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Cloning and characterization of a new asparagine-rich protein in *Plasmodium falciparum*

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Abstract A cDNA clone that encodes a *Plasmodium falciparum* asparagine (N)-rich protein (PfARP) was isolated through immunoscreening of an expression library. A 9.4 kb PfARP transcript was identified by Northern blot hybridization and the gene was localized on chromosome 1. The complete coding sequence (6666 bp) revealed a protein that contains clustered as well as randomly distributed N residues (24.3%), seven copies of a repeat sequence [DNT(D/N)(K/N)(V/L/M)] and multiple copies of tripeptide repeats within a 101 amino acid region containing 89 D/E residues. The PfARP was immunogenic in inbred and outbred mice and endemic sera revealed the presence of low-titer antibodies against PfARP. Anti-PfARP sera showed cytoplasmic and surface localization of apparently cross-reactive malarial antigens in different life-cycle stages (ring, trophozoite, schizont, and gametocytes). Although the biological function(s) of PfARP are not known, the observation that it is present in multiple parasite stages and that it is a target of natural immune response warrants further study of PfARP as an immune target.

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Nucleotide sequence data of the clones described in this paper is available in the GenBank under accession number AF047005

Introduction

Analysis of gene sequences in *Plasmodium falciparum* has suggested many novel and peculiar features of the encoded polypeptides. These include the presence of many repeat sequences (Kemp et al. 1987) and sequences rich in certain amino acids. For example, asparagine (N) is the most frequently (~12%) used amino acid followed by lysine (K) and glutamic acid (E) (9–10%) (Hyde and Sims 1987; Weber 1987; Saul and Battistutta 1988). A number of N-rich proteins (ARPs) have been identified in *P. falciparum* (Table 1). The roles played by these molecules in the parasite have not been defined; however, some ARPs appear to be associated with and localized on the surface of the parasite and parasitized red blood cells (PRBCs) (Dame et al. 1984; Stahl et al. 1986; Wahlgren et al. 1986; Franzen et al. 1989; Kun et al. 1991; Fidock et al. 1994). It has been proposed that the N-rich motifs might be the target of opsonizing antibodies which promote phagocytosis of the infected erythrocyte (Gysin et al. 1993; Barale et al. 1997a, b). The ARPs associated with PRBC membrane might possibly play some roles in PRBC cytoadherence and RBC rosetting which are linked to cerebral malaria. All these have prompted efforts to isolate the corresponding genes and evaluate their role in parasite biology.

The circumsporozoite protein (CSP) (29% N) and a threonine (T) and N-rich protein (STARP) (25% N) are two prominent sporozoite surface proteins in *P. falciparum* (Dame et al. 1984; Fidock et al. 1994). Similarly, partial clones for numerous N-rich proteins (designated as either ARP or CARP, Table 1), exhibiting extensive cross-reactivities with no clear function have been described in the blood stage parasites. Recent additions to this growing list are PRBC membrane associated antigens PfAARP1 (a N and D-rich protein 17% N), PfAARP2 (13% N) and PfAARP3 (Barale et al. 1997a, b). Since they contain N-rich repeat motif, their possible role as a target of cytophilic antibodies

Table 1 Asparagine-rich sequences from the blood stages of *Plasmodium falciparum*

No.	Clone	N (%)	Accession no.	References
1	ARP (AG319)	40	M24328	Stahl et al. 1986
2	CARP	30	M13021	Wahlgren et al. 1986
3	CL2122	23	M18825	Epping et al. 1988
4	10b	30	J03986	Franzen et al. 1989
5	E4	33	N/Aa	Ardeshir et al. 1990
6	C5	39		
7	G5	32		
8	R13	20		
9	R5	31		
10	Pfa 35-2	29	M59474	Nolte and Knapp 1991
11	Pfa 55-6	23	M59472	
12	Pfa 55-14	25	M59473	
13	PfK16	22	X53023	Kun et al. 1991
14	Pf12	25	X53015	
15	PfK14	28	X53022	
16	Pf221	29	X53017	
17	Pf297	59	X53020	
18	PfA18C1	28	X17485	Schreiber et al. 1989[b]
19	PfA52C11	39	X17489	
20	Clone 25C3	30	102334	
21	Clone 14C1	33	102332	
22	ARP-LSHTM	27	2149437	Li et al. 1997[b]
23	PfAARP1-3	13–17	Y08926, Y08924	Barale et al. 1997a, b

[a] No complete sequence available from publication or GenBank databases
[b] Unpublished, sequence submitted to GenBank

was proposed by these investigators. In this paper we describe the isolation and characterization of a cDNA clone encoding a novel *P. falciparum* asparagine-rich protein (PfARP). The complete sequence contains 24.3% N residues (clustered as well as scattered) and several repeat sequences. The studies described also demonstrate that this new PfARP is a target of human immune response during natural malaria infection.

