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The *Plasmodium vivax* rhoptry-associated protein 1

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Abstract

Rhoptries are cellular organelles localized at the apical pole of apicomplexan parasites. Their content is rich in lipids and proteins that are released during target cell invasion. *Plasmodium falciparum* rhoptry-associated protein 1 (RAP1) has been the most widely studied among this parasite species' rhoptry proteins and is considered to be a good anti-malarial vaccine candidate since it displays little polymorphism and induces antibodies in infected humans. Monoclonal antibodies directed against RAP1 are also able to inhibit target cell invasion in vitro and protection against *P. falciparum* experimental challenge is induced when non-human primates are immunized with this protein expressed in its recombinant form. This study describes identifying and characterizing RAP1 in *Plasmodium vivax*, the most widespread parasite species causing malaria in humans, producing more than 80 million infections yearly, mainly in Asia and Latin America. This new protein is encoded by a two-exon gene, is proteolytically processed in a similar manner to its *falciparum* homologue and, as observed by microscopy, the immunofluorescence pattern displayed is suggestive of its rhoptry localization. Further studies evaluating *P. vivax* RAP1 protective efficacy in non-human primates should be carried out taking into account the relevance that its *P. falciparum* homologue has as an anti-malarial vaccine candidate.

Keywords: Malaria; Rhoptry; *Plasmodium*; RAP1; Vaccine candidate

The invasive forms of parasites belonging to *Phylum apicomplexa* contain secretor organelles located on the apical pole; these have been named rhoptries, micronemes, and dense granules [1]. These organelles are actively involved during host cell invasion by secreting proteins located within them that aid in the parasite's adhesion and penetration [2]. Among the parasites grouped within this phylum, those belonging to the genus *Plasmodium* are able to cause malaria that is considered to be the main parasite disease worldwide, due to the huge morbidity and mortality which it produces [1].

Plasmodium falciparum, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae* are the four parasite species causing malaria in humans; the first two producing more than 90% of all cases [3].

Several *P. falciparum* rhoptry proteins involved in red blood cell invasion have been identified during the last 15 years; those belonging to the "high molecular mass complex" [4] (RhopH1, RhopH2, and RhopH3) and those from the "low molecular mass complex" (rhoptry-associated protein 1 (RAP1) [5], rhoptry-associated protein 2 (RAP2) [6], and the rhoptry-associated protein 3 (RAP3) [7]), being of particular importance, whilst only rhoptry-associated protein 2 (RAP2) has been described to date in *P. vivax* [8].

Plasmodium falciparum RAP1 is a 782 amino acid protein, localized at the rhoptry base, having an 82 kDa molecular mass and displaying very little polymorphism [1,5,9]. According to transcriptomic data [10], its encoding gene is transcribed during the last 6 h of this parasite's

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intra-erythrocyte cycle. Previous studies have pointed out this protein's importance in red blood cell invasion since monoclonal antibodies raised against RAP1 are able to inhibit *P. falciparum* in vitro invasion of erythrocytes [11]. Moreover, protection studies have shown that when non-human primates are immunized with RAP1, produced either as a recombinant protein or purified from cultured parasites, some monkeys are protected against experimental challenge with *P. falciparum*, demonstrating this protein's potential as an anti-malarial vaccine candidate [12,13].

The present study characterizes both the homologous gene and protein from *P. falciparum* rhoptry-associated protein 1 in *P. vivax* using the *P. vivax* genome database, its preliminary annotation data released by the Institute of Genomic Research (TIGR), and both molecular biology and immunology techniques.

Materials and methods

Parasites. *Plasmodium vivax* Vivax-Colombia-Guaviare I (VCG-I) strain [14], maintained in vivo by successive passages in splenectomized *Aotus nancymaae* monkeys kept at our primate station in Leticia (Colombia), was used as a source of DNA, RNA, and parasite lysate extraction. *P. vivax* infected RBCs were purified from a 3 ml blood sample taken from an infected animal using a 30–80% Percoll gradient (Sigma, St. Louis, MO).

