ELSEVIER

Molecular and Biochemical Parasitology 64 (1994) 219–232

MOLECULAR AND BIOCHEMICAL PARASITOLOGY

Cloning and characterization of a novel *Plasmodium falciparum* sporozoite surface antigen, STARP

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Received 22 September 1993; accepted 9 January 1994

Abstract

A novel *Plasmodium falciparum* sporozoite antigen, STARP (Sporozoite Threonine and Asparagine-Rich Protein), detected consistently on the surface of sporozoites obtained from laboratory strains and field isolates, has been identified and cloned, following a systematic approach aimed at isolating novel non-CS sporozoite surface antigens. The 2.0-kb STARP gene has a 5′ miniexon/large central exon structure and contains a complex repetitive region encoding multiple dispersed motifs and tandem 45- and 10-amino acid repeats. In sporozoites, transcription of the STARP gene has been conclusively demonstrated by reverse PCR and Northern blot hybridisation and the 78-kDa protein has been localized by immunofluorescence and immunoelectron microscopy to the sporozoite surface. STARP is also expressed in liver stages, as revealed by immunofluorescence assays using antisera raised either to the central repetitive region or the C-terminal non-repetitive region. Expression is also detected in early ring stages, though not in mature erythrocytic or sexual stages. Identification and elucidation of this novel antigen is a step forward in current efforts aimed at developing an effective preerythrocytic-stage malaria vaccine.

Key words: *Plasmodium falciparum*; Malaria; STARP; Sporozoite surface protein; Stage-specific expression; Vaccine candidate

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Note: Nucleotide sequence data reported in this paper have been submitted to the GenBank™ data base with the accession No. Z26314.

Abbreviations: STARP, sporozoite threonine and asparagine rich protein; CS, circumsporozoite; IFA, immunofluorescence assay; GST, glutathione-*S*-transferase; PCR, polymerase chain reaction; ORF, open reading frame.

SSDI 0166-6851(94)00012-C

1. Introduction

Vaccine development against the preerythrocytic stages of *Plasmodium falciparum* has been guided by the observation that protection against these stages could be induced in humans by immunisation with radiation-attenuated sporozoites (reviewed in [1]). This led to the identification of the immunodominant circumsporozoite (CS) protein [2], whose promise stemmed from results in rodent *Plasmodium* models showing that, under certain conditions, passive transfer of CS-specific monoclonal antibodies and cytotoxic T lymphocytes could confer protection (reviewed in [3]). However, attempts to induce protection in man using *P. falciparum* CS-based vaccines, despite recent improvements in their immunogenicity, have consistently provided only partial success (reviewed in [4–6]). It can be therefore be anticipated that additional antigens play a role in irradiated sporozoite-mediated protection and there is thus a recognisable need for identifying novel preerythrocytic stage antigens so as to investigate their potential ability to induce protection both naturally and artificially.

Initial experiments using human polyclonal and monoclonal antibodies directed to *P. falciparum* pre-erythrocytic stage antigens had previously shown us that multiple non-CS antigens were present on the sporozoite surface [7,8] and/or in liver stages (unpublished results). With the aim of directly identifying these antigens, we screened a λgt11 genomic expression library of *P. falciparum* with sera from 3 missionaries living in hyperendemic areas and under long-term, uninterrupted chloroquine prophylaxis, thus preferentially exposed to those antigens expressed in preerythrocytic stages. In this way, several preerythrocytic stage antigens, including the liver stage antigen LSA-1, have been identified [9,10]. We report here the characterisation of another of these antigens, chosen on the basis of its consistent expression on the surface of sporozoites from a variety of wild and laboratory isolates. We entitle this antigen STARP, for Sporozoite Threonine and Asparagine Rich Protein. As with the other *P. falciparum* sporozoite antigens so far described, namely CS [11,12], TRAP/SSP2 [13,14] and Pfs16 [15,16], expression of this antigen can also be detected during other stages of the life cycle. Indeed, results indicate that the STARP gene is expressed in sporozoites, liver stages and early erythrocytic stages.

2. Materials and methods

Parasites. *P. falciparum* salivary gland sporozoites were obtained from laboratory-maintained strains NF54 and 3D7 (as described in [15]) and wild Thai isolates [8]. *P. yoelii* and *P. berghei* sporozoites were isolated from *Anopheles stephensi* mosquitoes fed on parasitised mice. *P. vivax* sporozoites were obtained from infected *A. dirus* mosquitoes. *P. falciparum* liver schizonts were identified in liver biopsies of a Sapajou monkey (*Cebus apella*) infected with the African field isolate 730XI [17] and a chimpanzee (*Pan troglodytes*) infected with the strain NF54 [18]. Intraerythrocytic parasites were cultivated in vitro according to the method of Trager and Jensen [19]. Gametocytes and gametes were obtained in vitro as previously described [20].

Preparation of nucleic acids. Parasite DNA was purified either from saponin-lysed erythrocyte cultures [21] or directly from whole patient blood obtained from malaria-infected Senegalese individuals (A. Guanziroli, E. Bottius and P. Druilhe, manuscript in preparation). Asexual blood stage RNA was prepared from highly synchronised cultures as described by Chomczynski and Sacchi [22]. mRNA was enriched from RNA preparations of sporozoites, gametocytes or gametes, using oligo $(dT)_{25}$-coated Dynabeads according to the manufacturer's instructions (Dynal, Norway). Construction of a genomic DNA expression library in λgt11 has been described by Guerin-Marchand et al. [9].

DNA sequence analysis. λgt11 + DG21 DNA was prepared from a liquid phage lysate and the gel-purified *Eco*RI insert and internal *Fok*I subfragments subcloned into pUC or M13 vectors. These clones and sub-cloned fragments obtained by the polymerase chain reaction (PCR) were sequenced

from both strands by the dideoxynucleotide chain-termination method, using internal oligomers whenever necessary. Sequencing of cloned PCR fragments was always performed on at least three separate clones. In areas of polynucleotide runs, prone to nucleotide additions or deletions, at least 12 independent clones were sequenced prior to determining the correct sequence. All sequence data presented have been established from the *P. falciparum* clone T9/96.

