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Characterization of a *Plasmodium chabaudi* gene encoding a protein with glutamate-rich tandem repeats

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Abstract Several highly antigenic proteins containing tandem repeats rich in glutamic acid residues have been described in *Plasmodium falciparum*. However, relatively little information is available about analogous genes in rodent parasites. This report describes a 4.2-kb genomic DNA fragment from *P. chabaudi* with a deduced amino acid sequence that is predominantly glutamate-rich tandem repeats. Several different monoclonal antibodies raised against a 93-kDa *P. chabaudi* protein, which does not correspond to the cloned DNA fragment, recognize a recombinant protein expressed from the 4.2-kb DNA fragment. The only sequence similarities between these two genes are tandem repeats with a predominance of glutamate pairs followed by a hydrophobic residue. This repetitious-sequence motif may be the basis for the observed cross-reactivity. A similar motif has been demonstrated to be the basis for antibody cross-reactivity between glutamate-rich proteins of *P. falciparum*. The expression of multiple glutamate-rich proteins with cross-reacting epitopes may be a general phenomenon in *Plasmodium* species.

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Introduction

The characterization of malarial antigens is an important step towards understanding anti-malarial immunity and developing a malaria vaccine. Several *Plasmodium falciparum* blood-stage antigens have been identified by screening recombinant DNA libraries with immune sera. Many of these antigens contain blocks of tandem repeats that are rich in glutamic acid residues. Examples of glutamate-rich blood-stage antigens included Pf155/RESA (Coppel et al. 1984), GLURP (Borre et al. 1991), MESA (Coppel 1992), Ag332 (Mattei and Scherf 1992), Pf11-1 (Scherf et al. 1992), and D260 (Barnes et al. 1995). These proteins are highly antigenic, but little is known about their biological functions or their roles in anti-malarial immunity. Furthermore, many of these glutamate-rich proteins exhibit cross-reacting epitopes (Mattei et al. 1989; Ahlborg et al. 1991; Wahlin et al. 1992). Although several glutamate-rich proteins have been described in *P. falciparum*, little work has been done on similar proteins in rodent malaria species. Phosphoproteins containing high levels of acidic residues have been identified on the host membrane of *P. berghei*- and *P. chabaudi*-infected erythrocytes (Wiser et al. 1988). The best characterized of these acidic phosphoproteins is a 93-kDa *P. chabaudi* protein called Pc(em)93. Several investigators have independently identified proteins referred to as Pch105/RESA, Pc96, and Pc90 which are now known to be homologues of Pc(em)93 (Holmquist et al. 1990; Wiser et al. 1997). Cloning and sequencing the Pc(em)93 gene from three different *P. chabaudi* lines revealed tandem repeats rich in glutamic acid residues (Schmitt-Wrede et al. 1993; Wanidworanun et al. 1997; Giraldo et al. 1998). Although quite similar, the sequences of the tandem repeats vary between parasite lines. This report describes a distinct *P. chabaudi* gene encoding glutamate-rich tandem repeats which are recognized by monoclonal antibodies against Pc(em)93.

Materials and methods

Parasites

P. chabaudi chabaudi (strain 54X) was obtained from Dr. R. Walter (Bernhard Nocht Institut, Hamburg, Germany) and maintained by serial passage in CD1 outbred mice. Strain 54X was originally isolated by Dr. I. Landau (Landau and Killick-Kendrick 1966), acquired by Dr. P.I. Trigg (Trigg 1968) and then supplied to Dr. Walter. Mice were acquired from Charles Rivers Laboratories and maintained at the Tulane University Medical Center vivarium. Parasitemia was monitored by Giemsa-stained thin blood smears obtained from the tail. The IP-PC1 strain of *P. chabaudi chabaudi* was used in some of the studies and maintained as described elsewhere (Deleersnijder et al. 1992). Parasitized erythrocytes were passed over a CF-11 (Whatman) column and washed three times with Hanks' balanced-salts solution supplemented with 5 m*M* HEPES, pH 7.4 and 0.25% glucose. The cells were washed once in K-1 buffer (Wiser and Schweiger 1985) and resuspended in 10-20 times the initial packed cell volume. Infected erythrocytes were lysed with 0.01% saponin at 37 °C for 5 min and washed with K-1 buffer until hemoglobin free. The packed parasites were either immediately extracted for DNA or were stored at −75 °C until use.

