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Plasmodium vivax: Cloning and Expression of a Major Blood-Stage Surface Antigen

HERNANDO A. DEL PORTILLO,* JURG GYSIN,† DENISE M. MATTEI,* ELISABETH KHOURI,* PREETHI V. UDAGAMA,‡ KAMINI N. MENDIS,‡ AND PETER H. DAVID*

*Department of Immunology, Institut Pasteur, 28, Rue du Dr. Roux, Paris 75015, France; †Laboratoire d'Immunoparasitologie, Institut Pasteur, 94300 Cayenne, French Guyane; and ‡Department of Parasitology, Faculty of Medicine, University of Colombo, Sri Lanka

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DEL PORTILLO, H. A., GYSIN, J., MATTEI, D. M., KHOURI, E., UDAGAMA, P. V., MENDIS, K. N., AND DAVID, P. H. 1988. *Plasmodium vivax:* Cloning and expression of a major blood-stage surface antigen. *Experimental Parasitology* 67, 346–353. *Plasmodium vivax* is a highly prevalent malaria pathogen of man; the following report is the first to describe the cloning and expression of a major asexual erythrocytic stage antigen of this species. The screening of a genomic DNA expression library with a monoclonal antibody directed against a 200-kDa surface component (Pv200) of the more mature schizonts of *P. vivax* led to the selection of a recombinant bacterial clone which produced a fusion protein. Mouse and rabbit immune sera raised against the purified fusion protein recognized the 200-kDa parasite antigen on Western blots and reacted with the surface of segmenters by immunofluorescence. Sequencing of the 1.9-kb *P. vivax* DNA insert coding for this fusion protein revealed a 45–47% homology at the nucleotide level with the *P. falciparum* gene of a parasite surface antigen, Pf195, which has been shown to be a promising candidate for a malaria vaccine in primates and in man. © 1988 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Plasmodium vivax;* Blood-stage surface antigen; DNA sequence; Deoxyribonucleic acid (DNA); Indirect immunofluorescence antibody test (IFAT); Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE); Kilobase (kb); Kilo daltons (kDa); Molecular weight range (M_r); Base pair (bp).

INTRODUCTION

The strict species specificity of naturally acquired antimalarial protective immunity indicates that a vaccine against a given plasmodial species may not induce protection against another. However, most efforts aimed at developing a malaria vaccine have focused on *Plasmodium falciparum;* thus, relatively little is known about antigens of the other major human malarial parasite, *P. vivax*. The circumsporozoite protein is the only antigen of *P. vivax* that has been cloned to date (Arnot *et al.* 1985; McCutchan *et al.* 1985; de la Cruz *et al.* 1987) and characterization of gamete and asexual erythrocytic stage antigens has been achieved using monoclonal antibodies (Andrasiak *et al.* 1986; Barnwell 1986; Udagama *et al.* 1987; and Peiris *et al.* 1988).

A polymorphic 200-kDa component of the *P. vivax* schizont surface, defined by monoclonal antibodies (Udagama *et al.* 1987), appears to be analogous to a *P. falciparum* surface antigen, Pf195. The Pf195 antigen is present on the surface of the schizont as well as, in a processed form, on the surface of the merozoite (Holder and Freeman 1982). Several vaccination trials with Pf195 have led to a high level of protection against *P. falciparum* in nonhuman primates (Perrin *et al.* 1984; Hall *et al.* 1984; Cheung *et al.* 1986; Patarroyo *et al.* 1987; Siddiqui *et al.* 1986, 1987). Although Pf195 has been shown to be polymorphic, it contains portions which are conserved be-

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tween strains (McBride *et al.* 1985; Gentz *et al.* 1987; Tanabe *et al.* 1987) and protection induced by vaccination is not strain specific (Hall *et al.* 1984). Furthermore, the conserved regions of the amino terminus of Pf195 contain both B- and T-cell epitopes (Sinigaglia *et al.* 1988; Crisanti *et al.* 1988). In a recent vaccine trial on human volunteers, immunization with six polymerized peptides two of which were derived from the conserved amino-terminus regions of Pf195 conferred a significant level of protection against a challenge with *P. falciparum* asexual stages (Patarroyo *et al.* 1988). These results emphasize the potential role of Pf195 as a vaccine constituent against *P falciparum* asexual erythrocytic stages and it appeared important to further characterize the analogous antigen in *P. vivax,* Pv200. Here we report the cloning and expression of a large portion of the gene coding for this antigen.