Polymerase chain reaction and cloning of the amplified fragments. PCR reactions were carried out as described by Saiki [23], using the following primers (+ and − designates coding and noncoding strand respectively; numbers relate to the nucleotide positions indicated in Fig. 3A): 5′1 (+, 735–760); 5′2 (+, 759–791); 5′3 (+, 901–926); 5′4 (+, 982–1010); 5′5 (−, 1011–1034); 5′6 (+, 1122–1146); 5′7 (−, 1183–1208); 3′1 (+, 2383–2408); 3′2 (−, 2383–2408); 3′3 (+, 2430–2455); 3′4 (+, 2476–2499); 3′5 (+, 2611–2632); 3′6 (−, 2695–2724); 3′7 (−, 2728–2753).

To generate additional sequence flanking the DG21 sequence, 2×10^7 phages from the original T9/96 genomic DNA library were subjected to PCR, using a DG21-specific primer and a λgt11 primer (1 μM each) flanking the *Eco*RI cloning site. High molecular weight fragments were gel-purified and subcloned into a pCR (Invitrogen) or pUC vector. Positive clones were selected following hybridisation with an internal oligomer. One round of this approach generated an additional 150–350 bp of sequence. The complete 3′ coding sequence was obtained after two rounds (using internal oligomers 3′3 and 3′5). Verification of this 3′ sequence was performed by amplifying (with oligomers 3′4 and 3′7), subcloning and sequencing this region. In a similar manner, 350 bp of flanking 5′ sequence was also obtained (using the internal oligomer 5′7).

Reverse PCR was performed (on 1 μg of RNA) using either the thermostable rTth reverse transcriptase enzyme (Perkin Elmer Cetus) or a combination of the Superscript reverse transcriptase (BRL) and *Taq* polymerase (Amersham).

Hybridisation analysis. Genomic *P. falciparum* DNA (2 μg) was digested with restriction enzymes and size fractionated on a 0.8% agarose gel. PCR-amplified fragments were size fractionated on a 3% NuSieve GTG agarose gel. Standard Southern hybridisation techniques were employed [24]. Northern blot analysis, of 5–20 μg of each stage-specific RNA preparation, was carried out as previously described [25].

Expression of partial STARP sequences. The DG21 *Eco*RI fragment (bp 1151–2493, see Fig. 2B) was gel-purified from λgt11 + DG21 DNA. The complete 3′ non-repetitive region (bp 2383–2724, referred to as STARP3′) was PCR amplified from T9/96 DNA using primers 3′1 and 3′6 (containing additional 5′ *Bam*HI and *Eco*RI sites respectively). These were ligated into the expression vector pGEX [26], which directs the synthesis of foreign proteins as fusions with the C-terminus of the 26-kDa *Schistosoma japonicum* glutathione-*S*-transferase (GST). Recombinant GST + DG21, GST + STARP3′ and GST proteins were purified by affinity chromatography on glutathione agarose beads (Sigma) as described [26].

Production of antisera. 6–8-week-old female Swiss outbred mice were immunised subcutaneously with 15 μg of purified recombinant proteins, emulsified in complete Freund's adjuvant, and boosted at 4 and 6 weeks using incomplete Freund's adjuvant. Human antibodies from pooled human immune sera were affinity-purified on isopropyl-β-D-thiogalactopyranoside-induced confluent plaque lifts of the λgt11 + DG21 clone or, in the case of control sera, wild-type λgt11, as previously described [27].

Protein gels and immunoblotting. Parasite proteins were extracted in sodium dodecyl sulphate-containing sample buffer, subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis on 7.5% or 10% slab gels under reducing conditions and electroblotted onto nitrocellulose membranes. Immunological screening was essentially carried out as described previously [28], using mouse sera diluted 1/100 and then either a horseradish peroxidase-conjugated goat anti-mouse

IgG (Biosys, France) followed by a chemiluminescent reaction (Luminol kit, Amersham), or alternatively an alkaline phosphatase-conjugated rabbit anti-mouse IgG (Promega).

Immunofluorescent microscopy. Surface immunofluorescence assay (IFA) was performed on wet sporozoite preparations deposited on poly-L-lysine-coated slides [7]. Sporozoites lightly fixed with glutaraldehyde (0.05%, 2 min) were tested in parallel and yielded the same results; this method was used for the Thai isolates. Whilst these techniques have been previously established as only recognizing surface antigens, confirmatory surface IFAs assays were performed using freshly-dissected live sporozoites labeled in suspension. For the liver stages, both Carnoy-fixed material and 5 μm sections from unfixed liquid N_2-preserved material were studied [17]. Positive IFA reactions on hepatic schizonts were verified by subsequent Giemsa staining.

Immunoelectron microscopy. This was carried out as described by Aikawa and Atkinson [29]. Briefly, *P. falciparum*-infected *Anopheles* salivary glands were fixed in 0.1% glutaraldehyde, 0.1% formaldehyde solution and embedded in LR Gold resin (Poly Science Inc, Warrington, PA, USA). Sections were incubated with mouse anti-DG21 antisera and subsequently with goat anti-mouse IgG conjugated to gold particles (15 nm in diameter, Amersham). Sections were fixed in 2% glutaraldehyde solution, stained with uranyl acetate and lead citrate, and examined in a JEOL 100CX electron microscope.

3. Results

Identification and nucleotide sequence of the STARP gene. So as to select novel non-CS sporozoite surface antigens, we screened our 120 *P. falciparum* pre-erythrocytic stage recombinant clones with two human immune sera highly reactive by IFA on sporozoites (end-point titres of 1/1600 and 1/6400) though negative by enzyme-linked immunosorbent assay on the CS repeat antigen R32tet32. From the positive non-CS clones, human antibodies were affinity-purified and tested by surface IFA on sporozoites. This identified 34 clones, whose consistency of expression was further studied by IFA on 10 separate sporozoite isolates and 2 laboratory strains. One clone, named DG21, was selected for further study, since (in contrast to most other clones) all *P. falciparum* sporozoite isolates were positive; furthermore, for each *P. falciparum* isolate (in which one would expect multiple parasite populations) all sporozoites were labeled. Sporozoites of *P. vivax, P. yoelii* and *P. berghei* were negative. DG21 showed no cross-hybridisation or immunological cross-reactivity with any of the other original 119 clones.

