The primase domain of PfPrex is a proteolytically matured, essential enzyme of the apicoplast

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ABSTRACT

The apicoplast of *Plasmodium* is an essential organelle with its own circular genome that must be faithfully replicated and segregated to its progeny during parasite sporogony and schizogony. DNA replication proteins are not encoded by its genome. Instead, the replication machinery must be imported from nuclear-encoded genes. A likely apicoplast DNA replication factor, PfPrex, bears a bipartite leader sequence for apicoplast trafficking and contains several DNA replication-related enzymatic domains. Here we analyze the domain structure of PfPrex and examine its trafficking and maturation within the parasite. A minimal primase domain of PfPrex is shown to contain functional zinc-binding and TOPRIM-fold domains, which in a recombinant form are sufficient to produce RNA primers from a single-stranded DNA template. PfPrex is shown to be extensively proteolytically matured within the parasite, which effectively separates its functional domains. Gene targeting attempts to knockout the *Plasmodium yoelii* ortholog of Prex were unsuccessful, indicating the apparent essentiality of this protein to the parasite. Finally, over-expression in *Plasmodium falciparum* of PfPrex's trafficking and primase sequences yielded specific and dynamic localization to foci within the apicoplast. Taken together, these observations strongly suggest an essential role of PfPrex primase in the production of RNA primers for lagging strand DNA synthesis of the apicoplast genome.

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1. Introduction

Malaria is a leading cause of disease and death in the world. Over 300 million people are infected annually, resulting in approximately one million deaths per year [1,2]. While five *Plasmodium* species infect humans and produce similar symptoms, *Plasmodium falciparum* is the most virulent and causes nearly all deaths associated with this disease [1]. Moreover, populations of *P. falciparum* that have developed resistance to commonly used anti-malarial drugs are spreading, thus compounding this medical problem and increasing the need for new chemotherapeutic drugs.

P. falciparum contains 14 nuclear, linear chromosomes and two circular genomes contained in specialized organelles, which collectively comprise the most A + T-rich genome sequenced to date [2]. One of these organelles, termed the apicoplast, is an essential, non-photosynthetic plastid that retains type II fatty acid and isoprenoid biosynthetic functions [2–6]. The apicoplast genome is comprised of a circular ~35 kb dsDNA molecule that is replicated by both rolling circle and bidirectional D-loop mechanisms, reminiscent of the replication strategy of chloroplast genomes [7,8]. The initiation of DNA synthesis in this plastid genome occurs at both segments of a discrete, inverted repeat region, although what marks this region as an origin of DNA synthesis is not known [9,10]. Inhibitors of apicoplast DNA replication in the related species *Toxoplasma gondii* result in the loss of the apicoplast, and the "delayed death" of the parasite in the next cell division cycle [11,12]. Additionally, inhibitors of transcription from the apicoplast genome in *P. falciparum* result in the same death phenotype of the parasite [13]. These data, coupled with an evolutionarily distant origin of the organelle, make the apicoplast a promising target for the development of new anti-malarial drugs (recently reviewed by Dahl and Rosenthal [14]).

One attractive molecular target for drug development in the apicoplast is the putative DNA replicative machinery of its genome, termed Prex [15]. The *P. falciparum* orthologue of Prex (PfPrex,

PF14_0112) is a large (~235 kDa), multi-functional protein that is evolutionarily well-conserved among the *Plasmodium* genus and is present in other apicomplexans such as *Theileria*, *Babesia*, and *Toxoplasma* [16,17]. A bioinformatics analysis of PfPrex identified potential primase, helicase, exonuclease and polymerase domains, which were indirectly or directly confirmed experimentally by *in vitro* assays [15]. Fusion of the bipartite leader sequence from PfPrex to GFP demonstrated its apicoplast targeting, likely via the secretory system [15]. PfPrex was also shown to undergo proteolytic maturation of a large portion of its C-terminus, but the subcellular location of this process was not determined [15]. The fidelity of the polymerase activity (found in this processed C-terminal domain *in vivo*) was recently assessed biochemically using a recombinantly expressed protein, and was also found to rescue a temperature sensitive polymerase I growth defect in *Escherichia coli* [18]. Taken together, this arrangement in which multiple domains with related, complementary functions are expressed as a poly-protein and are subsequently proteolytically processed is reminiscent of expression strategies employed by viruses such as poliovirus, hepatitis C virus, and HIV-1 (for a review [19]). Inhibitors of the viral proteases responsible for the maturation of such poly-proteins have been shown to inhibit the subsequent enzymatic function of these proteins and are used in treating infections of these viruses [20,21].

