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Polymorphism of the Pf155/RESA Antigen among Field Isolates of *Plasmodium falciparum* in Thailand

ความหลากหลายของแอนติเจน Pf155/RESA ของเชื้อมาลาเรียชนิดฟัลซิปารัม ที่เก็บจากแหล่งระบาดในประเทศไทย

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ศึกษาความหลากหลายของยีนที่สังเคราะห์ Pf155/RESA ซึ่งเป็นแอนติเจนที่ผิวของเม็ดเลือดแดงที่ติดเชื้อมาลาเรียชนิดฟัลซิปารัมในระยะวงแหวน โดยการศึกษาทั้งจากไอโซเลตที่เลี้ยงไว้ในห้องปฏิบัติการและไอโซเลตที่เก็บจากแหล่งระบาดในประเทศไทย ยีนที่สังเคราะห์แอนติเจน Pf155/RESA ของเชื้อ F32 ซึ่งเป็นเชื้อจากอาฟริกา ถูกนำมาโคลนและหาลำดับเบสเพื่อใช้ในการเปรียบเทียบกับไอโซเลตอื่น ๆ พบว่ายีนนี้ไม่มีความหลากหลายในกลุ่มของเชื้อที่เลี้ยงไว้ในห้องปฏิบัติการ และความแตกต่างเพียงอย่างเดียวที่พบคือมีการเปลี่ยนแปลงของกรดอะมิโน 6 ชนิด บริเวณ variable region ของยีนนี้ในไอโซเลต FC27 อย่างไรก็ตาม เมื่อศึกษาเพิ่มเติมจากเชื้อที่เก็บจากแหล่งระบาดในประเทศไทยจำนวน 168 ตัวอย่าง พบว่ายีนนี้มีความหลากหลายมากขึ้น โดยแบ่งออกได้เป็น 10 กลุ่ม ซึ่งแต่ละกลุ่มจะพบในความถี่ที่ต่างกัน กลุ่มที่พบมากที่สุดคือ F32 พบร้อยละ 67 รองลงมาคือ กลุ่มที่มีความเหมือนกับกลุ่มแรกแต่มีการเปลี่ยนแปลงของกรดอะมิโนเพียงหนึ่งตำแหน่ง กลุ่มนี้พบร้อยละ 19 กลุ่มที่ 3 คือกลุ่มของ FC27 ซึ่งพบเพียงร้อยละ 6 สำหรับกลุ่มที่ 4 ถึง 10 จะพบรวมกันเพียงร้อยละ 8 จากการศึกษาครั้งนี้พบว่าความถี่ของความหลากหลายของยีน Pf155/RESA ในกลุ่มของไอโซเลตที่เก็บจากแหล่งระบาดจะสูงกว่าไอโซเลตที่เลี้ยงไว้ในห้องปฏิบัติการอย่างเห็นได้ชัด การที่เชื้อแต่ละสายพันธุ์มีรูปแบบของยีนนี้แตกต่างกันไปจะยังผลให้มีความได้เปรียบต่อการถูกคัดเลือกในการดำรงอยู่หรือไม่ยังไม่ทราบแน่ชัด

คำสำคัญ : พลาสโมเดียม ฟัลซิปารัม แอนติเจน Pf155/RESA
ความหลากหลายของแอนติเจน ความหลากหลายของยีน

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Abstract : Naowarat Seesod Kanchanakhan and Lena Aslund. 2002. Polymorphism of the Pf155/RESA antigen among field isolates of *Plasmodium falciparum* in Thailand. Thai J Health Res 16(2): 115-134.

The sequence diversity of the blood stage antigen Pf155/RESA in *Plasmodium falciparum* was investigated in laboratory strains of the organism as well as in fields isolates collected from endemic areas of Thailand. The gene was cloned from an African isolate F32 and its nucleotide sequence was determined. Comparison with other isolates revealed a striking sequence conservation except in the variable region where a 6 amino acid replacement has occurred in FC27 compared to the F32 strain. These sequence variants were the only ones found in the range of laboratory clones and isolates tested. However, analysis of 168 field samples from Thailand demonstrated a higher degree of polymorphism in this region. The sequence variants could be devided into 10 groups and occurred with differing frequency among the field isolates. The most prevalent variant was the F-32 type which was present in 67% of the samples. The second most frequent variant was identical to the F-32 type, except for one amino acid replacement and occurred in 19% of the patients. The FC-27 type, was only rarely present, having a frequency of about 6%. The remaining variants IV-X were found in just 8% of the field samples. Polymorphism in the parasite population is thus more frequent in field isolates than in parasites grown in vitro. Whether this reflects any selective advantage for the different variants is not known.