Plasmodium vivax genomic nucleotide sequence source. The partial *P. vivax Sal*I strain genomic nucleotide sequences used were downloaded from the TIGR web page (http://www.tigr.org/tdb/e2k1/pva1/).

Parasite DNA. *Plasmodium vivax* DNA was extracted from the monkey's *P. vivax*-infected blood sample using the Wizard DNA purification system (Promega, Madison, WI).

Cloning and sequencing. *Plasmodium vivax* VCG-I strain cDNA was used as template for PCR amplification. Primer design was based on the sequence of a putative transcript encoding a protein found by BLAST to be homologous with *P. falciparum* RAP1 in the *P. vivax* genome database reported at TIGR. The primers used encompassed the entire transcript; *Bam*HI and *Pst*I cloning sites were added to the forward (5′-CGGGATCCACTTGCGTAAGTTCCCTGC-3′) and reverse (5′-AACTGCAGTACTCCAATCGCTTGTAGA-3′) primers, respectively. PCR products were purified by Wizard PCR preps kit (Promega, Madison, WI) and cloned in pGEM-T vector (Promega, Madison, WI). Plasmid DNA from recombinant bacteria was purified using a Miniprep purification kit (Promega, Madison, WI). Cloned inserts were sequenced in an automatic sequencer (ABI PRISM 310 Genetic Analyzer, PE Applied Biosystems, Foster City, CA).

RNA extraction and cDNA synthesis. Total *P. vivax* RNA was extracted from infected *Aotus* monkey blood samples by the Trizol method [15] and treated with RQ1 RNase-free DNase (Promega, Madison, WI). One microgram of the extracted RNA was used for one-step RT-PCR using SuperScript III (Invitrogen, Carlsbad, CA) in 50 μl reactions according to manufacturer's recommendations. Briefly, cDNA was synthesized for 30 min at 58 °C and PCR was carried out for 40 cycles at the following temperatures: 94 °C for 15 s, 58 °C for 30 s, 68 °C for 150 s, and a final extension cycle of 68 °C for 5 min.

Peptide synthesis. Two 20-mer peptides, corresponding to the deduced *P. vivax* Sal-I RAP1 sequence, were synthesized. The sequences shown in one-letter, amino acid code were: ^{146}SSYSDYSAYDSGSASSVGSR^{165} and ^{532}KRDYTFLAFKTVCDKYVSHN^{551}. One glycine and one cysteine were added at both N- and C-termini of each peptide to allow further peptide polymerization. Peptides were synthesized by using standard *t*-Boc/Bzl solid-phase peptide synthesis strategy on a MBHA resin (0.15 meq.). The *t*-Boc was removed by 55% TFA in DCM (0.01% anisole). Amino acid coupling was carried out using fivefold molar excess aa/HOBt/DCC as coupling reagent and DMF as solvent. Coupling time was 1 h; re-coupling was used up to negative Kaiser test when necessary [16]. Side protection groups and peptide from resin were removed using HF low/high procedure [17]. Peptides were extracted, lyophilized, and finally characterized by RP-HPLC and MALDI-TOF MS.

Rabbit immunization. New Zealand white rabbits were provided by the Instituto Nacional de Salud (Bogota, Colombia), housed, fed, and handled in accordance with the recommendations made by the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA). One hundred and fifty micrograms of both polymerized peptides emulsified in Freund's complete adjuvant (FCA) were subcutaneously inoculated into three rabbits at multiple sites. The same amount of immunogen mixed with Freund's incomplete adjuvant (FIA) was inoculated as a booster on days 21 and 42 [18].

Recombinant protein expression and purification. The *Pv*RAP1 gene, previously cloned in pGEM-T vector, was cut with *Bam*HI and *Pst*I restriction endonucleases (New England Biolabs, Beverly, MA) according to manufacturer's instructions and further subcloned into pQE expression vector (Qiagen, Valencia, CA). This vector adds a six-histidine tag at the protein's N-terminal portion, enabling an easier purification process and immunodetection by anti-histidine monoclonal antibodies. Protein was purified using Ni–NTA metal affinity resin (Qiagen, Valencia, CA) in accordance with manufacturer's recommendations. Protein expression was verified by either sodium dodecyl sulfate–polyacrylamide gel electrophoresis stained with Coomassie blue or Western blot. Total protein amount was quantified by bicinchoninic acid assay.

