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Identification and characterisation of the *Plasmodium vivax* rhoptry-associated protein 2

Manuel A. Patarroyo [a,d,*], Oscar Perez-Leal [a], Yolanda Lopez [a], Jimena Cortes [b], Jose Rojas-Caraballo [a], Andromeda Gomez [a], Camilo Moncada [a], Jaiver Rosas [c], Manuel E. Patarroyo [a,d]

[a] *Molecular Biology Department, Fundacion Instituto de Inmunologia de Colombia, Bogota, Colombia*
[b] *Immunochemistry Department, Fundacion Instituto de Inmunologia de Colombia, Bogota, Colombia*
[c] *Chemistry Department, Fundacion Instituto de Inmunologia de Colombia, Bogota, Colombia*
[d] *Universidad Nacional de Colombia, Bogota, Colombia*

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Abstract

Plasmodium vivax is currently the most widespread of the four parasite species causing malaria in humans around the world. It causes more than 75 million clinical episodes per year, mainly on the Asian and American continents. Identifying new antigens to be further tested as anti-*P. vivax* vaccine candidates has been greatly hampered by the difficulty of maintaining this parasite cultured in vitro. Taking into account that one of the most promising vaccine candidates against *Plasmodium falciparum* is the rhoptry-associated protein 2, we have identified the *P. falciparum* rhoptry-associated protein 2 homologue in *P. vivax* in the present study. This protein has 400 residues, having an N-terminal 21 amino-acid stretch compatible with a signal peptide and, as occurs with its falciparum homologue, it lacks repeat sequences. The protein is expressed in asexual stage *P. vivax* parasites and polyclonal sera raised against this protein recognised a 46 kDa band in parasite lysate in a Western blot assay.

Keywords: Malaria; *Plasmodium vivax*; Rhoptry-associated protein 2; Vaccine candidate; Bioinformatics

Malaria remains as one of the infectious diseases causing greater morbidity and mortality worldwide. Recent studies have shown that more than 500 million people are infected each year, of which more than two million die, mainly children below 5 years of age in sub-Saharan Africa [1,2]. Four *Plasmodium* parasite species cause malaria in humans: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. Although *P. falciparum* generates the highest mortality, *P. vivax* is more prevalent in Asia and America, producing more than 75 million cases per year [3].

The development of anti-malarial vaccines has become a priority [4], since both parasites and transmitting vectors (*Anopheles* mosquitoes) have developed increasing resistance to both anti-malarial drugs and insecticides, respectively [5,6].

The *P. falciparum* genome sequencing project has been finished during the last few years [7], as well as studies describing this parasite's transcriptome [8,9] and proteome [10–12]. Nevertheless, these studies have been partly hindered for *P. vivax* due to the difficulty of culturing this parasite species in vitro [13]. To date, the *P. vivax* genome has been almost entirely sequenced, but no information has yet been published regarding the transcriptome or proteome.

Sequencing the *P. vivax* genome is being carried out by The Institute for Genomic Research (TIGR) with funds from the US Department of Defense and the National Institute of Allergy and Infectious Diseases [14]. As these data have become available to the scientific community it has allowed bioinformatic approaches to be used in characterising

* Corresponding author. Fax: +57 1 4815269.
E-mail address: mapatarr@fidic.org.co (M.A. Patarroyo).

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new *P. vivax* antigens which could become relevant in developing future anti-malarial vaccines or treatments.

Previous studies have shown high conservation in gene order and chromosome localisation amongst different *Plasmodium* species [15,16]. Taking this into account and considering the greater knowledge respecting *P. falciparum* antigens with potential use as vaccine candidates, our group has focused its efforts on identifying homologue antigens present in *P. vivax* [17,18].

The *P. falciparum* rhoptry-associated protein 2 (*Pf*RAP2) is a 398 amino acid protein encoded by a single exon gene located on this parasite's chromosome 5 [19]. This protein is discharged from the rhoptries into the forming parasitophorous vacuole during erythrocyte invasion by the parasite [20,21]. Previous studies aimed at identifying the possible role of *Pf*RAP2 in invading red blood cells (RBCs) have yielded somewhat conflicting results. Gene-targeted *P. falciparum* parasites, where a truncated form of RAP1 was expressed, were unable to localise RAP2 in the rhoptries but both their erythrocyte invasion and intracellular growth phenotypes were unaffected [22]. On the other hand, synthetic peptides derived from the *Pf*RAP2 sequence displayed high specific erythrocyte binding activity [23] and monoclonal antibodies against *Pf*RAP2 were able to inhibit the erythrocyte invasion process in vitro [24].