Key words : *Plasmodium falciparum*, Pf155/RESA Antigen, polymorphism, sequence variants

Introduction

Malaria is one of the most widespread tropical diseases. In attempts to develop a malaria vaccine, several antigens have been characterised and proposed as vaccine candidates. Among the antigens present in the asexual erythrocytic stage of *P. falciparum*, much interest has been focused on Pf155/RESA (Ring-infected erythrocyte surface antigen) (Perlmann, *et al.*, 1984; Coppel, *et al.*, 1984). The antigen is localised in the dense granules of the merozoite from which it is released into the parasitophorous vacuole during invasion and associates with the erythrocytic membrane (Aikawa, *et al.*, 1990; Culvenor, *et al.*, 1991; Vera Bravo, *et al.*, 2000). The major site of attachment to the membrane has been found to be the binding of RESA to spectrin, restricted to a domain of 48 amino acids within the antigen (Foley, *et al.*, 1991; Ruangjirachuporn, *et al.*, 1991; Foley, *et al.*, 1994). Another domain of the antigen shares homology with DnaJ, an *Escherichia coli* chaperone (Bork, *et al.*, 1992), suggesting that RESA is involved in the stabilisation of the erythrocyte membrane (Da Silva, *et al.*, 1994). The antigen has been proposed to be involved in the invasion event since both polyclonal and monoclonal anti-Pf155/RESA antibodies efficiently inhibit invasion of erythrocytes *in vitro* (Wahlin, *et al.*, 1984) and also inhibit parasite growth *in vitro* (Siddique, *et al.*, 1998). Partial protection has been induced in monkeys immunised with certain fusion polypeptides containing Pf155/RESA fragments (Collins, *et al.*, 1986). Sero-epidemiological studies indicate that the immune responses to Pf155/RESA may be protective and passive transfer of human antibodies against immunodomonants epitopes of the antigen has also been suggested to protect experimentally infected monkeys (Petersen, *et al.*, 1990; Riley, *et al.*, 1991; Berzins, *et al.*, 1991).

Like many other malarial antigens, the Pf155/RESA contains blocks of amino acid repeats (Coppel, *et al.*, 1984; Cowman, *et al.*, 1984; Favaloro, *et al.*, 1986). The 5' repeat region encodes degenerate units of 11 amino acids and the 3' region encodes an octapeptide repeated 5 times preceeding 36 repeats of a tetrapeptide with a few variants. Most anti-Pf155/RESA antibodies induced by natural infections are directed to these repetitive epitopes (Berzins, *et al.*, 1986; Aslund, *et al.*, 1987). However, reactivity to specific epitopes in the nonrepetitive regions has also been shown (Perlmann, *et al.*, 1989).

In view of the antigenic diversity found among several malarial antigens suggested for inclusion in a vaccine, it is of important to define invariant epitopes. The epitopes of Pf155/RESA seem to be conserved among isolates since no major difference in reactivity of antibodies could be detected (Berzins, *et al.*, 1986; Perlmann, *et al.*, 1989). The complete

nucleotide sequence of the gene encoding the antigen from the only one strain of *P. falciparum* (Favaloro, *et al.*, 1986) and partial sequences from two other isolates have been obtained (Cowman, *et al.*, 1984). A comparison of these sequences revealed that the gene is well conserved. However, for several other *P. falciprum* antigens a higher degree of polymorphism has been shown to exist in fields isolates than in laboratory strains (Suewin, *et al.*, 1991).

In order to investigate further the variability of the Pf155/RESA antigen, we have cloned the gene from F-32, a Tanzanian isolate of *P. falciparum*. The complete nucleotide sequence has been determined and compared with two previously published sequences, FC27 (Papua New Guinea) and NF7 (Ghana) isolates. Restriction enzyme analysis fragments, amplified by polymerase chain reaction (PCR), from six additional strains revealed no differences in restriction patterns except for a restriction fragment length polymorphism (RFLP) earlier reported, and a difference in the lengths of the C-terminal repeat block suggesting that the Pf155/RESA gene occurs in several different variants. This variability has been further demonstrated in a large number of fields samples from endemic areas in Thailand.

Materials and Methods

Parasites. Strains were cultivated *in vitro* essentially according to condition described by Jensen and Trager (Jensen and Trager, 1977). These strains included F32, Palo Alto, FCR3 (Africa); ITO4, B11 (South America); K1, Thai1 (Thailand); FC27, E12, D10 (Papua New Guinea); the clone HB3 (Honduras) and the IMTM22 (Brazil) clone 7G8. The clones TM142/CB1, TM142/CB7, CH150/7 and CH150/3 originated from Thailand. The clone 3D7 and isolate BRAZIL, PAK+ and Bonjul were kindly donated by Denise Mattei, Pasteur Institute, Paris.