Here we examine the domain structure and cellular processing of PfPrex and test whether Prex activities are essential. PfPrex is proteolytically processed into several fragments comprising each of its enzymatic domains. Bioinformatic and biochemical approaches allowed re-annotation of the functional domains of PfPrex, which correlates with sequences that are well conserved across orthologs of the *Plasmodium* species. As with well-characterized bacterial primases, a recombinant form of the complete primase domain from PfPrex includes a zinc-binding element that contains evolutionarily conserved CXXC sequence motifs. Consistent with its proposed activity, this domain is competent to transcribe short RNA primers. The primase domain localizes in distinct foci within the apicoplast as the parasite progresses through the intraerythrocytic asexual stages. Interestingly, the gene encoding Prex in the *Plasmodium yoelii* rodent malaria model could not be deleted, which supports its probable essential nature. Taken together, this characterization of Prex permits a greater understanding of the mechanisms underlying plastid replication in *Plasmodium* spp., while also providing potential targets for development of specific chemotherapeutic agents.

2. Materials and methods

2.1. Bacterial expression and purification of PfPrex primase derivatives

Sequences coding for PfPrex's TOPRIM domain alone (residues 336–465) or complete primase domain (comprised of its zinc-binding and TOPRIM domains, residues 115–465; Fig. 1) were PCR amplified from *P. falciparum* 3D7 genomic DNA, inserted between the NdeI and XhoI sites of pET28b+ (Novagen) and confirmed by sequencing. All primers used in this study are listed in Supplemental Table 1. Expression plasmids were transformed into the Rosetta2(DE3)pLysS strain of *E. coli* (Novagen). Significant quantities of soluble protein expression were only obtained by a multiple step induction process. In brief, bacteria were grown at 37 °C with shaking (220 rpm) until an OD_{600} of 0.7 was reached. The culture was then shifted to room temperature, shaking speed was reduced to 125 rpm, and chaperone expression was induced by the addition of benzyl alcohol (Sigma Aldrich) to 1 mM for 15 min [22]. Expression of PfPrex derivatives was then induced for 20 h by the addition of 0.5 mM (final) isopropyl β-D-1-thiogalactopyranoside (IPTG). Bacterial pellets were lysed by sonication in Nickel-NitriloTriAcetic acid (Ni-NTA) Loading Solution (25 mM HEPES pH 7.5, 500 mM NaCl, 150 mM sodium formate, 10 mM imidazole, 10% glycerol) with 1 mM (final) phenylmethylsulfonyl fluoride (PMSF) and 1 mM (final) benzamidine. The soluble fraction was separated from insoluble material by centrifugation (15,000 rpm, 4 °C, 20 min), and was then incubated in batch with Ni-NTA resin (Qiagen) on an orbital shaker for 1 h at 4 °C. The resin was applied to a column and washed extensively (>20 column volumes) with Ni-NTA Loading Solution, and then five column volumes of Ni-NTA Washing Solution (25 mM HEPES pH 7.5, 500 mM NaCl, 150 mM sodium formate, 250 mM dextrose, 50 mM imidazole, 10% glycerol). PfPrex derivatives were eluted by addition of five column volumes of Ni-NTA Elution Solution (25 mM HEPES pH 7.5, 500 mM NaCl, 150 mM sodium formate, 300 mM imidazole, 10% glycerol, 1 mM DTT). PfPrex derivatives were then dialyzed against SPFF Buffer A (50 mM MES pH 6.4, 500 mM NaCl, 150 mM sodium formate, 10% glycerol, 1 mM DTT), diluted 1:5 in SPFF Buffer A, and purified via SPFF ion exchange chromatography using a linear gradient over 10 column volumes of 8–100% SPFF Buffer B (50 mM MES pH 6.4, 1 M NaCl, 150 mM sodium formate, 10% glycerol, 1 mM DTT). PfPrex derivatives eluted between 15 and 40% SPFF Buffer B and collected protein was concentrated to ~30 μM. Purity of PfPrex derivatives was >90% as

Fig. 1. Schematic representation of PfPrex and its conserved enzymatic domains. Prex traffics to the apicoplast via an N-terminal bipartite leader sequence (BLS), and harbors evolutionarily conserved enzymatic domains with primase, helicase, exonuclease, and polymerase functions. The primase domain is here reannotated to include both the zinc-binding domain (ZBD) and the topoisomerase-primase (TOPRIM) domain. Two zinc-binding CXXC motifs (AA155–158, 182–185) are noted within the ZBD. Those portions of PfPrex with high and moderate levels of sequence identity and/or similarity to *Plasmodium berghei*, *P. yoelii*, and *Plasmodium chabaudi* are noted in red and pink, respectively, with the amino acid positions of their boundaries noted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

assessed by Coomassie Blue staining of a 12% SDS–polyacrylamide gel.

2.2. Generation of rabbit polyclonal antibodies to the TOPRIM domain of PfPrex

Bacterially expressed PfPrex TOPRIM was purified by Ni-NTA resin from inclusion bodies dissolved in 200 mM HEPES pH 7.5, 500 mM NaCl, 6 M GuHCl. The resin was washed with an additional 20 column volumes of buffer, and then five column volumes of the same solution supplemented with 50 mM imidazole. PfPrex TOPRIM was eluted with five column volumes of the same solution but containing 300 mM imidazole, and was found to be >90% pure. Protein was precipitated by excessive dilution in distilled water and was washed in 5 mM ammonium acetate prior to lyophilization. Pre-immune and post-immune sera were generated from two New Zealand white rabbits (Harlan BPS), and anti-TOPRIM antibodies were enriched by octanoic acid and ammonium sulfate precipitations and dialyzed into 1× PBS pH 7.2, 10% glycerol.