Genomic DNA isolation

Packed parasites were resuspended in 5–10 volumes of STE buffer (10 m*M* Tris, pH 8.0, containing 0.1 *M* NaCl and 1 m*M* EDTA) containing 0.5% sodium dodecyl sulfate (SDS) and 0.1 mg/ml proteinase K. Following a 2-h incubation at 50 °C, additional proteinase K (0.1 mg/ml) was added and the incubation continued overnight. Insoluble material was removed by centrifugation and the lysate was extracted with an equal volume of saturated phenol for 4–16 h with gentle rocking. Genomic DNA (gDNA) was precipitated from the aqueous phase with 2.5 volumes of 100% ethanol (−20 °C and swirled out with a curved pasteur pipette). The gDNA was dissolved in STE buffer, treated with RNase A (0.1 mg/ml) for 30 min at 37 °C and subjected to another round of proteinase K digestion, phenol extraction, and ethanol precipitation. This final gDNA precipitant was dissolved in 0.5–1 ml of 10 m*M* Tris, pH 8.0, containing 1 m*M* EDTA, and stored at 4 °C until use. DNA concentration and quality were determined spectrophotometrically at 260 and 280 nm. Only gDNA preparations with A_{260}/A_{280} ratios of 1.6–1.8 were used.

Southern blot analysis

Ten micrograms of gDNA was digested in a volume of 100 µl with 10–20 units of restriction enzyme using the recommended buffer. After 2 h of incubation, 10–20 additional units of restriction enzyme were added and the incubation continued overnight. The digested gDNA was precipitated and electrophoresed on 0.75–0.8% agarose gels. After electrophoresis, the DNA was denatured by subjecting the gel to two 15-min washes in a 0.5 *M* NaOH plus 1.0 *M* NaCl solution followed by neutralization with two 15-min washes in 0.5 *M* Tris, pH 7.4, containing 1.5 *M* NaCl. DNA was transferred to nylon membranes (Micron Separation) using a Posiblot apparatus (Stratagene) and cross-linked to the membrane with ultraviolet radiation (BioRad, GS Gene Linker). A 2-h prehybridization was carried out at 42 °C in 6× SSC solution (15 m*M* citrate, pH 7.0, containing 0.15 *M* NaCl) containing 5× Denhardt's reagent (1% Ficoll plus 1% polyvinylpyrrolidone plus 1% bovine serum albumin), 50% formamide, 0.2% SDS, and 100 µg/ml herring sperm DNA (Sigma). The ^{32}P-labeled probe was added and hybridization carried out overnight at 42 °C High-stringency washes were performed and the membranes exposed to X-ray film.

Radiolabeled probes were prepared from recombinant DNA inserts which were excised from plasmids with restriction enzymes and gel purified using the Gene Clean Kit (Bio 101). The inserts were labeled by random priming according to the manufacturer's instructions (US Biochemical). Briefly, 25–50 ng of insert DNA was labeled using 50 µCi of α-^{32}P-dTTP (NEN) and the probe was separated from unincorporated nucleotides by centrifugation through a Bio-gel P2 (Bio-Rad) column equilibrated with 10 m*M* Tris, pH 7.5, containing 1 mM EDTA.

Preparation and screening of gDNA libraries

A λgt11 expression library was prepared from *P. chabaudi* (IP-PC1 strain) gDNA digested with *Eco*RI under star conditions as previously described (Deleersnijder et al. 1992) and screened with monoclonal antibody (Mab)-43. Mab-43 was generated from a fusion of the NS0/U myeloma cell line with spleen cells from mice made hyperimmune by repeated *P. chabaudi* (strain IP-PC1) infection.

Genomic DNA from the 54X strain was digested with *Pst*I and electrophoresed. The region of the gel corresponding to approximately 4.2 kb was excised and the size-fractionated gDNA was purified using the Gene Clean Kit (Bio 101). The size-fractionated gDNA was ligated into the *Pst*I site of pBSIIKS+ (Stratagene). This library was screened by probing colony lifts with ^{32}P-labeled P26-R6 insert DNA.

DNA sequencing

Sequencing was performed by the Sanger dideoxy chain termination method using the Sequenase 2.0 kit (USB). Progressive unidirectional deletion clones were prepared using controlled exonuclease III digestion and sequenced. Sequence data were analyzed and managed with either PCGENE software (Intelligenetics) or MacDNASIS software (Hitachi). The GenBank database was searched for similar sequences using the BLAST program (Altschul et al. 1990) via the National Center of Biotechnology Information e-mail server.

