Endoplasmic Reticulum Stress Triggers Gametocytogenesis in the Malaria Parasite

Shweta Chaubey¹,², Manish Grover¹,², and Utpal Tatu³
From the Department of Biochemistry, Indian Institute of Science, Bangalore 560012, Karnataka, India

Background: Malaria pathogenesis depends on intricate functioning of ER secretory pathway; however, it lacks canonical UPR machinery to respond to ER stress.

Results: Malaria parasite fails to induce ER chaperones and converts to gametocytes upon ER stress.

Conclusion: Malaria parasite escapes ER stress by switching to the sexual stage.

Significance: ER stress may serve as a physiological cue for parasites to undergo gametocytogenesis.

The malaria parasite experiences a significant amount of redox stress during its growth in human erythrocytes and heavily relies on secretory functions for pathogenesis. Most certainly, the parasite is equipped with machinery to tackle perturbations in the secretory pathway, like the unfolded protein response pathway in higher eukaryotes. Our bioinformatics analysis revealed the complete absence of genes involved in the canonical unfolded protein response pathway in Plasmodium falciparum. Accordingly, the parasite was unable to up-regulate endoplasmic reticulum (ER) chaperones or ER-associated degradation in response to DTT-mediated ER stress. Global profiling of gene expression upon DTT treatment revealed a network of AP2 transcription factors and their targets being activated. The overall outcome was up-regulation of genes involved in protein export and the sexual stage of the parasite life cycle culminating in gametocytogenesis. Our results suggest that the malaria parasite uses ER stress as a cue to switch to the transmissible sexual stages.

The basic principles governing the folding of a protein are universal; however, the requirement of chaperones varies depending on the physiological needs of an organism. The proportion of the genome coding for the extracellular proteins is also variable among different organisms and so is the load on the endoplasmic reticulum (ER). The malaria parasite Plasmodium falciparum exports nearly 8% of its genome (~400 proteins) into the erythrocyte to bring about host cell remodeling and establishment of infection (1, 2). Amid the microaerophilic environment of the erythrocyte and temperature stress due to febrile episodes, how does the parasite manage such elaborate secretory functions?

The ER stress response pathway, commonly known as unfolded protein response (UPR), is a network of signaling events that is triggered when there is an imbalance between the protein folding load and ER folding machinery. It therefore aims to increase the folding capacity and thereby decrease the load on the ER (3–5). It is mediated by three ER-resident transmembrane proteins, namely IRE1, ATF6, and PERK. Normally, these proteins are sequestered in the ER by the chaperone BiP. BiP can be recruited by unfolded proteins in the ER resulting in the release and activation of these proteins. PERK phosphorylates eIF2-α resulting in inhibition of translation initiation. Ire1 is a kinase and endoribonuclease. Under ER stress, it is auto-phosphorylated and activates its endoribonuclease domain that processes the HAC1/XBP1 mRNA thereby allowing their translation to take place. HAC1/XBP1 thus formed along with ATF6 are transcription factors that induce expression of a range of proteins by binding to UPR elements (UPRE) and (or) ER stress elements (ERSE). Proteins involved in the protein folding and ERAD pathway become up-regulated so that misfolded proteins can be folded correctly or targeted for degradation. However, little is known about the UPR pathway in lower eukaryotes such as protozoan parasites (6, 7). Studies conducted until now have revealed the absence of a canonical UPR pathway, with only eIF2-α-mediated translational attenuation of an operational mechanism (6).

The malaria parasite experiences a significant amount of oxidative stress in its intra-erythrocytic life cycle because of heme detoxication, its own metabolism, and the immune system of the host (8–10). Oxidative stress disrupts the redox balance in the cell, thereby altering cellular homeostasis. In light of this fact, we also looked for the presence of proteins involved in regulating redox balance inside the cell. The malaria parasite lacks two major antioxidant enzymes, i.e. catalase and glutathione peroxidase. Also, the redox machinery is not as complex as compared with the mammalian system. There are only four homologues of PDI, and the number of thioredoxin domain-containing proteins such as thioredoxin reductase and glutaredoxin are also fewer in comparison with its mammalian counterpart (10–12). P. falciparum also lacks peroxiredoxin IV, a mammalian ER resident protein that acts as a sensor of perox-
ide levels and couples peroxide detoxification to disulfide bond formation (13). It has been reported that *P. falciparum*-infected erythrocytes efflux glutathione in substantially larger quantities than uninfected erythrocytes (14–16). This indicates that the parasite resides in an environment that involves extensive oxidative stress so that a large amount of glutathione accumulates in the parasite. This extra glutathione generated as a result of parasite homeostatic mechanisms is effluxed into its host environment.

Because protein trafficking is a central part of malaria pathogenesis, *P. falciparum* is likely to undergo a significant amount of ER stress accompanied by heat shock and changes in oxidative status. We have therefore examined the existence of UPR in *P. falciparum*. On a global scale, the parasite triggers a response involving induction of AP2 transcription factors and their targets, which culminates in stage transition and formation of gametocytes. Overall, our study sheds light on novel aspects of the ER stress response pathway in *P. falciparum* where switching over to the sexual stage seems to be the ultimate means to cope with ER stress.

EXPERIMENTAL PROCEDURES

**Bioinformatics Analysis**—The sequences of the major mediators of the UPR pathways, IRE1, XBP1/HAC1/bZIP60, ATF6, eIF2α, PERK, and ATF4, were collected from the human and yeast database. These were used as queries to carry out BLASTp (17) searches to identify the homologues present in the protozoa. The coding sequences of all the differentially expressed genes were obtained from PlasmoDB version 9.3 and TMHMM, and SignalP tools were used to predict the transmembrane domain and the signal peptide sequence (18, 19).

