During *Plasmodium falciparum* infection, host red blood cell (RBC) remodeling is required for the parasite’s survival. Such modifications are mediated by the export of parasite proteins into the RBC that alter the architecture of the RBC membrane and enable cytoadherence. It is probable that some exported proteins also play a protective role against the host defense response. This may be of particular importance for the gametocyte stage of the life cycle that is responsible for malaria transmission, since the gametocyte remains in contact with blood as it proceeds through five morphological stages (I to V) during its 12-day maturation. Using microarray analysis, we identified several genes with encoded secretory or export sequences that were differentially expressed during early gametocytogenesis. One of these, *PfGECO*, encodes a predicted type IV heat shock protein 40 (HSP40) that we show is expressed in gametocyte stages I to IV and is exported to the RBC cytoplasm. HSPs are traditionally induced under stressful conditions to maintain homeostasis, but *PfGECO* expression was not increased upon heat shock, suggesting an alternate function. Targeted disruption of *PfGECO* indicated that the gene is not essential for gametocytogenesis in *vitro*, and quantitative reverse transcriptase PCR (RT-PCR) showed that there was no compensatory expression of the other type IV HSP40 genes. Although *P. falciparum* HSP40 members are implicated in the trafficking of proteins to the RBC surface, removal of *PfGECO* did not affect the targeting of other exported gametocyte proteins. This work has expanded the repertoire of known gametocyte-exported proteins to include a type IV HSP40, *PfGECO*.

In order to survive within a red blood cell (RBC) during *Plasmodium falciparum* infection, the parasite must undergo major host cell remodeling. Such modifications are mediated by the export of a range of proteins of parasite origin, termed the exportome, across the parasitophorous vacuole (PV) to multiple sites within the infected RBC (22, 38, 58, 60). The exported proteins have been shown to affect nutrient uptake, alter the architecture of the RBC membrane, compromise membrane deformability, and facilitate the delivery of adhesins to the RBC surface. These latter modifications enable the infected RBCs to cytoadhere to endothelial cells and thus evade clearance by the host immune response (58). Proteins to be exported typically encode a conserved *Plasmodium* export element (PXEEL) (61) or host targeting signal (HT) motif (44) that is recognized and cleaved by the protease plasmepsin V within the endoplasmic reticulum (13, 14, 18, 48, 65, 75). The protein is released and transported to the PV membrane (PVM), where it passes through the *Plasmodium* translocon of exported proteins (PTEX) into the host RBC (30). Once in the host cell, some exported proteins will be trafficked to the RBC surface via either the Maurer’s clefts (1, 4, 9, 40, 51, 85, 91, 92) or the tubulovesicular network (TBV) (3, 39) to potentially assist in cytoadhesion, whereas others will remain in the RBC cytosol, possibly acting as chaperones.

As exported proteins need to cross several membranes to target to their final destinations, it is suggested that molecular chaperones, such as the heat shock protein 40-kDa (HSP40) family (also called DnaJ proteins), will be involved to help regulate protein transport (6, 55, 68). Traditionally, HSP40 proteins regulate the activity of HSP70 in a manner that facilitates the folding of proteins under both normal and stress response conditions (20, 34, 53, 54, 90). Members are divided into four distinct classes based on conservation of their four encoded domains: a DnaJ domain with a highly conserved His-Pro-Asp (HPD) motif, a Gly/Phe-rich region, a cysteine-rich zinc-binding domain, and a C-terminal substrate-binding domain (19). Type I HSP40 proteins encode all four domains, type II proteins lack the zinc-binding domain, and type III and IV have only the signature DnaJ domain, with type IV exhibiting variations in the HPD motif (16, 19, 90). The HPD motif is vital to stimulate the ATPase activity of HSP70, and its mutation abolishes interactions of the HSP40/HSP70 partnership, suggesting that type IV proteins may exert their function by a different partner or mechanism (16, 37, 43, 53, 62, 87, 93). In the *P. falciparum* genome, 18 of the 43 HSP40 family members bear a PXEEL/HT motif and are predicted to be exported (44, 61, 76). These include 11 of the 12 proteins belonging to the type IV class (16). Compared to other organisms, the type IV class is considerably expanded in *P. falciparum*; this may be to fulfill a specific function unique to the parasite (16).

