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***Plasmodium falciparum* GPI mannosyltransferase-III is functional with novel signature sequence**

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Abstract

The glycosylphosphatidylinositol (GPI) anchors of *Plasmodium falciparum* are indispensable for parasite survival since merozoite surface proteins-1, -2, -4, -5 and -10 crucial for erythrocyte invasion are GPI-anchored. Therefore, the GPI biosynthetic pathway can offer potential targets for novel anti-malarial drugs. Here, we characterized the putative *P. falciparum* PIG-B gene (*PfPIGB*) that encodes mannosyltransferase-III of GPI biosynthesis. *PfPIGB* mRNA is transcribed in a developmental stage specific manner. A protein corresponding to the expected size of PfPIGB is expressed by the parasite and is localized in the endoplasmic reticulum. Treatment of parasites with PfPIGB specific siRNA caused reduced GPI synthesis, affecting the PIG-B specific GPI intermediate. These data demonstrate that *PfPIGB* is functional and encodes for mannosyltransferase-III of the parasite GPI biosynthesis. The parasite PfPIGB is novel in that its signature sequence HKEHKI is unique and is only partially conserved compared to HKEXRF signature motif of mammalian PIG-B enzymes.

Keywords

Plasmodium falciparum; glycosylphosphatidylinositol anchors; biosynthesis; mannosyltransferase-III; characterization

Malaria caused by *Plasmodium falciparum* is a major health problem in many countries. About 40% of the global population is at risk to malaria, and in sub-Saharan Africa, millions of deaths, mostly among children less than 5 years old, occur every year [1]. The death toll due to malaria has been rapidly increasing because of drug resistance. Development of novel drugs targeting the essential metabolic pathways of the parasite and therapeutics/vaccine that prevent the infection and/or illness are urgently needed. In this context, glycosylphosphatidylinositol (GPI) biosynthetic pathway can offer important targets [2].

The GPIs of all organisms have a conserved glycan core, Man α 1–2Man α 1–6Man α 1–4GlcN α 1, covalently linked to *myo*-inositol of phosphatidylinositol (PI) at *O*-6 [3]. The mannose-3 of the glycan core is invariably substituted at *O*-6 with EtN-*P*, which is used for membrane anchoring of proteins through amide bond formation with the -COOH of the protein C-termini. GPIs from different organisms vary in the PI moiety in having acylated or nonacylated *myo*-inositol, and diacylglycerols, monoacylglycerols, alkyl/acylglycerols or ceramide [3]. In the case of *P. falciparum* GPIs, the conserved glycan core is substituted with an additional Man at *O*-2 of the third Man, and the PI comprised of diacylglycerol and *O*-2-acylated inositol moieties, both with variable fatty acids [4].

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The biosynthesis of GPIs occurs in the ER by the sequential addition of sugars to the PI by a coordinated action of glycosyltransferases, *N*-deacetylase, inositol acylase, EtN-*P* transferase, and several accessory proteins [5]. The preassembled GPIs are transferred en bloc to the C-termini of proteins that have GPI attachment signal sequence. In animals, hundreds of proteins including cell surface receptors, enzymes, cell adhesion molecules, and variant protective antigens are GPI anchored [4].

In intraerythrocytic *P. falciparum*, several functionally important cell surface proteins, including MSP-1, MSP-2, MSP-4, MSP-5 and MSP-10, are GPI anchored. These parasite proteins have been studied as invasion-blocking vaccine candidates [6]. Further, as in other parasites, *P. falciparum* expresses GPIs in large excess than the requirement for protein anchoring [7], leading to GPI-dependent recognition by the host immune system [8]. The parasite GPIs are thought to be the major factors responsible for malaria pathogenesis [4]. Therefore, the knowledge of GPI biosynthesis is important for understanding the parasite biology as well as in defining the role of GPIs in pathogenesis.

A recent bioinformatic study has suggested the presence of several putative genes of *P. falciparum* GPI biosynthesis [9]. However, thus far only *GPII*, a component of the multi-protein complex involved in the transfer of *N*-acetylglucosamine to PI, has been functionally characterized [10]. The other genes of the parasite GPI biosynthesis remain uncharacterized with regard to the expression of functional enzymes. Here, we studied the expression of the mannosyltransferase-III of *P. falciparum* GPI biosynthesis. We show that *P. falciparum* PIG-B (PfPIG-B) localized to ER and that it is functional.