Expression and immunoblotting of recombinant P26-R6

The P26-R6 insert DNA from the λgt11 clone was subcloned into pBSIIKS+ and progressive unidirectional nested deletions prepared from the 3′ -end of the gene. *Escherichia coli* (JM83 strain) transformed with the various recombinants was grown in Luria broth containing 50 µg/ml ampicillin until reaching an A_{600} of 0.5. The expression of the P26-R6 insert is possibly due to sequences near the 5′-end of the clone which exhibit homologies to the −35 element and TATA box of *E. coli* (GenBank accession number L21713). Samples corresponding to 10^8 bacteria were analyzed by SDS gel electrophoresis and immunoblotting as previously described (Wiser et al. 1997). The antibodies used in the immunoblotting were Mab-43 (described above), Mab-13.5 (Wiser et al. 1988), Mab-B7E10 (Gabriel et al. 1986), Mab-8F2 (Wanidworanum et al. 1987) and Mab-6C12 (Wanidworanum et al. 1987).

Results and discussion

Mab-43 recognizes an approximately 93-kDa *P. chabaudi* protein referred to as Pc(em)93 (Wiser et al. 1997). A λgt11 cDNA expression library was screened with Mab-43, and clone P26-R6, containing an *Eco*RI insert fragment of 2.1 kb, was obtained. Analysis of *P. chabaudi* gDNA by Southern blotting indicated that P26-R6 hybridized to a 4.2-kb *Pst*I gDNA fragment. Screening a *Pst*I size-fractionated gDNA library with the P26-R6 probe resulted in the cloning of the 4.2-kb fragment,

which was designated as clone Pc4.2. The insert fragment from clone Pc4.2, *P. chabaudi* gDNA digested with *Pst*I, and a sample of original size-fractionate gDNA were analyzed by Southern blotting on the same gel with the P26-R6 probe. All three hybridizing DNA fragments are the same size (data not shown); thus, no detectable recombination has occurred in clone Pc4.2.

The sequence of clone Pc4.2 exhibits a high degree of identity to clone P26-R6 (Fig. 1). Nucleotides 2–1412 of clone P26-R6 (GenBank accession number L21713) are identical to nucleotides 1508–2918 of clone Pc4.2 (GenBank accession number U35149) except for an adenine at nucleotide 1381 in clone P26-R6 versus a cytosine at nucleotide 2887 in clone Pc4.2. The guanine at nucleotide 1 in clone P26-R6 versus an adenine at nucleotide 1507 in clone Pc4.2 is likely explained by the recognition of AAATTC by *Eco*RI under star conditions (Polisky et al. 1975), and the subsequent conversion to GAATTC following ligation into the *Eco*RI site of λgt11. Nucleotides 1413–2112 of clone P26-R6 exhibit no homology to clone Pc4.2. The lack of homology in the 3′-region of clone P26-R6 suggests that clone P26-R6 may be the result of a cloning artifact. This supposition was tested by carrying out Southern blots with DNA probes derived from a unidirectional nested deletion mutant encompassing the 5′-region (ND10 probe) or derived from a *Dra*I/*Eco*RI fragment (*Dra*I probe) corresponding to the 3′-region (Fig. 1). The ND10 probe and the *Dra*I probe exhibit different hybridization patterns in that they recognize different restriction fragments (Fig. 2). In addition, there is a possible *Eco*RI star site (GAAGTC) beginning at nucleotide 1413 in clone P26-R6. The sequence alignments and Southern blots indicate that the P26-R6 insert DNA does not represent a contiguous piece of DNA in the *P. chabaudi* genome, but is the result of either a spurious ligation or an anomalous recombination. The nearly perfect identity between the overlapping regions of the two recombinant DNA clones is not unexpected since the IP-PC1 line was derived from the 54X line (Coombs and Gutteridge 1975; David et al. 1978) the respective sources of clones P26-R6 and Pc4.2.