Of the 11 potentially exported type IV HSP40 proteins in *P.
the RBC membrane comprised mainly of knob-associated histidine-rich protein (KAHRP) (59), which are protrusions of the RBC membrane of the type IV HSP40 PF10_0381 resulted in decreased knobs heat shock (21, 24, 27, 28, 35, 36, 74, 78). Targeted disruption iparticipate in cytoadhesion and stabilize the cytoskeleton against membrane, binds to spectrin, and is suggested to both partic-
take in the RBC remodeling (8, 11, 23, 89). Ring erythrocyte surface an-
gametocytes. Mature erythrocyte surface antigen (MESA)
neither nor RESA (PFA0110w) is similarly localized to the RBC
molecular gametocytes. In the present study, we demonstrate that PFL2550w is a soluble gametocyte-specific protein that
localizes to the RBC cytoplasm. Based on this expression pattern, we have named PFL2550w \textit{P. falciparum} gametocyte glythro-
cyte cytosolic protein (PIGECO) and have investigated a role
for the protein during sexual differentiation.

\textbf{MATERIALS AND METHODS}

\textit{P. falciparum} parasite culture, purification, and gametogenesis. Plasmodium \textit{falciparum} strain 3D7 was maintained in culture using standard procedures (86), and gametocytogenesis was induced as described by Ifediba and Vanderberg (47). In experiments where it was necessary to eliminate asexual stage parasites, gametocyte cultures were treated with 50 mM N-acetylglucosamine (NAG) (Sigma, St. Louis, MO) 6 to 8 days after the culture was initiated (66). Parasites were synchronized by sorbitol treatment (52), and gametocytes were purified using either a 70% Percoll gradient (GE Healthcare, Pittsburgh, PA) (49) or a MACS column (Miltenyi Biotec, Auburn, CA) (69). To perform the growth curve assay, parasite cultures were set at 0.2% rings and gametocytemia was assessed by counting daily Giemsa-stained smears for 14 days. To assay game-
etogenesis, an aliquot (0.5 ml) of parasites, containing mature stage V gametocytes was pelleted and resuspended in human serum to 50% hematocrit. After incub-
bation for 10 min at room temperature, the presence of exflagellating males was evaluated at 400\times magnification.

\textbf{Microarray analysis.} \textit{P. falciparum} strain 3D7 was transformed with pDT.Tg23.230-D1.356 (pD1.356) to study the role of gamete surface antigen \textit{Pf}230, and two independently transformed, pyrimethamine-resistant lines were obtained as described previously (32). One clonal line produced gametocytes (G\textsuperscript{+}), whereas the other was gametocyte deficient (G\textsuperscript{-}). RNA was isolated from synchronized G\textsuperscript{+} and G\textsuperscript{-} clones at parasitemias of 1.4\% and 0.9\% (G\textsuperscript{4+}) and then again 2 days later at parasitemias of 5.2\% and 5.5\%, respectively, and was used to generate fluorescently labeled cDNA as previously described (31). The cDNA was hybridized to the 70-mer oligonucleotide array designed by the DeRisi laboratory (Operon Technologies, Alameda, CA) (17) and assayed at the NIAID Microarray Research Facility, National Institutes of Health, Bethesda, MD.

\textbf{Northern blot analysis.} Gametocyte cultures were set at an asexual parasitemia of 0.2\%, and parasites were harvested once the parasitemia reached 5\% and then every other day until gametocyte stage V. RNA was isolated from parasites using TRIzol (Invitrogen, Carlsbad, CA) and the RNasey microkit (Qiagen, Valencia, CA), and the Northern blot was performed as previously described (31) using a \textit{PIGECO} (bp 91 to 691) random-primed labeled probe.

\textbf{Quantitative RT-PCR analysis.} Total RNA was isolated from parasites using TRizol reagent (Invitrogen) and the RNasey microkit (Qiagen), and cDNA synthesis was performed with the Quantitect reverse transcription kit (Qiagen). Gene-specific primers for quantitative reverse transcriptase PCR (RT-PCR) were designed with the Primer3 software (MIT) (see Table S1 in the supplemental material) and the Quantitect SYBR green PCR kit (Qiagen) was used for the reactions, which were run on a Bio-Rad iCycler iQ (Bio-Rad, Hercules, CA). The RNA transcript levels of the target genes were calculated from a standard curve derived from 5-fold serial dilutions of 3D7 genomic DNA (gDNA) and normalized to the value of the seryl tRNA synthetase housekeeping gene (\textit{PF07_0073}).

\textbf{Recombinant protein expression and antibody production.} A fragment of \textit{PIGECO} encompassing amino acids 23 to 469 was amplified by PCR from \textit{P. falciparum} 3D7 gDNA using primers TAATTCTAGAATTGTTATCATGAA
ATTITG and TGCATCTGAGACATTGATTTGGAGTAT (restriction sites underlined) and cloned into the XbaI and PflII sites of expression vector pH902. The recombinant protein was expressed in \textit{Escherichia coli} BL21(DE3) cells (Invitrogen) and purified as a maltose-binding protein (MBP) fusion using standard affinity chromatography. To raise polyclonal antiserum, mice were immunized monthly with 50 \mu g of recombinant protein emulsified in Ribi adju-
vant (Sigma) (31).