DNA isolation. DNA was extracted from asynchronous parasite cultures by a modification of a previously described method (Brawerman, *et al.*, 1972). After lysis of infected erythrocytes in 0.8% NH_4Cl, pH 7.4 for 10 min at 37 °C, the pelletted parasites were lysed in 0.15 M NaCl, 10 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$ and 0.6% NP40 on ice for 10 min. After centrifugation at 3000 rpm for 10 min the supernatant were removed and the pellet was incubated with 4% Sarkosyl and 1 mg/ml proteinase K at 37 °C for 1 h. After extraction with phenol and chloroform the DNA was precipitated and immediately dissolved in TE-buffer (10 mM Tris pH 7.6 and 1 mM EDTA).

Cloning of the Pf155/RESA gene. Four sets of oligonucleotides were synthesized in pairs generating overlaping fragments that covered the gene. The oligonucleotides (27-33 nt in length) were synthesized with restriction enzyme recognition sites suitable for cloning into the plasmid vector pUC19. Polymerase chain reaction was carried out as described (Saiki, *et al.*, 1988). A standard reaction with a volume of 100 µl was as follows: 10-30 ng of genomic DNA; 25 ng of each primer; 0.2 mM of each dNTP; 50 mM KCl; 10 mM Tris pH 8.4; 2.5 mM $MgCl_2$ and 2.5 Units of AmpliTaq polymerase (Perkin Elmer Cetus). The thermoprofile was 0.5 min in 94 °C, 2 min in 50 °C, 2 min in 72 °C and repeated for 30 or 35 cycles. The amplified products were cleaved with corresponding enzymes and subcloned into pUC19.

Restriction mapping of PCR products. For restriction enzyme analysis of amplified PCR products, 0.5 µCi of ^{32}P-labelled dCTP or dTTP was added prior to the amplification out of which 5 µl was used in each cleavage reaction and the products were separated on 10% PAGE and autoradiographed. Restriction fragment length polymorphism in exon 1 was assayed by *Rsa*I digestion of ^{32}P-labelled PCR fragments extending from the initiation codon of exon 1 to position 685.

DNA sequence analysis. The dideoxy chain termination methods was employed for sequence determinations (Sanger and Coulson, 1977). Between 2-5 clones generated from two independent amplifications were sequenced. The sequence were generated from both strands. The sources of the DNA sequences from two other isolates are Favaloro and colleagues (Favaloro, *et al.*, 1986) and Cowman and colleagues (Cowman, *et al.*, 1984). Solid phase sequencing was performed essentially as described (Hultman, *et al.*, 1989). As sequence primers, for the variable region oligonucleotides were synthesized with amino groups at the 5' end and biotinylated according to the method described (Hultman, *et al.*, 1989). Fifty µl of the PCR mixture were incubated with magnetic beads coupled to streptavidine (Dynabeads M-280-Streptavidine, Dynal AS, Norway). Both the immobilised and the eluted strands were sequenced using the Sequenase kit (USB, USA.). All the field samples were sequenced by the solid phase sequencing method.

Thai field isolates and samples. There were three sources of blood samples (i) cryopreserved infected red blood cells (ii) infected red blood cells from continuous culture and (iii) infected red blood cells of malaria patients collected directly from the field. Either isolates or clones from 168 blood samples were identified. All of these samples were collected from several endemic areas in Thailand during 1978 to 1994.

Preparation of samples. The templates were obtained by different methods; from all the freshly collected blood samples by immunomagnetic separation (IMS) (Seesod, *et al.*, 1993); from cryopreserved field samples by phenol extraction; and from *in vitro* cultures through boiling prior to PCR. DNA template preparation was performed by 3 procedures:

(i) Immunomagnetic separation: Ten µl of infected red blood cells, taken by fingerpuncture from malaria patients attending malaria clinics, were mixed with 90 µl of lysis buffer (0.5% NP40) and transported on ice for several hours depending on the distance from endemic areas to the laboratory in Bangkok. In the laboratory, 200 µl of beads coated with a monoclonal anti-MSP1 antibody were added and incubated at room temperature for 30 min, under gentle rotation (Seesod, *et al.*, 1993).

(ii) Boiling procedure: 20-30 µl of infected red cells from continuous culture were added with an equal volume of sterile water and boiled for 5-10 min. After spinning at 10,000 rpm for 10 min the supernatant was transferred to a new tube and used as a DNA template.

