Characterization of P0, a Ribosomal Phosphoprotein of *Plasmodium falciparum*

ANTIBODY AGAINST AMINO-TERMINAL DOMAIN INHIBITS PARASITE GROWTH*

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Arunava Goswami, Subhash Singh, Vilas D. Redkar, and Shobhona Sharma‡

From the Molecular Biology Unit, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay, 400 005 India

A cDNA expression clone of the human malarial parasite *Plasmodium falciparum*, APF4, which was reactive only to the immune sera and not to the patient sera, has recently been found to be the *P. falciparum* homologue of the P0 ribosomal phosphoprotein gene. A Northern analysis of the P0 gene revealed the presence of two transcripts, both present in all the different intrathyrocytic stages of the parasite life cycle. A 138-base pair amino-terminal domain of this gene was expressed as a fusion protein with glutathione-S-transferase in *Escherichia coli*. Polyclonal antibodies raised against this domain immunoprecipitated the expected 38-kDa P0 protein; affinity-purified using the expression clone APF4 also immunoprecipitated the same size protein from [35S]methionine-labeled *P. falciparum* cultures. Monospecific human immune sera affinity-purified using the expression clone APF4 also immunoprecipitated the same size protein from [35S]methionine-labeled *P. falciparum* protein extract. Purified IgG from polyclonal antibodies raised against the amino-terminal domain of P0 protein completely inhibited the growth of *P. falciparum in vitro*. This inhibition appears to be mainly at the step of erythrocyte invasion by the parasites.

It has been documented that people living in malaria-endemic areas acquire immunity to *Plasmodium falciparum* after repeated infections. The nature of this immunity is poorly understood at the molecular level. It is apparent from studies involving passive transfer of IgG from immune adults to the non-immune subjects that circulating antibodies do play an important role (1, 2). The specificity of these protective antibodies is as yet unknown. It has been shown that the antibodies present in immune adults recognize domains that are conserved in different strains of *P. falciparum* (3). However, it has also been documented that many malarial antigens possess repetitive protein domains, which evoke a strong antibody response. Many of these antibodies are non-protective, and the corresponding malarial epitopes are postulated to be immunoevasive or smokescreen domains (4, 5). Thus, to search for pan-specific and possibly protective antibodies, a differential immunoscreening of an erythrocytic stage-specific cDNA expression library of *P. falciparum* was carried out using malaria-immune and acute patient sera. This resulted in the identification of several novel cDNA clones, which reacted exclusively and yet extensively with immune sera samples (6). The clone APF4, which was reactive to the largest number of immune sera (80 out of 92), has been cloned and sequenced recently (7). This was found to be the *P. falciparum* gene homologue of the ribosomal phosphoprotein P0 (PfP0).

Ribosomal phosphoprotein P0 is considered to be related to the family of the acidic ribosomal phosphoproteins P1 and P2, because of the highly homologous carboxyl-terminal domain (8). Antibodies against this domain coprecipitate all three P proteins (9). P0 could be cross-linked to P1 and P2 protein in *Artemia salina* ribosomes (10), and these data, along with that from yeast cells (11), strongly indicate the existence of a (P1)2P0(P2)2 protein complex in the eukaryotic ribosomes. This complex has been compared with the bacterial complex L10(L7/L12)2, which forms the stalk of the large subunit at the GTPase domain along with the 23 S ribosomal RNA (12, 13). It has been documented that P0 protein is absolutely required for the ribosomal activity and cell viability in yeast (14). The conserved carboxyl-terminal domain of the P proteins is very antigenic and found to be the main antigenic target for sera reactivity of about 10–15% of patients of the autoimmune disorder systemic lupus erythematosus (15). Antibodies to this domain have also been detected in patients of suffering from diseases caused by protozoan parasites such as Chagas’ heart disease (16) and leishmaniasis (17). In this paper we report the characterization of this protein from *P. falciparum*, and we show for the first time that P0 is indeed a phosphoprotein. We also show that antibodies raised against the amino-terminal domain of this protein inhibits *P. falciparum* growth in vitro.

EXPERIMENTAL PROCEDURES

Materials—All reagents, unless otherwise specified, were purchased from Sigma. [α-32P]dATP, [35S]orthophosphoric acid were provided by the Board of Radiation Technologies, India. [32P]Methionine and [35S]Cysteine were purchased from Amersham International (Buckinghamshire, England).