\textbf{SDS-PAGE and Western blot analysis.} Parasites were lysed with NETT (150 mM NaCl, 5 mM EDTA, 0.5\% Triton X-100, 50 mM Tris, pH 8.0) in the presence of complete protease inhibitors (Roche, Indianapolis, IN), and sample buffer (Invitrogen) was added to the soluble fraction. Proteins were separated
by SDS-PAGE on a 4 to 20% polyacrylamide gel (Invitrogen) under either nonreducing or reducing conditions and transferred to nitrocellulose membrane (GE Healthcare) for Western blot analysis. Immunoblots were probed with either mouse anti-PfGECO (1:500), anti-Pfs16 (1:500) (31) or anti-skeleton binding protein 1 (SBP1; 1:500) (9) primary antibody followed by alkaline phosphatase-conjugated anti-mouse secondary antibody (Sigma) and detected using alkaline phosphatase substrate solution (Sigma).

Indirect immunofluorescence assay (IFA). Parasites were adhered to poly-L-lysine-coated coverslips for 30 min and then fixed in 1% formaldehyde and 0.005% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min. The parasites were washed with PBS, incubated for 10 min in 50 mM NH4Cl-PBS (Sigma), and permeabilized in 0.05% saponin (Sigma) for 20 min. After blocking in 0.2% fish skin gelatin-PBS (Sigma), parasites were probed with either mouse anti-PfGECO (1:100), anti-Pfg.748 (1:100) (31), anti-SBP1 (1:100) (31), anti-PfHSP101 (1:200) sera (30). The cells were washed in PBS, incubated with Alexa Fluor tetramethyl rhodamine isocyanate (TRITC)-conjugated anti-mouse Ig (1:2,000; Invitrogen) and/or Alexa Fluor 488-conjugated anti-rabbit IgG (1:2,000; Invitrogen) and then mounted in Vectashield (Vector Labs, Burlingame, CA) containing 4,6-diamidino-2-phenylindole (DAPI) and/or Alexa Fluor 488-conjugated anti-mouse IgG (1:2,000; Invitrogen) and then mounted in Vectashield (Vector Labs, Burlingame, CA) containing 4,6-diamidino-2-phenylindole (DAPI) and examined with a Leica fluorescence microscope.

Heat shock treatment of parasites. Gametocyte culture was set up at an asexual parasitemia of 0.2%, and 8 days later, when stage II gametocytes were first observed, they were divided into flasks that were incubated at either 37°C or 41°C for 2, 4, or 6 h. Total RNA was isolated and used for quantitative RT-PCR analysis as previously described using the primers in Table S1 in the supplemental material.

Targeted gene disruption. To disrupt PfGECO, two fragments (F1 and F2) from the 5' (bp 906 to 1) and 3' (bp 815 to 1555) ends of the gene, respectively, were cloned into the SacII/SpeI (F1) and NcoI/AvrII (F2) sites of Angel and stage II to V gametocytes (lanes II to V), and the blot was hybridized with a PfGECO probe. The ethidium bromide (EBr)-stained gel is shown as a loading control. (C) Quantitative RT-PCR was performed on RNA extracted from stage II wild-type 3D7 gametocytes using primers specific for PfGECO (blue), PfM7 (red), PfS16 (green), MESA (purple), and the seryl tRNA synthetase gene (gray). The transcript levels were calculated from a gDNA standard curve and normalized to the seryl tRNA synthetase housekeeping gene (seryl tRNA; PR07_0073). The data are shown as the gene expression relative to that of PfGECO and represent the mean value of three separate experiments performed in triplicate with standard error of the mean (SEM) with standard error bars.

**RESULTS**

PfGECO transcription is upregulated in early gametocytes. Previously, we performed a comparative expression analysis between a gametocyte-producing clone (G⁺) and one that was deficient in gametocytes (G⁻). We identified 11 P. falciparum genes that were upregulated (≥10-fold) in the G⁺ clone, 9 (82%) of which were predicted to be secreted or exported (S. Eksi et al., unpublished data). One such gene was the type IV HSP40, PfGECO, which was expressed on average 37.8 times higher in the G⁺ clone at low parasitemia (1.4 to 0.9%) and 55.5 times higher at high parasitemia (5.2 to 5.5%) than in the G⁻ clone (Fig. 1A). PfGECO was the only type IV HSP40 detected at this early gametocyte time point, suggesting that the parasite may differentially regulate the expression of members of this family throughout the life cycle.

