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Short communication

Plasmodial serine repeat antigen homologues with properties of schizont cysteine proteases[1]

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Proteases appear to be required for critical events in the erythrocytic life cycle of malaria parasites, including the rupture of erythrocytes by mature schizonts and the subsequent invasion of erythrocytes by daughter merozoites [1,2]. This conclusion is supported by studies showing that parasite rupture and invasion of erythrocytes are inhibited by serine and cysteine protease inhibitors [1] and that the proteolytic processing of late schizont-stage proteins is required for the completion of the erythrocytic cycle [3,4]. A number of schizont protease activities have been identified biochemically [2], but limitations on available quantities of protein have made it difficult to definitively characterize these proteases or to ascertain their specific biological roles.

The *Plasmodium falciparum* serine repeat antigen (known as SERA, SERA-1, SERP or P126 [5–7]) is being studied as a potential vaccine component [8]. A number of SERA homologues have been described, namely serine repeat protein homologue (SERPH [9] or SERA-2 [10]) and SERA-3 [10] from *P. falciparum* and five homologues from *Plasmodium vivax* [11]. SERA and SERPH have been localized to the parasitophorous vacuole of mature schizonts [9,12], and SERA fragments are released into the bloodstream near the time of erythrocyte rupture [12]. SERA and its homologues all contain a $\sim$30 kDa 'protease domain' that has similarity in sequence to papain-family cysteine proteases, particularly near highly conserved active site residues [13] (Fig. 1A). Taken together, available data suggest that SERA and SERPH may act as late schizont-

Abbreviations: SERA, serine repeat antigen; SERPH, serine repeat antigen homologue.

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[1] *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™ data base under accession numbers U59860, U59861, U59862 and AF052747.

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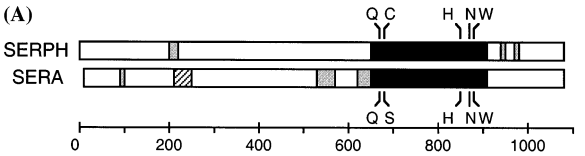
Table 1
Comparison of SERA family protease domains

Protein	Species	Protease domain identity (%)[a]		Papain active site Aas[b]		Identity with highly conserved papain family AAs (%)[c]	Reference
		SERA	SERPH	Cys_{25}	His_{159}		
SERA	*P. falciparum*	100	56.4	Ser	**His**	81.0	[6]
$SERA_{vivax}$-1	*P. vivax*	56.8	56.4	Ser	Leu	66.7	[11]
$SERA_{vivax}$-2	*P. vivax*	56.8	56.0	Ser	**His**	81.0	[11]
$SERA_{vivax}$-3	*P. vivax*	56.0	51.4	Ser	Leu	71.4	[11]
$SERA_{vivax}$-4	*P. vivax*	54.4	52.5	Ser	Leu	71.4	[11]
$SERA_{vivax}$-5	*P. vivax*	57.1	52.1	Ser	Leu	61.9	[11]
$SERA_{vinckei}$-3	*P. vinckei*	53.7	55.2	Ser	Met	71.4	[d]
SERPH	*P. falciparum*	56.4	100	**Cys**	**His**	90.5	[9]
$SERPH_{vivax}$	*P. vivax*	57.1	73.7	**Cys**	**His**	90.5	[d]
$SERA_{vinckei}$-1	*P. vinckei*	52.5	66.8	**Cys**	**His**	85.7	[d]
$SERA_{vinckei}$-2	*P. vinckei*	56.0	67.6	**Cys**	**His**	90.5	[d]

[a] The protease domains were aligned and amino acid (AA) identities with *P. falciparum* SERA and SERPH were calculated.
[b] The amino acid in the position of the active site cysteine$_{25}$ and histidine$_{159}$ of papain is shown for each protein using the standard three letter code. Amino acids that are identical to those in papain are in bold type.
[c] The percentage identities with papain for the most highly conserved 21 amino acids of papain-family proteases [19] are shown.
[d] This publication.

stage proteases required for erythrocyte rupture and/or invasion by malaria parasites.

