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Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method

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Abstract

The apicoplast and mitochondrion of the malaria parasite *Plasmodium falciparum* are important intracellular organelles and targets of several anti-malarial drugs. In recent years, our group and others have begun to piece together the metabolic pathways of these organelles, with a view to understanding their functions and identifying further anti-malarial targets. This has involved localization of putative organellar proteins using fluorescent reporter proteins such as green fluorescent protein (GFP). A major limitation to such an approach is the difficulties associated with using existing plasmids to genetically modify *P. falciparum*. In this paper, we present a novel series of *P. falciparum* transfection vectors based around the Gateway™ recombinatorial cloning system. Our system makes it considerably easier to construct fluorescent reporter fusion proteins, as well as allowing the use of two selectable markers. Using this approach, we localize proteins involved in isoprenoid biosynthesis and the posttranslational processing of apicoplast-encoded proteins to the apicoplast, and a protein putatively involved in the citric acid cycle to the mitochondrion. To confirm the localization of these proteins, we have developed a new immunofluorescence assay (IFA) protocol using antibodies specific to the apicoplast and mitochondrion. In comparison with published IFA methods, we find that ours maintains considerably better structural preservation, while still allowing sufficient antibody binding as well as preserving reporter protein fluorescence. In summary, we present two important new tools that have enabled us to characterize some of the functions of the apicoplast and mitochondrion, and which will be of use to the wider malaria research community in elucidating the localization of other *P. falciparum* proteins.

Keywords: *Plasmodium falciparum*; Apicoplast; Mitochondrion; Transfection; Green fluorescent protein; Immunofluorescence

1. Introduction

In the post-genomic era of *Plasmodium falciparum* the localization of the great majority of proteins remains uncertain. Although various computer tools are able to predict the cellular location of many proteins [1–3], hard evidence is only available for a minority of gene products [4]. A key technology for exploring protein trafficking and localization is the use of reporter molecules such as green fluorescent protein (GFP), and this approach has been valuable in *P. falciparum* [5–8]. This technique relies on the genetic manipulation of *P. falciparum* [9–11], but it still remains a fairly formidable task and technologies that simplify the process of creating transgenic parasites are most welcome. The process of creating a transgenic GFP expressing *P. falciparum* cell line is time consuming partly due to the extremely low transfection efficiency [12]. Additionally, part of the problem lies within the transfection vectors. Transfection vectors are typically large (often >10 kb), are low in copy number in *Escherichia coli*, and have repetitive AT-rich (>90%) portions interspersed with non-*P. falciparum* genes (encoding selectable markers and fluorescent proteins). As a result the assembled plasmids are inherently unstable in *E. coli*, complicating the assembly of vectors.

To circumvent some of these problems, we have designed several new plasmids based on Gateway™ recombinatorial

Abbreviations: PDF, peptide deformylase; CS, citrate synthase; ACP, acyl carrier protein; HSP60, heat shock protein 60 kD; IFA, immunoflourescence assay; GFP, green fluorescent protein

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cloning (Invitrogen). These vectors have been designed for expression of fluorescent protein fusions to allow cellular localization studies. The new vectors enable the use of two selectable markers, while also changing the relative orientation of the two expression cassettes. The orientation of the promoters driving the selectable marker and the protein of interest may be important because a recent study revealed that the calmodulin promoter used to drive the selectable marker is bi-directional and can affect the level of expression of the reporter protein [13].

Using this new series of vectors we investigated the localization of three *P. falciparum* proteins: two putative apicoplast proteins and one putative mitochondrial protein. *ispE* (formerly *ychB*) encodes 4-diphosphocytidyl-2C-methyl-D-erythritol kinase, an enzyme involved in isoprene chain biosynthesis [14,15] and predicted to be apicoplast targeted in *Plasmodium falciparum* [16]. Peptide deformylase (PDF) removes the formyl group from the initial methionine of proteins following prokaryotic-style translation [17]. Peptide deformylase occurs in bacteria and endosymbiotic organelles [18], and an apicoplast targeted version is predicted in *Plasmodium falciparum* [16]. Lastly, citrate synthase (CS) catalyses the first commited step of the citric acid cycle and is predicted to be mitochondrial [2]. GFP fusions were made for each gene and reporter localization examined.

After scrutinizing live transgenic parasites we wished to confirm the location of GFP via cross labelling with antibodies to known organellar markers. In our opinion, existing immunofluorescence protocols are suboptimal, so we developed a new protocol that provides a good level of antibody binding combined with superior morphology of both parasite and host cell. Using the new Gateway-based plasmids in conjunction with this new IFA protocol we establish that IspE and peptide deformylase are indeed located in the plastid, whereas citrate synthase resides in the mitochondrion.