2.3. Colorimetric detection of zinc ions by the PAR reagent

The amount of Zn^{2+} bound by PfPrex primase was determined spectrophotometrically by the use of 4-(2-pyridylazo)resorcinol (PAR), as previously described [23]. Briefly, the absorbance of PAR at 500 nm increases linearly with the addition of 1–8 μM zinc ions. Standard solutions of $ZnCl_2$ were diluted in PAR Assay Solution (50 mM HEPES pH 7.5, 3 M GuHCl) and were used to measure the amount of Zn^{2+} released from bacterially expressed and purified PfPrex primase (5 μM) when unfolded. Measurements were conducted in triplicate.

2.4. Direct primase enzymatic assay

The enzymatic activity of PfPrex primase was determined by direct measurement of $[\alpha\ ^{32}P]ATP$-incorporated RNA primers using a previously described method with modifications [24]. Primase reaction solutions (50 mM HEPES pH 7.4, 5 mM MgOAc, 1 mM spermidine, 200 mM NaCl, 10% glycerol, 0.5 mM CTP, 0.5 mM GTP, 0.5 mM TTP, 0.3 mM $[\alpha\ ^{32}P]ATP$) were incubated with 25 ng of a single-stranded dT_{50} oligonucleotide template and 10 μM (final) PfPrex primase or *E. coli* DnaG (positive control) at 37 °C for 1 h. Reactions were phenol/chloroform treated, and then purified using Bio-Gel P-6 spin columns (GE Healthcare) to remove unincorporated $[\alpha\ ^{32}P]ATP$. Formamide loading solution was added to each reaction, which were then heated to 95 °C for 5–10 min and loaded on a 7 M urea/20% SDS–polyacrylamide gel with a ^{32}P-labeled 4–20 nt molecular weight marker (4, 8, 12, 16, 20 nt long dT single-stranded DNA) run in parallel. Samples were electrophoresed at 4 °C, 250 V for 6–8 h. The gel was then dried onto Whatman paper and exposed to a storage phosphor screen. Signals were visualized by a Storm 860 PhosphorImager and ImageQuant 5.2 software (Molecular Dynamics).

2.5. Western blotting of P. falciparum lysates

Asynchronous *P. falciparum* (3D7 strain) parasites were grown in and isolated from human O+ erythrocytes by standard methods. Parasites were resuspended in 1× PBS pH 7.5 and lysed in an equal volume of 2× Sample Buffer (100 mM Tris–HCl pH 6.8, 4%, w/v SDS; 0.2%, w/v bromophenol blue; 20%, v/v glycerol) with sonication. Disulfide bonds were reduced by the addition of 5% (v/v, final) 2-mercaptoethanol. Lysates from a serial dilution of parasites were loaded on an 8% SDS–polyacrylamide gel with a molecular weight marker and 250 ng bacterially expressed and purified PfPrex primase loaded in parallel. Samples were transferred to

a PVDF membrane, probed with pre-immune or post-immune anti-TOPRIM rabbit sera, and visualized with a HRP-conjugated secondary antibody and SuperSignal West Pico ECL substrate (Thermo Scientific).

2.6. Indirect immunofluorescence assay (IFA)

P. falciparum (NF54 strain)-infected human RBCs (iRBCs) (4% parasitemia, 4% hematocrit) were processed for IFA using a previously described method [25]. Briefly, infected red blood cells (iRBCs) were pelleted initially (and between all steps) at 4000 rpm in a microcentrifuge at room temperature for 1 min. Cells were washed twice in 1× PBS, fixed in 1× PBS + 4% paraformaldehyde + 0.0075% glutaraldehyde for 30 min at room temperature, and permeabilized in 1× PBS + 0.1% Triton X-100 for 10 min at room temperature. A 1× PBS + 3% bovine serum albumin (BSA) blocking solution was applied at 4 °C overnight. A mouse anti-GFP monoclonal antibody (Roche, Cat# 11814460001) and a rabbit anti-*P. falciparum* acyl carrier protein (PfACP) polyclonal antibody (a kind gift of G. McFadden) were diluted in 1× PBS + 3% BSA solution at 1:200 and 1:100 respectively, and incubated with iRBCs for 1 h with end-over-end rotation at room temperature. Following two washes with 1× PBS + 3% BSA, Alexa Fluor-conjugated secondary antibodies (Invitrogen) specific to mouse IgG (Alexa Fluor 488, Green) and rabbit IgG (Alexa Fluor 594, Red) were diluted 1:500 in 1× PBS + 3% BSA and applied to iRBCs for 30 min with end-over-end rotation at room temperature with shielding from light. Nucleic acid was then stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in 1× PBS for 5–10 min at room temperature. Cells were washed three times with 1× PBS, and mixed 1:1 with VectaShield (Vector Laboratories) and applied to a glass slide and coverglass slip. Fluorescent and DIC images were acquired using a DeltaVision Spectris RT microscope (Applied Precision) using a 100× oil objective and were deconvolved using the softWoRx software package.

