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# Characterisation of the *Plasmodium vivax* Pv38 antigen

Alvaro Mongui [a], Diana I. Angel [a], Catalina Guzman [a], Magnolia Vanegas [a,b], Manuel A. Patarroyo [a,b,*]

[a] *Molecular Biology Department, Fundacion Instituto de Inmunologia de Colombia (FIDIC), Carrera 50 No. 26-20, Bogota, Colombia*
[b] *Universidad del Rosario, Calle 63D No. 24-31, Bogota, Colombia*

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ABSTRACT

This study describes the identification and characterisation of Pv38, based on the available genomic sequence of *Plasmodium vivax* and previous studies done with its *Plasmodium falciparum* homologue: Pf38. Pv38 is a 355 amino acid long peptide encoded by a single exon gene, for which orthologous genes have been identified in other *Plasmodium* species by bioinformatic approaches. As for Pf38, Pv38 was found to contain a s48/45 domain which is usually found in proteins displayed on gametocytes surface. The association of Pv38 with detergent-resistant membranes (DRMs), its expression in mature blood stages of the parasite (mainly schizonts) and the detection of its recombinant protein by sera from *Aotus* monkeys previously exposed to the parasite, were here assessed to further characterise this new antigen.


Malaria remains as one of the major public health problems worldwide, causing more than 2.5 million deaths yearly. The disease in humans is exclusively attributed to four species belonging to the genus *Plasmodium*: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*, with *P. falciparum* and *P. vivax* accounting for ~90% of all reported malaria cases [1]. Malarial symptoms are mainly due to the intra-erythrocytic parasite cycle, where merozoites invade red blood cells (RBCs) every 48–72 h. RBC invasion process is characterised by being complex and by including highly coordinated events [2] driven by a series of essential proteins localised in the apical secretory organelles [3]. Several studies have been performed aimed at characterising these proteins, including functional assays and protection studies to evaluate their suitability as potential anti-malarial vaccine candidates.

Current scientific and technological advances have led to more clearly elucidating pathogen-host interactions between *P. falciparum* and human host cells, counting amongst the most relevant ones: the complete genome sequencing of *P. falciparum* and the transcriptional and proteome profiles for its different developmental stages. Other studies have contributed to the identification of *P. falciparum* schizont proteins, mainly those localising in detergent-resistant membrane (DRM) regions [4,5]. Most of these proteins are either bound to the merozoite membrane by a GPI anchor or non-covalently associated with anchored proteins. Twelve GPI-anchored proteins in *P. falciparum* (MSP-1, -2, -4, -5, -8, -10, Pf12, Pf34, Pf38, Pf92, RAMA and ASP) have been described to date and most of them have been found to participate in *Plasmodium* parasites invasion of RBCs [6,7].

Pf38 (previously known as Pfs38) was initially identified in *P. falciparum* as a protein belonging to the Ps230 family [8,9]. This protein family (broadly characterised during the parasite's gametocyte stage) includes several surface proteins which are currently being evaluated as potential transmission-blocking vaccine candidates [10]. It has also been found that the gene encoding Pf38 is actively transcribed during late stages (schizonts) of the intra-erythrocyte cycle [11]. Another relevant finding concerns the strong recognition of the native Pf38 protein by serum antibodies from individuals naturally exposed to *P. falciparum* infection [4]. Moreover, when analysing the relative amount of proteins anchored by GPI to the merozoite's surface, only 11 proteins represent ~94% of the GPI-anchored-proteome, Pf38 contributing with an important ~5% [5].

In spite of the great advances done towards identifying new vaccine candidates against *P. falciparum*, similar studies in *P. vivax* have been limited mainly due to the difficulty of culturing the parasite *in vitro*. The complete sequencing of the *P. vivax* genome is currently being done by The Institute of Genomic Research (TIGR). Based on the results of this sequencing study, our group has focused on identifying *P. vivax* proteins which are homologous to those previously characterised in *P. falciparum* as being involved in RBC invasion. We have thus identified and characterised *P. vivax* surface (MSP-7, -8, -10) [12–14] and rhoptry proteins (RAP-1, -2, RhopH3) [15–17] to further assess their potential as vaccine candidates against this parasite species.