SDS–PAGE and Western immunoblotting. Blood samples were taken from a *P. vivax* infected monkey (having greater than 6% parasitemia) and passed through a CF11 cellulose column to isolate red blood cells. These were lysed with 0.25% saponin and washed three times with PBS to obtain parasites; they were then lysed with 5% SDS plus a protease inhibitor cocktail. Proteins in the extract were size-separated by electrophoresis in 14% SDS–PAGE and then electroblotted onto a nitrocellulose membrane. The membrane was blocked with 0.05% Tween 20 in TBS plus 5% skimmed milk and split into rows to be independently assayed against individual sera. Primary antibodies were detected with alkaline phosphatase (AP) conjugated anti-rabbit Ig.

Indirect immunofluorescence assays. Indirect immunofluorescence assays were performed as follows: red blood cells parasitized with *P. vivax* from *Aotus* monkeys were separated from leukocytes by using a CF11 cellulose column (Whatman, Maidstone, Kent, UK); the parasite's mature forms were then concentrated using a 30%–80% Percoll gradient (Sigma, St. Louis, MO). Twenty microliters of this schizont-parasitized sample was then sown in each well of glass 8-well multi-test slides (ICN, Irvine, CA). The sample was air-dried, fixed with 4% formaldehyde in PBS for 6 min at room temperature, briefly washed with PBS, and then permeabilized with 0.2% Triton X-100 in PBS (ICN, Irvine, CA) for 6 min. The slides were washed again twice with PBS.

Each well was then blocked for 15 min with 5% bovine serum albumin (BSA) in PBS. Rabbit polyclonal sera directed against the synthetic peptides were used as primary antibody at 1:100 dilution in PBS with 5% BSA. Fluorescein isothiocyanate (FITC), conjugated anti-rabbit Ig (Sigma, St. Louis, MO) was used as secondary antibody at 1:40 dilution in PBS with 5% BSA. An Olympus BX51 fluorescence microscope was used for reading immunofluorescence.

Nucleotide and amino acid sequence accession numbers. Nucleotide and amino acid sequences used in the present study have been reported in GenBank under Accession No. DQ311677.

Results

Identifying and characterizing the PfRAP1 P. vivax homologue

When searching the TIGR *P. vivax* genome database (http://www.tigr.org/tdb/e2k1/pva1/) using the

P. falciparum rhoptry-associated protein 1 (*Pf*RAP1) amino acid sequence as the BLAST query sequence, we identified a 2,031,768 bp chromosome segment containing a region encoding a putative protein having both high identity and similarity to the C-terminal fragment of *Pf*RAP1. According to the partial *P. vivax* genome annotation carried out by TIGR, this region encodes a putative protein (termed 38.m01528) having 740 amino acids and an estimated 85.6 kDa molecular mass. It was found that this protein is encoded by a 2223 bp transcript that, when aligned with the contig from where it was derived, showed that the *vivax* protein came from a gene comprised by two exons separated by a 212 bp intron, different to what occurs with *P. falciparum* RAP1 which is encoded by a single exon gene (Fig. 1).

The alignment of this new protein (*P. vivax* rhoptry-associated Protein 1, *Pv*RAP1) with its *P. falciparum* homologue shows 39% identity and 61% similarity. *Pv*RAP1 has a hydrophobic region at the N-terminal extreme that is a potential site for a signal peptide as indicated by the SignalP v3.0 software [19], similar to what occurs in *Pf*RAP1 [5].

Although *Pv*RAP1 does not contain the KSSSPS repeat present in *Pf*RAP1 [5], it does have the serine-rich region. *Pv*RAP1 has 7 cysteine residues while *Pf*RAP1 has 8 [5]; 6 of these were localized in the same position when both *vivax* and *falciparum* proteins were aligned (Fig. 1).

