Characterization of *Plasmodium falciparum* integral membrane protein Pf25-IMP and identification of its red blood cell binding sequences inhibiting merozoite invasion in vitro

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Abstract

The identification of proteins present on the surface of *Plasmodium falciparum*-infected red blood cells as well as of free merozoites has been widely considered as one of the main areas of research in the development of an antimalarial vaccine due to their involvement in the parasite's pathogenesis and invasion mechanisms. Major advances had been accomplished in this area thanks to the analysis of the reported genomic sequence of *P. falciparum*, allowing for the identification of genes encoding for putative integral membrane proteins. This study reports for the first time the transcription of the *MAL8P1.3* gene, which codifies for a 25-kDa integral membrane protein of *P. falciparum* (FCB-2 strain), namely, Pf25-IMP. Western blot and immunofluorescence assays using goat polyclonal sera indicate that this protein is expressed in erythrocytic asexual blood stages. A highly robust, sensible, and specific receptor–ligand interaction assay allowed identification of two high activity binding peptides (HABPs) derived from Pf25-IMP: 30577 (⁴¹YKTANENVKLASSLSRLSR⁶⁰) and 30583 (¹⁶¹LNKKTVVRKIAEGLGYTIVF¹⁸⁰). Both HABPs bound with high affinity to human red blood cells (RBCs), and such binding was susceptible to enzyme treatment with trypsin. A common RBC surface receptor of apparently 48 kDa was found for both HABPs, plus an additional 31-kDa receptor for HABP 30577. HABP 30577 inhibited merozoite invasion in vitro by 73%, while HABP 30583 showed a 59% inhibition at 200 μ M concentration. The data suggest a possible role of Pf25-IMP in merozoite invasion to RBCs and support its inclusion in further immunological studies for evaluating its potential as vaccine candidates.

Keywords: *Plasmodium falciparum*; Pf25-IMP; synthetic peptide; binding assay; red blood cell

Plasmodium falciparum malaria represents a serious threat for public health as it accounts for ~500 million clinical cases and is responsible for the death of around 1.5–2.7

million people each year worldwide (Suh et al. 2004). Although measures such as global control of its transmission vector, implementation of prevention programs, and development of new antimalarial drugs have provided a partial solution to malaria spread, the emergence of new drug-resistant *P. falciparum* strains as well as insecticide-resistant mosquitoes highlight the need for more effective control measures. Although several studies have demonstrated that it is possible to obtain various types of antimalarial

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vaccines (Richie and Saul 2002; Mahanty et al. 2003), there is currently not any approved vaccine, and so the search for new protection-inducing antigens continues.

The recent completion and publication of the entire *P. falciparum* genome sequence (Gardner et al. 2002; Bahl et al. 2003) has promoted the identification and characterization of the new parasite's proteins, particularly of those presented on merozoites and parasite-infected red blood cell (iRBC) membrane, since these proteins are possibly involved in the receptor–ligand interactions taking place during merozoite invasion into RBCs. Given that these surface proteins are exposed to the immune system during the parasite's intraerythrocyte development or during invasion, they have been considered potential vaccine candidates (Cowman et al. 2002; Mahanty et al. 2003).

Based on bioinformatic and molecular tools and receptor–ligand studies, we have characterized the *MAL8P1.3* gene (Bahl et al. 2003), seeking to identify new merozoite proteins being involved in the parasite's invasion of RBCs. The *MAL8P1.3* gene encodes a 25-kDa integral membrane protein, conserved in *P. falciparum* (Bahl et al. 2003) and named Pf25-IMP by us. Additionally, we have determined the *MAL8P1.3* gene expression during the asexual erythrocyte stage and its localization on the merozoite surface. We found two high activity-binding peptides (HABPs) derived from Pf25-IMP; both peptides had the ability to inhibit in vitro merozoite invasion of erythrocytes. These data provide evidence for the inclusion of these Pf25-IMP high affinity-binding peptides in further studies aiming toward the development of an antimalarial vaccine.

Results

Reverse transcription and DNA amplification

An 872-nt sequence containing a two-exon gene encoding a 218-amino-acid-long integral membrane protein was identified when analyzing the published *P. falciparum* genome sequence (Bahl et al. 2003; Fig. 1A). The transcription of the *MAL8P1.3* gene in infected erythrocytes (16, 21, and 36 h after invasion) was assessed by applying a reverse transcriptase PCR revealing that the gene was transcribed during all evaluated stages (Fig. 1B). RT-PCR revealed a single 604-bp band corresponding to the expected size, and further nucleotide sequencing confirmed this (data not shown). The obtained cDNA fragment was smaller than the one obtained by gDNA amplification (819 bp), demonstrating that, as predicted, intron splicing was occurring within this gene (Fig. 1B).