This study describes the identification and characterisation of the *P. vivax* Pv38 protein, by using bioinformatics tools, molecular biology techniques and immunochemical assays.

* Corresponding author. Address: Molecular Biology Department, Fundacion Instituto de Inmunologia de Colombia (FIDIC), Carrera 50 No. 26-20, Bogota, Colombia. Fax: +57 1 4815269.
*E-mail address:* mapatarr@fidic.org.co (M.A. Patarroyo).

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## Materials and methods

*The P. vivax genome sequence.* The partial nucleotide sequence of the *P. vivax* genome used in this study (SaI-1 strain) was obtained via the TIGR web page (http://www.tigr.org/tdb/e2k1/pva1/).

*Parasites. P. vivax* parasites (VCG-I strain) were used as DNA, RNA and parasite protein source [18]. The animals used in this study were treated according to regulations previously established by the Office for Protection from Research Risks (OPRR, Department of Health and Human Services, USA). Blood samples were obtained from monkeys according conditions established under the agreement with Corpo-Amazonia (resolution 00066, September 13th 2006). The greatest percentage of RBCs infected with late stage parasites (mainly schizonts) were extracted from 3–4 mL blood samples taken from infected animals using a Percoll discontinuous gradient [19].

*Extracting RNA and cDNA synthesis.* Parasite total RNA was extracted by using the Trizol method [20] and treated with RQ1 RNase-free DNase (Promega). One microgram of extracted RNA was used for RT-PCR, using the SuperScript III enzyme (Invitrogen) in 20 μL reactions. cDNA was thus synthesised for 60 min at 50 °C.

*Cloning and sequencing.* Primers were designed from the sequence of a putative transcript encoding the *P. vivax* Pv38 protein found by BLAST search in the reported genome for this species, using the Pf38 reported protein sequence as bait for finding the *P. vivax* cDNA homologue. Primers used covered the whole coding sequence (5′-ATGAGGCCCGCGAGG-3′ forward primer, 5′-CGCGCCCAGGA GAAG-3′ reverse primer). PCR amplifications from both genomic DNA and cDNA were then done with the Platinum *Pfx* DNA polymerase enzyme (Invitrogen) for 35 cycles at the following temperatures: 94 °C for 15 s, 58 °C for 30 s, 68 °C for 80 s and a final extension step at 68 °C for 5 min. PCR products were purified and cloned in pEXP5-CT/TOPO vector (Invitrogen, California, USA). Cloned insert integrity was confirmed by automatic sequencing in an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, California, USA).

*Peptide synthesis.* Two 20-amino-acid-long peptides were synthesised, based on portions of the Pv38 deduced sequence (SaI-1). Amino acid sequences (shown in single letter code) were: $^{34}$LSSSAKSVFLLNVAPGDTVV$^{53}$ and $^{312}$DRGEHSKHNFSSTTVTEREE$^{331}$. A glycine and a cysteine were inserted at the N- and C-termini of each peptide to allow polymerisation. Peptides were synthesised using the standard solid phase t-Boc/Bzl peptide synthesis strategy [21], lyophilised and then characterised by RP-HPLC and MALDI-TOF MS.

*Immunisation in rabbits and collecting their sera.* Each of two New Zealand rabbits received the polymerised synthetic peptide mix. The initial peptide dose (150 μg) was emulsified in Freund's complete adjuvant (FCA) (Sigma, Missouri, USA), 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, where the Pv38 gene was cloned, adds a six-histidine tag at the protein's C-terminal portion. The protein was purified under denaturing conditions using 6 M urea in a Ni + 2-NTA resin (Qiagen, California, USA). Expression was verified on a 12–14% polyacrylamide gel in the presence of SDS (SDS-PAGE) which was stained with Comassie blue or evaluated by Western blot. The pure recombinant protein fractions were pooled and exhaustively dialysed against PBS (1×) pH 7.4 (protein refolding step).