**Culturing and Stress Induction**—*P. falciparum* 3D7 was cultured in human O+ erythrocytes in complete RPMI 1640 medium (Sigma) supplemented with 0.5% (w/v) albumin II (Invitrogen), 0.2% (w/v) NaHCO3, 0.2% (w/v) glucose, 200 μM hypoxanthine, and 5 μg/liter gentamycin. HeLa cells were cultured in DMEM (Sigma), and Jurkat cells were cultured in RPMI supplemented with 10% (v/v) FBS. For inducing ER stress, *Plasmodium* cultures and HeLa and Jurkat cells were treated with DTT (Sisco Research Laboratories) to a concentration of 5, 10, or 20 mM for 1 h. The effect of thapsigargin was checked by incubating the parasites with 5 μM thapsigargin (Sigma) or an equal volume of ethanol for 5 h. The parasites were subjected to heat shock by incubating in a water bath at 42 °C for 1 h or 37 °C for the control.

**Cell Lysis and Western Blotting**—The parasites were purified on a 60% (v/v) Percoll (Sigma) gradient that enriches the late stages of the parasite, wherever required. The cells were resuspended in 10 times volume of PBS and lysed using 0.05% (w/v) of saponin in pH 7.4 PBS and incubated on ice for 10 min. After centrifugation at 4000 rpm for 10 min, the supernatant was used as the saponin lysate fraction, which includes proteins from the host RBC compartment as well as the parasitophorous vacuole. The pellet fraction, which consisted of the parasite proteins, was further lysed using 1% (v/v) Triton X-100 and 2 mM EDTA in PBS. All the lysis procedures were carried out in the presence of 1× protease inhibitor mixture (Roche Applied Science). Protein estimation was carried out using the Bradford method and 50 μg of each sample extracted in Laemmli buffer was separated on SDS-PAGE. Proteins were transferred to nitrocellulose membranes and then probed with the respective primary antibodies followed by goat horseradish peroxidase-conjugated secondary antibody (Bangalore Genei), and detection was carried out by enhanced chemiluminescence (GE Healthcare). The antibody dilutions used in the study are as follows: anti-PfBiP (1:2000, MR4); anti-human BiP, GRP94, PDI, and P-eIF2-α (1:1000, Sigma); β-actin from Sigma; anti-yeast eIF1-α (Sui 1) (1:1000).

**RNA Extraction and Evaluation**—RNA isolation was performed using the Qiagen mini kit with DNase treatment as per the manufacturer’s instructions. The concentration and purity of the RNA extracted were evaluated using the Nanodrop spectrophotometer (Thermo Scientific; 1000). The integrity of the extracted RNA was analyzed on the Bioanalyzer (Agilent; 2100). We considered RNA to be of good quality based on the 260:280 values (Nanodrop), rRNA 28 S:18 S ratios, and RNA integrity number (RIN-Bioanalyzer).

**Real Time PCR**—RNA amplification was performed in an iCycler iQ (Bio-Rad) using appropriately diluted cDNA and iQ SYBR Green supermix (catalog no. 170-8882). Primer pairs used for the qRT-PCR analysis are listed in Table 1. Expression levels of the genes were calculated relative to the amount of the constitutively expressed housekeeping gene actin.

**RNA Labeling, Amplification, and Hybridization**—The samples were labeled using Agilent Quick Amp kit (part number 5190-0442). 500 ng of total RNA was reverse-transcribed using oligo(dT) primer tagged to the T7 promoter sequence. cDNA thus obtained was converted to double-stranded cDNA in the same reaction. Furthermore, the cDNA was converted to cRNA in the *in vitro* transcription step using T7 RNA polymerase enzyme, and C3 dye was added into the reaction mix. During cRNA synthesis, the C3 dye was incorporated into the newly synthesized strands. The cRNA obtained was cleaned up using

| Gene | Primers |
|------|---------|
| PfHsp70–1 | Fwd, AAAGAAATCACTGCAAGAAC<br>Rev, ATCCATGTTCATCAGAAAT |
| PfBiP | Fwd, AATCTACAAGGCACAGAAT<br>Rev, AAAGCAGAGGAATACACT |
| PPD1 | Fwd, GAAAGTTGTTTGAGGACTG<br>Rev, TGCAATGATCAGGTCCTTT |
| PGRP94 | Fwd, TTGGTTCAGAATTTTGTAT<br>Rev, TGGATGACCTCTTTTACAT |
| PDer-1 | Fwd, TCCTTATTCATAGGGCTTT<br>Rev, TTCAATCTCTGSGGCTTCTT |
| PF13_0225 | Fwd, CNGATTAGAAGGACCAAA<br>Rev, CATGCAAGTTACATTCTGCA |
| PF07_0126 | Fwd, ACGATAGCAATGAGACCT<br>Rev, TGACTATCGCCAAACTATT |
| PFD0985w | Fwd, GCCCTGATGATCAGAAGAG<br>Rev, GCAAGATCCCTCTCTTATG |
| PF11_0404 | Fwd, GGAATAGAAGGGGAAACAA<br>Rev, ATGGTGTATGTTGTGTTCT |
| PfActin | Fwd, CTCAATCGTGTGAAATATAG<br>Rev, GTATTCTCCCTTTTGGTTACAT |
| PFD0210c | Fwd, CCTGACGACAATAGATTACCT<br>Rev, AAACCTATACCCCTCTTG |
| PF11_0045 | Fwd, AAAATGATGAGAGCAGCAG<br>Rev, TCCTGCTTCTTGAGGAAT |
| PF07_0089 | Fwd, GAGTACGATCTTACGGTATAT<br>Rev, TCACUAGAACTGATAGAAAGG |