Parasite Cultures—Asexual stages of FCR3 (Gambia) and the FCK2 (India) strains of *P. falciparum* were cultured in vitro at 37 °C in the presence of human erythrocytes of serological type O+ in complete medium (RPMI 1640 medium containing 28 mM NaHCO3, 25 mM HEPES, and supplemented with either 10% human serum or 0.5% Albumax (Life Technologies, Inc.) and 80 μg/ml Gentamycin sulfate) in sterile Petri dishes using the candle-jar method or sealed flasks flushed with 5% O2, 5% CO2, and 90% N2 gas mixture (18). For stage-specific RNA preparation and in vitro parasite growth inhibition assays, cultures were synchronized by sorbitol treatment according to the method of Lambros and Vanderberg (19). Intracellular parasites from each of the substages were liberated from infected erythrocytes by saponin lysis.
A GST reporter-based vector (pGEX-1) was used. GST-fusion Protein—follow manufacturer's protocol by estimating the volume of the signal. The gel-documentation system (Ultraviolet Products Inc.) as per manufacturer's protocol. Air-dried blots were then UV cross-linked in a UV cross-linker (Bio-Rad) for 2 min and pre-hybridized at 42 °C for 2–3 h. Hybridization was carried out overnight at 65 °C in 50% formamide by washing with 1 × saline/sodium/phosphate/EDTA, 0.1% (w/v) SDS followed by washes with 1 × saline/sodium/phosphate/EDTA, 0.1% SDS and 0.1 × saline/sodium/phosphate/EDTA, 0.1% SDS, and exposed to Fuji x-ray film for autoradiography. The quantitation was performed using the gel-documentation system (Ultraviolet Products Inc.) as per manufacturer's protocol by estimating the volume of the signal.

**Preparation of Nucleic Acids**—Total cellular RNA was extracted using a single step method described by Chomczynski and Sacchi (22). Genomic DNA was extracted from total erythrocytic stages of the parasite as described in detail elsewhere (6).

**Southern and Northern Hybridization**—Southern and Northern hybridization were performed with the radioactively labeled (α-32P)dATP 251-bp AP4 cDNA fragment as well as the 700-bp L-4-7 (carboxyl-terminal fragment of PFP0 protein) with specific activity of 2 × 106 cpm by following the membrane manufacturer’s protocol (Amersham International) in the presence of 50% formamide. Briefly, 2 μg of parasite genomic DNA cut with appropriate restriction enzymes (New England Biolabs Inc.) was electrophoresed in a 1.0% agarose gel. In the case of Northern hybridization, total RNA from the parasite was electrophoresed in formaldehyde containing 0.8% agarose gel. 0.69–9.44-kb RNA markers (Life Technologies, Inc.) were run and stained separately with ethidium bromide before transfer of the gel onto the membrane. Gels were transferred to Hybond N+ membrane (Amersham International) by capillary blotting following membrane manufacturer’s protocol. Air-dried blots were then UV cross-linked in a UV cross-linker (Bio-Rad) for 2 min and pre-hybridized at 42 °C for 2–3 h. Hybridization was carried out overnight at 65 °C for 15 min each with 2 × saline/sodium/phosphate/EDTA, 0.1% (w/v) SDS followed by washes with 1 × saline/sodium/phosphate/EDTA, 0.1% SDS and 0.1 × saline/sodium/phosphate/EDTA, 0.1% SDS, and exposed to Fuji x-ray film for autoradiography. The quantitation was performed using the gel-documentation system (Ultraviolet Products Inc.) as per manufacturer’s protocol by estimating the volume of the signal.

**Preparation of Amino-terminal Domain of P0 as a GST-fusion Protein**—A GST reporter-based vector (pGEX-1) was used an expression vector (23). A HindIII-EcoRI restricted 138-bp amino-terminal fragment of PFP0 (46–184 bp of GenBank™ accession number U56663) (7) was flushed at both ends with Klenow and subcloned in pGEX-1 restricted with SmaI. The Escherichia coli cells harboring the vector pGEX-1 and the recombinant containing PFP0-N insert were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 37 °C (23). The total cell lysates were run on a 12% denaturing SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue to show the fusion protein (PFP0-N). The polyacrylamide gel piece containing the fusion protein band was cut out and crushed in liquid nitrogen and dissolved in phosphate-buffered saline (8 g/liter NaCl, 0.2 g/liter KCl, 1.44 g/liter NaHPO4, 0.24 g/liter KH2PO4, pH adjusted to 7.4 with NaOH). The resulting slurry containing an estimated amount of 100 μg of protein was injected into two rabbits. Five boosts were given to each animal to generate antibodies with reasonable titer (>1000 as checked by enzyme-linked immunosorbent assay).

