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In Silico Characterization of *Plasmodium falciparum* and *P. yoelii* Translocon and Exoribonuclease II (RNase II) Identified in the Merozoite Rhoptry Proteome

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Abstract

In this study, we characterize *Plasmodium falciparum* proteins, exoribonuclease II (RNase II) and Translocon (PTEX150); encoded by genes PF3D7_0906000 and PF3D7_1436300, respectively, and selected from proteomic analysis of the *P. yoelii* merozoite rhoptries. The proteins were characterized bioinformatically using PlasmoDB, ExPasy and NCBI portals. The strategy for characterization was established on the basis of protein alignment for sequence identity, domain analysis and transcriptome analysis of the genes PF3D7_0906000 and PF3D7_1436300 among *Plasmodium* species and members of the phylum apicomplexa. The protein translocon (PTEX150) is a subunit of transport complex in the parasitophorous vacuole membrane (PVM) involved in protein export from the intracellular parasite into the host erythrocyte cytoplasm. The role of the translocon in *P. yoelii* is unknown. The ribonuclease binding (RNB-like) domains in RNase II are predicted to be highly conserved among *Plasmodium sp.* and other apicomplexans with different host cell specificities.

Keywords: *Plasmodium falciparum;* Structural proteomics; *Plasmodium yoelii;* Malaria bioinformatics; Rhoptries; Rhoptry proteins; Rhoptry proteome; Translocon; RNB-like domain

Introduction

Malaria still remains a significant economic and public health disease worldwide. The disease affects about 219 million people with an estimated 660,000 deaths [1,2]. Host erythrocyte invasion by the *Plasmodium* merozoite is dynamic and is mediated by ligand-receptor interactions involving *Plasmodium* rhoptry neck and body proteins [3-6] as well as microneme proteins, leading to a moving junction formation that assists merozoite entry into the erythrocyte [7-14]. Genomic and proteomic studies of *Plasmodium* asexual stages and merozoite rhoptries [15-17] generated large amounts of data demonstrating conservation of genes encoding proteins with potential involvement in establishing the early stages of parasitism in asexual stage parasite development within the host erythrocyte. In addition, proteins involved in erythrocyte invasion were identified [13,17]. Two rhoptry genes were selected for further characterization in this study; the RNAse II gene (PF3D7_0906000) and the Translocon gene (PF3D7_1436300). The level of gene organization and conservation, structural conformation and domain organization of both genes were studied. *In silico* analysis using PlasmoDB [18], GeneDB [19], ExPasy [20], PSortbII [21], National Center for Biotechnology Information (NCBI) and COBALT [22] Multiple Alignment Tool were employed to identify functional domains that could be validated in *in vitro* experimental studies in *P. falciparum* cultures. Both genes were characterized for features such as conservation profiles, domain architecture and alignment of sequences, both within *Plasmodium* species and among members of the phylum apicomplexa. The RNAse II and translocon genes were amplified by polymerase chain reaction (PCR) from *Plasmodium* genomic DNA using primers designed from DNA sequences obtained from PlasmoDB [18] for use in recombinant DNA construction. Due to the differences observed in localization of proteins involved in parasitophorous vacuole membrane (PVM) transport and exported proteins [23-25], gene mining approaches and bioinformatics were employed to examine data present in PlasmoDB [18], regarding gene and protein annotations for *P. falciparum* genes *PF3D7_0906000* and *PF3D7_1436300*. Predicted structural characteristics, functional domains and motifs from annotated data within the PlasmoDB [18] database were compared, in an attempt to develop testable experimental approaches that can identify specific conserved features that will expand our understanding of asexual stage host cell invasion and parasitism, identify new vaccine and drug targets and new diagnostic biomarkers.

Methods

Bioinformatics analysis

Genes *PF3D7_0906000* and *PF3D7_1436300* selected from proteome analysis of rhoptry proteins [18] were characterized bioinformatically. Gene *PF3D7_0906000* was selected because of the conserved RNB-like domains and the prospect for identifying catalytic domains with therapeutic potential. Gene *PF3D7_1436300* encodes a component of the *P. falciparum* translocon and was selected for investigation to better understand protein translocation across the parasitophorous vacuole membrane. The *in silico* strategy was established for data mining using the *Plasmodium* database [18]. The PlasmoDB [18] portal was used for analysis of gene, transcriptome and protein expression profiling of genes *PF3D7_1436300* and *PF3D7_0906000*. The mRNA expression from genes *PF3D7_0906000* and *PF3D7_1436300* based on the life cycle stage of the parasite in human and mosquito was predicted. The InterPro domain analysis

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helped in predicting functional domains in all the *Plasmodium* species. The protein paralogs and orthologs, FASTA sequences and average gene sequence identity were obtained from Orthomcl.org. The multiple sequence alignment for domains conserved by proteins among *Plasmodium* and apicomplexans was performed using the constraint-based multiple alignment tool (COBALT http://www.ncbi.nlm.nih.gov/tools/cobalt/) [22]. The software from ExPASy (www.expasy.org) proteomics were also used [20]. The PROSITE was used for predicting modification for the protein, active site analysis and domain prediction, PSORTb [21] III for the protein sub-cellular localization, HMMTOP for transmembrane predictions and SWISS-MODEL [26] repository for the prediction of secondary structure of protein and conserved domains. Conserved Domain Database tool and Conserved Domain Architecture Retrieval Tools were also used for predicting the domain sharing among all the apicomplexans.

