The human malaria parasite *Plasmodium falciparum* exports the ATP-binding cassette protein PFGCN20 to membrane structures in the host red blood cell

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Abstract

PFGCN20 is a member of the ATP-binding cassette family of proteins that is closely related to the yeast translational regulator Gcn20p. We have generated a polyclonal antibody against the N-terminal region of PFGCN20 and studied the cellular localization of PFGCN20 throughout the erythrocytic life cycle of *Plasmodium falciparum*. PFGCN20 was found to be present at all stages and a pronounced export of PFGCN20 into the erythrocyte was observed in the trophozoite and schizont stages. In the indirect immunofluorescence assay, PFGCN20 was found to display significant colocalization with antigens detected by the monoclonal antibody 41E11. In contrast, there was only a minimal overlap of PFGCN20 localization with EMP2 and HRP2. Immunoelectron microscopy demonstrated a pronounced accumulation of PFGCN20 in the lumen of the parasitophorous vacuole and deconvolution fluorescence microscopy showed membrane association with selective regions of a tubovesicular network in the red cell. We also observed a concentration of PFGCN20 in electron-dense plaques just underneath the parasite’s plasma membrane and an association of PFGCN20 with cytoplasmic vesicular structures within the parasite. The observed export of PFGCN20 and its association with the tubovesicular network in host red cells, may be indicative of the fact that PFGCN20 functions as ATP-binding subunit of an unknown multimeric ABC-transporter. The cytoplasmic localization of PFGCN20 in the parasite, however, suggests that the involvement of PFGCN20 in translational regulation or other cytoplasmic biological functions cannot be ruled out. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: *Plasmodium falciparum*; ABC protein; Malaria; Protein trafficking; Red blood cell

Abbreviations: ABC, ATP-binding cassette; EC, erythrocyte cytoplasm; EPM, erythrocyte plasma membrane; IF, indirect immunofluorescence; PBS, phosphate buffered saline; PFEMP2, *P. falciparum*-infected erythrocyte membrane protein 2; PFHRP2, *P. falciparum* histidine rich protein 2; PPM, parasite plasma membrane; PV, parasitophorous vacuole; PVM, parasitophorous vacuolar membrane; RBC, red blood cell; IRBC, infected red blood cell; SPBS, saponin containing phosphate buffered saline; TM, transmembrane domain; TPBS, triton containing phosphate buffered saline; TVM, tubovesicular network.

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1. Introduction

ATP-binding cassette (ABC) proteins form a large family of transmembrane transporters that are found in many prokaryotic and eukaryotic cells where they display a wide spectrum of substrate specificity [1]. The general structure of ABC-transporters is characterized by the presence of one or two ATP-binding domains each delineated by two highly conserved ATP-binding Walker motifs [2]. The second essential component of ABC transporters is represented by one or two clusters of membrane spanning \( \alpha \)-helices, which are believed to form the transmembrane pore facilitating the transporting activity. Eukaryotic ABC-transporters are characterized by the presence of transmembrane domains (TM) and ATP-binding sites on the same polypeptide chain, whereas many prokaryotic ABC transporters occur as multi subunit complexes where TM and ATP-binding sites are present on separate polypeptide chains. A small group of eukaryotic ABC proteins, including the yeast Gcn20p protein and elongation factor 3, resemble the bacterial ATP binding subunits but are not part of multimeric transporters [3,4]. Very little is known about the biological function of \( P. falciparum \) ABC-proteins, however, speculations about a possible role of ABC transporters in plasmodial drug resistance led to the cloning and partial characterization of three \( P. falciparum \) ABC protein genes: \( pfmdr1 \) [5], \( pfmdr2 \) [6] and \( pfgcn20 \) [7] encoding Pgh1, Pgh2 and PFGCN20, respectively.