In spite of *Pf*RAP2's role in RBC invasion not being clear, several data have suggested that it could be a potential anti-malarial vaccine candidate. Protective efficacy against *P. falciparum* challenge has been observed when *Saimiri sciureus* monkeys have been immunised with *Pf*RAP1 and *Pf*RAP2 purified from cultured parasites and emulsified with Freund's adjuvant [25,26]. In a later study, four out of six *Saimiri boliviensis* monkeys immunised with *Pf*RAP2 produced as a recombinant protein and emulsified in Montanide ISA720 were protected when challenged with the parasite [27].

Based on the above-mentioned potentially important advances in vaccine development, the present study describes the identification and immunochemical characterisation of the *Pf*RAP2 homologue in *P. vivax*.

Materials and methods

Parasites. P. vivax Vivax-Colombia-Guaviare I (VCG-I) strain [28], maintained in vivo by successive passages in splenectomised *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), following a previously described protocol [29].

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. P. 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. P. vivax VCG-I strain genomic DNA was used as template for PCR amplification. Primer design was based on the open reading frame (ORF) found by sequence homology in the partial *P. vivax* Sal-I strain genomic sequence reported at TIGR. The primers used encompassed the entire ORF; *Bam*HI and *Pst*I cloning sites were added to the forward (5′-CGGGATCCAAAGCGATAATTCTGATTTCCT-3′) and reverse (5′-AACTGCAGTTATGACTCCATACCTTTCTC-3′) primers, respectively. PCR products were purified by Wizard PCR preps Kit (Promega, Madison, WI) and cloned in the pGEM-T vector (Promega, Madison, WI). Plasmid DNA from recombinant bacteria was purified using the 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 *Plasmodium vivax* RNA was extracted from infected *Aotus* monkey blood samples by the Trizol method [30] 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 synthesised for 30 min at 55 °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 90 s, and a final extension cycle of 68 °C for 5 min. Another PCR amplification using Platinum *Taq* (Invitrogen, Carlsbad, CA) and 1 μg total RNA as template was used as negative control to discard genomic DNA presence. RT-PCR-amplified products were further cloned and sequenced similarly to that described above for PCR products amplified from *P. vivax* genomic DNA.

Peptide synthesis. Two 20-mer peptides, corresponding to the deduced *P. vivax* Sal-I RAP2 sequence, were synthesised. The sequences shown in one-letter, amino-acid code were: ^{49}RKILGNWVHFFFSHFSNPTD^{68} and ^{324}TYKMPNLKGLRLLKSFFRRK^{343}. One glycine and one cysteine were added at both N- and C-termini of each peptide to allow further peptide polymerisation. Peptides were synthesised 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 [31]. Side protection groups and peptide from resin were removed using HF low/high procedure [32]. Peptides were extracted, lyophilised, and finally characterised by RP-HPLC and MALDI-TOF MS.

Rabbit immunisation. New Zealand white rabbits were provided by the Instituto Nacional de Salud (Bogotá, 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 polymerised peptides emulsified in Freund's complete adjuvant (FCA) was 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 [33].

Recombinant protein expression and purification. The *Pv*RAP2 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. The recombinant plasmid was transfected into *Escherichia coli* M15 strain. 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 sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) stained with Coomassie blue or Western blot. Total protein amount was quantified by bicinchoninic acid assay.

SDS–PAGE and Western immunoblotting. Two different SDS–PAGE experiments were carried out. In the first, purified *Pv*RAP2, produced as a recombinant protein, was size-separated in 14% SDS–PAGE and then electroblotted onto a nitrocellulose membrane. In the second, blood samples were taken from a *P. vivax* infected monkey (having greater than 6% parasitaemia) and RBCs infected with mature parasites were purified by Percoll gradient as described above. They 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 15% SDS–PAGE and then electroblotted onto a nitrocellulose membrane. Both membranes were 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. Only Western blot results using sera from the rabbit displaying the best recognition pattern (rabbit 414) are shown.

Indirect immunofluorescence assays. Indirect immunofluorescence assays were performed as described elsewhere [34]. Briefly, the *Aotus* monkey *P. vivax*-infected cells were washed in PBS and then fixed with 4% electron microscopy (EM) grade paraformaldehyde and 0.0075% EM grade glutaraldehyde in PBS for 30 min. Fixed cells were washed once in PBS and then permeabilised with 0.1% Triton X-100/PBS for 10 min. Cells were washed again in PBS and then treated with 0.1 mg/ml sodium borohydride ($NaBH_4$)/PBS for 10 min. Following another PBS wash, cells were blocked in 3% BSA/PBS for 1 h. Rabbit polyclonal sera directed against the synthetic peptides were incubated as primary antibody at 1:80 dilution in PBS with 5% BSA for 1 h. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit Ig (Sigma, St. Louis, MO) was used as secondary antibody at 1:100 dilution in PBS with 5% BSA and incubated for 1 h. Slides were then washed in PBS and then mounted in 50% glycerol. Immunoreactivity was observed by using an Axioskop fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Images were recorded by an AxioCam MRm CCD camera (Carl Zeiss, Oberkochen, Germany).

