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Identifying and characterising the *Plasmodium falciparum* RhopH3 *Plasmodium vivax* homologue

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Abstract

Four *Plasmodium* species cause malaria in humans, *Plasmodium falciparum* being the most widely studied to date. All *Plasmodium* species have paired club-shaped organelles towards their apical extreme named rhoptries that contain many lipids and proteins which are released during target cell invasion. *P. falciparum* RhopH3 is a rhoptry protein triggering important immune responses in patients from endemic regions. It has also been shown that anti-RhopH3 antibodies inhibit *in vitro* invasion of erythrocytes. Recent immunisation studies in mice with the *Plasmodium yoelii* and *Plasmodium berghei* RhopH3 *P. falciparum* homologue proteins found that they are able to induce protection in murine models. This study described identifying and characterising RhopH3 protein in *Plasmodium vivax*; it is encoded by a seven exon gene and expressed during the parasite's asexual stage. *Pv*RhopH3 has similar processing to its homologue in *P. falciparum* and presents a cellular immunolocalisation pattern characteristic of rhoptry proteins.

Keywords: Malaria; *Plasmodium vivax*; Rhoptry; RhopH3; Vaccine candidate

Malaria, one of the most prevalent tropical diseases worldwide, is caused by four parasite species: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*, being the first two responsible for 90% of all malaria cases [1]. Several *P. falciparum* antigens belonging to either the pre-erythrocytic or the intra-erythrocytic stages of the parasite's life cycle have been identified during the last two decades and tested as vaccine candidates [2]. The antigens which have been chosen in producing an anti-malarial vaccine against intra-erythrocytic stages are mainly located on the merozoite surface or in the apical organelles, such as rhoptries, micronemes, and dense granules. Rhoptry proteins that are released during the invasion process have been classified into two protein complexes [3–6]. The Rhop-L low molecular weight complex (including the rhoptry-associated proteins, RAP) and the high molecular weight complex (Rhop-H), where 105, 135, and 150 kDa polypeptides (RhopH1, RhopH2, and RhopH3, respectively) have been identified, forming a non-covalent and stable association [7,8]. It has been proposed that the proteins belonging to both complexes are bound by a GPI membrane-anchored protein named RAMA (rhoptry associated membrane antigen) and thus targeted into the newly forming rhoptries as the parasite matures [9].

The RhopH3 encoding gene has been characterised into several *Plasmodium* species (*P. falciparum, Plasmodium yoelii,* and *Plasmodium berghei*) [10–13] and this protein is a leading vaccine candidate. Strong immune response against RhopH3 has been observed in malaria-infected people and anti-RhopH3 antibodies are able to inhibit

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merozoite *in vitro* invasion of red blood cells (RBC) [5,14]. In addition, RhopH3 protein has been shown to bind erythrocytes [3]. Immunisation studies using recombinant RhopH3 proteins from murine malarial parasites such as *P. yoelii* and *P. berghei* have shown that RhopH3 is not only immunogenic but is also able to protect mice against otherwise lethal challenge with these parasite species [15]. According to the above-mentioned characteristics, RhopH3 has become one of the leading candidates in the development of anti-malarial vaccines.

Despite the enormous advances achieved in identifying vaccine candidates against *P. falciparum*, equivalent studies in *P. vivax* (the second most prevalent parasite species around the globe causing malaria in humans) have been less successful, mainly due to the difficulty of maintaining this parasite in *in vitro* culture. However, the sequencing of the complete *P. vivax* genome (currently under final annotation by the Institute of Genomic Research, TIGR) has facilitated the identification of new *P. falciparum* homologue proteins in *P. vivax* involved in RBC invasion. We have previously identified and characterised three *P. vivax* merozoite surface proteins (MSP 7, 8, and 10) [16–18] and two rhoptry antigens (RAP1 and RAP2) [19,20] aiming at testing their immunogenicity and protection-inducing ability in the *Aotus* monkey model.

Here, we describe the identification and characterisation of RhopH3 in *P. vivax* by means of bioinformatics, molecular biology, and immunochemical studies.

Materials and methods

Parasites. Parasites (VCG-I strain) were cultured *in vivo* by successive passes in splenectomised *Aotus nancymaae* monkeys kept at our primate station in Leticia (Amazonas). The extraction of infected RBC (mainly at schizont stage) was done from 3–4 ml blood samples taken from infected animals, using a Percoll discontinuous gradient, following a previously described protocol [21].