2.7. Experimental animals

Female Swiss Webster (SW) mice were purchased from Harlan Laboratories (Indianapolis, IN) and used for routine parasite maintenance and transfection experiments. Animal handling was conducted in accordance with protocols approved by the Seattle Biomedical Research Institute Institutional Animal Care and Use Committee.

2.8. Attempted generation of Prex knockout P. yoelii parasites

The *P. yoelii* 17XNL (nonlethal) strain was transfected using standard methods [26]. Regions of homology (800–900 bp in length) located just 5′ and 3′ of the PyPrex open reading frame were PCR amplified, combined by SOE PCR [27], and inserted into the StuI site of pCR-Blunt (Invitrogen) for sequencing. The combined homology regions were released from pCR-Blunt by digestion with SacII and NotI and inserted into the same restriction sites of a B3d-derived plasmid. The B3d-PyPrexKO plasmid was linearized with SbfI, precipitated with ethanol, and 10 μg was transfected in duplicate with an Amaxa electroporator into purified schizonts in three independent trials. One day post-transfection, parasites were selected by virtue of a *T. gondii* dihydrofolate reductase (TgDHFR) expression cassette with pyrimethamine cycling (3 days on, ≥3 days off), transferred to another mouse, and selected again with pyrimethamine cycling. After parasitemia increased above 1%, parasites were harvested by passage over CF11 resin and released from RBCs with saponin. Genomic DNA from parasites was isolated and purified (QiAMP Blood DNA Mini Kit, Qiagen), precipitated with ethanol, and resuspended in ddH_2O. The presence of transgenic parasites was assessed by genotyping PCR.

2.9. Transfection of the NF54 strain of P. falciparum with a protein overexpression vector

The pDC2_hsp86 vector was digested with BglII and AvrII to allow the in-frame fusion of PfPrex AA1–465 with GFP, with expression driven by the constitutively active Pf hsp86 5′untranslated region (UTR) [28]. This plasmid was transfected into synchronous ring-stage *P. falciparum* NF54 parasites as previously described [29]. Transgenic parasites were selected by virtue of a human DHFR expression cassette by the inclusion of the anti-folate drug WR99210 in the growth media, and passaged by standard methods [30].

3. Results and discussion

3.1. Reannotation of the functional domains of PfPrex based upon conservation among Plasmodium species

PfPrex (PF14_0112) is a 2016-residue protein with ESTs present for all stages of the complex life cycle in many orthologs in *Plasmodium* species. Computational analyses predict that PfPrex is composed of several well-conserved domains encoding topoisomerase-primase (TOPRIM), helicase, exonuclease, and polymerase functions [31]. A discrete bipartite leader sequence (BLS) is predicted to be present at the N-terminus [32], which was previously shown to be sufficient to target GFP to the apicoplast [15]. Direct and indirect assays confirmed the presence of the predicted enzymatic activities *in vitro* using recombinant derivatives of the two halves of PfPrex [15]. As the primase domain of PfPrex bore similarity only to bacterial and phage proteins (Supplemental Fig. 1), we focused our efforts upon it due to its potential as a drugable target.

Initial efforts to express the TOPRIM domain (AA336–465) of PfPrex yielded low amounts of soluble protein that had no primase activity (data not shown). This prompted a more thorough comparison of PfPrex with its orthologs across *Plasmodium* species, as well as with well-characterized bacterial primases. As shown in Fig. 1, using cutoffs of >84% identity and >93% similarity (shaded red) and 55–68% identity and 80–85% similarity (shaded pink) allowed a precise prediction of the bounds of the conserved functional domains of PfPrex. Bacterial primases generally include a zinc-binding domain (ZBD), TOPRIM domain, and a helicase-binding domain (HBD). Bacteriophage can vary from this domain arrangement; for example, gene 4 of bacteriophage T7 encodes both a primase and helicase and lacks such an HBD [33]. The conserved regions in PfPrex include both an apparent ZBD and TOPRIM but a well conserved apparent HBD was not observed. As the primase and helicase domains of PfPrex are expressed on the same polyprotein, it is possible that the two domains are part of a single protein like that in T7. The putative ZBD region of PfPrex (AA118–295) includes two well conserved CXXC motifs that are common zinc-binding motifs. Based upon this analysis, we have re-annotated the primase domain to be composed of both the ZBD and TOPRIM domain (AA118–463).