(iii) Phenol-Chisam extraction: 400 µl of cryopreserved infected red blood cells were thawed at 42-45 °C and washed once with 1 ml PBS (10 mM Phosphate buffer, pH 7.4, 150 mM NaCl). After spinning at 13,000 rpm for 5 min the pellet was resuspended in 1 ml of cold PBS. After lysis with saponin to a final concentration of 0.05%, the parasites were recovered by centrifugation as above. The pellet was resuspended in 25 µl of digestion buffer (30 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0, 2% SDS, 10 µg/ml Proteinase K). Sterile water was added to a final volume of 100 µl and incubated at 55 °C for 4 hrs. Additional 300 µl of sterile water was added before phenol extraction. An equal volume of phenol, equilibrated with 0.1 M Tris-HCl, pH 7.0, was added and mixed gently. The mixture was spun at 13,000 rpm for 5 min, the aqueous phase was then reextracted as above with phenol/chisam (phenol/chloroform/isoamyl alcohol= 25/24/1). After precipitation, the DNA was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA pH 8.0).

Oligonucleotide sythesis. Two sets of oligonucleotides (outer, RIT405; 5'-CACAGCTTT AAATGCCGCTGAAC-3', and RIT406; 5'-ATCATGTTCTACATTTTCTTCAGC-3'; inner, RIT371, 5'-biotin-AATTGTTGTCAGATAATTCAGTAGATG-3',and RIT372, 5'-AATTGTTATCCGCTCACAATTGTTGTACATGTTCT GGTACATTTTC-3') were synthesized by phosphoramidite chemistry on an automated DNA synthesis machine (Gene Assembler Plus, Pharmacia LKB, Uppsala, Sweden) according to

the manufacturer's manual. The four primers are complementary to sequences in the gene encoding the Pf155/RESA *of P. falciparum*, amplifying the variable region (See below).

***In vitro* amplification.** The variable region of the Pf155/RESA gene was amplified by two sets of oligonucleotide primers in a nested procedure. The PCR mixture consisted of 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 Mm KCl, 0.1% Tween 20, 0.2 mM deoxynucleotide triphosphates, 0.2 μM primer and 1.0 U of AmpliTaq DNA polymerase (Cetus, Newwalk, Conn.) to a total volume of 50 μl. The PCR was performed by using a Perkin- Elmer Cetus instrument. The first amplification step was performed with the outer primer pair (RIT405 and RIT406) for 35 cycles. From this PCR, 5 μl was used as template in the inner amplification step with the primer pair (RIT371 and RIT372) for 30 cycles, generating the specific 246 bp fragment. The temperature cycle consisted of denaturation (96 °C, 0.5 min), annealing (60 °C, 1 min) and extension (72 °C, 2 mins). The amplified product was analysed by agarose gel electrophoresis.

Results

Cloning of the gene encoding the Pf155/RESA antigen. Four overlapping fragments covering the gene were amplified from genomic DNA of the *P. falciparum* isolate F32 and cloned into a plasmid vector (figure 1A). The nucleotide sequence of the complete gene was determined, spanning the two exons and the short intervening sequence of the gene. Sequence analyses of several clones containing the intron showed different lengths of the two polyT stretches, probably due to an effect of the Taq polymerase during the amplification. Clones generated from PCR fragments encoding the C-terminal repeats showed deletions containing different number of repeats. The sequence from this region was deduced from clones encoding the highest number of repeats.

Sequence comparison of three isolates. The deduced sequence from the strain F32 was compared to corresponding sequences from the strain FC27 and NF7 published earlier (Cowmwn, *et al.*, 1984; Favaloro, *et al.*, 1986). The sequence for the 5' region of the gene was available only from the FC27 isolate. Overall, the gene was found to be highly conserved at the nucleotide level in all three isolates. The 5' block of degenerate oligopeptide repeats was completely conserved in the three isolates. The region consists of 7 repeat units with a length of about 11 amino acids and shows similarities to the C-terminal repeats.