Materials and methods

Parasites

P. falciparum (3D7 strain) was cultured in RPMI 1640 using human O-positive blood and 10% O-positive human serum. Parasites were synchronized with 5% sorbitol [7].

Isolation of RNA and preparation of cDNA

The parasites were released from the mid-trophozoite stage culture (300 µl) with 20–25% parasitemia by 0.05% saponin treatment [7]. The parasite pellet was dissolved in 1 ml of TRIzol (Invitrogen) and RNA was isolated. RNA (1 fg) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) [11].

PCR Amplification of the PfPIG-B

PfPIG-B was identified by BLAST search of the *P. falciparum* genome database (www.PlasmoDB.org) using the conserved motifs of human PIG-B amino acid sequence as query. The full-length *PfPIG-B* ORF was amplified from cDNA by PCR using high fidelity *Taq* DNA polymerase (Invitrogen) and primers 5'-ATGATTTACAATGACATTTTAACATTATG-3' (forward), and 5'-TTAGGAAGGAACCCTCTTGAATATG-3' (reverse).

PfPIG-B cloning

The *PfPIG-B* ORF DNA was ligated into pSTBlue-1 AccepTor vector and positive clones containing full-length inserts were screened by restriction analysis [11]. The clones were confirmed by sequencing. The sequence data has been submitted to Genbank (Accession No. **AY576000**).

Analysis of PfPIG-B mRNA

Parasite RNA (5 fg each), isolated as described above from parasites at different time intervals, was separated on a 0.74% formaldehyde/1.2% agarose gels and transferred onto Zeta-Probe nylon membranes (Bio-Rad) [11]. A [³²P]dCTP labeled 616 bp DNA fragment corresponding to 195–810 nt of *PfPIG-B* ORF, obtained by PCR using 5'-

ATGGGAATGGGAACCTTGTG-3' (forward), and 5'-

ATGATGATCATTCCTTCTTTC-3' (reverse) primers, was used as the probe for Northern hybridization. The Northern analysis was performed as described previously [11].

Preparation of siRNA

A 616 bp PCR product corresponding to 195–810 nt region of the *PfPIG-B* ORF was amplified using the primers with T7 promoter (bold): 5'-

CGGTAATACGACTCACTATAGGGAGAATGGGAATGGGAACCTTGTG-3' (forward) and 5'-

CGGTAATACGACTCACTATAGGGAGAATGATGATCATTCCTTCTTTC-3' (reverse). A 566 bp (882–938 nt) PCR product of *P. falciparum* erythrocyte membrane protein 1 gene (*PfEMP1*) (PlasmoDB ID PFE1640w) amplified using primers, 5'-

CGGTAATACGACTCACTATAGGGAGATGATTCCTCCAAGAAGAAGAC-3' (forward) and 5'-

CGGTAATACGACTCACTATAGGGAGAAATATCATTCTGACATCCAGG-3' (reverse), with T7 promoter (bold), was used for the preparation of control dsRNA. The dsRNAs were synthesized by *in vitro* transcription using T7 RNA polymerase (Fermentas).

The DNA template was removed by treatment with 3 units DNase I (Novagen). The dsRNAs were precipitated with LiCl and digested with human recombinant dicer enzyme (Gene Therapy Systems, San Diego, CA) according to the manufacturer's instructions.

Treatment of parasites with siRNA and metabolic labeling

The synchronized parasite culture (10 µl) at the ring stage (12–15% parasitemia) in 24-well culture plates was treated with siRNA (10–20 fg) in 400 µl incomplete medium at 37 °C with occasional shaking. After 2 h, 600 µl medium containing 5% Albumax (Invitrogen) was added, and incubated at 37 °C. Untreated parasites, and those treated with lipofectamine, and nonspecific siRNA were used as controls. The parasites were metabolically labeled at 30 h postinvasion with 50 fCi of [³H]GlcN (Amersham Biosciences) for 6 h and harvested.

