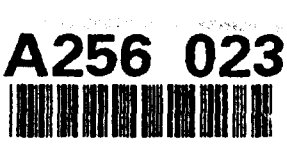

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Characterization of the gene encoding sporozoite surface protein 2, a protective *Plasmodium yoelii* sporozoite antigen

William O. Rogers, Miriam D. Rogers, Richard C. Hedstrom* and Stephen L. Hoffman

Malaria Program, Naval Medical Research Institute, Bethesda, MD, USA

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Sporozoite surface protein 2 (SSP2) is a 140-kDa, protective sporozoite surface protein from *Plasmodium yoelii* distinct from the circumsporozoite protein (CSP). A genomic clone containing the SSP2 gene was isolated and sequenced to determine its size, structural organization and deduced primary amino acid sequence. The coding sequence consists of a single, long open reading frame encoding 826 amino acids. The overall structure of SSP2 is similar to that of the CSP, consisting of a central region of immunogenic amino acid repeats flanked by non-repetitive sequence. SSP2 has one copy of a thrombospondin repeat motif in common with several cell adhesion molecules as well as with the CSP and the thrombospondin related anonymous protein (TRAP) of *P. falciparum*. Additionally, SSP2 shares substantial sequence similarity to TRAP, suggesting that TRAP is the analogue of SSP2 in *P. falciparum*.

Key words: Malaria, *Plasmodium*; Sporozoite; Antigen

Introduction

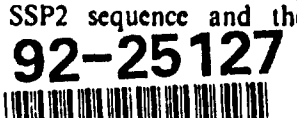
Efforts to develop a pre-erythrocytic stage malaria vaccine have focused almost entirely on the circumsporozoite protein (CSP)[1]. The CSP is the predominant protein on the surface of the infective malaria sporozoite. It is well known that immunization of humans or animals with radiation attenuated sporozoites induces solid sterile immunity to malaria and both humoral and cellular immune responses to the CSP [2-6]. Monoclonal antibodies (mAbs) [7,8,10] and cytotoxic T cells [9] directed against the CSP are protective in passive transfer. Nonetheless, it has not been possible to induce active immunity with recombinant or synthetic vaccines based on the CSP alone [8,11-17] comparable to that achieved by immunization with irradiated sporozoites. We have therefore attempted to identify additional sporozoite surface antigens which might be combined with the CSP in a multicomponent vaccine. We recently described a new sporozoite surface antigen, sporozoite surface protein 2 (SSP2), from *Plasmodium yoelii* [5,18]. Monoclonal antibodies directed against SSP2 recognize a 140-kDa protein in sporozoite extracts. Sequence analysis of a 1.5-kb genomic DNA fragment encoding part of SSP2 revealed an immunogenic series of repeating amino acids and a region of similarity to the region II domain of the CSP [1]. Mice immunized with P815 mouse mastocytoma transfectants expressing the partial SSP2 sequence and the CSP were

Correspondence address: William O. Rogers, Malaria Program Naval Medical Research Institute Bethesda, MD 20889-5055, USA. Tel.: (301) 295-1776; Fax: (301) 295-6171.

Present address. U. S. Naval Medical Research Unit 3, Cairo, Egypt

Note: Nucleotide sequence data reported in this paper have been submitted to the GenBank™ data base with the accession numbers M84732 and M84733.

Abbreviations: CSP, circumsporozoite protein; TRAP, thrombospondin-related anonymous protein; mAb, monoclonal antibody.



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protected against challenge with infective sporozoites [19]. We report here the characterization of a genomic clone containing the complete SSP2 gene.

Materials and Methods

Parasites and DNA isolation. *P. yoelii* 17X (NL) parasites were maintained and DNA isolation performed as described previously [20].

Genomic library construction and screening. A *P. yoelii* genomic library was constructed using 2.0–7.0-kb fragments generated by partial DNAse I digestion as previously described [18]. Purified insert DNA from λgMSY4 was nick translated and used to screen the library under standard high stringency conditions [21]. Five positive clones were isolated, and one, λPySSP2.10 was found to contain a 4.7-kb insert which overlapped both ends of λgMSY4.