**Preparation of Monospecific Human Sera—AP4 cDNA clones in Agt11, as well as wild type Agt11, were grown on E. coli strain Y1090 to lytically induced C′-fragment containing plasmid. Mutant Agt11 (Agt11ΔC′) cloned (Agt11ΔC′ International) soaked in isopropyl-β-D-thiogalactopyranoside was overlaid on these phase plates and grown for an additional 12–16 h. The membranes were washed three times with 1 × TBS-T (200 μM Tris, 50 mM NaCl, 0.05% Tween 20, pH 7.5). A pool of six reactive human immune sera (1:100 dilution) used for the differential immunoscreening (6) was first incubated overnight at 4 °C with membrane saturated with the wild type Agt11 lysate. Then the sera was incubated overnight with several membranes containing the lysate of recombinant AP4. Monospecific antibodies against AP4 were eluted with 5 mM glycine from the membrane, neutralized with 1 M Tris, and then dialyzed overnight with three changes of TBS-T. Resulting monospecific antibody solution against AP4 was concentrated using an Amicon concentrator (Amicon Inc.), reconstituted to the original volume of the human sera with 1 × TBS-T, and used for immunoprecipitation.

**Preparation of [35S]Methionine, [35S]Cysteine, and [32P]-Labeled Proteins from P. falciparum and Immunoprecipitation Studies**—Asynchronous cultures of P. falciparum containing 10–12% parasitemia were washed twice with methionine- and cysteine-free RPMI 1640 medium and resuspended at a final hematocrit of 5% in the same medium supplemented with 10% human serum. 100 μCi/ml of both radiolabeled methionine and cysteine were added to the culture medium and incubated for 4 h at 37 °C under normal culture conditions with occasional shaking. After labeling, the cells were washed and extracted as described elsewhere (24). In the case of [32P]-labeling, 1 mCi/ml radiola beled orthophosphoric acid was neutralized using 1 N NaOH added to the washed parasites at 5% hematocrit (about 10% parasitemia) in the RPMI 1640 medium supplemented with 10% human serum. 100 μCi/ml of both radiolabeled methionine and cysteine were added to the culture medium and washed five times with phosphate-buffered saline before addition of 10–12% parasitemia. 100 μCi/ml of both radiolabeled methionine and cysteine were added to the culture medium and incubated for 4–6 h at 37 °C under normal culture conditions with occasional shaking. After labeling, the cells were washed and extracted as described elsewhere (24). In the case of [32P]-labeling, 1 mCi/ml radiolabeled orthophosphoric acid was neutralized using 1 N NaOH added to the washed parasites at 5% hematocrit (about 10% parasitemia) in the RPMI 1640 medium supplemented with 10% human serum. 100 μCi/ml of both radiolabeled methionine and cysteine were added to the culture medium and incubated for 4–6 h at 37 °C under normal culture conditions with occasional shaking. After labeling, the cells were washed and extracted as described elsewhere (24). In the case of [32P]-labeling, 1 mCi/ml radiolabeled orthophosphoric acid was neutralized using 1 N NaOH added to the washed parasites at 5% hematocrit (about 10% parasitemia) in the RPMI 1640 medium supplemented with 10% human serum.
scopic examination of 10,000 red blood cells. In each experiment, the number of rings, trophozoites, and schizonts were counted separately. Total parasitemia was estimated as the sum total of the rings, trophozoites, and schizonts.

RESULTS

A Southern blot of genomic DNA from the HB3 strain of *P. falciparum*, probed with the 251-bp APf4 insert, showed that it hybridizes with the 6.1-, 13-, 1.5-, and 11-kilobase pair band when the DNA was restricted with EcoRI, Clai, DraI, and HaeIII, respectively (Fig. 1, panel A). The gene has an internal EcoRI site, and this was demonstrated by probing the same blot with a 700-bp fragment, L-4-7, representing the carboxyl-terminal part of the protein (7), which lit up the same bands for all restriction digests except that of EcoRI, where it showed a 7.0-kilobase pair band (Fig. 1, panel B). Southern analysis of genomic DNA from two other *P. falciparum* strains, FCR3 and NF54, was also performed with these two probes, and no significant restriction fragment length polymorphism was observed. These results show that the PfP0 is coded by a single gene and is well conserved in different strains of the parasite.

