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A RECOMBINANT HYBRID PROTEIN AS ANTIGEN FOR AN ANTI-BLOOD STAGE MALARIA VACCINE: A STUDY ON THE CONSERVATION OF A PROTECTIVE COMPONENT

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Recently we have shown that two hybrid proteins expressed in Escherichia coli *confer protective immunity to Aotus monkeys against an experimental* Plasmodium falciparum *infection (Knapp et al., 1992). Both hybrid proteins carry a sequence containing amino acids 631 to 764 of the serine stretch protein SERP (Knapp et al., 1989b). We have studied the diversity of this SERP region in field isolates of* P. falciparum. *Genomic DNA was extracted from the blood of six donors from different endemic areas of Brazil and West Africa. The SERP region encoding amino acids 630 to 781 was amplified by polymerase chain reaction (PCR) and sequenced. Only conserved amino acid substitutions in maximally two positions of the analyzed SERP fragment could be detected which supports the suitability of this SERP region as a component of an anti-blood stage malaria vaccine.*

Key words: *Plasmodium falciparum* – malaria vaccine – serine-stretch protein SERP – field isolates

Several approaches have been taken in order to identify candidate antigens for a vaccine against the blood stage of human malaria. In one approach polypeptides from *Plasmodium falciparum* were selected by monoclonal antibodies inhibiting the in vitro growth of the parasite (Perrin & Dayal, 1982). Those proteins were purified and tested for their ability to induce protective immunity in monkeys. Complete or partial protection could be demonstrated for protein bands with molecular weights of 200, 140 and 41 kD (Perrin et al., 1984, 1985).

The 200 kD antigen is a predominant protein of schizonts recognized by patient sera. During the release of merozoites from infected erythrocytes it is processed to products of 83, 42, 36 and 19 kD which are the main protein components found on the merozoite surface (Lyon et al., 1986). Therefore this protein was called the merozoite surface antigen I (MSAI). MSAI exhibits antigenic polymorphism, however it contains conserved regions (Peterson et al., 1988) We have isolated a DNA fragment coding for part of the conserved N-terminal sequences which was shown to carry two T-cell epitopes (Crisanti et al., 1988).

By screening of a λgt11 library from *P. falciparum* schizont cDNA with an antiserum raised against the protective 140 kD protein band two corresponding phage clones were isolated. Their insert DNAs were used to identify and clone a genomic fragment which carries the entire sequence coding for a protein of 113 kD characterized by a stretch of 37 serine residues which therefore was called the serine stretch protein SERP (Knapp et al., 1989b). Different protein regions of SERP were expressed in *Escherichia coli* and used to determine the capacity of human lymphocytes to recognize these subfragments. We defined one region of SERP which was recognized by human lymphocytes very efficiently. Synthetic peptides covering this SERP fragment were used to induce proliferative responses of lymphocytes, allowing the identification of two T-cell epitopes on a narrow region of SERP (Roussilhon et al., 1990). This region of the SERP antigen was expressed in *E. coli* and tested for its protective property in Aotus monkeys. After challenge infection with *P. falciparum* parasites a partial protective effect of the recombinant antigen which covers the SERP region carrying the T-

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cell epitopes was observed (unpublished results). Recently protective immunity induced in Aotus monkeys has also been shown for recombinant SERP fragments originating from the N-terminal region by Inselburg et al. (1991). Therefore this antigen is considered to be a candidate for a malaria vaccine.

Using an antiserum raised against the protective 41 kD protein band for screening of λgt11 libraries, we have isolated 16 phage clones coding for different gene products (Knapp et al., 1989a). Three of these clones, 41-2, 41-3 and 41-7, were analyzed in more detail and the complete coding regions were isolated. The insert DNA of the 41-7 phage clone was used to isolate a genomic clone which codes for a 41 kD protein, the fructose disphosphate aldolase of *P. f*alciparum (Knapp et al., 1990). This enzyme is a main component of the protective 41 kD protein band. However, a recombinant aldolase could not protect Aotus monkeys (Herrera et al., 1990) which suggests that the aldolase is not the protective constituent of the 41 kD protein band. The insert DNA of the phage clone 41-3 was used to isolate the entire gene (Knapp et al., 1991b). The molecular weight of the deduced amino acid sequence has been calculated to be 41.2 kD and therefore the 41-3 protein could well be a component of the protective 41 kD protein band. However, further investigations are necessary to analyze whether this protein is a candidate for the development of an anti-blood stage malaria vaccine.