Anti-sera production, Western blotting, and immunofluorescence assays (IFA)

Schizont-stage parasites were evaluated to determine whether mRNA transcription was associated with protein expression. Pre-immune and post-third-inoculation goat sera were adsorbed with *Escherichia coli* and *Mycobacterium smegmatis* lysates, as well as with the SPf66 vaccine (Patarroyo et al. 1987) coupled to Sepharose columns for eliminating nonspecific reactivities. The absorbed goat sera were then tested against *P. falciparum* lysates by

Figure 1. (A) Schematic representation of *MAL8P1.3* gene, including its neighboring genes and its Pf25-IMP expression product protein. The 657-nt mature mRNA is formed following removal of the intron region and translated into a 218-amino-acid polypeptide. Two predicted transmembrane (TM) helical regions are boxed in gray. The localization of HABP 30577 and 30583 sequences are shown. (B) Transcription of *MAL8P1.3* gene. cDNA amplified products from *Plasmodium falciparum* FCB2 strain at 16, 21, and 36 h (lanes 1,3,5) and gDNA (lane 7). Negative control (lanes 2,4,6). (M) 100-bp molecular weight markers.

Western blotting, showing that goat's pre-immune sera did not recognize parasite proteins, whereas sera from a goat immunized with polymeric peptides elicited antibodies specifically recognizing a 28-kDa protein, almost coinciding with the estimated weight of 25 kDa for Pf25-IMP (Fig. 2A). Interestingly, the 28-kDa protein was no longer recognized when the immune sera (III-20) was preincubated with the inoculated peptides. Immunofluorescence assays (IFA) using goat anti-IMP-Pf25 antibodies recognized a protein in *P. falciparum* schizont-iRBCs showing the characteristic staining pattern of the merozoite's membrane proteins (Fig. 2B; Black et al. 2001, 2003). No fluorescence was observed in *P. falciparum* iRBCs incubated with goat's pre-immune sera (data not shown).

Red blood cell binding assays

The specific binding activity to RBCs was assessed by performing binding assays with the 11 synthetic peptides covering the total length of Pf25-IMP (*MAL8P1.3*), and two peptides covering the protein's sequence between residues 151 and 170. Peptide binding activity corresponds to the slope of the specific binding curve (total binding minus nonspecific binding), which is defined as the ratio between bound peptide (in picomoles) and added peptide (in picomoles). HABPs were defined as being those peptides showing an activity $\geq 2\%$, indicating that these peptides recognized more than 200 specific binding sites per cell at low radiolabeled peptide concentrations (200 nM) (Urquiza et al. 1996; Rodriguez et al. 2000; Curtidor et al. 2001).

Two erythrocyte HABPs were found in Pf25-IMP: 30577 ($^{41}\text{YKTANENVKLASSLSDRLSR}^{60}$) and 30583

($^{161}\text{LNKKTVVRKIAEGLGYTIVF}^{180}$) (Fig. 3A). HABP 30577 was located in the amino terminal following a region predicted as being a transmembrane domain (Combet et al. 2000). HABP 30583 localized toward the carboxy-terminal region, having eight amino acid residues (173–180) included within a second transmembrane predicted domain (Figs. 1A, 3A; Combet et al. 2000), suggesting that only those residues residing outside the transmembrane helix are involved in RBC binding (shown in bold type above).

Peptides 36070 ($^{161}\text{LNKKTVVRKIAEY}^{172}$) and 36071 ($^{151}\text{KCEYKKQKEKLNKKTVVRKI}^{170}$), both containing part of the HABP 30583 sequence (shown in bold type), showed high specific binding activity with even higher activity than HABP 30583 (Fig. 3B).

HABP affinity constant determination

Saturation assays and Hill analysis (Hulme 1993) were performed for HABPs 30577 and 30583 (Fig. 3C). Peptide 30583 had an affinity constant value of 179 nM, Hill coefficient of 1.6, and around 239,000 binding sites per cell, suggesting a strong HABP–RBC interaction with positive cooperativity. HABP 30577 binding was not saturable under assay conditions.

