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Plasmodium falciparum AARP1, a Giant Protein Containing Repeated Motifs Rich in Asparagine and Aspartate Residues, Is Associated with the Infected Erythrocyte Membrane

JEAN-CHRISTOPHE BARALE,[1] DANIEL CANDELLE,[1]† GÉRALDINE ATTAL-BONNEFOY,[1] PIERRE DEHOUX,[2] SERGE BONNEFOY,[1]‡ ROBERT RIDLEY,[3]§ LUIZ PEREIRA DA SILVA,[1] AND GORDON LANGSLEY[1]*

Unité de Parasitologie Expérimentale, Département d'Immunologie,[1] and Unité des Intéractions Bactéries-Cellules,[2] Institut Pasteur, 75724 Paris, France, and Department of Molecular Biology, University of Edinburgh, Edinburgh, Scotland[3]

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During *Plasmodium falciparum* asexual intraerythrocytic development, the host's cell plasma membrane is modified by the insertion of parasite proteins. One or more of these modifications mediate the cytoadherence of infected erythrocytes to host vascular endothelium. However, these surface antigens can be the target of cytophilic antibodies which promote phagocytosis of the infected erythrocyte. It has been proposed that antibodies directed to epitopes rich in asparagine play an important role in this process, which has promoted efforts to isolate the corresponding gene(s). We describe here *P. falciparum* asparagine- and aspartate-rich protein 1 (PfAARP1), a new giant (circa 700-kDa) protein associated with the infected erythrocyte membrane which is rich in asparagine and aspartate residues due to the presence of nine blocks of repeats. Topology analysis predicts that PfAARP1 has multiple transmembrane domains and at least five external loops. Human antibodies immunopurified against a sequence composed exclusively of asparagine and aspartate amino acids derived from PfAARP1 label the surface of the infected erythrocyte, demonstrating that such motifs are exposed. Interestingly, external loop 4 of PfAARP1 contains repetitions of these residues, and their possible role as a target of cytophilic antibodies is discussed.

The malaria parasite *Plasmodium falciparum* invades and develops within human erythrocytes, where infection leads to the generation of specific antibodies and to the slow acquisition of protective clinical immunity, termed premunition (35). The protective antibodies are believed to be directed against accessible epitopes located on the surface of either merozoites or infected erythrocytes. This observation has led to the use of hyperimmune sera from protected adults to isolate a number of membrane-associated parasite antigens (2). Malaria antigens associated with the infected erythrocyte membrane have also been widely studied due to their expected role in mediating cytoadherence. This phenomenon is the capacity of infected erythrocytes to adhere to host vascular endothelium and is thought to be linked to cerebral malaria (35).

Among the most studied groups of parasite proteins are those associated with electron-dense protrusions, or "knobs" (30). At least three antigens, KAHRP, PfEMP2-MESA, and PfEMP3 compose the knob structure without being exposed at the surface (14). The only parasite antigen clearly demonstrated to be exposed is PfEMP1 (29). The latter is encoded by a multigene family (*var* genes), where expression of different *var* genes leads to antigenic variation (7, 47, 49). Antigenic variation should impair an efficient immune response to the parasite protein which mediates cytoadherence (31). Knobs are far from the only alteration to the host erythrocyte; the cytoskeleton is also subjected to modification. Although the precise functions of both MESA-PfEMP2 and RESA remain to be elucidated, they are thought in some way to modify the erythrocyte cytoskeleton (11, 13, 16).

Two erythrocyte membrane-associated antigens (Pf11-1 and Pf332) are megadalton proteins containing repeated amino acids rich in glutamate residues (33). Whereas Pf11-1 is expressed exclusively in gametocytes, Pf332 is an asexual-stage specific antigen located in Maurer's clefts (23, 45). Due to the presence of diglutamic acid motifs, it has been proposed that Pf11-1 and Pf332 are involved in a cross-reactive epitope network which includes RESA, FIRA, and D260 (6, 32). In a way different from antigenic variation, this type of cross-reactive network may also function to impair an efficient immune response (3). Another group of related antigens which have been proposed to be membrane associated are those rich in asparagine, including the circumsporozoite protein (12, 17, 27, 48, 52). However, no clear function has yet been assigned to any of the above-mentioned blood stage proteins.

We present here the characterization of a new membrane-associated giant protein, *P. falciparum* asparagine- and aspartate-rich protein 1 (PfAARP1). PfAARP1 shares with PfAARP2 and PfAARP3 (5) the presence of repeated motifs ([E/N]NDDD) rich in asparagines and, more importantly, in aspartic acid. We show that human antibodies affinity purified on an asparagine- and aspartate-rich peptide derived from PfAARP1 label the surface of the infected human erythrocytes. The relevance of this observation in relation to PfAARP1's membrane localization is discussed.

* Corresponding author. Mailing address: Unité de Parasitologie Expérimentale, Département d'Immunologie, Institut Pasteur, 75724 Paris, France. Phone: 33 1 45688922. Fax: 33 1 40613185. E-mail: langsley@pasteur.fr.

† Present address: Câtedra de Morfofisiologia I, Area Ciencias de la Salud, UNEFM, Coro, Edo, Falcô, Venezuela.

‡ Present address: Department of Microbiology and Immunology, Stanford University Medical School, Stanford, CA 94305.