An additional antigen found by cross-reaction with the antiserum raised against the 41 kD protein band was identified as the histidine alanine rich protein HRPII (Knapp et al., 1988). This protein is characterized by an extended repeat region consisting of the tripeptide Ala-His-His and the tripeptide Ala-Ala-Asp (Wellems & Howard, 1986; Knapp et al., 1988). The HRPII antigen was shown to be released from the infected red blood cell (Howard et al., 1986). However, data from cell surface radioiodination have shown that HRPII also stays associated with the outer surface of the erythrocyte membrane (Rock et al., 1987). A recombinant antigen expressing parts of the repeat region of HRPII was shown to protect Aotus monkeys against a parasite challenge (Knapp et al., 1988). Therefore the HRPII antigen was considered to be another candidate for the development of a malaria vaccine.

From the recombinant antigens we have tested so far for their protective potential in Aotus monkeys HRPII and SERP were shown to be the best candidates for the development of an anti-blood stage vaccine. We have expressed two hybrid proteins in *E. coli* containing selected partial sequences of both antigens. Antibodies raised against both hybrid proteins in rabbits and Aotus monkeys recognize the corresponding schizont polypeptides. Aotus monkeys immunized with either of the two hybrid proteins were shown to be protected against an experimental *P. falciparum* infection (Knapp et al., 1991a; Knapp et al., 1992).

A demand for the efficacy of a subunit vaccine is that its antigenic moieties are well conserved among the parasites of different areas. One of the components of both protective hybrid proteins is a region containing amino acids 631 to 764 of the antigen SERP. The gene coding for SERP was cloned from two different *P. falciparum* laboratory strains (Knapp et al., 1989b; Bzik et al., 1988), and the SERP region which is part of both protective hybrid antigens was shown to be identical between the two strains. However, recently a significant difference in the sequences of a malarial antigen from parasite field isolates, compared to laboratory strains was reported by Kimura et al. (1990). These data suggest that investigations based on cultured parasites may not reflect the real in vivo situation.

Therefore we have studied the diversity of the SERP antigen from parasite field isolates and in the first place we have focused on the SERP region from amino acid position 631 to 764 which is common to both protective hybrid proteins. Genomic DNA was extracted from the blood of three donors from Brazil and three donors from Senegal. A SERP fragment coding for the amino acids 630 to 781 was analyzed following polymerase chain reaction and DNA sequencing. We could demonstrate that SERP shows maximally two conserved amino acid substitutions in the region investigated. These data support the suitability of the SERP fragment containing amino acids 631 to 764 for the development of an anti-blood stage malaria vaccine.

MATERIALS AND METHODS

Genomic DNA of *P. falciparum* from blood of infected humans was isolated from three

Brazilian patients (B1, B2, B3) and three patients from Senegal (S14, S15, S16) according to the method of Kimura et al. (1990). The oligonucleotides p1 (5' ATGGAATTCTTACAAATTATTGAAGAT 3', EcoRI site at the 5' end) and p2 (5' AAAGGATCCCCAATATGGACCCCAACTGTTTCTTAC 3', BamHI site at the 5'end) were used to amplify a region of the SERP gene from nucleotide position 2641 to 3096 from genomic DNA isolated from the blood of the infected humans. The polymerase chain reaction was carried out under standard conditions using the Gene-AMP™ kit of Perkin Elmer-Cetus. The amplified DNA was digested with the restriction enzymes EcoRI and BamHI and introduced into the pKS Bluescript vector (Stratagene) according to standard techniques (Sambrook et al., 1989). Both strands of the insert DNAs of the six different plasmids obtained were sequenced by the chain termination procedure using the sequenase system from USB (Cleveland, OH).