In a previous study, we reported that the overall structure and distribution of the ATP-binding cassettes of PFGCN20 revealed a close similarity to the group of ATP-hydrolyzing subunits of bacterial ABC transporters [7]. Indeed, 43% homology was detected with the ATP binding \( vga \) protein from \( Staphylococcus aureus \) that had previously been shown to be part of a membrane-bound multimeric transporter involved in resistance to virginiamycin A-like antibiotics [8]. However, the closest protein homologue with known function of PFGCN20 was the yeast Gcn20p protein. Gcn20p is part of the yeast Gcn protein family. It was shown recently that Gcn20p together with Gcn1p, Gcn2p and Gcn4p plays a major part in the induction of amino acid synthesis under conditions of nutritional starvation [3,9–11]. In the present paper, we have analyzed the intracellular localization of PFGCN20 to better understand the physiological function of this ABC-protein. We observed that PFGCN20 is transported into the host cell and that PFGCN20 in both the parasite and its erythrocyte is present in a membrane-associated form.

2. Materials and methods

2.1. \( P. falciparum \) cultures

\( P. falciparum \) strains FAC8 and 3D7 were cultivated as previously described [12]. Briefly, RPMI 1640 medium (Gibco) was supplemented with human serum of the AB blood group (Sigma) (10% v/v), hypoxanthine (50 mg l\(^{-1}\)), glucose (2 g l\(^{-1}\)) and HEPES (26 mM). Purified human erythrocytes were added to 5% hematocrit. Parasites were grown at 37°C, under controlled gas conditions (3% O\(_2\), 4% CO\(_2\), 93% N\(_2\)).

2.2. DNA cloning

The entire genomic DNA segment contained in GenBank accession number \# U37225 was reconstructed into the pBluescript KS plasmid. A 595 bp \( HindIII/EcoRV \) fragment extending from nucleotide position (nt pos) 624 to nt pos 1219 of the coding region of \( pfgcn20 \) was cloned into the \( pQE30 \) expression vector (\( pQE30-624:1219 \)). Two synthetic oligonucleotides (5\% -ACGTGGATCCATGATGGAAGAACTTT-3\% , and5\% -CCCTTACTGGTTGTGG-3\%) were used to amplify the first 519 bp of the \( pfgcn20 \) coding region by PCR. The 5\% oligonucleotide contains an ‘in frame’ \( BamHI \) site immediately upstream of the initiation codon which was used together with the \( pfgcn20 \) internal \( HindIII \) site at nt pos 417 to clone the amplified fragment into the \( BamHI/ HindIII \) digested pQE30-624/1219 plasmid, upstream of the 595 bp insert. This construct was re-digested with \( HindIII \) and ligated with the \( pfgcn20 \) internal 207 bp \( HindIII \) fragment (nt pos 417 to nt pos 624 of \( pfgcn20 \) coding region) to
obtain a 1219 bp 5′ pfgen20 fragment in frame with the 6xHis-tag present in pQE30 (pQE30-0/1219). The accuracy of the cloning procedure was confirmed by DNA sequencing of the entire insert and flanking vector regions.

2.3. Antibody production and immunoblotting

The E. coli strain JM109 was used for expression of the insert contained in pQE30-0/1219. The resulting polypeptide (PFGCN20-45) was purified on a Ni²⁺ Agarose (Qiagen, Mississauga, Canada) column followed by SDS PAGE separation, eluted from the gel and used for immunization of rabbits. The resulting antisera were affinity purified using a PFGCN20-45 coupled HiTrap® NHS-activated column according to the manufacturer’s specifications (Pharmacia Biotech, Mississauga, Canada).

For immunoblotting, P. falciparum parasites were isolated from in vitro cultures using Percoll gradient centrifugation as described previously [13]. Uninfected human erythrocytes, and P. chabaudi infected mouse erythrocytes were washed three times in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.74 mM KH₂PO₄). Cells were lysed in reducing buffer (SDS PAGE sample buffer) for 30 min at room temperature (RT) and aliquots corresponding to 10 µg of total proteins were loaded into each lane on the 7.5% SDS PAGE gel [14]. Size separated polypeptides were transferred onto nitrocellulose membranes and probed with the AF43 antibody at a dilution of 1:10 000. Antibody binding was visualized by using an enhanced chemiluminescence immunoblot kit according to the manufacturer’s specifications (Amersham, Arlington Heights, IL, USA).