Parasitic cell culturing

Plasmodium falciparum strains were cultured in type A+ human erythrocytes (Interstate Blood Bank, Memphis TN. USA) at 5% hematocrit and 20% parasitemia and maintained *in vitro* according to the method of Trager and Jensen [27] in RPMI 1640-Hepes media supplemented with 10% human serum (Interstate blood bank). Schizont pellets were collected by treating *Plasmodium*-infected erythrocytes with 10mM Tris HCl pH 8.8 and centrifuging at 15000 rpm for 15 min [28]. Parasite pellets were stored at -70°C after adding protease inhibitor (aprotinin) to the pellets. The parasite pellets were used for protein extraction and genomic DNA isolation.

DNA isolation

Plasmodium falciparum pellets with approximately 20% parasitemia was used. Pellets were treated with 600 µl of 10 mM Tris-HCl of pH 7.6, 50mM EDTA pH 8.0, 0.1% SDS and 1mg/ml proteinase K, incubated overnight at 50°C. Phenol: chloroform extractions were performed twice and supernatants were collected into an eppendorf tube. Chloroform extraction was performed to remove excess phenol. Precipitation of DNA was done using 0.1 vol of 5 M sodium acetate and 1 vol of isopropanol by incubating at room temperature for 15 minutes. DNA was centrifuged at 14000 rpm for 10 minutes, to collect the DNA pellet followed by a 70% ethanol wash to remove excess salt from the DNA. The DNA was stored in dH_20 at -20°C.

Polymerase chain reaction (PCR), cloning and protein expression

Plasmodium falciparum genes *PF3D7_1436300* (*PfA0680, Pfc140344*) and *PF3D7_0906000* (*PFI0295c*) previously identified in proteome studies [17] were amplified using the polymerase chain reaction (PCR). Amplification of the genes was performed using primers designed with appropriate restriction sites for cloning using New England BioLabs Inc. [29]. Appropriate restriction sites were included in the primers to permit cloning of the gene sequence into the multiple cloning sites of the expression plasmid pT7CFE1-CHis (Thermo Fischer). Genomic DNA from *P. falciparum* strains 3D7 and from FCR3 were used as templates. The PCR products were size fractionated on 1% agarose gels, DNA bands were excised from the gels and purified by Freeze and Squeeze DNA Gel Extraction Spin Columns (Bio-Rad Laboratories. Hercules CA). The pT7CFE1-CHis expression vector and gel purified DNA were then separated on a 1% agarose gel, followed by gel purification of the digested DNA. Ligation of the digested plasmid vector and PCR products were carried out at 12°C overnight (Thermolyne Thermokool, Barnstead).

Expression of proteins from genes

***In vitro* human cell free expression system:** The Pierce® Human *in vitro* protein Expression Kit for DNA templates (Thermo Scientific. Rockford. IL) was employed for protein synthesis from the cloned DNA fragments using transcription and translation kit components following the manufacturer's protocols. Proteins expressed using pT7CFE1-CHis expression vector have a C-terminal His tag. Twenty microliters (20 µl) of transcription mixture containing 2 µl of the recombinant plasmid DNA was incubated for 75 min at 30°C. After incubation, 2 µl of transcript mixture containing transcribed mRNA was mixed with 25 µl of the translation mixture followed by incubation at 30°C for 90 min. The Pierce® 1 – step Human Coupled *in vitro* translation (IVT) Protein Expression Kit for DNA templates (Thermo Scientific, Rockford, IL) was also employed for protein synthesis from the cloned DNA fragments, using the components provided in the kit and following manufacturer's protocols.

RNase II and translocon PCR amplification

Genomic DNA was isolated from *P. falciparum* strains Dd2, 3D7, FCR3, HB3, K1 and D10, *P. yoelii* and *P. berghei* using established protocols [30]. These strains were selected to check and determine the conservation of these proteins across different species of plasmodia. Isolated DNA was analyzed on a 0.7% agarose gel. The genomic DNA from these strains was used as the source of template DNA for amplification of the RNase II and translocon genes using *P. falciparum* primers. Amplified DNA was purified from the agarose gel, digested with appropriate enzymes and cloned into the plasmid vector pT7CFE-Chis (Thermo Scientific Pierce, Rockford, IL 61105 USA) for *in vitro* protein expression. Transcription and translation was carried out *in vitro* in a cell free expression system using both the two step and the single step protocol [30]. The *in vitro* translated (IVT) reaction product was analyzed using western blotting. The expressed protein was fractionated on a 10% SDS-PAGE gel and analyzed by western blotting using antiserum 676, a whole rhoptry-specific antibody [31] and antiserum 685, specific for SERA (serine rich antigen) [32], a parasitophorous vacuole protein. Normal rabbit serum (NRS) was used as a negative control.

SDS-PAGE and Western blotting

Translated proteins were separated on 10 % SDS-PAGE gels and transferred to nitrocellulose paper (NCP) using a semi-dry western blotting chamber for 2h. Nitrocellulose paper was blocked with 2% non-fat milk and incubated with specific antibodies.