Materials and Methods

Parasites. The *Plasmodium vivax* Belem strain, adapted to the squirrel monkey, was used throughout this study. Most leukocytes were removed by passing infected blood through a CF11 cellulose powder column (Whatman) as described by Homewood and Neame (1976). Parasites were then concentrated by centrifugation on a discontinuous 40–60% Percoll (Pharmacia) gradient as described by Ihalamulla and Mendis (1987). Such parasite preparations were used for the production of monoclonal antibodies, Western blots, and immunofluorescence. For DNA extraction, remaining leukocytes were removed as follows: the enriched parasite preparation was incubated for 20 min at room temperature in a 1/20 dilution of rabbit anti-human leukocyte antisera (see below); after washing in RPMI 1640 (Flow Laboratories) the cells were suspended in RPMI 1640 and passed through a 2-ml Sepharose 6B-protein A column (Sigma). the flowthrough parasite preparation was shown to contain less than one leukocyte per 10,000 parasites, as judged by microscopic examination of Giemsa-stained thin films.

Production of monoclonal antibody 6H1D11. Monoclonal antibody 6H1D11 was derived from Balb/C mice immunized with purified asexual erythrocytic stages essentially as described by Kohler and Milstein (1975). Briefly, mice were immunized with three doses of 10^8 parasites each administered intravenously at 3-week intervals. Three days after last immunization, spleen cells were fused by polyethylene-glycol treatment with the P3U1 myeloma cell line (Kearny *et al.* 1979). Selection of positive hybridomas was performed by indirect immunofluorescence using *P. vivax* parasites.

IFAT and Western blotting. IFAT and Western blotting using *P. vivax* parasites as antigen were performed as described in Udagama *et al.* (1987).

Construction and immunoscreening of a P. vivax *genomic DNA expression library in λgt11. P. vivax* genomic DNA was used to construct an expression library in λgt11 according to the method of Mattei *et al.* (1988). Briefly, DNA was partially digested with DNase I in the presence of Mn^{2+}, as to obtain DNA fragments of about 2 kb. After repair and addition of *Eco*RI linkers, the DNA was ligated to *Eco*RI-digested dephosphorylated λgt11 vector (Promega). Immunoscreening of approximately 10^5 recombinants was made with a 1/50 dilution of monoclonal antibody 6H1D11 followed by iodinated protein A (Amersham) as described by Mattei *et al.* (1988).

Mouse and rabbit antisera. Mouse (Balb/C) and rabbit immune sera directed against the fusion protein Pv200 were obtained by immunizing animals with recombinant products purified by electroelution from preparative SDS–PAGE of bacterial extracts (Hudson *et al.* 1983). Three intradermal injections of 200 μg each of purified antigen emulsified in Freund's adjuvant were administered at 3-week intervals and animals were bled 5 days after the last injection.

Rabbit anti-human leukocyte immune sera were obtained by immunizing animals with human peripheral blood lymphocytes purified on Ficoll–Paque (Pharmacia) (Fotino *et al.* 1971). Three injections of 10^7 cells were performed at 3-week intervals, the first subcutaneously with cells emulsified in Freund's complete adjuvant, the others intravenously with whole cells and no adjuvant. Animals were bled a week after the last inoculation. Serum was heat-inactivated at 56 C for 30 min and absorbed six times by incubation with washed human erythrocytes (v/v) for 30 min at room temperature.