DNA sequencing. Phage DNA of λPySSP2.10 was prepared from liquid lysates by standard methods. Insert DNA was released by *Eco*RI digestion and the inserts were cloned into M13mp18 and pUC18. Overlapping clones spanning the insert were generated in pUC18 and M13 using exonuclease III digestion [22]. Single and double stranded templates were sequenced using the dideoxy method [23] and Sequenase (U.S. Biochemical Corp., Cleveland, OH). Sequence analysis was carried out using Genepro 4.2 and DNASIS software.

Results

A λgt11 DNase I genomic library was screened with the 1.5-kb fragment of the SSP2 gene contained in λgMSY4. Five positive clones were obtained. One, λPySSP2.10, contained a 4.7-kb insert which included within it the complete sequence of the λgMSY4 sequence. The 4.7-kb insert was subcloned into pUC18 and M13mp18. Nested deletions were prepared using exonucleaseIII [22] and the complete sequence determined by the Sanger dideoxy method [23].

Fig. 1 shows the sequence of the 4.7-kb insert of λPySSP2.10. A single long open reading frame is present and includes the previously described sequence of λgMSY4 [18]. The AT content of the coding and noncoding regions, 63.2% and 80.7% respectively, are similar to those found in other *Plasmodium* genes [24]. The sequence encodes a polypeptide containing 826 amino acids with a calculated molecular weight of 91 300. Several possibilities may account for the discrepancy between this calculated molecular weight and the observed molecular weight, 140 000 [5,18]. First, the gene might contain additional exons. However, no additional long open reading frames were found either 700 bp 5′ to the initiation codon or 1500 bp 3′ to the first in frame stop codon. No *Plasmodium* consensus intron boundary sequences [24] were found in the flanking regions. A large intron could extend beyond the region we have sequenced, but previously described *Plasmodium* introns have been less than 600 bp long. Second, SSP2 may be a glycoprotein, and indeed, there are several consensus *N*-glycosylation sites in the sequence (Fig. 1). Finally, the protein may migrate anomalously in SDS-PAGE gels, perhaps as a result of its very high proline content.

The deduced amino acid sequence of SSP2 is shown in Fig. 1 and a map of the sequence in Fig. 2. Like a number of other *Plasmodium* surface antigens [24], the deduced amino acid sequence contains tandem repeats of simple amino acid repeats and is particularly rich in proline (18.0%) and asparagine (21.2%). The general structure of SSP2 is similar to that of the CSP (Fig. 2). There is a central region of short, repeated peptide sequences flanked on both sides by non-repetitive sequence. Hydrophobicity analysis [25] identified a putative N-terminal hydrophobic leader [26] as well as putative transmembrane and cytoplasmic domains [27] near the carboxy terminus (Figs. 1 and 2). It is interesting, however, that while SSP2 has both a transmembrane domain and a