2.4. Cell fractionation

P. falciparum FCR-3 in human red blood cells at approximately 50% parasitemia and uninfected human red blood cells were pelleted and washed twice with PBS prior to being resuspended in a hypotonic Mg²⁺/Tris–HCl solution containing 10 mM Tris–HCl (pH 7.5), 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride and 1 × protease inhibitor cocktail (complete™, Boehringer, Mannheim, Germany). Red blood cells were incubated for 15 min at 4°C and subsequently homogenized with a Dounce homogenizer (100 strokes). The homogenate was spun for 10 min at 2,500 × g to collect unbroken parasite cells and the resulting supernatant was spun for 30 min at 25 000 × g. The crude membrane pellet was washed with Mg²⁺/Tris–HCl solution and then resuspended in 200 µl of Mg²⁺/Tris–HCl. The total protein content of the membrane fraction was estimated using a modified Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Immunofluorescence

Strain FAC8 cultures, grown to 5% parasitemia, were washed with PBS at 37°C. Smears of the washed cultures were prepared on ProbeOn™ MCPlus microscope slides (Fisher Biotech, Montreal, Canada) and air-dried immediately. The cells were fixed with 2 or 4% paraformaldehyde in PBS for 60 and 20 min, respectively. Quenching with 1 mg ml⁻¹ NaBH₄ in PBS was performed three times for 15 min at RT, prior to permeabilization of red blood cells with 0.1% Triton X100 in PBS (TPBS) for 30 min at RT. Identical results of PFGCN20 localization were obtained when infected red blood cells were permeabilized with 0.05% saponin as described under 2.7. The samples were then blocked using 5% BSA in TPBS for 60 min at RT. Primary antibody binding was carried out at 4°C for 12 h. The primary and secondary antibodies used in these studies are summarized in Table 1. Goat Anti Rabbit IgG-FITC and Goat Anti Mouse-FITC antibodies were purchased from BioRad, Hercules, CA, USA; Donkey Anti Rabbit IgG-Cy™3 and Goat Anti Mouse IgG-Cy™3 antibodies were purchased from Jackson Immunoresearch Laboratories, Westgrove, PA, USA; Goat Anti Rat-FITC antibodies were purchased from Pierce, Rockford, IL, USA. The secondary antibody incubations were performed for 30–60 min at RT. The labeled red blood cells (RBC) were subsequently washed with PBS, mounted with the SlowFade™ antifade kit (Molecular Probes, Eu-
Table 1
Antibodies used in immunofluorescence studies

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilutions for FITC</th>
<th>Secondary antibody</th>
<th>Dilutions for FITC</th>
<th>Antigens</th>
<th>Ref.</th>
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</thead>
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<td>1:100</td>
<td>AntiRabbitIgG</td>
<td>1:400</td>
<td>PFGCN20</td>
<td>This study</td>
</tr>
<tr>
<td>1D6</td>
<td>1:200</td>
<td>AntiMouse IgG</td>
<td>1:400</td>
<td>PFHRP2</td>
<td>[40]</td>
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<tr>
<td>4H9.1</td>
<td>1:100</td>
<td>AntiMouse IgG</td>
<td>1:400</td>
<td>PFEMP2/RESA</td>
<td>[20]</td>
</tr>
<tr>
<td>41E11</td>
<td>1:50 n.u.</td>
<td>AntiRat IgM</td>
<td>1:300</td>
<td>P/332, D260, P/155/RESA</td>
<td>[22]</td>
</tr>
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</table>

gene, OR, USA) and visualized by conventional fluorescence microscopy using the Zeiss Axioskop fluorescent microscope. The mAb 4h9.1 mouse anti PFEMP2 (P. falciparum-infected erythrocyte membrane protein 2), mAb 1D6 mouse anti PFHRP2 (P. falciparum histidine rich protein 2) and Rat IgM 41E11 antibody were a kind gift from D. Baruch (NIH, MD, USA).