2. Materials and methods

2.1. Construction of a series of new P. falciparum transfection vectors

pENTR3C (Invitrogen) was chosen as the basis for our ENTR vector. We initially digested this plasmid with *Eco*RI to remove the death gene and chloramphenicol selectable marker. We further modified this vector by removing the *Pst*I restriction enzyme site by cutting open the vector, digesting back the sticky ends using S1 nuclease and ligating back onto the blunt ends. This produced a vector we call pENTR3C(Δ*Pst*I). The *P. berghei* DHFR-TS 3′ terminator was amplified from the pHH2 vector [8] with the primers ATCGGCGGCCGCGGATATGGCAGCTTAATGTTCGTTTTTC (*Not*I site underlined) and ATCGCTCGAGTACCCTGAAGAAGAAAAGTCCGATGATGTTGTGG (*Xho*I site underlined), and inserted into the *Not*I/*Xho*I sites of pENTR3C(Δ*Pst*I). This produced the vector we dubbed pB (for *berghei* terminator). We next amplified DsRed from the vector pDsRed1-1 (Clontech), using the primers AGTCGGATCCAGATCTGCTAGCCCTAGGATGGTGCGCTCCTCCAAGAACGTCATCAAGGAGTTCATGC (*Bam*HI, *Bgl*II and *Avr*II sites underlined) and AGTCGAATTCATGGTACCCTTAAGCTGCAGCAGGAACAGGTG (*Eco*RI, *Kpn*I and *Pst*I sites underlined), and ligated this into the *Bam*HI/*Eco*RI sites of pB. This produced the vector pRB (for DsRed). GFPmut2 was digested from pHH2 [8] with *Avr*II and *Pst*I and ligated into the *Avr*II and *Pst*I sites of pRB to produce the vector pGB (for GFP). Finally, the *P. falciparum* Hsp86 5′ region was amplified from pHH2 using the primers AGTCGTCGACGGAAAGGGGCCATTGGATATATA (*Sal*I site underlined) and AGTCAGATCTTTTATTCGAAATGTGGGAAG (*Bgl*II site underlined) and ligated into the *Sal*I and *Bgl*II sites of pGB. This produced the vector we called pHGB (for Hsp86 promoter; Fig. 1A).

Destination vectors were produced by cloning the Gateway attR cassette into the pHH1 [19] and pCBM vectors [20]. The attR cassette contains the necessary att sites to enable recombination, as well as a chloramphenicol resistance marker and a *ccdB* death gene that only allows propagation of resulting vectors in bacterial strains resistant to the effects of CcdB. The *ccdB* gene functions as a negative selectable marker for successful recombination events, whereby upon a successful event, the *ccdB* gene is replaced in the destination vector by the pENTR expression cassette, enabling plasmids to be retained by bacterial strains sensitive to CcdB (Fig. 1B and C). The attR1/2 cassette was blunt-end cloned into the *Eco*RV site of pHH1 [19], and colonies that contained the attR cassette in a head-tail orientation relative to expression of the variant form of human DHFR (hDHFR) were identified and isolated (Fig 1B). This variant form of hDHFR confers resistance to the drug WR99210 [21] while its expression is controlled by the *P. falciparum* calmodulin 5′ region. This vector was consequently named pCHD-1/2 (Calmodulin 5′ driving hDHFR, - referring to the orientation of the att cassette and 1/2 for attR sites 1 and 2)(Fig. 1B).

To produce the blasticidin-S resistant destination vectors, the Gateway attR cassette was amplified using the primers CGTAACTAGTGTCAACTCTTGAGGAACCC (*Spe*I site underlined) and CGTACCGCGGGAGTTCGAGATCGGTTCCCA (*Sac*II site underlined), and ligated into the *Spe*I and *Sac*II sites of pCBM (a gift of C. Ben-Mamoun, University of Connecticut, [20]). This results in the attR cassette being placed in a head-to-tail arrangement relative to the expression of the blasticidin-S deaminase (BSD) gene, which is under control of the HrpIII promoter (Fig 1C). This plasmid was consequently named pHrBl-1/2 (HrpIII 5′ driving blasticidin-S deaminase in the head-to-tail arrangement with attR sites 1 and 2).

2.2. Construction of GFP fusion transfection vectors

To produce C-terminal GFP fusions to the genes encoding our proteins of interest, we ligated them into the *Bgl*II and