To confirm upregulation in gametocytes, the transcription profile of PfGECO was assessed by Northern blot analysis. RNA was harvested from a wild-type 3D7 gametocyte culture every 2 days starting on day 6 after setup, when the culture contained both asexual parasites and a subpopulation committed to gametocytogenesis (Fig. 1B). The culture was treated with NAG from day 6 to 9 to eliminate asexual stage parasites to ensure that the transcript detected was attributable to gametocytes. PfGECO RNA was detected at low levels in asexual parasites and gametocyte stages II and III and then tapered off at stage IV and had disappeared at stage V gametocytes. Together, the results show that PfGECO expression is highest in early gametocytes and is consistent with the profile reported in the gametocyte transcriptome and mass spectrometry analysis (79, 94).

To compare the RNA expression level of PfGECO to other genes transcribed during gametocytogenesis, including PfM7 (31), PfS16 (50), and MESA (10), quantitative RT-PCR was...
FIG. 2. PfGECO expression profile in gametocytes. (A) Schematic representation of the PfGECO protein, showing the signal sequence (black box), PEXEL/HT motif (red box), and mutated DnaJ domain (green box). Residue numbers are shown above the schematic. The fragment (amino acids 23 to 459) used to raise polyclonal antiserum is indicated. (B) Western blot of gametocyte (lane G), asexual parasite (lane A), and uninfected RBC (lane R) proteins extracted probed with anti-PfGECO serum. (C) PfGECO-probed Western blot of protein extracts from a gametocyte time course where parasites were collected every 2 days as the parasites progressed from asexual (lane A) to gametocyte stages I to V (lanes I to V) and gametes (lane Gm). (D) Western blot of wild-type gametocytes (lane G) and uninfected red blood cells (lane R) probed with either anti-PfGECO or anti-Pfs16 serum. (E) Protein extracts from wild-type (lane WG) and saponin-treated (lane SG) gametocytes were immunoblotted and probed with anti-PfGECO serum.

performed on asynchronous wild-type 3D7 gametocytes (Fig. 1C). The results were normalized to the seryl tRNA synthetase housekeeping gene (PF07_0073), and PfGECO was shown to have lower RNA levels than Pf748, Pfs16, MESA, and the seryl tRNA synthetase gene. These results show that PfGECO RNA transcripts are in low abundance during gametocytogenesis.

**PfGECO is predominantly expressed in gametocytes.** PfGECO encodes a 469-amino-acid protein that is predicted to contain a signal sequence, a PEXEL/HT motif, and a DnaJ domain with a mutated HPD motif (Fig. 2A). The presence of a PEXEL/HT motif and a DnaJ domain are characteristic of the HSP40 protein family, and the mutated HPD motif classifies PfGECO into the type IV category. Comparing PfGECO to the other predicted exported *P. falciparum* type IV HSP40 proteins, the only common features are the PEXEL/HT motif and the mutated DnaJ domain (see Fig. S1 in the supplemental material) (76). Of the 11 proteins, only two (PfGECO and PF0700925w) have a signal sequence beginning at the N terminus, whereas it is recessed ~45 amino acids in the other 9 proteins. PfGECO-specific polyclonal antibodies were generated against the full-length protein minus the signal sequence for use in subsequent immunochemical assays (Fig. 2A).

To confirm protein expression in gametocytes, a Western blot was performed with protein extracts from asynchronous wild-type 3D7 asexual and gametocyte parasites under both nonreduced and reduced conditions and probed with anti-PfGECO serum. A 55-kDa product was detected only in the gametocyte lane, which is consistent with the 55.7-kDa predicted protein size of PfGECO (Fig. 2B). The same-sized product was detected in both the nonreduced and reduced samples, indicating that PfGECO is not coupled to another protein by disulfide bonds and that the disruption of disulfide bonds does not significantly alter the size of the protein. There was no reactivity in the uninfected RBCs, confirming the parasite specificity of the antibody, or in the asexual parasites, suggesting that PfGECO is predominantly expressed in the gametocyte stage. To determine the time course of PfGECO protein production throughout gametocytogenesis, wild-type 3D7 gametocytes were collected every 2 days starting from an asexual parasitemia of 10% and continuing until stage V was induced to form gametes (Fig. 2C). The 55-kDa PfGECO product was detected in gametocytes from stage I to stage V, with the highest levels of protein detected in parasites at stages I to III. There is a faint 55-kDa product in the asexual parasite lane, suggesting that PfGECO expression may begin as early as a committed schizont. There was no reactivity in the gametes. The PfGECO serum also detected a second product at 70 kDa that mirrors the timing of the 55-kDa product but is present at much lower quantities. The composition of this 70-kDa band is unclear, but it may indicate that full-length PfGECO migrates at 70 kDa and its processed form at 55 kDa. *P. falciparum* proteins are often detected at higher than expected sizes by Western blotting due to their unusually slow migration by SDS-PAGE (2, 67). To compare the protein expression levels of PfGECO to those of the gametocyte protein Pfs16, a Western blot was performed on asynchronous gametocytes from wild-type 3D7 (Fig. 2D). PfGECO was detected in lower quantities than Pfs16, which is consistent with the RNA transcript analysis.