Rabbit antisera #676 [31] specific for *P. falciparum* merozoite rhoptries, and antiserum #685 specific for the *P. falciparum* parasitophorous vacuole protein, SERA (serine rich antigen) [32] were used in western blotting. Goat anti-rabbit antibody conjugated to horse radish peroxidase (HRP) was used as secondary antibody at a dilution of 1:1000. Goat anti-rabbit antibodies were diluted 1:1000 in 2% milk in 1X blot buffer. Normal rabbit serum was used as control. *Plasmodium falciparum* schizont protein extracts were separated on the gel as control.

Immunoelectron microscopy

Localization of the rhoptry proteins using peptide antibodies was carried out by performing immunoelectron microscopy (IEM). Thin smears of *P. yoelii* schizont-infected erythrocytes were fixed for 30 min at 4°C with 1 % formaldehyde, 0.1% gluteraldehyde in 0.1 M phosphate

buffer, pH 7.4 was washed, dehydrated and embedded in LR white resin (Polyscience Inc., Warrington, PA) as described [31]. Thin sections were incubated with peptide specific antisera in PBS-BSA-Tween 20 (PBT) at 1:50 or 1:100 dilution. Negative controls included, normal rabbit serum or PBT used as primary antibody. Fifteen nm gold-labeled anti-rabbit IgG (Amersham Life Science) diluted 1:20 in PBT was used as secondary antibody. Samples were examined in a Zeiss CEM902 electron microscope (Zeiss Oberkochen, Germany).

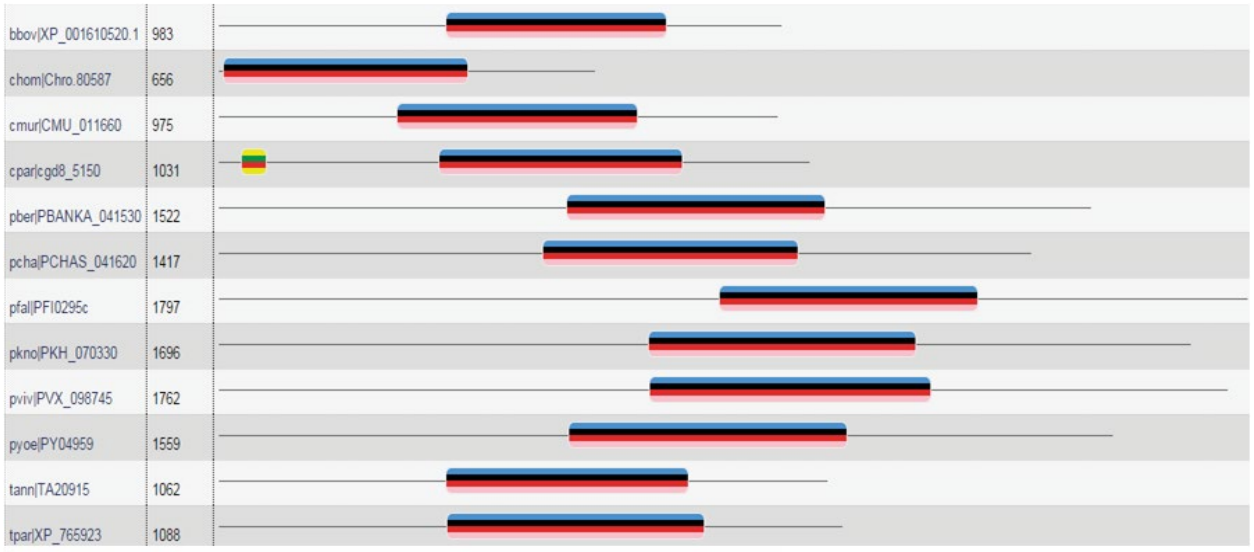
Results and Discussion

Bioinformatic analysis of *PF3D7_0906000*

The gene *PF3D7_0906000* expresses a putative RNase II, a ribonuclease binding (RNB)-like protein of approximately 214 kDa found in *Plasmodium* and in other members of the apicomplexan phylum such as *Theileria, Babesia* and *Cryptosporidium*. Data annotated in PlasmoDB [18] shows that RNase II characterized by the presence of RNB-like protein domains is conserved across *Plasmodium* species PlasmoDB [18]. The gene *PF3D7_0906000*, located on chromosome 4 is a 5515 bp gene with one intron and two exons. The gene has a paralog in the *P. falciparum* IT strain *PFIT_0906200*, of size 5537 bp with one intron, located on chromosome 9. *PF3D7_0906000* has orthologs in *Babesia bovis* T2Bo (XP_001610520), *Cryptosporidium hominis* TU502 (Chro.80587), *C. muris* RN66 (CMU_011660), *C. parvum* Iowa II (cgd8_5150), *P. berghei* ANKA (PBANKA_041530), *P. chabaudi chabaudi* (PCHAS_041620), *P. knowlesi* strain H (PKH_070330), *P. vivax* Sal-1 (PVX_098745), *P. yoelii yoelii* 17XNL (PY04959), *Theileria annulata* strain Ankara (TA20915) and *T. parva* strain Muguga (XP_765923). PVX_098745 and XP_765923 are conserved hypothetical proteins. PY04959 is predicted as a rhoptry protein. The average protein identity among *Plasmodium sp.* and members of the phylum apicomplexans is 32.8% (OrthoMCL) as shown in Figure 1. Mass spectroscopy evidence obtained from *in silico* annotations in PlasmoDB [18] shows that RNase II is found in nuclear and cytoplasmic fractions of trophozoites and schizonts [33,34]. The RNB-like domain is predicted to possess putative RNase II enzyme activities. Putative RNase II activity is thought to be present in *P. falciparum*, *P. vivax*, *P. knowlesi*, *P. berghei, P. chabaudi* and *P. yoelii*. The protein is listed as hypothetical in *P. cyanomolgi*, strain B and as a rhoptry protein in PY04959 (*P. yoelii* 17XNL). RNase II is synthesized by both the sporozoite and merozoite stages of the parasite [15]. There are orthologs predicted among the sequences of *Plasmodium* species and *Escherichia coli* where the enzyme activity was originally described [35]. However, *in silico* analysis revealed a sequence similarity of the following amino acid residues, HFTSPIRRYPD at different positions on *Plasmodium species* and *E. coli*. The RNB-like sequence homology analysis of *E. coli* RNase II reveals a sequence similar to the cold shock domain (CSD) that functions as an RNA chaperone in bacteria and is involved in regulating translation in eukaryotes [36]. RNase II family of enzymes possesses multiple roles, including biogenesis of organelles, growth and viability as well as RNA metabolism. Members of the RNase II like family exist as part of a multi-subunit exosome complex or function independently of the complex [35]. The secondary structure of the enzyme rather than the protein sequence activity is critical for enzyme activity [35]. The predicted structure of two *Plasmodium* orthologs is shown in Figure 2. *PF3D7_0906000* is annotated to express a product involved in ribosome assembly and other metabolic activities PlasmoDB [18].