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. DQ130066.

Results and discussion

PvRAP2 gene identification

Taking the *Pf*RAP2 amino-acid sequence (GenBank Accession No. CAA41577) as query sequence, we used the Basic Local Alignment Search Tool (BLAST) to search the *P. vivax* partially sequenced genome (available at TIGR) for a homologous encoded protein. A similar 1203 bp ORF was thus found within a 495,033 bp contig. This ORF encodes a protein having an estimated 46.6 kDa molecular mass.

The *Pv*RAP2 gene encodes a 400 amino acid protein (just two amino acids more than *Pf*RAP2). As can be seen in Fig. 1, when both proteins are aligned, they display 43% identity and 64% similarity. Similar values have been reported for other *P. vivax* antigens when compared to their falciparum homologues [17,35]. Additional features are shared amongst *Pv*RAP2 and *Pf*RAP2. A putative signal peptide comprising the first 21 *Pv*RAP2 N-terminal amino acids, followed by a cleavage site after glycine 21, were found using SignalP v3.0 software [36], similar to what occurs in *Pf*RAP2 [19] (Fig. 1). *Pv*RAP2 is also a basic protein having a calculated isoelectric point (p*I*) of 8.77 (*Pf*RAP2 p$I = 8.9$) and lacks repetitive elements as predicted by REP v1.1 software [37]. The four cysteine residues present in both *Pv*RAP2 and *Pf*RAP2 were found to be located in the same position in the alignment (Fig. 1).

Similarity amongst those genes neighbouring *Pv*RAP2 in the contig and those neighbouring *Pf*RAP2 in *P. falciparum* chromosome 5 was studied in order to confirm gene synteny. As can be seen in Fig. 2, the ORFs of both *Pv*RAP2 and *Pf*RAP2 are orientated in the same direction. Also, both genes located downstream of RAP2 in both *P. falciparum* and *P. vivax* displayed a high degree of identity and similarity; they were orientated in the same direction. Nevertheless, the gene found upstream of *Pv*RAP2 showed no similarity with the two genes found in the corresponding *P. falciparum* region. It has been found in a previous study that *P. falciparum* has an additional copy of the *Pf*RAP2 gene which has been named *Pf*RAP3 [38]. This gene is located upstream of *Pf*RAP2 and it has been proposed that it arose from a gene duplication process. It seems that *P. vivax* has just one copy of *Pv*RAP2, as occurs in other *Plasmodium* species other than *P. falciparum*, such