**TABLE 1**

List for primers used for the qRT-PCR analysis

Fwd is forward, and Rev is reverse.
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Qiagen RNeasy columns (Qiagen, catalog no. 74106). The concentrations and amount of dye incorporated were determined using Nanodrop. Samples that passed the quality check for specific activity were taken for hybridization. 600 ng of labeled cRNA were hybridized on the array using the Gene Expression Hybridization kit (part number 5190-0404; Agilent) in SureHyb Chambers (Agilent) at 65 °C for 16 h. Hybridized slides were washed using Agilent Gene Expression wash buffers (part number 5188-5327). The hybridized, washed microarray slides were then scanned on a G2565C scanner (Agilent Technologies).

Microarray Data Analysis—Images were quantified using Feature Extraction Software (Version 11.5, Agilent). The feature-extracted raw data were analyzed using GeneSpring GX Version 12.0 software from Agilent. Normalization of the data was done in GeneSpring GX using the 75th percentile shift (percentile shift normalization is a global normalization, where the locations of all the spot intensities in an array are adjusted). This normalization takes each column in an experiment independently, and computes the nth percentile of the expression values for this array, across all spots (where n has a range from 0 to 100 and n = 75 is the median). It subtracts this value from the expression value of each entity and is normalized to specific control samples. Significant genes up- and down-regulated showing 1-fold and above within the samples with respect to control samples were identified. Student’s t test p value was calculated for the replicate samples. Differentially regulated genes were clustered using hierarchical clustering based on Pearson’s coefficient correlation algorithm to identify significant gene expression patterns. Genes were classified based on functional category.

Metabolic Labeling and TCA Precipitation—An asynchronous Plasmodium falciparum culture was subjected to treatment with DTT at a final concentration of 10 mM or mock-treated in the presence of 200 μCi of [35S]cysteine and [35S]methionine mix obtained from the Board of Radiation and Isotope Technology (BRIT). The infected erythrocytes were purified and lysed using saponin to obtain parasite pellet and lysate fractions. The lysates were resolved on a 10% SDS-PAGE and analyzed by autoradiography. For determining the TCA-precipitable radioactivity, the parasite pellet obtained from the DTT-treated and control sample were identified. Student’s t test p value was calculated for the replicate samples. Differentially regulated genes were clustered using hierarchical clustering based on Pearson’s coefficient correlation algorithm to identify significant gene expression patterns. Genes were classified based on functional category.

Detection of ROS—To detect endogenous ROS production, asynchronous parasite cultures were incubated with increasing concentrations of DTT (1, 2, 5, and 10 mM) or an equivalent amount of water for 1 h.

The cultures were then incubated with 10 mM peroxide-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate acetyl ester for 30 min at RT in the dark with mild shaking. In the presence of intracellular H2O2, nonfluorescent membrane-permeable 2′,7′-dichlorodihydrofluorescein diacetate acetyl ester is converted to impermeable fluorescent 2′,7′-dichlorofluorescein. ROS production was thus monitored by measuring 2′,7′-dichlorofluorescein emission at 530 nm with excitation at 488 nm using a spectrophotometer.

Difference Gel Electrophoresis (DIGE) Labeling and Two-dimensional Gel Electrophoresis—DIGE labeling was performed as described previously (20). The parasite pellets obtained from control and treated cultures were suspended in 50 μl of two-dimensional lysis buffer with appropriate protease inhibitors. The samples were incubated at RT for 1 h with constant mixing. The lysate thus obtained was estimated for protein concentration. Equal amounts of each protein preparation were taken, and labeling was performed with DIGE minimal dye fluoros (GE Healthcare) for 30 min on ice in the dark using 0.4 nmol of CyDye/50 μl of protein (Cy5-control and Cy3-treated). An internal standard having equal amounts of control and treated samples was labeled with Cy2 in the same way. The labeling reaction was stopped by addition of 1 μl of 10 mmol/liter lysine per 0.4 nmol of CyDye. All the three samples were mixed together such that total protein concentration is 150 μg. The volume of the mixed solution was made up to 125 μl by adding the required amount of DeStreak (GE Healthcare) solution.

The solution was used to rehydrate a 7-cm IGE strip with a 4–7 pH range (GE Healthcare) overnight at RT in the dark. Isoelectric focusing was performed using standard program followed by SDS-PAGE.
Bioinformatics Analysis Reveals Lack of UPR Machinery in P. falciparum—Host transition events challenge protozoan parasites with a variety of stressful conditions, and thus fidelity of protein folding and trafficking must be properly ensured. This implies that these parasites must have a well-developed UPR pathway like higher eukaryotes. We performed BLASTp search for the major mediators of the UPR pathway, i.e. IRE1, XBP1/Hac1/bZIP60, ATF6, eIF2-α, PERK, and ATF4 using the sequence from Homo sapiens as the query (Table 2) against the three groups of protozoan parasites, Amoebazoa, Excavata, and SAR (21). A homologue for Ire1 was found to be present in all the three amoebozoan parasites namely Acanthamoeba, Entamoeba, and Dictyostelium. Among the excavates, only Trichomonas showed the presence of Ire1, whereas in the SAR group only Tetrahymena and Blastocystis possessed an Ire1 homologue. There are three transducers of IRE1-mediated eIF2-α phosphorylation, namely PERK and ATF4, which perform the same function in different eukaryotic lineages, i.e. mammals, yeast, and plants, respectively. Homologues for none of them could be identified in any of these protozoans. Similarly, all these organisms lacked the homologue for ATF4 and ATF6 as well. This indicates that transcriptional responses associated with the canonical UPR pathway are completely absent from protozoa. However, the translational attenuation pathway seems to be well conserved as all of them have a homologue for eIF2-α and a PERK-related protein except in Entamoeba, Tetrahymena, and Reticulomyxa. This suggested that protozoa may rely on translational attenuation to tackle unfolded protein accumulation.