The Expasy [20] and Swiss-PROT [21] predicted RNB-like domain to possess DIS3 like-exonuclease II activity and a subunit in the exosome complex of exonucleases. The 13 distinct secondary structures of RNB-like domains were predicted by SWISS-PROT Workspace as shown in Figure 3. The domain is present in all species examined, though there is no sequence or secondary structure identity among the domains.

The RNB-like domain analysis among all the apicomplexans



Accession	Symbol	Description	Count	Legend
PF00773	RNB	RNB domain	12	
PF08206	OB_RNB	Ribonuclease B OB domain	1	

Figure 1: RNB domain architecture seen in *Plasmodium* and some members of apicomplexa. (orthoMCL conserved domain architecture) Source: Plasmodb.org.

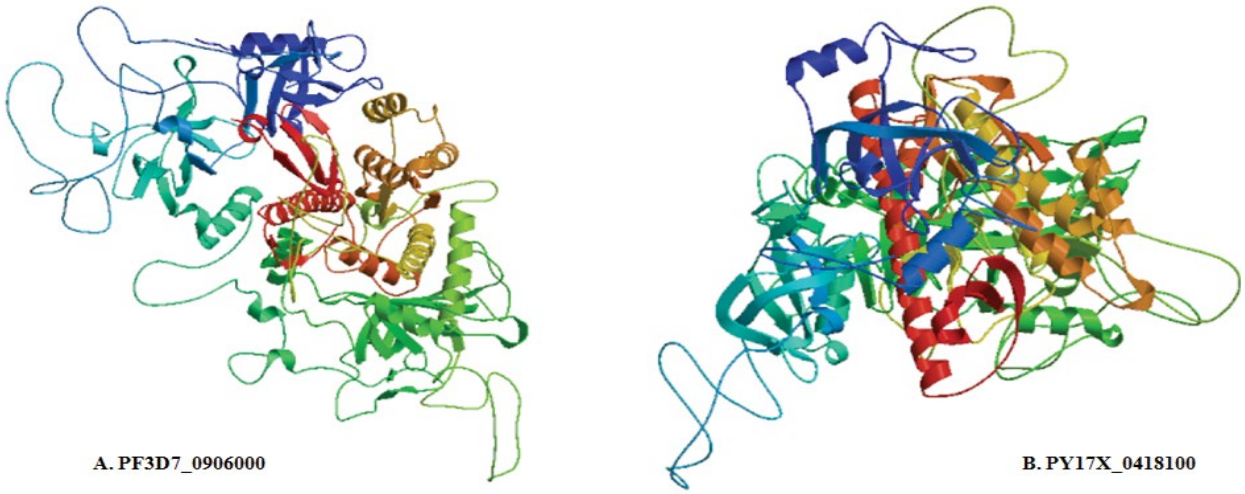


Figure 2: Prediction and comparison of secondary structure of the protein RNase II between *P. falciparum* (PF3D7_0906000) [37] and *P. yoelii* (PY17X_0418100) source: SWISS-PROT workspace.

was performed using COBALT [22] for the sequence identity among the amino acids in the catalytic region of the exonuclease II and shown in Figure 4. Recent studies demonstrate through various immunohistochemical techniques, localization studies and crystallography, that *P. falciparum* RNase II, a non-canonical exoribonuclease, regulates *var* gene expression, thereby controlling parasite virulence [37]. Immunofluorescence localization identifies the *P. falciparum* RNase II enzyme at the nuclear periphery [37]. The ribosome processing activity of the enzyme is thought to act on some components of the ribosomes such as SnoRNP, 90S and 60S particles. RNase II encoded by *PF3D7_0906000* is thought to influence the overall quality control of mRNA biogenesis PlasmoDB [18]. The *P. yoelii* gene *PY17X_0418100*, ortholog of *P. falciparum PF3D7_0906000* located on chromosome 4 has 4888 bp with one intron, encodes a protein of approximately 183 kDa, with putative RNB-like protein and RNase II activity PlasmoDB [18].