Enzymatic treatment and cross-linking assays

The membrane of RBCs was submitted to different enzyme treatments to assess the effect of each treatment over the HABP–RBC interaction. (Fig. 4). Treatment with trypsin significantly reduced HABPs 30577 and 30583 specific binding 29% and 12%, respectively, compared to

Figure 2. (A) Western blot assays. Immunoblot of *P. falciparum* (FCB2 strain) schizont lysate incubated with pre-immune goat serum (lane 1) or immune serum (III-20) (lane 2) obtained from goat B70 inoculated with Pf25-IMP polymeric peptides. (Lane 3) Immune serum (III-20) preincubated with inoculated polymer peptides. All sera were preadsorbed on a Sepharose column coupled to *M. smegmatis*, *E. coli*, and SPF-66 vaccine (Patarroyo et al. 1987, 1988). The immune (III-20) sera recognized a 28 kDa band in the schizont stage erythrocytes lysate (lane 2). (B) Immunofluorescence assay with fixed *P. falciparum*-parasitized erythrocytes incubated with immune serum (III-20), showing a grape-like immunofluorescence pattern characteristic of merozoite membrane proteins in late-schizont stages. The pre-immune B-70 serum did not show reactivity (data not shown).

Figure 3. (A) Specific binding profile of Pf25-IMP peptides to RBCs. Each black bar represents the ratio (shown as a percentage) between added radiolabeled peptide and bound radiolabeled peptide. This ratio, the so-called binding activity, is obtained from the slope of the specific binding graph. Peptides having binding activity $\geq 2\%$ were considered to be HABPs. Tyr was added to the carboxy-terminal of those peptides not containing this residue within their sequence. (B) Binding assay of peptide 36070 analog to HABP 30583 and peptide 36071 overlapping 30582 and 30583 sequences. (C) Saturation curves for HABP 30583. Increased quantities of labeled peptide were added in the presence or absence of unlabeled peptide. The curve (outset) represents specific binding. In the Hill plot (*inset*), the X-axis represents log free peptide, and the Y-axis represents log $(B/B_{\max} - B)$; (B) bound peptide.

control. Treatment with chymotrypsin affected specific binding of HABP 30577 by 10% and HABP 30583 by 20%, while treatment with neuraminidase increased both HABPs' binding to RBCs up to 50%. The cross-linking assay showed that HABPs 30577 and 30583 bound to the RBCs membrane (Fig. 4). Both peptides bound strongly and specifically to a 48-kDa RBC membrane protein, but 30577 weakly recognized an additional 31-kDa RBC membrane receptor. Radiolabeled peptide interaction with these receptors was inhibited when the binding was performed in the presence of unlabeled peptide, indicating that specific binding had taken place (Fig. 4).

Cross-competition assays

The results of cross-linking and enzyme treatment assays suggested a common 48-kDa membrane receptor for both HABPs. Cross-competition assays using radiolabeled and unlabeled HABPs were performed to determine whether HABPs could displace each other. The assay evidenced that unlabeled HABP 30577 inhibit binding of radiolabeled HABP 30583 in a concentration-dependent manner (Fig. 4), whereas unlabeled HABP 30583 had a minimum effect on radiolabeled HABP 30577 binding (Fig. 5).

Figure 4. (A) Binding assays of Pf25-IMP-derived HABPs with enzyme-treated erythrocytes. Binding of peptide 30577 and 30583 were compared between enzyme-treated and untreated RBCs. Each bar represents the percentage of specific binding activity shown by peptides upon incubation with chymotrypsin (light gray), trypsin (white), and neuroaminidase (gray). Untreated erythrocytes were used as controls (black). (B) Covalent cross-linking assay for HABPs 30577 and 30583 to erythrocyte surface receptor molecules. Binding with radiolabeled HABPs was performed in the absence (total binding, lanes 1,3) or presence of unlabeled HABPs (nonspecific binding, lanes 2,4). Cross-linked proteins with labeled peptides were visualized on Imaging Screen K (BioRad Quantity One, Quantitation Software). The proteins' apparent molecular weights were determined by using molecular weight markers (New England Bio Labs).

Circular dichroism measurement

Circular dichroism (CD) analysis was used for obtaining general information regarding HABP's secondary structure. CD profiles (30% TFE/water) for HABPs 30577 and 30583 revealed a helical type-ordered structure with double minima at 208, and 220, and 190 nm maximum ellipticity, characteristic of an α -helical conformation (Fig. 6). Deconvolution of CD spectra data estimated an $\sim 92\%$ α -helical content in both HABPs structures.