To correct for mutations generated by PCR the amplified DNA fragments were sequenced directly according to a modified protocol of Richardt & Woo (1991). The PCR products were electrophoresed through a 1.2% agarose gel to remove the PCR primers, excised and purified using Quiagen tips (Diagen, Düsseldorf, F.R.G.). Approximately 200 ng of the purified PCR products were annealed to 10 ng of oligonucleotides p1 and p2, respectively, by boiling for 6 min and allowing to cool to room temperature for 1 min. Sequencing was performed according to the sequenase system from USB with the following exceptions: The extension reaction was carried out on ice for 3 min and the labeling-mix was diluted 1:10.

RESULTS

Isolation of a gene region of the SERP gene from different parasite strains — The oligonucleotides p1 and p2 were used to amplify a SERP gene region from genomic DNA of *P. falciparum* isolated from three different Brazilian patients and three different patients from Senegal to investigate the allelic polymorphism of this SERP fragment from parasites of different areas. The amplified region codes for the amino acid residues 630 to 781 of SERP (Knapp et al., 1989b), containing the residues 631 to 764 which are common to two hybrid antigens shown to confer protective immunity to Aotus monkeys (Knapp et al., 1992). Fig. 1 shows the amplified fragments of the three Brazilian and the three Senegal parasite isolates. No variation in the size of the amplified fragments could be detected among the different parasite isolates suggesting that no deletion or insertion has occurred.

Cloning and sequencing of amplified fragments of the SERP gene from different parasite isolates – To analyze the variation of the amplified DNA fragments originating from six different isolates in more detail, the fragments were inserted into the pKS vector for DNA sequencing. Additionally instead of cloned isolates the entire populations of PCR products have been subjected to sequence analysis to correct for artifactual mutations generated by PCR. The nucleotide sequences and the deduced amino acid sequences obtained from the six different DNA fragments are shown in Fig. 2. The oligonucleotides p1 and p2 used for the amplification cover the nucleotide positions 2641 to 2667 and 3067 to 3096 of the SERP gene (Knapp et al., 1989b), respectively; consequently single nucleotide substitutions could not be detected in these regions and variation could only be investigated for the nucleotides 2668 to 3066 coding for the amino acid residues 639 to 771. This region contains the residues 631 to