Analysis of GPIs

The GPIs from [³H]GlcN-labeled siRNA-treated and untreated parasite cultures were isolated as described previously [7]. The washed parasite pellets were extracted 4 times with 100 µl CHCl₃/MeOH/water (10:10:3, v/v/v). Aliquots of GPIs containing equal amounts of radioactivity were analyzed by HPTLC using CHCl₃/MeOH/water (10:10:2.4, v/v/v) as reported previously [7]. The nature of the [³H]GlcN-labeled glycolipids was confirmed by their susceptibility to nitrous acid and α-mannosidase [12].

Production of antiserum against PfPIG-B specific peptide

Antiserum against PfPIG-B peptide, WTNLKERRNDHHFDYENN (amino acids 259 to 277) was produced in mice. The antiserum was analyzed for the peptide-specific IgGs by ELISA. The preimmune serum was analyzed in parallel as a control.

Immunoprecipitation of PfPIG-B and Western blotting

PfPIG-B was immunoprecipitated from the lysates, prepared from the parasite pellet (0.2 ml) obtained by saponin treatment of the late trophozoite stage culture, using mouse anti-PfPIG-

B peptide antiserum and Protein A-agarose beads. The immunoprecipitates were analyzed by Western blotting with 1:400 diluted anti-PfPIG-B peptide antiserum, 1:2000 diluted HRP conjugated goat anti-mouse IgG, and chemiluminescence substrate.

Localization of PfPIG-B by immunofluorescence analysis

Thin smears of infected and uninfected erythrocytes from *P. falciparum* culture at the trophozoite stage on glass slides were fixed in 2 % paraformaldehyde, permeabilized as reported [13], probed with 1:800 diluted anti-PfPIG-B peptide antiserum followed by 1:400 of rat anti-GRP antibodies (MR4, ATCC). The slides were then treated with a mixture of DAPI, 1:400 diluted Alexa Fluor 568-conjugated goat anti-mouse IgG, and Alexa Fluor 488-conjugated goat anti-rat IgG. Slides incubated with preimmune serum and anti-GRP antibodies were used as controls. The slides were examined by fluorescence microscopy.

Results

BLAST search of the *P. falciparum* genome database, using the highly conserved regions of the human PIG-B protein sequence as query, identified a single ortholog hypothetical protein MAL13P1.210, on chromosome 13 of the parasite. The full-length *PfPIG-B* cDNA was cloned into pSTBlue1 vector and used as template for the preparation of dsRNA and probe for Northern analysis. Analysis of *PfPIG-B* expression during the 48 h parasite life cycle showed little or no mRNA until 24 h postinvasion, whereas high levels of mRNA was synthesized during the mid to late trophozoite stage (24–36 h postinvasion) with maximal level expression between 30 and 36 h postinvasion (Fig. 1A). The level of mRNA was markedly low between 36–48 h postinvasion. These results are consistent with the reported timing of GPI biosynthesis in *P. falciparum*, i.e., during the trophozoite stage (28–40 h) with peak level by 40 h postinvasion [7].

Immunoprecipitation using the PfPIG-B peptide antiserum followed by Western analysis showed a single band of molecular mass ~84 kDa (Fig. 1B), suggesting that the mRNA of PfPIG-B is translated. Protein of similar molecular size was not precipitated with preimmune serum. The calculated molecular mass of PfPIG-B, based on the predicted amino acid sequence and subtracting for 41 amino acid residues of putative signal sequence, is ~90 kDa. These data suggest that the mRNA of PfPIG-B is translated.

If PfPIG-B were to be a mannosyltransferase of the GPI biosynthesis, then the enzyme must be localized in the ER. Immunofluorescence analysis using PfPIG-B peptide-specific antibodies showed that the parasite-infected erythrocytes express the protein around the parasite nucleus (Fig. 2). The stained pattern colocalized with that observed when stained with antiserum against the 78-kDa glucose-regulator protein (GRP) of *P. falciparum*. Previously GRP has been shown to be present in the lumen of the parasite's ER [14]. These results agree with the gene being parasite *PIG-B*.