3.2. Recombinant derivatives of PfPrex primase can be expressed in soluble form and purified from E. coli Rosetta2(DE3)pLysS when cells are stressed by benzyl alcohol

Using our re-annotated domain structure of PfPrex, an expression vector encoding for a protein with both the ZBD and TOPRIM domain ("PfPrex primase", AA115–465) was created. Initial expression attempts using the Rosetta2(DE3)pLysS strain of *E. coli* yielded multiple smaller protein species that bound directly or indirectly to Ni-NTA resin. Tandem mass spectroscopic analysis revealed these to be premature translation termination products of PfPrex, with the major species identified as the bacterial chaperone protein
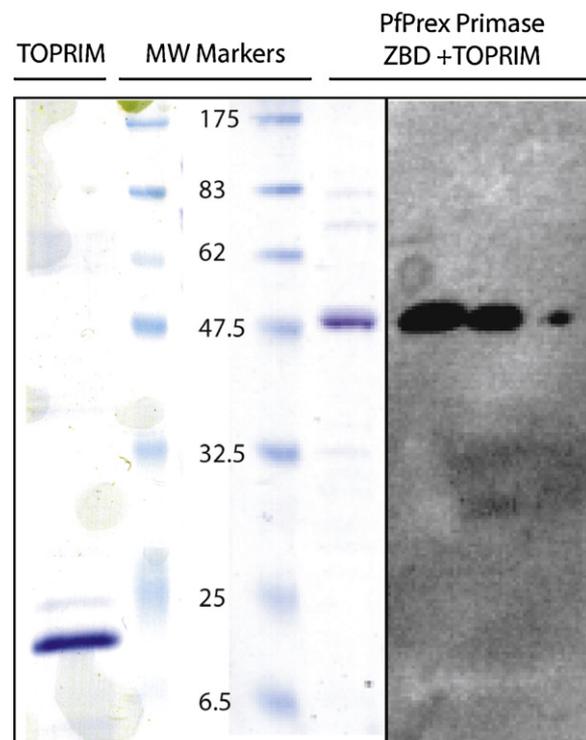

Fig. 2. Soluble recombinant TOPRIM domain and ZBD + TOPRIM domains of PfPrex can be purified from chaperone-induced bacteria. Two derivatives of the primase domain of PfPrex were expressed in Rosetta2(DE3)pLysS bacteria during chaperone-induction by the addition of 1 mM benzyl alcohol. Derivatives expressing either the TOPRIM domain alone or the ZBD + TOPRIM domains together were purified using standard methods to >90% purity as per Coomassie Blue staining of a 12% SDS–polyacrylamide gel (left). Rabbit polyclonal antibodies generated to the TOPRIM domain specifically react with a serial dilution of bacterial lysate harboring the complete primase domain (right).

GroEL (data not shown). To facilitate the production of full-length PfPrex primase derivative, protein expression was induced overnight at room temperature in the presence of benzyl alcohol, a potent membrane fluidizer and chaperone inducer [22]. Only in these conditions was PfPrex primase expressed in significant quantities in a soluble form. PfPrex primase was purified to >90% purity from the soluble fraction (Fig. 2). A rabbit polyclonal antibody raised against the TOPRIM domain (AA336–465) reacted specifically with PfPrex primase in a serial dilution of bacterial lysates, as well as with purified protein (Fig. 2).

3.3. PfPrex's primase domain contains a functional ZBD

Purified PfPrex primase was tested to determine whether zinc was bound to the protein as predicted. Using an absorbance assay [23], PfPrex primase was found to have zinc bound at a 1:1 molar ratio (Fig. 3). As the only source of this zinc was from the bacteria used to overexpress PfPrex primase, zinc is most likely incorporated into the structure of the protein as it folds. We predict that the ZBD participates in sequence-specific priming by PfPrex primase, as has been observed with the T7 phage primase [34].

3.4. Recombinant PfPrex primase transcribes RNA primers from a ssDNA template

We next wanted to determine if recombinant PfPrex primase was able to catalyze DNA-dependent RNA synthesis. Previous work by Seow et al. had used a Klenow fragment extension assay to
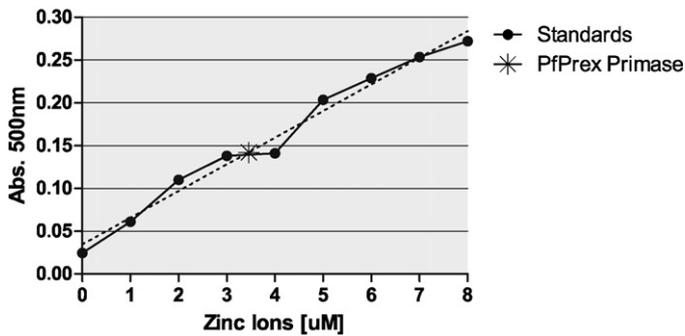
Fig. 3. PfPrex's primase domain binds zinc. A 5 μM solution of PfPrex ZBD + TOPRIM was unfolded in 50 mM HEPES pH 7.5, 3 M GuHCl and the amount of zinc ion released was observed by the change in A_{500} in the presence of 100 μM PAR. Values were normalized against a serial dilution of 1–8 μM zinc ions, which fell within the linear range of detection ($R^2 = 0.9817$). The asterisk ($A_{500} = 0.143 \pm 0.007$, 3.46 μM) represents the average signal observed over three replicates.

