Biosynthesis of GDP-fucose and Other Sugar Nucleotides in the Blood Stages of Plasmodium falciparum*

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Carbohydrate structures play important roles in many biological processes, including cell adhesion, cell-cell communication, and host-pathogen interactions. Sugar nucleotides are activated forms of sugars used by the cell as donors for most glycosylation reactions. Using a liquid chromatography-tandem mass spectrometry-based method, we identified and quantified the pools of UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, GDP-mannose, and GDP-fucose in Plasmodium falciparum intraerythrocytic life stages. We assembled these data with the in silico functional reconstruction of the parasite metabolic pathways obtained from the P. falciparum annotated genome, exposing new active biosynthetic routes crucial for further glycosylation reactions. Fucose is a sugar present in glycoconjugates often associated with recognition and adhesion events. Thus, the GDP-fucose precursor is essential in a wide variety of organisms. P. falciparum presents homologues of GDP-mannose 4,6-dehydratase and GDP-L-fucose synthase enzymes that are active in vitro, indicating that most GDP-fucose is formed by a de novo pathway that involves the bioconversion of GDP-mannose. Homologues for enzymes involved in a fucose salvage pathway are apparently absent in the P. falciparum genome. This is in agreement with in vivo metabolic labeling experiments showing that fucose is not significantly incorporated by the parasite. Fluorescence microscopy of epitope-tagged versions of P. falciparum GDP-mannose 4,6-dehydratase and GDP-L-fucose synthase expressed in transgenic 3D7 parasites shows that these enzymes localize in the cytoplasm of P. falciparum during the intraerythrocytic developmental cycle. Although the function of fucose in the parasite is not known, the presence of GDP-fucose suggests that the metabolite may be used for further fucosylation reactions.

Malaria is a global health problem caused by protozoan parasites of the genus Plasmodium and transmitted by the Anopheles mosquito. Approximately half of the world’s population is at risk of infection, and there are around 200 million cases annually, leading to more than half a million deaths each year (1). Most of the people who die are children living in resource-poor countries in sub-Saharan Africa. Among the five Plasmodium species that cause malaria in humans (Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale, and Plasmodium knowlesi), P. falciparum is the most deadly and is responsible for the majority of the malaria-linked deaths in sub-Saharan Africa (1). Chemotherapy is one of the central strategies for malaria treatment, but unfortunately, drug resistance to commonly used antimalarial drugs has spread very rapidly (2, 3). The implementation of new approaches to prevent and fight malaria has greatly benefited from the sequencing of the parasite genome (4) and the development of improved tools for functional genomics (5–8). However, this area of research remains greatly limited by our incomplete knowledge of parasite biology (9). Therefore, it is critical to promote research efforts that probe the basic biochemistry and cell biology of Plasmodium with the aim of characterizing essential proteins, metabolic pathways, and other processes that could be suitable for intervention and to validate new candidate drug targets.

Glycosylphosphatidylinositol (GPI)§ anchors (10) represent the major carbohydrate modification described in P. falciparum cell surface proteins (11). Several of these GPI-anchored glycoproteins are essential for parasite invasion and virulence (12), and GPI anchors are generated via a complex synthetic pathway (13). Recently, Bushkin et al. (14) presented new evidence demonstrating the existence of functional N-glycosyla-

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The abbreviations used are: GPI, glycosylphosphatidylinositol; Man, mannoside; Fuc, fucose; TSR, thrombospondin type 1 repeat.
tion in the intraerythrocytic stages of *P. falciparum*. In general, protozoan parasite N-glycosylation patterns are atypical of those described in higher eukaryotes (15, 16). In *P. falciparum*, the secondary loss of enzymes related to the biosynthesis of N-glycosylation precursors and the quality control of glycoprotein folding in the endoplasmic reticulum (17, 18) result in a very unusual N-glycosylation (14) that, if essential, could be therapeutically exploitable. To our knowledge, despite some controversy (19–24), O-glycans have never been unequivocally described in *P. falciparum*. Thus, although there have been significant efforts to understand the glycobiology of the parasite, several questions on this topic remain unanswered.