*SDS-PAGE and Western blot.* The malaria parasite was lysed with a solution containing 5% w/v SDS, 1 mM EDTA, 10 mM PMSF and 10 mM iodoacetamide. Lysate proteins were size-separated by electrophoresis and then transferred to a nitrocellulose membrane. The Western blot was carried out as described elsewhere [17].

*Recognition of recombinant protein by sera from Aotus previously infected by P. vivax.* Polysorb plates were loaded with Pv38 recombinant protein (1 μg/well), incubated overnight at 4 °C and washed thrice with PBS-0.05% v/v Tween 20 (PBS-Tween). Plates were blocked at 37 °C for 1 h with 5% w/v skimmed milk in PBS-Tween. A pool of sera from *Aotus* monkeys which had had several prior episodes of *P. vivax* malaria was added in duplicate in a 1:100 dilution and incubated for 1 h at 37 °C. Plates were then washed thrice with PBS-Tween. A 1:10,000 dilution of goat anti-*Aotus* IgG antibodies coupled to peroxidase were added as secondary antibody, followed by incubation for 1 h at 37 °C. Excess peroxidase-coupled antibody was removed by washing thrice with PBS-Tween. Plates were revealed by using a TMB Microwell Peroxidase Substrate System kit (KPL Laboratories, Washington, USA) and absorbance was read at 620 nm.

*Isolation of detergent-resistant membranes (DRMs).* Parasitised RBCs, mainly in schizont stage, were treated with 0.2% w/v saponin and then washed thrice with PBS. Two different aliquots of parasite were separately suspended in ice-cold TNET buffer (1% w/v Triton X-100, 25 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5) plus protease inhibitors. Each aliquot was treated as follows: (1) incubated in ice for 30 min and centrifuged at 10,000*g*, 4 °C for 10 min and (2) kept at 37 °C for 30 min and spun at 10,000g at room temperature for 10 min. Insoluble pellet from each sample was then suspended in TNET buffer (50 μL), incubated at 37 °C for 30 min and centrifuged at 10,000g at room temperature for 10 min. All samples were resolved by SDS-PAGE and immunoblotting was then carried out using a pool of hyper-immune sera from rabbits previously immunised with the Pv38-derived synthetic peptides.

*Indirect immunofluorescence assays.* A fresh parasite sample was stained by using rabbit polyclonal antibodies as a primary antibody and goat anti-rabbit IgG conjugate labelled with fluorescein isothiocyanate as secondary antibody, following a previously described protocol [12]. Fluorescence was analysed using an Olympus BX51 microscope.

*Accession number.* Nucleotide and amino acid sequences described in the present study have been reported in the GenBank database under the accession number EU305671.

## Results and discussion

### *Identifying the P. vivax Pv38 encoding gene*

The Pv38 protein was identified in *P. vivax* by using the amino acid sequence of its homologue in *P. falciparum* (Pf38) as bait (GenBank accession no. AAK57742). A search was made in the *P. vivax* genome database (currently hosted at TIGR) by using a local alignment tool (tBlastn). The PlasmoDB and Sanger Institute databases were also analysed, searching for Pf38 orthologue genes in other *Plasmodium* species. A 521,442 bp chromosome segment was found in *P. vivax* (ctg_7027) containing the *pv38* gene. Both *Plasmodium yoelii* and *Plasmodium knowlesi* had a gene similar to Pf38; however, the search was inconclusive for *Plasmodium chabaudi* and *Plasmodium berghei* as their contigs had not been assembled yet, thereby hindering a more thorough analysis.