indirectly demonstrate that the N-terminal half of PfPrex contains a functional primase domain [15]. Here we measured primase activity directly by observing the incorporation of [α ^{32}P]ATP into RNA polymers produced using a dT_{50} single-stranded DNA template. As seen in Fig. 4, RNA primers are produced by PfPrex and the positive control bacterial primase *E. coli* DnaG. In the absence of other components of replisome, it has been shown that EcDnaG can create RNA primers up to 60 nt in length, which we observe as well [35]. PfPrex primase is also sufficient to transcribe RNA primers, which form in a variety of lengths >20 nt as evidenced by a smear of products. While a preferred initiation trinucleotide was not determined here due to the use of a dT_{50} single-stranded DNA template, it is not surprising that PfPrex primase produces a RNA polymer from this template due to the extremely A–T rich content of the *P. falciparum* genome. Therefore it is likely that the primase domain of PfPrex is capable of producing RNA primers to facilitate lagging-strand DNA synthesis within the parasite as well.

3.5. Testing the possible essential nature of Prex in P. yoelii

Having characterized PfPrex biochemically, we next sought to characterize it within the context of the parasite. The rodent malaria parasite *P. yoelii* is an excellent model system to utilize as it resembles *P. falciparum* in its infectivity, in the kinetics of the immune response against it, and in its overall genomic structure [26,36,37]. Moreover, a rapid and efficient transfection procedure is available and the complete genome sequence is known, making such assessments relatively quick and straightforward [26,36]. To this end, the *P. yoelii* syntenic ortholog of Prex (PyPrex, PY00163) was targeted for gene deletion. Regions of homology to the 5′UTR and 3′UTR of PyPrex were inserted into the pB3d-DsRed plasmid [38], linearized to expose the regions of homology for double-strand DNA homologous recombination, and transfected into the non-lethal 17XNL strain of *P. yoelii*. Across three independent experiments each of which was conducted in duplicate, only wild-type parasites were recovered after pyrimethamine drug cycling as determined by genotyping PCR of the resulting parasites (Supplemental Fig. 2). As a knockout parasite was never observed, this likely indicates that PyPrex is an essential protein, as would be predicted by its constitutive life-cycle expression pattern. While an indirect experiment, the repeated inability to disrupt the PyPrex gene is strongly indicative of its essentiality in the bloodstage.
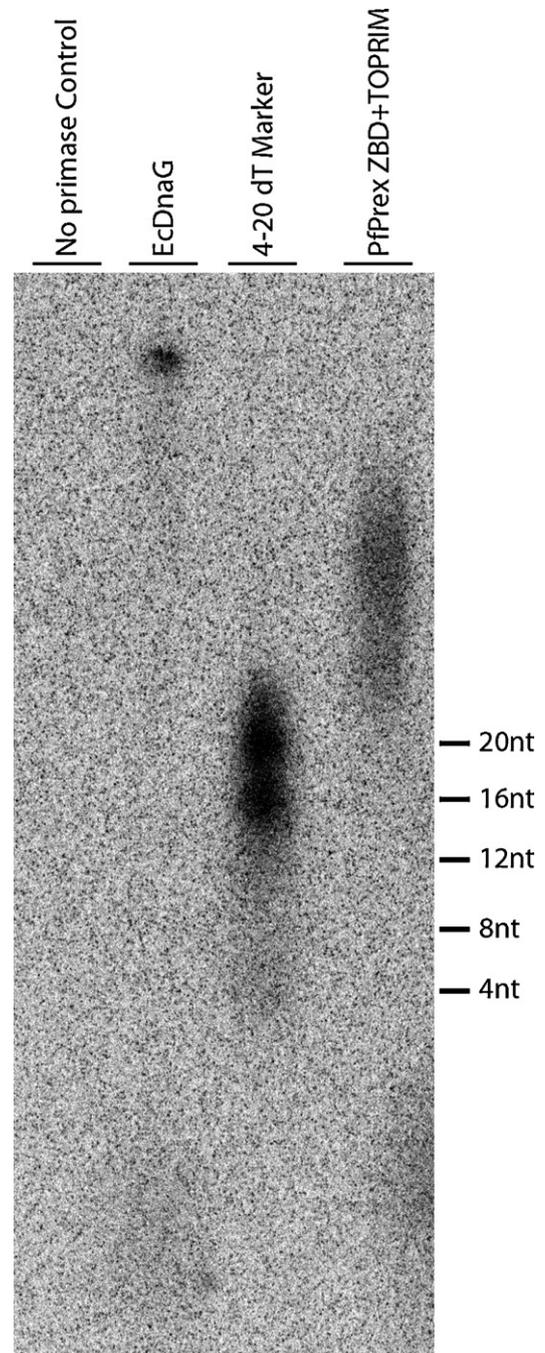