The analysis also revealed that P. falciparum completely lacks homologues for proteins involved in the transcriptional responses (IRE1, XBP1, ATF6, and ATF4), whereas a weak homologue of the translation-attenuating protein PERK is present. We further looked for the presence of UPRE and ERSE in P. falciparum genes by using the mammalian consensus sequence TGACGTGG(A/G) and CCAAT(N)CCACG, respectively (22–24). We analyzed the upstream sequence (up to 1 kb) of the major ER chaperones Bip, GRP94, and PDI and did not find any sequence similar to UPRE or ERSE sequences (Table 3). The parasite is known to possess a minimal ERAD pathway suggesting that protein degradation processes are not as elaborate as in mammals (25). This indicates that the parasite is unable to up-regulate ER chaperones in response to stress. Conversely, the parasite might have evolved an alternative mechanism to do the same, similar to Giardia, which shows the canonical UPR involving up-regulation of ER chaperones and ERAD despite the lack of the conventional eukaryotic machinery for UPR (26).

**P. falciparum Is Hypersensitive to ER Stress**—In the absence of robust UPR machinery, it is possible that the parasite would readily succumb to ER stress. Studies on UPR in yeast as well as mammalian cells have heavily relied on DTT as a perturbant of ER homeostasis. DTT-mediated disruption of disulfide formation is known to result in accumulation of unfolded proteins in the ER thereby triggering the unfolded protein response. Therefore, to examine the sensitivity of the malaria parasite to UPR, P. falciparum cultures were treated with a wide range of DTT concentrations for a fixed time interval, and tritiated hypoxanthine incorporation assay was performed to determine the number of viable parasites as described under “Experimental Procedures.” In parallel, the experiment was also performed with a mammalian cell line (Jurkat-T cells). As shown in Fig. 1A, P. falciparum is indeed hypersensitive to ER stress as compared with mammalian cells. The IC_{50} value obtained for P. falciparum is 0.45 mM, which in turn is 5-fold less than mammalian cell control (IC_{50} = 2.1 mM).

To check the effect of DTT on the growth of the malaria parasite, P. falciparum culture was treated with a sub-lethal concentration (0.2 mM), and change in parasitemia was monitored over the course of 3 days (72 h) (Fig. 1B). Although the parasitemia continuously increased in the untreated culture reaching up to 14% in 72 h, the parasitemia in treated culture declined with time attaining a 4-fold low as compared with the starting value by 36 h. With time, the effect of DTT was reduced, and with a continuous change of medium lacking DTT every 24 h, DTT was nearly removed from the culture. Thus, we.

### RESULTS

#### Bioinformatics Analysis Reveals Lack of UPR Machinery in P. falciparum

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### TABLE 2

| Protozoan classification | Organism | IRE1 | XBP1/Hac1/bZIP60 | ATF6 | PERK | ATF4 |
|--------------------------|----------|------|------------------|------|------|------|
| Amoebazoa                | Entamoeba histolytica | +    | -                | -    | -    | -    |
|                          | Acanthamoeba castellani | +    | -                | +    | +    | +    |
|                          | Dictyostelium discoideum | +    | -                | -    | +    | -    |
| Excavata                 | Trichomonas vaginalis | +    | -                | -    | -    | +    |
|                          | Leishmania donovani | -    | -                | -    | +    | -    |
|                          | Giardia lamblia | -    | -                | -    | -    | +    |
|                          | Trypanosoma brucei | -    | -                | -    | -    | +    |
| SAR (Stramenopiles, Alveolata, and Rhizaria) | Blastocystis hominis | +    | -                | -    | -    | +    |
|                          | Tetrahymena thermophila | +    | -                | -    | -    | -    |
|                          | Toxoplasma gondii | -    | -                | -    | +    | -    |
|                          | Theileria annulata | -    | -                | -    | -    | +    |
|                          | Plasmodium falciparum | -    | -                | -    | -    | +    |
|                          | Reticulomyxa filosa | -    | -                | -    | -    | -    |

#### Status of UPR pathway in P. falciparum in comparison with the mammalian system

| Components of UPR pathway | Mammals | Plasmodium |
|---------------------------|---------|------------|
| Mediators of transcriptional response | IRE1, XBP1, ATF6, ATF4 | Absent |
| UPRE | TGACGTGG/A | Absent |
| ERSE | CCAAT(N)CCACG | Absent |
| Translational attenuator | PERK | PERK |

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can see that after 36 h, the parasitemia started increasing in the treated culture as well and attained the value reached by untreated culture in the initial 36 h. This further confirms that reduction in the parasitemia observed was due to the effect of DTT only. We also looked at the morphological changes brought about by treatment of the parasites with DTT by observing Giemsa-stained blood smears. When the treatment was carried out at the ring stage, the parasites appeared as irregular shrunk dot-like structures. Upon carrying out the treatment on trophozoites and schizonts, the morphological changes were pronounced. The parasites appeared as compacted and rounded structures that stained darker than the healthy parasites (Fig. 1C).