The 1.35-kb DG21 insert comprises a single, uninterrupted open reading frame (ORF) which includes the 1.2-kb central repetitive region of the STARP gene. Restriction enzyme analysis indicated that this gene is present in only one copy in the genome (Fig. 1A). Pulse Field Gradient Electrophoresis gels of K1 and FCR-3 parasite DNA were used to locate this gene to chromosome 8, as revealed by hybridisation of a chromosome blot with a DG21 probe (unpublished data) and chromosome 8-specific probes [30]. Low-stringency hybridization of a rodent malaria chromosome blot (*P. berghei, P. yoelii, P. chabaudi, P. vinckei*) with a DG21 probe did not detect any homologous sequences in these species (unpublished data).

To estimate the size of this gene, a Southern blot was made of *P. falciparum* DNA digested by mung bean nuclease which, under appropriate formamide concentrations, liberates either gene-sized fragments or smaller fragments resulting from enzyme cleavage within an intron [31]. Hybridisation of this blot with the DG21 insert detected a band at approximately 2.0 kb under conditions of digestion in 40% formamide and a 1.85-kb band with 45% formamide (Fig. 1B). This data suggested that the DG21 fragment did not cover the entire STARP gene, whose size was estimated to be in the order of 2.0 kb.

The remainder of the gene sequence and flanking regions was obtained by using two approaches. The complete 3′ sequence and part of the 5′ sequence was obtained using a PCR technique (see Materials and Methods). This revealed a long

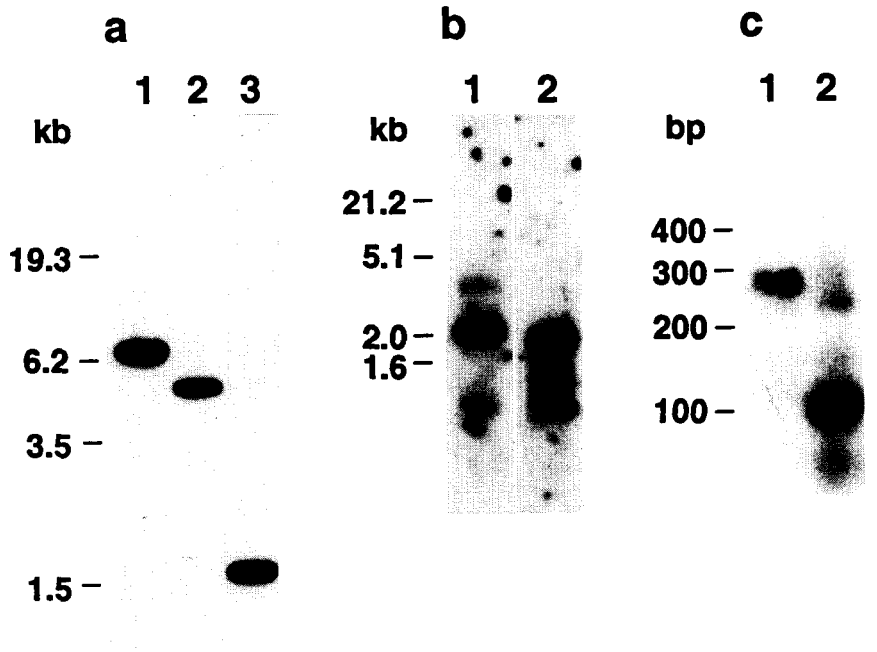

Fig. 1. Restriction and mung bean enzyme and reverse PCR analysis of the STARP gene locus. (A) Southern blot of *P. falciparum* T9/96 DNA digested by *Sca*I (lane 1), *Sca*I/*Xba*I (lane 2) and *Hin*cII (lane 3) and hybridized with a STARP probe amplified using oligomers 5'3 and 5'7 (located as shown in Fig. 2B). (B) NF54 DNA was Mung bean-digested under conditions of 40% (lane 1) and 45% formamide (lane 2) and the resulting blot hybridized with a radiolabeled DG21 probe. These conditions were judged as appropriate since rehybridization of this blot with a CS probe labeled CS gene-sized bands. (C) Reverse PCR was performed on NF54 sporozoite mRNA, using the primers 5'2 and 5'5 specific to coding regions spanning the intron, and the product (lane 2) electrophoresed alongside the product of a control PCR reaction on genomic NF54 DNA (lane 1). After transfer to nylon, the blot was probed with an end-labeled primer 5'4, located internally in exon 2.

open reading frame (bp 982–2724, see Fig. 2A) containing no putative methionine initiation codons and interrupted at the 5' end by a stop codon and a highly AT-rich region, indicating the presence of a 5' intron. The second approach was to subclone a 5' *Hin*cII fragment chosen by restriction map analysis. Sequence of this 1.6-kb fragment revealed the likely presence of a 5' miniexon (bp 735–806), starting from a putative methionine initiation codon. No alternative ORFs that could function as a miniexon were found further upstream. The coding nature of this 5' miniexon and the existence of the intron were confirmed by reverse PCR on sporozoite RNA, using primers derived from exons 1 and 2 (Fig. 1C).

A restriction enzyme cleavage map of the STARP gene locus and the overall organisation of this gene are shown in Fig. 2A and 2B respectively. Confirmation of the two-exon structrure came from analysis of full-length cDNA amplified by reverse PCR on ring-stage RNA (using oligomers 5'1 and 3'6) (unpublished data). Sequence analysis of reverse PCR products generated from both sporozoite and ring-stage RNA showed furthermore, that splicing of this intron (bp 807–981) from RNA transcripts is identical in both of these stages. The 5' and 3' noncoding regions are very A + T-rich (88% and 89% respectively), as is the intron (91%), whilst the A + T content of the coding regions is 77%.