Open reading frames (ORFs) were predicted (by GenScan and GeneComber software) [22,23] for the chromosome fragments containing the genes encoding Pf, Py, Pk and Pv38 to elucidate their organisation, structure and to verify adjacent genes' integrity. As seen in Fig. 1, the *pf, py, pk* and *pv38* genes were localised in chromosome regions homologous in these four *Plasmodium* species, since downstream and upstream genes shared relatively high identity (*I*) and similarity values (*S*) (at the amino acid level) and conserved the same ORF orientation. These genes thus displayed

*I*: 24–73% and *S*: 36–90% for *P. falciparum vs. P. vivax*; *I*: 25–73% and *S*: 37–88% for *P. falciparum vs. P. knowlesi*, and *I*: 55–89% and *S*: 64–95% for *P. vivax vs. P. knowlesi*. These values agreed with those reported in the literature where *P. vivax* and *P. knowlesi* are more closely related [24]. *P. yoelii* genes were excluded from this analysis since the equivalent contig has not been fully assembled. It should be stressed that, even though the regions being analysed had a high homology, they were not necessarily localised in the same chromosome in all species.

The previously characterised Pf38 protein has 349 amino acids encoded by a 1050 bp gene and an estimated 40.6 kDa molecular weight ($M_w$). It has been predicted that Pf38 orthologues in *P. yoelii* and *P. knowlesi* (Py38 and Pk38) would be 344 and 408 amino acid proteins, encoded by 1035 bp (MALPY00761: from 5789 to 6823 bp) and 1227 bp (chr10: from 1,160,417 to 1,161,643 bp) long genes, respectively. Similarly, as here reported, the gene encoding Pv38 has 1068 bp and encodes a 355 amino acid protein having an estimated 40.5 kDa $M_w$ (Fig. 2A). An N-terminal signal peptide and a C-terminal GPI anchor were predicted in the Pv38 sequence by bioinformatics tools [25,26].

An alignment of the Pf, Pk, Py and Pv38 protein sequences revealed an overall 20.6% identity, 83.5% similarity and a greater degree of conservation towards the central region (*I*: 30.3% and *S*: 99.2%), this latter corresponding to the s48/45 domain (Pfam accession no. PF07422) (Fig. 2A) of Ps230 family proteins. This domain is found in proteins expressed on the parasite's gametocyte surface, playing an important role during fertilisation [27]. The s48/45 domain has a particular cysteine pattern leading to the formation of three disulfide bridges as follows: Cys 1 and 2, Cys 3 and 6, and Cys 4 and 5 (Fig. 2A) [28,29]. It can be seen that the six cysteines from the s48/45 domain are conserved in Pf, Py, Pk and Pv38 and that the four additional cysteines localised towards the protein's N-terminus constitute a partial s48/45 domain.

### *Pv38 is transcribed in blood-stage parasites*

Sequence comparison between *pv38* genomic DNA and cDNA amplification products (Fig. 2B) allowed confirming that this gene is encoded by a single exon. Two nucleotide substitutions were found when comparing the sequences obtained from the