To further confirm the effect of DTT on the parasites, we performed biochemical experiments to show reduction of disulfide bonds and generation of ROS upon DTT treatment. We resorted to conventional two-dimensional diagonal SDS-PAGE. For this, the metabolically labeled in vivo DTT- and mock-treated culture lysates were sequentially resolved by nonreducing followed by reducing SDS-PAGE as described under “Experimental Procedures.” Fig. 1D shows a prominent diagonal line that represents the majority of proteins that do not contain disulfide bonds. This was further evidence of the fact that samples run in reduced form in both the dimensions showed spots only along the diagonal of the gel. The proteins that form disulfide bonds have either slower (intra-disulfide linkage) or faster (inter-disulfide linkage) electrophoretic mobility in the second dimension thereby migrating either above or below the diagonal, respectively. The off-diagonal spots (spot no. 1–3 and 6) recede to the diagonal indicative of their complete reduction upon DTT treatment. Spots 7 and 8 were not detected upon the treatment indicative of their resistance to the concentration of DTT used for the treatment. The result provides biochemical evidence that the time and concentration of DTT used in the experiment indeed causes reduction of some disulfide-bonded cellular proteins. The correlation between DTT-induced ER stress and ROS production was examined by fluorometric assay using 5-(and-6)-carboxy-2’,7’-dichlorofluorescein diacetate. Fig. 2E shows accumulation of intracellular fluorogenic 2’,7’-dichlorofluorescein in response to DTT treatment in a dose-dependent manner, indicating generation of ROS. These experiments are indicative of effective DTT treatment.

P. falciparum Is Unable to Up-regulate BiP in Response to ER Stress—Next, we examined the status of BiP, GRP94, and PDI upon exposure of the parasite to DTT. Effect of thapsigargin
and heat shock was also analyzed as thapsigargin is also a well known inducer of ER stress, and heat shock brings about general stress to the cell. We excluded tunicamycin treatment as it is well established that the malaria parasite lacks N-glycosylation machinery (27, 28).

qRT-PCR analysis was performed from RNA isolated from cells subjected to heat shock or DTT treatment as described under “Experimental Procedures.” As we can see in Fig. 2A, no change was observed at the transcript level for bip, pdi, and grp94 upon heat shock or with DTT treatment. As reported previously, pfhsp70-1 expression was induced by nearly 3-fold upon heat shock and thus served as a positive control (29). These results were further validated at the protein level. Toward this, infected erythrocytes were isolated and subjected to different stress conditions followed by analysis by immunoblotting as described under “Experimental Procedures.” As shown in Fig. 2B, no induction in the levels of BiP was observed upon incubating the cells with DTT. Under the same set of conditions, mammalian cells showed 1.5–2-fold induction of BiP as reported previously for both DTT and thapsigargin treatment. Fig. 2C shows that there was no induction in the levels of PfBiP even on using high concentrations of DTT. No induction was observed at the protein level for PDI and GRP94 as well (Fig. 2D). We also used thapsigargin, which blocks the ER calcium ATPase channel, to induce ER stress upon the parasites. In this case again, we could not observe the induction in the level of BiP (Fig. 2E). On subjecting the cells to heat shock, a general cellular stress, again we could not observe any change in the levels of BiP. However, as reported earlier, PfHSP70-1 showed greater than 2-fold induction (Fig. 2F). It has recently been reported that P. falciparum possesses a minimal ERAD pathway as well (25). Therefore, we looked for the induction of

FIGURE 2. Biochemical analysis of UPR pathway in P. falciparum. A, qRT-PCR-based analysis of fold change in gene expression in P. falciparum upon exposure to heat shock (41 °C/1 h) and DTT treatment (10 mM/1 h), using actin as the reference gene. B, immunoblot-based analysis of BiP expression upon DTT treatment (10 mM/1 h) in Plasmodium and Jurkat and HeLa cells. Bottom right panel indicates the quantitation of the levels of PfBiP observed in the Western blot. C, immunoblot-based analysis of PfBiP expression with increasing concentrations of DTT (5–10 mM). D, immunoblot-based analysis of PDI and GRP94 expression in Plasmodium upon DTT treatment (10 mM/1 h). E, immunoblot-based analysis of PfBiP expression upon treatment of Plasmodium with thapsigargin (5 μM/5 h). F, effect of heat shock on the expression of PfBiP and PfHsp70-1 at the protein level; C, control; HS, heat shock; Rec, recovery. Right panel indicates the quantitation of the levels of PfBiP and PfHsp70-1 observed in the Western blot. G, qRT-PCR based analysis of fold change in gene expression of PfDerlin-1 upon exposing P. falciparum to DTT treatment, using actin as the reference gene.
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Derlin-1, an ERAD protein, upon DTT treatment by performing qRT-PCR as described under “Experimental Procedures.” As shown in Fig. 2G, we did not observe any change in the transcript levels of Derlin-1 upon DTT treatment.