Blood stage expression data contained in PlasmoDB [18] demonstrates that *PY17X_0418100* is expressed during late schizonts in the erythrocyte stage. SignalP portal predicted that this protein lacks a signal peptide, and therefore would not enter into the secretory pathway. PSORTII, localization program predicted *PY17X_0418100* to be either a nuclear protein without transmembrane domain predicted by HMMTOP. The PROSITE portal predicted lysine at N-terminus, glutamate and asparagine rich segment in the middle of the sequence and aspartate rich at the C-terminus. PROSITE portal also identified enzyme modification sites on the protein such as, casein kinase phosphorylation, N-glycosylation, myristoylation, protein kinase C phosphorylation, tyrosine kinase phosphorylation, cAMP and cGMP dependent protein kinase phosphorylation, amidation and leucine zipper motif site. A second *P. yoelii* gene *PY04959* ortholog of *PF3D7_0906000* and paralog of *PY17X_0418100* shares common characteristics such as its paralog. However, protein annotation in PlasmoDB [18] showed the protein has 60 additional amino acids at the N-terminus and 16 additional amino acids closer to C-terminus between 1121 and 1200 amino acids region. Unlike *PY17X_0418100*, with a predicted RNB-like domain, *PY04959* is predicted to encode a rhoptry protein in PlasmoDB [18] and rabbit antisera prepared against peptides of the PY04959 protein localize it to the rhoptries [6]. Due to the multifunctional activity of RNase II, the predicted presence of the enzyme within organelles associated with merozoite invasion and parasitophorous vacuole formation may implicate the enzyme in early events regulating ribonucleotide recycling and transcript levels, including mRNA function, post invasion [35]. Exoribonucleolytic activity could be investigated in stage specific blood stage parasite extracts including isolated rhoptries to identify enzyme activity and substrate specificity. Exoribonuclease activity was demonstrated in recombinant *P. falciparum* exoribonuclease catalytic domain of PfRNase II [37]. However, enzyme activity in parasite crude extracts or homogenates will also be needed to determine substrate specificities and other conditions required for optimal enzyme activity during the blood stage.

Bioinformatic analysis of *PF3D7_1436300*

PF3D7_1436300 (previously known as *PF14_0344* and ortholog of *P. yoelii PY02301* Py17XNL), encodes a protein annotated as a translocon protein (PTEX150) in *P. faciparum, P. berghei,* and *P. chabaudi,* but a hypothetical protein in *P. vivax, P. yoelii* and in *Theileria parva muguga.* The gene has six orthologs in PlasmoDB [18]. PTEX150 is part of a *Plasmodium* transport protein complex comprising PEXEL, PTEX88, EXP2 and HSP70 [23,38,39]. PTEX150 has a putative endoplasmic reticulum (ER) signal sequence that is found in most species of *Plasmodium* that infect humans and in *Theileria*, another member of the apicomplexan phylum [39]. The specific sequence of PTEX150 that interacts with the export machinery has not been described. However, through multiple expectation maximization for motif elicitation (MEME) and motif alignment search tool (MAST) in the *P. falciparum* database PlasmoDB [18,40], the presence of conserved motifs of 11 amino acids RxSRILAExxx in the vacuolar transport sequences (VTSs) of parasite proteins that are transported to the erythrocyte cytoplasm via the parasitophorous vacuole transport system was identified [23]. The primary sequence pattern RxSRILAExxx is observed in over 250 parasite proteins [40]. Mass Spectroscopy evidence from PlasmoDB [18] showed that the translocon gene product is found in both the nuclear and cytoplasmic fractions of trophozoite and schizont stages of the 3D7 strain of *Plasmodium*. The protein is expressed at