The P. vivax genome sequence. The partial nucleotide sequence of the *P. vivax* genome used in this study (Sal-I strain) was obtained via the TIGR web page (http://www.tigr.org/tdb/e2k1/pva1/).

Cloning and sequencing. Plasmodium vivax DNA and RNA were extracted as previously described [19,20]. cDNA was used as template for PCR amplification. The primers were designed from the sequence of a putative transcript which encoded the *P. vivax* RhopH3 protein found by BLAST search in the genome reported for this specie, using the reported *Pf*RhopH3 protein sequence as bait for finding its *P. vivax* homologue. The primers used covered the whole transcript (5′-ATGCGAAGC AAGCTCTTTGT-3′ forward primer, 5′-CGTTTCGGACGGGGAGG-3′ reverse primer). PCR products were purified by using a Wizard PCR preps kit (Promega, Wisconsin, USA) and cloned in pEXP5-CT/TOPO vector (Invitrogen, California, USA). Recombinant plasmid DNA was purified using a Miniprep purification system kit (Promega, Wisconsin, USA). Cloned insert integrity was confirmed by automatic sequencing in an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, California, USA).

Extracting RNA and cDNA synthesis. Parasite total RNA was extracted by using the Trizol method [22] and treated with RQ1 RNase-free DNase (Promega, Wisconsin, USA). One microgram of extracted RNA was used for RT-PCR, using the SuperScript III enzyme (Invitrogen, California, USA) in 20-μl reactions, according to manufacturer's recommendations. cDNA was thus synthesised for 60 min at 50 °C, and PCR amplification was then done with the Platinum *Pfx* DNA polymerase enzyme (Invitrogen, California, USA) for 35 cycles at the following temperatures: 94 °C for 15 s, 58 °C for 30 s, 68 °C for 3 min, and a final extension step at 68 °C for 5 min. Additional PCR was carried out using non-reverse transcribed RNA as template (negative control) for discarding genomic DNA contamination.

Peptide synthesis. Three 20-amino-acid-long peptides were synthesised, based on portions from the *P. vivax* RhopH3 deduced sequence (Sal-I). The amino acid sequences, shown in single letter code, were: ^{186}KIYLSSVG TPTSALKNLYLN^{205}, ^{299}RDDVHLVKPQSVWGIPLFTT^{318}, and ^{792}SA GVGTVSTHSPATAARMGL^{811}. A glycine and cysteine were inserted at the N- and C-termini of each peptide to allow polymerisation. The peptides were synthesised using standard solid phase t-Boc/Bzl peptide synthesis strategy [23]. The peptides were lyophilised and then characterised by RP-HPLC and MALDI-TOF MS.

Immunisation in rabbits and collecting their sera. Three rabbits (435, 455, and 456) were subcutaneously inoculated in multiple sites on day 0. Each rabbit received the polymerised synthetic peptide mix. The initial dose was 0.5 mg of emulsified peptides in Freund's complete adjuvant (FCA), whilst the same amount of peptide mixed with Freund's incomplete adjuvant (FIA) was inoculated on days 21 and 42 as booster. Sera were collected before the first immunisation (pre-immune sera) and 21 days after the third immunisation (hyper-immune sera).

Recombinant protein expression and purification. pEXP5-CT/TOPO vector (Invitrogen, California, USA), where the *Pv*RhopH3 gene was cloned, adds a six-histidine tag to the protein's C-terminal portion, thereby facilitating purification and immunodetection by anti-histidine monoclonal antibodies. The protein was purified in denaturing conditions using 6 M Urea in Ni^{2+}–NTA resin (Qiagen, California, USA), according to manufacturer's recommendations. Its expression was verified on 8–10% polyacrylamide gel in the presence of SDS (SDS–PAGE) which was stained with Coomassie blue or evaluated by Western blot. The total amount of protein was determined by bicinchoninic acid assay.

SDS–PAGE and Western blot. The malarial parasite, purified from *Aotus* monkeys' total blood, was lysed with a solution containing 5% SDS, 1 mM EDTA, 10 mM PMSF, and 10 mM iodoacetamide. The proteins in lysate were size-separated on 8–10% polyacrylamide gel in the presence of SDS and then transferred to a nitrocellulose membrane. The Western blot was carried out as described elsewhere [19,20].