Red blood cell invasion inhibition

The role of Pf25-IMP HABPs in *in vitro* merozoite invasion into RBCs was examined using HABPs and one low activity binding peptide (LABP) (Puentes et al. 2005). The addition of LABP to synchronized late-stage schizont cultures before the merozoite's release from

iRBCs had no effect on merozoite invasion, whereas adding HABP 30577 inhibited invasion by 73% and HABP 30583 by 59% at 200 μM (Table 1). Similarly, the ability of anti-IMP-Pf25 for inhibiting merozoite invasion was assessed using goat B70 hyper-immune serum, finding a 67% inhibition at a 1:10 dilution and no inhibition with goat's pre-immune serum (Table 1).

Polymorphism analysis of FCB-2/FVO and PAS-2 strains

Amplified products of each of the regions encoding peptides 30577 and 30583 in the FCB-2, FVO, and PAS-2 strains were visualized on agarose gels, detecting a specific band of ~ 400 bp for the region encoding peptide 30577 and of ~ 171 bp for the one encoding 30583 (Fig. 7A). Although a 438-bp size was expected for the former peptide based on the genomic region of the 3D7 strain, whose sequence was used for synthesizing primers, such difference results from the lack of a 40-bp region within the 216-bp intron in the three strains being evaluated when compared to the 3D7 strain.

Seeking to assess the degree of polymorphism lying specifically in the region containing our HABPs, the sequences obtained for both protein fragments from the three *P. falciparum* strains being analyzed were thus aligned using Clustal W software and compared to the 3D7 sequence (Combet et al. 2000). For the region encoding HABP 30577 a conserved 398-bp fragment was observed in each of the strains. Figure 7B shows an amino acid multiple sequence alignment of the 3D7 strain with peptides belonging to the FCB-2, FVO, and PAS-2 strains where a 100% identity is seen among strains, indicating that both peptides are highly conserved in the three strains being evaluated.

Similarly, the second alignment performed for fragment 30583 with clones from each strain revealed a 171-bp conserved sequence in each of the three strains, which presented a 100% identity with the region encoding

Figure 5. Cross-competition assays. Inhibition of radiolabeled HABP binding to RBC in the absence or presence of increasing concentrations (0–120 μM) of unlabeled peptide. All assays had a $<10\%$ standard deviation.

Figure 6. CD profile of Pf25-IMP HABPs. The spectrum was obtained for each HABP from averaging three scans taken in aqueous TFE (30% [v/v]) solution. The results were expressed as mean residue ellipticity $[\Theta]$, the units being degrees $\times$ cm² $\times$ dmol⁻¹ according to the $[\Theta] = \Theta_{\lambda}/(100lc)$ function where Θ_{λ} represents measured ellipticity, l is the optical path length, c is the peptide concentration, and n is the number of amino acid residues in the sequence (Sreerama et al. 1999).

30583 in the 3D7 strain. This result corresponded to that obtained by PCR amplification.

Discussion

This study was directed toward characterizing the *P. falciparum* integral membrane protein Pf25-IMP, which is encoded by the *MAL8P1.3* two-exon gene located in chromosome 8 of the *P. falciparum* 3D7 strain and encoding for a 218-amino-acid-long protein. According to our results, the *MAL8P1.3* gene is expressed in asexual intra-erythrocyte stages in the *P. falciparum* FCB-2 strain (Figs. 1, 2). The immunofluorescence assay shows that Pf25-IMP is expressed on the surface of merozoites (Fig. 2B), thus agreeing with predictions assigning a membrane protein localization to Pf25-IMP (Gardner et al. 2002). Although its primary amino acid sequence does not contain the canonical signal sequence (ss), the presence of a possible cleavage site between residues ²⁸CVS-VE³² (Bendtsen et al. 2004) suggests that the N-terminal region could be harboring a recessed hydrophobic signal sequence playing a role functionally similar to that of a sequence signal (Cooke et al. 2004). Additionally, some other *P. falciparum* proteins displayed on the surface of merozoites and iRBCs but lacking of a hydrophobic N-terminal region have been reported, thus indicating the existence of an alternative transport mechanism possible through the interaction with other proteins or of other exportation motives still not described (Nacer et al. 2001; Aruna et al. 2004; Cooke et al. 2004).

Further characterization of the Pf25-IMP showed that hyper-immune anti-serum (III-20) from an immunized goat with the polymerized peptide reacted with a band of ~28

kDa in the late-schizont *P. falciparum* lysate Western blot, which approaches the 25-kDa molecular weight predicted for this protein (Fig. 2A). This 28-kDa band was no longer recognized when the same hyper-immune anti-serum was preincubated together with inoculated peptide, thus ascertaining the specificity of the peptide's recognition and discarding any cross-reactivity with other parasite proteins.