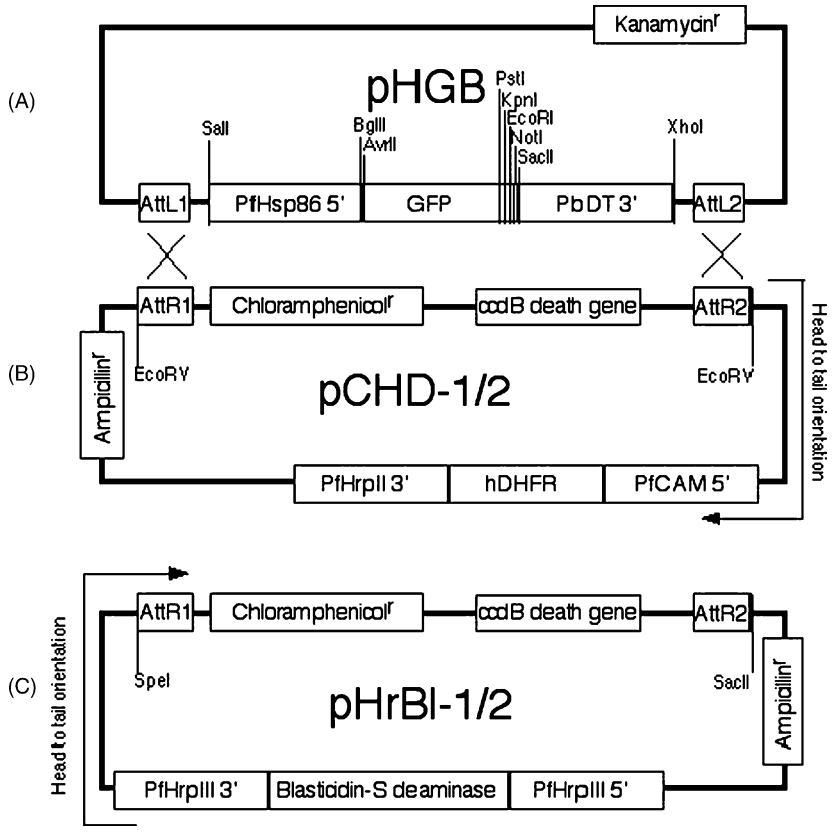

Fig. 1. A schematic diagram of new transfection vectors based on Gateway^{TM} recombinational cloning. (A) The pHGB entry clone. Unique cloning sites are available both N- and C-terminal of GFP. GFP is driven by HSP86 5′ promoter region and terminated by PbDT3′. (B) pCHD-1/2 destination clone containing the cassette responsible for expression of hDHFR, the gene mediating resistance to WR99210 treatment. This plasmid also contains attR1 and attR2 sites and appropriate positive and negative selectable markers for selection of the correct recombinational events. After the recombination reaction, expression cassettes are orientated in a head-to-tail fashion. (C) pHrBl-1/2 expresses blasticidin-S deaminase which is under the control of HRPIII 5′ and 3′. Other features are similar to pCHD-1/2, with pHrBl-1/2 also expressing the two cassettes in a head-to-tail fashion.

*Avr*II sites of pHGB (Fig. 1A). The *IspE* leader was amplified using the primers CCCAGATCTTATTATGAATCAGTTCTTAAACC (*Bgl*II site underlined) and GCTCCTAGGAAAAAAAGATCGCTTTTTTAAAT (*Avr*II site underlined), the full length peptide deformylase gene using the primers GATCAGATCTATGTTGATGTATTATTCACTTTTCC (*Bgl*II site underlined) and GCTACCTAGGTAGGGCTGGTTCTTCTGAGTG (*Avr*II site underlined), and the citrate synthase leader sequence using the primers ACTGAGATCTAAAATGGAAGGAATAAGATACCTATCATGC (*Bgl*II site underlined) and ACTGCCTAGGTTTCAAAATATTCATAATAACAGATTCTTC (*Avr*II site underlined).

To produce the final transfection vector, Gateway LR clonase reactions were performed according to the manufacturer's instructions (Invitrogen). Briefly, approximately equal concentrations of ENTR and destination vectors (300 ng) were mixed in LR reaction buffer, to which LR clonase enzyme mix was added. We recombined the CS(l)-pHGB and IspE(l)-pHGB vectors with the pCHD-1/2 plasmid, to confer WR99210 resistance, whereas the PDF(f)-pHGB vector was recombined into the blasticidin-S resistant pHrBl-1/2 plasmid. This reaction was incubated at 25 °C for 1 h, and stopped by incubation in proteinase K at 37 °C for 10 min. Plasmids were transformed into electrocompetent *E. coli* cells sensitive to CcdB. Resulting plasmids were checked for correct recombination by restriction digest analysis and purified from large bacterial cultures using a Qiagen Maxi kit. Curiously, we found that plasmid copy numbers of the resultant vectors appeared to be much higher than that of pHH2 despite their similar sizes.

2.3. Parasite transfection

The D10 strain of *P. falciparum* was used for all transfections. Parasites were grown in human O+ red blood cells at 4% hematocrit in RPMI 1640 supplemented with Albumax (GibcoBRL) to a final concentration of 0.5% and gassed with 5% $CO_2$and 0.5% O_2 in N_2 at 37 °C as described previously [22]. Parasite growth rate was measured before transfection, which proceeded only when parasites were multiplying at a rate of at least 20 times every 48 h. Transfection was carried out either by electroporation of ring stage parasites [10,23] or by red blood cell loading [24] using 100 μg of plasmid DNA. Cells were allowed to grow in the absence

of drug through one erythrocytic cycle (48 h) after which either 5 nM of WR99210 or 2.5 μg/ml blasticidin-S was added to the culture medium as appropriate. We found 2.5 μg/ml blasticidin-S to be the IC90 of wild type D10 parasites, and parasites stably transfected with BSD displayed a resistance window of four to five times this concentration (not shown).

