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Characterization and antigenicity of the promising vaccine candidate *Plasmodium vivax* 34 kDa rhoptry antigen (Pv34)

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ABSTRACT

This study describes the identification of the *Plasmodium vivax* rhoptry antigen Pv34 whose sequence was obtained based on homology comparison with the *Plasmodium falciparum* Pf34. The *pv34* gene product was characterized by molecular biology and immunological techniques. Additionally, association of Pv34 to detergent-resistant microdomains (DRMs), expression in late blood-stage parasites and recognition of recombinant Pv34 (rPv34) by sera from *P. vivax*-infected *Aotus* monkeys and patients was assessed. Lymphoproliferation and cytokine secretion was also evaluated in individuals living in malaria endemic areas. Altogether, the data support carrying out further studies to assess the immunogenicity and protection-inducing ability of rPv34 as component of a multi-antigenic, multi-stage vaccine against vivax malaria.

1. Introduction

Malaria has been the most important parasitic disease throughout history causing about 300–500 million clinical cases each year, 1.5–2.7 million of which die as a consequence of the disease [1]. Additionally, more than 2 billion people live in the 88 countries where the disease is endemic, indicating that ~48% of the world's population is currently at risk of acquiring the disease [2]. The situation is further aggravated by the gradual emergence of parasite strains resistant to antimalarial drugs, as well as of insecticide-resistant *Anopheles* mosquito populations, which make it imperative to develop an effective antimalarial vaccine.

Of the four *Plasmodium* species causing malaria in humans, *Plasmodium falciparum* is responsible for the largest annual number of clinical cases mainly in Africa [3]. This has by itself encouraged a large number of studies carried out over the last few decades on the biology and pathology of this species, most of which have focused on the development of an effective vaccine to control this scourging disease.

In parasites of the genus *Plasmodium*, the identification of possible vaccine targets has been mainly focused on parasite surface antigens and proteins contained inside apical organelles, mainly in rhoptries and micronemes. In general, these apical organelles are shared by all members of the phylum Apicomplexa since they emerged as an early evolutive trait [4] and various proteins contained inside these organelles are relatively conserved among the different parasite genera; however, each genus' tropism for invading an specific cell line (*e.g.* erythrocytes, lymphocytes and epithelial cells) has led to the functional specialization of members belonging to this unique pool of proteins to fulfill different roles during target-cell invasion [5].

Proteins of the low molecular weight complex (LMW) (RAP1, RAP2 and RAP3), the high molecular weight complex (HMW) (RhopH1, RhopH2 and RhopH3), the rhoptry-associated membrane antigen (RAMA) and reticulocyte binding ligands (RBLs) are among the best characterized *P. falciparum* rhoptry antigens. It has been suggested that these proteins might play an important role in host cell selection, erythrocyte binding or parasitophorous vacuole formation [6].

Recent studies have enabled the identification of other parasite rhoptry proteins based on their localization on detergent-resistant microdomains (DRMs), as in the case of the Pf34 protein [7]. Same as other well-studied erythrocyte-stage vaccine candidates (*e.g.* MSP1, MSP2, MSP4 and MSP5), Pf34 contains a glycosylphos-

Abbreviations: Pv34, *P. vivax* 34 kDa rhoptry antigen; rPv34, recombinant *P. vivax* 34 kDa rhoptry antigen; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SaI-1, Salvador 1 strain; VCG-1, Vivax Colombia Guaviare 1 strain; DRMs, detergent-resistant microdomains; PBMCs, peripheral blood mononuclear cells.

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phatidylinositol (GPI)-anchor, which is a common characteristic shared by putative erythrocyte invasion proteins [8]. It has been also demonstrated that the gene encoding Pf34 is actively transcribed in late-erythrocyte stages [9], showing strong correlation with the expression of the native protein during the schizont stage. Moreover, the significant recognition of Pf34 (obtained as a recombinant protein) by hyperimmune sera from patients living in malaria endemic areas [7], highlights Pf34 as an attractive candidate for a vaccine against *P. falciparum* malaria.

On the other hand, advances in the identification and characterization of vaccine candidates in *Plasmodium vivax* (the second most prevalent *Plasmodium* species with predominant distribution in Asia and South America) has been notably delayed due to the difficulty of maintaining a long-term culture of this species *in vitro* given its preference for invading human reticulocyte subpopulations (accounting for only 1–2% of the total blood cell count). This phenomenon together with the absence of endothelial cytoadherence explains the milder symptoms seen in *P. vivax* malaria compared to malaria caused by *P. falciparum*. The unavailability of a standardized continuous culture considerably delayed the completion of the *P. vivax* genome [10] and transcriptome (of the intraerythrocytic cycle) [11] compared to *P. falciparum*, and is reflected in the current absence of proteomic data.

Aiming at developing a vaccine against *P. vivax* malaria, our research group has focused on the identification of new antigens based on homology comparison with previously characterized *P. falciparum* antigens. Using the just until recently partial *P. vivax* genomic sequence and a *P. vivax* strain adapted to *Aotus* monkeys [12], we have been able to identify various surface proteins (MSP-7, -8, -10 and Pv41) [13–16] as well as several rhoptry proteins (RAP-1, -2, RhopH3 and Pv38) [17–20], which are likely to play an important role in parasite invasion to red blood cells and are currently being tested as vaccine candidates.