A Northern blot of stage-specific total RNA from the asexual stages of *P. falciparum*, probed with the 251-bp APf4 fragment, showed a dominant 3.0-kb-size fragment in every substage (Fig. 2). However, a second band, about 2.0 kb in size, was also seen in all stages. A quantitative determination of the ratio of these transcripts showed that the 3.0-kb message was 2.1 ± 0.26-fold as abundant as the 2.0-kb message in each of the stages. However, the 3.0-kb transcript was about 2.0- and 1.5-fold greater in abundance in the trophozoites compared with the rings and the schizont stages, respectively. The coding region of the P0 gene is 957 bp. However, the gene seems to possess a long 5′-untranslated region of about 1.4 kb in length, as observed by the size of cDNA clones isolated (7). Polymerase chain reaction studies using primer sequences within the coding region amplify the same size of fragments when genomic DNA is used as a template (data not shown), and therefore, there are no introns within the coding sequence. However, the presence of introns in the 5′-untranslated region is yet to be determined.
Fig. 3A shows the expression of the GST-fusion protein of the amino-terminal domain of the *P. falciparum* P0 protein (PfP0-N) in the total *E. coli* cell lysate. The 46-amino acid stretch (17–62 amino acids) from the 138-bp HindIII-EcoRI fragment (7) produced the expected 31-kDa GST-fusion protein. Polyclonal rabbit antibodies, with a titer of >1000, were raised against PfP0-N and used for immunoprecipitation, immunofluorescence, and growth inhibition studies.

This antibody immunoprecipitated a single 38-kDa phosphoprotein from the \textsuperscript{35}S- and \textsuperscript{32}P-labeled *P. falciparum* proteins (Fig. 3B). Control antibodies such as rabbit preimmune sera and rabbit polyclonal antibodies raised against GST did not show any band (data not shown). To ascertain that the human immune sera originally used for the differential screen actually recognized the PfP0 protein domain in the \textit{LPf4} expression clone, monospecific human immune sera was affinity-purified using \textit{LPf4} expression clone and used for immunoprecipitation analysis. This also brought down the 38-kDa P0 protein from \textsuperscript{35}S-labeled parasite proteins (Fig. 3B). Human immune sera affinity-purified against the control phage \textit{agt11} did not show any reactivity with the parasite extract (Fig. 3B). Western blots of the recombinant PfP0-N protein with immune and patient sera samples showed that the recombinant protein was detected only by immune sera samples and not by patient sera samples (data not shown).

Rabbit antibodies raised against PfP0-N were used for IFA studies with different asexual stages (Fig. 4, A and B) as well as gametocytic stages of the parasite (Fig. 4, C and D). Rabbit preimmune sera and polyclonal antibodies against GST were used as control. The control antibodies showed no staining with parasites, whereas the anti-PfP0 antibody showed staining with all the different developmental stages of the parasite. In some of the gametocytic stages, intense staining of some subcellular domains were noticed (Fig. 4D), but the significance of this observation is not clear. The localization of the antigen was predominantly intracellular, however there appeared to be some staining at the surface as well. Preliminary experiments with biochemically fractionated parasite extracts do show antibody reactivity to the particulate fractions on Western blots (data not shown). When tested on *Toxoplasma gondii*, an apicomplexan parasite closely related to *Plasmodium*, it was observed to stain the surface, as confirmed by double staining with surface and internal markers of *Toxoplasma*.

It has been reported that the protective human immune sera inhibits the \textit{in vitro} growth of *P. falciparum* only in the presence of monocytes (2). However, these experiments were performed with mixed specificities of immunoglobulins. To ascertain whether anti-PfP0 antibody inhibits the growth of *P. falciparum* in the presence or absence of monocytes, \textit{in vitro} inhibition studies using synchronized parasites starting from the ring stages were performed (Fig. 5). The antibodies raised against PfP0-N completely inhibited the growth of the parasites over a 48-h period (Fig. 5, panel A). Preimmune sera and sera raised against an irrelevant \textit{P. falciparum} GST-fusion protein showed no inhibition in the growth of the parasites. Cultures without any IgG and with sera raised against GST protein showed normal growth (data not shown). 4–6 independent experiments were performed for each treatment, and...
the figure represents an average of these experiments. The presence of monocytes were found to stimulate the parasite growth, but the inhibition of growth of *P. falciparum* by the antibody was independent of the presence of monocytes (Fig. 5A).

The growth inhibition studies were assayed by microscopically counting at least 10,000 red blood cells for each time point. The counting assay was performed rather than the hypoxanthine-uptake assay, because the uptake of hypoxanthine by the monocytes would cause errors in the assay. Also, starting with a synchronized culture and counting the different substages at every time point would show the development of the substages of the parasite with time. Cultures with synchronized ring stages were the starting point, and every 6 h the different substages of the parasite were counted. In the presence of the control antibody, the parasites progressed through the ring, trophozoite, and schizont stages in about 24 h, and subsequently there was an increase in the ring stages, indicating the invasion of fresh red blood cells (Fig. 5B, top panel). However, in the presence of anti-PfP0 antibody, even though the parasites developed from rings to trophozoite and schizont stages, fresh infection of red blood cells did not take place as the number of ring stages remained close to zero until 48 h in culture (Fig. 5B, lower panel). The same profile was observed for the distribution of the parasite substages irrespective of the presence (Fig. 5B) or absence (data not shown) of monocytes.