The sequence of the STARP gene and flanking regions, as determined from both strands, is shown in Fig. 3. This sequence predicts a mature polypeptide of 604 residues with a molecular mass of 67.0 kDa, rich in asparagine (25.2%) and threonine (19.3%). Negatively and positively charged

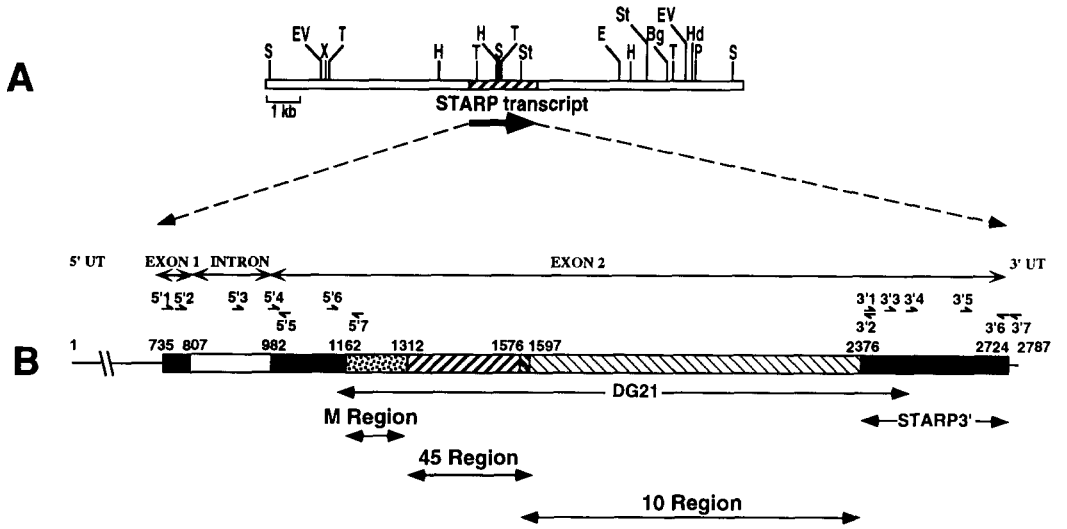

Fig. 2. Restriction enzyme cleavage map and gene structure of the STARP gene locus. (A) Summary of the restriction enzyme cleavage data for the clone T9/96. Bg = *Bgl*II; E = *Eco*RI; EV = *Eco*RV; H = *Hin*cII; Hd = *Hin*dIII; P = *Pst*I; S = *Sca*I; St = *Sty*I; T = *Taq*I; X = *Xba*I. No *Xho*I or *Bam*HI sites are present. This map was made using the enzymes *Sca*I, *Hin*cII and *Sty*I, which cut only once in the gene. (B) STARP gene organization showing the two-exon structure, the location of the oligonucleotide primers used, the starting nucleotide number of each region, the location of the DG21 and STARP3′ fragments and the central repetitive region (comprised of the M, 45 and 10 regions). This is shown as an enlargement of the STARP locus delineated in (A).

residues account for 8.9% and 9.9% respectively. Due to its amino acid composition, this protein contains a very high number of potential N-linked glycosylation (Asn-X-Ser/Thr) sites, 38 in all. The N-terminal amino acid sequence consists of an uncharged and hydrophobic stretch of 13 residues (amino acids 8–20), preceded by a short positively charged region and followed by a charged and polar region. This is characteristic of a signal sequence peptide [32]. Another hydrophobic region is located at the C-terminus, consisting of two short uncharged hydrophobic stretches of 14 and 11 residues (amino acids 569–582 and 593–603 respectively), separated by a rather hydrophilic run of 10 amino acids.

The central region of the molecule is strongly hydrophilic and contains a complex repetitive structure, which can be subdivided into three regions (see Fig. 3B): the 'M' or mosaic region (amino acids 85–134), the '45' region (amino acids 135–229) comprised of 2 repeats of 45 amino acids and the '10' region (amino acids 223–489) comprised of 26 repeats of 10 amino acids, with the latter two regions slightly overlapping. For the 45 region, the two repeats present are perfectly conserved at the amino acid (and nucleotide) level. In contrast, the repeats in the 10 region vary in length (from 9 to 11 amino acids) and are highly degenerate, with 24 of the 26 repeats showing a unique sequence. The single shared repeat sequence (STDNNNTKTI) is found in 3 tandem copies (amino acids 440–470; the residues preferentially conserved throughout the 10 region are underlined). With respect to the M region, this is essentially composed of a series of motifs, short runs of between 9 and 3 residues that are found scattered in this region and elsewhere in the 45 and 10 amino acid repeats. Indeed, only a single run of 7 residues (PESSSTY, amino acids 92–98) is not found repeated elsewhere. Additional motifs are also found shared by the 45 and 10 regions. These dispersed motifs are summarised in Table 1.

The STARP repeat region is polymorphic. As an initial means of investigating whether the central repeat region is polymorphic between strains, this region was amplified from a variety of laboratory-

