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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.
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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′-ATGCGAAGCAAGCTCTTTGT-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}KIYLSSVGTPTSALKNLYLN^{205}, ^{299}RDDVHLVKPQSVWGIPLFTT^{318}, and ^{792}SAGVGTVSTHSPATAARMGL^{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)

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