Materials and methods

Parasites and parasite culture

Asexual stages of *P. falciparum* were cultured in vitro as described (Trager and Jansen 1976) and synchronized using 5% sorbitol (Lambros and Vanderberg 1979). Gametocytes of *P. falciparum* were cultured as described by Carter and Miller (1979) and modified by Ifediba and Vanderberg (1981).

Immunoscreening of cDNA expression library

A cDNA expression library (UNI-ZAP XR) made from mRNA purified from enriched gametocytes (NF54) was screened using pooled antisera from four rabbits immunized with gametes and zygotes of *P. falciparum*. The antiserum was subjected to immunoadsorption with the lysate of *E. coli* infected with wildtype phage prior to immunoscreening of library. A total of 5×10^5 plaque forming units were screened and the immuno-reactive clones obtained after the tertiary screen were rescued as pBluescript phagemid. Based on initial sequence analysis (T3 and T7, pBluescript primers), two clones (3-1 and 22-1) were chosen for further analysis.

DNA sequence, Southern and Northern analysis

Complete sequence of the two cDNA clones in both the orientations was obtained from the overlapping truncated clones obtained by the "Erase-a-Base" system (Henikoff 1984) using the dideoxy chain termination method (Sequenase kit, United States Biochemicals) (Sanger et al. 1977). The sequence data were analyzed by various programs provided in (1) MacVector 6.0 (Oxford Molecular) and (2) Dnastar (Dnastar, Madison, Wis., USA). Various sequence databases in the GenBank or at the Sanger Center were used for BLAST analyses (www.ncbi.nlm.nih.gov, www.sanger.ac.uk). A recent search at the Sanger center (14 April 1999 update) identified a clone (MAL1P2_00218; Acc = AL031745) providing the complete coding sequence (6666 bp) of the cloned PfARP gene.

Genomic DNA was extracted from the parasite pellet using the cesium chloride (CsCl) method as described (Dame and McCutchan 1983). Total RNA, isolated using "RNA isolation kit" (Stratagene), was separated on 1% agarose gels run under denaturing conditions using formamide and formaldehyde in MOPS buffer and transferred to nitrocellulose membranes (Sambrook et al. 1989). The blots were hybridized at 65 °C in $6 \times$ SSC/$5 \times$ Denhardt's/1% SDS, washed at 60 °C in $0.2 \times$ SSC/0.1% SDS and exposed to Kodak XAR film at −70 °C for autoradiography.

Production of antisera against PfARP

DNA insert of the clone 3-1 and a 5′ ~700 bp fragment corresponding to amino acid residues 1493–2222 and 1493–1723, respectively, were cloned into expression vectors pRSET C and A (Invitrogen, Calif.) yielding clones translationally in frame with the metal binding domain $(His)_6$ sequence of pRSET. The ORF and orientation of the inserts were confirmed by PCR and DNA sequencing. The recombinant *E. coli* BL21(DE3) were induced with 0.4 mM IPTG and the expressed proteins were purified using Nickel (Ni^{+2}) column (Novagen, Madison, Wis., USA). Partially purified fusion protein obtained by nickel-chromatography was further purified by electro-elution (Bio-Rad, Electro-Eluter Model 422) as described (Uparanukraw et al. 1993). SDS was removed from the electroeluted protein by circulating through Extracti-Gel D column (Pierce), and protein concentration estimated by the BCA method (Pierce). Clone 3-1 and the 700 bp inserts were also cloned in the VR1020 plasmid (Vical, San Diego) and used as DNA immunogens for antibody production.

Outbred female ICR and CD-1 mice (6–8 weeks) (Harland Bioproducts for Science, Me., USA and Charles River Laboratories, Bar Harbor, Me.) were immunized (i.p.) with Ni^{+2} column purified fusion protein (50 μg) in complete Freund's adjuvant (CFA) followed by two booster immunizations (21 day intervals) with 25 μg of electro-eluted protein in incomplete Freund's adjuvant (IFA). For DNA immunization, five ICR mice were injected i.m. in right and left tibialis anterior muscles with 50 μg/mouse of plasmid DNA dissolved in PBS and boosted twice on day 42 and day 77, respectively. Tail bleeds were taken 7–10 days prior to immunization as well as 10 or 20 days after immunization and the sera stored at −20 °C.

Parasite labeling, immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

Parasites (5–10% parasitemia) were biosynthetically labeled using $Tran^{35}S$-label (ICN Biomedicals), extracted in Triton X-100 in the presence of a cocktail of six protease inhibitors, and tested in immunoprecipitation (Kumar et al. 1991). Approximately 200,000 TCA insoluble counts were used with 10 μl antisera for immunoprecipitation. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), enhanced by soaking the gel in 1 M salicylic acid, dried and exposed with X-ray films at −70 °C for autoradiography.