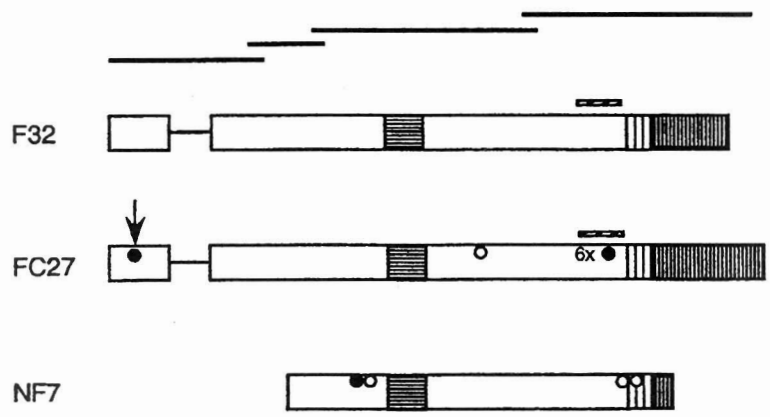


Figure 1A. Schematic comparison of the gene encoding the Pf155/RESA. The gene was cloned by PCR amplification of the regions indicated by black bars. The complete nucleotide sequence of the gene from the F32 isolate was determined. The sequence is compared with the Pf155/RESA sequences of the FC27 and NF7 isolates previously reported (Favaloro, *et al.*, 1986; Cowman, *et al.*, 1984). Hatched bars indicate the regions of oligopeptides present in the antigen. Nucleotide substitutions (O) and amino acid replacements (●) in comparison to the F32 isolate are indicated in the figure. The NF7 sequence starts at position 1027. The first exon spans the region from position 2 to 197 and the second exon starts at position 400. The *Rsa*I site is indicated by an arrow. The polymorphic region is indicated with a black bar and the six nucleotide substitutions and amino acid replacements occurring within this region are indicated.

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          958                                                997
F32-type  GGKRNDKKAKKYDIQDVVGYIMHGISTINKEMKNQNENV
          ----------------------------------------
FC27-type GGKRNDKKSKNFDIKDIVGYIMHGISTINTEMKNQNENV
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F32-type				FC27-type
Africa	South America	Asia	Europe	Papua New Guinea
F32	7G8	K1	3D7	FC27
Palo Alto	Brazil	Thai1		E12
Bonjul	HB3	TM142/CB1		D10
FCR3	FCBR	TM142/CB7		
PAK+	ITG24	CH150/3		
	ITO4	CH150/7		
	B11	CAMP		

Figure 1B Region of sequence polymorphism in Pf155/RESA.
The short region indicated by amino acid position comprising the six amino acid replacements is shown. The sequenced clones and isolates are listed according to which type of variant they possess.

The C-terminal differed, however, in the number of the tetrapeptide repeats, especially in comparison to the African isolate NF7, which terminates with a totally unrelated sequence. The five octapeptide repeats in the C-terminal (starting at position 2881) are perfectly conserved between F32 and FC27. This region is followed by 30 tetra peptide repeat units in F32 and 36 units in the isolate FC27. The amino acid sequences of the repeats are well conserved although some amino acid changes and several silent nucleotide substitutions occur.

A single base substitution confers a restriction fragment length polymorphism. One single base substitution was found when the nucleotide sequence of exon 1 was compared in the F32 and the FC27 isolates (Figure 1A). This substitution at position 51 also gives rise to an amino acid replacement from a Gly in FC27 to an Asp in F32. This substitution leads to a restriction fragment length polymorphism (RFLP) by abolishing a *Rsa*I recognition site. This is in agreement with earlier reported findings by Favaloro and colleaques (Favaloro, *et al.*, 1986), where five isolates could be assigned to two groups based on this polymorphism. The RFLP was confirmed in several different laboratory strains by restriction of an amplified fragment covering exon 1 (data not shown). The exon 1 from the FC27 isolate contained one *Rsa*I site which was abolished in F32 strain. The isolates Palo Alto and Thai1 showed the same pattern as F32, suggesting that they belong to the same allelic group (data not shown).

A region of sequence polymorphism in the RESA gene. When compared to the FC27 sequence, only two nucleotide substitutions were found up to position 2760 in the F32 sequence. The first substitution is at the polymorphic *Rsa*I site discussed above and the second base substitution, occurring at position 1773, is a silent change in the third codon position. This remarkable conservation between the isolates is lost in the region 5' of the C-terminal repeats. Within this short stretch of 70 bp, 6 base substitution occur, each giving rise to an amino acid replacement (Figure 1B). The substitutions are as follows: at nucleotide position 2764 (T to G) (Ser to Ala) at position 2772 (T to A) (Asn to Lys), at position 2774 (T to A) (Phe to Tyr), at position 2782 (A to C) (Lys to Gln), at position 2788 (A to G) (Ile to Val) and at position 2828 (C to A) (Thr to Lys). The NF7 and F32 are alike in this region. However, differences between F32 and NF7 are seen downstream in this region at position 2857 where an A to C substitution has occurred, not giving rise to any amino acid replacement. Furthermore, of two base substitutions at positions 1335-6 (AT to TA in NF7) the first is a silent substitution while the second leads to an amino acid

replacement (Ser to Thr). In addition, the C-terminal regions in NF7 and F32 are very different in terms of number of repeats.