**PfGECO is exported to the RBC cytoplasm.** The presence of a signal sequence and PEXEL/HT motif and the absence of a transmembrane domain imply that PfGECO is exported from the parasite to the host RBC in a soluble form. To assess this, asynchronous wild-type 3D7 gametocytes were purified and half were treated with saponin to lyse the RBC (Fig. 2E). PfGECO was detected in the wild-type gametocytes but not in the saponin-treated gametocytes, consistent with the protein being exported to the host RBC cytosol which is removed upon saponin hemolysis. The result also suggests that PfGECO remains soluble in the RBC cytoplasm following export. Additional treatment of gametocytes to isolate detergent-resistant microdomain (DRM) proteins confirmed that PfGECO remains in the soluble fraction and does not become membrane bound (see Fig. S2 in the supplemental material).

To more directly determine the subcellular localization of PfGECO and corroborate its timing of expression, IFA was performed. Results could be obtained only when the parasites were fixed with 1% formaldehyde and 0.005% glutaraldehyde, a combination that has previously been shown to be necessary when localizing soluble proteins (81). The majority of stage I to IV gametocytes stained positive for PfGECO, with the protein located exclusively in the host RBC and not in the parasite itself (Fig. 3). In stages I and II, the protein is seen in a dense punctate pattern, and as the amount of RBC material decreases in stages III and IV, the pattern becomes more rim-like. PfGECO was detected very weakly in some stage V ga-
metocytes and not at all in others, consistent with the protein no longer being expressed. As found by immunoblotting, PfGECO was not detected in gametes, nor was the protein present in asexual parasites (see Fig. S3 in the supplemental material).

To confirm that the PfGECO-positive parasites were gametocytes, colocalization studies with Pfs16 were performed. Pfs16 is one of the earliest gametocyte proteins expressed, first appearing in committed schizonts and localizing to the PVM in stages I to V (50). PfGECO is always in the host RBC external to Pfs16 staining; even when the PfGECO staining becomes a rim-like pattern it is outside Pfs16 (Fig. 4A). MESA is known to be expressed in both asexual parasites and gametocytes and localizes to the RBC membrane (10). Colocalization studies with PfGECO and MESA antisera show that MESA localizes to the RBC surface of all gametocyte stages and that this staining pattern is external to PfGECO (Fig. 4B).

Maurer’s clefts are parasite-generated secretory organelles located in the infected RBC cytoplasm and are important for the trafficking of exported parasite proteins to the host RBC membrane (1, 4, 9, 40, 51, 85, 91, 92). Antibodies against the Maurer’s cleft resident protein, skeleton binding protein 1 (SBP1), were used to compare the PfGECO pattern with the morphology of the clefts throughout gametocyte development. In gametocyte stages I to V, SBP1 had a discrete punctate pattern that localized both within and at the periphery of the host RBC consistent with previous studies (see Fig. S4 in the supplemental material) (12, 32, 46, 63). This pattern is different from PfGECO’s more diffuse staining within the RBC cytoplasm, suggesting that PfGECO does not localize to the Maurer’s clefts.

PfGECO is not affected by heat shock. Classically, HSP expression is elevated with increased temperature, and HSPs act as chaperones to prevent aggregation and promote the correct folding of proteins (70). To investigate if PfGECO is induced with elevated temperature, quantitative RT-PCR was performed on RNA isolated from stage I/II gametocyte cultures that were grown under normal (37°C) and heat shock (41°C) conditions for 2, 4, or 6 h. These time points were selected as during a primary malaria infection the duration of a febrile illness in patients is typically between 2 and 6 h. The RNA transcript levels of PfGECO and PfHSP70-1 (PF08_0054), which was included as a positive control, were normalized to the seryl tRNA synthetase housekeeping gene (Fig. 5). PfHSP70-1 transcripts were detected at 37°C growth and increased approximately 3-fold after heat shock at all time points tested, consistent with its classification as an HSP. In contrast, PfGECO transcript levels did not increase upon heat shock at the 2-, 4-, or 6-h time point, indicating that it does not function as an HSP.