2.6. Immunoelectron microscopy

FAC8 infected erythrocytes at 5% parasitemia were washed twice in PBS and subsequently once in sodium cacodylate buffer (0.1 M sodium cacodylate, 4.54 mM CaCl₂, and 204 mM sucrose) at 37°C. Cell were fixed in 4% paraformaldehyde containing 0.5% glutaraldehyde, 0.1M sodium cacodylate and 2 mM calcium chloride for 2 h at RT, and than embedded in LR White resin. Ultrathin sections were blocked with 5% BSA in TPBS and incubated with AF43 (dil. 1:10-20 in TPBS) for 12 h at 4°C. After washing in TPBS the sections were incubated with 16 nm gold labeled Goat Anti Rabbit IgG (dil. 1:100) (Jackson Immunoresearch Laboratories, Westgrove, PA, USA), washed in TPBS and viewed in a Joel electron microscope. Control incubations of sections with secondary antibody only did not result in detectable immunogold labeling.

2.7. Membrane staining, and deconvolution microscopy

FCR-3 infected red blood cells (IRBC) were washed with serum free RPMI 1640 medium and incubated with 20 µM Bodipy-Ceramide (Bodipy®FL C₂₅-ceramide; N-(4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-s-indacene-3-pentanoyl) sphingosine; Molecular Probes, Eugene, OR, USA) in RPMI 1640 containing 2 mg ml⁻¹ BSA at 37°C for 30 min. The cells were washed in PBS, attached to poly-L-lysine precoated slides and fixed with 1% formaldehyde in PBS. For the membrane/PFGCN20 co-staining experiments, the labeled cells were further permeabilized in 0.05% saponin for 20 min and blocked with 0.1% fish skin gelatin in 0.1% saponin PBS (SPBS). Primary antibody was added at a dilution of 1:100 in SPBS and incubated for 1 h at 37°C. Slides were washed with SPBS and binding of the primary antibody was visualized using the Goat Anti Rabbit IgG- Cy™5 conjugates at a dilution of 1:100 for 60 min at 37°C. The samples were examined in a Delta Vision microscope connected to a work station (Applied Precision, Seattle, WA, USA). Twenty to thirty 200 nm optical sections were stored, deconvoluted [15], and images were further processed using the Adobe® Photoshop®, San Jose, CA, USA.

3. Results

3.1. Generation and characterization of PFGCN20-specific polyclonal antibody (AF43)

The 5' segment of pfecn20 (0-1219 bp) was cloned 'in frame' into the His-tag expression vector PQE31 and expressed in E. coli. A 45 kDa polypeptide (PFGCN20-45) was purified as described in Section 2 and used for immunization of rabbits. Immune sera (AF43) were affinity purified and used for immunoblotting and cellular
localization studies. Total cell lysates of partially purified *P. falciparum* parasites were employed for immunoblotting analysis with purified AF43 antisera. A single prominent 95 kDa band was detected in three *P. falciparum* strains (ICI, B6, and FAC8) while no signal was observed in uninfected red blood cells (Fig. 1 A). The apparent size of the protein revealed by AF43 in SDS PAGE (95 kDa) is in good agreement with the size of PFGCN20 predicted from the amino acid sequence (95.5 kDa). The presence of PFGCN20 in several independent strains of *P. falciparum* suggests that the protein is likely to be conserved across the species. The AF43 antibody, however failed to detect any protein in *Plasmodium chabaudi* infected mouse blood (Fig. 1 B). These results demonstrate a high degree of specificity of AF43 for PFGCN20 expressed by *P. falciparum*. We also prepared crude membrane fractions of *P. falciparum* infected red blood cells and detected significant co-purification of PFGCN20 with membrane fractions (Fig. 1 C). By contrast, only a weak signal of PFGCN20 was detectable in heavily concentrated supernatants of membrane preparations (data not shown). These results suggest that at least a subfraction of PFGCN20 may be physically associated with membranes in *P. falciparum* infected erythrocytes.