Sugar nucleotides, the essential intermediates in carbohydrate metabolism and glycoconjugate biosynthesis, are activated forms of sugars produced by the cell as donor precursors for most of the glycosylation reactions. They are formed in two main ways: by a salvage pathway involving “activation” of the sugar using a kinase and a pyrophosphorylase or by a *de novo* pathway involving the bioconversion of an existing sugar or sugar nucleotide. Specific *P. falciparum* metabolic databases (25), based on the parasite genome sequence, predict the conservation of the *de novo* biosynthetic pathways for UDP-N-acetyl-glucosamine, GDP-mannose, GDP-fucose, and UDP-glucose. Most of the predicted open reading frames (ORFs) that encode for the enzymes involved in these pathways are also present in other *Plasmodium* species (*P. vivax*, *P. knowlesi*, *Plasmodium chabaudi*, *Plasmodium yoelii*, and *Plasmodium berghei*) (26), suggesting that they are conserved and encode for proteins playing important roles in the parasite. Indeed, UDP-N-acetyl-glucosamine and GDP-mannose (through its product dolichol-phosphomannose) are essential donor substrates for the biosynthesis of GPI structures (27). Because sugar nucleotides are the “basic building blocks” of glycoconjugates, the conservation of their biosynthetic metabolic routes strongly suggests the existence of further downstream glycosylation reactions in which these metabolites are involved (i.e. glycan biosynthesis).

In this work, we identify and quantify the sugar nucleotides present in different stages of the intraerythrocytic life cycle of *P. falciparum* using a liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assay, and we present the results in the context of the functional metabolic pathways reconstructed from the genome of the parasite. We demonstrate that *P. falciparum* is unable to take up significant amounts of tritiated fucose from the culture media. Furthermore, we show that the putative GDP-fucose biosynthesis enzymes are functional in *vitro*, by expressing them and detecting GDP-fucose production. Endogenous forms of these enzymes localize to the cytosol of *P. falciparum*. We show that the genes encoding these enzymes are expressed at low abundance, and their levels are modulated throughout the red blood cell stages of the parasite. Finally, we also demonstrate that the parasite expresses a protein o-fucosyltransferase homolog, and schizont extracts incorporate tritiated GDP-fucose.

**Experimental Procedures**

**Parasites and Parasite Culture—** *P. falciparum* 3D7 (obtained from MR4-ATCC) parasites were cultured with human erythrocytes (3–4% hematocrit) in RPMI medium (Sigma) supplemented with 10% AB+ human serum, incubated at 37 °C in an atmosphere of 92% N2, 3% O2, and 5% CO2 using standard methods (28). For sugar nucleotide analysis and labeling experiments, parasites were grown in RPMI medium supplemented with 0.5% Albumax II (Invitrogen) to avoid possible variations due to the uses of different human serum batches. Parasite growth was monitored by counting the infected erythrocytes in Giemsa-stained thin blood smears under light microscopy.

**Sugar Nucleotide Metabolic Routes in *P. falciparum***

Sugar nucleotides—Osmotic lysis of red blood cells of sorbitol-synchronized cultures at different stages of the intraerythrocytic life cycle (rings, trophozoites, and schizonts, approximately 18, 35, and 44 h postinvasion, respectively) and 10–12% parasitemia was performed by resuspending erythrocyte pellet twice in 60 volumes of cold erythrocyte lysis buffer (10× stock solution: 0.15 m NaCl, 0.1 m KHCO3, 0.01 m EDTA). Non-infected red blood cells were lysed and included as controls in every analysis to discard noise due to the detection of sugar nucleotides carried away from the erythrocytes. The suspension was incubated on ice until lysis was completed (~10 min) (29). Pellets were washed three times with cold phosphate-buffered saline (PBS), and sugar nucleotide analysis was performed as described elsewhere (30). Briefly, parasite pellets were lysed in 70% ethanol in the presence of 20 pmol of GDP-glucose internal standard, and sugar nucleotides were extracted using Envi-Carb columns (31). Sugar nucleotides were then analyzed using LC-MS/MS, using multiple-reaction monitoring for detection. HPLC conditions were adapted from Ref. 31, and acetonitrile was added postcolumn to produce stable electrospray ionization. The peak areas for each sugar nucleotide, along with their empirically determined molar relative response factors and the known amount of internal GDP-glucose, were used to quantify sugar nucleotides. Analyses were performed on three different sugar nucleotide extracts.

**Metabolic Labeling of Parasite with Tritiated Sugars—** Parasite cultures at 10–12% parasitemia were synchronized to the ring stage and then washed and resuspended in RPMI medium supplemented with Albumax II. Cells were incubated at 3–4% hematocrit for 16–18 h, until the trophozoite stage. The parasites were then washed with glucose-free RPMI supplemented with Albumax II and 20× stock fructose, and they were metabolically labeled with 3H-sugars (50 μCi/ml) for 1 or 4 h, adapting the conditions used in previous work (23, 32, 33). Triplicates of each condition were included, and controls were “mock-labeled” for a few seconds using medium at 4 °C supplemented with an excess (100-fold) of the unlabeled (cold) sugar. Labeled cultures and controls were washed three times with PBS at 4 °C and treated with saponin to release parasites. Free parasites were washed again three times with PBS at 4 °C and lysed, and the radioactivity was measured by liquid scintillation counting.