Since Pf25-IMP had not been implicated in RBC invasion before, eleven 20-mer nonoverlapping peptides encompassing the entire Pf25-IMP sequence were synthesized with the purpose of determining those regions specifically binding to RBCs and assessing their effect on merozoite in vitro invasion (Fig. 3A).

As a result two HABPs derived from Pf25-IMP were found: HABP 30577 (⁴¹YKTANENVKLASSLSDRLSR⁶⁰) localizing toward the N terminus following a predicted transmembrane domain (residues 15–35) (Combet et al. 2000) and HABP 30583 (¹⁶¹LNKKTIVVRKIAEGLGYTIVF¹⁸⁰) localizing toward the C terminus. Eight of the 20 residues comprising the former peptide (¹⁷³GLGYTIVF¹⁸⁰) belonged to a second transmembrane predicted domain (Combet et al. 2000), while the remaining residues (¹⁶¹LNKKTIVVRKIAEY¹⁷²) extended outside the transmembrane helix, possibly indicating that these residues were the ones involved in peptide–RBC interaction. For ascertaining the above, two additional peptides were synthesized: peptide 36070 (¹⁶¹LNKKTIVVRKIAEY¹⁷²), containing exclusively the 12 residues extending outside the helix, and overlapping peptide 36071 (¹⁵¹KCEYKKQKEKLNKKTIVVRKI¹⁷⁰), which included the C-terminal region of the 30582 moderate binding activity peptide (Fig. 3). Both peptides showed high specific binding activities to RBCs, strongly supporting the involvement of the common region (¹⁶¹LNKKTIVVRKI¹⁷⁰) lying outside the transmembrane helix in specific binding to RBCs. Additionally, HABP 30583 binding was saturable, showing around 239,000 binding sites per cell and presenting high affinity values (K_d 182). HABP 30577 was not saturable under the same experimental conditions.

Table 1. Invasion inhibition assays with Pf25-IMP derived HABPs and anti-IMP-Pf25 raised in goat 870

Activity binding	Peptide	Invasion inhibition (%)	
		200 μ M	100 μ M
High	30577	73 $\pm$ 3	3 $\pm$ 1
	30583	59 $\pm$ 1	3 $\pm$ 1
Low	31120	3 $\pm$ 1	0 $\pm$ 1
B70 pre-immune goat serum	1:10		20
B70 immune goat serum	1:10		68
Chloroquine (0.5 mg/mL)		100 $\pm$ 1	
EGTA		100 $\pm$ 1	

Mean $\pm$ SD from three experiments.

Figure 7. (A) PCR amplification of regions encoding peptides 30577 and 30583. (Lanes 1,11) 50-bp molecular weight marker. (Lanes 2,5,8) PCR of 30577 in FCB-2, FVO, and PAS-2 strains, respectively. (Lanes 3,6,9) PCR of 30583 in FCB-2, FVO, and PAS-2 strains, respectively. (Lanes 4,7,10) Negative controls. (B) Amino acid alignment of peptides 30577 and 30583 sequence with the sequence of the 3D7 strain and one clone of each strain being studied.

The probable nature of their RBC membrane receptors was evaluated in binding assays with enzyme-treated RBCs (using neuraminidase, trypsin, and chymotrypsin). Trypsin cleavage of glycophorins A and C, considerably decreasing specific binding of HABP 30577 as well as 30583 (Fig. 4A), suggests a possible glycoprotein-type receptor (Pasvol 1984; Baum et al. 2005). The low effect of chymotrypsin treatment on HABPs' specific binding to RBCs together with the increased binding activity, shown by treating RBCs with neuraminidase, supposes sialic-acid-independent binding receptors for both HABPs, such as the yet undescribed type "X" receptor (Okoyeh et al. 1999).

Cross-linking assays revealed that HABPs 30577 and 30583 bind to 48-kDa RBC membrane receptor/receptors and that HABP 30577 bind to an additional 31-kDa receptor, both establishing HABP-RBC membrane receptor-specific interactions, since unlabeled peptide inhibited cross-linking of radiolabeled peptide to RBC membrane receptor (Fig. 4B). These results, together with the similar behavior pattern shown in binding assays to enzyme-treated RBCs (Fig. 4), supposes a common 48-kDa receptor for both HABPs, possibly glycophorin A (GPA) (~47 kDa) (Perkins and Holt 1988). It was also observed that binding sites for HABP 30583 (239,000) were exclusively associated to the 48-kDa receptor while HABP 30577 binding sites were distributed between 31- and 48-kDa receptor molecules. These results correlate with the non-saturable binding of peptide 30577, and thus suggest the presence of at least two different sialic-acid-independent binding receptor molecules for HABP 30577.