PfGECO is not required for gametocyte production. The transcription of PfGECO early in the gametocyte life cycle was
suggestive that it may be important for gametocyte production. To determine if PfGECO was essential for gametocytogenesis, the gene was disrupted using pFCU/H9004 PfGECO, which when integrated into the genome by a double-crossover event should knock out the gene (Fig. 6A). Two independent transfections (T1 and T2) were performed, and two parasite clones from each were selected for further analysis. The PfGECO locus was disrupted in all four clones, as demonstrated by the appearance of the expected 1.1-kb and 0.88-kb products (primer pairs p5/2 and p3/8, respectively) and the loss of 1.1-kb and 1.0-kb endogenous fragments (primer pairs p5/6 and p7/8, respectively) (Fig. 6B).

Loss of protein expression in the ΔPfGECO clones was confirmed by immunoblotting and IFA. Anti-PfGECO serum detected protein in the wild-type gametocytes but not in the ΔPfGECO clones (Fig. 6C). The absence of both the 55-kDa

FIG. 4. PfGECO is external to Pfs16 and internal to MESA. Colocalization IFA was performed on gametocyte stages I to IV using anti-PfGECO serum (red) and either anti-Pfs16 (green) serum (A) or anti-MESA (green) serum (B). Also shown is the parasite nucleus stained with DAPI (blue), the bright-field images (BF), and the merged images.
and 70-kDa products in the ΔPfGECO clones indicates that the latter is a form of PfGECO and its detection is not due to nonspecific binding. The blot was then reprobed for the early gametocyte protein PfS16, and a product of the expected size was detected in all gametocyte lanes, confirming that the lack of PfGECO signal in the disrupted clones was not due to an absence of gametocytes in the sample (Fig. 6C). Colocalization IFA was performed on ΔPfGECO clones T1-F10 and T2-F5 using antibodies specific for PfGECO and PfS16 (Fig. 6D). While PfS16 could be detected at the PVM in all gametocyte stages tested, PfGECO was negative. Together, these results confirm that PfGECO was successfully disrupted.

The observation of viable and healthy gametocytes following disruption of PfGECO implies that the gene is not essential to gametocytogenesis. The ΔPfGECO clones were able to make both female and male gametocytes, with the latter capable of exflagellation. Further, there was no obvious difference in growth rates between the ΔPfGECO clones and wild-type parasites under standard in vitro culture conditions (Fig. 6E).

**PfGECO disruption does not affect the export of gametocyte proteins.** Although PfGECO was shown not to be necessary for gametocyte production, the gene-disrupted parasite line could now be used to investigate the function of PfGECO.

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**FIG. 5.** PfGECO expression is not affected by heat shock. Quantitative RT-PCR was performed on RNA extracted from wild-type 3D7 gametocytes grown for 2, 4, or 6 h at 37°C or 41°C using primers specific for PfGECO (blue) and PfHSP70-1 (red). The transcript levels were calculated from a gDNA standard curve and normalized to the value of the seryl tRNA synthetase housekeeping gene (seryl tRNA). The data plotted represent the mean results of two separate experiments performed in triplicate with standard error of the mean (SEM) error bars. Significant differences in expression levels (P < 0.05) are indicated by an asterisk.

**FIG. 6.** Generation of *P. falciparum* ΔPfGECO parasites. (A) Schematic representation of the PfGECO gene disruption strategy. The pFCUΔPfGECO plasmid contains two PfGECO fragments (F1 and F2, blue boxes) flanking the human dihydrofolate reductase cassette (hDHFR, pink box) and a cytosine deaminase-negative selectable marker (CD, green box). Following transfection, integration of the plasmid into the genome by double-crossover homologous recombination would result in disruption of PfGECO. The arrow represents the translational start site of PfGECO, and the primers used to test integration and the expected products are indicated. (B) PCR analysis was performed on two parasite clones from each of the two transfections (T1 and T2) using the primers depicted in panel A to test for double-crossover (CO) integration and wild-type PfGECO. Lane 1, T1-F10; lane 2, T1-G6; lane 3, T2-F5; lane 4, T2-F7; lane 5, 3D7; lane 6, no DNA. (C) Western blots with gametocytes from wild-type 3D7 (3D7g) and the ΔPfGECO clones T1-F10, T1-G6, T2-F5, and T2-F7 probed with either anti-PfGECO serum or anti-PfS16 serum. Also included are asexual parasites from 3D7 (3D7a) and uninfected RBCs as negative controls. (D) Colocalization IFA with stage I to IV gametocytes from ΔPfGECO clones T1-F10 and T2-F5 using anti-PfGECO (red) and anti-PfS16 (green) sera with DAPI-stained nuclei (blue). Bright-field (BF) and merged images are shown. (E) Growth curve assay comparing the percentage of gametocytemia of the ΔPfGECO clones T1-F10, T2-F5, and T2-F7 to that of wild-type 3D7. The results shown are the mean values with standard error of the mean (SEM) error bars from three replicates.
Since the traditional role for HSP40 proteins is as chaperones, a possible role for PfGECO could be to assist with the correct folding and transport of other proteins exported during gametocytogenesis. Using IFA we examined whether the absence of PfGECO affected the correct localization of known exported proteins Pfg.748, MESA, and SBP1 in gametocytes (10, 31). In the wild-type strain 3D7, Pfg.748 localized to the PV in stage I gametocytes as previously reported (Fig. 7A). In ΔPfGECO clones T1-F10 and T2-F5, Pfg.748 was still being correctly localized to the PV. Similarly, MESA was targeted to the RBC surface and SBP1 to the Maurer’s clefts in both wild-type 3D7 and the ΔPfGECO clones (Fig. 7B and C). These results suggest that the export and localization of Pfg.748, MESA, and SBP1 are not dependent on PfGECO.