3.2. Intracellular localization of PFGCN20 by indirect immunofluorescence microscopy

Indirect immunofluorescence (IF) analysis employing AF43 with IRBC demonstrated that PFGCN20 was expressed by early ring stages where it appeared in discrete compartments on the periphery of the parasite (Fig. 2 A–B). As was expected from the immunoblot analysis, there was a lack of AF43 staining in uninfected RBC. In late ring stages, a homogenous IF stain of the parasite cell appeared in addition to the punctuate signal, preserved from the previous stage. In addition, PFGCN20 became detectable throughout the erythrocyte cytoplasm (EC) in the form of distinct large spherical AF43 labeled structures (Fig. 2 C). In the late trophozoite and early schizont stages, a significant portion of the AF43 IF signal became localized on the cytoplasmic face of the erythrocyte plasma membrane (EPM; Fig. 2 D). Although the majority of PFGCN20 was detected as relatively large structures, smaller aggregates were also revealed (Fig. 2 C–D). Growth progression of the parasite through the trophozoite stage was characterized by the increasing concentration of PFGCN20 containing clusters in the EC (data not shown).
Fig. 2.
In schizonts, PFGCN20 was detected in the EC with at least the same intensity as in late trophozoites. However, the IF staining pattern in the EC was finer and more dispersed. (Fig. 2 E–F). A diffuse IF background stain of IRBC suggested the presence of PFGCN20 in the parasite cell and/or the parasitophorous vacuole (Fig. 2 F). There was no IF signal when: (i) nonpermeabilized IRBC were probed with AF43 antibody (Fig. 2 G); (ii) AF43 was preincubated with PFGCN20-45 polypeptide (Fig. 2 H); or (iii) when IRBC were incubated with secondary antibody only or with rabbit preimmune sera and secondary antibody (data not shown). Taken together, the immunofluorescence data suggest that PFGCN20 is present throughout the entire asexual erythrocytic life cycle of *P. falciparum*. While there appeared to be a constant export of PFGCN20 into the EC, a significant portion of PFGCN20 was detectable within or on the surface of the parasite from early ring to late schizont stages Table 1.

### 3.3. Colocalization of PFGCN20 with known plasmodial antigens

A large number of plasmodial proteins have been recently shown to be transported across the parasite plasma membrane/parasitophorous vacuolar membrane (PPM/PVM) two-membrane envelope of the parasite into the EC or to be secreted from the IRBC. Evidently, such protein transport may follow several distinct protein trafficking pathways that involve non-membranous protein ‘packets’ of various size, multiple vesicle populations [16] and the tubovesicular network (TVM) formed as an extension of the PVM into the EC [17–19]. To further elucidate the localization of PFGCN20, we compared the intracellular immunofluorescence pattern of...
PFGCN20 and three plasmodial antigens, known to be exported outside of the parasite cell: PFHRP2, PFEMP2, and Mab 41E11 antigen.

PFHRP2 was shown to be transported into the EC in the form of small protein aggregates before being excreted out of the IRBC [16,20]. In agreement with previous findings, the PFHRP2 specific monoclonal antibody 1D6 detected a diffuse spotted immunofluorescence pattern in the EC that starts at several hours post invasion and persists throughout the cycle. The double staining analysis with AF43 and 1D6 suggested that the PFHRP2 trafficking pathway is distinct from structures to which PFGCN20 is localized (Fig. 3). The export of PFGCN20 into the EC appeared to begin later in the trophozoite stage than PFHRP2, and there was no pronounced overlap between AF43 and 1D6 staining in the trophozoite and schizont stages (Fig. 3). This was most evident when a large number of cells were analyzed.

The large plasmodial protein (≈ 300 kDa) designated PFEMP2 also called MESA (mature-parasite-infected erythrocyte surface antigen) has been shown to localize to the cytoplasmic side of the EPM and to be associated with cytoskeletal proteins [21]. It was recently suggested that PFEMP2 is transported to the EPM mainly by lipid-free packets of varying size and that only a minor PFEMP2 fraction is associated with vesicular structures [16]. Double staining showed a clearly different protein localization profile of PFEMP2 and PFGCN20 throughout the P. falciparum life cycle (Fig. 4). During the ring stage, when PFGCN20 was detected as a particulate stain inside the parasite, no PFEMP2 could be detected. In the trophozoite stage, a distinct filamentous localization of PFEMP2 was detected, accompanied by a few large spherical focal patches. A modest overlap of these latter formations with PFGCN20 IF stain was observed (Fig. 4). In the schizont stage, the PFGCN20 signal persisted, while that of PFEMP2 decayed and there was no colocalization of these proteins (Fig. 4).