We have also confirmed that those genes flanking *Pv*RAP1 in *P. vivax* are homologous to those flanking *Pf*RAP1 in *P. falciparum* (Fig. 2).

PvRAP1 is transcribed during asexual stage life-cycle

Red blood cells from an *Aotus* monkey parasitized with the *P. vivax* VCG-I strain were used as a source of parasite DNA and RNA. RNA extracted from *P. vivax* mature blood-stage parasites was treated with DNAse and amplified by one-step RT-PCR to study *Pv*RAP1 gene transcription. As can be seen in Fig. 3, a ~2220 bp product was obtained in the RT-PCR (lane 3), having a smaller size when compared to that obtained when the genomic DNA was amplified using the same primer set (~2430 bp) (lane 1). This result is in agreement with the presence of the ~210 bp intron within this gene. No amplification was observed in the negative control (lane 2). The product amplified in the RT-PCR was then cloned and confirmed by sequencing. The sequence from the *Pv*RAP1 gene obtained from the VCG-1 strain showed the following differences with respect to that from the *Sal*I strain available in TIGR database. It has a 12 bp insertion encoding the

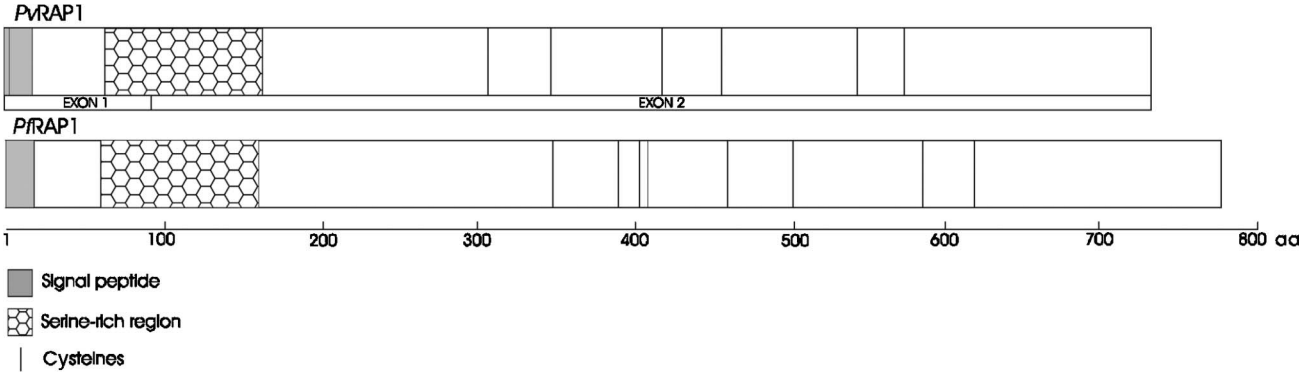

Fig. 1. Scale diagram showing the structural characteristics of RAP1 in both *P. vivax* and *P. falciparum*. The putative signal peptide sequences, the serine-rich regions, and the cysteines are indicated in the figure, as well as those regions encoded by each exon in *P. vivax*. The positional conservation of cysteine residues is not apparent in the figure since *Pf*RAP1 is bigger than *Pv*RAP1.