Confocal microscopy assays. RBC infected with mature blood-stage parasites (mainly schizonts) were used for confocal microscopy, after being washed thrice with PBS to remove any Percoll remaining after extraction. A previously reported methodology was followed for this purpose [24]. Briefly, 500 μl of extracted parasites were fixed with 4% paraformaldehyde and 0.0075% glutaraldehyde for 30 min and then washed thrice with 0.9% saline solution. Samples were then permeabilised with 0.1% Triton X-100 (ICN, California, USA) for 10 min and washed again in saline solution. 0.1 mg/ml sodium borohydride was then added for 10 min. After washing the slides thrice more with saline solution, each sample was blocked for 1 h with 3% bovine serum albumin (BSA) in PBS. Rabbit polyclonal antibodies directed against synthetic peptides at 1:40 dilution in PBS with 3% BSA were used as primary antibody and incubated for 30 min. After washing thrice, goat anti-rabbit IgG conjugate labelled with fluorescein isothiocyanate (Sigma, Missouri, USA) diluted 1:40 in PBS with 5% BSA was used as secondary antibody for 30 min. An Olympus FluoView confocal laser scanning microscope IX81—FV1000 was used for reading immunofluorescence.

Accession number. The accession number for the nucleotide and amino acid sequences used in the present study has been reported in GenBank as follows: EF566468.

Results and discussion

Identifying the gene encoding RhopH3 protein in P. vivax

The database holding the *P. vivax* genome sequencing project (TIGR) was searched for identifying the *P. falciparum* RhopH3 (GenBank Accession No. CAD51739)

homologue in *P. vivax* using the tblastn tool. This search allowed us to identify a chromosomic region of 1,198,945 bp (ctg_7052) where the *pvrhoph3* gene was included. However, the precise location of the *pvrhoph3* encoding sequence was difficult to assess at first, since this gene has two short exons at the beginning. An open reading frame (ORF) prediction of a ctg_7052 54-kbp segment (using GenScan and GeneComber software) [25,26] was thus carried out to overcome this problem and neighbouring genes were analysed too. Such analysis revealed that the entire *pvrhoph3* gene has 4159, 2673 bp being the encoding sequence. As shown in Fig. 1, *pvrhoph3* preserves the same exon-intron structure as its *P. falciparum* homologue (PlasmoDB Gen ID No. PFI0265c), the latter having 4049, 2694 bp of which are the encoding sequence.

RhopH3 from both parasite species is mainly encoded by a 7 exon gene, having lengths of 64, 110, 958, 63, 57, 780, and 638 bp in *P. vivax* and 63, 111, 961, 63, 57, 771, and 668 bp in *P. falciparum*. The six intron sequences span a total of 1355 bp in *P. falciparum* and 1489 bp in *P. vivax*. According to the above-mentioned gene structure, the *P. vivax* RhopH3 protein has 890 amino acids.

This protein displayed an estimated molecular weight (MW) of 102482.3 Da, being only seven amino acids shorter than *Pf*RhopH3 (estimated to be 104.5 kDa MW). The *Pv*RhopH3 and *Pf*RhopH3 amino acid alignment showed a 52.7% identity (I) and 68.6% similarity (S), considered to be high values when compared to other parasite proteins involved in erythrocyte invasion [17,19,27]. An N-terminal hydrophobic region which could serve as a signal peptide was found when the protein's whole sequence was analysed. The predicted cleavage site for this peptide is located between amino acids 24 and 25 (VLG-RE) as assessed by SignalP v3.0 software [28], similar to what has already been reported in *Pf*RhopH3 (VWG-KD) [11].

Gene organisation analysis of *rhoph3* neighbouring genes for *P. falciparum* and *P. vivax* has revealed that *rhoph3* is localised in homologous chromosome regions in both parasite species, since upstream and downstream genes shared high identity (31.8–79.9%) and similarity (40.9–89.1%) values and displayed the same ORF orientation (Fig. 1). Although, the chromosomic region comprising all these genes was slightly longer in *P. vivax* than in *P. falciparum*, each gene tends to be smaller in the former parasite species.