Handunnetti et al., [22] showed that rat monoclonal antibody 41E11 recognizes three large plasmodial proteins (Pf332, D260 and Pf155/ RESA) that are exported into the EC via a shared trafficking pathway. Fig. 5 summarizes the relative distribution of PFGCN20 and Mab 41E11 antigens. As seen in the immunofluorescence assay, during the ring stage, both PFGCN20 and
41E11 showed a particulate staining pattern in the parasitic cell. However, compared to PFGCN20, Mab 41E11 labeled finer structures closer towards the surface of the parasite cell. The transport of both PFGCN20 and MAb 41E11 labeled proteins into the EC starts in the early trophozoite stage and continues to the schizont stage. There was significant overlap between the two in the trophozoite stage which became somewhat reduced in the schizont stage. While it is not possible to conclude from these results that 41E11 antigens and PFGCN20 localize to the same cellular structures there was a clear and pronounced correlation in the cellular distribution between 41E11 antigens and PFGCN20. At all stages, the diffuse PFGCN20 IF signal in the vicinity of the parasite cell was never detected for the 41E11 antigens.

3.4. Immunoelectron microscopy of PFGCN20 in IRBC

In agreement with the light microscopy IF studies, at the electronmicroscopy level the PFGCN20 immuno-gold stain was observed in the parasite cytoplasm, the PPM/PVM, and the EC. The intraparasitic localization of PFGCN20 was found in four different subcellular regions (Figs. 6 and 7). First, accumulation of gold particles associated with vesicular structures was readily observed within the parasite (Fig. 6 A). The observed vesicular structures were distinct from the food vacuole (data not shown). Second, PFGCN20 was occasionally detected at the cytoplasmic side of the nuclear membrane, suggesting a possible association of PFGCN20 with the plasmodial rough endoplasmic reticulum (Fig. 6 B). Third, a cytoplasmic immunogold stain that was not associated with clearly defined cellular structures was mainly detected in young stages of parasites (Fig. 6 C). This pattern was fairly consistent and is unlikely to represent non-specific precipitation of gold particles. Finally, the most pronounced accumulation of immunogold particles was found at the periphery of the parasite, primarily in the ring stage (Fig. 7 A–F). In this stage, PFGCN20 was found to be concentrated in electron-dense plaques just underneath the PPM (Fig. 7 A–B). In the trophozoite and schizont stages, PFGCN20 was most readily detected in the lumen of the parasitophorous vacuole (PV) or in PV protrusions (Fig. 7 C–F). Although a subpopulation of immunogold particles appeared in close association
with the PVM, many other particles were localized in the lumen of the PV extensions (Fig. 7 C–F). The appearance of the membrane structures detected in the trophozoite and schizont stages suggested that PFGCN20 is present in vesicles budding off the PVM and/or in TVM extensions of the PVM.