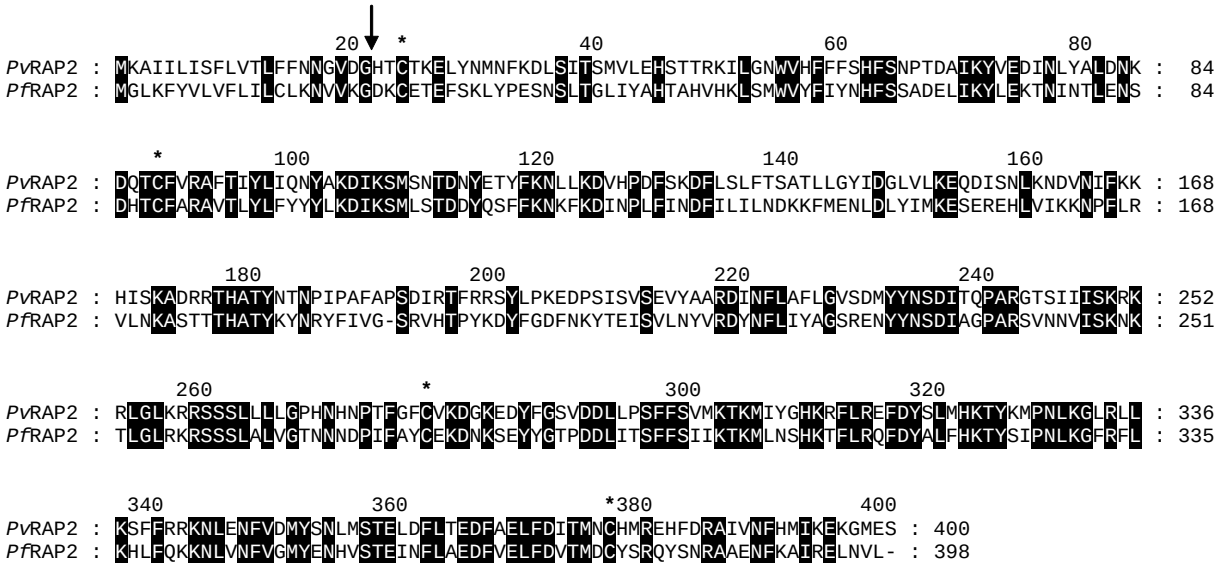

Fig. 1. *Pf*RAP2 and *Pv*RAP2 amino-acid alignment. Black highlighting indicates identical residues. (*) The four cysteines present in both mature peptides are conserved. The vertical arrow indicates the signal peptide cleavage position.

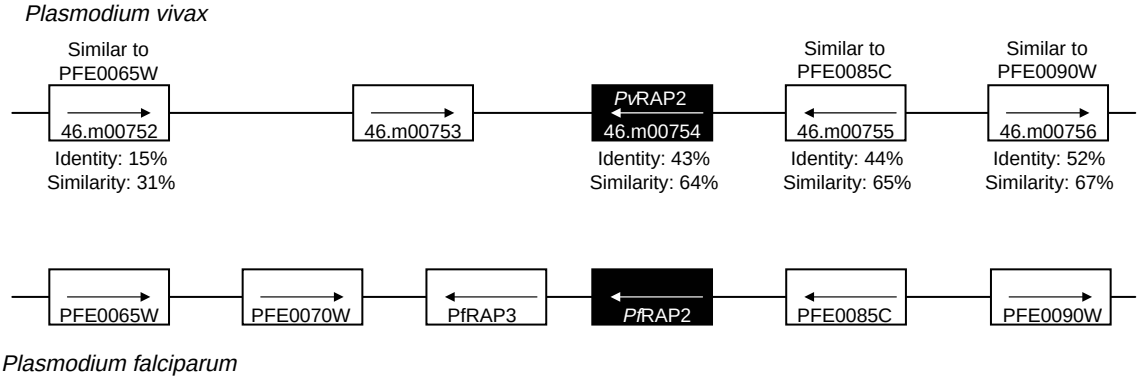

Fig. 2. Schematic diagram comparing *P. falciparum* RAP2 and its neighbouring genes' localisation in chromosome 5 and the corresponding region in *P. vivax*. Arrows indicate the direction of each ORF. *P. vivax* genes have been named according to the partial *P. vivax* genome annotation carried out by TIGR. Identity and similarity percentages are displayed below each *P. vivax* ORF.

as *P. chabaudi*, *P. yoelii*, and *P. berghei* [38]. Upstream of this non-homologous stretch, *P. vivax* genes start displaying similarity to the corresponding *P. falciparum* ones again (Fig. 2). Taken together, these data suggest that gene synteny is preserved in the locus encoding RAP2 gene in both parasite species.

PvRAP2 gene is transcribed in blood-stage parasites

*Pv*RAP2 gene transcription was studied by extracting RNA from parasites obtained from blood taken from a *P. vivax*-infected *Aotus* monkey. After RNA was extracted, it was treated with DNAse to avoid genomic DNA contamination. As observed in Fig. 3, a single ~1200 bp amplification band was obtained in the RT-PCR assay. No DNA contamination was present, since no band was amplified in the negative control. As positive control, *P. vivax* genomic DNA was used as template for PCR. A single ~1200 bp amplification band was observed, similar to that obtained in the RT-PCR.

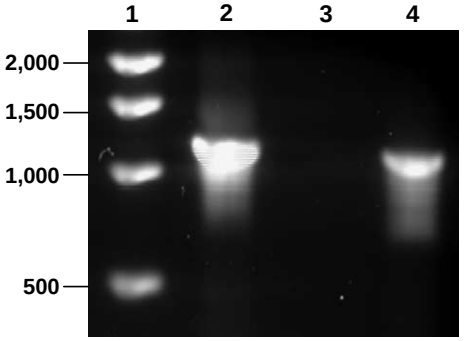

Fig. 3. One-step RT-PCR analysis of total RNA isolated from *P. vivax* asexual stage parasites. Size standards (lane 1) are indicated in basepairs at the left-hand side of the panel. Primers corresponding to the predicted 5′ and 3′ ends of the *Pv*RAP2 ORF were used in one-step RT-PCR on DNase-treated total RNA from asexual stage *P. vivax* parasites (lane 2). Negative control for the RNA sample is shown in lane 3. The same PCR was performed with *P. vivax* genomic DNA as template as positive control (lane 4).