2.4. Western blot analysis

Protein samples for western blot analysis were prepared by culturing transgenic parasites to a high parasitemia, treating with 0.15% saponin (Sigma) in PBS for 10 min on ice and then pelleting the parasites through centrifugation. The parasite cell pellet was washed several times in PBS, lysed in reducing sample buffer and boiled for 2 min. Prepared samples were then run on a 12% polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were blocked in 5% non-fat skim milk powder in PBS for an hour and then probed with anti-GFP (1:500; Roche). Primary antibody binding was detected by a goat-anti-mouse antibody conjugated to horseradish peroxidase (Pierce).

2.5. A new fixation method for P. falciparum

We were not satisfied with the quality of preservation achieved with existing *P. falciparum* fixation methods so we chose to develop our own. Cells were washed once in PBS then fixed with 4% EM grade paraformaldehyde (ProSciTech) and 0.0075% EM grade glutaraldehyde (ProSciTech) in PBS for 30 min. Fixed cells were washed once in PBS and then permeabilized with 0.1% Triton X-100/PBS for 10 min. Cells were then washed again in PBS and then treated with ∼0.1 mg/ml of sodium borohydride ($NaBH_4$)/PBS for 10 min to reduce any free aldehyde groups. Following another PBS wash, cells were blocked in 3% BSA/PBS for one hour. Anti-acyl carrier protein (ACP) antibody (diluted 1/500; [8]) or anti-*E. coli* HSP60 antibody (GroEL; diluted 1/5000; a gift from Sabine Rospert) was added and allowed to bind for a minimum of 1 h in 3% BSA/PBS. Cells were washed three times in PBS for 10 min each to remove excess primary antibody. AlexaFluor goat anti-rabbit 594 secondary antibody (Molecular Probes) was added at 1:1000 dilution (in 3% BSA/PBS) and allowed to bind for an hour while cells settled onto a previously flamed coverslip coated with 1% polyethylenimine (PEI; Sigma). Cells were washed three times in PBS and mounted in 50% glycerol with 0.1 mg/ml of 1,4-diazabicyclo[2,2,2]octane (DABCO, Sigma). The coverslips were then inverted onto a glass microscope slide, mounted and sealed.

2.6. Comparison of the new P. falciparum fixation method with existing methods

Our new protocol was compared with published fixation methods, using time-lapse video microscopy (supplementary information). Protocols included: (a) blood smear followed by treatment with cold methanol [7], (b) 0.1% paraformaldehyde followed by treatment with 0.1% saponin and 0.1% foetal calf serum [25], (c) 1% paraformaldehyde followed by treatment with 0.05% saponin (Sigma), then blocking in 0.2% fish skin gelatine (Sigma) [26], and (d) 4% paraformaldehyde, permeabilization with 0.1% Triton X-100 [27] and blocking with 3% BSA.

2.7. Microscopy

Both live and fixed samples for each of the three GFP expressing cell lines were observed and captured on a Leica TCS 4D confocal microscope. Live mitochondrial staining was visualised by treating erythrocytes infected with live CS(l)-GFP expressing parasites with 20 nM of MitoTracker® Red CM-H_2XRos (Molecular Probes).

3. Results

3.1. A new set of transfection vectors for P. falciparum

We decided to base our new vectors on the recombination-based cloning found in Invitrogen's Gateway™ series of plasmids. The Gateway system is based around a site-specific recombination reaction, whereby a sequence of interest is cloned into a so-called "entry" vector (pENTR), which is then recombined with a so-called "destination" vector (pDEST) to produce the desired expression vector. This system of cloning promotes the easy assembly of a variety of expression vectors in one cloning step. In our case, we designed the entry vector to contain a fluorescent reporter protein (GFP) flanked by a promoter (the *P. falciparum* Hsp86 5′ region) and a terminator (the *P. berghei* DHFR-TS 3′ region) (Fig. 1A). Since the resulting entry vector has only one of the two expression cassettes compared to traditional *P. falciparum* transfection vectors, it is considerably smaller, has a higher yield in *E. coli*, and is easier to ligate into. The entry vector we describe here is designed to enable the addition of a target sequence to the N-terminus of GFP under expression of the *P. falciparum* Hsp86 promoter (Fig. 1A). However, we have also produced modifications of this vector that express other reporter proteins and promoters, and have also created target sequences fused to the C-terminus of GFP using the downstream multiple cloning site (van Dooren and McFadden, unpublished).

The destination vectors we have developed are essentially modifications of the existing *P. falciparum* pHH1 [19] and pCBM vectors [20], which encode resistance to WR99210 and blasticidin-S, respectively (Fig. 1B and C). Upon performing the Gateway recombination reaction using the vectors described above, a *P. falciparum* expression vector is produced that expresses GFP under control of the Hsp86 promoter and encodes resistance to either WR99210 or blasticidin-S depending on the choice of destination vector (Fig. 1).