Furthermore, cross-competition assays evidenced that inhibition effect between both HABPs was not reciprocal, since HABP 30583 binding was considerably inhibited by HABP 30577, whereas HABP 30583 had no appreciable effect over 30577 binding to its receptors (Fig. 5). These results together with cross-linking results suggest that HABP 30577 inhibition of HABP 30583 is related to the presence of a common 48-kDa-membrane receptor for both HABPs. Finding diverse binding sites for *P. falciparum* proteins correlates with the well-described alternative pathways used by the parasite for invading host cells, which involve different parasite ligands and RBC receptors (Mitchell et al. 1986; Perkins and Holt 1988). Such RBC membrane receptors have been reported to be sialic acid dependent and acid independent (Okoyeh et al. 1999; Duraisingh et al. 2003), and include proteins such as Band 3 (Goel et al. 2003), GPA, GPB, GPC, and the still noncharacterized "X, Y, Z, and E" receptors (Dolan et al. 1994; Sim et al. 1994; Mayer et al. 2001; Rayner et al. 2001; Duraisingh et al. 2003).

The PHD prediction (Rost and Sander 1994; Combet et al. 2000), suggesting a helical structure for most of the HABP 30577 sequence and the central region of HABP 30583, was confirmed by CD data revealing α -helical-like features in both HABPs' conformational structures (Fig. 6). Additionally, the deconvolution of CD data (Compton and Johnson Jr. 1986; Sreerama et al. 1999) showed a large percentage of α -helical features in both HABP structures, thus suggesting that the region containing the two helical HABPs is being exposed and thereby is accessible for establishing molecular interactions with RBC receptors. We expect that additional studies will determine whether a structure-binding activity relationship exists the same as the structure-protection relationship found for some peptides in previous work (Cifuentes et al. 2003; Espejo et al. 2004; Salazar et al. 2004).

Once the expression and localization of Pf25-IMP was established during the asexual intra-erythrocytic development, the functional role of this protein during RBC invasion was evaluated in inhibition assays with HABPs 30577 and 30583. These peptides inhibited merozoite invasion by 73% and 59%, respectively, at 200 μ M, whereas low binding activity peptide had no effect at all in invasion (Table 1). The same inhibitory effect was seen with anti-Pf25-IMP antibodies (1:10 dilution), which inhibited merozoite invasion to RBCs by 68%, possibly due to a steric blockage of these antibodies since the immunized peptide 28794 (C^{21} GNILVIDLFTVSVEFFNKHFTFG 40 C) lies contiguous to HABP 30577 (residues 41-60) (Fig. 3). These findings thus highlight the involvement of Pf25-IMP in merozoite invasion to RBCs.

Most malaria proteins performing a functional role within the erythrocyte-invasion cycle are highly polymorphic, which hampers their inclusion in a vaccine

design (Escalante et al. 1998; Volkman et al. 2002). Such an event made it relevant to evaluate the degree of polymorphism lying in those regions where peptides 30577 and 30583 are located in three *P. falciparum* strains, such as FCB-2, FVO, and PAS-2 (Fig. 7). Interestingly, it was found that the region containing these two peptides were conserved among the different evaluated strains, which supports the inclusion of the Pf25-IMP in future immunological studies. Nevertheless, some HABPs or sequences of *P. falciparum* reported proteins participating in the invasion process contain sequences being conserved but poorly immunogenic or protective in the *Aotus* model.

We have developed a robust methodology that consists of the use of conserved HABPs sequences as templates for the design of analog peptides whose immunologic characteristics are improved by such modifications, thus rendering them into suitable candidates of a new antimalarial, multicomponent, subunit-based vaccine (Salazar et al. 2002, 2004; Torres et al. 2003). Therefore, the results of this study support including Pf25-IMP, or its derived HABPs, in further immunological studies for evaluating their potential as vaccine candidates.

Materials and Methods

Extraction and purification of genomic DNA from P. falciparum

The *P. falciparum* (FCB-2 strain) erythrocyte ring stage parasites were obtained from a sorbitol synchronized culture and maintained as described previously elsewhere (Trager and Jensen 1978; Lambros and Vanderberg 1979). A 200 μ L culture showing 30% parasitemia was lysed using 0.2% saponin. Genomic DNA (gDNA) was extracted from parasite isolates and purified using Wizard Lambda Preps DNA Purification System (Promega).