In order to further investigate the occurrence of the two sequence variants, the nucleotide sequence of the polymorphic region was determined by direct genomic sequencing of additional twenty isolates and clones of *P. falciparum*. The results which are shown in Figure 1B demonstrate that the F32 variant was present in all but the clones D10 and E12, which originate from the FC27 isolate. No additional base substitutions were found in the sequenced region. The result suggests that the F32-type is common in strains of different geographical regions.

Restriction mapping of PCR products encoding Pf155/RESA from different isolates. In order to study further the conservation of the gene encoding Pf155/RESA in several additional isolates, overlapping fragments were amplified from genomic DNA of 6 different isolates and clones, all propagated in culture *in vitro*. The isolates were Palo Alto, K1 and 4 clones originated from Thailand; TM142/CB1, TM142/CB7, CH150/3 and CH150/7. The fragments were digested with several restriction enzymes (data not shown). No size differences were detected between the generated fragments in any of the six strains in comparison to F32 except for a *Rsa*I/*Mse*I cleavage where fragment spanning the 3' repeat region showed a slight difference in length. This is most likely due to a variation in the number of repeats present in the C-terminus of the gene. The results indicate that no larger deletions or insertions have occurred within the gene in the analysed strains and that the Pf155/RESA is well conserved between isolates, consistent with the sequence comparison above.

Sequence variability in field isolates from Thailand. To investigate the distribution of the two sequence variants of the Pf155/RESA gene and possible additional polymorphisms among field isolates, the variable region identified above was amplified and sequenced in a large number of samples. The patient samples were collected from several endemic areas in Thailand. In addition, several samples previously collected were also analysed. The location and the name of the areas are shown in table 1 and Figure 2, respectively. In total, 168 patient samples were used in the study. After sequencing the amplified region it was evident that the sequence variants could be divided into 10 groups (depicted by roman letters in Figure 3A). The variant I represents the F32-type described above and the variant II is the FC27-type. Three of the groups vary in more than one base, out of which variant II is most diverse with 6 base substitutions. Interestingly, all of the base substitutions give rise to amino

acid replacements except in the case of group X in which the single mutation generate a stop codon. The amino acid replacements for the corresponding regions are shown in Figure 3B.

Table 1 Distribution of variants among field samples

District	I	II	III	IV	V	VI	VII	VIII	IX	X	I+II	I+III	I+III+IV	sum
Sriracha	7	2	4									3		16
Trat	49	5	6	4		1					1	4	1	71
Tak	4		1											5
Trop-Med	1			1								1		3
Chantaburi	1													1
Pongnamroon	1													1
Kanchanaburi	1													1
Thongpapoom	42	3	6	2	1		2	1	1	1	1	10		70
sum	106	10	17	7	1	1	2	1	1	1	2	18	1	168



Figure 2 Map of Thailand showing the locations of the endemic areas from where the field isolates were collected.

The distribution of the sequence variants among the analysed field samples is shown in Table 1. All of the locations where samples were collected are endemic areas with various degree of transmission. However, the samples were collected at different occasions, regardless of seasons. Most of the samples were collected from the areas Trat and Thongpapoom, representing distinct geographical locations. Three of the samples were taken from patients admitted to the hospital for Tropical diseases, Faculty of Tropical Medicine in Bangkok. Twenty one out of the 168 samples demonstrated mixed infections of variants I+II, I+III or I+III+IV, respectively, out of which the combination of variant I+III was the most common. All the different variants occurred in the samples from Trat and Thongpapoom, however, variants V, VII, VIII, IX and X were present in the Thongpapoom isolates exclusively and variant VI was only found in the Trat samples.