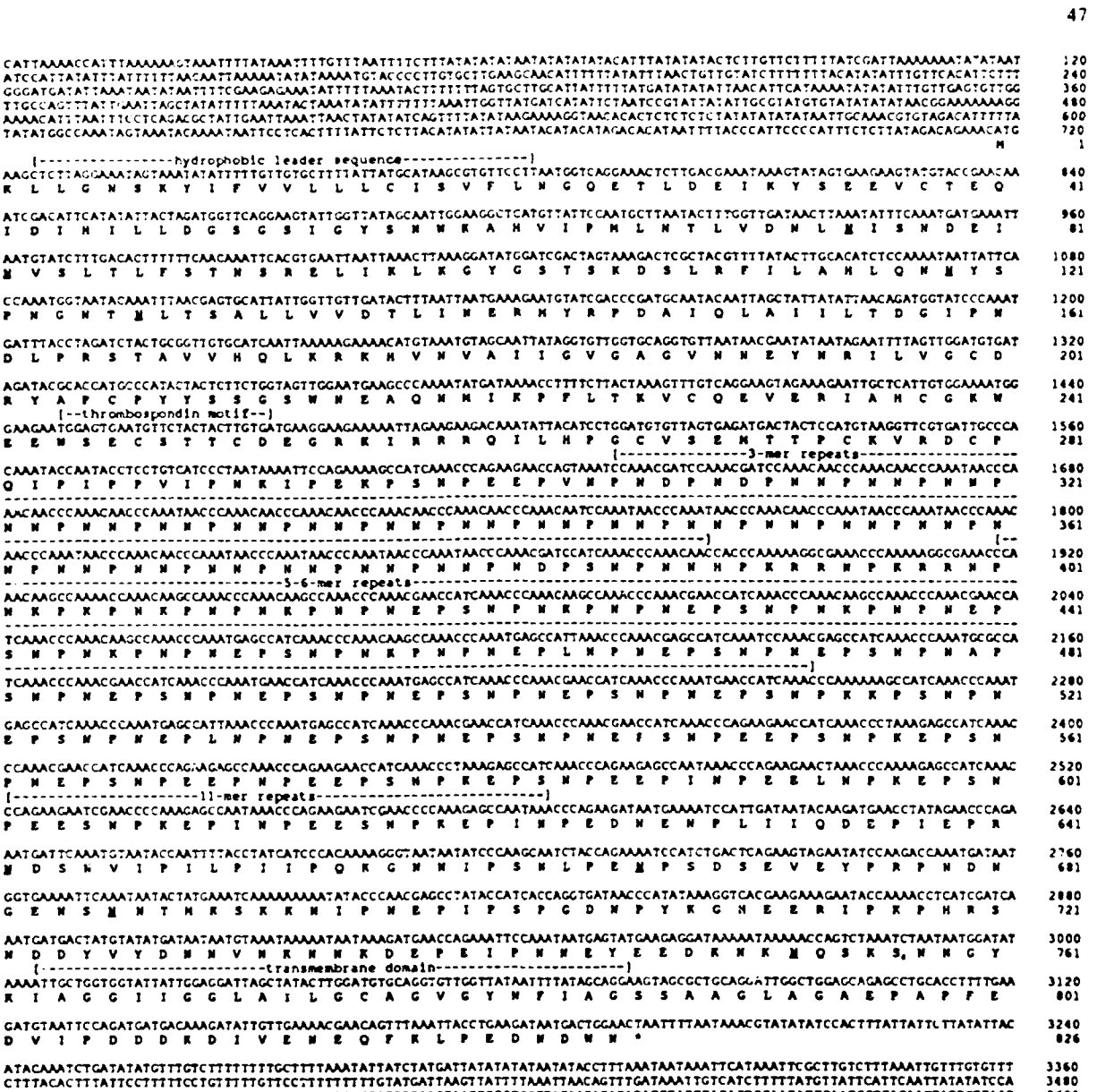

Fig. 1. Sequence of the 4.7-kb insert of λPySSP2.10. The inferred amino acid sequence of SSP2 is shown below the open reading frame. The location of the conserved thrombospondin motif, of the repeated peptide motifs, and of the putative hydrophobic leader and transmembrane domain is indicated. Potential *N*-glycosylation sites are underlined.

cytoplasmic domain, the CSP has only a membrane anchor, suggesting that these 2 proteins interact differently with the membrane.

The arrangement of short amino acid repeats in SSP2 is complex. There is first a region of 23 perfect repeats of the tripeptide PNN, followed by 3 degenerate copies, PND PSN PNN. There

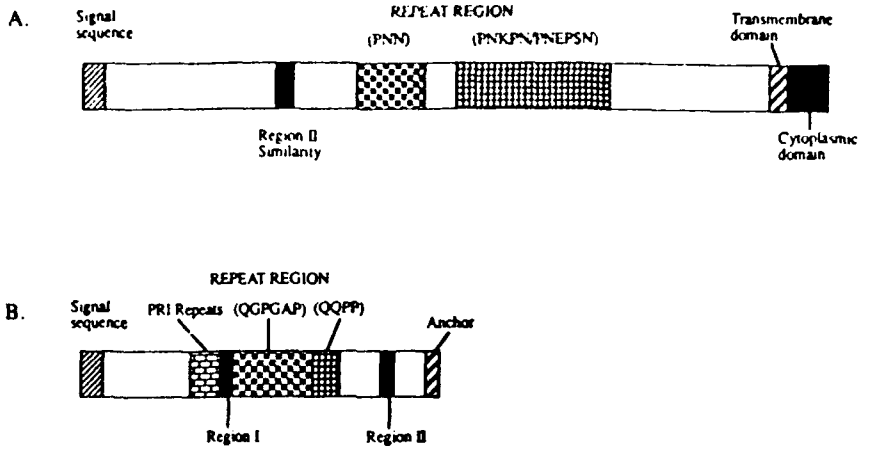

Fig. 2. Structure of SSP2 and CSP. Shown are schematic diagrams of the sequence of SSP2 (A) and the *P. yoelii* CSP (B) [33]. The diagrams are drawn approximately to scale.