3.2. Analysis of organellar targeted GFP cell lines

Microscopic examination of the resulting transfectants reveals distinct subcellular GFP localization for all constructs. As with other cell lines expressing GFP in the apicoplast [8], IspE(l)-GFP also fluoresces within a characteristically small and round compartment early in the infection cycle (Fig. 2A), which then elongates (Fig. 2B) and branches (not shown) at trophozoite and shizont stage prior to splitting into numerous individual spots—one for each daughter merozoite (not shown). Interestingly, in contrast, PDF(f)-GFP fluorescence was not as reliable a marker. We observed small round apicoplasts in only a proportion of early stage parasites (Fig. 2C and D). Later stages of parasite development were completely devoid of fluorescence (not shown). The fluorescence pattern of the CS(l)-GFP expressing parasites was present in a slender rod-like structure in early stage parasites (Fig. 2E) before growing in length around the periphery of the parasite (Fig. 2F) and then branching extensively throughout in later stages (not shown). MitoTracker, a dye that specifically labels mitochondria, co-localized with the GFP at all stages (Fig. 2E and F), confirming that CS(l)-GFP is localized to the mitochondria.

Analysis of these transgenic parasites by anti-GFP western blots showed that PDF(f)-GFP and IspE(l)-GFP have two different GFP molecular mass species (Fig. 2G, lanes 1 and 3) characteristic of apicoplast targeted proteins [8]. CS(l)-GFP however contains only a single band consistent with the size of mature GFP alone (Fig. 2G, lane 2).

3.3. Comparison of different P. falciparum fixation methods

A selection of methods is available for fixation of *P. falciparum* for IFA. These range from the simple air-dried blood smears fixed by cold methanol treatment [7], to the somewhat more sophisticated method of fixing cells in suspension using a range of concentrations of paraformaldehyde in conjunction with different detergent treatments. We set out to create our own protocol to achieve better structural preservation of both the erythrocyte and parasite while still retaining the antigenicity of the target proteins. Other cell types are known to fix well with a combination of glutaraldehyde and paraformaldehyde, and we hoped that this combination would improve the structural preservation of *P. falciparum*. We used 4% paraformaldehyde as a base solution and tested a range (0.05–0.5%) of commonly used glutaraldehyde concentrations. Although these protocols gave excellent structural preservation, such fixation prevented antibodies binding to their antigens (data not shown). In a corollary experiment, we found that the use of paraformaldehyde in the absence of glutaraldehyde afforded good antibody binding, but poor cellular fixation (supplementary material, Suppl 4). A dilution series was carried out to determine a level of glutaraldehyde concentration that allowed good antibody binding while still preserving cellular structure. It was found that a remarkably low concentration of glutaraldehyde (0.0075% in 4% paraformaldehyde in phosphate buffered saline [PBS]) resulted in the best combination of cellular integrity and antibody labelling. Fortuitously, this fixation regime also maintained GFP fluorescence (Fig. 3).

Using time-lapse video microscopy we compared the integrity of cells using this new protocol with other published methods (described in Section 2.4). We found the cold methanol method to be harsh, providing poor structural preservation while also promoting a great amount of autofluorescence (supplementary information, Suppl 1). Formaldehyde treatment alone afforded better structural preservation, but a common theme in all protocols tested is red blood cell membrane disintegration after detergent treatment (supplementary material, Suppl 2, 3, 4). Our method indicates that a tiny amount of glutaraldehyde is necessary and sufficient to allow both excellent structural preservation and strong antibody binding (supplementary material, Suppl 5).

3.4. IspE(l)-GFP and PDF(f)-GFP localize to the apicoplast

To confirm the predicted localization pattern of the GFP-fusion proteins we performed an immunofluorescence assay (IFA) using our new method for fixing and permeabilizing cells (see Section 3.3). Indeed, antibodies raised to acyl carrier protein—a resident apicoplast protein-co-localized with both IspE(l)-GFP (Fig. 3A and B) and PDF(f)-GFP (Fig. 3C and D). Conversely, antibodies to *E. coli* HSP60 (GroEL) co-localised with CS(l)-GFP (Fig. 3E and F), which is consistent with the MitoTracker labelling (Fig 2E and F) and confirms that this bacterial antibody is a robust marker for the *P. falciparum* mitochondrion.

4. Discussion

The original GFP transfection vectors of *P. falciparum* are large, low copy in *E. coli* and hard to work with. We have successfully created a new set of *P. falciparum*-specific vectors using the recombination-based cloning offered by Invitrogen's Gateway series of plasmids. These vectors are smaller in size, promote easy construction of GFP fusions and allow two parasite selectable markers to be used. Our vectors yielded GFP expressing parasites faster than the traditional pHH2 plasmid. We believe this improved rate reflects the change to a head-to-tail orientation of the two expression cassettes [13]. Recently O'Donnell et al. [12] and others [28] were able to reduce the time for the emergence of transgenic parasites by the addition of the telomere clustering element rep20. We have recently ligated a rep20 element, into the *Xho*I site of the pHGB vector, and preliminary results suggest that this further reduces the time necessary for drug resistant parasites to emerge, typically 3 weeks.