Expression of type IV HSP40 genes in PfGECO-disrupted clones. The lack of a growth phenotype upon disruption of PfGECO raised the possibility that another member of the type
IV HSP40 protein family may be compensating for the deletion. To examine this, RNA was extracted from stage II gametocytes from wild-type 3D7 and the ΔPfGECO clones (T1-F10, T2-F5, and T2-F7) and used in quantitative RT-PCR to compare expression of the 11 type IV HSP40 genes (Fig. 8). As expected, PfGECO transcription was detected only in wild-type 3D7 and not in the PfGECO-disrupted clones. For most of the type IV HSP40 genes there was an increase in their transcript levels in the ΔPfGECO clones compared to that in wild-type 3D7, but the difference was not significant, showing that the disruption of PfGECO does not lead to overexpression of another type IV HSP40 gene. All of the parasite lines had similar levels of PfS16 transcription, indicating that the changes in expression detected were gene specific and not due to differences in gametocyte numbers. PfS16 was the most abundant transcript detected in the gametocyte samples, followed by MESA, PFA0675w, PFA0110w (RESA), and PfGECO; this finding is in accordance with the transcriptome data available (94).

DISCUSSION

During the early stages of gametocytogenesis, there is an upregulation of exported proteins, suggesting that the parasite specifically requires these proteins for its development or survival (31, 79). In this study, we expand the repertoire of known gametocyte-exported proteins to include PfGECO. We have demonstrated by Northern blotting that PfGECO is transcribed from asexual stages until stage III gametocytes and, using protein-specific antibodies, have shown that PfGECO is predominantly expressed in stage I to IV gametocytes. Protein expression was minimal in stage V gametocytes and asexual parasites and absent in gametes. The transcriptional profile suggests that PfGECO may first be expressed in committed schizonts and that protein production begins after committed merozoites invade and begin remodeling the RBC. Protein expression continues until stage IV, when levels decrease before disappearing as the gametocyte matures into a gamete. The expression of PfGECO so early in the gametocyte life cycle suggested that it may play a role in initiating gametocytogenesis or be required to modify the RBC to host the developing gametocyte. However, disruption of PfGECO showed that the gene was not necessary for either gametocyte production or in maintaining the morphological shape of the gametocyte during its maturation. Instead, PfGECO may function in the in vivo survival of the gametocyte during its sequestration.

By IFA analysis we show that PfGECO is indeed exported to the RBC cytoplasm, as predicted by the presence of a PEXEL/HT motif. Here it remains as a soluble protein, unlike other exported type IV HSP40 proteins such as MESA and RESA, which bind to components of the RBC cytoskeleton (8, 28, 35, 36, 74, 89). Several P. falciparum genes are predicted to be soluble exported proteins, but only a small subset of these are known to reside freely in the RBC cytoplasm, including HRP2 and REX3 (58). Many of the others, such as PF0920w and PF13_0073, have not been localized but have been shown to play a role in RBC rigidity, which may promote cytoadherence and contribute to the virulence of the parasite (59). Since the timing of PfGECO expression correlates with the duration of gametocyte sequestration it is feasible that it could similarly play a role in the deformability of the infected RBC. In a large screen of exported proteins in the asexual blood stage, three soluble proteins were shown to participate in the trafficking of the adhesin PfEMP1 to the RBC surface (59). PfGECO was also investigated in this study, but its disruption was shown not to affect PfEMP1 targeting in asexual stages, which is not surprising considering we have shown that the protein is predominantly expressed in gametocytes and not asexual parasites (59). It is still possible that PfGECO could function in assisting the export of PfEMP1, as PfEMP1 is known to be expressed in stage I and II gametocytes and has been suggested to contribute to the cytoadherence of early gametocyte stages (29). However, preliminary experiments indicate that deletion of PfGECO does not affect adherence of early gametocytes to human microvascular endothelial cells expressing CD36, implying that PfEMP1 is being correctly trafficked to the RBC surface (see Fig. S5 in the supplemental material). The observation that late stage II to IV gametocytes do not have knobs and preferentially bind to receptors other than CD36, such as ICAM-1, CD49c, CD166, and CD164, was suggestive that ligands other than PfEMP1 may be involved in adherence of the later-stage gametocytes (29, 71, 72). Additional adhesion studies, perhaps using bone marrow stromal cells that have been shown to support binding of gametocytes (72), are required to determine if PfGECO plays a role in facilitating the adhesion of these late-stage gametocytes.