In *P. falciparum*, a homologue of the protein PERK is present suggesting that attenuation of protein synthesis upon ER stress possibly takes place in the parasite, and this is normally brought about by phosphorylation of the eIF2-α (30, 31). To determine whether this pathway is operational in the parasite, immunoblotting with anti-phosphorylated eIF2-α was performed. Fig. 3A shows greater than 4-fold induction in the levels of phosphorylated eIF2-α in DTT-treated cells, which is quantitated in the lower panel, thereby confirming the presence of this pathway as a part of parasite UPR. Inhibition of protein synthesis was further confirmed by an *in vivo* labeling experiment wherein the cells were labeled with radioactive [35S]cysteine/methionine mix during the course of DTT treatment. Total parasite lysates were prepared, separated by SDS-PAGE, and analyzed by phosphorimaging. Fig. 3B shows a significant reduction in the overall protein synthesis upon DTT treatment, as revealed by a decrease in the number and intensity of bands observed. The Coomassie-stained profile of the two samples served as the loading control (Fig. 3B, left panel). The TCA-precipitable radioactivity revealed a 50% decrease in the protein synthesis upon DTT treatment in comparison with the untreated control (right panel).

**ER Stress Induces a Cascade of AP2 Transcription Factors**—Having established the inability of the malaria parasite to increase the abundance of the ER chaperones upon being challenged with ER stressors, it was of interest to investigate the gene expression profile in response to DTT treatment. We induced ER stress on cultures with 10% parasitemia by treatment with 10 mM DTT for 1 h. After incubation, the parasite pellet was obtained from both treated as well as control samples; RNA was extracted, and microarray analysis was carried out. The experiments were carried out in biological duplicates. The expression levels for nearly 5000 genes were analyzed for both sets of samples. 155 genes were found to be up-regulated in response to DTT treatment, out of which 66 showed greater than 2-fold induction on log scale (Fig. 4A). In yeast, treatment with DTT leads to the up-regulation of nearly 400 transcripts (33). The numbers of genes found to be down-regulated were 81, qRT-PCR-based validation of the differential expression of a few randomly picked genes revealed similar induction levels as obtained in microarray analysis (data not shown). All the differentially expressed genes were further characterized by using conventional algorithms such as SignalP, SMART, and BLASTp to predict signal peptides, protein transmembrane, and functional domains, if any (supplemental Tables S1, a and b, and S2, a and b).

We looked at the gene expression levels of the known UPR targets. In congruence with our biochemical analysis, we did not find up-regulation of ER chaperones as well as ERAD proteins. Functional grouping of the up-regulated genes revealed a large number of genes encoding hypothetical proteins (Fig. 4B). The genes encoding exported proteins represent yet another abundant class. These include RIFINS, STEVOR, FI KK kinases, and PHIST domain-containing proteins. No folding enzymes or chaperones were found to be up-regulated upon treatment. Notably, we found some of the genes specific to the sexual stages of the malaria parasite to be up-regulated. These genes belonged to the family of secreted ookinete protein, CPW-WPC, and 6-cysteine proteins. In addition, we also found genes belonging to the family of DEAD/DEAH box helicases to be up-regulated. Translation repression mediated by these helicases has been shown to play a crucial role in the sexual development of the parasite (34). This hinted at the possibility of a directed stage transition in response to the ER stress in *P. falciparum*.

We examined the involvement of any other transcriptional regulator specific to DTT-induced ER stress. We identified four genes belonging to the family of AP2 (Apetala-2) transcription factors. AP2 are specific transcription factors that are possessed by apicomplexa and bring about regulation of developmental processes and stress response (35–37). We carried out qRT-PCR for all these four genes to confirm the up-regulation of

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**FIGURE 3. DTT-mediated ER stress results in translational attenuation in *P. falciparum*.** A, immunoblot-based analysis of eIF2-α phosphorylation upon subjecting the cells to 10 mM DTT treatment. Bottom panel indicates the quantitation of the levels of phosphorylated eIF2-α obtained in Western blot. B, effect of DTT treatment on global protein synthesis analyzed by [35S]cysteine and -methionine incorporation. Upper panel shows the autoradiograph of the proteins resolved by SDS-PAGE and the lower panel shows Coomassie-stained gel as the loading control (left panel). Parasite proteins were TCA-precipitated to determine incorporated radioactivity through scintillation counting (right panel). The data shown represents mean disintegrations/min (dpm).
expression. The expression levels were measured in DTT-treated versus untreated cultures using actin as the reference gene. As can be seen in Fig. 4C, all four AP2 transcription factors were up-regulated upon DTT treatment. PF11_0404 showed a 4-fold increase in expression, whereas the other three genes were induced by 2-fold.

On comparing our list of up-regulated genes with that of the previously known targets of AP2 factors (38), we found that an entire network of AP2 factors is up-regulated upon DTT-mediated ER stress (Fig. 4D). PF13_0235 is the initiating factor that up-regulates nine genes and another AP2 factor PF11_0404. These nine genes belong to various functional classes such as exported proteins, DNA/RNA-processing proteins, and genes specific to the sexual stage of the parasite. PF11_0404 in turn up-regulates 23 genes along with PFD0985w and PF07_0126 AP2 transcription factors. PF07_0126 up-regulates three genes, whereas PFD0985w up-regulates 53 genes along with the other three AP2 transcription factors, PF13_0235, PF11_0404, and PF07_0126, ensuring feedback regulation (dotted arrows) of the entire cascade.