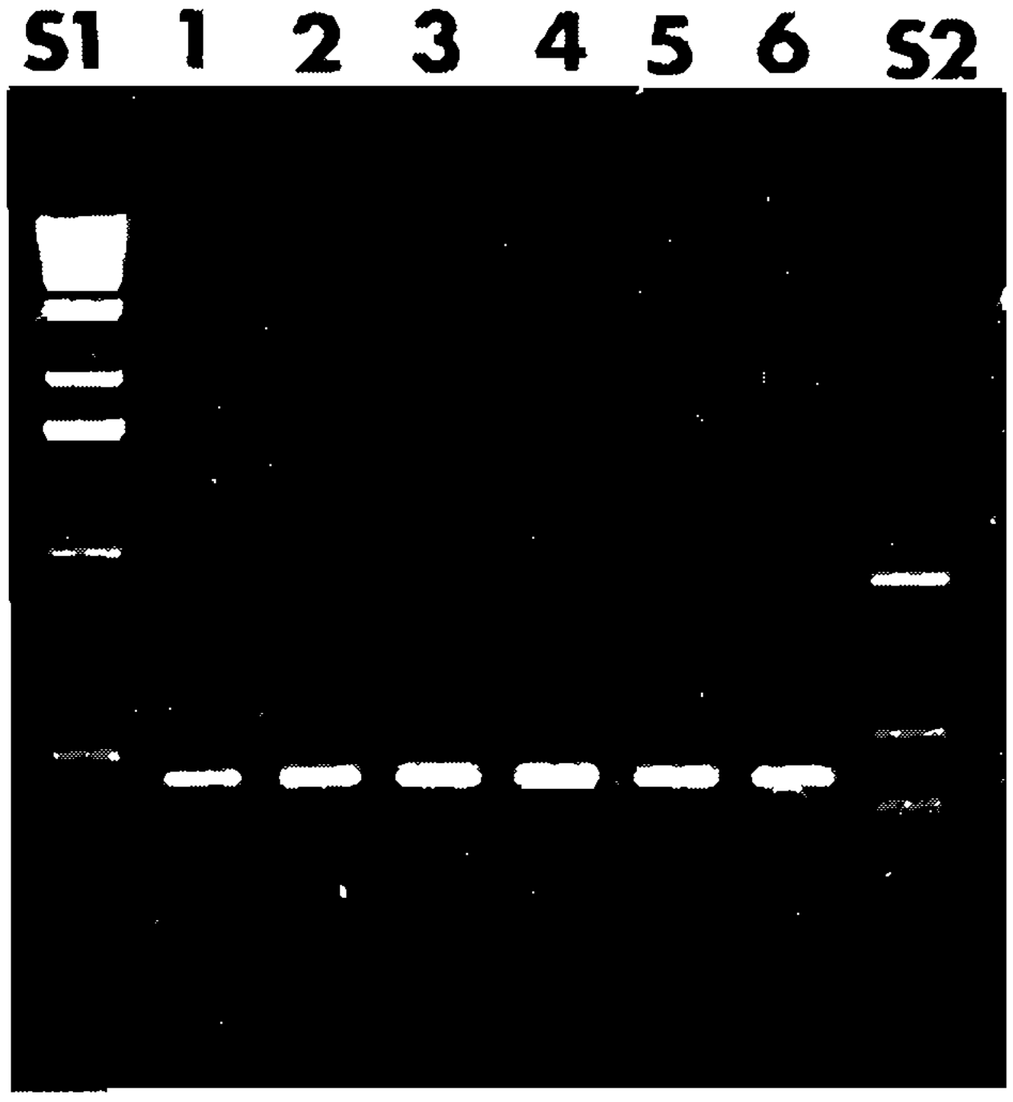

Fig. 1: polymerase chain reaction analysis of the SERP gene region coding for the amino acids 630 to 781 from three Brazilian (B1, B2, B3) and three west African (Senegal; S14, S15, S16) field isolates of *Plasmodium falciparum*. 1/20 of each PCR reaction was applied on a 1.8% agarose gel and stained with ethidium bromide after electrophoretic separation. Lanes: 1, B1; 2, B2, 3, B3; 4, S14; 5, S15; 6, S16; S1 1kb ladder from BRL (size marker); S2, pUC18 DNA digested with DdeI (size marker).

A

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FCBR    ATGGAATTCTTACAAATTATTGAAGATTATGGATTCTTACCAGCAGAATCAAATTATCCATATAACTATGTGAAAGTTGGAGAACAATGTCCAAAGGTAG  2740
FCR-3   ................................................................................................

B1      ................................................................................................
B2      ................................................................................................
B3      ................................................................................................
S14     ................................................................................................
S15     ................................................................................................
S16     ................................................................................................

        AAGATCACTGGATGAATCTATGGGATAATGGAAAAATCTTACATAACAAAAATGAACCTAATAGTTTAGATGGTAAGGGATATACTGCATATGAAAGTGA  2840
        ...........G....................................................................................
        ...........G....................................................................................
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        ...........G....................................................................................

        AAGATTTCATGATAATATGGATGCATTTGTTAAAATTATTAAAACTGAAGTAATGAATAAAGGTTCAGTTATTGCATATATTAAAGCTGAAAATGTTATG  2940
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        GGATATGAATTTAGTGGAAAGAAAGTACAGAACTTATGTGGTGATGATACAGCTGATCATGCAGTTAATATTGTTGGTTATGGTAATTATGTGAATAGCG  3040
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        AAGGAGAAAAAAATCCTATTGGATTGTAAGAAACAGTTGGGGTCCATATTGGGGA                                          3096
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        ........................G
        ........................G
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B

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FCBR    MEFLQIIEDY GFLPAESNYP YNYVKVGEQC PKVEDHWMNL WDNGKILHNK    679
FCR-3   .......... .......... .......... .......... ..........

B1      ..........V ..........V ..........V ..........V ..........
B2      ..........V ..........V ..........V ..........V ..........
B3      ..........V ..........V ..........V ..........V ..........
S14     ..........V ..........V ..........V ..........V ..........
S15     ..........V ..........V ..........V ..........V ..........
S16     ..........V ..........V ..........V ..........V ..........

        NEPNSLDGKG YTAYESERFH DNMDAFVKII KTEVMNKGSV IAYIKAENVM    729
        .......... .......... .......... .......... ..........