PvRAP2 is encoded by a single exon

Amplified products obtained from both *P. vivax* DNA and RNA were further cloned and sequenced. No differences were observed when DNA and RNA sequences were compared, confirming that *Pv*RAP2 is encoded by a single exon, as occurs with its *P. falciparum* homologue [19]. A single nucleotide difference was observed when *Pv*RAP2 obtained from the VCG-I strain was compared to the Sal-I strain sequence available at TIGR. This non-synonymous substitution was present in nucleotide position 575 and led to an amino acid change from a tyrosine (in Sal-I) to a serine (in VCG-I). Very low polymorphism has been reported in the *Pf*RAP2 gene [19]. Although, according to our results, the same phenomenon seems to be occurring in *Pv*RAP2, sequencing of additional *P. vivax* isolates is required to confirm this assumption.

PvRAP2 is expressed during asexual stage

Polyclonal rabbit sera raised against two synthetic peptides based on part of the *Pv*RAP2 amino-acid sequence were produced in order to carry out further immunochemistry assays leading to determining *Pv*RAP2 protein expression in blood-stage parasites. As the rabbits were being immunised, the complete *Pv*RAP2 was being expressed and purified as a recombinant protein in *E. coli*.

Purified recombinant *Pv*RAP2 was run on a SDS–PAGE and electroblotted into a nitrocellulose membrane. As can be seen in the Western blot assay shown in Fig. 4, a single band was recognised by rabbit 414 hyper-immune serum but not by its corresponding pre-immune serum, suggesting that those antibodies raised against the *Pv*RAP2-derived synthetic peptides were able to recognise the complete *Pv*RAP2 when expressed as a recombinant molecule. Although the molecular mass of the band being recognised is a little higher than that expected, it is worth noting that the expressed recombinant includes the signal peptide and some extra amino acids added by the expression vector.

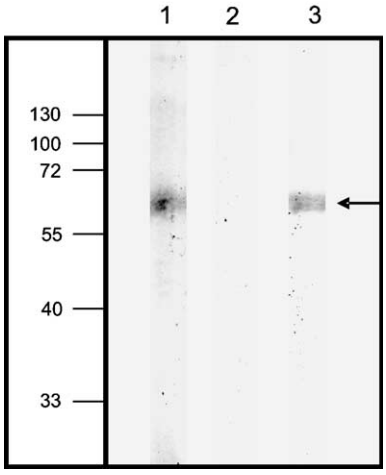

Fig. 4. *Pv*RAP2 recombinant protein was size-separated by SDS–PAGE, electroblotted, and tested with rabbit sera raised against two *Pv*RAP2 synthetic peptides. Lane 1: purified recombinant *Pv*RAP2 recognition by anti-histidine monoclonal antibody (positive control); lane 2: purified recombinant *Pv*RAP2 recognition by rabbit 414 pre-immune serum; lane 3: purified recombinant *Pv*RAP2 recognition by rabbit 414 hyper-immune anti-*Pv*RAP2 serum. Molecular mass standards are indicated on the left-hand side of the panel in kilodaltons.

Plasmodium vivax protein lysate obtained from an infected *Aotus* monkey's blood was run on SDS–PAGE and electroblotted into a nitrocellulose membrane. Western blot analysis using rabbit 414 pre-immune and hyper-immune sera was carried out. Fig. 5 shows that hyperimmune serum recognised a 46 kDa band in *P. vivax* parasite lysate. The molecular mass of the band being recognised was in agreement with the expected *Pv*RAP2 migration pattern. As previously described for *Pf*RAP2 [19], apart from signal peptide cleavage, no further proteolytical processing seems to occur in *Pv*RAP2.

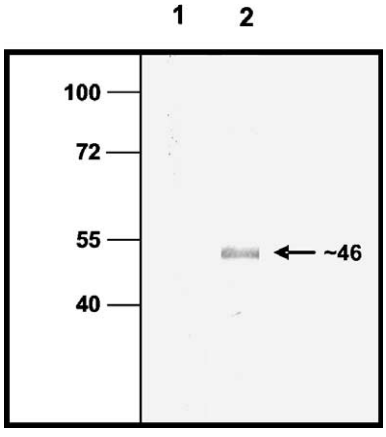

Fig. 5. *Plasmodium vivax* parasite lysate was size-separated by SDS–PAGE, electroblotted, and tested with rabbit sera raised against *Pv*RAP2 recombinant protein. Lane 1: parasite lysate recognition by pre-immune rabbit 414 serum; lane 2: parasite lysate recognition by rabbit 414 anti-*Pv*RAP2 serum. Molecular mass standards are indicated on the left of the panel in kilodaltons. On the right-hand side, the arrow indicates the detected band and its estimated molecular mass is shown in kilodaltons.