Traditionally, HSPs are activated by stressful conditions encountered by the host, such as elevated temperature, chemical stress, or oxidative injury, and serve to restore cell homeostasis. We found that whereas PfHSP70-1 expression increased when the temperature was raised from 37°C to 41°C, PfGECO transcript levels remained constant under heat shock conditions,
suggested that PfGECO does not act as a classical HSP. Although PfGECO may not defend the parasite against the febrile episodes encountered during infection, it could still provide a protective role for the gametocyte throughout its extended development. It takes a gametocyte 12 days to mature into a stage V that is released into the circulation, compared to the 2-day asexual life cycle. It is reasonable that the gametocyte may express additional proteins and develop alternative mechanisms to that of the asexual parasite to protect itself from the immune system during its long sequestration.

PfGECO encodes an HPL in its DNA domain instead of the HPD found in canonical HSP40 that is crucial for activating the ATPase activity of HSP70, resulting in its classification as a type IV HSP40 (16). It is feasible that PfGECO may have an alternate binding partner, and to investigate this we performed immunoprecipitation studies, but no interacting proteins were verified (see Fig. S6 in the supplemental material). There is still the possibility that PfGECO interactions exist but that they are short-lived or of low avidity, making them difficult to detect. Such interactions would support a role for PfGECO in the transient trafficking of other exported parasite proteins to their final destination within the host RBC. Although IFA performed on PfGECO disruptant clones showed that the known gametocyte-exported proteins Pfg.748, MESA, and SBP1 were still being correctly targeted, PfGECO may facilitate the export of proteins yet to be identified. Alternatively, PfGECO may act more generally in recruiting proteins exported during gametocytenogenesis to a gametocyte-specific chaperone for protein folding and assembly (59).

The lack of an obvious deleterious phenotype in the gametocyte life cycle upon PfGECO disruption demonstrated that the parasite had the capacity to adapt to the loss of the gene under the normal conditions of in vitro growth. The accumulation of RNA transcripts of the other type IV HSP40 genes was examined to determine whether the mutant parasite could replace the lost function of one member of the family by increasing the expression of another. In P. falciparum, the phenomenon of redundancy of function has been proposed for other gene families, including the RAP complex (5), and the PfRH protein family (83). However, there was no significant difference in the transcript levels of the other type IV HSP40 genes between the wild-type 3D7 and the PfGECO disrupted clones that would suggest a redundancy of function. A similar conclusion of redundancy was reached with the digestive vacuole plasmepsin family when single-gene-knockout mutants had no effect on growth of the asexual blood stage (64) but it too was not associated with an increase in the RNA levels of the remaining intact genes (15, 64).

The more that exported proteins are confirmed in the gametocyte, the more we begin to understand about the exportome of this stage of the parasite. It has been shown previously and confirmed in this study that MESA and SBP1 are expressed in gametocytes and localize to the RBC plasma membrane and Maurer's clefts, respectively. It is likely that as these proteins have the same localization pattern in gametocytes as they do in asexual parasites, they will perform similar functions. This suggests that the gametocyte maintains many of the hallmarks of protein export from the asexual blood stage. The confirmation of Pfg.744, Pfg.748, PFA0670c, and now PfGECO as gametocyte-specific exported proteins supports the hypothesis that the exportome is altered in this stage. This may be to facilitate the changes in shape that the gametocyte goes through during its five stages of development or to provide protection for the gametocyte throughout its life cycle. The demonstration that of all the type IV HSP40 genes only PfGECO is specifically upregulated in the early gametocyte stages, and that protein production continues until stage V, when gametocytes reenter the circulation, suggests that it has a distinct role during gametocyte sequestration.

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