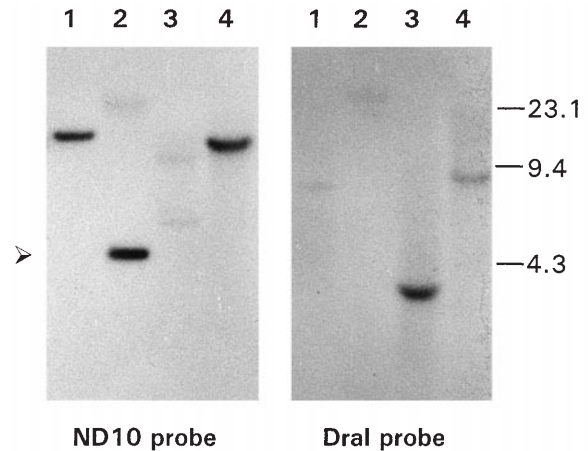
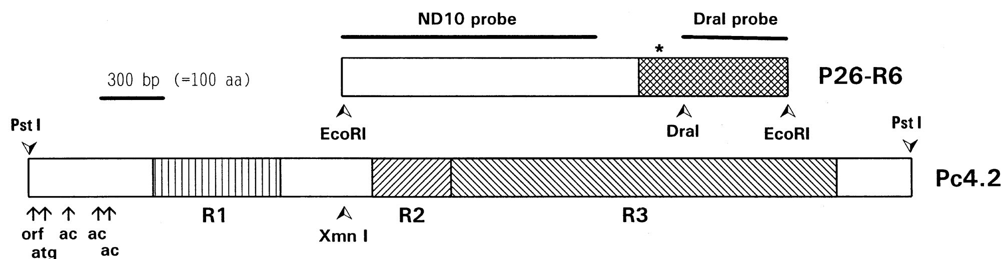


Fig. 2 Southern blot analysis. *Plasmodium chabaudi* gDNA was digested with *Hind*III (*lanes* 1), *Pst*I (*lanes* 2), *Nsi*I (*lanes* 3), and *Eco*RI (*lanes* 4) and electrophoresed as duplicate samples on the same gel. After electrophoresis, the DNA was transferred to a nylon membrane, and the duplicate blots were probed with either the *ND10* or the *DraI* probes (Fig. 1) as indicated. The *arrowhead* denotes the position of the 4.2-kb *Pst*I fragment (*lane* 2)

Clone Pc4.2 contains a single open reading frame (ORF) beginning at nucleotide 24 and continuing throughout the remainder of the sequence (Fig. 1), indicating that clone Pc4.2 is missing the 3′-end of the gene. Regarding the 5′-end of the gene, the first in-frame initiation codon is at nucleotide 81. This ATG does not have the characteristics of other *Plasmodium* start codons since it lacks adenines at positions −1 and −3 (Saul and Battistutta 1990; Robson and Jennings 1991). Furthermore, no typical eukaryotic promoter elements, such as TATA boxes or CAP sites, are detected upstream of this ATG. Other in-frame methionines (positions 1065, 1239, and 1375) are within the tandem repeats and therefore probably do not represent start codons. Splice junctions and branch points of *Plasmodium* introns are conserved with other eukaryotes (Brown and Coppel, 1991) and several potential splice acceptor sequences are observed at nucleotides 191–192, 332–333, 383–384, 426–427, and 440–441 (Fig. 1). However, no potential splice donor sequences are found. The sequence data are

Fig. 1 Schematic of clones P26-R6 and Pc4.2. Overlap between clone P26-R6 and clone Pc4.2 is indicated by the *open area* and the *cross-hatching* shows nonhomologous sequence. Stop codons are found throughout the nonhomologous sequence in all three reading frames and the *asterisk* denotes the position of the first in-frame stop codon. The *thick lines* denote the regions corresponding to a unidirectional nested deletion mutant (*ND10 probe*) and a *Dra*I/*Eco*RI fragment (*DraI probe*) which are used as probes in Southern blotting (Fig. 2). Three distinct blocks of tandem repeats, designated *R1*, *R2*, and *R3*, are observed in clone Pc4.2. A single open reading frame beginning at nucleotide 24 (*orf*) and continuing throughout clone Pc4.2 is observed. The positions of an in-frame ATG at nucleotide 81 (*atg*), three potential splice acceptor sites (*ac*), and some relevant restriction sites are denoted



most consistent with the 5′-end of clone Pc4.2 being within an intron and the presumptive splice donor being upstream of the 5′-*Pst*I restriction site.