Detection by indirect immunofluorescence assays and immunoelectron microscopy

Parasite cultures enriched in asexual-blood stages or gametocytes were spotted onto eight well Toxo slides (Bellco Glass), fixed in methanol and incubated with different dilutions of various antisera for 1 h at room temperature. The slides were washed in cold PBS and incubated with 1:50 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse Ig (A, G, M; heavy and light chains) (Cappel) for 30 min., mounted in 90% glycerol for microscopy. For immunoelectron microscopy (IEM), *P. falciparum* (asexual and sexual) infected erythrocytes were fixed and processed as described previously (Aikawa and Atkinson 1990). Thin sections were incubated for 30 min in PBS containing 1% bovine serum albumin fraction V (BSA) and 0.01% Tween 20. Grids were then incubated for 2 h at room temperature with mouse antisera (1:100) in PBS-BSA-Tween 20 (PBT). After washing in PBT, the grids were incubated for 1 h in 5 or 15 nm gold labeled goat-anti-mouse IgG (Amersham Life Science, Arlington, Ill., USA) diluted 1:20 in PBT followed by staining with uranyl acetate and lead citrate. Samples were examined in Zeiss CEM902 electron microscope (Zeiss, Oberkochen, Germany).

Detection of antibodies to PfARP in human sera

Sera samples from smear positive *P. falciparum* patients attending the Pong Nam Ron Community Hospital, Chanthaburi Province, Thailand were shipped frozen. Normal human sera (NHS) were obtained from blood donors in the blood bank in the United States. Partially purified fusion protein of the PfARP was fractionated by 10% SDS-PAGE mini gel under reducing conditions and then transferred onto nitrocellulose membrane. After blocking in 0.3% Tween-20, the membrane was assembled in the Miniblotter (Immunetics, Cambridge, Mass.). The human sera samples diluted (1:10) in TTS buffer (10 mM TRIS, 150 mM NaCl, 0.05% Tween-20, pH 7.6) were added into individual tracks and incubated overnight at 4 °C with rocking. After extensive washing of individual lanes with TTS, the blot was removed, rinsed with TTS and then reacted with HRP-conjugated goat anti-human immunoglobulin A, G, M (1:1000) (Cappel, West-Chester, Pa., USA) at room temperature for 1–2 h with rocking. The blots were washed again and finally developed using 4-chloro-naphthol (Sigma) as substrate. The reaction was stopped by washing the blot extensively with distilled water. A χ^2 test was used for statistical analysis.

Results

Isolation of cDNA clones 3-1 and 22-1 and characterization

A total of 30 positive clones were obtained from immunoscreening 5×10^5 plaque forming units of *P. falciparum* cDNA expression library with rabbit antisera raised against gametes and zygotes. The insert sizes of these clones detected by PCR analysis using the primers T3 and T7 were found to vary from 230 bp to 3169 bp. Twenty-four clones hybridized with *P. falciparum* HSP70 (Kumar et al. 1991) or 27 kDa (Lobo et al. 1994) genes and excluded from further analysis. Phagemids were excised from the remaining six clones and partially sequenced using T3 and T7 primers. None of these revealed sequence identity to any previously sequenced *Plasmodium* gene. Two of these, clone 3-1 and 22-1, were chosen for further characterization because both were identical except 3-1 was 94-bp longer at the 5′-end, and both contained a 3′ polyadenylation signal sequence (AATAAA), 9 bp downstream of the termination sequence (TAA) and 37 bp upstream from the poly $(A)_{18}$ tail. The complete sequence of the insert did not reveal identity or similarity to any previously sequenced *Plasmodium* gene or any other available sequences except to clone 0405m3, a genomic DNA sequence tag of 323-bp from *P. falciparum* dbEST database and another 267-bp of 498-bp genomic DNA fragment (M1J1e12.s1t) of *P. falciparum* chromosome 1 short contigs from a then unfinished data sequence (March 1998) of malaria genome project, thus extending the 5′-end of clone 3-1 by 231 bp. This and Northern blot analysis (below) suggested that these clones represented only part of the complete sequence. A recent search of the *P. falciparum* (3D7, a clone of NF54) genome database at the Sanger Center resulted in the identification of complete sequence (MAL1P2_00218; Acc = AL03174, open reading frame of 6666 bp) for the two cDNA clones. The sequence of the two cDNA clones was identical to the sequence of the genomic clone.

Clone 3-1 encodes a new PfARP

Initial analysis of clone 3-1 had revealed a single large open reading frame (2184 bp) terminated by a TAA stop codon encoding a polypeptide containing N-rich regions. The deduced amino acid sequence of the 6666 bp open reading frame suggests a protein of apparent molecular weight of 255 kDa and isoelectric point of 5.44. The predicted protein sequence (Fig. 1A) contains small and large clusters of N residues (24.3%) randomly distributed over the polypeptide chain and a 101 amino acid region rich in D or E amino acids (89%) displaying multiple possible tripeptide repeat sequences. Another noteworthy feature of the deduced amino acid sequence is the presence of seven copies of a hexapeptide repeat sequence [DNT(D/N)(K/N)(V/L/M)]. The hydrophilicity plot (Fig. 1B) shows the extremely hydrophilic overall nature of the encoded protein product.