We have successfully established three GFP fusion cell lines using this new versatile set of *P. falciparum* transfection

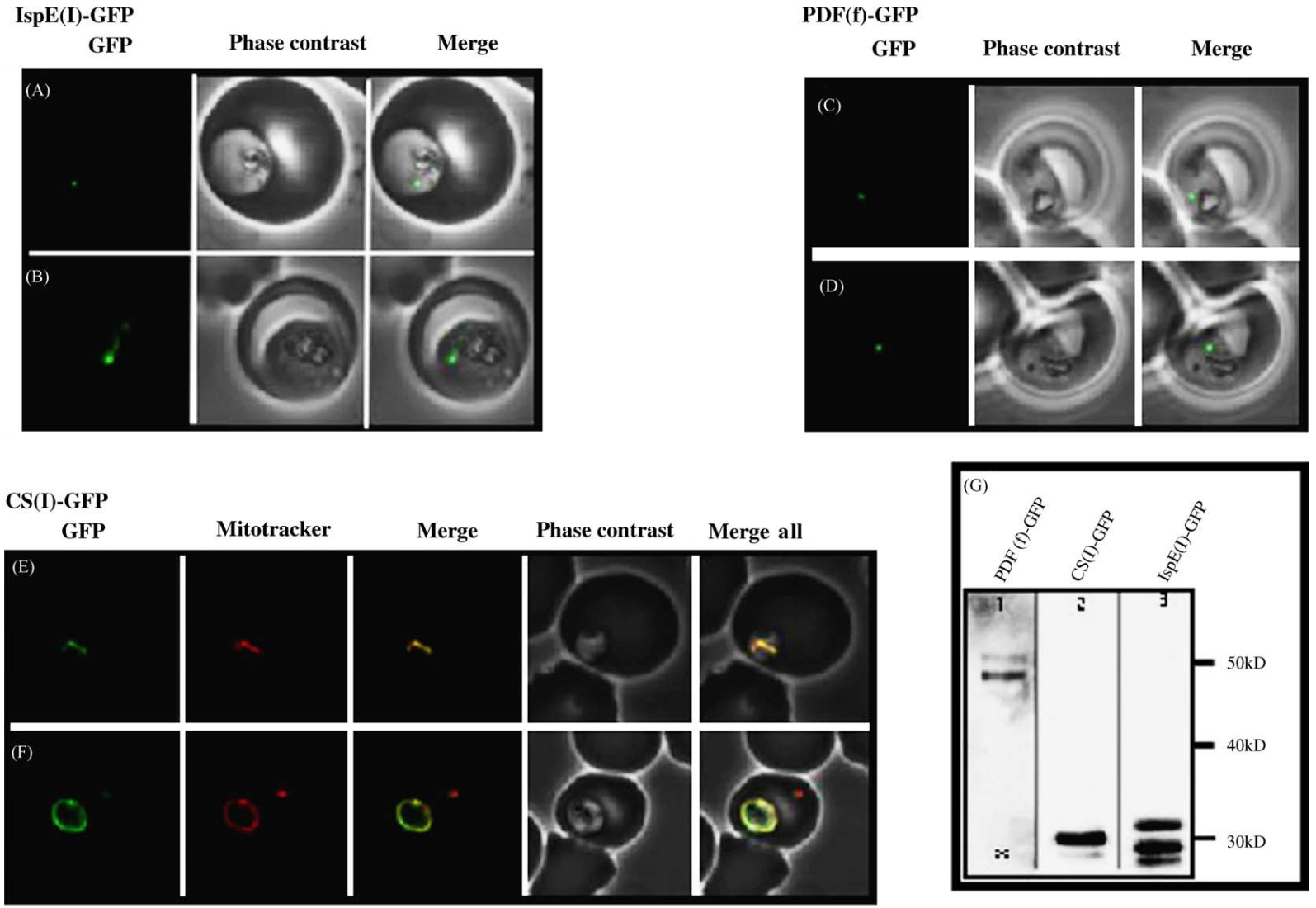

Fig. 2. Localization and western blot analysis of 4-diphosphocytidyl-2C-methyl-D-erythritol kinase (IspE), peptide deformylase (PDF) and citrate synthase (CS) using the new *Plasmodium falciparum* transfection vectors. IspE(l)-GFP (A and B) and PDF(f)-GFP (C and D) localize to a distinct single intracellular punctate spot, reminiscent of an apicoplast localization [8]. CS(l)-GFP (E and F) fluoresces as a more elongated intracellular structure in early stage parasites, which co-localizes with MitoTracker. Western blot analysis (G) reveals that PDF(f)-GFP and IspE(l)-GFP are proteolytically processed in a similar fashion to other characterised apicoplast proteins [8]. PDF(f)-GFP is expressed at very low levels, and therefore the western blot was overexposed (*) to reveal both GFP bands. CS(l)-GFP has an expected band size of a mature protein, and reveals no obvious pre-protein form, suggesting that leader processing is more rapid than in apicoplast targeting. IspE(l)-GFP and CS(l)-GFP also have a lower molecular weight protein band expected to be a GFP degradation product [7,8].