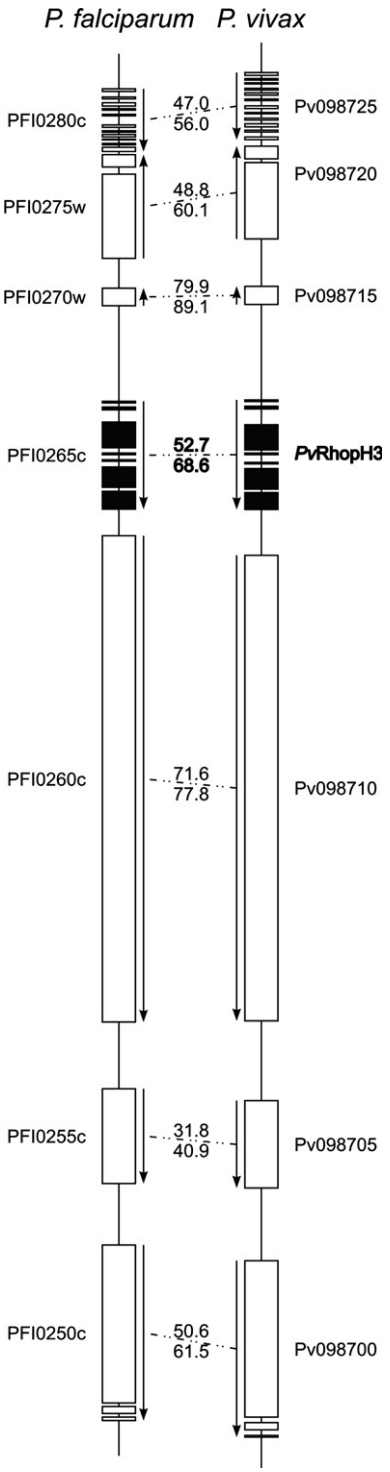

Fig. 1. Scale schematic diagram of the chromosome localisation of genes encoding RhopH3 and adjacent genes for *P. falciparum* and *P. vivax*. The direction of the ORF and the exon organisation is shown in each gene. The *P. falciparum* chromosome 13 fragment analysed corresponded to 53 kb (235–288 kb) length, whilst it was 54 kb (138–192 kb) for *P. vivax*. Identity and similarity values are shown for the proteins derived from these genes.

PvRhopH3 is transcribed in blood-stage parasites

Previous analysis of the *pfrhoph3* transcriptional profile (http://malaria.ucsf.edu/comparison/index.php) has shown that this gene starts being transcribed 20 h after parasite invasion (trophozoite stage), reaching its maximum at 38 h (schizont stage) [29,30]. PCR amplification of the *Pv*RhopH3 coding sequence confirmed the presence of this transcript in mature *P. vivax* blood stages. As observed in Fig. 2, a product having the expected weight (~2673 bp) was obtained from cDNA amplification. Likewise, a product having the expected weight (~4159 bp) was observed upon genomic DNA amplification, confirming the presence

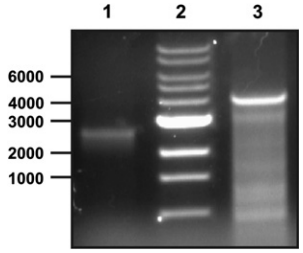

Fig. 2. PCR amplification of the RhopH3 gene in *P. vivax*. Lane 1, the RT-PCR analysis of *Pv*RhopH3 ORF using the described primers. Lane 2, the molecular weight markers. Lane 3, the PCR for *Pv*RhopH3 from genomic DNA using the same set of specific primers.

of intron regions within the gene. Three independent RT-PCRs were carried out followed by cloning. Gene sequencing from each independent reaction was thus done. Four nucleotide substitutions were observed when comparing the Sal-I reference strain (available at TIGR homepage) with the monkey-adapted VCG-I strain. These substitutions (at nucleotide positions 172, 174, 667, and 1568) led to three amino acid changes as follows: glutamic acid for asparagine at amino acid position 58 (E58N), arginine for glycine at position 223 (R223G) and serine for asparagine at position 523 (S523N). It is worth noting that none of these substitutions fell in the synthetic peptide sequences analysed for evaluating protein expression.

As previously described, cysteines are essential for correct *Pf*RhopH3 conformation and for this protein's interaction with other Rhop-H complex proteins [8]. *Pv*RhopH3 cysteine localisation was highly conserved when compared to its *P. falciparum* homologue in the 3D7 strain. Thirteen out of the fourteen cysteines were conserved between the two proteins and in *Pv*RhopH3, these were encoded by exons 2, 3, and 6 (Fig. 3A). Exon 3 encoded most of the cysteines, eight in *Pf*RhopH3, and seven in *Pv*RhopH3 (different to the six predicted in a previous study [15]).