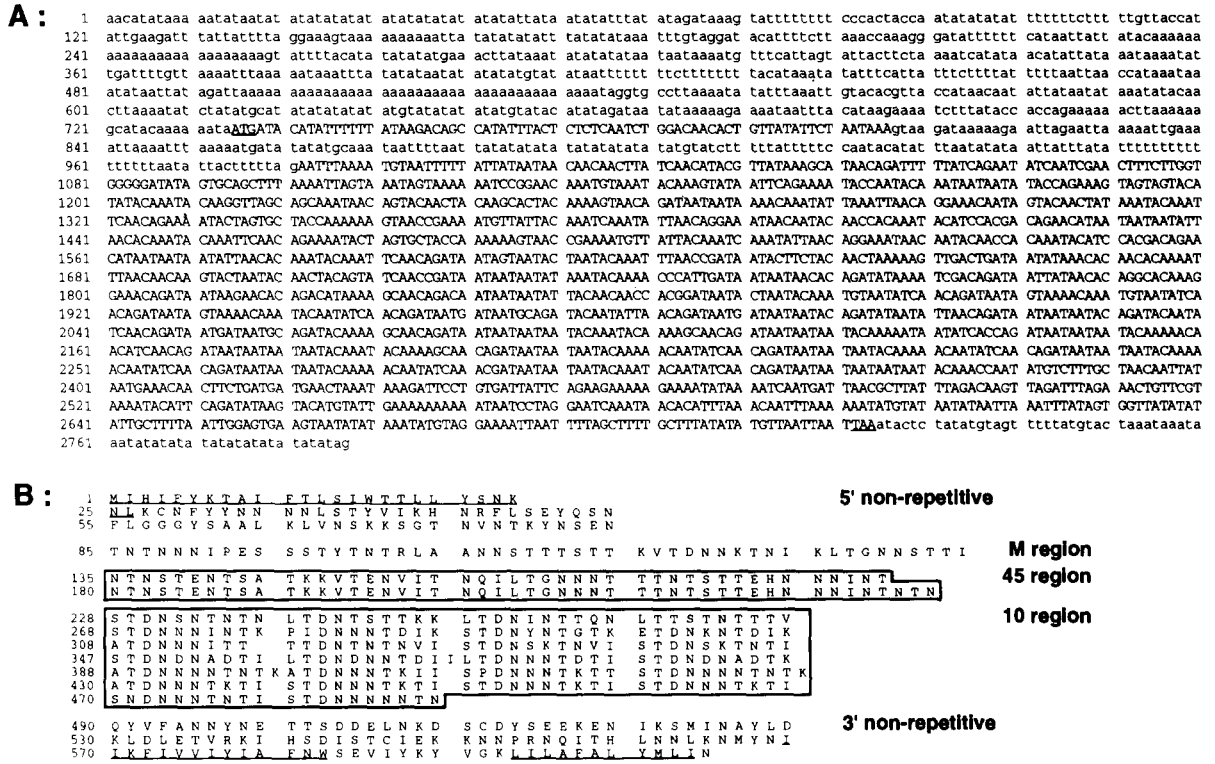

Fig. 3. Sequence of the STARP gene (clone T9/96). (A) Nucleotide sequence of the STARP gene. The coding regions are shown in capital letters while the non-coding regions are indicated in lower case. The initiation codon and stop-codon are underlined. Nucleotide 1 corresponds to the start of the subcloned (1.6 kb) 5′ *Hin*cII fragment. (B) Amino acid sequence showing the designated regions of the protein. The predicted signal peptide, encoded in majority by exon 1 (amino acids 1–24), is underlined, as are the C-terminal hydrophobic regions.

MOTIF	REGION	COPY N°
KVTDNNKTN	M	1
KVTENV I TN	45	2
TDNSKTN	10	2
TDNN	10	10
NTSATKKVT	45	2
NTSTTKKLT	10	1
LAANNSTT	M	1
LTGNNSTT	M	1
LTGNNNTT	45	2
NNNT	10	12
NTNTN	M	1
NTNTN	45	2
NTNTN	10	3
TNTST	45	2
TNTTT	10	2

MOTIF	REGION	COPY N°
NNNI	M	1
NNNI	45	2
NNNI	10	2
TSTT	M	1
TSTT	45	2
TSTT	10	1
TNGI	45	2
TNTI	10	2
TNVI	10	1
NTTT	45	2
NTTT	10	2
NINT	45	2
NINT	10	1
TNT	M	2
TNT	45	2
TNT	10	8

Table 1 Sequence motifs present in the STARP central repetitive region

maintained strains as well as from African field isolates (from Dielmo, Senegal). In the laboratory strains only a single band was amplified, of approximately the same size (1.29 kb) for T9/96, NF54 and 3D7 parasites, whilst this region is approximately 100 bp smaller in the Palo Alto strain (Fig. 4). Interestingly, two bands, corresponding to two distinguishable STARP polymorphs present in vivo, were observed in 4 of the 7 field isolates; in all 4 isolates both bands were of approximately the same size (about 1.37 and 1.26 kb). Restricted size polymorphism was thus detectable in the repeat region of the STARP gene. In contrast, PCR studies on these DNA preparations revealed a size conservation in the 5′ and 3′ non-repetitive coding regions of the gene (unpub-

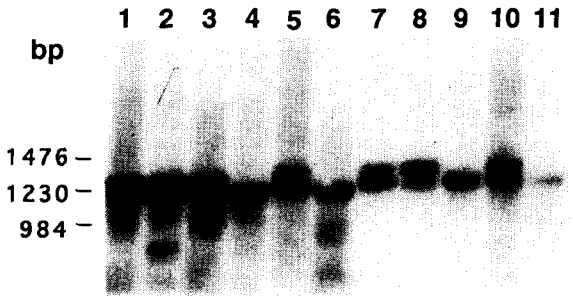

Fig. 4. Size polymorphism in the central repetitive region. The repetitive region of the STARP gene was amplified by PCR, using the flanking oligomers 5'6 and 3'2, from 4 laboratory-maintained strains: T9/96 (lane 1), 3D7 (lane 2), NF54 (lane 3), Palo Alto (FUP/CB) (lane 4), and from 7 separate field isolates from Dielmo, Senegal: I754 (lane 5), I1951 (lane 6), I756 (lane 7), I368 (lane 8), I1660 (lane 9), I649 (lane 10), I1148 (lane 11). After gel electrophoresis and Southern transfer, these were hybridized with a radiolabeled DG21 probe, showing a size polymorphism ranging from 1.2 to 1.4 kb in these strains and isolates. Minor, lower molecular weight products, likely resulting from faulty PCR amplification of the full-length STARP repetitive region, are occassionally observed, as seen in lanes 2 and 3.

lished data).

Detection of STARP in sporozoites. We have investigated expression of the STARP gene in sporozoites by several means. Reverse PCR on sporozoite mRNA, using primers from exons 1 and 2, generated a predominant band of 101 bp, corresponding to the predicted size of the spliced cDNA and clearly distinguishable in size from the intron-containing band of 276 bp amplified from genomic DNA (see Fig. 1C). Hybridization of a Northern blot of sporozoite RNA with a DG21 probe revealed a faint band at approximately 2.6 kb (Fig. 5A), confirming transcription of this gene in sporozoites.