To establish whether PfPIG-B is a functional enzyme, we studied the effect of the gene-specific siRNA on the parasite GPI biosynthesis. Northern analysis of RNA isolated from siRNA treated parasite culture showed a significant decrease (~50%) of PfPIG-B mRNA. The siRNA-treated parasites synthesized GPIs at 10–20% lower levels than that by untreated parasites as assessed by metabolic labeling with [³H]GlcN at 30 h postinvasion, suggesting the target (GPIs) specific effect. HPTLC analysis of the purified GPIs showed accumulation of Man₂-GlcN-PI intermediate in siRNA-treated samples as compared to untreated parasites (Fig. 3A; compare lane 2 with lane 1; also see lane 1 in panel B). Treatment of parasite cultures with a nonrelevant siRNA, prepared from dsRNA from *PfEMP1*, did not show accumulation of Man₂-GlcN-PI (Fig. 3C). Upon treatment of the GPIs with jack bean α -mannosidase, the accumulated intermediate was converted into inositol-acylated GlcN-PI, confirming that it is Man₂-GlcN-

PI (Fig. 3B). These results suggest that *PfPIG-B* gene is functional and encodes the mannosyltransferase-III of the GPI biosynthetic pathway (Fig. 3D).

The sequence analysis of the cloned *PfPIG-B*, revealed a 2361 bp ORF and the deduced protein sequence (786 amino acids) showed the presence of glycosyltransferase domains characteristic of Alg9-like mannosyltransferase family, which includes mannosyltransferase enzymes of GPI biosynthetic pathway. PfPIG-B shows basic isoelectric point of 8.95 similar to the basic pI of PIG-B from human, mouse and yeast. PfPIG-B showed 24, 24, 21 and 17% sequence homology, respectively, with PIG-B from human, *Arabidopsis*, mouse and *Saccharomyces cerevisiae*. Kyte and Doolittle Hydropathy plot showed the presence of several potential transmembrane domains (TMDs) (Fig. 4A) [15]. Consistent with these results, analysis by TopPred 2 revealed the presence of 10 potential TMDs with structural organization similar to that proposed for the PIG-B of other organisms (Fig. 4B) [16]; the cytoplasmic and luminal orientations of various loops are similar (Fig. 4B) [17]. The NH₂- and C-terminal hydrophilic motifs are predicted to be in the cytoplasmic side. The long hydrophilic loop between TMD 1 and 2 contain highly conserved DE, Q, E, WEW, R, and P amino acid residues, and the loop is in the predicted luminal side. The loop between TMD 4 and 5 with the conserved RT amino acid residues are present in the predicted cytoplasmic side. Similarly, the loop containing highly conserved residues F, N, and G residues and that with HKEXBaHy (where 'Ba' is basic amino acid and 'Hy' is a hydrophobic amino acid) motif are in the predicted cytoplasmic side. As in the case of PIG-B of other species, the predicted cytoplasmic hydrophilic C-terminal motif is well conserved and contains putative ER membrane retention signal, KRVP (Fig. 4B). In PIG-B of various species, the conserved HKEXRF motif is the signature sequence for the enzyme. Interestingly, in PfPIG-B, the corresponding motif is only partially conserved; the corresponding peptide sequence in PfPIG-B is HKEHKEI (Fig. 4C). However, the corresponding non-conserved amino acids H and I in this motif represent, respectively, basic and hydrophobic residues, similar to PIG-B of other species. Sequence analysis using NCBI Conserved Domain Database Search program indicated that the 34–124 NH₂-terminal amino acid region and the 315–521 middle regions of PfPIG-B show close similarity to $\alpha(1-2)$ mannosyltransferase family enzymes of both the *N*-glycan and GPI biosynthesis (Fig. 4D). However, the conserved domains covering amino acid 34–106 and 346–655 resemble more closely the $\alpha(1-2)$ mannosyltransferase-III of the GPI biosynthesis rather than that of the *N*-glycan biosynthesis (not shown), indicating that the parasite protein is encoded by *PfPIG-B* of the GPI biosynthetic pathway.