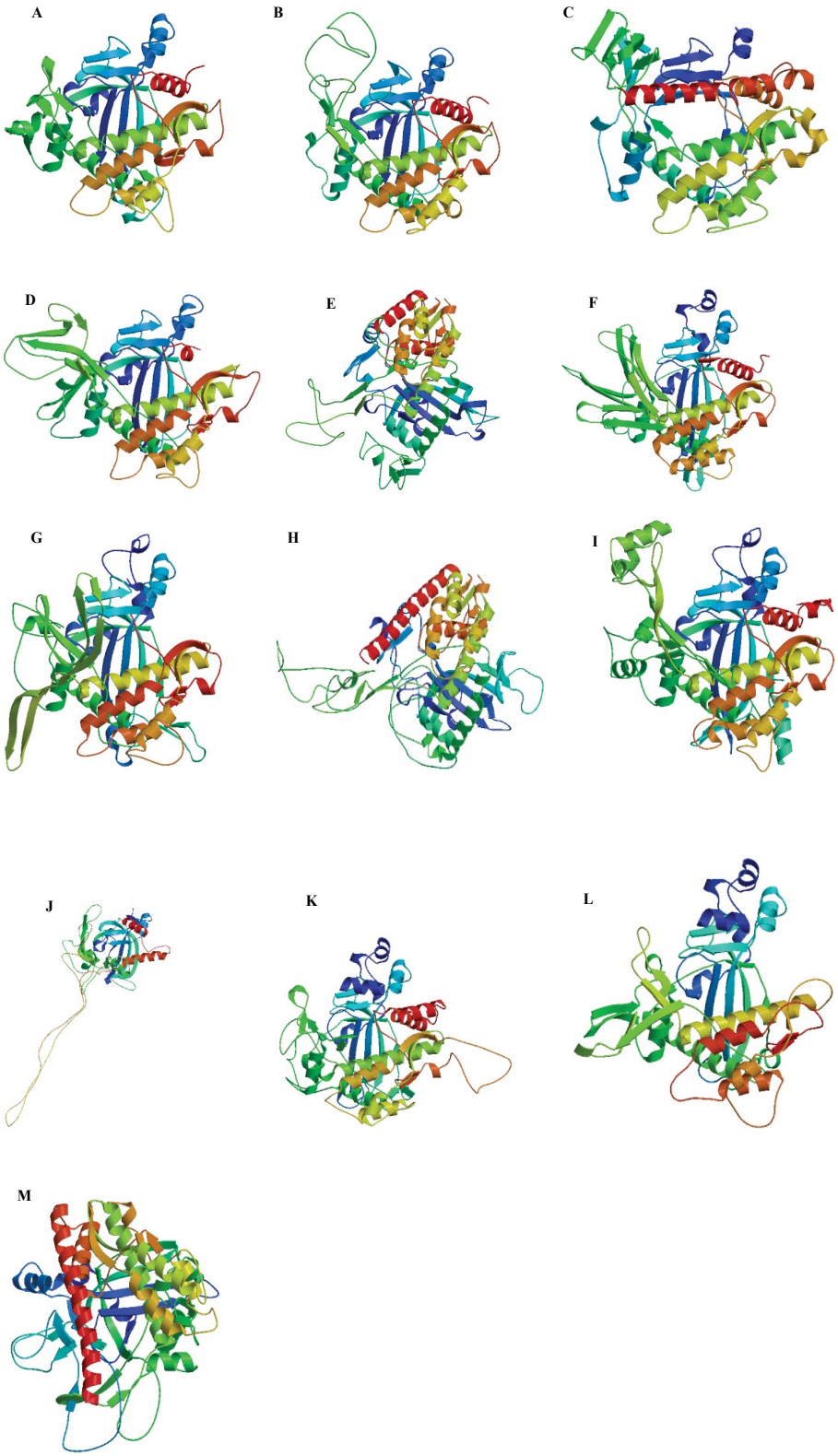


Figure 3: Showing the secondary structure of the RNB domain that is conserved among *Plasmodium sp.* and other apicomplexans. **A:** *Babesia bovia* T2Bo (bbov|XP_001610520.1) **B:** *Cryptosporidium hominis* TU502 (chom|Chro.80587) **C:** *Cryptosporidium muris* RN66 (cmur| CMU_011660) **D:** *Cryptosporidium parvum* Iowa II (cpar|cgd8_5150) **E:** *Plasmodium berghei* str. ANKA (pber|PBANKA_041530) **F:** *P. chabaudi chabaudi* (pcha|PCHAS_041620) **G:** *P. falciparum* 3D7 (pfal|PFI0295c) **H:** *P. knowlesi* str. H(pkno|PKH_070330) **I:** *P. vivax* Sal-1 (pviv|PVX_098745) **J:** *P. yoelii yoelii* str. 17XNL (pyoe|PY04959) **K:** *Theileria annulata* str. Ankara (tann|TA20915) **L:** *Theileria parva* str. Muguga (tpar|XP_765923) **M:** *P. yoelii* Str. YM (PYYM_0418100)

Figure 4: COBALT analysis of RNB domain that is conserved among the *Plasmodium sp.* and other apicomplexans. Similarity is indicated in **pink**. **Query_10001:** *Babesia bovia* T2Bo (bbov|XP_001610520.1) **Query_10002:** *Cryptosporidium hominis* TU502 (chom|Chro.80587) **Query_10003:** *Cryptosporidium muris* RN66 (cmur| CMU_011660) **Query_10004:** *Cryptosporidium parvum* Iowa II (cpar|cgd8_5150) **Query_10005:** *Plasmodium berghei* str. ANKA (pber|PBANKA_041530) **Query_10006:** *P. chabaudi chabaudi* (pcha|PCHAS_041620) **Query_10007:** *P. falciparum* 3D7 (pfal|PFI0295c) **Query_10008:** *P. knowlesi* str. H(pkno|PKH_070330) **Query_10009:** *P. vivax* Sal-1 (pviv|PVX_098745) **Query_10010:** *P. yoelii yoelii* str. 17XNL (pyoe|PY04959) **Query_10011:** *Theileria annulata* str. Ankara (tann|TA20915) **Query_10012:** *Theileria parva* str. Muguga (tpar|XP_765923).

all stages of the parasite post-invasion. At present, the full scope of proteins exported across the PVM is unknown. Proteins utilizing the *Plasmodium* exported element (PEXEL) and PEXEL negative exported proteins (PNEP) have been identified [41-43]. Exported proteins are trafficked to the Maurer's clefts in the erythrocyte cytoplasm in *P. falciparum* infected erythrocytes. The *P. yoelii* gene *PY02301*, ortholog of *PF3D7_1436300* encodes a protein structurally similar to PTEX150. However, there is no evidence to suggest that *P. yoelii* contains Maurer's clefts. Intracellular membrane networks similar to the Maurer's cleft as well as intracytoplasmic vesicles have been described in the cytoplasm of rodent *Plasmodium* infected cells [25]. Experiments to isolate intracytoplasmic membranes and vesicles for investigating protein distribution will identify differences, if they exist, in the distribution of proteins involved in intracellular trafficking across the PVM. The differences in the localization of *P. yoelii* EXP-2 within cytoplasmic vesicles and the PVM, suggests that proteins participating in the export of proteins across the PVM may have different distribution with the host cell cytoplasm of different *Plasmodium* species [25].