§ Present address: Pharma Research Preclinical Infectious Diseases, Pharma Division, F. Hoffmann-La Roche, CH-4070 Basel, Switzerland.

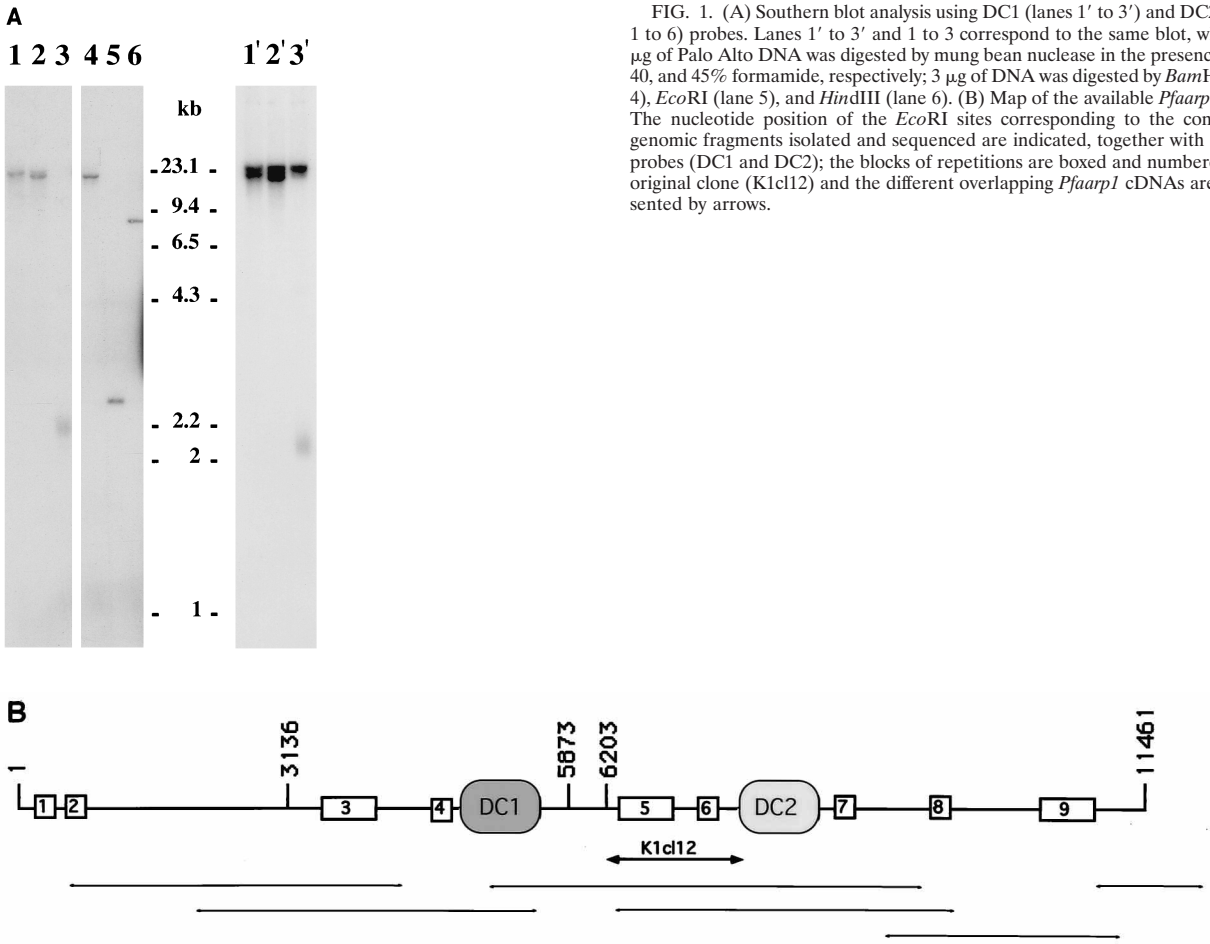

FIG. 1. (A) Southern blot analysis using DC1 (lanes 1′ to 3′) and DC2 (lanes 1 to 6) probes. Lanes 1′ to 3′ and 1 to 3 correspond to the same blot, where 10 μg of Palo Alto DNA was digested by mung bean nuclease in the presence of 35, 40, and 45% formamide, respectively; 3 μg of DNA was digested by *Bam*HI (lane 4), *Eco*RI (lane 5), and *Hin*dIII (lane 6). (B) Map of the available *Pfaarp1* locus. The nucleotide position of the *Eco*RI sites corresponding to the contiguous genomic fragments isolated and sequenced are indicated, together with the two probes (DC1 and DC2); the blocks of repetitions are boxed and numbered. The original clone (K1cl12) and the different overlapping *Pfaarp1* cDNAs are represented by arrows.

MATERIALS AND METHODS

Parasite culture. *P. falciparum* Palo Alto and 3D7 were maintained as described by Trager and Jensen (50). Synchronous cultures were obtained by alanine treatment (10) of parasitized erythrocytes, which were subsequently centrifuged and resuspended in 2 volumes of prewarmed (37°C) 0.3 M L-alanine in 10 mM HEPES for 3 min. Lysis was stopped by adding an excess of RPMI-serum; after centrifugation, the parasites were again placed in in vitro culture.