To calculate the amount of sugar incorporated into the glycoprotein and glycolipid compartments, after the labeling, free parasites were further lysed, and the proteins were precipitated with 10% trichloroacetic acid and filtered (glycoproteins), or the glycolipids were extracted with organic solvents (glycolipids) (34).
Expression and Purification of GDP-fucose Biosynthesis Genes in *Escherichia coli*—Codon harmonization was used for the production of heterologously expressed *P. falciparum* GDP-mannose 4,6-dehydratase (PfGMD, gene ID PF3D7_0813800) and GDP-L-fucose synthase (PfFS, gene ID PF3D7_1014000) in *E. coli* (35). Codon-harmonized genes (Genscript) were cloned into the pGEX 6P expression vector (GE Healthcare) and expressed in BL21 (DE3) *E. coli* cells. 3-Liter cultures were grown to an A<sub>600</sub> of 0.6 and induced overnight at 18 °C with 100 μM isopropyl-β-D-thiogalactopyranoside. Cells were harvested and lysed in PBS including 0.1 mg/ml lysozyme in the presence of Complete protease inhibitor mixture (Roche Applied Science). Lysis was ensured by sonication (Misonix Micoson XL 2000). 0.5% Triton X-100 was added, and, after a 30-min incubation at 4 °C, the lysate was clarified by centrifugation at 12,000 × g for 20 min, and the supernatant was added to 1 ml of PBS-washed GST beads (GE Healthcare) and incubated overnight at 4 °C. The beads were collected in chromatography columns, washed with >10 bead volumes of PBS before three elutions of 1 ml with 10 mM glutathione in 50 mM Tris-HCl, pH 8. The eluates were pooled, dialyzed with PBS, and concentrated in 10,000 nominal molecular weight limit centrifugal filter units (Amicon, Millipore).

**GDP-fucose Biosynthesis Assay**—Assay conditions were adapted from elsewhere (36–38). Reactions were performed in 25 μl (final volume) of 100 mM MOPS, pH 7.0, 100 mM NaCl, 10 mM dithiothreitol, 5 mM EDTA, 1 mM GDP-mannose (Sigma), 0.4 mM NADPH, and 1 mM NADP. The reaction was started by adding 1.5 mg/ml of the recombinant PfGMD enzyme, and the reaction was left to proceed for 3 h at 37 °C. Recombinant PfFS (1.5 mg/ml) was then added, and the concentration of NADPH was adjusted to 1.5 mM. For experiments without the PfFS enzyme, only NADPH was added at this point. Reactions were stopped after 2 h at 37 °C by heating to 100 °C for 2 min, and then the samples were filtered to remove insoluble material, and sugar nucleotides were analyzed by HPLC (31), including standards for every HPLC series of experiments.

For LC-MS/MS analysis, HPLC conditions were adapted from Ref. 39, using a porous graphitic carbon column (Hypercarb, 100 × 2.1 mm, 5-μm particle size, Thermo Scientific) and MS-compatible mobile phases. Starting buffer was 0.1% formic acid, brought to pH 9.0 with ammonia, followed by a 36-min gradient from 10 to 50% acetonitrile at a flow rate of 100 μl/min. Detection was performed by negative mode electrospray ionization-MS on an API3000 triple quadrupole LC-MS/MS mass spectrometer (PE-Sciex) with a declustering potential of −50 V, focusing potential of −300 V, collision energy of 30 V, and source temperature of 375 °C.

Cloning and Expression of Epitope-tagged Versions of PfGMD and PfFS in *P. falciparum*—Gene PfGMD was amplified from *P. falciparum* genomic DNA using primer pARL1F-GMD-KpnI (CGCGGTACCTAGCGAGTTGCTTTAATC) and pARL1R-GMD-PstI (CGGCCTGAGTTGCTTTTTACCCA- TTT). PfFS was amplified from *P. falciparum* cDNA using primer pARL1F-FS-KpnI (CGCGGTACCATGACACGAAT- TTGCTTTTTC for seryl-tRNA synthetase) and pARL1R-FS-PstI (CGGCCTGAGTTGCTTTTTACCCA-TTT). *P. falciparum* genes were cloned in the KpnI-PstI site of transfection vector pARL1a-3HA (40) under the control of the pfCRT promoter region (41). *P. falciparum* were transfected as described previously (41). Briefly, 150 μg of each plasmid was used to electroporate (310 V, 950 millifarads) 200 μl of infected red blood cells at >5% parasitemia, synchronized for ring stage parasites. Transfected parasites were selected on 2 nM of WR99210 drug, and resistant parasites appeared in culture from 25 to 35 days after drug application.