3.5. Colocalization of membranes and PFGCN20

To evaluate the membrane association of PFGCN20 suggested by biochemical and ultrastructural evidence in further detail the distribution of the protein was examined in IRBCs labeled with Bodipy-Ceramide. Consecutive optical sections were imaged in the DeltaVision deconvolution fluorescence microscope. As shown in Fig. 8, PFGCN20 was detected in association with lipid labeled (green) tubovesicular elements of the TVM network in the infected red cells cytoplasm. The overlap between red (PFGCN20) and green (lipid) signal is indicated by orange-yellow. Maximal levels of membrane association were detected in the periphery of the parasite and on the erythrocyte plasma membrane (EPM). Protein clusters that were apparently lipid free were also detected. In the trophozoite stage, three main features of membrane associated PFGCN20 could be distinguished (Fig. 8). First, a large proportion of the EC located PFGCN20 stain was associated with the TVM. However, only at very specific points was there tight overlap between TVM domains and PFGCN20 (Fig. 8).
Fig. 8. Localization of PFGCN20 to membranes in the red cell. Infected erythrocytes were labeled with Bodipy-ceramide and AF43 and examined in a DeltaVision deconvolution microscope (see Section 2). Green delineates lipid staining, red indicates the distribution of PFGCN20 and yellow-orange shows the relative overlap between the two. Three consecutive optical sections 400 nm apart are shown for an early stage trophozoite (A–C), a late stage trophozoite (D–F) and a schizont (G–I). Arrowheads indicate the periphery of the red cell, short thin arrows indicate labeling of tubovesicular elements of the TVM, and long thin arrows indicate the presence of PFGCN20 at the infected red cell membrane. (Bar corresponds to 5 µm. Abbreviation: P, parasite).
strongest membrane association of PFGCN20 was observed in the immediate vicinity of the parasite that mainly corresponds to PPM/PVM and its nearest TVM extensions. Finally, a significant fraction of PFGCN20 was found along the cytoplasmic side of the EPM over extended patches. This association of PFGCN20 with the EPM was most pronounced in late schizont stage IRBCs (Fig. 8 G–I). Taken together, these results show that PFGCN20 in the EC, as in the parasite itself, can be found in a membrane-associated form(s).

4. Discussion

In the present study, we have analyzed the cellular distribution of the plasmodial PFGCN20 protein. We have observed that PFGCN20 is present during the entire erythrocytic life cycle of *P. falciparum* and that the protein is exported into the cytoplasm of IRBCs from the early trophozoite to the late schizont stages. In an attempt to better understand the PFGCN20 localization, we studied the co-localization of PFGCN20 with three other plasmodial proteins that are thought to be exported into the IRBC cytoplasm by divergent pathways: PFEMP2, PFHRP2, and the Mab 41E11 antigens. PFEMP2 and PFHRP2 are predominantly transported in the form of non-membranous protein aggregates with non-overlapping spatial distribution while the Mab 41E11 antigens are believed to travel in large membrane enclosed vesicles [16]. We observed significant overlap of PFGCN20 with Mab 41E11 antigens, especially during the trophozoite stages of the plasmodial life cycle. In contrast, there was minimal colocalization between PFGCN20 and PFHRP2 or PFEMP2. These results support the idea that protein trafficking in IRBC follows distinct and well-delineated transport pathways. Since in the RBC the cytoskeleton is largely confined to the periphery of the cell, this conclusion raises the interesting question of which cellular structures represent the molecular framework for the spatial organization of these pathways. At least a partial answer to this question may be derived from the membrane and PFGCN20 colocalization studies.

In these experiments, we noticed a pronounced tendency of EC located PFGCN20 to organize in a spatial pattern that was defined by TVM structures (Fig. 8). It remains unknown whether the association of PFGCN20 with TVM structures reflects the PFGCN20 transporting pathway and/or import properties of the TVM.