In this report we compare previously described SERA homologues and additional homologues that we have identified from *P. vivax* and the murine malaria parasite *Plasmodium vinckei*. We used PCR with consensus cysteine protease primers to screen *P. vivax* genomic DNA (kindly provided by D. Kaslow) for papain-family protease genes (see [14] for a description of the primers used). We amplified portions of a gene encoding a typical papain-family protease of *P. vivax* [14] and also a second gene. The DNA encoding this second gene was used to screen an erythrocytic-stage *P. vivax* (Salvador I strain) λZAP cDNA library (kindly provided by M. Kieffer) and isolate a gene encoding a SERA homologue ($SERPH_{vivax}$; GenBank accession number AF052747). We also identified genes encoding three SERA homologues from a *P. vinckei* genomic DNA library [15] ($SERA_{vinckei}$ 1–3; GenBank accession numbers U59860, U59861 and U59862). Our comparison of the protease domains of described SERA homologues (the sequence of the SERA-3 protease domain is not available) identified two subclasses of proteins, represented by *P. falciparum* SERA and SERPH (Table 1). The SERA subclass includes SERA, five previously described *P. vivax* proteins ([11]; $SERA_{vivax}$ 1–5) and $SERA_{vinckei}$-3. These proteins share $\sim$55% identity in their protease domains. They also share papain-family sequence motifs, but, as noted previously for SERA [16,17], the active site cysteine (Cys_{25} in the papain numbering system) is replaced by a serine, and, in most cases, the active site histidine (His_{159}) is replaced by leucine or methionine. The three other papain family active site residues, Gln_{19}, Asn_{175} and Trp_{177} [18], are conserved in SERA and all described SERA homologues. The SERPH subclass is represented by SERPH, the newly identified *P. vivax* homologue $SERPH_{vivax}$, and two *P. vinckei* homologues $SERA_{vinckei}$-1 and $SERA_{vinckei}$-2 (Fig. 1B). Compared to the SERA subclass, SERPH-subclass proteins share greater ($\sim$70%) amino acid identity within the protease domain, and they uniformly have conservation of papain family active site amino acids. Considering the 21 amino acids that are conserved in $\geq$95% of 82 evaluated papain-family proteases [19], identity with papain



(B)

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                 622          D R          Q  CG C                                  C
SERPH_vivax      SKYCNYEYCDRWKDKTSCISNIEVEEQGNCGLCWVFASKLHLETIRCMRGYGHFRSSALYVANCS
                 ||||||||| ||:|||||||:|||||||||||||:|||||| ||||||||||||||||||||||
SERPH            SKYCNYEYCNRWRDKTSCISQIEVEEQGNCGLCWIFASKLHFETIRCMRGYGHFRSSALYVANCS
                 |:||||:|| || ||||||| |:|||||||| |||:|||||| ||||||||:|| | |||||||||
SERA_vinckei-1   SRYCNYDYCHRWQDKTSCISNIDVEEQGNCSLCWLFASKLHLETIRCMRGFGHNRGSALYVANCS
                 ||||| ||| || |||||:|||:||||| | :||:||||||||||||||:|| | |||||||||
SERA_vinckei-2   SRYCNKDYCDRWKDKTSCLSNIEVEEQGECGVCWVFASKLHLETIRCMRGYGHYRSSALYVANCS

                 687   GC  G                          PY        C
SERPH_vivax      KRNPKDICTVGSNPIEFLKIVQDTGFLPLEADHPYLHKNAGNECPAPKENWINLWGNSKLLFHKM
                 || | | |  ||||:|||:|: :  |||||   ||    ||| ||     | |||| :|||| |
SERPH            KRKPIDRCEEGSNPLEFLRILDEKKFLPLESNYPYSYTSAGNSCPKLPNSWTNLWGDTKLLFNKK
                  |     |: ||||:|||:||:  ||||||||:||       :|||  | |||:|| ||||:|
SERA_vinckei-1   ERTADNVCDVGSNPIEFLKILEKNKFLPLESNFPYVWKNVSDTCPKPANNWTNIWGNTKLLYNNM
                 :|    :||:|||||||| ||   |:||||||:||    |  :||   | |||:||  ||||
SERA_vinckei-2   DRNKSEICDIGSNPIEFLQILHDRKYLPLESNYPYAYYRVGGSCPSTKNSWTNLWGKNKLLYFKS

                 752                                                      Y
SERPH_vivax      YG-HFMSYKGFISYESSHFKDNMHIFIDLIKREVQNKGSVIIYIKTKDVIGYDFNGRGVHNMCGDK
                     ::  ||||| |:| || || :|||::|||||||||||||||| |||||||||:||| ||||:
SERPH            VH-RYIGNKGFISHETSYFKNNMDLFIDMVKREVQNKGSVIIYIKTQDVIGYDFNGKGVHSMCGDR
                     :|  :|||   : ||  |||:|||:||||: |||||| |||| ||| |||||: :  :||
SERA_vinckei-1   YG-EFIQHRGFIAYNSRYFAKNMDVFIDIVKREIRNKGSVIAYIKTRDVIEYDFNGRYISNICGHS
                    :|    ||||  |  :   :|  | |:| |:|||||| ||||  ||:|||||: | |:||
SERA_vinckei-2   RSIDFTSSYGFIALSSSNYQDDLDTYIQIIKNEVRNKGSVIIYIKTENVIDYDFNGKIIHNLCGDD