is then a short segment of non-repetitive sequence, followed by 3 tandem copies of the pentamer PNKP(K/N), 4 copies of PNKPN alternating with 4 copies of PNEPSN, and 18 tandem degenerate copies of PNEPSN. These tandem repeats are followed by a short region

```
A.

SSP2  MKLLGNSKYIFVVLLLCISVFL-NGQE----TLDEIKYSEEVCTEQIDIHILLDGSGSIG      55
      :  ::: ::. .: :.   .:: :: .      .:::::::::: .:.:. .: : ::::
TRAP  MNHLGNVKYLVIVFLIFFDLFLVNGRDVQNNIVDEIKYSEEVCNDQVDLYLLMDCSGSIR      60

SSP2  YSNWKAHVIPMLNTLVDNLNISNDEINVSLTLFSTNSRELIKLKGYGSTSKDSLRFILAH      115
        ::  : .:    :. .:::.    : . . .:: : .:.:.:   .:  :.    :.
TRAP  RHNWVNHAVPLAMKLIQQLNLNDNATHLYVNVFSNNAKEIIRLHSDASKNKEKALIIIRS      120

SSP2  LQNNYSPNGNTNLTSALLVVDTLINERMYRPDAIQLAIILTDGIPNDLPRSTAVVHQLKR      175
      :     : : :::: ::: :   .:.:  :  : :: .:::::::  .  :       :
TRAP  LLSTNLPYGRTNLTDALLQVRKHLNDRINRENANQLVVILTDGIPDSIQDSLKESRKLSD      180

SSP2  KHVNVAIIGVGAGVNNEYNRILVGCDRY-APCPYYSSGSWNEAQNMIKPFLTKVCQEVER      234
      . : .:. :.: :.:  .:: ::::    . :  :    :    : : ::   :: :::.
TRAP  RGVKIAVFGIGQGINVAFNRFLVGCHPSDGKCNLYADSAWENVKNVIGPFMKAVCVEVEK      240

SSP2  IAHCGKWEEWSECSTTCDEGRKIRRRQILHPGCVSEMTTPCKVRDCP                  281
       : :: :.::: :: ::  : . :.: ::: :: ::    :    ::
TRAP  TASCGVWDEWSPCSVTCGKGTRSRKREILHEGCTSEIQEQCEEERCP                  287


B.

SSP2  SNNGYKIAGGIIGGLAILGCAGVGYNFIAGSSAAGLAGAEPAPFEDVIPDDDKDIVENEQ      816
      : : ::::::: :::::.:.::::.: :.    :   :: :::::.. . ..::::. : ::
TRAP  SDNKYKIAGGIAGGLALLACAGLAYKFVVPGAATPYAG-EPAPFDETLGEEDKDLDEPEQ      550

SSP2  FKLPEDNDWN                                                      826
      :.:::.:.::
TRAP  FRLPEENEWN                                                      560
```

Fig. 3. Alignment of the N-terminal (A) and C-terminal (B) regions of *P. yoelii* SSP2 and *P. falciparum* TRAP. ':' indicates identical amino acids, '.' indicates conservative substitutions. The thrombospondin repeat motif is underlined.

in which short repeat motifs, PEE and PSN, are interspersed with non-repetitive sequence. Finally, there is a tandem duplication of the 11-mer, PEESNPKEPIN. All of the repeat sequences in SSP2 are clearly distinct from the repeats in the *P. yoelii* CSP, QGPGAP and QQPP. The only common feature which the SSP2 repeats share with the CSP repeats is the general structure PXXPXX, which might be expected to impart to the repeat domains of both proteins a structure rich in β-bends [28].

SSP2 shares sequence motifs with several plasmodial proteins and molecules involved in cell adhesion. Thrombospondin, the CSP region II, properdin, the terminal complement components and the thrombospondin related anonymous protein share similarities based around the nonapeptide, WSPCSVTCG [29,30]. This sequence is found in 3 copies in thrombospondin, 6 copies in properdin and one copy in all CS proteins sequenced to date. A similar sequence, underlined in Fig. 1, is also found in SSP2. In SSP2 this thrombospondin motif is found amino terminal to the central repeat region, while the analogous sequence in the CSP is found in Region II, carboxy terminal to the repeats.