Reverse transcription PCR

Total RNA from isolated parasites was extracted using Trizol reagent (Invitrogen). Two micrograms of total RNA were treated with RQ-DNAse (Promega) to avoid gDNA contamination and used as template for RT-PCR by using the forward primer D5 (5'-ATGAAAAGTATTATAAATAACCG-3') and reverse primer D7 (5'-ATATTAAGAACCATTACAAAGC-3') according to the SuperScript III OneStep RT-PCR system guidelines (Invitrogen). Primers were designed based on the reported *MAL8P1.3* sequence. Two microliters of gDNA were PCR amplified in a 25- μ L reaction consisting of 1U Taq polymerase (Promega), 1 $\times$ Taq polymerase reaction buffer, 1.5 mM MgCl₂, 0.2 μ M dNTPs, and 0.4 μ M of each primer (D5/D7) under the following thermocycling conditions: initial 5-min denaturing cycle at 95°C, followed by 40 cycles consisting of 1 min annealing at 55°C, 1 min extension at 72°C, and 1 min denaturing at 95°C. A final 5-min extension cycle was run at 72°C. A control reaction was carried out under the same conditions using DNAse-treated

RNA as template. Amplified products were visualized by 1% agarose gel electrophoresis and ethidium bromide staining.

Producing goat anti-Pf25-IMP serum

A goat (B70) nonreactive to the *P. falciparum* lysate was subcutaneously inoculated in the inguinal region with a mixture of two polymeric synthetic peptides, namely, 28794 (CG²¹NILVIDLFTVSVEFFNKHTF⁴⁰GC) and 28796 (CG¹⁰¹VLEVCAEEEDGENEIRITMK¹²⁰GC), which represent the amino and central portion of Pf25-IMP [*MAL8P1.3*], respectively, with the purpose of obtaining large quantities of anti-Pf25-IMP serum. Immunizations were done emulsifying the two polymeric peptides (500 μ g each) in Freund's complete adjuvant on day 0 and incomplete adjuvant on days 20 and 40. Bleeding was performed on days 0 (pre-immune), 20 (I-20), and 40 (II-20) for evaluating antibody production. Final bleeding was carried out on day 60 (III-20) for collecting anti-Pf25-IMP serum. Immunizations as well as bleeding were done following the Colombian Ministry of Public Health guidelines for handling animals.

Goat sera adsorption with E. coli and M. smegmatis lysate and SPf66 Sepharose

E. coli (DH5 α strain) protein lysate was obtained from overnight culture in Luria-Bertani medium, then washed, resuspended, sonicated for 2 min at 4°C, and spun for 10 min at 4500 $\times$ g. *M. smegmatis* proteins were obtained from 5-d-old Middlebrook 7H9 broth culture, washed, suspended, sonicated for 10 min (as described above), and spun for 10 min at 4500 $\times$ g. Both pellets were suspended individually in coupling buffer (1.0 M NaHCO₃, 0.5 M NaCl, pH 8.3). The suspended lysate and synthetic SPf66 vaccine (Patarroyo et al. 1987, 1988) were collected and used individually for coupling to CNBr-activated Sepharose 4B (Pharmacia Biotech) according to the manufacturer's recommendations. The goat serum (pre-immune and immune) was preadsorbed with *E. coli* Sepharose, *M. smegmatis* Sepharose, and SPf66 Sepharose affinity columns for eliminating non-specific cross-reactivities. Briefly, each serum (5 mL) was added to Sepharose affinity columns (5 mL) and left in a gently rotating/shaking mode for 30 min at room temperature. This procedure was done twice using a new lysate Sepharose affinity column each time; the anti-serum was stored at -70°C until further use.

Western blotting

Proteins from *P. falciparum* intra-erythrocyte schizont lysate were separated in a discontinuous SDS-PAGE system (1 mg/mL lysate was loaded per gel) by using an acrylamide gradient (7.5%–15% [w/v]). Following SDS-PAGE, the proteins were transferred to a nitrocellulose membrane (Hybond 203c, Pharmacia) using the semidry blotting technique (Kyhse-Andersen 1984). After being blocked with 5% skimmed milk in TBS, nitrocellulose membranes (0.4 mm) were incubated for 1 h with 1:100 dilution of the polyclonal pre-immune and immune goat B-70 sera in TBS-T blocking solution (1% Tween 20 and 5% skimmed milk in TBS). In a parallel assay, immune serum was preincubated with inoculated polymer peptides before being incubated with the nitrocellulose membrane. One hour's incubation with 1:5000 alkaline phosphatase-conjugated anti-goat IgG antibody (ICN) was carried out after five TBS-T washes.

The reaction was then developed with NBT/BCIP (Promega); commercial molecular mass markers (NEB) were used for estimating the relative molecular mass.