Discussion

Our conclusion that *PfPIG-B* encodes $\alpha(1-2)$ mannosyltransferase-III of *P. falciparum* GPI biosynthesis is supported by the following evidence: (i) Northern analysis showed an mRNA of the size predicted for the transcript of *PfPIG-B* gene. The gene is transcribed in a parasite developmental stage-specific manner. The pattern of mRNA expression is very similar to the previously observed pattern of GPI biosynthesis during the intraerythrocytic *P. falciparum* development; GPI synthesis occurs exclusively during the maturation of trophozoites and no GPI biosynthesis was evident during the ring stage. (ii) Immunoprecipitation of the parasite lysates and western analysis using antibodies raised against PfPIG-B peptide showed a ~84 kDa protein. These results agree with the calculated molecular mass of the putative PIG-B. (iii) Immunofluorescence analysis of parasite, localized PfPIG-B protein to the ER, where the GPI biosynthesis occurs. These results strongly suggest that the putative *PfPIG-B* is a functional gene.

The results of RNAi studies demonstrate that *PfPIG-B* encodes a functional enzyme. As shown in Fig. 3, PfPIG-B-specific effect was evident by siRNA treatment, as revealed by the accumulation of distinctively noticeable amounts of Man₂-GlcA-PI intermediate. This effect

is likely due to either RNAi or antisense mechanisms. Although parasite lacks RNAi machinery as indicated by the absence of components of RNAi in parasite genome database [18,19], some studies have reported successful gene-specific RNAi effect [20–24]. Recently, it has been shown that both uninfected and parasite-infected red blood cells contain high levels of human miRNA, particularly mir-451 miRNA [25]. Since parasite-specific miRNA were not evident in parasites, it was suggested that erythrocytes contain a low level of RNAi machinery, which could be used by parasites [25]. Thus, the observed RNAi effect in this study as well as the previously reported siRNA-dependent degradation of target specific mRNAs is likely due to the transport of RNAi enzymes from host cells. It is possible that RNAi components present in fresh erythrocytes are taken up by the parasite that degraded PIG-B mRNA by RNAi process. This suggestion agrees with the observed low efficiency of the RNAi effect and with the fact that mature erythrocytes do not produce RNAi machinery *de novo* and thus are expected to have only low levels of RNAi components. Alternatively, it is possible that the antisense strand of siRNA has inhibited PfPIG-B mRNA translation as suggested by previous studies [24,26, 27]. Regardless of the mechanism involved, our results indicate that the *PfPIG-B* gene studied here is functional and encodes for α (1–2)mannosyltransferase-III of *P. falciparum* GPI biosynthesis.

The primary structure and structural organization of the functional PfPIG-B characterized in this study are consistent with the enzyme belonging to the α (1–2)mannosyltransferase family enzymes of the *N*-glycan and GPI biosynthesis. Similar to the other members of this family, PfPIG-B has multiple TMDs. The predicted topological orientations of the N-terminal and C-terminal motifs as well as the various hydrophilic loops with the highly conserved amino acids residues and peptide motifs are similar to those for other members of this family. However, a novelty of PfPIG-B is that the signature peptide motif HKEXBaHy is only partially conserved. Furthermore, despite its unique signature motif, PfPIG-B clearly distinguishes the related Smp3 sub family enzymes, the α (1–2)mannosyltransferases that catalyze the attachment of Man-4 of the GPIs, that have HQEXRF signature motif [17]. Overall, the structural organization of PfPIG-B resembles closely those of the other members of the α (1–2)mannosyltransferase family, particularly the PIG-B of the GPI biosynthesis.

Considering that the amino acid sequence identity is 24% between human and *P. falciparum* PIG-B, and their signature motifs are significantly distinct, it is likely that the active sites for human and parasite enzymes are considerably different. Therefore, detailed structure-activity studies should reveal novel targets for the development of drugs for malaria.

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Abbreviations

GPI	glycosylphosphatidylinositol
MSP	merozoite surface protein
Man	mannose
GlcN	glucosamine
PI	

phosphatidylinositol

Man₂-GPI

Man α 1–6Man α 1–4GlcN α 1-PI

Man₃

Man1 α –2Man α 1–6Man α 1–4GlcN α 1-PI

Man₄-GPI

Man α 1–2(Et-*P*-6)Man1 α –2Man α 1–6Man α 1–4GlcN α 1-PI

EtN-*P*

ethanolamine phosphate

PIG

phosphatidylinositolglycan like

SSC

sodium saline citrate

HPTLC

high performance thin-layer chromatography

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Fig. 1.