Western blotting of NF54 sporozoites revealed intense labeling of a polypeptide of 78 kDa (Fig. 5B), also detected in 3D7 sporozoites (unpublished data). A higher MW band, either a precursor or differently-glycosylated form of the STARP protein or a cross-reactive molecule, is also faintly recognized at about 88 kDa. No band was recognized using either sera from mice immunised with GST alone or pre-immune sera. Control anti-CS

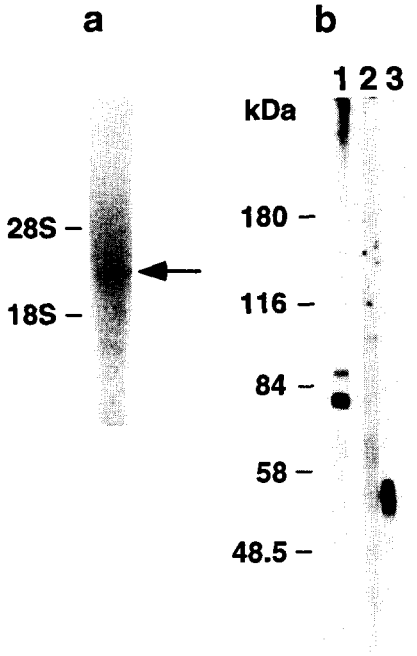

Fig. 5. Expression of the STARP gene in sporozoites. (A) 5 μg of sporozoite RNA was electrophoresed on a 1.5% formaldehyde-agarose gel and the Northern blot hybridized with a DG21 probe. (B) Protein extracts from 3.5×10^5 NF54 sporozoites were subjected to SDS-PAGE, Western blotted and probed with anti-DG21 antiserum (lane 1), control anti-GST antiserum (lane 2) and mAb22, an anti-CS monoclonal antibody (lane 3).

antibodies labeled the CS protein of 51–54 kDa.

Sequence analysis predicts that STARP is transported to the surface of sporozoites. In order to verify this experimentally, surface IFA and immunoelectron microscopy was performed. IFA was performed on freshly-dissected non-fixed sporozoites either in suspension or attached to poly-L-lysine slides (a 'wet' IFA), using mouse anti-DG21 antisera. This revealed distinctive surface labeling. The same image was also observed with mouse sera raised against the C-terminal non-repetitive region, i.e. anti-STARP3' antisera (unpublished data). Interestingly, the surface labeling pattern varied between sporozoites, with some showing staining over nearly all the sporozoite, reinforced in one or two patches (as shown in Fig. 6A), whilst others showed a single patch of bright staining over the centre of the sporozoite or

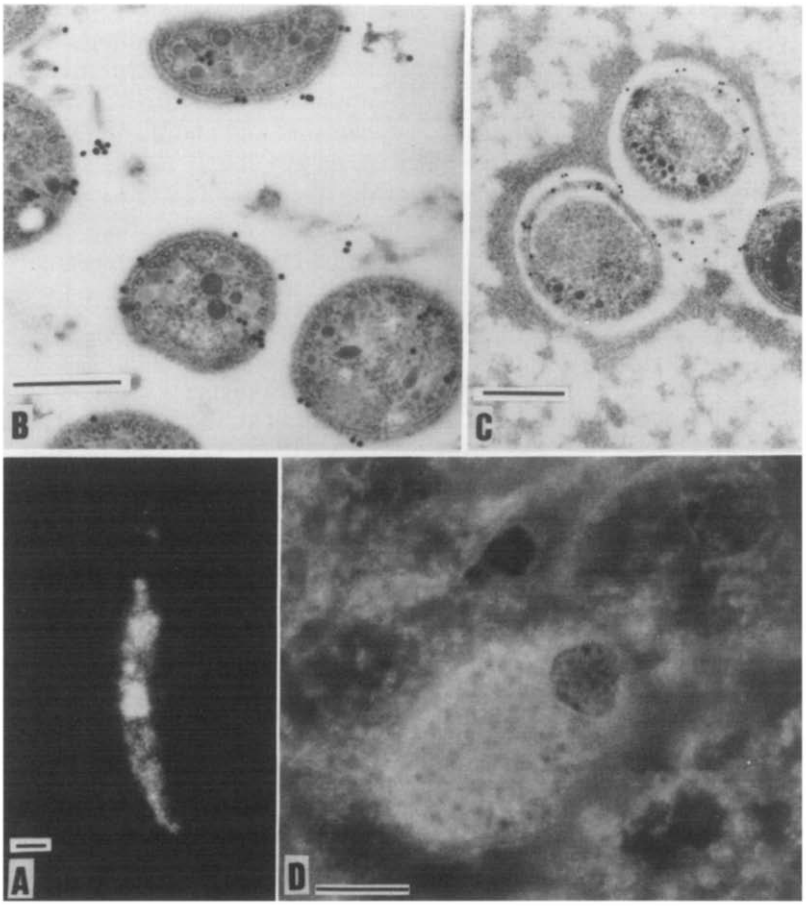

Fig. 6. Indirect immunofluorescence and immunoelectron microscopy of *P. falciparum* preerythrocytic stages. (A) Immunofluorescence image of non-fixed NF54 sporozoites labeled with anti-DG21 antisera. (B,C) Immunoelectron micrograph of NF54 sporozoites stained with (B) mouse anti-DG21 antisera and (C) human affinity-purified monospecific anti-DG21 antibodies, and counterstained with immunoglobulin gold. This shows clusters of gold labeling on the surface of the sporozoite as well as some clusters associated with the inner membrane surface and the cytoplasm. (D) Immunofluorescence image of an in vivo-derived liver schizont labeled with anti-STARP3′ antisera. The host nucleus is seen squeezed into the top right corner of the parasitized hepatocyte and appears to be surrounded by the parasite. Inside the parasite, numerous developing liver merozoites are discernible as small, non-fluorescing circular objects. A non-infected, non-fluorescing cell with its nucleus can be seen diagonally above. Paler areas surrounding the parasitized hepatocyte result from characteristically non-homogenous Evan's blue staining of the host tissue. Control pre-immune and anti-GST antisera were negative. (A,B,C) Bar represents 1 μm. (D) Bar represents 10 μm.

covering an extremity. Immunoelectron microscopy using either mouse antisera or human affinity-purified antibodies also localized the STARP protein to the sporozoite surface (Fig. 6B and 6C respectively).