Genomic arrangement of PfARP and conservation in *P. falciparum* isolates

Sequence identity of clone 3-1 to the fragments of the chromosome 1 short contigs indicated that the PfARP gene is located on the chromosome 1 in *P. falciparum* genome. This was further confirmed by hybridization of 3-1 probe to chromosome 1 separated by pulse-field gel electrophoresis (not shown). Clone 3-1 insert DNA also hybridized to restricted (*Eco*R1 and *Rsa*1) *P. falciparum* (NF54) genomic DNA with expected banding pattern. PCR analysis of genomic DNAs from the various *P. falciparum* clones and isolates (NF54, HB-2, HB-3, 7G8, VietPf, ItG2F6, T2, N1 and LE5) with two pairs of internal primers of clone 3-1 (CAGTATGTA-

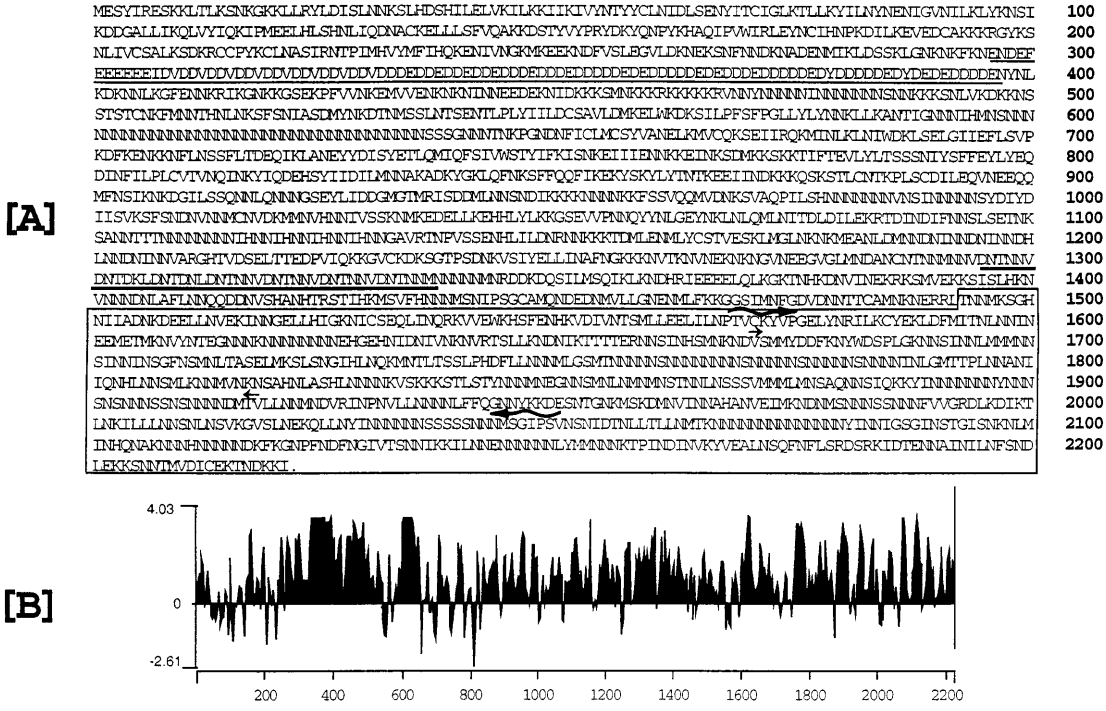

Fig. 1 **A** Deduced amino acid sequence of N-rich PfARP. The various regions of interest are: D/E- rich (residues 296–396, *thin underline*), the hexa-peptide repeat region (residues 1295–1336, *thick underline*) and sequence of the clone 3-1 (residues 1493–2222, *box*). Matching pairs of *arrows* denote the regions of gene fragments amplified by PCR from the various isolates of *P. falciparum*. **B** Graphical representation of hydrophilicity of PfARP

AATATGTACC/GATGGTATTCCGCTCATA and GATGTCAGTATGATGTATG/CAATAACACCG-TC-ATATC) also indicated that these regions (1441 and 753 bp, respectively) of the PfARP gene are conserved in size (results not shown).