        .......... .......... .......... .......... ..........
        .......... .......... .......... .......... ..........
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        GYEPSGKRVQ NLCGDDTADH AVNIVGYGNY VNSEGERRSY WIVRNSWGPY WG 781
        .......... .......... .......... .......... .......... ..

        .......... .......... .......... .......... ..........M
        .......... .......... .......... .......... ..........M
        .......... .......... .......... .......... ..........M
        .......... .......... .......... .......... ..........M
        .......... .......... .......... .......... ..........M
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Fig. 2: nucleotide (A) and amino acid (B) sequence alignment of the PCR amplified SERP fragments (nucleotides 2641 to 3096) coding for the amino acids 630 to 781 of SERP from the laboratory strain FCBR (Knapp et al., 1989b) with the homologous regions of the laboratory strain FCR-3 (Bzik et al., 1988) and the field isolates B1, B2, B3, S14, S15 and S16. The position of the oligonucleotides used for polymerase chain reaction are overlined. The SERP region included in both hybrid proteins is indicated by asterisks.

674 which are common to two protective hybrid antigens (Knapp et al., manuscript submitted). The SERP fragment of the three Brazilian isolates (B1, B2, B3) show two base pair exchanges when compared with the laboratory strains: position 2752 is changed from A to G and position 3066 is changed from T to G resulting in the amino acid substitutions Met_{667} → Val and Ile_{771} → Met, respectively. The three field isolates from Senegal (S14, S15, S16) also have changed the Ile_{771} residue to a Met residue based on the same nucleotide substitution as found for the Brazilian isolates, whereas only one isolate (S16) carries the Met_{607} → Val amino acid substitution.*

In comparison to the nucleotide sequence data obtained by direct sequencing of the PCR fragments some of the cloned DNA fragments show additional base pair exchanges which could be the result of PCR artefacts: strain B3 carries a C instead of a T in position 3005 and the cloned fragments of the Senegal strains S15 and S16 have changed the A in position 2795 to a G and the G in position 3004 to an A, respectively.

DISCUSSION

In search of a vaccine against the blood stage of *P. falciparum*, we have designed two hybrid proteins combining partial sequences of the antigens SERP (Knapp et al., 1989b), HRPII (Knapp et al., 1988) and MSAI (Crisanti et al., 1988), and we have shown that both can protect Aotus monkeys from an experimental *P. falciparum* infection (Knapp et al., 1991a; Knapp et al., 1992). Although the partial sequences of both hybrid antigens originate from *P. falciparum* strains different from the challenge strain their antigenic variation needs an investigation in more detail to consider the real field situation. Both hybrid proteins carry the C-terminal 189 amino acid residues of the HRPII antigen and a protein region of SERP from amino acid position 631 to 764.

In this study we have shown that the SERP component common to both protective hybrid proteins is well conserved among different field isolates of *P. falciparum* originating from different endemic areas. All the field isolates analyzed show a Ile_{771} → Met substitution in a position contained only in one of the hybrid antigens (Knapp et al., 1991a). In the region from amino acid position 631 to 764 only one conserved amino acid substitution has been detected: four of the six field isolates analyzed show a Met → Val substitution in position 667.

We could not detect any significant difference in the variations between the isolates from Brazil and Senegal, suggesting that variation of the analyzed SERP fragment is limited to only two positions in field isolates from different endemic areas of the world. Both amino acid substitutions are conserved and therefore they should not influence the secondary structure of the protein. A recombinant protein containing amino acids 631 to 684 of SERP and two synthetic peptides covering amino acids 646 to 660 and 676 to 690, respectively, were recognized by human lymphocytes very effectively (Roussilhon et al., 1990). Regions containing B-and T-cell epitopes have been reported to be subject to considerable antigenic variation. In this sense, the conservation of this sequence containing at least two T-cell epitopes needs to be emphasized. The conservation of the SERP protein region from amino acids 631 to 764, the presence of T-cell epitopes in this region and the data obtained from vaccination of Aotus monkeys suggest this protein region of SERP to be a suitable component of an anti-blood stage malaria vaccine.

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*Note added in proof: the base exchange from T to G in position 3066 is caused by a PCR artefact as analyzed by direct sequencing of extended DNA fragments.

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