Genomic and cDNA library screenings. The original clone K1cl12 was isolated by immunoscreening of a genomic expression library by using a rabbit polyclonal antiserum raised to rhoptry-specific proteins (41). In enzyme-linked immunosorbent assays (not shown), this serum reacted with the K1cl12-specific NNDDD peptide. The genomic expression clone was then used to screen an *Eco*RI genomic library cloned in λ1149 (18). In this way, the corresponding 5,258-bp *Eco*RI fragment was isolated and sequenced. This fragment was used to screen a cDNA library cloned in pcDNAII (38), and the specific cDNA clones were isolated and sequenced. The relevant cDNA was then used to rescreen the genomic *Eco*RI library; in this way, the four contiguous *Eco*RI fragments representing 11,461 bp were isolated and sequenced.

***P. falciparum* DNA preparation and Southern blotting.** Mature parasites obtained by in vitro culture were lysed by 0.05% saponin treatment, and DNA was prepared from free parasites as described previously (44). Four micrograms of genomic DNA was digested with restriction endonucleases under the conditions recommended by the manufacturers. Mung bean nuclease (Amersham) digestions were done as described by McCutchan et al. (34). After ethanol precipitation, digested DNA was electrophoresed through 0.9% agarose gels and transferred to a nylon membrane (Hybond N^+; Amersham). DC1 and DC2 probes were PCR fragments amplified with the following oligonucleotides containing substituted bases (lowercase) to create *Bam*HI and *Eco*RI sites (underlined) for expression cloning: DC1a (5′-AATTCTCTggATCcAATGTTAATG-3′), DC1b (3′-GTATATATCGGAGAATTcTAAATATAG-5′), DC2a (5′-GTGTAGGGG aTccAGATTTTATTTG-3′), and DC2b (3′-CGTAGATTAGAATTcTATCCTG -5′).

Probes were [α-^{32}P]dATP labeled by random priming (Megaprime; Amersham). Membranes were hybridized overnight at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–2.5% milk–0.1% sodium dodecyl sulfate (SDS) and extensively washed at 65°C (see figure legends). Alkaline dehybridizations were performed according to Amersham protocols.

Chromosome separation. 3D7 and Palo Alto chromosome blocks were prepared as described previously (25) and migrated on a contour-clamped homogeneous electric field apparatus (Pharmacia). Chromosomes were separated in a 0.8% agarose (chromosomal grade; Bio-Rad) in 0.5× Tris-borate-EDTA; pulse time was ramped from 90 to 300 s for 24 h at 95 V followed by ramped switch time from 300 to 720 s for 24 h at 85 V (22). After the migration, the gel was stained in ethidium bromide, photographed, and then incubated in 0.4 M HCl before alkali transfer onto Hybond N^+ (Amersham).

PCR amplification, subcloning, and DNA sequencing. PCRs were performed in a Hybaid Thermalcycler apparatus, using a final concentration of 1 μM each oligonucleotide and the buffer conditions provided by the manufacturer (Amersham). The following cycles were used: hot start at 95°C for 2 min; *Taq* polymerase addition at 80°C; and 30 cycles at 90°C for 20 s, 50°C for 1 min, and 72°C for 2 min (the last cycle included an extension of 10 min at 72°C). An aliquot of the PCR mixture was electrophoresed on a 1.5% agarose gel. Uniquely amplified fragments of interest were subcloned into pCRII vector as instructed by the manufacturer (InVitrogen) except that unincorporated nucleotides and salts were removed by Chroma-Spin column (Clontech) centrifugation. When more than one DNA band was amplified, the fragment of interest was purified by using GeneClean (Bio 101) before ligation into pCRII. Recombinant clones were sequenced by using a U.S. Biochemical kit and [^{33}P]dATP.

Inferred amino acid sequence analysis and topology prediction. The DNA and inferred amino acid sequences were analyzed on the mainframe computer run by the Institut Pasteur Computer Services, using the Genetics Computer Group software package, version 8.0 (15). Database searches were carried out with the BLAST (1) family of programs in the Genetics Computer Group software package. Detection of a potential PEST signature was performed with the PEST-FIND program (39) implemented as a network service in the Biomedical Informatics Unit at http://www.biu.icnet.uk/projects/pest/. Evaluation of potential transmembrane domains was performed with the PHD topology algorithm im-

plemented as a network service in EMBL at http://www.embl-heidelberg.de/predictprotein/Dab/phd_htmtop.html) (42).

Immunization, affinity selection of antibodies, immunoblotting, and indirect fluorescent antibody (IFA) analysis. Antibodies were obtained by immunization of Biozzi mice (9) with the nonrepetitive residues contained in the glutathione *S*-transferase (GST)–DC1 fusion protein which was generated by amplification with DC1a and DC1b primers and cloned in frame (see above) into pGEX-1 (Pharmacia). For the first injection, mice were subcutaneously immunized with 10 μg of the GST-DC1 fusion protein in complete Freund's adjuvant and subsequently boosted five times with the same amount of recombinant protein in incomplete Freund's adjuvant. Mice were bled 8 days after each immunization.

Human hyperimmune sera that recognize the GST-DC1 fusion protein, the 19-residue peptide NNDDD (YNNDDDNDDDNDDNNDDNN is derived from repetitive block 6), and the LSA1 peptide were preselected by enzyme-linked immunosorbent assay. Immunoselection of human antibodies from positive serum was achieved by incubation with GST-DC1 fusion protein or peptide-saturated nitrocellulose filters. After extensive washing in phosphate-buffered saline (PBS; 10 mM potassium phosphate, 145 mM NaCl [pH 7.4]) containing 0.1% Tween 20, antibodies were eluted with HEPES-magnesium buffer as described previously (51). Selected antibodies were dialyzed overnight against an excess of PBS and conserved in PBS–50% fetal calf serum.