**Indirect Immunofluorescence Assays**—Cultured *P. falciparum* transgenic lines were washed in PBS and then fixed with 4% EM grade paraformaldehyde and 0.075% EM grade glutaraldehyde in PBS (42). Fixed cells were permeabilized with 0.1% Triton X-100 in PBS and blocked for 1 h at room temperature in 3% PBS-bovine serum albumin (PBS-BSA). Samples were incubated overnight with primary antibody (rat anti-HA (1:10; Roche Applied Science) or rabbit anti-HSP70 (1:50; Stress-Mark) (43) diluted in 3% PBS-BSA, followed by a 1-h incubation with secondary antibody (anti-rat conjugated with Alexa Fluor 488 or anti-rabbit conjugated with Alexa Fluor 594 (1:200, Invitrogen)) diluted in 3% PBS-BSA. Nuclei were stained for 1 h with 4,6-diaminido-2-phenylindole (DAPI; 2 mg/ml diluted in PBS) during the secondary antibody incubation. Confocal microscopy was performed using a laser scanning confocal microscope (TCS-SP5; Leica Microsystems) at the microscopy scientific and technical services facility of the Universitat de Barcelona.

**RNA Preparation and Quantitative Real-time PCR**—Tight synchronization of parasites was achieved by Percoll purification of schizonts followed by sorbitol lysis 5 h later, to obtain a population of a defined age window of 0–5 h postinvasion. RNA was purified using the TRIzol method at the ring, trophozoite, and schizont stage, and cDNA was synthesized by reverse transcription performed using random hexamers and SuperScript-III reverse transcriptase (RT; Invitrogen) according to the manufacturer's instructions and including controls without RT. All quantitative PCRs were performed using PowerSYBR Green Master Mix (Invitrogen), and expression values were calculated using the relative standard curve method. Results are expressed in arbitrary units, relative to a genomic DNA standard curve, and were normalized against seryl-tRNA synthetase (PF3D7_0717700). The primers used were GGTGATTCACCTAAAGGAAAA and TTCATAACCGACGCGCATGAGCTTTGATAT for PfGMD, CTGTATTTTCCCTGTTAAATTTCTTAC and GATGTTACACGCCTGACATT for PfFS; TTTTTTTAAAGTGTAGACCGATTTGAGGTAT and AAAAAAGTCCCTAAATATACACCTCT for PfoFLUT2, and AAGTACGGGATCATCGTGTTT and TCTGGCACAATCTCCATAA for seryl-tRNA synthetase.

**Preparation of *P. falciparum* Cell Extracts and [H]GDP-fucose Incorporation Assays**—Percoll-purified infected red blood cells (36–42 h postinvasion) grown at 10–12% parasitemia were saponin lysed and washed three times with PBS. The cell pellet was placed on ice and homogenized for 1 h at (10<sup>7</sup> cells/ml) with 1% Nonidet P-40, 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 10 mM MnCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 1× protease inhibitor mixture (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 μg/ml N<sup>-</sup>-tosyl-L-lysine choloromethyl ketone. Inactivated extracts were boiled for 20 min.
**Sugar Nucleotide Metabolic Routes in P. falciparum**

**FIGURE 1.** Sugar nucleotide biosynthetic pathways identified in the genome of *P. falciparum*. The numbers refer to the enzymes and known or candidate genes described in Table 1. Predicted known fates of sugar nucleotide donors according to the glycoconjugates described in *P. falciparum* are in italic type (marked with a question mark if the fate is unknown). Sugar nucleotides identified in this study are boxed. Dotted lines indicate confirmed salvage pathways, and sugars, taken up from the medium, are underlined. The discontinuous arrow (step 4) represents the glucosamine-phosphate N-acetyltransferase activity (EC 2.3.1.4) for which a candidate gene is not yet identified. HK, hexokinase; G6PI, glucose-6-phosphate isomerase; GFPT, glucosamine-fructose-6-phosphate aminotransferase; GNA, glucosamine-phosphate N-acetyltransferase; PAGM, phosphoacetylglucosamine mutase; UAP, UDP-N-acetylglucosamine pyrophosphorylase; MPI, mannose-1-phosphate isomerase; PGM, mannose-1-phosphate guanyltransferase; PMM, phosphomannomutase; MPG, mannose-1-phosphate guanylyltransferase; GMD, GDP-mannose 4,6-dehydratase; FS, GDP-L-fucose synthase; PAGM, phosphoglucomutase; UGP, UTP-glucose-1-phosphate uridylyltransferase; GALE, UDP-glucose 4-epimerase; GK, galactokinase. The suggested pathway for the biosynthesis of UDP-Gal through the activity of the UDP-sugar pyrophosphorylase enzyme is indicated with a gray discontinuous arrow (see “Discussion”).

with 3 mM urea, 0.1 mM dithiothreitol, and 5 mM EDTA. 2 μCi of [3H]GDP-fucose (American Radiochemical Chemicals, Inc.) was added to the extract, and the reaction was incubated at 37 °C for 1 h. Proteins were TCA-precipitated and washed three times with cold 100% acetone, and tritium incorporated into the TCA pellets was measured by scintillation counting.