The results of our experiments unambiguously established that in an infected red cell PFGCN20 is found in both the parasite and the host cell. To move between these two cellular compartments, PFGCN20 must cross the PPM and PVM. It is interesting to note that PFGCN20, like a number of *P. falciparum* secreted proteins [23], does not have a classical export signal. Two models of protein transport mechanisms across the PPM/PVM complex were recently proposed. In the first model, it was suggested that malarial proteins are translocated into the EC at regions where the PPM and the PVM fuse into a junction [16]. In this case, chaperones from the heat shock family are suggested to be responsible for the maintenance of the appropriate conformation of the proteins translocated across the fused membranes into the erythrocyte cytosol [24]. The second model, suggested by Elmendorf and Haldar [17], proposes independent transport of plasmodial proteins across the PPM and PVM bilayers. In this model, proteins are first recruited into the parasitophorous vacuole before being distributed to their final destination in the TVM and/or EC at specific stages of the life cycle [18,19,25]. We have shown that during the ring stage PFGCN20 is localized at the periphery of the parasite cytoplasm in electron-dense plaques. It is possible that these structures represent specialized compartments of the parasite involved in recruitment and possibly transport of exported proteins. It is noteworthy that these electron-dense plaques were not in close juxtaposition of PPM and PVM. The relationship of these electron dense structures with a recently described secretory pathway employing an alternative endoplasmatic reticulum-like structure is presently unknown [26]. In the trophozoite stage, PFGCN20 was also detected in the lumen of the PV and in ‘budding off’ type formations of the PVM. These results suggest that PFGCN20 translocation across the PPM occurs
independently of its transport across the PVM. We also observed a tight association of PFGCN20 with specific regions of the TVM including spots of high PFGCN20 concentration in the distal tubular TVM compartment. If such points of tight association between PFGCN20 and the TVM represent specific points of PFGCN20 translocation across TVM membranes or are a result of PFGCN20 acting as a subunit of a putative TVM transporter is presently not known. Although we cannot formally exclude the possibility that PFGCN20 detected in the PV has been re-imported from the EC via the TVM, a simpler explanation is that we detected export of PFGCN20 via the PV and TVM into the EC.

Previous DNA sequence analysis of pfgcn20 revealed that the encoded protein displayed a high degree of homology with certain subunits of multimeric bacterial ABC transporters and yeast Gcn20p, a protein involved in the control of translation initiation [7]. Thus, a prediction of PFGCN20 function based on the known functions of homologous proteins in other species was not conclusive. The localization of PFGCN20 within the parasite cell is consistent with the proposal that PFGCN20 may function as a translation modulator similar to its yeast Gcn20p homologue which is known to be associated with ribosomes and to act as translational regulator of Gcn4p [10,11]. However, two points argue against an exclusive function of PFGCN20 in translational control. First, a significant portion of PFGCN20 is exported from the parasite into the EC and so far there is no reported translational activity in the RBC. Second, the region of lowest homology between PFGCN20 and Gcn20p is in the N-terminal part of the protein. It was recently shown that the N-terminal segment of Gcn20p is sufficient for its function as translational regulator [11]. Thus, the molecular basis for a functional homology between the yeast and the plasmodial proteins is not as strong as one would predict from the overall high degree of similarity between the two proteins. We are presently evaluating the functional homology between PFGCN20 and Gcn20p using gene transfection and functional complementation in a Gcn20p deficient strain of S. cerevisiae.

The most striking observations of our study were the consistent localization of PFGCN20 to a large variety of membrane organelles. Within the parasite, a significant portion of PFGCN20 is associated with vesicular structures of unknown identity. Following export of the protein into the PV, PFGCN20 was observed in close association with the PVM and selected regions of the TVM network. Finally, there was tight association between the erythrocytic plasma membrane and PFGCN20 that was most pronounced during the schizont stage of the parasite’s life cycle. It is tempting to speculate that these findings indicate an interaction of PFGCN20 with a so far unknown transmembrane protein(s), and that PFGCN20, like its bacterial homologues, is indeed the ATP-binding and -hydrolyzing subunit of a multimeric ABC transport system. In prokaryotic organisms, ABC proteins play a major role in import of nutrients [27–29] and ions, as well as in the excretion of bacterial toxins (hemolysin) [30] or enzymes (metalloproteases, lipases) [31,32]. For malaria, it was shown that plasmodial infection of RBCs induces an active uptake of many nutrient substances including amino acids [33], nucleotides [34], and lipids [35], while other, potentially harmful metabolites are efficiently extruded from IRBCs [36–38]. The PV and the TVM were shown to be essential for such transport [9,39]. These observations suggest the necessity for specific transmembrane transporters in the TVM network and the plasma membranes of both IRBC and the parasite. The results of our experiments and the general properties of ABC transporters suggest PFGCN20 as candidate for involvement in the complex transport network necessary for the intracellular survival of P. falciparum in its host cell. Further studies addressing the possible interaction of PFGCN20 with plasmodial transmembrane proteins are presently in progress and should help to identify the biological role of PFGCN20.

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