Soluble protein extracts were prepared from washed infected erythrocytes, resuspended in water, and lysed by successive freeze-thawing. After centrifugation, the supernatants and the pellets containing soluble and insoluble proteins, respectively, were separated on an SDS–7.5% polyacrylamide gel (28). Polypeptides were transferred to nitrocellulose and incubated in 50 mM Tris-HCl (pH 8)–150 mM NaCl–0.5% Triton X-100–5% nonfat milk powder for 1 h at room temperature in the presence of mouse sera diluted 1:100 or affinity-purified antibodies diluted 1:2. Membranes were then washed and incubated in a 1:4,000 dilution of peroxidase-coupled (Biosys) secondary antibodies or a 1:10,000 dilution of alkaline phosphatase-coupled (Promega) secondary antibodies in 50 mM Tris-HCl (pH 8)–150 mM NaCl–0.2% Triton X-100–2.5% nonfat milk powder. Detection was performed after washing, using enhanced chemiluminescence products (Amersham) or nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate substrates (Promega).

For immunofluorescence assays, immunopurified antibodies were incubated with air-dried fixed parasites for 30 min in a humidified chamber at 37°C. Slides were washed three times for 10 min each in PBS and incubated with goat anti-human immunoglobulin G and immunoglobulin M fluorescein-conjugated antibodies (Immunotech) in the presence of 50 μg of propidium iodide per ml. Slides were then washed in PBS, sealed in the presence of 0.1% *p*-phenylenediamine–0.5% glycerol as an antifading agent, and subsequently analyzed with a Leica microscope at 1,000-fold magnification under UV light. Surface immunofluorescence analysis was done according to the same protocol except that mature parasites were enriched by plasma gel flotation (36) and then extensively washed in PBS (parasites were centrifuged gently for 5 min at 500 × *g*) before incubation of live parasites in suspension together with antibodies. In both cases, the second fluorescein-coupled anti-human antibodies were preabsorbed for 1 h (37°C) on noninfected erythrocytes and were used as a negative control. Human sera from which antibodies had been selected were used at a dilution of 1/20 as a positive control.

Nucleotide sequence accession numbers. The GenBank/EMBL accession number of the *Pfaarp1* sequence is Y08926. The *Pfaarp2* and *Pfaarp3* sequences are also available (Y08924 and Y08925, respectively [5]).

RESULTS

***Pfaarp1* is a unique gene with a single open reading frame of circa 20 kb.** Originally, a partial genomic clone of 1.4 kb (K1cl12 [Fig. 1B]) was isolated as a false positive when a rabbit polyclonal serum raised against the low-molecular-weight rhoptry (RAP) complex was used to screen for the 80-kDa rhoptry antigen, RAP-1 (40). To characterize PfAARP1, we cloned (Fig. 1B) and sequenced over 11 kb of its gene from the K1 isolate. Confirmation that the genomic DNA sequence is indeed coding came from the isolation and sequencing (see Materials and Methods) of overlapping cDNAs covering 90% of the available *Pfaarp1* locus (Fig. 1B).

Mung bean nuclease combined with formamide denaturation cuts *P. falciparum* DNA at very A+T-rich noncoding regions and is used to identify the size of parasite genes (34). As can be seen from the mung bean nuclease Southern blot (Fig. 1), two probes (DC1 and DC2 [Fig. 1B]) from different parts of *Pfaarp1* hybridize to the same fragment of approximately 20 kb. We are confident that the circa 20-kb fragment (Fig. 1A, tracks 2′ and 2) is derived from complete mung bean nuclease digestion, since the same blot was hybridized with two

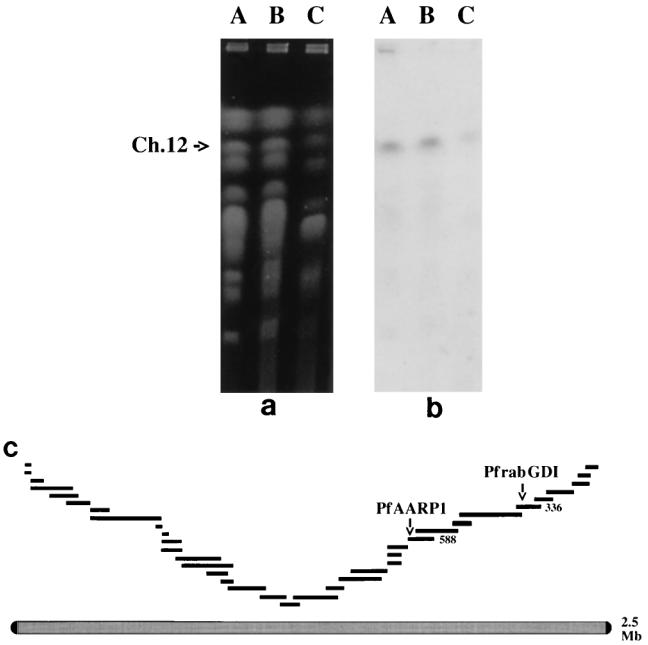

FIG. 2. (a and b) Chromosomal localization of *Pfaarp1* in *P. falciparum* Palo Alto (lanes A), 3D7 (lanes B), and FCR3 (lanes C). The *Pfaarp1*-specific DC1 probe hybridizes to chromosome 12 (Ch.12) of all parasite strains. (c) Locations of *Pfaarp1* and *Pfrab*GDI on their respective YACs.

other genes (*Pfaarp2* [5] and 96tr/GBP130), and specific fragments of the expected size were identified (data not shown). At 45% formamide (Fig. 1A, lanes 3 and 3′) mung bean nuclease cuts within the gene such that an approximately 2-kb smear is identified with each probe. When restricted genomic DNA is probed at high stringency (0.1× SSC–0.01% SDS at 65°C) *Pfaarp1* behaves as a unique gene (Fig. 1A, lanes 4 to 6).