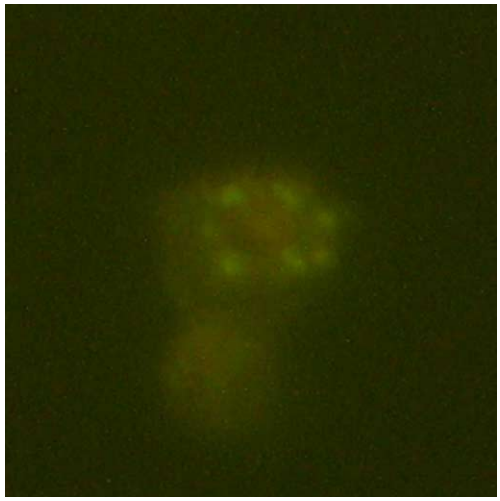

Fig. 6. Indirect immunofluorescence assay using rabbit 414 anti-*Pv*RAP2 serum showing parasite labelling of a fixed *P. vivax* schizont. The punctate fluorescence pattern observed is characteristic of rhoptry proteins.

PvRAP2 displays a typical rhoptry localisation pattern as determined by IFA

A blood sample obtained from a *P. vivax*-infected *Aotus* monkey was used as a source for fresh parasitised RBCs. Immunofluoresence microscopy was thus carried out using the rabbit 414 hyper-immune anti-*Pv*RAP2 polyclonal antiserum as primary antibody. As can be seen in Fig. 6, a punctate fluorescence pattern was observed. Previous studies have shown that this staining pattern is typical of rhoptry proteins [38–40]. No fluorescence was observed when pre-immune rabbit 414 serum was used (data not shown).

Rhoptry discharge of a membranous material into the forming parasitophorous vacuole is one of the molecular events occurring during RBC invasion by merozoites [20,21]. Three proteins (rhoptry-associated proteins 1, 2, and 3) located in the rhoptry base have been identified in *P. falciparum* and have been grouped under the name of the "low-molecular-mass complex" [19,38,41].

Previous data obtained by studying RAP2 in *P. falciparum* have shown this protein's potential as an anti-malarial vaccine candidate. *Pf*RAP2, either extracted from cultured parasites or produced as a recombinant protein, has induced protection against experimental challenge with *P. falciparum*-infected RBCs in immunised monkeys [25–27]. In addition, *Pf*RAP2 displays very low polymorphism [19] and peptides derived from its primary amino-acid sequence are able to specifically bind RBCs [23].

The *P. falciparum* RAP2 homologue has been identified and characterised in *P. vivax* in the present study. This is the first member of the "low-molecular-mass complex" to be described in *P. vivax*. Taking into account the results obtained with its *P. falciparum* homologue as a potential vaccine candidate, we believe that further protection studies should be carried out using *Pv*RAP2 as a vaccine candidate against *P. vivax* malaria.