DNA sequencing. The 1.9-kb *P. vivax* DNA insert from the positive λgt11 clone was subcloned into the *Eco*RI/*Pst*I site of Bluescript vector and a series of overlapping clones were generated by exoIII/mung bean nuclease deletions according to the manufacturer's instructions (Stratagene). The complete nucleotide sequence was determined by the Sanger dideoxy method (Sanger *et al.* 1977), and a search for homologies with sequences contained in the Los Alamos databank was made as described in Lipman and Pearson (1985).

Results

Monoclonal antibody 6H1D11 was selected as possibly reacting with the equiva-

lent in *Plasmodium vivax* of the *P. falciparum* Pf195 schizont/merozoite antigen based on the following observations: (i) On Western blots of *P. vivax* asexual stages, 6H1D11 recognized a parasite antigen of 200 kDa, Pv200 (Fig. 1-I). (ii) By IFAT with asexual erythrocytic stages of *P. vivax*, 6H1D11 was found to produce a distinct "grape-like" pattern of reactivity with the rim of the more mature stages of the parasite (segmenters) (Fig. 1-II). (iii) When different field isolates of *P. vivax* from Sri Lanka were examined by Western blots with 6H1D11, Pv200 was shown to exhibit size and epitope polymorphism (data not shown). Accordingly, this monoclonal antibody was used to screen a *P. vivax* genomic DNA expression library in λgt11. Screening of 10^5 recombinants led to the selection of one positive clone, Pv200, expressing a large β-galactosidase fusion protein (195 kDa) highly reactive with 6H1D11.

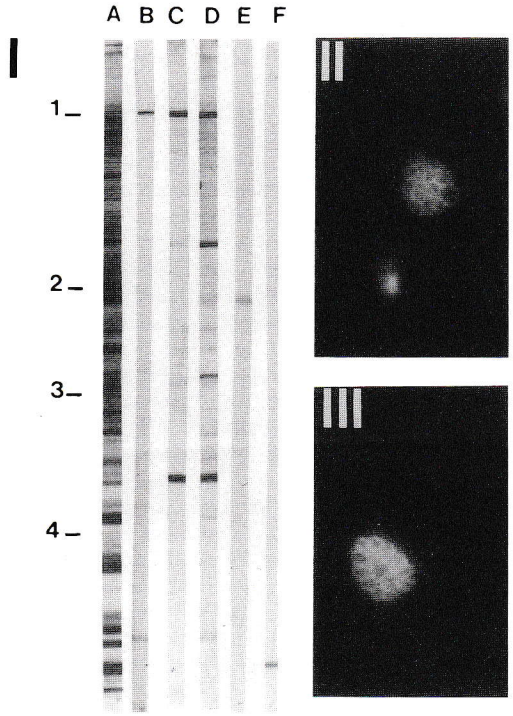

FIG. 1. Reactivity of the monoclonal and polyclonal antibodies against the *Plasmodium vivax* 200-kDa schizont/merozoite antigen. Fig. 1-I: Western blots of SDS–PAGE resolved extracts of asexual stages of *P. vivax*. Nitrocellulose strips onto which *P. vivax* antigen had been electroblotted were reacted with human hyperimmune serum (A), with monoclonal antibody 6H1D11 used to screen the genomic DNA library (B), with mouse (C), and rabbit (D), immune sera raised against purified Pv200 recombinant fusion protein, and with rabbit (E), and mouse (F), preimmune sera. Prestained molecular weight markers (Bethesda Research Laboratories): (1) myosim (220,000); (2) phosphorylase *b* (97,400); (3) bovine serum albumin (68,000); (4) ovalbumin (43,000). Fig. 1-II and III: Indirect immunofluorescence pattern of acetone fixed *P. vivax* parasites (segmenters) reacted with monoclonal antibody 6H1D11 (Fig. 1-II) or mouse immune serum raised against Pv200 recombinant fusion protein (Fig. 1-III).