***Pfaarp1* is centrally located on chromosome 12.** The chromosomal location of *Pfaarp1* was determined by hybridization to pulsed-field gel separated *P. falciparum* chromosomes of three different isolates (Fig. 2a). A single chromosomal band was identified in each isolate (Fig. 2b). Consistent with its location on chromosome 12, *Pfaarp1*-specific oligomers were used to identify by PCR the corresponding yeast artificial chromosome (YAC) (49a) whose position on the chromosome contig had already been determined (46). Interestingly, for a gene encoding a repetitive antigen, *Pfaarp1* is located even more toward the center of the chromosome than a recently identified housekeeping gene, *Pfrab*GDI (4) (Fig. 2c).

Partial sequence of the *Pfaarp1* gene indicates that Pf AARP1 is potentially a multifunctional transmembrane giant (>700-kDa) protein. Consistent with the mung bean nuclease digestion, which suggests a coding region of approximately 20 kb (Fig. 1A, lanes 1 to 3), the 11,461 bp of genomic DNA encode a single open reading frame containing no stop codons, which corresponds to 3,820 amino acids (Fig. 3). We have thus determined a more than 400-kDa region of the predicted 700-kDa PfAARP1 polypeptide sequence, which, although partial, exhibits a number of interesting features. First, DNA homology matrix analysis indicates that the available locus contains nine different blocks of internally repetitive sequences (Fig. 1B). These encode amino acid repeats; the consensus of each block and its position in the locus are given in Table 1.

As its name suggests, PfAARP1 is an asparagine- and as-

FIG. 3. Inferred amino acid sequence in single-letter code of PfAARP1. Hypothetical transmembrane segments (TM 1 to TM 10), blocks of amino acid repeats (BR1 to BR9), charge clusters (+/−), and PEST and VIP signatures are underlined. Signatures, transmembrane segments, and charge clusters were detected as described in Materials and Methods.

partate-rich protein due in part to the presence of these two residues in eight of the nine different blocks of repetitions (Table 1). Even though *Pfaarp1* appears to be a unique gene (Fig. 1 and 2), we have nonetheless recently demonstrated that the repetitions encoding the NNDDD motifs (Table 1, blocks 3 and 6) define a new gene family and have identified two members, *Pfaarp2* and *Pfaarp3* (5), encoding ENDDD and E(E/N)DDD repeats, respectively. Several previously

TABLE 1. Consensus motifs of the nine blocks of amino acid repetitions of PfAARP1[a]

Block	Consensus motif
BR1	$(NNNT)_3(NNNNNNT)(NNNT)(NNNNNNK)$
BR2	$(MIIKMKVLKIFH)_4$
BR3	$(MDD)(NDDDNDD)_2(NDDD)_4(NNDDD)_{11}(NDD)$
BR4	$(KKYID)_3$
BR5	$(MTSDNINE/K)_{14}$
BR6	$(N)_4(NNDDD)(NDDD)(NDD)(NNDD)(NNDDD)(N)_3$
BR7	$(DNIYG)_6$
BR8	$(N)_{25}$
BR9	$(D[G/D]NKIL)_{10}(N)_{11}$

[a] The locations of the blocks are given in Fig. 1B. The consensus motif of the corresponding amino acids is also given; subscripts denote the number of times each motif in parentheses is repeated.

described malaria genes might also belong to the *Pfaarp* family (27). Moreover, it is noteworthy that the P-cation ATPase (26) has a series of DNIYG repeats 85% identical to those present in block 7 of PfAARP1.

In addition to these blocks, PfAARP1 contains 10 different regions having the characteristics of transmembrane domains (TM 1 to 10 in Fig. 3). A vasoactive intestinal peptide (VIP)-glucagon family signature common to a family of polypeptidic hormones involved in glycogenesis and vasodilatation can also be identified. Furthermore, there is a PEST motif which defines a hydrophilic stretch of ≥12 amino acids containing at least one proline (P), one glutamate (E) or aspartate, and one serine (S) or threonine (T), which confers on proteins their rapid degradation. The locations of these different domains are given on the computer-predicted topology map of PfAARP1 (Fig. 4). The consequences of such a topology and the significance of the various motifs and domains are discussed in more detail later (see Discussion).