PvRhopH3 is expressed during P. vivax asexual blood stage

Rabbit polyclonal antibodies were obtained by immunising the animals with a mixture of three synthetic peptides derived from the *Pv*RhopH3 predicted primary amino acid sequence (Fig. 3A). Sera ability to recognise the protein was assessed by Western blot against the purified *Pv*RhopH3 recombinant protein (Fig. 3B). As can be observed, this assay showed a ~105 kDa recognition band when either immune rabbit sera or anti-histidine monoclonal antibodies were used. *P. vivax* parasite lysate was run on SDS–PAGE, transferred to nitrocellulose membrane and then incubated with rabbit sera to evaluate *Pv*RhopH3 protein expression. A previous study of *P. falciparum* RhopH3 has shown that this protein is produced as a 110-kDa precursor in trophozoite stage and then gradually processed to a 100-kDa protein as the parasite matures [3]. Our Western blot showed that ~100- and ~105-kDa bands were recognised by the three hyperimmune rabbit sera, the smaller band better being recognised by serum # 456 (Fig. 3C). This result strongly suggests that *Pv*RhopH3 undergoes a similar proteolytic cleavage to that displayed by its *P. falciparum* homologue. Despite the difficulty in experimentally identifying *Pv*RhopH3 cleavage site, evidence about its *P. falciparum*

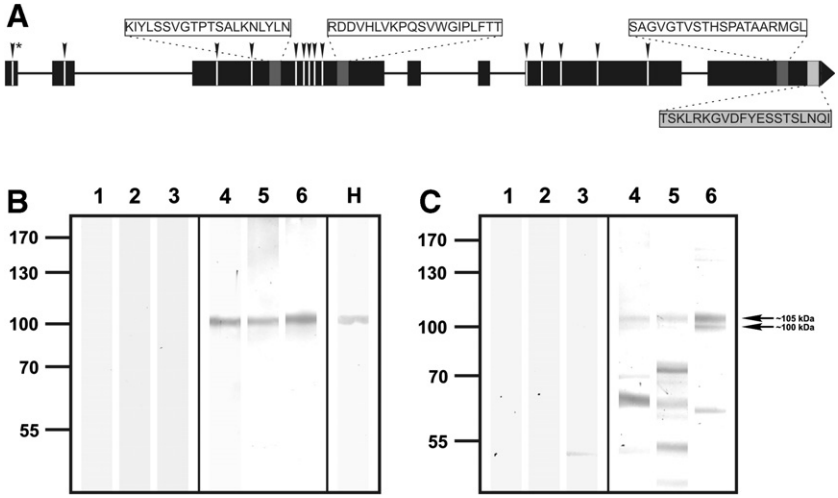

Fig. 3. Scheme of *Pv*RhopH3 gene, localisation of synthetic peptides, and detection of the protein with polyclonal antibodies. (A) Organisation of the gene encoding *Pv*RhopH3, showing the localisation of the cysteines (arrows) on the exons. A cysteine residue is highlighted (*) on being present only in *P. vivax* since the remaining 13 are conserved with *Pf*RhopH3. The localisation of the synthetic peptides used in this study are shown (text in white boxes). The peptide sequence towards the C-terminal extreme of *Pv*RhopH3 (text in grey box) includes a potential cleavage site [31]. (B) Western blot of the purified *Pv*RhopH3 recombinant protein. Lanes 1–3, recombinant *Pv*RhopH3 detected with pre-immune sera from rabbits 435, 455, and 456. Lanes 4–6, recombinant *Pv*RhopH3 detected with hyper-immune sera from rabbits 435, 455, and 456, respectively. Lane H, recognition of purified *Pv*RhopH3 using anti-histidine monoclonal antibody. (C) *P. vivax* parasite lysate was size-separated by SDS–PAGE, electroblotted, and tested with the rabbit sera raised against the synthetic peptides. Lanes 1–3, recognition of pre-immune sera from rabbits 435, 455, and 456 in parasite lysate. Lanes 4–6, recognition of hyper-immune sera from rabbits 435, 455, and 456 in parasite lysate, respectively.