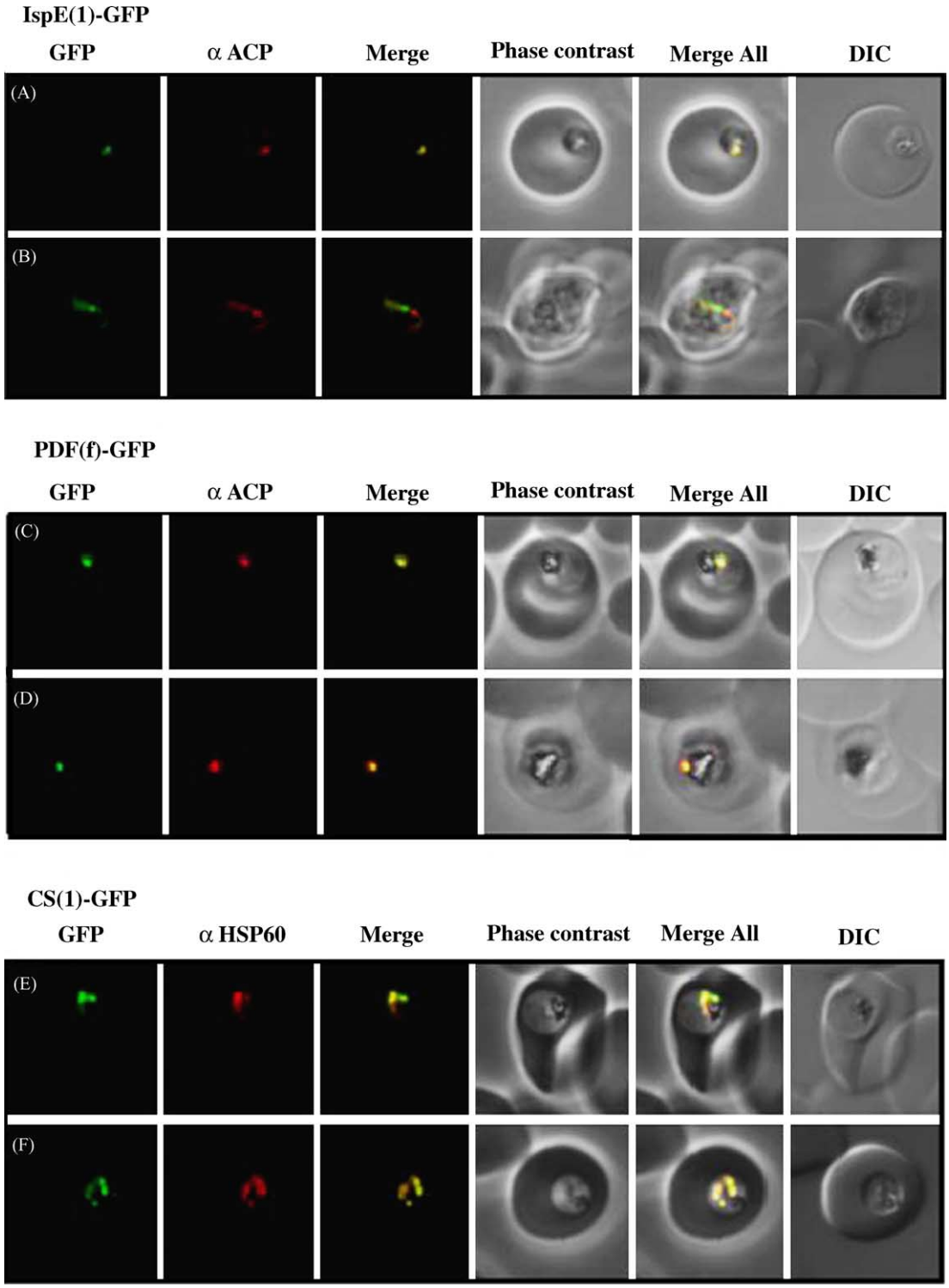

Fig. 3. Immuno-localization of GFP chimeras in transgenic parasites using a new *Plasmodium falciparum* cell fixation protocol. IspE(l)-GFP and PDF(f)-GFP were fixed and incubated with anti-ACP rabbit anti-serum. IspE(l)-GFP (A and B) co-localizes with anti-ACP. PDF(f)-GFP also co-localizes with anti-ACP (C and D), although it sometimes appears to localize within a sub-region of the ACP labelling (D). Stable CS(l)-GFP expressing cells (E and F) were incubated in the presence of anti-Hsp60 from *E.coli*. Overlay of GFP with Hsp60 suggests this Hsp60 antibody is a useful mitochondrial marker. Transmission images were captured in both phase contrast and differential interference contrast (DIC) to highlight the integrity of cells fixed under these new conditions.

vectors and shown with aid of a new cell fixation protocol that IspE and peptide deformylase localize to the apicoplast whereas citrate synthase localizes to the mitochondrion (Figs. 2 and 3).