The present study shows the expression of the Pf34 homolog in *P. vivax*, here denoted as Pv34, its subcellular localization in late-intraerythrocytic parasite life-cycle stages, its recognition by sera from *P. vivax*-infected patients and *Aotus* monkeys when expressed as a recombinant protein (rPv34), its ability to stimulate proliferation of peripheral blood mononuclear cells (PBMCs) from individuals with a history of *P. vivax* malaria and the resulting cytokine profile.

2. Materials and methods

2.1. Bioinformatics analysis

The *P. vivax* SaI-1 strain genome (available at http://www.tigr.org/tdb/e2k1/pva1/) was scanned by tBlastn using Pf34 as query sequence (GenBank accession no. CAD49234.1; PlasmoDB accession no. PFD0955w) in order to confirm the sequence of the gene encoding Pv34. The sequence yielding the highest score was selected as the putative Pv34 sequence. Sanger Institute and J. Craig Venter Institute (JCVI) databases holding partial genome sequences from other plasmodial species were also screened searching for *pf34* or *pv34* homologous genes. Moreover, open reading frames (ORFs) adjacent to *pf34*, *pv34* and *pk34* were analyzed using GenScan and GeneComber [21,22]. Identity and similarity values between *P. vivax*–*P. falciparum* and *P. vivax*–*P. knowlesi* peptide sequences were obtained using the ALignX tool from the VectorNTI Suite 9 bioinformatics software package (Invitrogen, California, USA). The presence of a signal peptide and a GPI-anchor site was determined by using SignalP 3.0 [23] and FragAnchor [24], respectively. Tandem repeats in the Pv34 sequence were identified using the XSTREAM server [25]. The presence of lineal B epitopes in Pv34 was determined using the Bepipred (at a default 0.35 threshold and 75% of specificity) [26]. Parker's antigenicity, solvent accessibility and hydrophilicity values were evaluated using the Antheprot software [27].

2.2. Animal handling

All animals used in this study (rabbits and *Aotus* spp. monkeys) were kept at FIDIC's primate station in Leticia, Amazonas and taken care according to procedures previously established by the Office for Protection from Research Risks (OPRR, Department of Health and Human Services, USA), under the constant supervision of a primatologist. Immunization and bleeding procedures on *Aotus* monkeys were carried out in agreement with the conditions stipulated by CorpoAmazonia (resolution 00066, September 13th 2006). Ten wild-caught *Aotus* monkeys were experimentally infected with *P. vivax* (as described below) and their parasitemia levels were assessed daily by Acridine orange staining until monkeys had developed 3–5% parasitemias and 5–7 schizonts were observed per field under the microscope. Monkeys were immediately treated whenever parasitemias were $\geq$5% or before if recommended by the supervising primatologist. Treatment consisted of orally administered pediatric doses of Chloroquine (10 mg/kg on the first day and 7.5 mg/kg per day until day 5) and Primaquine (0.25 mg/kg starting on day 3 and until day 5). Once assuring total clearance of parasites from blood and excellent health conditions, monkeys were released back into their natural habitat close to the site where they had been captured with the supervision of CorpoAmazonia officials. All procedures were approved by our institute's ethical committee.

2.3. Isolation of P. vivax parasites

Parasites from the VCG-1 (Vivax Colombia Guaviare 1) strain were cultured by successive passes in *Aotus* monkeys as previously described elsewhere [12]. Briefly, infected red blood cells (primarily at the schizont stage) were extracted from 3 to 4 mL *Aotus* blood samples using a discontinuous Percoll gradient (GE Healthcare, Uppsala, Sweden), according to a previously described protocol [28]. The isolated parasite pellet was used in either of the following procedures: (1) RNA extraction, (2) genomic DNA extraction, (3) total protein extraction, (4) DRM isolation or (5) immunofluorescence assays.

2.4. RNA extraction, cDNA synthesis and genomic DNA isolation

Parasite RNA was isolated by the Trizol methodology [29] and used for reverse transcription assays. In brief, 1–5 μg RNA was synthesized into cDNA by using the one-step RT-PCR SuperScript III kit (Invitrogen), according to the manufacturer's recommendations. Genomic DNA was isolated from a parasite pellet resuspended in 300 μL 1× phosphate-buffered saline (PBS) by using the UltraClean Blood DNA Isolation kit (MO BIO, California, USA). The integrity of the purified RNA and genomic DNA was examined by electrophoresis in agarose gels.

2.5. Cloning and sequencing

Based on the SaI-1 strain *pv34* nucleotide sequence, specific forward (5′-ATGATGAATGTTTTCTTCTGC-3′) and reverse (5′-GCTGAGCAGAAAGGCGAT-3′) primers were designed to amplify the entire *pv34* gene from both cDNA and genomic DNA. These primers were included in PCR reactions with the Platinum *Pfx* DNA polymerase enzyme (Invitrogen). PCR amplification was carried out according to the following temperature profile: 1 cycle at 94 °C for 2 min, 35 cycles of 56 °C for 30 s, 68 °C for 80 s and 94 °C for 10 s, and a final extension step at 68 °C for 5 min. Each amplified fragment was purified by using the Wizard PCR preps Kit (Promega, Wisconsin, USA). Only *pv34* PCR products obtained from cDNA