Detection of STARP in liver and asexual blood stages. Owing to the very low numbers of liver stages produced in in vitro and in vivo models, it is not possible to explore the expression of the STARP gene by Western or Northern analysis. Nevertheless, the possible expression of STARP in liver stages was explored by performing IFA on immature (day 5) *P. falciparum* liver schizonts obtained from a Sapajou monkey infected with an African field isolate, using anti-STARP3′ antisera

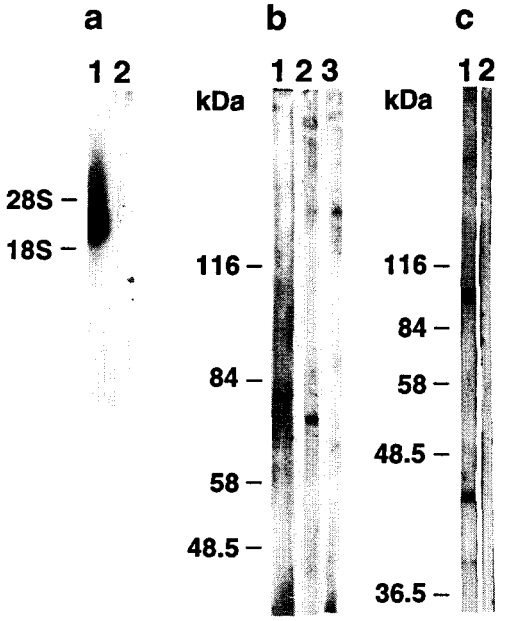

Fig. 7. Investigation of erythrocytic stage expression of the STARP gene. (A) 20 μg of RNA from synchronized ring-stages (lane 1) and schizonts (lane 2) was electrophoresed on a 1.5% formaldehyde-agarose gel, transferred and hybridized with a DG21 probe. (B) Immunoblot of 2×10^7 ring stage NF54 (lane 1) and Palo Alto (lanes 2, 3) parasites labeled with anti-STARP3′ antisera (lanes 1, 2) and control anti-GST antisera (lane 3). (C) Immunoblot of 2×10^7 NF54 schizonts labeled with anti-DG21 (lane 1) and anti-GST (lane 2) antisera. No bands were labeled with anti-STARP3′ antisera.

(Fig. 6D) and anti-DG21 antisera (data not shown). Both revealed staining throughout the parasite cytoplasm between the developing liver merozoites. These antisera gave a similar pattern of positive IFA labeling of mature (day 7) liver schizonts from a chimpanzee infected with the NF54 strain (unpublished data). Positive staining was seen for all 30 schizonts examined.

We have examined whether STARP is expressed during the erythrocytic cycle. Hybridization with a DG21 probe of separate RNA preparations made from synchronized ring-stage and schizont-stage cultures revealed a clear band at about 2.6 kb in the rings, whilst no band could be detected in schizonts (Fig. 7A). Subsequent hybridization of a Northern kinetic blot (samples prepared from synchronized parasites at 7 evenly-spaced time-points over one cycle) with a DG21 probe detected a faint signal only in young (8 h) ring-stage parasites (unpublished data). Western blot analysis of synchronized ring-stage parasites revealed a faint band at about 78 kDa in NF54 parasites and about 73 kDa in Palo Alto parasites (Fig. 7B). These molecular masses correspond to the major band of 78 kDa detected in NF54 sporozoites. Western blotting of more mature asexual blood-stage parasites repeatedly failed to detect a band of this MW and, only in the case of anti-DG21 sera, labeled other, presumably cross-reactive polypeptides (migrating at about 96 kDa and a pair of polypeptides of varying relative intensity migrating at about 44 and 39 kDa, Fig. 7C).

Northern hybridization of RNA prepared from gametes and gametocytes, using a DG21 probe, gave no detectable signal in blots which otherwise gave a strong signal when hybridized with a Pfs16 probe. Similarly, no STARP cDNA could be detected either by reverse PCR from gamete mRNA or by PCR from a gamete cDNA library (unpublished data). This indicates that the STARP gene is not expressed during the sexual stages.

4. Discussion

We here report the identification, cloning and stage-specificity of a novel *P. falciparum* sporozoite surface antigen, STARP, initially chosen based on its consistent expression on the surface of sporozoites from various isolates. The STARP gene spans 2.0 kb and has a two-exon structure. The potential methionine start codon at the start of the 5′ miniexon is located just downstream of multiple stop codons and is preceded by an adenine at positions −3 and −1 (relative to the ATG), which agrees with consensus sequences found at equivalent positions of initiation sites for other *P. falciparum* genes ([21] and references therein). These two exons are separated by a 175-bp intron with typical consensus splice donor/acceptor sites ([33] and references therein).

The STARP gene encodes a mature polypeptide of 604 residues, rich in asparagine and threonine. This protein is distinct from the previously described malaria asparagine-rich antigens and

screening of the Genbank protein data base (version 78) detected no significant homology with these or other protein entries.

The N-terminal sequence of STARP is characteristic of a signal peptide, whose hydrophobic core is encoded by the 5′ miniexon. Interestingly, all previously-characterized *P. falciparum* antigens containing such a 5′ mini-exon structure have been found to be exported proteins whose signal peptide is either partially or fully encoded by a miniexon (reviewed in [34]). The only other hydrophobic region resides at the carboxy terminus, though its unusual structure does not correspond to a classical transmembrane anchor structure [35]. Due to the scarcity of sporozoite material, it has not yet been possible to investigate whether STARP is anchored to the sporozoite surface membrane via a phosphatidyl-inositol-phosphate moiety.