**RESULTS**

Sugar Nucleotide Biosynthetic Pathways Predicted in *P. falciparum*—Based on the current knowledge of *P. falciparum* sugar biochemistry (11, 14, 23, 32) and the predicted metabolic pathways (25), we have reconstructed the sugar nucleotide biosynthetic routes present in *P. falciparum* (Fig. 1). The enzymes and genes involved in these metabolic routes are shown in Table 1. Bioinformatic analysis suggests that sugar nucleotides are made by conventional eukaryotic de novo routes from glucose 6-phosphate, although other salvage pathways also exist (Fig. 1). Conserved pathways for the biosynthesis of UDP-GlcNAc and GDP-mannose agree well with the monosaccharide content of known glycoconjugates in *P. falciparum* consisting of glucosamine (derived from GlcNAc) and Man residues present in GPI anchors and N-glycans (11, 14). Furthermore, the detection of UDP-GlcNAc, UDP-Glc, GDP-Man, and GDP-Fuc is consistent with the presence of known or candidate sugar nucleotide biosynthetic enzymes encoded in the genome of *P. falciparum* (25). We were surprised to find that pools of UDP-galactopyranose can be identified at the different blood stages of the parasites, although no apparent candidates for UDP-glucose-4′-epimerase can be detected in the genome of the parasite. The possible metabolic route for the biosynthesis of this sugar nucleotide is discussed below.

**Metabolic Labeling of Parasites with Tritiated Sugars**—To assess the presence of carbohydrate salvage routes in the parasite *P. falciparum*-infected erythrocytes, cultures were metabolically labeled with tritiated Man or Fuc in medium containing 20 mM d-fructose (32, 34, 44, 45). Whereas [3H]Man was incorporated as described previously (23), [3H]Fuc was not significantly taken up by the parasite (Fig. 3A), contributing to less than 15% of the average pool amount, and we did not detect a significant incorporation into its glycoproteins or glycolipids (Fig. 3B). This indicates that the main source of GDP-Fuc for *P. falciparum* intraerythrocytic life stages is through the bio-
Sugar Nucleotide Metabolic Routes in *P. falciparum*

**TABLE 1**

| Step no. | Enzyme name | Enzyme no. | Putative *P. falciparum* homologues |
|----------|-------------|------------|-----------------------------------|
| 1        | Hexokinase (HK) | EC 2.7.1.1 | PF3D7_0624000                     |
| 2        | Glucose-6-phosphate isomerase (G6PI) | EC 5.3.1.9 | PF3D7_1436000                     |
| 3        | Glucosamine-fructose-6-phosphate aminotransferase (GFPT) | EC 2.6.1.16 | PF3D7_1025100                     |
| 4        | Glucosamine-phosphate N-acetyltransferase (GNA) | EC 2.3.1.4 | No gene identified                  |
| 5        | Phosphoacetoglucosamine mutase (PAGM) | EC 5.4.2.3 | PF3D7_1130000                     |
| 6        | UDP-N-acetylglucosamine pyrophosphorylase (UAP) | EC 2.7.7.23 | PF3D7_1343600                     |
| 7        | Mannose-6-phosphate isomerase (MPI) | EC 5.3.1.8 | PF3D7_0801800                     |
| 8        | Phosphomannomutase (PMM) | EC 5.4.2.8 | PF3D7_1017400                     |
| 9        | Mannose-1-phosphate guanyltransferase (MPG) | EC 2.7.7.13 | PF3D7_1420900                     |
| 10       | GDP-mannose 4,6-dehydratase (GMD) | EC 4.2.1.47 | PF3D7_0813800                     |
| 11       | GDP-L-fucose synthase (FS) | EC 1.1.1.271 | No gene identified                 |
| 12       | GDP-Fucase (PAGM) | EC 5.4.2.3 | PF3D7_1014000                     |
| 13       | UTP-glucose-1-phosphate uridylyltransferase (UGP) or UDP-sugar pyrophosphorylase (USP) | EC 2.7.7.9 or EC 2.7.7.64 | PF3D7_0517500                     |
| 14       | UDP-glucose 4-epimerase (GALE) | EC 2.7.1.6 | No gene identified                 |

* Step numbers refer to those shown in Fig. 1.
* Gene ID numbers underlined are as identified and annotated in the *P. falciparum* genome (25, 46), and those in boldface type and underlined have been functionally characterized in this paper.
* Glucosamine-6-phosphate isomerase (EC 5.3.1.9) enzyme, PF3D7_1436000 has been crystallized, but to our knowledge its activity has not been published (77).
* GDP-Fucase (EC 2.7.1.6) enzyme, PF3D7_1025100 has been crystallized, but to our knowledge its activity has not been published (77).