Detection of RNA transcript in the parasite

Northern blot analysis was performed on equal amounts of total RNA isolated from asexual and sexual stages to determine expression of PfARP in *P. falciparum*. As shown in Fig. 2B, an approximately 9.4 kb RNA band was identified in the asexual parasites (lane a). A similar size RNA was identified when two 3-1 specific fragments [rich or poor in AAT (a codon preferentially used for N amino acid residue in PfARP) sequences] were used as probes (data not shown). In two of five experiments, the clone 3-1 probe also hybridized with several diffuse smaller RNA bands from the gametocytes (Fig. 2B,

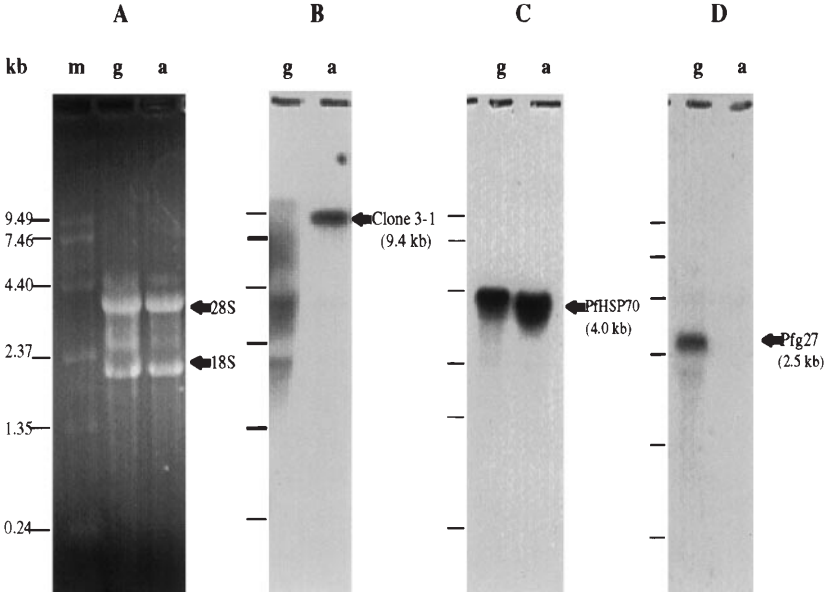

Fig. 2A–D Detection of RNA transcript for the gene of clone 3-1. **A**. Total RNA (10 μg) from the asexual parasites (*lane a*) and gametocyte RNA (*lane g*) were analyzed by 1% formaldehyde agarose gel and stained with 1 μg/ml ethidium bromide. RNA molecular weight marker, 0.24–9.5 kb RNA ladder (BRL) are shown in *lane m*. **B**. RNA samples in **A** were transferred onto nitrocellulose membrane for hybridization with ^{32}P-labeled PCR product of clone 3-1. **C**. The blot in **B** was stripped to remove the probe and re-hybridized with ^{32}P-labeled PfHSP70 gene. **D**. The blot in **C** was stripped once again and re-hybridized with ^{32}P-labeled probe for Pfg27

lane g). Sequential hybridization of the same blot with PfHSP70 (transcribed in both asexual and sexual stages) (Kumar et al. 1991) (Fig. 2C) and Pfg27 (only expressed in gametocytes) (Lobo et al. 1994) (Fig. 2D) demonstrated comparable good quality of RNA from asexual and sexual stages used in these Northern analyses. Apparent absence of 9.4 kb transcript in gametocytes could thus be due to low transcription of the PfARP gene in these stages.

Expression of recombinant proteins and immunization of mice

Initial attempts to express the full-length insert of cDNA clone 3-1 in pRSET were unsuccessful. However, a 700 bp fragment (amino acid residues 1493–1723) was successfully expressed as $(His)_6$ fusion protein, although the level of expression was quite low. The recombinant fusion protein (~33 kDa), confirmed by western blotting [using anti-$(His)_6$ sera], was purified using nickel columns by elution with 60–80 mM imidazole (Fig. 3A, lane 2). Partially purified PfARP revealed two dominant doublets of 33/30 kDa and 26/22 kDa among other proteins. The 33/30 kDa doublet was further purified by electro-elution after SDS-PAGE (Fig. 3A, lane 3). Mouse antisera against recombinant proteins were strongly reactive while those against DNA immunogens were weakly reactive with various purified recombinant proteins.

Detection of PfARP cross-reactive proteins in *P. falciparum* asexual and sexual stages

