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Plasmodium falciparum GPI mannosyltransferase-III has novel signature sequence and is functional

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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 PfPIG-B is expressed by the parasite and is localized in the endoplasmic reticulum. Treatment of parasites with PfPIG-B specific siRNA caused reduction in GPI synthesis, affecting the PIG-B specific GPI intermediate. These data demonstrate that *PfPIG-B* is functional and encodes mannosyltransferase-III of the parasite GPI biosynthesis. The parasite PfPIG-B is novel in that its signature sequence HKEHKI is unique and is only partially conserved as compared to HKEXRF signature motif of mammalian PIG-B enzymes.

Keywords: *Plasmodium falciparum*; Glycosylphosphatidylinositol anchors; Biosynthesis; PIG-B gene; Characterization; Mannosyltransferase-III of GPI biosynthesis

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, Man1-2Man1-6Man1-4GlcN1, 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].

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

Abbreviations: GPI, glycosylphosphatidylinositol; MSP, merozoite surface protein; Man, mannose; GlcN, glucosamine; PI, phosphatidylinositol; Man_2-GPI, Manα1-6Manα1-4GlcNα1-PI; Man_3, Man1α-2Manα1-6Manα1-4GlcNα1-PI; Man_4-GPI, Manα1-2(Et-*P*-6)Man1α-2Manα1-6Manα1-4GlcNα1-PI; EtN-*P*, ethanolamine phosphate; PIG, phosphatidylinositolglycan like; SSC, sodium saline citrate; HPTLC, high performance thin-layer chromatography.

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acylase, EtN-*P* transferase, and several accessory proteins [5]. The preassembled GPIs are transferred enbloc 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 *GPI1*, 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. Plasmodium 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 cell pellet) 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 μg) 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 (http://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 μg 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 [^{32}P] 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′-**GCGTAATACGACTCACTATAGGGAGA**ATGGGAATGGGAACCTTGTG-3′ (forward) and 5′-**GCGTAATACGACTCACTATAGGGAGA**ATGATGATCATTCCTTCTTTC-3′ (reverse). A 566 bp (8822–9387 nt) PCR product of *P. falciparum* erythrocyte membrane protein 1 gene (*PfEMP1*) (PlasmoDB ID PFE1640w) amplified using primers, 5′-**GCGTAATACGACTCACTATAGGGAGA**TGATTCCTCCAAGAAGAAGAC-3′ (forward) and 5′-**GCGTAATACGACTCACTATAGGGAGA**AAATATCATTCTGACATCCAGG-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 μg) 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 μCi of [6-^{3}H]GlcN (Amersham Biosciences) for 6 h and harvested.

Analysis of GPIs. The GPIs from [^{3}H]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 $CHCl_3$/MeOH/water (10:10:3, v/v/v). Aliquots of GPIs containing equal amounts of radioactivity were analyzed by HPTLC using $CHCl_3$/MeOH/water (10:10:2.4, v/v/v) as reported previously [7]. The nature of the [^{3}H]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, WTNLKERRNDHHFDTYENN (amino acids 259–277) 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 prepara-