The recombinant product was purified by preparative electroelution from SDS–PAGE and used to immunize rabbits and mice. Both species produced antisera which gave an identical "grape-like" pattern of immunofluorescence as did the original 6H1D11 with segmenters (Fig. 1-III). On Western blots, these sera recognized the 200-kDa parasite component (Fig. 1-I); the other lower M_r components recognized could constitute degradation or processing products of Pv200 such as those described in the case of Pf195 (Holder and Freeman 1982). Both by immunofluorescence and on Western blots, anti-Pv200 immune sera did not cross-react with *P. falciparum* parasites (data not shown).

To further confirm that Pv200 shared analogies with Pf195, the 1.9-kb *P. vivax* DNA insert from the original λ clone was sequenced (Fig. 2). The insert presents an open reading frame coding for 636 amino acid residues in frame with β-galactosidase. It has a G + C content of 47%, an estimated molecular weight of 72,179 Da, three potential glycosylation sites, and 15 repeated glutamine residues at its carboxy terminus. This sequence was then searched for homologies (Lipman and Pearson 1985) with sequences contained in the Los Alamos databank. The highest level of DNA homology, 45–47% was observed with the Pf195 genes from five different *P. falciparum* isolates (Holder *et al.* 1985; Mackay *et al.*

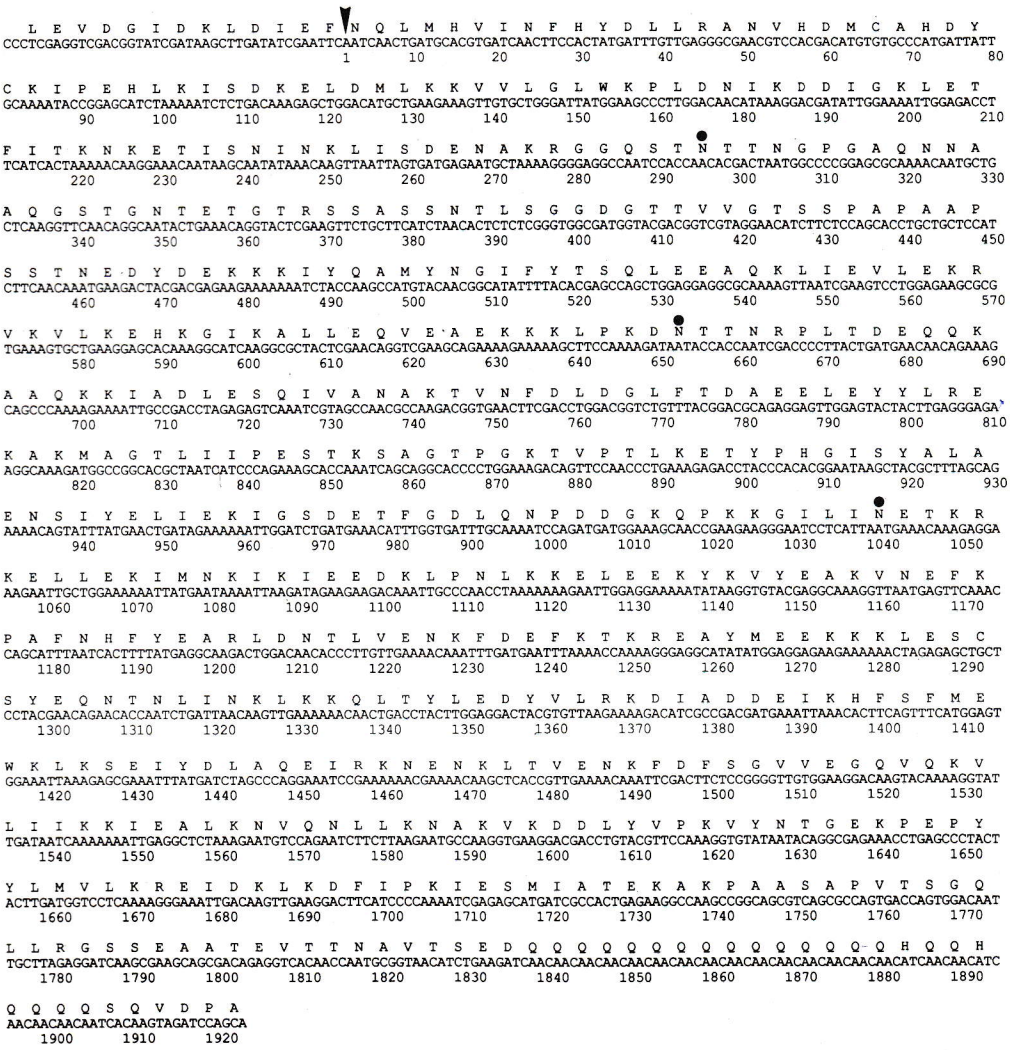

FIG. 2. Nucleotide and deduced amino acid sequences of the Pv200 clone. The arrow marks the limit between the β-galactosidase and the *Plasmodium vivax* insert. Potential *N*-glycosylation sites are denoted by circles.

1985; Weber *et al.* 1986; Tanabe *et al.* 1987; Certa *et al.* 1987; Weber *et al.* 1988). In contrast, when these comparisons were made at the amino acid level, only a 17% homology was detected among them (Fig. 3).

DISCUSSION

Plasmodium vivax is the most widely distributed human malarial pathogen and around 20 million cases a year are reported globally; however, molecular studies on this parasite have only recently been undertaken. (Reviewed in David *et al.* 1988). *P. vivax* cannot be maintained in continuous culture and the necessity to obtain parasite material from patients or infected monkeys has somewhat hindered research on an anti-*P. vivax* vaccine.

Results reported on the vaccination of primates and of human volunteers against *P. falciparum* asexual erythrocytic stages