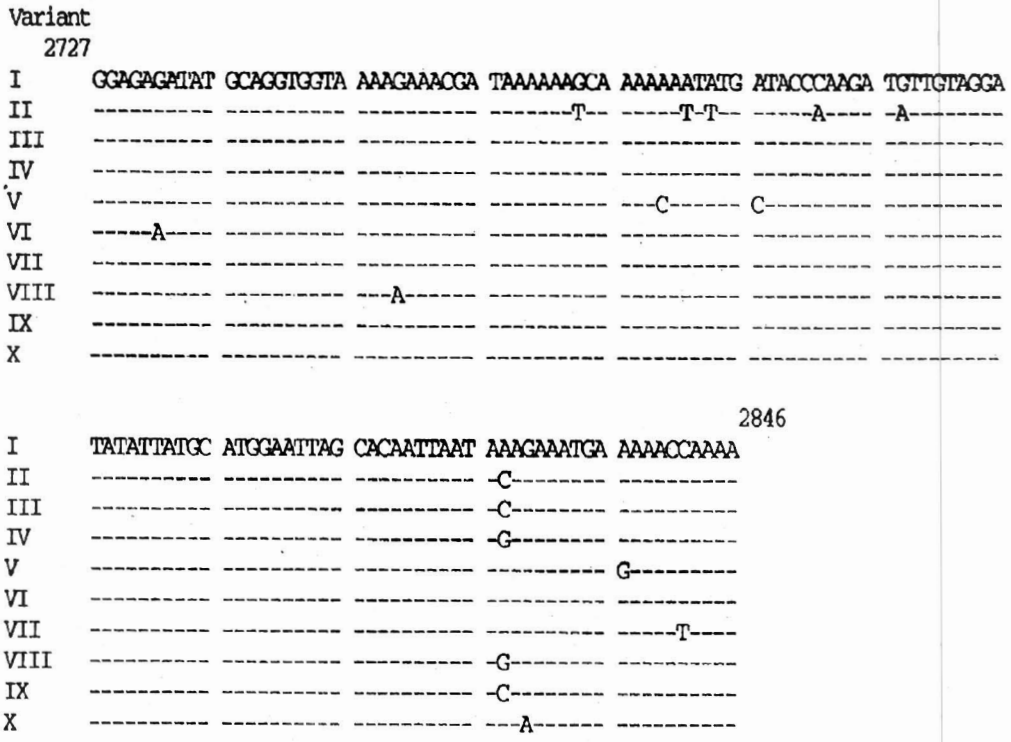


Figure 3A Sequence polymorphism among field isolates. The nucleotide sequence obtained from the different patient samples could be divided into 10 variants, numbered I-X. Only the base substitutions are indicated. The numbers indicate the nucleotide position in the F32 sequence.

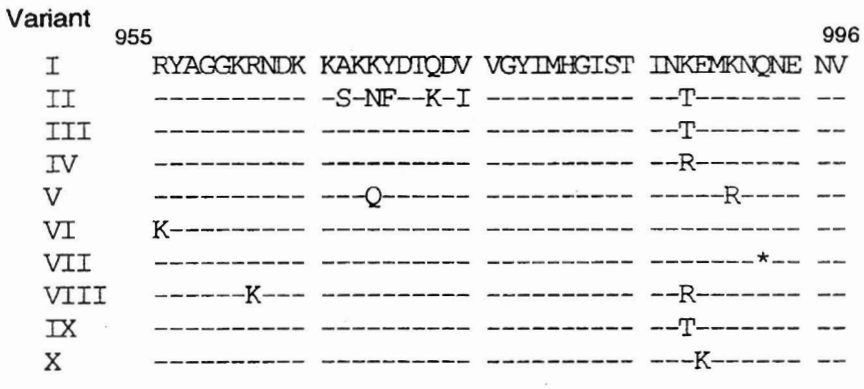


Figure 3B Sequence of the variants. The amino acid sequences of the ten variants I-X are shown. Variant I represents the F32 type and variant II represents the FC27 type. Only the amino acid replacements are indicated. The nucleotide substitution in variant VII generates a stop codon (*). The numbers indicate the amino acid position in the F32 sequence.

The most frequent allele of Pf155/RESA in the field isolates is the variant I, representing the F32-type sequence (see Table 2). This variant is present in 67% of field isolates, including the samples with mixed infections. The second most frequent allele (19%) is variant III which is identical to the F32-type except for one amino acid replacement of a lysine to a threonine residue (see Figure 3B). The FC27-type (variant II) is present in only 6% of the field samples from Thailand. The variants IV-X are much less frequent and represented in less than 8% of the samples studied. These results are consistent with the finding that the Pf155/RESA allele of the F32-type is most frequent in lab strains as well as in field isolates. Furthermore, a higher degree of sequence polymorphism is found among the field isolates than in laboratory strains as have been demonstrated for other malarial antigens.

Table 2 Frequency of the variants

Variant	Frequency (%)	Variant	Frequency (%)
I	66.84	VI	0.52
II	6.31	VII	0.52
III	18.94	VII	0.52
IV	4.21	IX	0.52
V	1.05	X	0.52

Discussion

We have studied the extent of sequence variability in the gene encoding Pf155/RESA, a blood stage antigen from *Plasmodium falciparum*, which is considered as a vaccine candidate. The gene was cloned from the African isolate F32 and the complete nucleotide sequence was obtained. The gene showed a high degree of sequence conservation when compared to sequences of isolates analysed earlier (Cowman, *et al.*, 1984; Favaloro, *et al.*, 1986) except for a short region juxtaposed to the C-terminal repeats. The conservation of the PF155/RESA sequence was further demonstrated by the lack of microheterogeneity in restriction enzyme digests of the gene from several parasite strains, demonstrating that no large deletions or insertions have occurred. These results revealed a high degree of sequence conservation in the gene encoding Pf155/RESA, consistent with the

reported lack of antigenic diversity in Pf155/RESA from different strains of *P. falciparum* (Perlmann, *et al.*, 1987).