Identification of the circa 700-kDa PfAARP1 protein. To identify PfAARP1, two separate fragments (DC1 and DC2 [Fig. 1B]) which do not include repeats were expressed as GST fusion proteins and used to immunize mice. In preliminary immunofluorescence experiments, the two sera gave identical patterns, and we show here the results obtained with antibodies raised against GST-DC1. These mouse antibodies recognized a very large polypeptide migrating significantly above the 200-kDa size marker (Fig. 5, lane 2) expressed by young trophozoites. Human antibodies, obtained from Senegalese patients, immunopurified on the GST-DC1 fusion protein also recognized a single large protein (Fig. 5, lane 3). In both cases, PfAARP1 migrates close to the border between the stacking and separating gels. No reaction was observed with these antibodies on nonparasitized erythrocyte protein extracts (Fig. 5, lane 1), with young ring-stage parasites (Fig. 5, lane 4), or with the preimmune mouse serum (data not shown).

Although difficult to estimate correctly, the very large size of the polypeptide identified by both mouse and human antibodies is consistent with the size of the gene estimated by the mung bean nuclease analysis (Fig. 1) and amino acid sequence (Fig. 3). Taken together, these results show that PfAARP1 is a new giant protein encoded by *P. falciparum*. The difficulty in identifying the native PfAARP1 protein is probably linked to its unusual size (>700 kDa), combined with the presence of both multiple transmembrane domains and a PEST sequence, since

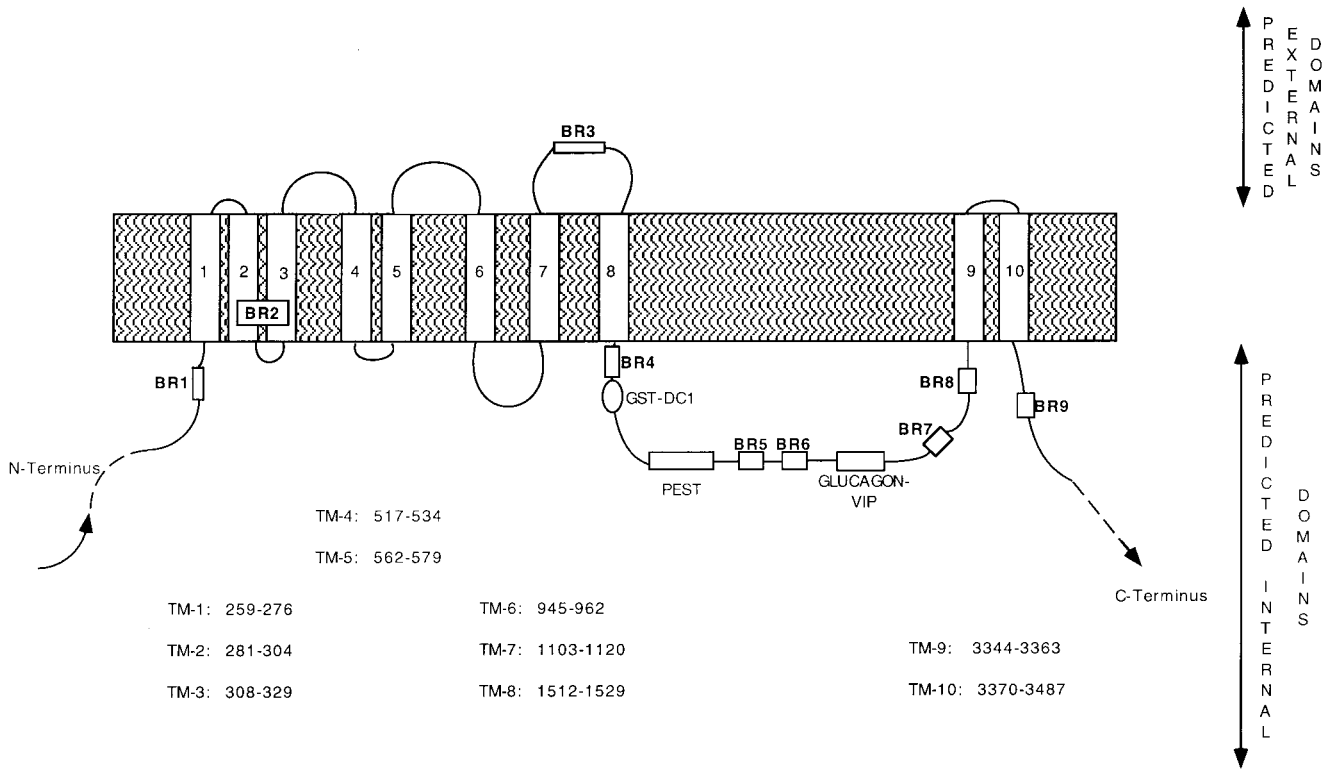

FIG. 4. Topology map of the partial PfAARP1 sequence, generated by using the PHD algorithm as described in Materials and Methods. Positions of the 10 transmembrane domains (TM-1 to TM-10) are given in the diagram, and their precise locations are indicated below. The nine blocks of amino acid repeats (BR1 to BR9) are boxed. The positions of the PEST and VIP signatures are also indicated, together with the cytosolic location of the GST-DC1 fusion protein.

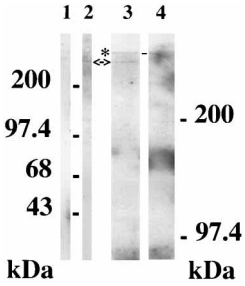

FIG. 5. Western blot analysis of PfAARP1. SDS protein extracts prepared from noninfected erythrocytes (lane 1), young rings (lane 4), and trophozoites (lanes 2 and 3) were separated on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Mouse antibodies raised against GST-DC1 fusion protein (lanes 1 and 2) and human antibodies isolated from hyperimmune sera on the GST-DC1 fusion protein (lanes 3 and 4) react with a high-molecular-weight protein (arrow). The asterisk indicates background due to the border between the stacking and separating gels.

the latter is known to mediate rapid protein degradation by proteasomes (36).