**TABLE 2**

| Sugar nucleotide | Rings* | Trophozoites* | Schizonts* | Lysed red blood cells* |
|------------------|--------|---------------|------------|------------------------|
| UDP-Glc          | 0.53 ± 0.12 | 1.78 ± 0.38 | 1.96 ± 0.29 | 0.02                   |
| UDP-Gal          | 0.09 ± 0.04 | 0.42 ± 0.06 | 0.61 ± 0.12 | <0.01                  |
| UDP-GlcNAc       | 0.34 ± 0.04 | 1.35 ± 0.26 | 1.31 ± 0.11 | 0.02                   |
| GDP-Man          | 0.31 ± 0.06 | 0.69 ± 0.08 | 0.74 ± 0.06 | <0.01                  |
| GDP-Fuc          | 0.27 ± 0.01 | 0.53 ± 0.02 | 0.17 ± 0.02 | <0.01                  |

* Amounts are indicated in pmol/10^7 cells.
* S.D. values are at least 1 order of magnitude smaller than the calculated amount and are not included.

FIGURE 2. Specific sugar nucleotide levels in different blood stages of *P. falciparum*. Values of UDP-Glc (open bars), UDP-Gal (black solid bars), UDP-GlcNAc (striped bars), GDP-Man (dotted bars), and GDP-Fuc (gray solid bars) indicate the average of three different extractions. Error bars, S.D. The last set of bars represent the levels of sugar nucleotides measured on osmotically lysed non-infected red blood cells (RBCs; used as control). Values are indicated in pmol/10^7 cells. Analyses were performed in triplicate, and mean values ± S.D. are shown. Statistically significant differences (one-way analysis of variance Tukey’s post-test) between stages are shown with an asterisk (*, p < 0.05, rings versus trophozoites or schizonts) or a dot (●, p < 0.05, trophozoites versus schizonts).

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| GDP-Man          | 0.31 ± 0.06 | 0.69 ± 0.08 | 0.74 ± 0.06 | <0.01                  |
| GDP-Fuc          | 0.27 ± 0.01 | 0.53 ± 0.02 | 0.17 ± 0.02 | <0.01                  |

* Amounts are indicated in pmol/10^7 cells.
* S.D. values are at least 1 order of magnitude smaller than the calculated amount and are not included.

conversion of an existing sugar/sugar nucleotide, which is consistent with the apparent absence of genes encoding enzymes involved in the GDP-fucose salvage pathway in the *P. falcipa-
Although there are no commercial standards available to ana-
lyze the transition of GDP-4-keto-6-deoxy mannose for identi-
fication, the fragmentation of this novel product produced a
major product ion at m/z 442 corresponding to a [GDP-H]-,
strongly resembling the fragmentation of GDP-deoxyhexoses
(Fig. 4B, bottom panels) (30, 36).

To assess the effect of time and GDP-Man and PfGMD/PfFS
concentration on the production of GDP-Fuc, we performed
different activity assays where sugar nucleotides were mea-
sured by reverse phase HPLC. GDP-Fuc production was
dependent on time and the initial concentration of GDP-Man
(Fig. 4C). Interestingly, at the PfGMD and GDP-Man concen-
trations tested (1.5 mg/ml and 1 mM, respectively), PfFS did not
become rate-limiting at 1 mg/ml, whereas decreasing the initial
concentration of PfGMD to 1 mg/ml reduced the relative yield
of GDP-Fuc to a 70%.

PfGMD and PfFS Are Expressed during P. falciparum Intra-
erythrocytic Life Cycle and Their Protein Products Localize in
the Cytosol of the Parasite—Real-time quantitative RT-PCR
analysis of PfGMD and PfFS RNA transcripts was performed at
the different asexual stages of P. falciparum (rings, trophozo-
ites, and schizonts), and expression values were calculated
using the relative standard curve method. In both cases, the
highest expression levels were found at the schizont phase (Fig.
5), in agreement with what had been observed previously in
other studies and with what is annotated in P. falciparum met-
abolic databases (25, 48, 49). Nevertheless, transcription of both

FIGURE 3. [3H]Sugars incorporated by P. falciparum trophozoites. A, amounts of mannose (dotted bars) and fucose (gray solid bars) indicate the average of
three different determinations at different times. Error bars, S.D. Values are indicated in pmol of sugar/10^7 cells. t_0 controls were mock-labeled for a few seconds
using medium at 4 °C supplemented with an excess (100-fold) of the unlabeled (cold) sugar. B, amount of sugar incorporated to the glycoproteins (left; determinations performed in triplicate; error bars, S.D.) or glycolipids (right) of P. falciparum. [3H]incorporated is indicated in pmol of sugar/10^7 cells. C, [3H]GlcN
(dashed line) and [3H]GlcNAc (dotted line) incorporated by P. falciparum trophozoites. L-Glc, which is not incorporated by the parasite, is included as a negative
control of incorporation (solid line). Determinations were performed in triplicate at three different times, and mean values ± S.D. are shown. t_0 controls were
generated as in A.
genomes through the whole intraerythrocytic cycle is ~10-fold lower than that of the seryl-tRNA synthetase housekeeping gene expression taken as a reference (48), with much lower PfFS levels in the ring stage (Fig. 5).