The short polymorphic region comprised 6 nucleotide substitutions relative to the FC27 isolate. All these substitutions gave rise to amino acid replacements. The region was identical in F32 (Tanzania) and the African isolate NF7 (Ghana) (Cowman, *et al.*, 1984) as well as in the reported Palo Alto isolate (Kun, *et al.*, 1994). Sequence analysis of the region from several different isolates and clones, showed that the F32-type was much more predominant than the FC27-type, which was found only in two clones originating from the FC27 isolate (Papua New Guinea). The predominance of the F32-type among the laboratory strains and clones analysed could also be due to acquired genetic changes or selective growth of parasites during long-time cultivation *in vitro.* Another possible explanation for the high frequency of the F32-type is evidently cross-contamination of cultures in the laboratory (Robson, *et al.*, 1992).

The prevalence of the variants among field isolates were studied in blood samples, collected from several endemic areas in Thailand. All the sequence variants obtained from 168 samples could be grouped into nine categories, out of which the variant I and variant II represented the above described types F32 and FC27, respectively. However, the existence of additional variants of the Pf155/RESA gene can not be excluded since the nucleotide sequences of the field samples only covered the short polymorphic region. Regardless, these results demonstrate a higher sequence diversity in the wild parasite population.

The prevalence of alleles for a several antigens has been shown to vary in natural parasite population both geographically (Creasey, *et al.*, 1990) and with time (Babiker, *et al.*, 1995). The variant I (F32-type) was shown to be by far the most prevalent allele and present in almost 70% of the samples analysed. The FC27-type (group II) occurs rarely, at a frequency of about 6% in field isolates. This finding correlates well with the distribution found among the laboratory strains. The F32 allele was also more frequent in patient isolates from Indonesia (Kun, *et al.*, 1994). However, none of the other variants, III or IV-IX, were found in any of the laboratory strains. This could reflect a selective growth of subpopulations from field isolates when establishing *in vitro* culture (Thaithong, *et al.*, 1988; Viriyakosol, *et al.*, 1994) or simply accumulation of mutations. All the additional nucleotide substitutions found in the groups were nonsynonymous except in the case of variant X, in which the substitution generates a stop codon. Non-synonymous substitutions have been found in several surface antigens and suggested to imply a high mutation rate and

a rapid selection of mutants (McConkey, *et al.*, 1992). Interestingly, nonsynonymous substitutions, at least in the case of CSP in *P. falciparum*, have been shown to be concentrated to regions of T-cell determinants (McCutchan, *et al.*, 1992; Hughes, 1991; Jongwutiwes, *et al.*, 1994). These polymorphisms have been suggested to favor selection for the parasite in order to evade T-cell recognition.

Interestingly, the polymorphic motif is located immediately upstream of the C-terminal repeats, in the region exhibiting amphiphatic properties, thought to be of importance for eliciting cellular immune responses (Perlmann, *et al.*, 1987; Kabilan, *et al.*, 1988). Thus, peptides which flank and shortly overlap this region have been shown to induce both T-cell and B-cell responses in individual donors primed to *P. f*alciparum by natural infection (Perlmann, *et al.*, 1989; Troye-Blomberg, *et al.*, 1989; Rzepczyk, *et al.*, 1988). Single amino acid replacements in some of these peptides have indeed been found to affect the magnitude of the antibody response (Perlmann, *et al.*, 1989). In fact, in sera from 15 Liberian immune donors reacting with peptides covering part of the polymorphic region in group I (aa 862 V, aa 875 K) and Group II (exchanges in corresponding positions are I and T, respectively) resulted in an immune reactivity which, on average, was 3 times higher for the group II (FC27) than for group I peptide (F32). When these sera were further analysed for reactivity with short synthetic peptides (6-8 aa) a notable difference was seen in the recognition of peptides containing the variable residue at position 987 (K/T). While all sera reacted with the peptides containing the F32 motif KEMKNQ, virtually no reactivity was seen with the corresponding FC27 peptide containing the motif TEMKNQ (Ahlborg, *et al.*, unpublished results). This discrepancy may reflect a more frequent exposure to parasites carrying the F32 allele but could also reflect a difference in immunogenicity of the two different motifs.

The differences in immunoreactivity to the allelic variants I (F32) and II (FC27), detected in the *in vitro* assays, could be part of a variant-specific immunity. However, as previously reported, the immunodominant regions of Pf155/RESA appear to be invariant and the relative importance of the variability described herein for immune protection is unknown.

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