**Proteomic Analysis Confirms Up-regulation of Sexual Stage-specific Gene Products upon DTT-mediated ER Stress**—Following gene expression analysis, we wanted to perform global proteomic profiling to examine the changes that occur at the protein level in response to DTT treatment. Toward this, two-dimensional DIGE or two-dimensional difference gel electrophoresis was performed. Two-dimensional DIGE allows quantitative measurement of relative protein abundance between samples containing multiple proteins. The control and DTT-treated samples were labeled with Cy5 (red) and Cy3 (green) dyes, respectively (Fig. 5A). A protein reference pool (internal standard) was made by taking equal amounts of lysate from control and DTT-treated samples and labeling the mixture with Cy2. Equal amounts of both the samples were mixed with internal standard, and two-dimensional gel electrophoresis was performed. The images obtained were analyzed by DeCyder software. The ratio between the fluorescence signals of individual proteins was used to determine the relative differences in protein expression profiles. A total of 609 spots were confidently discerned with each having a suitable three-dimensional landscape representation. The relative abundance of each protein was determined based on the peak intensity of these three-dimensional landscape images. Of the 609 spots identified, 26 spots showed up-regulation (Fig. 5B).
5B) and 47 spots showed down-regulation (data not shown). Spots with values ±2.5 were considered to be exclusively present in the either case. Using the software Image Master Platinum, molecular weights and isoelectric points were predicted for the spots, and putative identification for the corresponding protein was done using PlasmoDB. The supplemental Table S4, a and b gives the list of putative differentially expressed proteins along with their pI, molecular weights, and fold change values.

Of the 26 up-regulated proteins found in DIGE, five were up-regulated at the transcript level as well as revealed by the microarray data. The fold change values for these proteins were comparable at both the mRNA and protein levels. These proteins included three previously uncharacterized proteins, a transcription factor, and a protein involved in pyridoxine biosynthesis. Genes encoding 11 out of the other 21 proteins were not detected in the microarray. This group primarily included proteins and metabolic enzymes. However, the genes encoding the remaining 10 proteins were detected in the microarray, but no fold change was observed for them upon treatment. This indicates that these proteins are “preferentially translated” upon DTT treatment. This set included proteins involved in vesicular transport, zinc finger domain-containing proteins, and a stress-induced translation initiation factor, SUI1. Three-dimensional landscape representations of three different proteins, which become up-regulated upon DTT treatment, are shown (Fig. 5C). The SUI1 homologue in yeast is known to be induced upon thapsigargin induced ER stress (39). Therefore, we performed immunoblot analysis to validate the effect of DTT on SUI1 expression in Plasmodium. It was observed that Sui1 becomes up-regulated by 2.5-fold upon treatment of P. falciparum cultures with DTT (Fig. 5D).

Interestingly, SUI1 (PF08_0079) and the zinc finger domain containing protein PF08_0056 have previously been shown to be specific to the gametocyte stage of the parasite life cycle (40). Preferential expression of proteins involved in vesicular trafficking found by DIGE complements the increased expression of exported proteins found in our microarray analysis. Overall, the gene and protein expression patterns described above seem to direct transition to the sexual stage of the parasite.
ER Stress Induces Gametocytogenesis—Intrigued by the up-regulation of transcripts specific to sexual stages of the malaria parasite, we examined whether there was a link between UPR and gametocytogenesis in malaria. Toward this, P. falciparum strain 3D7A was used as it is known to form gametocytes under in vitro culturing conditions. The ring stage parasites were treated with two different concentrations of DTT (200 μM for 48 h or 10 mM for 1 h) along with control cells that were incubated with an equivalent amount of water. After the incubation period was over, cells were washed with incomplete medium to completely remove DTT and were resuspended in the complete medium. The medium was changed every 24 h, and the parasites were monitored daily by preparing thin Giemsa-stained smears. On day 6, the numbers of gametocytes formed were counted against the asexual stages. As can be seen in Fig. 6A, the percentage of gametocytemia increased by 2-fold in culture treated with 200 μM DTT (n = 144) and by 2.3-fold in 10 mM DTT-treated culture (n = 165) in comparison with the control (n = 73). Thapsigargin also brought about 1.7-fold induction in the numbers of gametocytes formed. To further confirm this observation, we performed the same assay with a clinical isolate of P. falciparum capable of forming gametocytes. We could reproducibly observe a 2-fold induction in the case of all the three treatments as follows: 200 μM DTT (n = 188), 10 mM DTT (n = 183), and 5 μM thapsigargin (n = 182) in comparison with the control (n = 91) (Fig. 6B). The gametocytes were distinguishable from the asexual parasites from day 3 (stage I/stage II), day 5 (stage III), day 7 (stage IV), and day 9 (stage V) of the assay set up on day 0 (treatment). D, qRT-PCR-based analysis of fold change in gene expression of PFD0210c, PF11_0045, and PF07_0089 sexual stage-specific genes, upon exposing P. falciparum to DTT treatment, using actin as the reference gene.
the cell surface of the zygote/ookinete stage parasites and is identified as a candidate for the transmission-blocking vaccine (42, 43). However, it is known that the mRNA for these genes is transcribed much earlier in the life cycle, i.e. in the gametocyte stage. The expression of these two genes was increased by 7-fold. Previously, it has been shown that the increase in protein export and the reduction in adhesion properties are the early steps toward gametocyte formation (40, 44). Our gene expression data show up-regulation of multiple exported proteins and down-regulation of PfEMP1 upon treatment with DTT. Overall, switching over to sexual stages seems like a mechanism that the parasite has evolved to combat ER stress, as the kind of response initiated is similar to that involved during gametocytogenesis.

**DISCUSSION**

Eukaryotic cells have evolved intricate mechanisms to cope with environmental stress. In addition to the classical heat shock response, which helps them combat general environmental stress, they have also developed UPR to counter challenges to their secretory capacity, including protein folding in the ER. Although the above stress response pathways were originally described in model systems such as yeast and fruit fly, they are particularly relevant to the life cycle of protozoan parasites that cope with the challenging host cell environment for their growth and survival.