Fig. 4. A PfPrex primase fragment that includes both the ZBD and TOPRIM domains is sufficient for primase activity. Ten micromolar solutions of the purified recombinant PfPrex primase or *E. coli* DnaG were incubated at 37 °C with a dT(50) template and [α ^{32}P]ATP/rNTP mix for 1 h to produce radiolabeled RNA primers. Reaction products were phenol/chloroform treated, purified by size-exclusion chromatography (Bio-Gel P-6), and then electrophoresed through a 7 M urea/20% denaturing polyacrylamide gel. The dried gel was exposed to a storage phosphor screen and signals were visualized by a Storm 860 PhosphorImager and ImageQuant 5.2 software.

3.6. PfPrex is proteolytically matured into separate domains in vivo

PfPrex is encoded by a large open reading frame composed of several related functional domains, which is reminiscent of the polyprotein strategy employed by several viruses [19]. It was previously shown that the C-terminal exonuclease/polymerase ("Exo/Pol") domain of PfPrex is proteolytically separated *in vivo* by Western blotting [15]. In order to assess if any further
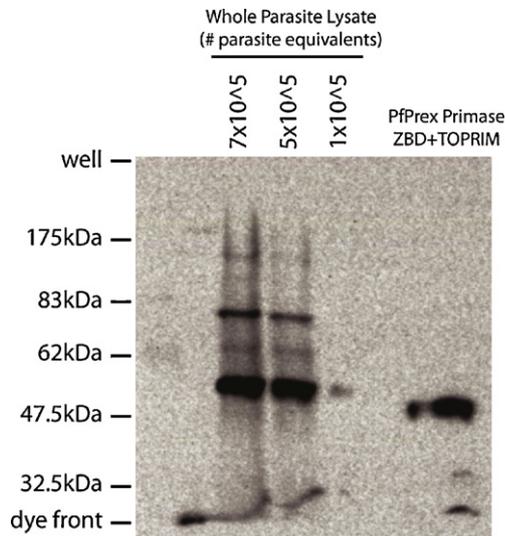
Fig. 5. PfPrex is proteolytically processed in multiple locations *in vivo*. Whole parasite lysates or purified, bacterially expressed PfPrex primase were electrophoresed through an 8% SDS–polyacrylamide gel, transferred to a PVDF membrane, and probed with rabbit polyclonal anti-PfPrex TOPRIM and a HRP-conjugated secondary antibody. Reactive species corresponding to proteolytically processed forms of PfPrex were visualized by standard ECL reagents. No specific signals were recognized by preimmune sera from parasite lysate nor from purified PfPrex primase (Supplemental Fig. 3). Pre-stained size markers were run in parallel, and are denoted to the left of the blot.

from the parasite lysate. The major species migrated slightly more slowly than did recombinant PfPrex primase, which could reflect a protein cleaved downstream of its bipartite leader sequence and/or between the primase and helicase domains. Other larger protein species observed also carry the epitopes from the TOPRIM domain, and may correspond to intermediate forms of PfPrex. For instance, it is possible that the band observed at ~80 kDa could represent the primase–helicase portion of PfPrex prior to proteolysis. Thus, the extensive proteolytic maturation observed with PfPrex separates it minimally into its primase and Exo/Pol functional domains, and by extension also separates its helicase domain as well. Such proteolysis of PfPrex may have many possible functions, including allowing regulation of enzymatic activities and/or providing a means to obtain the necessary/optimal stoichiometries of each component in subsequent complexes. Further work is warranted to appropriately explore the role of these proteolytic processes. As with viral polyproteins, if proteolytic processing of Prex is prevented or inhibited its enzymatic functions might be compromised, and thus the putative maturase of Prex might constitute a good therapeutic target.