Data mining of PlasmoDB and other data bases such as ExPaSy, PSORTb, SWISSPROT-workspace, GeneDB, National Center for Biotechnology Information (NCBI) and COBALT: Multiple Alignment Tool was used to analyze *P. falciparum* genes *PF3D7_ 1436300* and *PF3D7_0906000* [18-22,44]. Both genes were characterized for features such as conservation profiles, domain architecture and alignment of sequences. Both genes are conserved in most species of *Plasmodium* infecting humans, rodents and nonhuman primates as well as members of the phylum apicomplexa. The proteins are also expressed early upon host invasion. The RNB protein domains of RNase II are generally conserved across *Plasmodium* species but protein identity across species is approximately 30%. The amino acid identity is about 40% across species for the translocon protein. This gene has an RNB-like domain containing putative RNAse II enzyme activities such as ribosome assembly and other metabolic activities (PlasmoDB). RNase II is thought to influence the overall quality control of mRNA biogenesis and RNA editing (Plasmodb.org) [18,36]. It will be important to explore the role of this gene in *Plasmodium* species as this enzyme may be a potential target for drug therapy.

The *PF3D7_ 1436300*, gene is annotated as a translocon in *P. faciparum, P. berghei,* and *P. chabaudi,* but as a hypothetical protein in *P. vivax, P. yoelii* and in *Theileria parva muguga.* It is conserved within the genus *Plasmodium* in terms of the type of residues present but polymorphism is significantly high with only 30% identity. Based on data from immuno-fluorescence, immunoelectron microscopy and gene knockout studies, *PF3D7_1436300* encodes a putative translocon PTEX150 (PlasmoDB) [39]. HSP101 associates with PTEX150 to transport nascent peptides during protein biogenesis from the PVM to the erythrocyte cytoplasm with destinations to the erythrocyte membrane [39]. PTEX150 is part of a *Plasmodium* transport protein complex comprising PEXEL, PTEX88, EXP2 and HSP70 [23,39,42,43]. PTEX150 has a putative endoplasmic reticulum (ER) signal sequence that is found in most species of *Plasmodium* that infect humans and in *Theileria*, another member of the apicomplexan phylum [39]. *PY02301* is annotated to encode a protein of unknown function in *P. yoelii.* No conserved domain architecture was found both within the *Plasmodium* species and other members of the apicomplexa with orthologs for the gene such as in *Theileria p. muguga.*

To verify the *in silico* predictions *in vitro*, the conservation of genes *PF3D7_0906000* and *PF3D7_1436300* were demonstrated by PCR amplification from genomic DNA of *P. falciparum* strains Dd2, 3D7, FCR-3, K1, HB3 and 7G8. The conservation of the gene was also analyzed in *P. yoelii* and *P. berghei* using the primers shown in Table 1. Agarose gel electrophoresis of PCR products shows that genes *PF3D7_0906000* and *PF3D7_1436300* are conserved in different *P. falciparum* species and strains. PCR products (results not shown) for *P. falciparum* stains 3D7 and FCR are shown in Figure 5.

Immunoelectron microscopy

In order to characterize rhoptry proteins identified in proteome studies, peptides were synthesized as MAPs and used for immunizations as described previously [6]. Peptide antibodies reacted with rhoptries in merozoites (Figure 6). Peptide specific antibodies reacted within the bodies of merozoite rhoptries in immunoelectron microscopy. Antibody reactivity was not observed on other organelles such as dense granules or micronemes (Figure 6).

Expression of recombinant translocon and RNase II proteins by one step *in vitro* human cell free expression system

As an alternative to recombinant protein expression in *E. coli*, *in vitro* human cell free expression was used to express rhoptry proteins [30]. Genomic, plasmid and amplified DNA were analyzed on 0.8% and 1% agarose gels respectively (data not shown). The plasmid and PCR amplified fragments were successfully digested with the appropriate restriction enzymes, separated on agarose gels and also successfully purified by the freeze and squeeze method (Bio-Rad). Gel purification was followed by ligation of digested PCR fragments and plasmid vector at 12°C. Due to small amounts of recombinant plasmid DNA recovered, PCR was used to verify the clones. Cloning was successful

Gene ID	Protein	Primer Sequence for PCR
PF3D7_1436300	Translocon component	Fwd ***XhoI-*** AATAATAACAATCATAATAATAAG Rev ***BamHI-*** ATTATCATCAGGTTTAGCTAATTTTC
PF3D7_0906000	RNB-like	Fwd ***XhoI-*** ATGTTAGGTCAAAAAAACACAAATA Rev ***BamHI-*** TGTTATTTGCTTTTTGTTTTGAAAA Fwd ***XhoI-*** ATATATATTTTTACCATAATACTATG Rev ***BamHI-*** ACCTATTTTCATGTCAGGAAAATAA

Table 1: List of primers used for PCR.

