SERA5 and SERA6 through-
out this study) were described more than 10 years ago, and numerous publications further characterizing these molecules have emerged in the ensuing period. Somewhat surprisingly, it is now apparent that the genome contains at least nine SERA-like genes, eight in a cluster on chromosome 2 and one on chromosome 9 (24) (PlasmoDB data base). Other Plasmodium species also appear to contain relatively large SERA gene fam-
ilies (25, 26). Our interest in the work described here was to begin to explore the biological purpose of the P. falciparum SERA genes by analyzing expression profiles and potential functional redundancy across this family.

Our first observation was that all SERA genes appear to be
expressed and co-regulated at the mRNA level. Both RT-PCR and microarray experiments highlighted the accumulation of mRNA of SERA family members in mature blood stage forms of the parasite. It did not appear, however, that all members were expressed to equal levels. Microarray analysis performed using the 3D7 parasite line revealed very little increase in transcription in mature stages of SERA genes located at the periphery of the chromosome 2 cluster (particularly SERA1, -2, and -8) when comparing mRNA prepared from immature parasites (ring stage) with that from mature forms (trophozoite-schizont stages). In contrast, all other SERA genes showed a significant increase in transcription by this analysis, with SERA5 showing the greatest increase in expression. We note that the absolute values obtained here should be treated with some caution, however, since the technical limitations of microarray (e.g. different target sequences may favor adherence to the glass slide and/or hybridization to the probe) do not always allow for accurate quantitation of transcription increase.

Consistent with the microarray data, we detected some evi-
dence of protein expression in blood stages of the molecules encoded by genes centrally located in the chromosome 2 SERA locus as well as by the SERA9 gene. Species that corresponded to SERA4–SERA6 were easily detected in three laboratory lines tested (D10, 3D7, and W2meF). SERA5 and SERA6 have of course already been described in the literature, but SERA4 has not been detected previously. This protein is synthesized as a 100-kDa protein that co-localizes with SERA5 in the parasi-

tophorous vacuole. Whether it is subjected to complex proteo-

tylic processing events like SERA5 remains to be investigated (13). An ~85-kDa species was detected with rabbit anti-SERA9 antibodies; however, we have not yet confirmed the identity of this polypeptide with independently derived mouse antibodies. Although we did not detect other SERA proteins in parasite extracts with the specific antisera raised for this study, anti-
bodies specific for SERA2 and -3 recombinant proteins were detected in pooled groups of serum from P. falciparum immune individuals. This suggests that these family members may also be expressed in at least some parasite lines. It remains possible that SERA2 and -3 are also expressed in the laboratory lines tested here, but the antibodies raised to the N-terminal fusion proteins do not recognize the corresponding native parasite proteins. Notably, however, the ability to disrupt these genes is consistent with an absence of expression in the D10 laboratory line.

With respect to the targeted gene disruption experiments, a strong correlation was evident between those genes that could be disrupted and those that showed little or no evidence of protein expression in the laboratory line tested (summarized in Table I). SERA2, -3, -7, and -8 were all disrupted by single crossover recombination technology. This process relies on the gene targeting event having little or no effect on blood stage growth, since the methodology depends on parasites possessing integrated forms of the plasmid outgrowing those containing episomally replicating forms (30, 42, 43, 45). Hence, SERA2, -3, -7, and -8 are all either not involved, or play a redundant role, in the erythrocytic cycle.

In contrast, it did not appear possible to disrupt SERA4, -5, and -6 despite repeat attempts in both the D10 and 3D7 para-
site lines. Together with the strong expression of the proteins encoded by these genes, this result implicates an important role for some or all of these genes in blood stage growth in these laboratory parasite lines. We were unable to disrupt the SERA1 gene in the one attempt shown in this paper. Although the possibility remains that SERA1 is playing a role in the erythrocytic cycle, given that SERA1 mRNA showed little in-
crease in expression in mature stages and that SERA1 protein

![Diagram](image-url)
expression was not evident in laboratory or field isolates, we consider this unlikely and suspect that repeat attempts at targeting this gene may be successful.

Taken together, the data presented here suggest that whereas all genes in the chromosome 2 SERA cluster have the capacity to be expressed late in the blood stage cycle, those toward the center (SERA4–SERA6) appear to be important to the replication of the laboratory lines tested. Low or absent expression and/or the ability to disrupt the peripherally located genes in the cluster (SERA1–SERA3 and SERA7 and -8) raises the possibility that some of these may be pseudogenes. This is even more likely in the case of SERA8 that appears to possess a frameshift mutation that induces a premature stop codon (24). As alluded to above, however, it appears that at least some of these “dispensable” genes (most notably SERA2 and -3) may be expressed at a protein level in at least some parasite lines. Another possibility, not addressed here, is that some SERAs are expressed at different stages of the parasite life cycle. Whereas the biological role of the SERA family remains unknown, it is clear from this study that only a subset of the family are necessary to normal blood stage growth of the parasite. It is interesting that this subset includes members that possess catalytic serine (SERA4 and SERA5) and cysteine (SERA6) residues consistent with a (presumably different) role in the erythrocytic cycle for both types of protease. The intriguing possibility remains, however, that there is functional redundancy among the SERAs and that expression profiles may alter in relation to certain stimuli such as immune pressure and/or red blood cell diversity.

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