The presence in STARP of tandem repeats of oligopeptide sequences is a characteristic shared by many *P. falciparum* antigens, in which the repeat regions often contain the major B-cell epitope(s) [36]. In marked contrast to the perfectly conserved 45 amino acid repeats of STARP, the 10 amino acid repeats are highly degenerate. These degenerate repeats may create a panoply of related epitopes, in a manner similar to the gametocyte-specific antigen Pf11-1 [37]. In spite of this high level of degeneracy, most of the 10 amino acid repeats in STARP are predicted to maintain a β-turn structure, suggesting that structural rather than primary sequence constraints might be operating on this region. This is reminiscent of the CS protein, where the only known repeat variant (NVDP) is optimal in maintaining the β-turn structure inherent in the NANP repeats (Andre Tartar, personal communication), and may reflect a general mechanism operating on repeat regions of *P. falciparum* antigens.

The third type of repeat structure present in STARP is defined by the mosaic region – a composite of sequence motifs that reappear in degenerate forms in a seemingly random, non-contiguous order throughout all 3 regions. The overall structure of STARP's central repetitive region would seem to result from extensive duplication and rearrangement events. Not surprisingly, this central region showed size polymorphism between different parasite populations, a phenomenon which appears to be typical for many repetitive *P. falciparum* antigens [36].

We have investigated the stage specificity of the STARP gene products. Such characterisation is important as it provides the base for future studies aimed at evaluating the potential role of the STARP antigen in eliciting stage-specific immune defense mechanisms.

Reverse PCR and Northern blot hybridization both demonstrate that the STARP gene is transcribed in sporozoites. Reverse PCR data was particularly conclusive, since the miniexon enables size discrimination between RNA reverse transcripts and genomic DNA. On immunoblots of sporozoites, the STARP protein migrates at about 78 kDa. The DNA sequence predicts a molecular mass of 67 kDa and the reduced mobility may be either related to the hydrophilic nature of this antigen, as has been noted for other hydrophilic *P. falciparum* proteins, or alternatively may result from post-translational modifications of the native STARP protein.

IFA and immunoelectron microscopy studies using a series of mice and human antibodies detect STARP associated with the sporozoite surface. IFA studies reveal an unusual surface distribution which, in contrast to the even distribution of the CS and several of our partially-characterized sporozoite antigens, is predominantly localized to discrete regions of the surface. Non-homogeneous surface localization has also been observed with anti-SSP2/TRAP antibodies [14,38]. STARP however differs notably from TRAP/SSP2 in that all freshly-dissected, unfixed sporozoites were positive by IFA using anti-STARP antibodies, whereas only 5–10% were reported to show surface fluorescence with anti-TRAP/SSP2 antibodies [14]. The consistent surface expression of STARP may thus provide an effective target for sporozoite-specific antibodies.

In liver stages, the expression of STARP was examined by IFA on immature and mature liver schizonts from two separate *P. falciparum* isolates. This revealed consistent positive labeling with antibodies raised to either the repetitive region or the C-terminal non-repetitive region. The

intense labeling observed with anti-STARP antibodies suggests that STARP is actively produced at the liver stage, in contrast to the CS antigen which persists in minor quantities during liver schizogony ([39]; our unpublished observations). Evidence arguing for the potentially critical role in preerythrocytic-stage immunity of antigens expressed during the liver stage has come from in vitro studies suggesting that protection induced by irradiated sporozoites is dependent upon the transformation of sporozoites into young hepatic trophozoites [40]. STARP may provide a target for the large variety of immune mechanisms that in rodent models have been found to be active against this stage (reviewed in [41]).

During the intraerythrocytic cycle, STARP gene products have only been detected at the ring stage. Northern hybridization revealed a band of 2.6 kb, the same size as that detected in sporozoite RNA. By immunoblotting STARP was detected in ring stages only when large numbers of parasites were used, thus contrasting with the readily detectable presence of STARP transcripts. This difference may be related to low translational activity or rapid protein breakdown and degradation. The observed differences in the molecular mass of the STARP protein in NF54 and Palo Alto ring-stage parasites (78 kDa and 73 kDa respectively) reflects the central repeat region polymorphism seen at the nucleic acid level. Minor bands detected by anti-DG21 antisera in schizont extracts may well correspond to other asparagine-rich *P. falciparum* antigens synthesized by mature erythrocytic stage parasites. Indeed, cross-reactions between asparagine-rich proteins are well-documented phenomena [42,43]. The finding that intra-erythrocytic expression of STARP is limited to the initial stage of the erythrocytic cycle is intriguing and its significance for the parasite remains to be elucidated. It is worthwhile noting that low-level expression of a sporozoite surface antigen during the erythrocytic stage has also been reported for TRAP/SSP2 [13] and CS [44].

The use of irradiated sporozoites, whilst not feasible on a large scale, has already shown that an effective preerythrocytic-stage malaria vaccine can be found. For this purpose, there is now a recognized need to more comprehensively characterize the malarial antigens expressed on the sporozoite surface and/or in liver stages. Our study represents the first step in the identification and characterization of a novel *P. falciparum* sporozoite surface antigen, STARP, which in a recombinant form proved immunogenic in mice. The consistent surface expression of STARP on sporozoites of various isolates and its expression in the liver stage, a stage which may well be critical in pre-erythrocytic stage immunity, supports the need to assess its potential involvement in naturally-acquired or artificially-induced immunity.

5. Acknowledgements

We are grateful to Serge Bonnefoy, Hasnaa Bouharoun-Tayoun, Odile Mercereau-Puijalon, Andy Waters and the late Richard Beaudoin as well as the Department of Medical Microbiology, University Hospital Nijmegen, The Netherlands, for their helpful participation during the course of this study and would like to sincerely thank Odile Mercereau-Puijalon, Artur Scherf and Gordon Langsley for critically reading the manuscript. This work received financial support from the Science and Technology for Development Programme of the Commission of the European Communities, the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and in part by the USAID (for M.A. and T.K.).

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