Construction and transfection of the IspE(l)-GFP fusion shows a fluorescence pattern that co-localizes with ACP (Fig. 3A and B), confirming apicoplast localization for this isopentenyl diphosphate synthesis enzyme [29]. Recently, Jomaa et al have shown that fosmidomycin, a well-known inhibitor of isoprene chain formation, inhibits *P. falciparum* growth, and that its target DOXP reductoisomerase (IspC) localizes to the apicoplast in *T. gondii* [30]. Our data demonstrates that another enzyme in this pathway, IspE, localizes to the apicoplast and is the first localization of an isoprene biosynthesis enzyme in *P. falciparum*. This data supports the broader notion that isoprenoid biosynthesis occurs within the apicoplast [29].

PDF(f)-GFP fusion protein localizes the apicoplast. However, PDF(f)-GFP does not fluoresce in all cells, and, in those that do, often localizes to a sub-region of the apicoplast suggesting that this protein may be somehow internally compartmentalized. It is not clear however whether this localization is biologically meaningful or an artifact of transgenic over-expression. In other systems, peptide deformylase mediates the co-translational removal of formyl-methionine from growing peptide chains, and we might speculate that peptide deformylase localizes to a sub-region of the apicoplast involved in protein translation. Alternatively, the fusion protein may be misfolded and insoluble. This potentially interesting phenotype is currently being more closely investigated within our lab. Peptide deformylase is the target of the antibacterial agent actinonin in prokaryotes [17], and has been shown to inhibit recombinantly expressed peptide deformylase from *P. falciparum* as well as inhibiting parasite growth in culture [31]. Our localization of peptide deformylase in *P. falciparum* strongly suggests that the apicoplast is the target of actinonin.

Citrate synthase is the initial enzyme of the mitochondrial citric acid (or tricarboxylic acid) cycle, and our studies show a mitochondrial location for this enzyme in *P. falciparum*. However, it remains to be established whether or not a citric acid cycle occurs in *P. falciparum* mitochondria. Studies of asexual stages of *P. falciparum* find no clear biochemical evidence for the existence of a citric acid cycle [32]. Moreover, despite the existence of genes for all the enzymes necessary for a complete citric acid cycle in *P. falciparum*, there is evidence that some of these may not function in such a process [33,34]. We have shown here that the citrate synthase homologue of *P. falciparum* localizes to the mitochondrion, which suggests that *P. falciparum* is capable of catalyzing the first step of the citric acid cycle within this organelle. However, further work needs to be carried out to determine the role of citrate synthase in the biochemistry of the *P. falciparum* mitochondrion. Our new vectors will help elucidate the role of citrate synthase and also provide a generic tool for verifying the location of other enzymes that may be involved in the citric acid cycle.

In previous studies we have shown that the cellular pool for a given apicoplast targeted protein is made up of two molecular mass species on western blots [8]. We have also shown that the upper band is a precursor that is proteolytically cleaved to produce the lower molecular mass, mature form [35]. This cleavage event has been proposed to occur within the apicoplast through the activity of a stromal processing peptidase homologue [35]. The existence of two bands of IspE(l)-GFP and PDF(f)-GFP fusion proteins (Fig. 2G lanes 1 and 2) is wholly consistent with this pattern of precursor and mature forms of an apicoplast targeted protein. In contrast, CS(l)-GFP transgenic parasites only show one major band (Fig. 2G lane 3), which is the predicted size for the mature protein alone. This single band is consistent with mitochondrially-targeted proteins having their transit peptides cleaved much sooner after translation than the step-wise processing of apicoplast-targeted proteins [35].

IFA imposes conflicting demands for cell preservation. The cells have to be preserved in a state as close to living as possible whilst at the same time it is necessary to make the cells sufficiently porous in order that antibodies and conjugates can diffuse into and out of the cellular structures. Moreover, the fixation should not be so complete that antigens become unrecognisable to the antibodies. In this study we determined that a remarkably low concentration of glutaraldehyde combined with formaldehyde provided optimal preservation and labelling. The protocol also affords the option of visualising a reporter protein in conjunction with an IFA localization of a known organelle protein thereby confirming the identity of the structure to which the reporter protein is targeted. Our new vectors will simplify reporter protein experiments and allow more rapid localization of gene products in parasites. However, it should always be remembered that reporter proteins are an artificial means of localizing gene products and potentially prone to artefacts. A superior approach is IFA localizations or immuno-electron microscopy using antisera to the native protein.

Hsp60 is a highly conserved molecular chaperone found in bacteria and the endosymbiotic organelles of eukaryotes. Recently, Sato and Wilson have shown that *P. falciparum* contains two Hsp60 homologues, one of which localizes to the mitochondrion, the other to the apicoplast [28]. We obtained an antibody to Hsp60 (GroEL) from *E. coli*, and this serum decorated a single 60 kD band on a western blot of *P. falciparum* proteins (data not shown). Immunofluorescence labeling with the Hsp60 antibody revealed co-localization with CS(l)-GFP in the mitochondrion (Fig 3E and F), which in turn co-localizes with MitoTracker (Fig. 2E and F). This *E.coli* Hsp60 antiserum will serve as a useful *P. falciparum* mitochondrial marker for future studies.

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