**DISCUSSION**

Earlier we had reported the cloning of the full-length gene of PfP0 from the 7G8 strain of *P. falciparum* (7). The largest cDNA clone had a coding region 957 bases long, an unusually long 5’-untranslated region of at least $-1.4$ kb, and a 3’-untranslated region of $-50$ bases, making it a total of at least $-2.4$ kb in size. The 3.0-kb transcript therefore matches with this size. It is not clear whether the second transcript of $-2.0$-kb size is a processed transcript or a degradation product. The transcripts are twice as abundant in the trophozoite stages, indicating that the expression of this gene may be regulated.

The predicted molecular mass of the full-length gene sequence is 37.5 kDa. The rabbit sera raised against the recombinant protein domain of the P0 gene recognized a 38-kDa *P. falciparum* protein from both 32P- and 35S-labeled cultures, which matches with the expected size of the deduced P0 protein. The monospecific immune sera also recognized the same size protein. These immunoprecipitation studies showed that the antibodies raised against the PfP0-N recognize a protein of the predicted size, that it is indeed a phosphoprotein, and that antibodies against PfP0-N are present among malaria-immune people and not in patients.

The P0 protein was first identified as a member of the P family of proteins through coprecipitation studies using antibodies against the conserved carboxy-terminal region (9, 15). Although definitive studies have been performed to demonstrate that P1 and P2 are phosphoproteins (27), there has been no direct evidence that P0 is a phosphoprotein. 32P-Labeled cultures of yeast cells showed a large number of phosphoribosylproteins (28), of which the 41-kDa protein has been assumed to be the P0 protein (9). Thus, for the first time, through immunoprecipitation studies using anti-P0 antibodies, we are demonstrating that P0 is indeed a phosphoprotein.

Autoantibodies against the conserved carboxy-terminal domain of the P0 proteins have been found in 10–15% of patients with an autoimmune disorder, systemic lupus erythematosus (15). These autoantibodies have been implicated as a cause for the psychotic disorders among these patients. Immune response has also been documented against P0 protein in chronic Chagas’ disease (16) and leishmaniasis (17). However, in each of these cases the antibody has been reported against the carboxy-terminal domain. The systemic lupus erythematosus patient sera have been tested for reactivity against the amino-terminal domain of the P2 protein but did not show any response (15). The reactivity of the malaria-immune sera to P0 is widespread (87% of immune sera samples) (6) and is directed toward the P4 region which is the amino-terminal domain of the PfP0 protein (7). Thus this immune response toward P0 in malaria-immune persons appears to be different from that observed for the systemic lupus erythematosus patients.

The mechanism of the passive clearance of the parasites in malaria patients with IgG of malaria-immune persons is not clearly understood. The IgG may be specifically interacting and blocking crucial parasite domains, or it may be attaching to the parasitized cells and allowing other cellular components of the immune system to clear the parasites. The latter has been supported by studies in which a pool of immune human sera inhibited the *in vitro* growth of *P. falciparum* only in the presence of monocytes (2). However, these experiments were performed with mixed specificities of immunoglobulins. Anti-PF0-N antibody inhibits the growth of *P. falciparum* irrespective of the presence or absence of monocytes, indicating a direct block of parasite invasion. The mechanism behind the inhibition of P. falciparum growth with anti-PF0-N antibodies is unclear given the predominant cytoplasmic localization of the P0 protein. The inhibition is unlikely to be due to the inhibition of the *P. falciparum* ribosomal activity as the development from the ring to the schizont stages were nearly normal in the presence of anti-PF0-N antibody over a period of 24 h. The growth inhibition by anti-PF0-N IgG clearly seems to act at the erythrocyte-invasion step. While the IFA results do indicate a surface component in addition to the cytoplasmic localization of P0 in the trophozoites and gametocytic stages, whether there is a surface localization of this protein in the merozoite stage is yet to be determined. Interestingly, the presence of an antigenic determinant related to the carboxy-terminal of P0 protein has been localized to the surface of hepatoma, neuroblastoma, and human fibroblast cells (29).

What this surface determinant of P0 protein may be and what role it may play in the erythrocyte invasion by the malarial parasite remains to be established.

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Arunava Goswami, Subhash Singh, Vilas D. Redkar and Shobhona Sharma

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