The intraerythrocytic life cycle of *P. falciparum* marks the symptomatic phase of malaria. The growth of the parasite in the erythrocyte creates an environment that is vulnerable to redox perturbation and subsequently ER stress. This could be attributed to the following factors: (i) oxidative stress associated with heme detoxication (10); (ii) efflux of glutathione from the parasitized RBCs (14–16); (iii) elevated levels of homocysteine in the plasma of malaria patients (45); and (iv) missing peroxygen IV in the ER of the parasite that performs the important function of coupling peroxide detoxication to disulfide bond formation (13). Together, these factors predispose the parasite to ER stress.

The ability to respond and cope with stressors that challenge the protein folding and trafficking capacity of the ER is thought to be universally present. Indeed the main components (IRE1, XBP1, ATF6, and ATF4), which transduce the stress signal from ER to the nucleus, are well conserved among metazoans. However, many protozoa seem to lack one or more of these components and yet thrive in the daunting host cell environment that often causes ER stress.

Our *in silico* analysis revealed a lack of orthologs for XBP1, IRE1, ATF6, and ATF4 in *P. falciparum*. Also, the search for the UPRE (TGACGTG(G/A)) and ERSE (CCAATN9CCACG) in the upstream region of *pfBip*, *pfgrp94*, and *pfpdi* did not give any success. Lack of these factors and their cognate binding sites suggested an inability of the malaria parasite to mount a transcriptional response against the burden of misfolded proteins in the ER. Analysis of PfBIP, PfGRP94, and PFPDI at both transcript and protein levels indeed confirmed the lack of induction of these chaperones in response to DTT and thapsigargin-induced ER stress. However, the presence of a PERK homologue in *Plasmodium* suggested the existence of translational regulation in response to ER stress. This was indeed confirmed by phosphorylation of eIF2-α in response to DTT-induced ER stress.

The results discussed above suggested that the human malaria parasite lacks the two most important mechanisms of the UPR pathway but has retained the ability to arrest translation upon ER stress. It is important to mention here that a related protozoan, namely *Giardia lamblia*, shows up-regulation of ER chaperones and ERAD upon ER stress despite the lack of canonical UPR mediators. This response has been shown to be mediated by *Giardia*-specific stress regulatory elements (26). *Plasmodium* is unique in terms of lacking the canonical UPR mechanisms as well as the alternative mechanism described in *Giardia*.

To gain insights into global responses triggered in response to ER stress in *P. falciparum*, we resorted to microarray analysis. Our transcriptome analysis revealed up-regulation of 155 genes. Noticeable among these were the genes belonging to the AP2 family of transcription factors. Based on the previously known target genes, AP2 factors alone accounted for about 60% of the 155 genes induced upon DTT exposure. Involvement of the AP2 family of transcription factors may emerge as a common mechanism among protozoans as *Toxoplasma* is also known to involve AP2 factors in response to tunicamycin-induced ER stress (32).

In terms of functional pathways, genes coding for exported proteins, those involved in host cell remodeling, and genes known to be specifically expressed in sexual stages were found in the list of genes induced upon DTT exposure. However, cytoadhesion-mediating *pfemp1* genes were down-regulated upon ER stress. Our two-dimensional DIGE-based proteomic analysis confirmed up-regulation of additional sexual stage-specific gene products.

The above described transcriptomic and proteomic responses involving up-regulation of gametocyte-specific genes, genes encoding for exported proteins, and down-regulation of cytoadherence genes were reminiscent of the expression profiles exhibited by the sexual stage of the parasite (40, 44). We therefore conjectured that ER stress might induce gametocytogenesis in *P. falciparum*. Indeed, our quantitative analysis of gametocyte induction confirmed the above observation. We found greater than 2-fold gametocyte induction in parasites treated with DTT. We also observed a 1.8-fold induction in the gametocyte numbers on subjecting the parasites to treatment with thapsigargin, an alternative ER stressor. The observation was also validated with a clinical isolate of *P. falciparum*. In addition to throwing light on mechanisms used by the human malaria parasite to counter ER stress, our study highlights the potential use of DTT, a common laboratory reagent, to induce gametocytogenesis in *P. falciparum*.

Although it is widely known that stress brings about a switch to sexual stages in *P. falciparum*, the molecular triggers involved in this process are not fully known. Gametocytogenesis is generally known to be induced in conditions that are detrimental for asexual growth of the parasite and has been induced in vitro by exposure to spent medium, erythrocyte lysate, cholera toxin, as well as antimarial drugs like chloroquine (46, 47). However, there is a lack of consensus among
these reports with regard to the extent of gametocyte induction and their underlying molecular mechanisms. Our study for the first time links a cellular stress response mechanism to induction of sexual stages in the life cycle of the malaria parasite.

Our results show that the malaria parasite utilizes novel mechanisms to cope with perturbations to its secretory pathway. Instead of launching transcriptional induction of ER chaperones, it chooses to convert into gametocytes that can be taken up by the insect vector during a blood meal, thereby initiating the sexual part of the *Plasmodium* life cycle. It appears that ER stress–induced gametocytogenesis could be a physiological adaptation to ensure the continued propagation of the parasite in an alternative host. It is important to emphasize here that the molecular cues used by the parasite to convert into sexual stages in *vivo* remain unclear. In light of our observation, it appears plausible that ER stress may be one such cellular cue utilized by the parasite to trigger transition to the sexual stage.

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