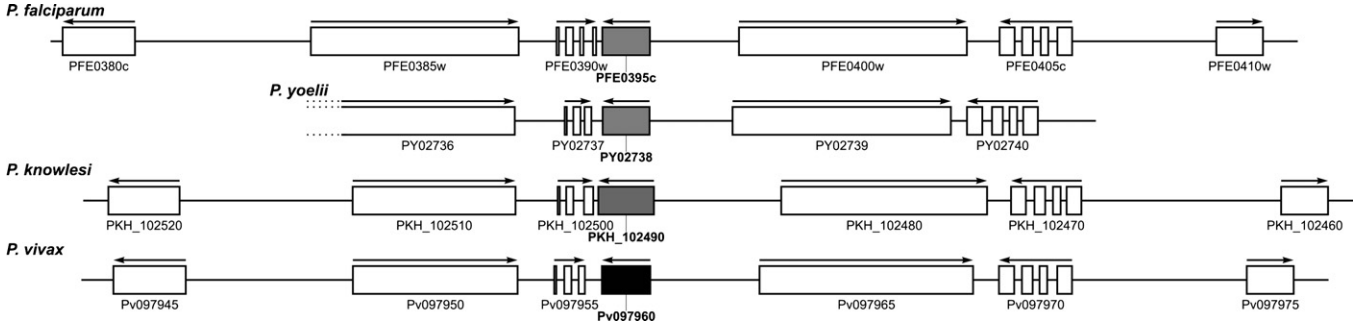


**Fig. 1.** Scale diagram showing the localisation of genes encoding Pf, Py, Pk (in grey) and Pv38 (in bold) and adjacent genes in the *P. falciparum*, *P. yoelii*, *P. knowlesi* and *P. vivax* chromosomes, respectively. PlasmoDB accession numbers, ORFs orientation and exon organisation of each gene are indicated. The analysed fragments comprise 27.5 kbp from *P. falciparum* chromosome 5 (316,501–344,000 bp), the complete length of the assembled *P. yoelii* contig (16,664 bp) (MALPY00761), a 28 kpb region belonging to *P. knowlesi* chromosome 10 (1,145,001–1,173,000 bp) and a 27.5 kbp from the *P. vivax* contig ctg_7027 (386,501–414,000 bp).

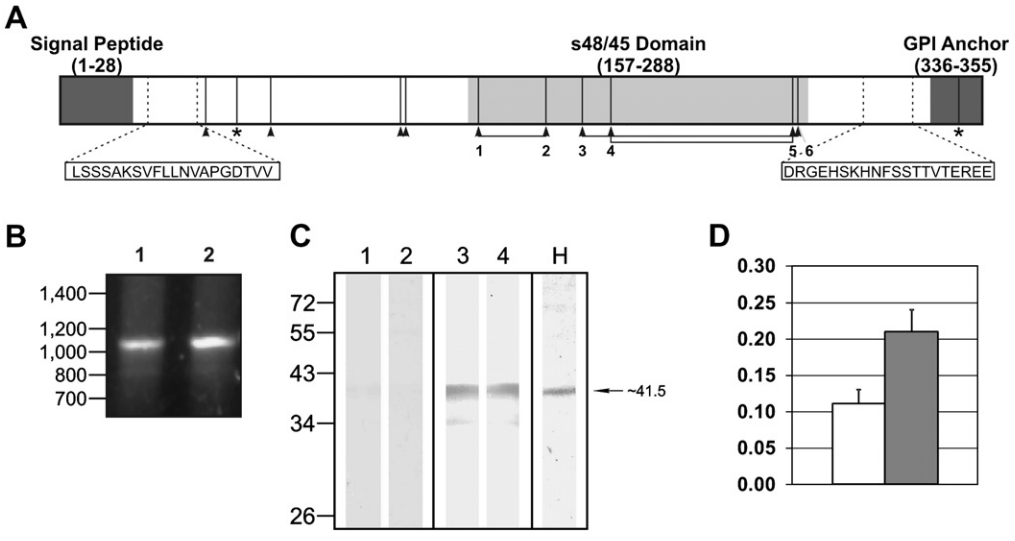


**Fig. 2.** (A) Schematic representation of the complete Pv38 protein showing the signal peptide, the s48/45 domain and the GPI anchoring site. Conserved cysteines' localisation amongst the four *Plasmodium* species (arrows) and cysteines present only in *P. vivax* (asterisks) together with the localisation of the synthetic peptides used in this study (white boxes) are shown. Numbers and arrows below the s48/45 domain depict the three disulfide bridges and the cysteines involved in them. (B) PCR amplification of *P. vivax pv38* from cDNA and genomic DNA. Lane 1, *pv38* RT-PCR. Lane 2, *pv38* PCR from genomic DNA. (C) Western blot of purified Pv38 recombinant protein. Lanes 1 and 2, recognition of Pv38 protein by pre-immune rabbit sera. Lanes 3 and 4, detection of Pv38 protein by hyper-immune rabbit sera. Lane H, recognition of purified Pv38 using anti-histidine monoclonal antibody. The estimated weight of the protein when including the signal peptide and the histidine tag is shown. (D) ELISA recognition of purified recombinant Pv38 protein by sera from *Aotus* monkeys with prior *P. vivax* malaria episodes. The white bar shows Pv38 recognition when extracted under denaturating conditions. The grey bar shows recognition of the refolded protein.