The ORF of clone Pc4.2 encodes 159 kDa of a protein with particularly high levels of glutamate (14.6%), asparagine (11.5%) and isoleucine (10.5%). Approximately 80% of the Pc4.2 sequence is composed of three distinct blocks of tandem repeats (Figs 1, 3). Repeat block one (R1) contains five tandem repeats of either 38 or 33 amino acids and one partial copy of the repeat. Repeat block two (R2) contains five degenerate repeats of 25 amino acids and repeat block three (R3) contains 26 tandem repeats of 24 or 25 amino acids interrupted twice by a related 14-amino-acid sequence. The R2 and R3 repeats are contiguous and exhibit partial homology. All of the repeat blocks contain highly conserved glutamate residues throughout the respective repeats. Interestingly, some of these glutamates in the R2 and R3 repeats are spaced so that a glutamate residue occurs every 11–13 amino acids throughout the entire repeat block with only three exceptions (Fig. 3).

Clone Pc4.2 exhibits little homology to Pc(em)93 sequences (GenBank accession numbers L01040, U58989, and U80896) even though it was isolated as a result of screening expression libraries with an anti-Pc(em)93 Mab. The validity of the Pc(em)93 clones has been corroborated (Giraldo et al. 1998). Furthermore, the size of the clone Pc4.2 ORF, which is probably still missing sequence from both ends, is too large to be Pc(em)93. Therefore, clone Pc4.2 is not the gene for Pc(em)93 but represents another glutamate-rich protein which is recognized by Mab-43. Other anti-Pc(em)93 Mabs have also been tested for reactivity against clone P26-R6. Mab-13.5 and Mab-6C12 react with clone P26-R6, whereas Mab-8F2 and Mab-B7E10 do not (Table 1). Clone P26-R6 expresses 37 amino acids at the C-terminus which are not part of the clone Pc4.2 sequence (Fig. 1). It is unlikely, though, that the observed cross-reactivity is due to recognition of these 37 amino acids since absolutely no homology nor similarity is observed between these residues and the Pc(em)93 sequence (data not shown). However, the tandem repeats of clone Pc4.2 and Pc(em)93 do exhibit similarities (see below) suggesting that the cross-reactivity is due to the glutamate-rich tandem repeats.

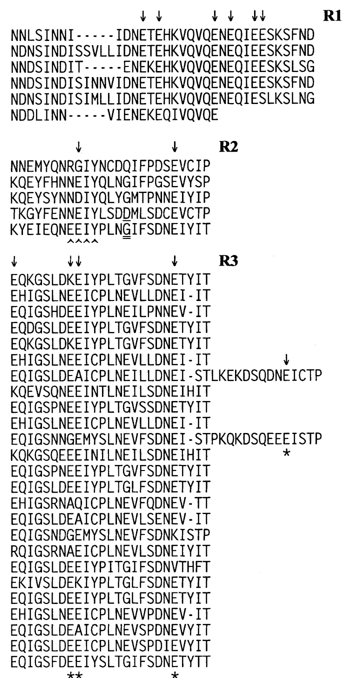


Fig. 3 Deduced amino acid sequence of Pc4.2 tandem repeats. The deduced amino acid sequence of repeat block one (*R1*), repeat block two (*R2*), and repeat block three (*R3*) is shown. The *arrows* denote highly conserved glutamate residues within the repeats and the *asterisks* are below glutamates that are evenly spaced every 11–13 amino acids. The *underlined residue* denotes the last amino acid of a unidirectional nested deletion mutant which does not express a protein recognized by Mab-13.5. The *double underline* denotes the last residue of the smallest nested deletion mutant which expresses a protein recognized by Mab-13.5. The ^^^^ denote an EEIY sequence within this 25-amino-acid region which is conserved in the R3 block

The cross-reactivity of these Mabs extends to other malarial proteins. For example, screening an expression library with Mab-B7E10 resulted in the cloning of clone Pch105e (Snounou et al. 1988) which also reacts with some of the other anti-Pc(em)93 Mabs (Holmquist et al. 1990). The sequence of clone Pch105e (GenBank accession number AF019972) is not homologous to either Pc(em)93 or clone Pc4.2, but encodes yet another protein rich in asparagine (16.2%) and glutamate (13.1%). In addition, some of the anti-Pc(em)93 Mabs are known to recognize proteins from other *Plasmodium* species. For example, Mab-B7E10 (Holmquist et al. 1988) and Mab-7C6 (Wanidworanum et al. 1989) cross-react with Pf155/RESA, and Mab-7C6 recognizes proteins from *P. vivax* and *P. cynomolgi* (Wanidworanum et al. 1989).