                      ↓                          ↓ ↓
                 817  H     G                   N W    G  G           C
SERPH_vivax      --TPDHAANIVGYGNYINTKGVKRSYWIVRNSWGYYWGNEGTFKVDMLGPKGCKYNFIHTAVVFKV
                   ||||||||:|||||| || |||||::|||| |||| || |:||||||| | |||||| | ||:
SERPH            --TPDHAANIIGYGNYINKKGEKRSYWLIRNSWSYYWGDEGNFRVDMLGPKNCLYNFIHTVVFFKL
                    |||| |||||||||  |||||:|||||||| |||| ||||:|| ||| ||  ||||||: ||:
SERA_vinckei-1   --HPDHAVNIIGYGNYISDKGEKRTYWLIRNSWGYYWGHEGNFKVDALGPDNCFHNFIHTVMVFKI
                    |||  |||||||||||||:||||||||||||| |||||||  ||  |  ||||||: ||:
SERA_vinckei-2   NDDADHAAAIIGYGNYISDKGEKRSYWLIRNSWGYYWGDEGNFKVDMYGPRHCKYNFIHTVVFFKL
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Fig. 1. (A) Diagrammatic alignment of *P. falciparum* SERA and SERPH. The predicted protease domains are black, and the positions of five active site papain family residues are labeled with the single-letter amino acid code. The serine stretch of SERA is cross-hatched and gaps added to provide optimal alignment are stippled. The horizontal bar shows amino acid numbers. (B) Alignment of the protease domains of SERPH and closely related homologues from *P. vivax* ($SERPH_{vivax}$) and *P. vinckei* ($SERA_{vinckei}$-1 and $SERA_{vinckei}$-2). Numbering is for SERPH. The single letter amino acid code is used, and identities (|) and conservative substitutions (:) are labelled. Papain-family active site amino acids [18] are marked by arrows. Amino acids that are conserved between papain and $\geq 95\%$ of 82 evaluated papain family proteases [19] are shown above the aligned sequences, and amino acids that conform to the papain sequence at these sites are underlined.

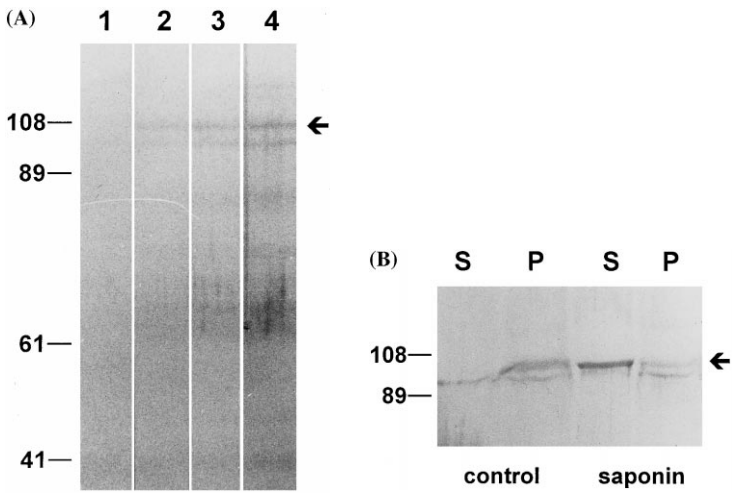

Fig. 2. Characterization of $SERA_{vinckei}$-1. (A) Identification of $SERA_{vinckei}$-1 in schizonts. Blood was collected from *P. vinckei*-infected mice and schizont-infected erythrocytes were isolated with a Percoll gradient [21]. Parasite proteins were separated by SDS-PAGE under reducing conditions and blotted onto an Immobilon-P membrane (Millipore), and immunoblotting was performed with control antisera generated against acrylamide alone (1:500 dilution; lane 1), and with antisera generated against $SERA_{vinckei}$-1 diluted at 1:2000 (lane 2), 1:1000 (lane 3), and 1:500 (lane 4). Membranes were washed, incubated with alkaline phosphatase-conjugated goat anti-mouse serum, washed, and evaluated by colorimetric detection as previously described [20]. For both immunoblots, molecular weight standards (kDa) are labeled, and the arrow identifies the 105 kDa protein recognized by the anti-$SERA_{vinckei}$-1 serum. (B) Localization of $SERA_{vinckei}$-1 to the parasitophorous vacuole. Schizont-infected erythrocytes (10% hematocrit in Hanks' balanced salt solution) were incubated in the absence (control) or presence of 0.05% saponin (5 min, 37°C), which disrupts erythrocyte and parasitophorous vacuole membranes, but not the parasite plasma membrane [22]. After centrifugation (5 min, $13000 \times g$, 37°C) pellets (P) were resuspended in the same volume as the supernatants (S), and both samples were analyzed by immunoblotting with anti-$SERA_{vinckei}$-1 serum at 1:1000 dilution. The localization of $SERA_{vinckei}$-1 to the pellet in control schizonts and the supernatant in saponin-treated parasites indicates that it is located in the parasitophorous vacuole.

is also greater among SERPH-subclass than SERA-subclass proteins (Table 1).