VCG-I-adapted strain and the Sal-1 reference strain (available in TIGR). The substitution in nucleotide 209 involved switching an arginine for a leucine in position 70 (R70L), whilst the one in position 969 was synonymous.

*Pv38 recombinant protein expression and its recognition*

Two synthetic 20-amino-acid-long peptides were designed and chemically synthesised based on the Pv38 predicted sequence (Fig. 2A); they were then used in rabbit immunisation assays for obtaining polyclonal antibodies recognising the complete protein. Sera's ability to react against purified Pv38 protein was assessed by Western blot (Fig. 2C), evidencing the recognition of a single ~41.5 kDa band.

Alternatively, antibodies' ability to recognise the Pv38 recombinant protein was assessed using sera from monkeys who had been previously infected with *P. vivax* malaria. The ELISA revealed that monkeys' sera recognised better the Pv38 protein when it was refolded than when it was denatured (Fig. 2D). This showed that antibodies produced in *Aotus* monkeys by the experimental malarial infection recognised mainly Pv38 conformational epitopes, thus suggesting that if this protein was to be tested as a vaccine candidate against *P. vivax*, it would be better to use the recombinant protein obtained either under non-denaturing conditions or as a refolded protein.

*Pv38 is expressed in P. vivax asexual stages*

When parasite proteins were obtained by using an SDS solution, rabbit hyper-immune sera recognised a band at ~37.4 kDa corresponding to Pv38 in the immunoblot (corresponding to the predicted Pv38 $M_w$ without the signal peptide) (Fig. 3A). On the other hand, Pv38 association with DRMs was evaluated by determining whether the protein localised in the parasite's insoluble fraction upon being suspended in cold non-ionic detergents [30]. Pv38 was partially insoluble in TNET buffer at 4 °C, but completely soluble at 37 °C (Fig. 3A), suggesting that as occurs with its *P. falciparum* homologue, the Pv38 protein is localised in DRMs.

Pv38 showed the typical fluorescence pattern of proteins localising in rhoptries (Fig. 3B), agreeing with the punctate fluorescence pattern of Pf38 which is known to be localised in these organelles [4].

The following evidence found here leads to considering Pv38 as a potential anti-malarial vaccine candidate against *P. vivax*: (1) its expression during the late stages of the parasite's intra-erythrocytic cycle (mature schizonts), like its *P. falciparum* homologue, (2) its association with merozoite's DRMs, since some DRM-associated proteins are currently being considered as vaccine candidates against this parasite species (e.g., *Pv*MSP1) [31], and (3) the enhanced recognition of the refolded protein by sera from *Aotus* monkeys previously exposed to *P. vivax* malaria. It would be therefore convenient to perform future Pv38 immunisation assays using *Aotus* monkeys as an animal model.