IFA studies indicate that PfAARP1 is translocated into the erythrocyte cytoplasm, while epitopes defined by the NNDDD peptide are exposed at the surface of infected erythrocytes. IFA analysis was performed with both mouse and human antibodies raised against or affinity selected on the GST-DC1 fusion protein. The IFA pattern obtained on fixed parasites with both antibody preparations being identical, we show in Fig. 6 the image obtained with air-dried trophozoite-infected erythrocytes labeled by the human affinity-purified antibodies. The fluorescence (Fig. 6C) is markedly delocalized from the parasite nucleus (shown by propidium iodide DNA staining [Fig. 6B]), indicating that PfAARP1 is translocated into the cytoplasm of the infected erythrocyte. Moreover, PfAARP1-specific fluorescence appears very close to the erythrocyte membrane (Fig. 6C), while the propidium iodide nuclear staining remains visible. No surface immunofluorescence staining, however, was obtained on parasites in suspension, using the anti-GST-DC1 antibodies (data not shown), implying that this part of the PfAARP1 protein is not exposed, which gives credence to its cytosolic localization on the predicted topology map (Fig. 4).

Given that a number of asparagine-rich proteins have been proposed to be associated with the infected erythrocyte membrane (17, 27, 52) and that recombinant proteins derived from these clones are recognized by opsonizing antibodies (20), we investigated whether PfAARP1 repeated NNDDD epitopes