Evaluation of biological features of $SERPH_{vivax}$ has not yet been possible, but the presence of the gene in an erythrocytic-stage cDNA library indicates that it is expressed in asexual parasites. To allow a comparison of known features of SERA and SERPH with those of a *P. vinckei* homologue, we generated antisera against the *E. coli*-expressed protease domain of $SERA_{vinckei}$-1. The protein was expressed in BL21(DE3)pLysS strain *E. coli* and affinity purified as previously described [15]. Purified $SERA_{vinckei}$-1 was then electrophoresed on an SDS-PAGE gel, and CD-1 mice were immunized intraperitoneally with 50–100 μg of the protein in gel slices as previously described [20]. Control mice were immunized in the same fashion with gel slices not containing protein. Mouse sera were collected 5–7 days after the last of three immunizations. Immunoblotting studies showed only low-level reactivity of the antisera with proteins from *P. vinckei* parasites (mostly rings and trophozoites) that were not life cycle stage-selected (not shown). When schizont-stage parasites were purified on Percoll gradients (the upper-most layer on a 62% Percoll gradient contained almost exclusively schizont-infected erythrocytes [21]) and their proteins evaluated by immunoblotting, however, specific reactivity was seen between antisera from each of four immunized mice and *P. vinckei* proteins of M_r 100 and 105 kDa (Fig. 2A). The antisera also reacted with proteins of similar size from *Plasmodium berghei* and *Plasmodium chabaudi* (not shown). Thus, as is the case with SERA and SERPH, $SERA_{vinckei}$-1 appears to be expressed specifically in schizont-stage parasites as a protein of >100 kDa. Anti-$SERA_{vinckei}$-1 serum may have recognized a dimer

rather than a single protein band due to cross-reactivity with a related protein (e.g. another SERA homologue) or to partial proteolysis of $SERA_{vinckei}$-1.

To evaluate the intracellular localization of $SERA_{vinckei}$-1, immunoblots containing proteins from saponin-treated *P. vinckei* parasites were evaluated (Fig. 2B). Saponin selectively lyses erythrocyte and parasitophorous vacuole membranes, but not the parasite plasma membrane [22]. Without saponin treatment, $SERA_{vinckei}$-1 localized primarily to pellets containing intact infected erythrocytes. In contrast, when parasites were treated with saponin, $SERA_{vinckei}$-1 localized primarily to a soluble fraction. Thus, disruption of erythrocyte and parasitophorous vacuole membranes liberated $SERA_{vinckei}$-1 from parasites. This result strongly suggests that $SERA_{vinckei}$-1 is localized in the parasitophorous vacuole, as are SERA and SERPH.

Our results show that a set of SERA homologues is present in multiple plasmodial species. The SERA class can be divided into two subclasses. SERA-subclass proteins have similarities with papain family cysteine proteases in a ~ 30 kDa protease domain, but important active site amino acids are not conserved. Thus, SERA may be a unique protease that utilizes a serine molecule for catalysis within the context of a cysteine protease backbone. Alternatively, SERA and closely related proteins may have other functions, perhaps including protease-like activities, such as binding to peptides late in the erythrocytic cycle. The SERPH subclass exhibits a higher degree of sequence conservation in the protease domain, and all papain family active site residues are conserved. The proteins are much less similar in sequence to papain than are typical papain family proteases, however, and the protease domains are located within large proteins that do not show homology with papain family enzymes outside of the protease domain. Nonetheless, the conservation of active site sequences across plasmodial species suggests that this group of proteins has cysteine protease activity. Furthermore, the presence of these proteins in multiple plasmodial species, their stage specific expression in schizonts, and their localization to the parasitophorous vacuole all suggest that they function as proteases with roles in erythrocyte rupture and/or invasion.

Note added in proof

The full sequence of *P. falciparum* chromosome 2, which was recently released, includes consecutive genes encoding SERA, SERPH and six additional homologues. Two homologues share with SERPH the conservation of the papain active site cysteine, and four homologues share with SERA the presence of serine at this location.

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