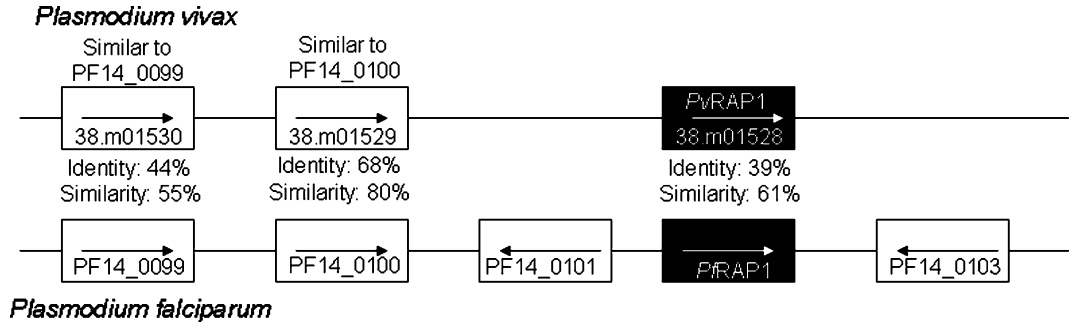

Fig. 2. Diagram depicting the chromosome regions encoding *Pf*RAP1 and *Pv*RAP1. *P. vivax* genes have been named according to the code assigned by TIGR in the partial genome annotation. Arrows indicate the direction of each open-reading frame (ORF). Identity and similarity percentages respecting their *P. falciparum* homologues are indicated below each *P. vivax* gene.

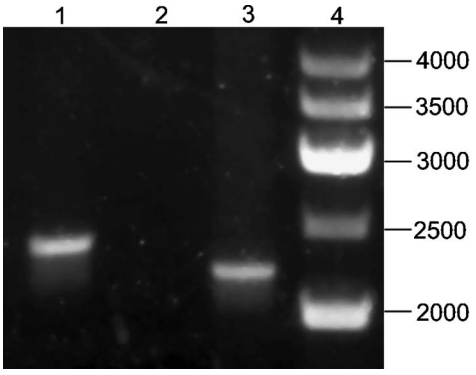

Fig. 3. PCR of RAP1 in *P. vivax* using gDNA and cDNA as templates. Lane 1 shows the PCR of RAP1 using *P. vivax* genomic DNA as template. Lane 2, Negative control. Lane 3 shows the one-step RT PCR of DNase-treated total *P. vivax* RNA using the same specific primers. Lane 4 shows size standards in basepairs.

SKSG sequence located within the serine-rich region; there are eight point mutations, four of them being synonymous whilst the remainder produce amino acid changes N331K, T342I, S555N, and L714F.

PvRAP1 is expressed during the P. vivax asexual stage

Polyclonal antibodies were produced against two 20-residue long synthetic peptides in rabbits, each peptide being derived from the amino acid sequence of the newly identified protein. Serum quality was verified by ascertaining its ability to detect recombinant *Pv*RAP1, as can be seen in Fig. 4A. Lysed *P. vivax* parasites extracted from red blood cells from an infected monkey were run on SDS–PAGE, followed by immunoblotting. The antibodies raised against the synthetic peptides reacted against four bands of around 88.5, 81, 76, and 69.5 kDa (Fig. 4B). This figure shows that *Pv*RAP1 seems to be proteolytically processed in a similar fashion to *Pf*RAP1[20] which undergoes an N-terminal proteolytic process, generating 86, 82, 70, and 67 kDa bands.

IFA revealed PvRAP1 presence in the parasite

A sample of *Aotus* blood parasitized with the adapted *P. vivax* VCG-I strain was taken from a monkey kept at our primate station in Leticia, Colombia; this was used for determining *Pv*RAP1 cellular localization. Immunofluorescence microscopy of formaldehyde-fixed *P. vivax* parasites was carried out using anti-*Pv*RAP1 rabbit antibodies. Fluorescence appeared in a punctate pattern in mature schizonts (Fig. 5). This staining pattern is typical of rhoptry proteins [21].

Discussion

The rhoptries from parasites belonging to the *Plasmodium* genus are paired, pear-shaped organelles, localized at the merozoites' apical pole. They are rich in proteins and