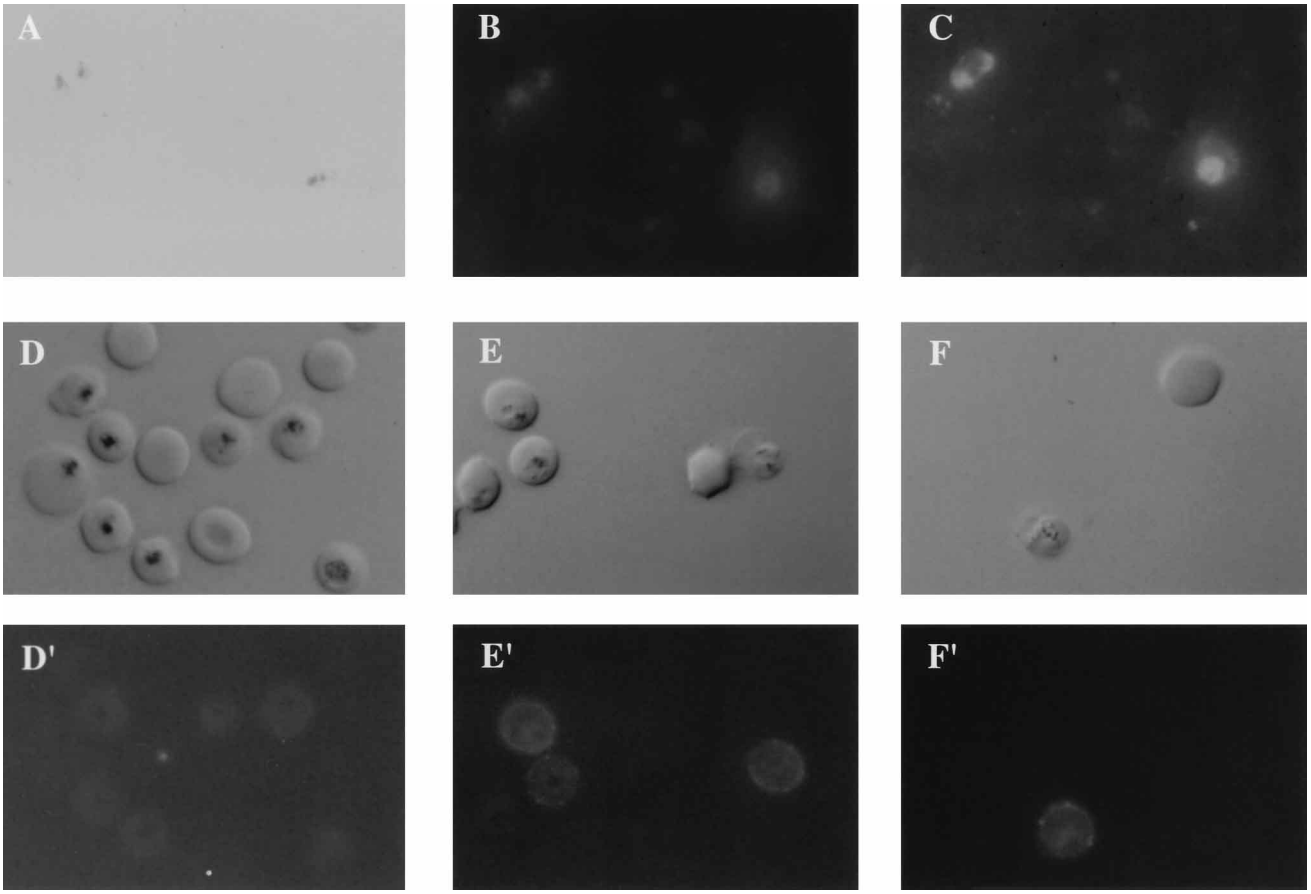

FIG. 6. IFA analysis (magnification, ×940) of PfAARP1. (A to C) Air-dried fixed parasites were incubated with human antibodies selected on GST-DC1. Panel A shows the phase-contrast picture corresponding to the same field as in panels B and C. Fluorescein-labeled second antibodies were incubated with propidium iodide to localize the parasite nucleus (B). Panel C shows the fluorescein pattern given by antibodies affinity purified on GST-DC1; propidium iodide label of the nucleus is still visible. Panels D to F are phase-contrast images (Nomarski pictures) of the same fields as in panels D′ to F′. In panel D′, as a negative control, parasites in suspension were incubated with human antibodies immunoselected on a peptide derived from LSA1 antigen (19). Panel E′ represents a positive control of surface immunofluorescence, using the same human serum from which the antibodies were affinity purified. Panel F′ displays the positive surface immunofluorescence on parasitized erythrocytes, using human antibodies affinity purified on the NNDDD peptide; noninfected erythrocytes appear negative.

are exposed on the surface of the infected erythrocyte. Human antibodies were therefore selected on the NNDDD peptide and used in surface immunofluorescence assays performed with live infected erythrocytes in suspension (Fig. 6). A clear surface immunofluorescence labeling was obtained with human antibodies selected on the NNDDD peptide (Fig. 6F′). The pattern is similar to that obtained when a human hyperimmune serum is used (Fig. 6E′). In contrast, no surface labeling was obtained (Fig. 6D′) when human antibodies were selected on a peptide derived from repeats of the liver stage-specific antigen LSA1 (19). Thus, epitopes contained within the NNDDD repetitions are exposed on the surface of parasitized erythrocytes.

DISCUSSION

We have isolated and characterized a new gene (*Pfaarp1*) that exhibits an open reading frame of approximately 20 kb which encodes a giant membrane protein of circa 700 kDa. PfAARP1 is not the first malaria protein of this size to be described, since both Pf11-1 and Pf332 are megadalton proteins which are associated with the membrane of the infected erythrocyte (33). A characteristic that distinguishes *Pfaarp1* from Pf11-1 and Pf332 is its chromosomal location. Unlike the loci of the two other large genes, the *Pfaarp1* locus is telomere distal (Fig. 2), being located even more centrally than the housekeeping gene *Pfrab*GDI, encoding a Rab-specific effector protein (4). Since subtelomeric regions of malaria chromosomes are known to be unstable (37, 46) and have led to the deletion of Pf11-1 (45) or translocation of Pf332 (21), the central location of *Pfaarp1* reduces the likelihood of its inactivation by chromosome subtelomeric rearrangements.

The unique amino acid sequence of PfAARP1 contains a number of different motifs present in other proteins. First, there is a PEST sequence which is expected to be cytosolic (Fig. 4) and moreover is associated with a potential phosphorylation site. This finding argues for functional significance of the PfAARP1 PEST sequence, since PEST-mediated degradation by proteasomes can involve phosphorylation (39). Second, in the same cytosolic loop as the PEST motif, there is a glucagon-VIP family signature. In mammals, members of this family of polypeptidic hormones are usually involved in regulating glycemia (8). It is interesting to speculate that if correctly processed, PfAARP1 (or another, unidentified malaria antigen) could yield a parasite-derived peptide hormone that might interfere with the host's homeostasis.

A topology map of the partial PfAARP1 sequences suggests that PfAARP1 is a membrane-associated protein with 10 transmembrane domains giving rise to five external and four internal loops (Fig. 4). Taken together with the IFA pattern obtained with human antibodies immunopurified on the GST-DC1 fusion protein, this observation suggests that PfAARP1 is associated with the erythrocyte membrane. Results of IFA analyses performed on live parasites in suspension, using human antibodies affinity purified on the NNDDD peptide, indicate that such epitopes are exposed at the surface of the infected erythrocyte (Fig. 6). The topology map strongly suggests that the PfAARP1-specific NNDDD epitopes potentially exposed are those contained in external loop 4 (Fig. 4). It is noteworthy that similar NDDD motifs are also present in PfEMP1 (7, 49). Therefore, NNDDD repetitions may define the first cross-reactive network of epitopes exposed at the erythrocyte surface, reminiscent of the immunodominant repeated (NANP) epitope present on sporozoites (53). Since the NNDDD peptide defines epitopes of immunological interest, we have initiated an epidemiological study in Burkina Faso, and preliminary results indicate that the humoral response against the peptide correlates with age and hence exposure to the parasite (24).

In conclusion, the PfAARP1 NNDDD motifs identify a new family of antigens that include PfAARP2 and PfAARP3 and distinguish them from the previously identified asparagine-rich antigens. Importantly, the NNDDD epitopes are exposed at the surface of the parasitized erythrocyte. In contrast, PfAARP1 polyasparagine stretches are not predicted to be exposed, although their presence could explain the previous identification of asparagine-rich antigens by sera raised against infected erythrocyte membranes. Interestingly, the PfAARP1 topology map suggests that four other loops, in addition to the one containing the NNDDD repetitions, are potentially exposed at the surface. This prediction can now be tested experimentally, using specific peptides and recombinant proteins derived from each loop, and these reagents should be useful in efforts to dissect the function of PfAARP1.

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