In order to identify the *P. falciparum* polypeptide represented by the cloned PfARP, the mouse antisera were used to immunoprecipitate Triton X-100 extracts of mixed asexual stage parasites of *P. falciparum* (NF54) labeled with ^{35}S methionine/cysteine. The antisera against partially purified recombinant PfARP immunoprecipitated a large number of polypeptides ranging in size from 35 to 245 kDa with at least 16 distinct bands (apparent MW: 245, 241, 200, 190, 170, 162, 157, 148, 138, 127, 122, 105, 75, 47, 41, 35 kDa) (Fig. 3B, representative of three experiments). A comparable complex pattern was obtained with all the different antisera (raised against partially purified fusion protein, electro-eluted 33/30 kDa doublet and DNA immunogens). A dominant 75 kDa band present in immunoprecipitation carried out with all sera including pre-immune sera is most likely HSP70 of *P. falciparum* (Kumar et al. 1991). In the next series of experiments synchronous asexual and sexual stages of *P. falciparum* were labeled and used in immunoprecipitation analyses. The pattern of immunoprecipitated polypeptides increased in complexity during asexual growth and development of parasites. The doublet of 162/157-kDa was present in ring and trophozoite stages; however, these two bands disappeared or were weak in the schizont. On the other hand, a protein band of 170-kDa and a doublet of 148/138-kDa were seen in the schizont but not in the ring and trophozoite.

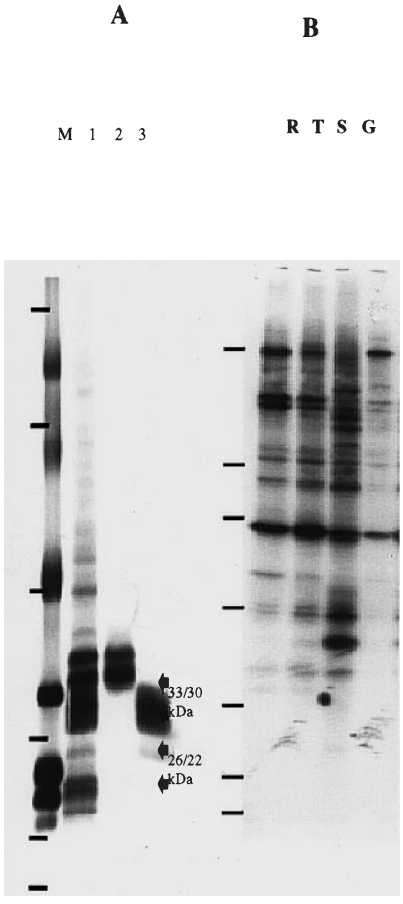

Fig. 3 **A** Purification of recombinant PfARP. Ni^{+2} column partially purified fusion protein was concentrated and loaded onto a 5–15% SDS-PAGE gel for further purification by electro-elution. The electro-eluted samples were analyzed by 5–15% SDS-PAGE under reducing conditions: *lane M* prestained high molecular weight standards (BRL); *lane 1* 100 × concentrated partially purified protein; *lanes 2 and 3* doublet of 33/30- and 26/22-kDa, respectively isolated by electro-elution. **B** Biosynthesis of PfARP in synchronized stages of parasites (*R* ring form; *T* trophozoite; *S* schizont; *G* gametocyte). Antisera from mice immunized with Ni^{+2} column purified fusion protein were used and immunoprecipitated proteins were analyzed by 5–15% gradient SDS-PAGE under reducing conditions. The positions of the molecular weight markers (214.2, 111.4, 74.3,45.5,29.5,18.3 and 15.4 kDa, BRL) are shown on the *left* side

The presence and localization of anti-PfARP-reactive proteins in parasites was further investigated by indirect immunofluorescence assay. While all the sera were positive in the immunofluorescence assay, antisera against recombinant proteins (partially purified or electro-eluted) gave much higher titers as compared to antisera elicited by DNA-immunogen, and were used for subsequent studies. The reactivity with asexual stages was much stronger than with sexual parasites. No reactivity was seen with uninfected red blood cells present in the same slide. The intensity of fluorescence at a given antibody dilution increased progressively between rings, trophozoites and schizonts (Fig. 4A). In all the stages, the fluorescence was