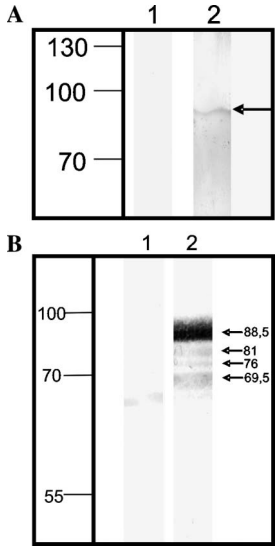

Fig. 4. *Plasmodium vivax* RAP1 detection with polyclonal antibodies. (A) Western blot of purified recombinant *Pv*RAP1 recognized by polyclonal antibodies against the two synthetic peptides. Lane 1, recombinant *Pv*RAP1 detected with pre-immune sera. Lane 2, recombinant *Pv*RAP1 detected with hyper-immune sera. Size standards are indicated in kilodaltons on the left-hand side. (B) *P. vivax* parasite lysate was size-separated by SDS–PAGE, electroblotted, and tested with rabbit sera raised against two *Pv*RAP1 synthetic peptides. Lane 1, parasite lysate recognition by pre-immune sera. Lane 2 parasite lysate recognition by hyper-immune sera. Arrows indicate the bands and the estimated molecular mass.

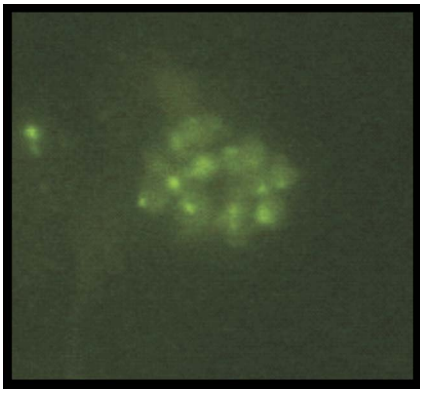

Fig. 5. Indirect immunofluorescence assay using hyper-immune serum against two *Pv*RAP1 synthetic peptides showing parasite labeling of a fixed *P. vivax* schizont. The punctate fluorescence pattern observed is characteristic of rhoptry proteins.

lipids which are released during red blood cell invasion, meaning that their content is implicated in both host cell invasion and parasitophorous vacuole formation and preservation [1].

Plasmodium falciparum has been the most studied of the four parasite species causing malaria in humans as it causes more clinical and fatal cases around the world than the other species combined [3].

Researchers have been able to culture this parasite in vitro during the last few decades [22], leading to greater knowledge being accumulated regarding its molecular, structural, and functional properties.

Some of the previously identified and characterized rhoptry proteins have been found to actively participate in red blood cell invasion due either to their ability to bind to red blood cells or because monoclonal or polyclonal antibodies raised against them can inhibit in vitro invasion of target cells [23].

Plasmodium falciparum rhoptry-associated protein 1 is localized at the rhoptry base and it is thought that it is implicated in invasion due to the above [11]; more importantly, it has been found that its inoculation confers protection in non-human primates [13]. *Pf*RAP1 is a non-polymorphic protein [9] and its antigenicity has been widely studied in human communities exposed to *P. falciparum* [24–28]. Such studies have revealed that this protein is antigenic in most people during naturally occurring parasite infection, thereby presenting it as a good vaccine candidate.

This work has described identifying and characterizing RAP1 in *P. vivax*. This new protein shares some structural characteristics with its homologue in *P. falciparum* such as: having a signal peptide, the absence of transmembrane regions, a serine-rich N-terminal region, and positionally conserved cysteine residues.

Different to what occurs in homologous proteins identified in *P. vivax* and *P. falciparum*, where the structure of the encoding gene is preserved, *Pv*RAP1 is encoded by a two-exon gene while its *falciparum* homologue is encoded by a single exon gene [5].

The new protein seems to undergo a proteolytic processing similar to that which occurs in *P. falciparum* where there is evidence of a sequential amino acid loss in the N-terminal extreme [20]. Its localization within the rhoptries was inferred from the punctate pattern observed in the immunofluorescence assay.

Recent *P. falciparum* gene transcription studies during the blood stage cycle have indicated that RAP1 must be considered to be a good vaccine candidate as it is expressed at the same time as other proteins implicated in invasion [10,29]. Bearing this in mind, as well as the above, we consider that RAP1 could be a good candidate for a vaccine against *P. vivax*; further protection assays must therefore be carried out using this new protein to immunize non-human primates.

Acknowledgments

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