Analysis of *PfPIG-B* gene transcripts and protein. Panel A: Northern blot analysis of *PfPIG-B* mRNA transcripts. Lanes 1–7: RNA from parasites harvested at 12, 18, 24, 30, 36, 42, and 48 h, respectively. The sizes of the standard RNA markers are shown in the left. The bottom panel shows ethidium bromide-stained 28S and 18S RNA bands as loading controls. Panel B: Western blot analysis of PfPIG-B. Immunoprecipitates from parasite cell lysates were analyzed by Western blotting using 5–10% gradient SDS-polyacrylamide gels and nitrocellulose membranes. Lane 1: Precipitate with PfPIG-B antiserum. Lane 2: Precipitate with normal mouse serum. The positions of the protein molecular mass standards (kDa) are indicated on the left and the arrow on the right side indicates the position of the immunoprecipitated protein. The lower molecular mass protein bands (~50 kDa and below) represent the IgG heavy and light chains.

Fig. 2.

Localization of PfPIG-B by immunofluorescence analysis. Upper panel: (A) DAPI; (B) DAPI plus mouse normal serum; (C) DAPI plus rat anti-GRP antibodies; (D) merged images of (B) and (C). Lower panel: (E) DAPI; (F) DAPI plus mouse antiserum against the PIG-B peptide; (G) DAPI plus rat anti-GRP antibodies; (H) merged images of (F) and (G).

Fig. 3.

HPTLC analysis of GPIs synthesized by siRNA-treated *P. falciparum*. (A) Lanes 1: GPIs from untreated parasites. Lane 2: GPIs from parasites treated with PfPIG-B siRNA. (B) Lane 1: GPIs from PfPIG-B siRNA-treated parasites. Lane 2: GPIs from PfPIG-B siRNA-treated parasites after incubation with jack bean μ -mannosidase. The Man₂-GlcN-PI in panel A (lane 2) and panel B (lane 1) is shown by arrowheads. (C) GPIs from parasites treated with PfEMP1 siRNA. In each lane, equal amounts of radiolabeled GPIs were analyzed. Therefore, the proportions of GPIs in treated and untreated parasites appear similar even though the total amounts of GPIs synthesized by the siRNA-treated parasites were 10–20% lower than those synthesized by untreated parasites. (D) Schematic of GPI biosynthetic pathway; mannosyltransferase-III (PIG-B) is underlined. Gn-PI, GlcN-PI; Gn-PI*, GlcN-PI without inositol acylation; M₂Gn-PI, Man μ 1–6Man μ 1–4GlcN μ 1-PI; M₃Gn-PI Man1 μ –2Man μ 1–6Man μ 1–4GlcN μ 1-PI; M₄Gn-PI, Man μ 1–2Man1 μ –2Man μ 1–6Man μ 1–4GlcN μ 1-PI; EM₃Gn-PI, EtN-*P*-6Man1 μ –2Man μ 1–6Man μ 1–4GlcN μ 1-PI; EtM₄Gn-GPI, Man μ 1–2(EtN-*P*-6)Man1 μ –2Man μ 1–6Man μ 1–4GlcN μ 1-PI; ManT, mannosyltransferase.

Fig. 4.

Structural organization and conserved regions of PfPIG-B. (A) Kyte-Doolittle hydropathy plot of PfPIG-B over a window length of 17 [15]. (B) TMDs of PfPIG-B predicted by the TopPred 2 program [16]. The open rectangles are helical TMDs in the lipid bilayer (gray horizontal band). The highly conserved amino acid residues and the peptide motifs in various loops are indicated. The putative ER membrane retention signal is boxed. C, cytosol; L, lumen of the ER. (C) Homology comparison of the 483–516 amino acid region of PfPIG-B. The putative signature sequence motif of PIG-B, HKEXBaHy, is boxed. (D) Schematic representation of the regions of the parasite enzyme that show well conserved homology with the animal ALG9 and PIG-B, the $\mu(1-2)$ mannosyltransferases of the *N*-glycan and GPI biosynthesis, respectively.