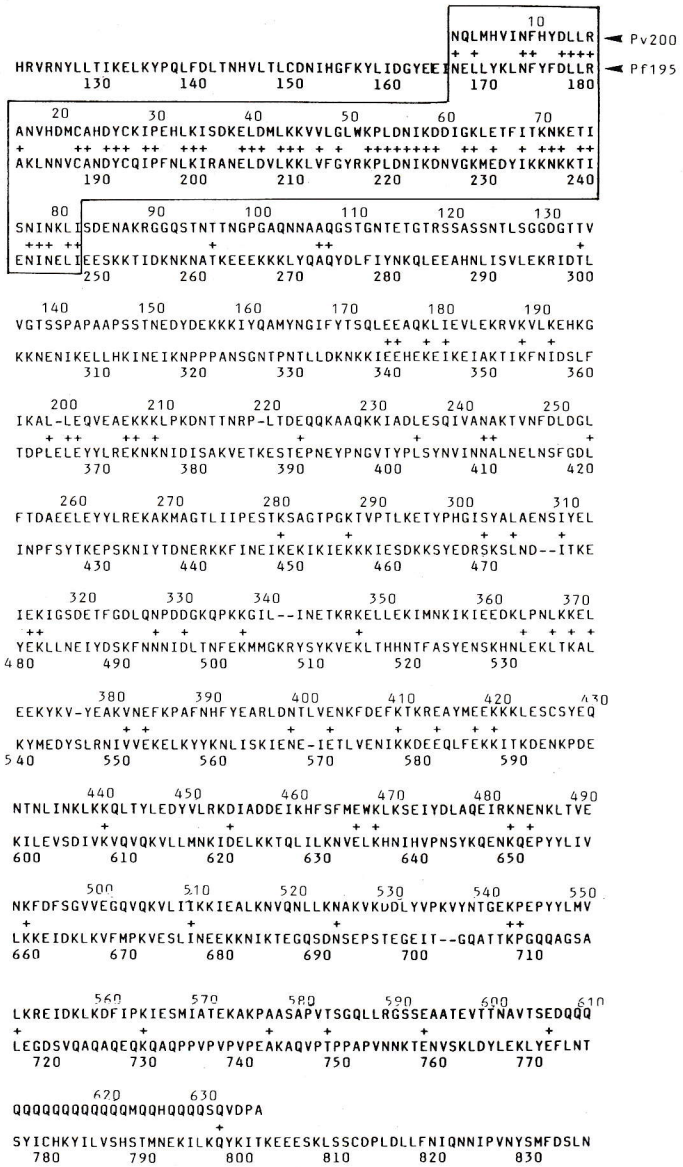

FIG. 3. Amino acid sequence comparison between the Pv200 clone and the Pf195 gene from the K1 strain (McKay *et al.* 1985). The comparison was obtained by the program of Lipman and Pearson (1985). Similar homologies and locations were observed with Pf195 gene sequences from other strains (Holder *et al.* 1985; Weber *et al.* 1986; Certa *et al.* 1987; Tanabe *et al.* 1987; and Weber *et al.* 1988). The boxed portion corresponds to the conserved region between *Plasmodium vivax* and *P. falciparum*.

have confirmed the importance of a parasite surface antigen, Pf195, as a vaccine candidate. The Pf195 antigen presents several characteristics. (i) It is present on the intraerythrocytic schizont; this leads to a typical "grape-like" pattern of fluorescence with anti Pf195 antibodies. (ii) It is processed to smaller M_r components. (iii) It exhibits size as well as epitope polymorphism in different strains. Our results show

that Pv200 shares these properties with Pf195, strongly suggesting that the two antigens are analogous.

The nucleotide sequence of the 1.9-kb *P. vivax* DNA insert coding for a predicted 72179 kDa polypeptide of the Pv200 antigen confirmed this analogy. Comparison between DNA sequences of the *P. vivax* clone and of the gene coding for Pf195 in five different isolates showed a 45–47% homology; this is high considering that Pf195 is a polymorphic antigen and that portions of its sequence differ markedly between isolates (no significant homology was detected between the Pv200 sequence and any other sequence of the databank). However, there are several differences between the Pv200 and the Pf195 sequences and their coding products. (i) Unlike the published sequence of Pf195 genes, the sequence of Pv200 contains a 15 × 3-bp repeat coding for 15 glutamine residues in its 3′ end (Fig. 2). (ii) the Pf195 gene contains an average G+C content of 26% in contrast to the coding sequences of Pv200 which contain an average G+C content of 47%. A similar G+C content has been reported for the coding region of the *P. vivax* circumsporozoite gene (Arnot *et al.* 1985; McCutchan *et al.* 1985; de la Cruz *et al.* 1987). These values thus reflect the differences in total genomic DNA composition of these two parasite species (McCutchan *et al.* 1984). (iii) There is no cross-hybridization between *P. falciparum* DNA and the Pv200 insert in Southern blots under stringent conditions (data not shown). This may be explained by the fact that the homologies between the Pv200 and Pf195 sequences are highly scattered as aligned by the computer program. (iv) In contrast with the homology seen at the nucleotide level, only a 17% homology was observed when deduced amino acid sequences of Pv200 and Pf195 were compared (Fig. 3); neither immunofluorescence nor Western blotting could detect any cross-reactivity between the two antigens. Interesting, a sig-nificant percentage (45%) of this amino acid homology resides between residues 1 and 82 of the *P. vivax* sequence. This delimits a zone in the amino-terminus part of the antigen that has been conserved not only in the different *P. falciparum* strains, but also between *P. vivax* and *Plasmodium falciparum,* two otherwise distantly related species (McCutchan *et al.* 1984). Such conservation suggests the association of an important functional role with this portion of the molecule.

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