Searching databases with the Pc4.2 nucleotide and deduced protein sequences did not reveal any highly homologous sequences; thus, Pc4.2 represents a previously undescribed gene. A limited amount of similarity, however, is observed between Pc4.2 and several different *P. falciparum* glutamate-rich and/or asparagine-rich proteins, with Ag332 (Mattei and Scherf, 1992) and MESA (Coppel 1992) being the most notable. The

Table 1 Reactivities of anti-Pc(em)93 monoclonal antibodies with non-Pc(em)93 recombinant DNA clones. *Line* indicates the *Plasmodium chabaudi* line used to generate the Mab (*Pca* P. *c. adami*). *Escherichia coli* expressing clone *P26-R6* were analyzed by immunoblotting with the indicated Mab. The reactivity against clone *Pch105e* was derived from Holmquist et al. (1990) (*nd* not determined)

Mab	Line	Recombinant DNA Clone	
		Pc26-R6	Pch105e
13.5	54X	+	nd
43	IP-PC1	+	+
B7E10	AS	–	+
8F2	Pca	–	–
6C12	Pca	+	nd

similarities between the deduced Pc4.2 sequence and the sequences of Ag332 and MESA are due to resemblances in the tandem-repeat sequences. In particular, the tandem repeats of Ag332 and MESA both have numerous glutamate pairs followed by hydrophobic residues (e.g., EEV and EEI). The EEI motif is also found throughout the R3 repeats of Pc4.2 (Fig. 3). Antibodies against glutamate-rich proteins from *P. falciparum* often cross-react with other antigens. For example, Mab-33G2 primarily recognizes Ag332, but cross-reacts with Pf11.1 and Pf155/RESA (Mattei et al. 1989). The optimal epitope recognized by Mab-33G2 is VTEEI with the pair of glutamates followed by a hydrophobic residue being particularly important (Ahlborg et al. 1991). Similar motifs are found in the repeats of Ag332, Pf11.1, and Pf155/RESA, accounting for the cross-reactivity of Mab-33G2.

Unidirectional deletion mutants prepared from the 3′-end of clone P26-R6 were used to map the epitope recognized by Mab-13.5. Immunoblotting of *E. coli* expressing the nested deletion mutants demonstrated that the last residue of the smallest nested deletion recognized by Mab-13.5 is a glycine in the last repeat in the R2 block (Fig. 3). The last residue in the largest nested deletion not expressing an epitope recognized by Mab-13.5 is an aspartate in the preceding repeat. Therefore, the epitope recognized by Mab-13.5 is mapped to a 24-amino-acid region between the aspartate and glycine. The most striking sequence within this 24-amino-acid region is the EEIY, which is conserved throughout the R3 repeats and is similar to the EEIV and EENY sequences found in the repeats of the Pc(em)93 (Giraldo et al. 1998). These tetrapeptide sequences are similar to the sequences responsible for the cross-reactivity of antibodies with different *P. falciparum* glutamate-rich proteins with tandem repeats discussed above. Although the epitopes recognized by Mab-13.5 and the other *P. chabaudi*-specific Mabs have not been precisely mapped, it is plausible that the glutamate pairs in association with hydrophobic residues contribute to the antibody cross-reactivities.

In summary, several *P. falciparum* proteins containing tandem repeats rich in glutamic acids have been described. Relatively few proteins with similar properties are known in other *Plasmodium* species. This report describes a previously unreported *P. chabaudi* gene that contains tandem repeats particularly rich in glutamic acid and asparagine. As also observed in the *P. falciparum* glutamate-rich proteins, the deduced amino acid sequence of this protein contains pairs of glutamates followed by a hydrophobic residue. This motif appears to be the basis for the cross-reacting determinants with other glutamate-rich proteins. Therefore, the expression of numerous proteins with glutamate-rich tandem repeats that exhibit cross-reacting epitopes may be a general feature of *Plasmodium* species. The functions of these glutamate-rich proteins are not known, but have been speculated to divert the immune system or serve as a 'smokescreen' (Anders 1986). In other cases, such as MESA (Coppel 1992) and Pc(em)93 (Giraldo et al. 1998), the proteins have been proposed to play a structural role in the modification of the host erythrocyte membrane.

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