The N-terminal and C-terminal regions of SSP2 bear a remarkable similarity to TRAP which extends well beyond the similarity to the thrombospondin motif. Fig. 3A shows an alignment of the N-terminal regions of SSP2 and TRAP. Over a region of 281 amino acids, there is 43% similarity at the amino acid level. Ten of 11 cysteine residues are conserved, the only exception being a single cysteine in the putative hydrophobic leader of SSP2. Fig. 3B show the alignment of the C-terminal regions of the 2 proteins. Over a region of 71 amino acids, there is 56% identity at the amino acid level. SSP2 and TRAP may be members of a protein family involved in interaction between the sporozoite and erythrocytic stages of *Plasmodium* and the cells of the host.

Discussion

SSP2 is a new, non-CSP, 140-kDa sporozoite surface antigen from *P. yoelii* [18]. We have recently observed that immunization of mice with a combination of P815 mouse mastocytoma transfectants expressing the CSP and the original 1.5-kb fragment of SSP2 [18] are protected against challenge with infective *P. yoelii* sporozoites [19]. SSP2 and its presumed homologs in the human *Plasmodium* species are therefore important vaccine candidates. We have here reported the complete sequence of the *P. yoelii* SSP2 gene.

The deduced amino acid sequence of SSP2 shares a number of characteristics with other *Plasmodium* surface antigens in general and with the CS protein in particular. First, it is characterized by a central repeat region consisting of tandem repeats of several different short peptide sequences. As in the case of the CSP repeats from many *Plasmodium* species, the amino acids used in the SSP2 repeats are chosen from a restricted set of amino acids (P,N,E,Q,G,D,A,R,V for CSP repeats and P,N,E,K,S,I for the SSP2 repeats). As in the *P. yoelii* CSP, there are several different repeat units in the repeat region, however, the organization of the repeats in SSP2 is somewhat more complex than that in the CSP. There are 2 major repeat regions, one consisting of tandem repeats of the tripeptide PNN, the second consisting of 2 basic repeat units, PNKPN and PNEPSN. The units are intercalated in the general structure AAAABABABABBBB, where A = PNKPN and B = PNEPSN. This organization could have arisen from an ancestral 11-mer repeat unit AB = PNKPNPNEPSN by duplication of the component 5- and 6-mers at the amino and carboxy terminal ends of an ancestral 11-mer repeat region. One would expect that over the course of evolutionary time the central, alternating AB repeats could be eliminated by homologous recombination, resulting in a simpler AAAAABBBBBB organization, which is in fact observed in a number of CSP repeat regions [31–33]. The SSP2 repeat region may, therefore, represent an intermediate step in a general mechanism in *Plasmodium* antigen genes by which an ancestral tandem duplication of a relatively long sequence evolves into a

repeat region characterized by tandem repeats of 2 or more different short peptide sequences.

Sporozoites which have been inoculated into the mammalian host progress rapidly from the circulation to infect hepatocytes. It is likely that rapid homing to the liver requires specific cell-cell interaction between the sporozoite and hepatocytes, Kupffer cells, or endothelial cells in the hepatic circulation. It is thus interesting to find that SSP2 shares a sequence with the cell adhesion molecules, thrombospondin, properdin, and the terminal complement components, as well as with the CSP Region II and another *Plasmodium* antigen, thrombospondin related anonymous protein (TRAP). These thrombospondin motifs are centered on the nonapeptide WSPCSVTCG, and are found in 3 copies in thrombospondin, 6 copies in properdin, and one copy in all CS proteins sequenced to date [29,30]. SSP2 may also have a role in cell-cell interactions between the sporozoite and the mammalian host.

SSP2 bears a striking similarity to TRAP which extends considerably beyond the thrombospondin repeat motif. The first 281 amino acids of SSP2 and TRAP have a 43% similarity at the amino acid level. Ten of 11 cysteines in the amino terminal sequence of SSP2 are identically conserved in TRAP, the only exception being a single cysteine in the putative hydrophobic leader of SSP2. A region of 56% identity extending over 71 amino acids is found at the carboxy terminus. The similarity in overall structure, as well as the striking amino acid sequence similarities at the amino and carboxy termini strongly suggest that TRAP is the *P. falciparum* analogue of SSP2. It is interesting that has a large repeat region, while TRAP, an apparently closely related protein, has none. If SSP2 and TRAP are indeed analogous proteins with the same function, the absence of repeats in TRAP may call into question the functional importance of repeats in *Plasmodium* antigens generally.

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