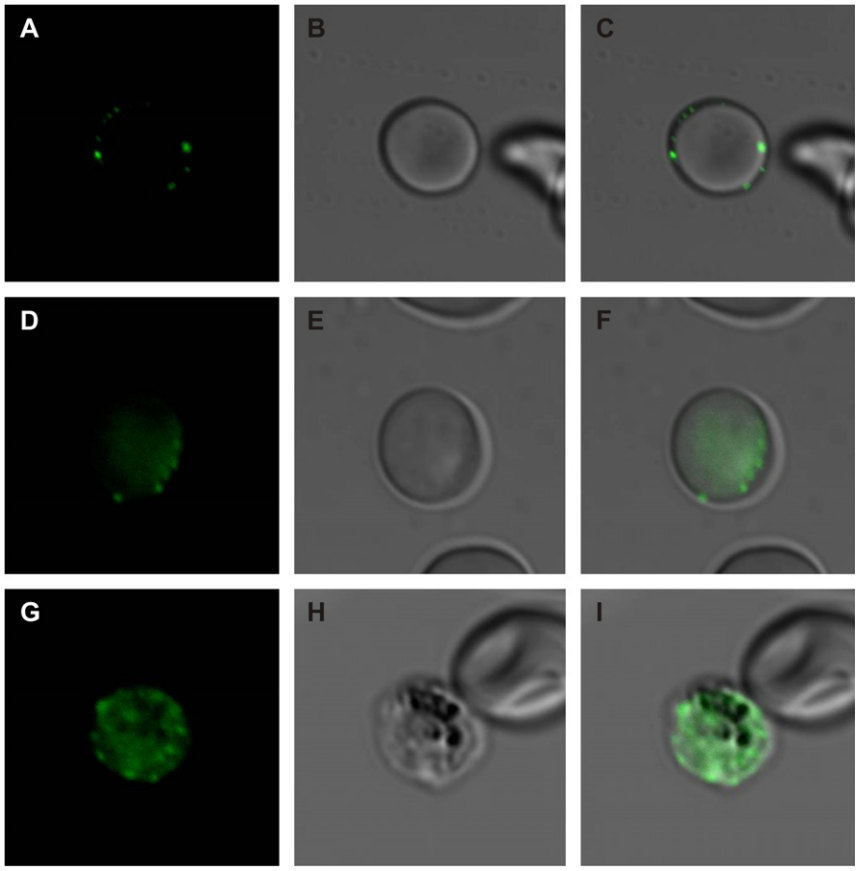

Fig. 4. Immunolocalisation by confocal microscopy of *Pv*RhopH3 protein in the parasite using polyclonal antibodies. (A–C) *P. vivax* merozoites in early contact with healthy RBC. (D–F) *Pv*RhopH3 protein release on the surface of RBC in the step prior to invasion. (G–I) Parasite in schizont stage, showing the localisation of *Pv*RhopH3 within infected RBC. The fluorescence punctate pattern is characteristic of rhoptry proteins. The figure shows the fluorescence images, differential interference contrast (DIC), and the merging of both.

homologue indicates that cleavage takes place 45–50 amino acids from the C-terminus [31] and the same phenomenon might be occurring in *Pv*RhopH3. It is important to point out that RhopH3 exon 7 encoded the protein region displaying the lowest identity and similarity values (27.4% and 45.1%, respectively) when comparing *P. falciparum* and *P. vivax*. However, at the end of this encoded region there was a segment where values rose (I: 55%; S: 75%) and the *Pv*RhopH3 cleavage site is more likely to lie within this segment (amino acids 845–864) (Fig. 3A). Although the biological implications of this cleavage event remain unknown, it has been suggested that C-terminal processing of RhopH3 might modify the localisation of the high molecular weight protein complex within rhoptries and possibly its later destination in newly invaded RBC [31]. Localisation studies using confocal microscopy were performed to confirm the expression (Fig. 4). *Pv*RhopH3 was localised around the RBC upon early contact between the parasite and RBC. Once the parasite invaded and reached the trophozoite stage, a faint fluorescence was detected intracytoplasmically and RBC surface stain decreased. At schizont stage, forming merozoites could be observed within the RBC and a punctate fluorescence pattern (typical of rhoptry-localised proteins [9,32,33]) appeared.

In the present study, the *P. vivax* RhopH3 protein and its encoding gene have been identified and characterised. Future studies aimed at testing this new protein's immunogenicity and protection-inducing capacity in *Aotus* monkeys will shed light on its potential as an anti-*P. vivax* vaccine candidate.

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