Acknowledgments

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References

[1] B. Greenwood, T. Mutabingwa, Malaria in 2002, Nature 415 (2002) 670–672.
[2] R.W. Snow, C.A. Guerra, A.M. Noor, H.Y. Myint, S.I. Hay, The global distribution of clinical episodes of *Plasmodium falciparum* malaria, Nature 434 (2005) 214–217.
[3] B. Sina, Focus on *Plasmodium vivax*, Trends Parasitol. 18 (2002) 287–289.
[4] T.L. Richie, A. Saul, Progress and challenges for malaria vaccines, Nature 415 (2002) 694–701.
[5] F. Chandre, F. Darrier, L. Manga, M. Akogbeto, O. Faye, J. Mouchet, P. Guillet, Status of pyrethroid resistance in *Anopheles gambiae sensu lato*, Bull World Health Organ. 77 (1999) 230–234.
[6] R.G. Ridley, Medical need, scientific opportunity and the drive for antimalarial drugs, Nature 415 (2002) 686–693.
[7] M.J. Gardner, N. Hall, E. Fung, O. White, M. Berriman, R.W. Hyman, J.M. Carlton, A. Pain, K.E. Nelson, S. Bowman, et al., Genome sequence of the human malaria parasite *Plasmodium falciparum*, Nature 419 (2002) 498–511.
[8] Z. Bozdech, M. Llinas, B.L. Pulliam, E.D. Wong, J. Zhu, J.L. DeRisi, The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*, PLoS Biol. 1 (2003) E5.
[9] K.G. Le Roch, Y. Zhou, P.L. Blair, M. Grainger, J.K. Moch, J.D. Haynes, P. De La Vega, A.A. Holder, S. Batalov, D.J. Carucci, et al., Discovery of gene function by expression profiling of the malaria parasite life cycle, Science 301 (2003) 1503–1508.
[10] L. Florens, M.P. Washburn, J.D. Raine, R.M. Anthony, M. Grainger, J.D. Haynes, J.K. Moch, N. Muster, J.B. Sacci, D.L. Tabb, et al., A proteomic view of the *Plasmodium falciparum* life cycle, Nature 419 (2002) 520–526.
[11] N. Hall, M. Karras, J.D. Raine, J.M. Carlton, T.W. Kooij, M. Berriman, L. Florens, C.S. Janssen, A. Pain, G.K. Christophides, et al., A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses, Science 307 (2005) 82–86.
[12] E. Lasonder, Y. Ishihama, J.S. Andersen, A.M. Vermunt, A. Pain, R.W. Sauerwein, W.M. Eling, N. Hall, A.P. Waters, H.G. Stunnenberg, et al., Analysis of the *Plasmodium falciparum* proteome by high-accuracy mass spectrometry, Nature 419 (2002) 537–542.
[13] L.K. Basco, J. Le Bras, Short-term in vitro culture of *Plasmodium vivax* and *P. ovale* for drug-susceptibility testing, Parasitol. Res. 80 (1994) 262–264.
[14] J. Carlton, The *Plasmodium vivax* genome sequencing project, Trends Parasitol. 19 (2003) 227–231.
[15] J.M. Carlton, M.R. Galinski, J.W. Barnwell, J.B. Dame, Karyotype and synteny among the chromosomes of all four species of human malaria parasite, Mol. Biochem. Parasitol. 101 (1999) 23–32.
[16] J.M. Carlton, R. Vinkenoog, A.P. Waters, D. Walliker, Gene synteny in species of *Plasmodium*, Mol. Biochem. Parasitol. 93 (1998) 285–294.
[17] O. Perez-Leal, A.Y. Sierra, C.A. Barrero, C. Moncada, P. Martinez, J. Cortes, Y. Lopez, L.M. Salazar, J. Hoebeke, M.A. Patarroyo, Identifying and characterising the *Plasmodium falciparum* merozoite surface protein 10 *Plasmodium vivax* homologue, Biochem. Biophys. Res. Commun. 331 (2005) 1178–1184.
[18] O. Perez-Leal, A.Y. Sierra, C.A. Barrero, C. Moncada, P. Martinez, J. Cortes, Y. Lopez, E. Torres, L.M. Salazar, M.A. Patarroyo, *Plasmodium vivax* merozoite surface protein 8 cloning, expression, and characterisation, Biochem. Biophys. Res. Commun. 324 (2004) 1393–1399.
[19] A. Saul, J. Cooper, D. Hauquitz, D. Irving, Q. Cheng, A. Stowers, T. Limpaiboon, The 42-kilodalton rhoptry-associated protein of *Plasmodium falciparum*, Mol. Biochem. Parasitol. 50 (1992) 139–149.
[20] M. Aikawa, L.H. Miller, J. Johnson, J. Rabbege, Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite, J. Cell Biol. 77 (1978) 72–82.
[21] L.H. Bannister, G.H. Mitchell, G.A. Butcher, E.D. Dennis, Lamellar membranes associated with rhoptries in erythrocytic merozoites of *Plasmodium knowlesi*: a clue to the mechanism of invasion, Parasitology 92 (Pt. 2) (1986) 291–303.
[22] D.L. Baldi, K.T. Andrews, R.F. Waller, D.S. Roos, R.F. Howard, B.S. Crabb, A.F. Cowman, RAP1 controls rhoptry targeting of RAP2 in the malaria parasite *Plasmodium falciparum*, EMBO J. 19 (2000) 2435–2443.
[23] R. Lopez, J. Valbuena, H. Curtidor, A. Puentes, L.E. Rodriguez, J. Garcia, J. Suarez, R. Vera, M. Ocampo, M. Trujillo, et al., *Plasmodium falciparum*: red blood cell binding studies using peptides derived from rhoptry-associated protein 2 (RAP2), Biochimie 86 (2004) 1–6.
[24] L.H. Perrin, E. Ramirez, P.H. Lambert, P.A. Miescher, Inhibition of *P. falciparum* growth in human erythrocytes by monoclonal antibodies, Nature 289 (1981) 301–303.
[25] L.H. Perrin, B. Merkli, M.S. Gabra, J.W. Stocker, C. Chizzolini, R. Richle, Immunization with a *Plasmodium falciparum* merozoite surface antigen induces a partial immunity in monkeys, J. Clin. Invest. 75 (1985) 1718–1721.
[26] R.G. Ridley, B. Takacs, H. Etlinger, J.G. Scaife, A rhoptry antigen of *Plasmodium falciparum* is protective in *Saimiri* monkeys, Parasitology 101 (Pt. 2) (1990) 187–192.
[27] W.E. Collins, A. Walduck, J.S. Sullivan, K. Andrews, A. Stowers, C.L. Morris, V. Jennings, C. Yang, J. Kendall, Q. Lin, et al., Efficacy of vaccines containing rhoptry-associated proteins RAP1 and RAP2 of *Plasmodium falciparum* in *Saimiri boliviensis* monkeys, Am. J. Trop. Med. Hyg. 62 (2000) 466–479.
[28] Y. Pico de Coana, J. Rodriguez, E. Guerrero, C. Barrero, R. Rodriguez, M. Mendoza, M.A. Patarroyo, A highly infective *Plasmodium vivax* strain adapted to *Aotus* monkeys: quantitative haematological and molecular determinations useful for *P. vivax* malaria vaccine development, Vaccine 21 (2003) 3930–3937.
[29] V. Fernandez, Separation of *Plasmodium falciparum* mature stages in Percoll/sorbitol gradients, in: I. Ljungström, H. Perlmann, M. Schlichtherle, A. Scherf, M. Wahlgren (Eds.), Methods in Malaria Research, MR4/ATCC, Manassas,Virginia, 2004.
[30] P. Chomczynski, A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples, Biotechniques 15 (1993) 532–534, See also pp. 536–537.
[31] V.K. Sarin, S.B. Kent, J.P. Tam, R.B. Merrifield, Quantitative monitoring of solid-phase peptide synthesis by the ninhydrin reaction, Anal. Biochem. 117 (1981) 147–157.
[32] J.P. Tam, W.F. Heath, R.B. Merrifield, SN 1 and SN 2 mechanisms for the deprotection of synthetic peptides by hydrogen fluoride. Studies to minimize the tyrosine alkylation side reaction, Int. J. Pept. Protein Res. 21 (1983) 57–65.
[33] F.M. Mallon, M.E. Graichen, B.R. Conway, M.S. Landi, H.C. Hughes, Comparison of antibody response by use of synthetic adjuvant system and Freund complete adjuvant in rabbits, Am. J. Vet. Res. 52 (1991) 1503–1506.
[34] C.J. Tonkin, G.G. van Dooren, T.P. Spurck, N.S. Struck, R.T. Good, E. Handman, A.F. Cowman, G.I. McFadden, Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method, Mol. Biochem. Parasitol. 137 (2004) 13–21.
[35] C.G. Black, J.W. Barnwell, C.S. Huber, M.R. Galinski, R.L. Coppel, The *Plasmodium vivax* homologues of merozoite surface proteins 4 and 5 from *Plasmodium falciparum* are expressed at different locations in the merozoite, Mol. Biochem. Parasitol. 120 (2002) 215–224.