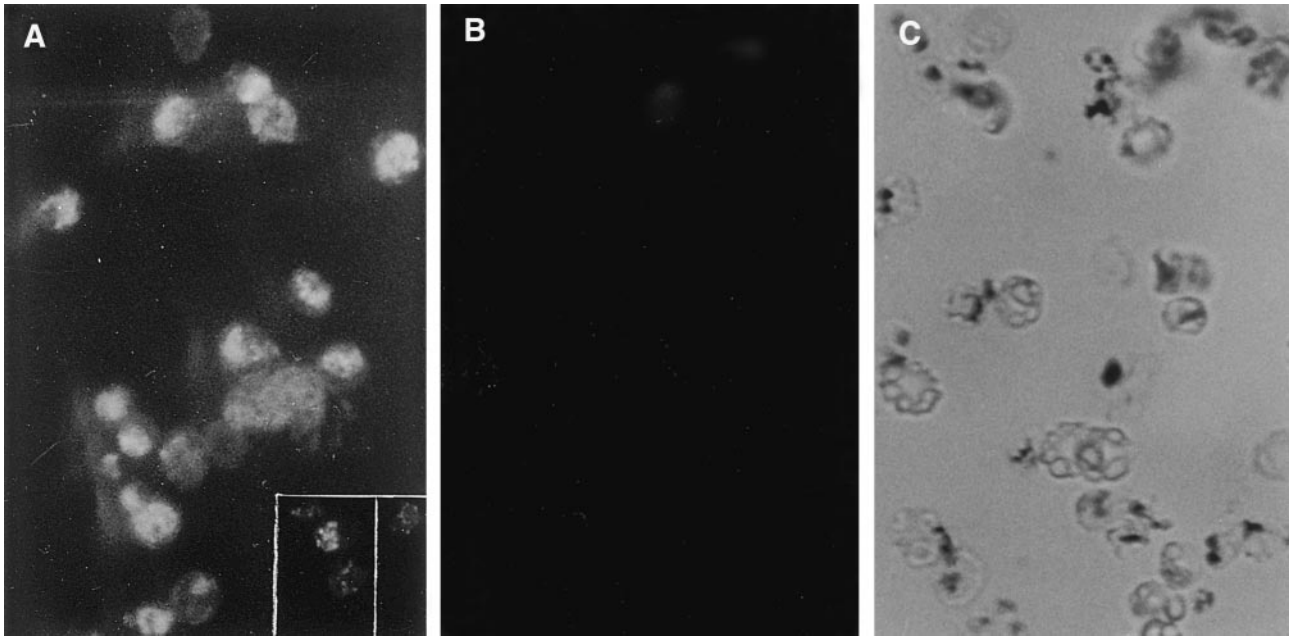

Fig. 4A–C Localization of the anti-PfARP reactivity on asexual parasite stages by indirect immunofluorescence. **A** Incubation with 1:100 dilution of antisera raised against the electro-eluted 33/30-kDa; *insets* show parasites with punctate pattern, **B** represents incubation with pre-immune mouse sera at the same dilution, **C** shows the phase-contrast micrograph of the same field as in **B** and showing malaria pigments in the parasites. The slides were examined under oil immersion at 1000× magnification

localized on the surface as well as in the cytoplasm of infected erythrocytes with a punctate distribution pattern (Fig. 4A, insets). A similar, however less intense pattern was obtained with gametocyte stages (not shown). The immunofluorescence assays were repeated 5–7 times using various antisera at multiple dilutions (1:10–1:1000). The IEM studies using the antisera against the electro-eluted 33/30-kDa recombinant protein further confirmed the observation in immunofluorescence assays. The gold particles were distributed on the surface and in the cytoplasm of infected erythrocyte and parasite nucleus (Fig. 5). The pre-immune sera used as a control was negative in IEM (data not shown).

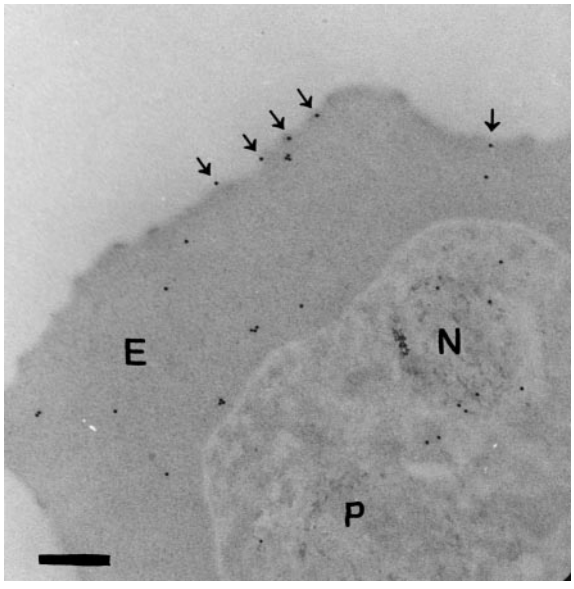

Fig. 5 Immunoelectron microscopy with anti-PfARP sera. The antisera raised against the electro-eluted 33/30-kDa protein was incubated with *P. falciparum* asexual stage (*P* parasite; *E* erythrocyte; *N* nucleus). An *arrow* indicates the surface location. *Bar* = 1.2 μm

Antibody response to PfARP in malaria-infected individuals

Human sera from malaria patients of different age groups who experienced multiple or at least a single infection in malaria-endemic areas of Thailand were tested by immunoblotting for anti-PfARP reactivity. Normal sera of blood donors from the USA blood bank (non-endemic control) were used as a negative control. The antibody titers were low in the sera from malaria-infected individuals. These results also indicated that the percentage antibody reaction to the PfARP (33/30 and 26/22 kDa doublets) increased with age: 14.3% (0–10 years), 58.8% (11–20 years), 69.7% (21–40 years) and 100% (40+ years) (Table 2). Of 12 normal adult human sera only one showed a weak reactivity in the Western blot test (Table 2). The differences among the various groups were statistically significant ($\chi^2 = 41.82$, $P < 0.01$).