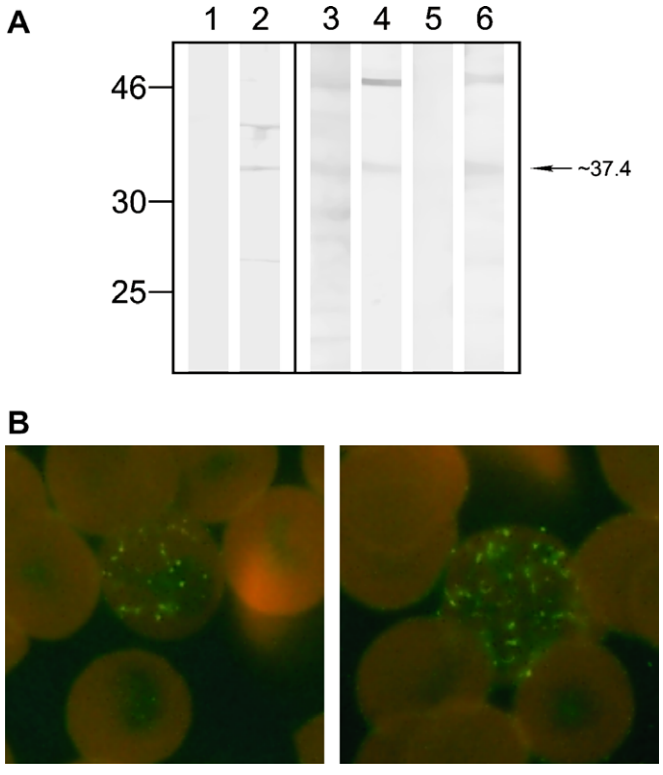


**Fig. 3.** (A) Size-separated and electrotransferred *P. vivax* parasite lysate tested with the rabbit serum raised against synthetic peptides. Lane 1, recognition of the protein by pre-immune rabbit serum. Lane 2, Pv38 detection with hyper-immune rabbit serum. Western blot detection of Pv38 in DRMs using hyper-immune serum. Lanes 3 and 5, Pv38 recognition in the *P. vivax* insoluble fraction upon being suspended at 4 and 37 °C, respectively. Lanes 4 and 6, Pv38 recognition in the *P. vivax* soluble fraction upon being suspended at 4 and 37 °C, respectively. The estimated weight for the native protein without including the signal peptide is shown. (B) Pv38 immunofluorescence pattern in *P. vivax* schizonts.

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## References

[1] 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.
[2] A.F. Cowman, B.S. Crabb, Invasion of red blood cells by malaria parasites, Cell 124 (2006) 755–766.
[3] W. Souza, Secretory organelles of pathogenic protozoa, An. Acad. Bras. Cienc. 78 (2006) 271–291.
[4] P.R. Sanders, P.R. Gilson, G.T. Cantin, D.C. Greenbaum, T. Nebl, D.J. Carucci, M.J. McConville, L. Schofield, A.N. Hodder, J.R. Yates 3rd, B.S. Crabb, Distinct protein classes including novel merozoite surface antigens in Raft-like membranes of *Plasmodium falciparum*, J. Biol. Chem. 280 (2005) 40169–40176.
[5] P.R. Gilson, T. Nebl, D. Vukcevic, R.L. Moritz, T. Sargeant, T.P. Speed, L. Schofield, B.S. Crabb, Identification and stoichiometry of glycosylphosphatidylinositol-anchored membrane proteins of the human malaria parasite *Plasmodium falciparum*, Mol. Cell. Proteomics 5 (2006) 1286–1299.
[6] L. Wang, N. Mohandas, A. Thomas, R.L. Coppel, Detection of detergent-resistant membranes in asexual blood-stage parasites of *Plasmodium falciparum*, Mol. Biochem. Parasitol. 130 (2003) 149–153.
[7] P.R. Sanders, G.T. Cantin, D.C. Greenbaum, P.R. Gilson, T. Nebl, R.L. Moritz, J.R. Yates 3rd, A.N. Hodder, B.S. Crabb, Identification of protein complexes in detergent-resistant membranes of *Plasmodium falciparum* schizonts, Mol. Biochem. Parasitol. 154 (2007) 148–157.
[8] T.J. Templeton, D.C. Kaslow, Identification of additional members define a *Plasmodium falciparum* gene superfamily which includes Pfs48/45 and Pfs230, Mol. Biochem. Parasitol. 101 (1999) 223–227.
[9] J. Thompson, C.J. Janse, A.P. Waters, Comparative genomics in Plasmodium: a tool for the identification of genes and functional analysis, Mol. Biochem. Parasitol. 118 (2001) 147–154.
[10] G. Pradel, Proteins of the malaria parasite sexual stages: expression, function and potential for transmission blocking strategies, Parasitology 134 (2007) 1911–1929.
[11] 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.
[12] A. Mongui, O. Perez-Leal, S.C. Soto, J. Cortes, M.A. Patarroyo, Cloning, expression, and characterisation of a *Plasmodium vivax* MSP7 family merozoite surface protein, Biochem. Biophys. Res. Commun. 351 (2006) 639–644.