To assess the subcellular location of PfGMD and PfFS proteins, HA-tagged versions of the genes were transiently expressed under the control of the crt promoter using the pARL1a-3HA expression vector (41). Immunofluorescence microscopy using anti-HA antibodies produced a typical cytosolic distribution in the different stages of the intraerythrocytic life cycle (Fig. 6). To confirm this, we also stained the cells using anti-HSP70 antibody, a commonly used cytosolic marker (43). The merged image shows co-localization, indicating that the expressed HA-tagged PfGMD and PfFS are localized in the cytosol of P. falciparum through the different stages of the parasite asexual life cycle.

P. falciparum Schizonts Express a Protein o-Fucosyltransferase (PoFUT2) Homolog and Incorporate [3H]GDP-Fuc in a Cell-free Assay—The P. falciparum genome presents a homolog of PoFUT2 that, in other organisms, is involved in the O-fucosylation of thrombospondin type 1 repeat (TSR) domains (50). Because this gene (PF3D7_0909200) is the most suitable candidate to encode for a fucosyltransferase activity, we performed real-time quantitative RT-PCR analysis of PfPoFUT2 at the different blood stages of P. falciparum. Despite the low levels, a peak of expression was observed at the schizont phase of
the parasite (Fig. 7A) coinciding with the maximum expression of \textit{PfGMD} and \textit{PfFS} genes. Furthermore, \textit{P. falciparum} schizont extracts incorporated $[^3]$H]GDP-Fuc, strongly suggesting that fucosylation processes are active in the late stages of the asexual life cycle of the parasite (Fig. 7B).

**DISCUSSION**

Carbohydrate structures that decorate the surface of cells play important roles in the biology of host-pathogen interactions. In the particular case of the malaria parasite, glycan structures associated with the parasite itself appear to be limited to the parasite proteins (23) or into the \textit{P. falciparum} genome, and trypted galactose is not significantly incorporated into the parasite proteins (23) or into the $\alpha$-galactose moieties of digalactosyl diglycerides (64).

Bioinformatic analysis (25) and our own work suggest that UDP-GlcNAc, the direct donor for all GlcNAc transfersases, is made in \textit{P. falciparum} by the conventional eukaryotic de novo route from glucose 6-phosphate (Fig. 1), although a salvage pathway also exists, via the action of hexokinase (GlcN $\rightarrow$ GlcN-6-P) (22, 23). With more than 30% similarity and $e$ values of $<10^{-55}$, however, although galactose competes for \textit{P. falciparum} PfHT1 hexose permease (45) and its incorporation into the parasite galactolipids has been reported (62, 63), there is not a clear galactokinase candidate in the \textit{P. falciparum} genome, and trypted galactose is not significantly incorporated into the parasite proteins (23) or into the $\alpha$-galactose moieties of digalactosyl diglycerides (64).