Discussion

The gene encoding a new PfARP was isolated by immunoscreening of a *P. falciparum* cDNA expression library using rabbit polyclonal sera against gametes and

Table 2 Antibody response to the PfARP during natural *P. falciparum* infection. ($\chi^2 = 41.82$, $P < 0.01$)

Serum group	No. tested	No. positive	% positive
Normal sera	12	1	8.3
Immune sera			
0^+–10 years	7	1	14.3
11–20 years	17	10	58.8
21–40 years	33	23	69.7
40^+ years	11	11	100.0

zygotes. The cDNA clones contained a 3′ putative polyadenylation signal sequence (AATAAA) located 9 bp downstream from the stop codon (TAA) and 37 bp upstream from the poly $(A)_{18}$ tail, indicating that the cDNA clones correspond to the 3′-end of the PfARP gene. Since the size of mRNA for PfARP revealed by Northern blot analysis was much larger (9.4 kb) than the cloned fragment (2258 bp) it was concluded that the clones did not represent the complete gene for PfARP. Indeed, a recent search of the malaria genome database at the Sanger Center identified a 6666 bp long open reading frame with 100% sequence identity. The difference between the size of the mRNA transcript and the open reading frame is most likely due to long 5′ and 3′ untranslated regions.

PfARP is a new N-rich antigen of *P. falciparum*. The nucleotide and the deduced amino acid sequence of clone 3-1 did not show identity or similarity to any previously sequenced *Plasmodium* protein including other known N-rich proteins of *P. falciparum* or to any other available sequences in GenBank. In order to characterize the protein in the parasite, we attempted to express the full-length PfARP clone in *E. coli*. However, we were able to detect weak expression of only the 5′ portion 700-bp fragment of the cDNA clone. We do not know whether a preferential bias for codon for N residues is the reason for poor expression. The codon usage in *P. falciparum* deviates markedly from that reported for other species (Maruyama et al. 1986) and differs significantly for highly expressed genes in both *E. coli* and in yeast (Sharp and Li 1986; Sharp et al. 1986). In particular, in *P. falciparum* the codon most frequently used for the amino acid N is AAU, while AAC is strongly favored in highly expressed genes in both *E. coli* and yeast (Saul and Battistutta 1988). Analysis of codon usage in PfARP revealed that AAU was used 460 of 540 times as compared to only 80 of 540 times for AAC.

The various antisera raised against the recombinant protein and the DNA immunogen detected a large number of parasite polypeptides ranging in size from 35 to −245 kDa. Moreover, a similar complex polypeptide pattern was seen when these antisera were tested in Western blotting (not shown) or in immunoprecipitation experiments with synchronized asexual and sexual stages of *P. falciparum*. These observations on extensive cross-reactivity with antisera against recombinant protein made it impossible to identify a specific PfARP. As suggested by the northern blot analysis (9.4 kb transcript size) and by the large open reading frame (2222 amino acids) it is reasonable to assume that the actual PfARP must be a large polypeptide (∼250 kDa). Other features, especially a rich acidic amino acid domain and clusters of K residues, could also result in abnormal behavior of PfARP during SDS-PAGE. In addition, we cannot rule out the possibility that some of the immunoreactive bands could result from some form of processing of protein in the parasite. Other investigators (Stahl et al. 1986; Wahlgren et al. 1986; Franzen et al. 1989; Ardeshir et al. 1990; Nolte and Knapp 1991) have also had difficulty in characterizing parasite proteins rich in N residues. Mattei et al. (1989) were unable to raise specific antibodies to a recombinant *P. falciparum* antigen, called Pf332, because of the presence of cross-reactive epitopes on several parasite proteins. Although the primary sequence of PfARP is not identical, and does not contain any similar or conserved sequence to other N-rich proteins in *P. falciparum*, the possibility that they might still cross-react due to N-rich epitopes, or some similarity in the secondary structure of polypeptides, cannot be excluded. The immunofluorescence and IEM studies using the antisera against PfARP further confirmed the expression of the immunoreactive epitopes on the surface of infected RBC as well as in the cytoplasm and nucleus of parasites. Since the deduced amino acid sequence of full-length PfARP lacks any transmembrane or putative membrane anchor sequences, further studies using antisera against multiple specific regions will be necessary to confirm the localization observed in these studies.

In summary, our studies have indicated that PfARP, a new N-rich protein, is present in various stages of parasitized RBC, is conserved and is a target of antibodies during natural malaria infections. Thus identification of an antigen that crosses the stage barrier might present an attractive candidate for inclusion in a malaria vaccine. However, further studies with high titer and affinity-purified anti-PfARP antibodies in functional assays, i.e. growth inhibition assays, binding to endothelial cells in culture will be necessary to establish the biological role of PfARP in the erythrocytic parasite stages.

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