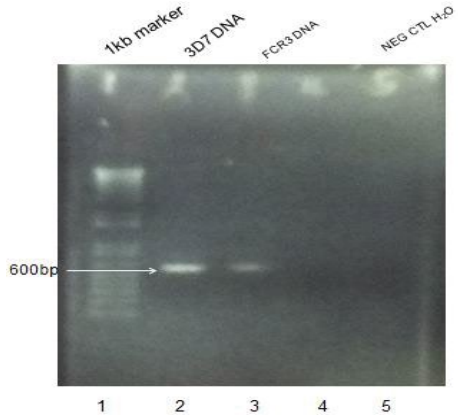


Figure 5: 1.0 % agarose gel of PCR amplified PF3D7_1436300 fragments. The expected 600bp fragment of DNA was amplified from 3D7 and FCR3 strains.

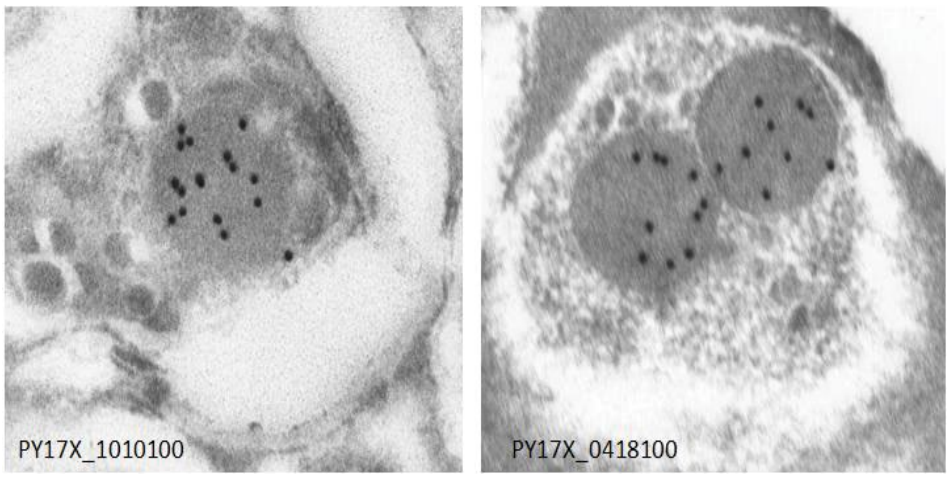


Figure 6: Indirect immunoelectron microscopy (IEM) using peptide specific antibodies and *P. yoelii* segmented schizonts. A. *P. yoelii* schizont-infected erythrocytes fixed and processed as described were incubated with peptide specific antibodies against PY17X_1010100 (PY02301) and PY17X_0418100 (PY04959) were processed for IEM as described [31]. Merozoites were reactive with antibody as shown. Gold particles (15 nm) label the body of the rhoptries. Micronemes (Mn) were not stained.

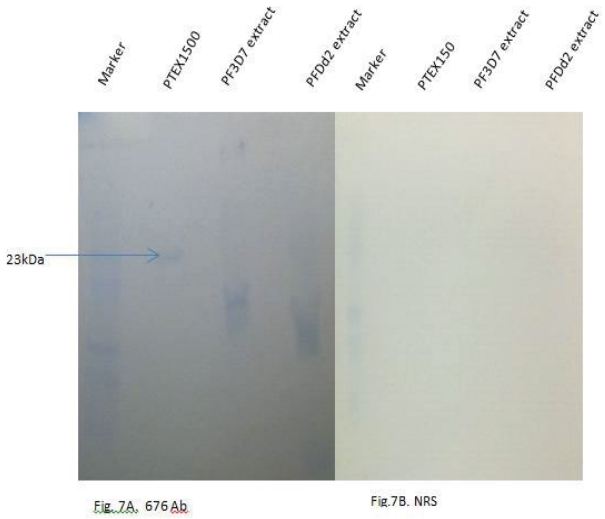


Figure 7: Western blot analysis of expressed 23kDa translocon protein detected with rhoptry specific antibody 676. The normal rabbit serum did not react with the translated protein.

for three genes as PCR re-amplification of gene fragments from the cloned plasmid was observed on 1% agarose gels (data not shown). The plasmids ligated with genes *PF3D7_0906000* and *PF3D7_1436300* were transcribed and translated *in vitro*. The recombinant proteins PF3D7_0906000 (*P. falciparum* ortholog of PY17X_0418100) and PF3D7_1436300 (*P. falciparum* ortholog of PY02301) were expressed along with Maurer's cleft protein (PF3D7_0114100) using one step *in vitro* human cell free expression systems, followed by purification of expressed recombinant proteins using Ni- chelating resin via batch method. Translated recombinant rhoptry proteins were separated on 10% SDS-PAGE gels and analyzed by western blotting using polyclonal antisera #676 specific to whole rhoptries of *P. falciparum* merozoites [31]. Antisera #676 identified recombinant rhoptry proteins. Normal rabbit serum did not show any reactivity (Figure 7A and 7B). Antisera 685 did not react with the recombinant protein (results not shown).

Conclusion

The early secretion of both PTEX150 and RNB-like proteins soon after merozoite invasion, as well as their persistence throughout the blood stage suggests that both proteins may play a role in the parasite's ability to invade and establish infection in the red blood cells. Taken together, both genes and their protein products could serve as useful vaccine candidates and drug targets because they are highly conserved in all *Plasmodium* species, particularly those species infecting humans.

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