[13] 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.
[14] 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.
[15] O. Perez-Leal, A. Mongui, J. Cortes, G. Yepes, J. Leiton, M.A. Patarroyo, The *Plasmodium vivax* rhoptry-associated protein 1, Biochem. Biophys. Res. Commun. 341 (2006) 1053–1058.
[16] M.A. Patarroyo, O. Perez-Leal, Y. Lopez, J. Cortes, J. Rojas-Caraballo, A. Gomez, C. Moncada, J. Rosas, M.E. Patarroyo, Identification and characterisation of the *Plasmodium vivax* rhoptry-associated protein 2, Biochem. Biophys. Res. Commun. 337 (2005) 853–859.
[17] A. Mongui, O. Perez-Leal, J. Rojas-Caraballo, D.I. Angel, J. Cortes, M.A. Patarroyo, Identifying and characterising the *Plasmodium falciparum* RhopH3 *Plasmodium vivax* homologue, Biochem. Biophys. Res. Commun. 358 (2007) 861–866.
[18] 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.
[19] P.M. Andrysiak, W.E. Collins, G.H. Campbell, Concentration of *Plasmodium ovale*- and *Plasmodium vivax*-infected erythrocytes from nonhuman primate blood using Percoll gradients, Am. J. Trop. Med. Hyg. 35 (1986) 251–254.
[20] 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. 536–537.
[21] R.A. Houghten, General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids, Proc. Natl. Acad. Sci. USA 82 (1985) 5131–5135.
[22] C.B. Burge, S. Karlin, Finding the genes in genomic DNA, Curr. Opin. Struct. Biol. 8 (1998) 346–354.
[23] S.P. Shah, G.P. McVicker, A.K. Mackworth, S. Rogic, B.F. Ouellette, GeneComber: combining outputs of gene prediction programs for improved results, Bioinformatics 19 (2003) 1296–1297.
[24] L. Kedzierski, A.A. Escalante, R. Isea, C.G. Black, J.W. Barnwell, R.L. Coppel, Phylogenetic analysis of the genus Plasmodium based on the gene encoding adenylosuccinate lyase, Infect. Genet. Evol. 1 (2002) 297–301.
[25] 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.
[26] B. Eisenhaber, P. Bork, F. Eisenhaber, Prediction of potential GPI-modification sites in proprotein sequences, J. Mol. Biol. 292 (1999) 741–758.
[27] M.R. van Dijk, C.J. Janse, J. Thompson, A.P. Waters, J.A. Braks, H.J. Dodemont, H.G. Stunnenberg, G.J. van Gemert, R.W. Sauerwein, W. Eling, A central role for P48/45 in malaria parasite male gamete fertility, Cell 104 (2001) 153–164.
[28] R. Carter, A. Coulson, S. Bhatti, B.J. Taylor, J.F. Elliott, Predicted disulfide-bonded structures for three uniquely related proteins of *Plasmodium falciparum*, Pfs230, Pfs48/45 and Pf12, Mol. Biochem. Parasitol. 71 (1995) 203–210.
[29] D.L. Gerloff, A. Creasey, S. Maslau, R. Carter, Structural models for the protein family characterized by gamete surface protein Pfs230 of *Plasmodium falciparum*, Proc. Natl. Acad. Sci. USA 102 (2005) 13598–13603.
[30] D.A. Brown, E. London, Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes?, Biochem Biophys. Res. Commun. 240 (1997) 1–7.
[31] C.A. Barrero, G. Delgado, A.Y. Sierra, Y. Silva, C. Parra-Lopez, M.A. Patarroyo, Gamma interferon levels and antibody production induced by two PvMSP-1 recombinant polypeptides are associated with protective immunity against *P. vivax* in Aotus monkeys, Vaccine 23 (2005) 4048–4053.