3.7. PfPrex primase traffics to the apicoplast and forms multiple foci in vivo

Because PfPrex is proteolytically matured, we sought to determine the localization of PfPrex primase within the parasite. To this end, we used the hsp86 promoter to overexpress PfPrex primase with its bipartite leader sequence intact (AA1–465), and with GFP fused to its C-terminus. Indirect immunofluorescence microscopy of asynchronous parasite cultures revealed a strong co-localization of PfPrex primase with the apicoplast resident acyl carrier protein (ACP) in early ring stages, as well as with DAPI signal derived from staining of the apicoplast genome (Fig. 6, upper row). However, as parasites progressed through the asexual blood stage schizogony, two discrete foci of PfPrex primase formed in trophozoites, which
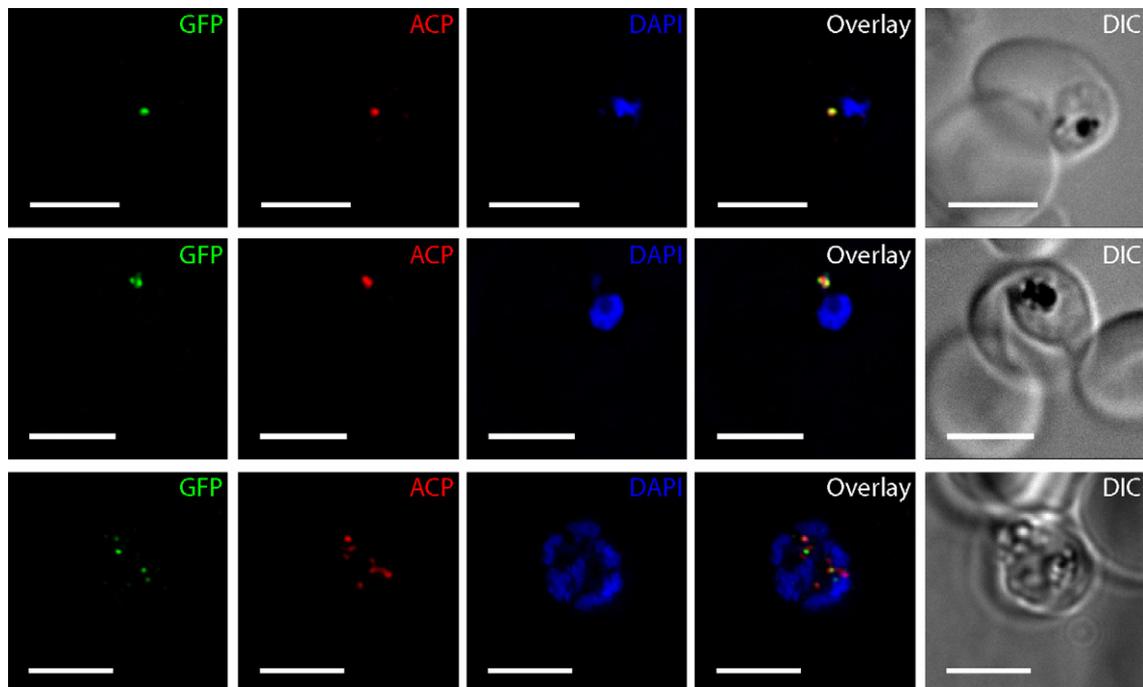
proteolytic maturation of PfPrex occurs in the parasite, we used a rabbit polyclonal antibody specific to the TOPRIM domain of PfPrex to probe lysates from RBCs infected with an asynchronous population of *P. falciparum* (Fig. 5). When compared with the pre-immune sera (which yielded no reactive bands, Supplemental Fig. 3), anti-TOPRIM antibody reacted specifically with recombinantly expressed PfPrex primase, as well as with several protein species

Fig. 6. PfPrex primase traffics to the apicoplast and forms two foci during the intraerythrocytic cycle. The complete primase domain (AA1–465) of PfPrex was genetically fused to GFPmut2 and expressed from a nuclear plasmid in *P. falciparum* NF54 parasites. Indirect immunofluorescence microscopy with DAPI and antibodies against GFP and PfACP demonstrates clear apicoplast localization of PfPrex throughout the intraerythrocytic cycle. Differential interference contrast (DIC) images show typical appearances of both the parasite and the host RBC. Two foci appear within the apicoplast during the early trophozoite stage and then segregate to daughter merozoites during schizogony. Scale bar is 5 μm.

flanked and partially overlapped the anti-ACP signal (middle row). These foci became less intense as the apicoplast underwent branching, and subsequently they reformed in late-stage schizonts when foci paired with daughter merozoites (lower row).

As was observed with the bipartite leader sequence alone, PfPrex primase also conclusively traffics to the apicoplast and changes its localization therein over the course of the asexual intraerythrocytic life cycle. It is also noteworthy that overexpression of PfPrex AA1–465 had no negative impact upon the parasite, permitting such an analysis. We postulate that PfPrex associates with other components of the apicoplast replisome, and contributes to the replication of its 35 kb circular genome by provision of RNA primers for DNA synthesis. The two discrete foci observed in trophozoites may therefore be stationary replication loci used to duplicate the plastid genome into the several copies eventually needed for each daughter merozoite.

3.8. Conclusions

In this study, we have characterized several important aspects of PfPrex *in vitro*, as well as in its native context. Specifically, we have demonstrated that the re-annotated primase domain of PfPrex (AA115–465) is composed of both a functional ZBD and a TOPRIM fold domain. This recombinant form of PfPrex is sufficient to transcribe RNA primers from a single-stranded DNA template. When coupled with its apparent essentiality, apicoplast localization and proteolytic separation from the other enzymatic domains of PfPrex *in vivo*, these observations implicate a role of PfPrex primase in the production of RNA primers for the replication of the apicoplast genome. Further inspection of the other enzymatic domains of PfPrex, along with the role of its extensive proteolytic maturation, will continue to build upon our understanding of the replicative processes of *Plasmodium*'s apicoplast and its genome. Moreover, inhibition of PfPrex's enzymatic functions or its proteolytic processing may prove to be good therapeutic targets.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2011.08.002.

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