[36] J.D. Bendtsen, H. Nielsen, G. von Heijne, S. Brunak, Improved prediction of signal peptides: SignalP 3.0, J. Mol. Biol. 340 (2004) 783–795.
[37] M.A. Andrade, C.P. Ponting, T.J. Gibson, P. Bork, Homology-based method for identification of protein repeats using statistical significance estimates, J. Mol. Biol. 298 (2000) 521–537.
[38] D.L. Baldi, R. Good, M.T. Duraisingh, B.S. Crabb, A.F. Cowman, Identification and disruption of the gene encoding the third member of the low-molecular-mass rhoptry complex in *Plasmodium falciparum*, Infect. Immun. 70 (2002) 5236–5245.
[39] J.T. Clark, R. Anand, T. Akoglu, J.S. McBride, Identification and characterisation of proteins associated with the rhoptry organelles of *Plasmodium falciparum* merozoites, Parasitol. Res. 73 (1987) 425–434.
[40] R.F. Howard, H.A. Stanley, G.H. Campbell, R.T. Reese, Proteins responsible for a punctate fluorescence pattern in *Plasmodium falciparum* merozoites, Am. J. Trop. Med. Hyg. 33 (1984) 1055–1059.
[41] R.G. Ridley, B. Takacs, H.W. Lahm, C.J. Delves, M. Goman, U. Certa, H. Matile, G.R. Woollett, J.G. Scaife, Characterisation and sequence of a protective rhoptry antigen from *Plasmodium falciparum*, Mol. Biochem. Parasitol. 41 (1990) 125–134.