The identification of UDP-Gal is somewhat puzzling. The \textit{P. falciparum} genome lacks candidate genes for a UDP-glucose 4-epimerase (EC 5.1.3.2) that can produce UDP-galactose via the epimerization of UDP-glucose or a galactose-1-phosphate uridylyltransferase activity (EC 2.7.7.12) (25). Thus, two main activities can be proposed for the production of this sugar nucleotide. Either an enzyme with UTP-glucose-1-phosphate uridylyltransferase activity (EC 2.7.7.9) presents a weak galactose-1-phosphate uridylyltransferase activity (EC 2.7.7.10), as described in mammals (55, 56), or a broad substrate range UDP-sugar pyrophosphorylase (EC 2.7.7.64) is present in the genome of \textit{P. falciparum}, as described in plants or \textit{Leishmania major} (57–61). UDP-sugar pyrophosphorylase is an enzyme that can nonspecifically utilize UTP and glucose 1-phosphate or galactose 1-phosphate to produce UDP-glucose or UDP-galactose and pyrophosphate. Interestingly, searches with functionally characterized UDP-sugar pyrophosphorylase orthologues from plants and \textit{L. major} identify a match, PF3D7_0517500, with more than 30% similarity and an e value of $<10^{-55}$. However, although galactose competes for \textit{P. falciparum} PfHT1 hexose permease (45) and its incorporation into the parasite galactolipids has been reported (62, 63), there is not a clear galactokinase candidate in the \textit{P. falciparum} genome, and trypted galactose is not significantly incorporated into the parasite proteins (23) or into the $\alpha$-galactose moieties of digalactosyl diglycerides (64).
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![FIGURE 7. P. falciparum late blood stages express PfPoFUT2 and incorporate [³H] GDP-fucose. A, real-time quantitative RT-PCR results are expressed in arbitrary units and normalized against seryl-tRNA synthetase. Data are representative of two independent RNA extractions per stage, assayed in triplicate. Statistically significant differences (one-way analysis of variance Tukey’s post-test) between stages are shown with an asterisk (*, p < 0.05, rings versus trophozoites or schizonts) or a dot (○, p < 0.05, trophozoites versus schizonts). B, detergent homogenates of late trophozoite/schizont stage parasites were incubated with [³H]GDP-fucose before (gray solid bar) and after inactivation (open bar). The experiment was repeated two times on different days, and [³H]GDP-fucose incorporation was assayed in triplicate. Data are expressed as mean values ± S.D. (error bars).

Along with the labeling of GPI-anchored proteins using tritiated glucosamine (Fig. 3C) (23, 63), strongly suggests that the UDP-GlcNAc metabolic route (25) (Fig. 1) is active in *P. falciparum*. Most likely the UDP-GlcNAc de novo pathway from glucose is the most important in vivo for the parasite, because glucosamine is not an abundant free sugar in the mammalian or insect host. Because the glucosamine moiety, derived from GlcNAc by de-N-acetylation (27), is present in GPI structures that serve as membrane anchors for many important surface antigens of the parasite invasive stages (12, 66), the inability to identify a candidate gene encoding for the glucosamine-phosphate N-acetyltransferase activity is very intriguing because this pathway is potentially targetable for selective anti-malarial drug design.

The presence of the fucose donor GDP-Fuc in the intracellular stages of *P. falciparum* is not surprising, because the parasite contains homologues of the enzymes involved in the biosynthesis of this precursor from GDP-Man. We have shown that *PfGMD* and *PfFS* are active in vitro (Fig. 4), and they are expressed through the parasite’s asexual life cycle (Fig. 5) (67). The GDP-Fuc pools in *P. falciparum* are, on average (Table 2), comparable with the pools of other protozoan parasites, such as trypanosomatids (30), including *T. brucei*, for which this metabolite is essential (36). The apparent lack of agreement with the amount of the GDP-Fuc pool in the schizont stage of the parasite when the *PfFS* gene is more expressed may be due to the specific demand of the metabolite at that stage. Because sugar nucleotides are donors for glycosylation reactions, the rates of turnover and the amount of the pools detected may change, reflecting their utilization. The localization of both enzymes in the cytoplasmic compartment (Fig. 6), as aldolase and other enzymes involved in the metabolism of carbohydrates (68), agrees well with the identification of a putative GDP-Fuc transporter in the *P. falciparum* endoplasmic reticulum/Golgi apparatus (69), where N- and O-glycosylation processes occur. The location and function of a putative fucose-containing glycan in *P. falciparum* remain enigmatic, and biosynthetic labeling with tritiated fucose is not feasible, because there is no prominent GDP-Fuc salvage pathway in the parasite, and/or hexose transporters do not efficiently take up fucose (Fig. 3). However, the labeling of parasite extracts after incubation with modified precursors opens a door to the identification of putative fucose-containing glycoconjugates (Fig. 7B). Interestingly, the C-terminal region of *P. falciparum* circumsporozoite surface protein (CS), in which the RTS,S malaria vaccine is based (70), and other proteins of the parasite (71) contain TSR domains. TSR domains are generally O-fucosylated in higher eukaryotes by PoFUT2, and the fucose residue can be further modified by the addition of β1–3 glucose (72–74). The C-terminal region of CS, containing the TSR domain, was recently expressed in HEK293T cells and structurally analyzed, showing the presence of a fucose and a glucose residue (75, 76). The expression of PfPoFUT2 in *P. falciparum* (Fig. 7A) and its conservation in other *Plasmodium* species raise the possibility of the presence of a mechanism of O-fucosylation of CS and other TSR-containing proteins in the parasite.

In summary, in this work, we have reported the first evidence of the presence of sugar nucleotides in the blood stages of *P. falciparum*, and we have described the active metabolic routes involved in their biosynthesis. In addition, we have characterized the de novo route of GDP-Fuc, a metabolite that may probably be involved in the biosynthesis of novel fucosylated glycans not yet described in the malaria parasite.

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