Immunofluorescence assay

Immunofluorescence microscopy of schizont-stage parasites was conducted as described elsewhere (Tonkin et al. 2004). Briefly, the cells were washed in PBS, and then fixed with 4% electronic microscopy (EM) grade paraformaldehyde and 0.0075% EM grade glutaraldehyde in PBS for 30 min. Fixed cells were washed once in PBS, and then permeabilized with 0.1% Triton-X-100/PBS for 10 min. Cells were washed again in PBS, and then treated with 0.1 mg/mL of sodium borohydride (NaBH₄)/PBS for 10 min. Following another PBS wash, cells were blocked in 3% bovine serum albumin BSA/PBS for 1 h, and then incubated with polyclonal pre-immune (B70 PI) or hyper-immune goat (B70) anti-serum for 30 min. After three washes, cells were incubated with FITC-conjugated rabbit anti-goat IgG as secondary antibody for 30 min, washed in PBS, and then mounted in 50% glycerol. Immunoreactivity was observed using an Axioskop fluorescence microscope (Carl Zeiss). Images were recorded by an AxioCam MRm CCD camera (Carl Zeiss).

Peptide synthesis and binding assays

Eleven 20-mer nonoverlapping peptides corresponding to the complete *P. falciparum* Pf25-IMP protein amino acid sequence (MAL8P1.3) were chemically synthesized by solid phase multiple synthesis (Merrifield 1963); BHA resin (0.7 meq/mg) and t Boc amino acids were employed. Peptides were cleaved by the low-high HF technique (Tam et al. 1983) and analyzed by HPLC and MALDI TOF mass spectrometry (TOF proteins, Bruker). A tyrosine was added at the carboxy-terminal of those peptides that did not contain this residue within their sequences to enable their radiolabeling. Synthesized peptides are shown in Figure 3A in one-letter code.

All peptides were radiolabeled according to a previously described process with few modifications (Urquiza et al. 1996; Rodriguez et al. 2000; Curtidor et al. 2001): Five microliters of 2.6 μ M purified peptide were reacted with 10 μ L of 1.8 mM chloramine T and 5 μ L of 4.7 μ M Na¹²⁵I (ICN) having 100 μ Ci/mL specific activity, in HBS buffer (pH 7.4). The reaction was stopped by adding 20 μ L of 9.4 mM sodium bisulphite. The radiolabeled peptide was separated from subproducts by passage through Sephadex G-10 columns (Pharmacia); these peptides had 1–10 μ Ci/nmol specific activity.

The radiolabeled peptides were tested in a highly robust, specific, and sensitive receptor–ligand interaction assay originally developed for human erythrocyte binding assays. Briefly, erythrocytes (2×10^8 cells) obtained from healthy donors were washed in HBS buffer until the buffy coat was removed. The erythrocytes were then incubated with increasing radiolabeled peptide concentrations (10–200 nM) in the absence (total binding) or presence (nonspecific binding) of unlabeled peptide (16 μ M). Samples reached a 200 μ L final volume by the addition of HBS and were incubated for 90 min at room temperature (Urquiza et al. 1996; Rodriguez et al. 2000; Curtidor et al. 2001). After incubation, cells were washed two times with HBS, and bound cell radiolabeled peptide was quantified in an automatic gamma counter (4/200 plus ICN Biomedicals). The binding assays were performed in triplicate. Two additional peptides were synthesized: peptide 36070 containing part of

HABPs 30583 sequence and peptide 36071 overlapping peptide 30582 as well as HBP 30583 (Fig. 3B). Both peptides were evaluated in RBC binding assays according to the aforementioned protocol.

Saturation assays

A modified erythrocyte binding assay was used to ascertain saturation with two Pf25-IMP derived HABPs: 10^7 – 10^8 cells were used at 170 μ L final volume; radiolabeled peptide concentrations ranged between 40 and 860 nM. Unlabeled peptide concentration was 35 μ M. Cells were washed with HBS and bound cell radiolabeled peptide was quantified in an automatic gamma counter (4/200 plus ICN Biomedicals) (Urquiza et al. 1996; Rodriguez et al. 2000; Curtidor et al. 2001).

Cross-competition assay

HABPs 30577 and 30583 were evaluated in cross-competition assays following a previously described protocol with few modifications (Valbuena et al. 2004). Briefly, 2×10^8 RBCs in HBS were incubated with radiolabeled HABPs (28 nM) in the presence of increasing concentrations of the other nonlabeled HBP (0–120 nM) for 90 min at room temperature. Following incubation, nonbinding peptide was removed by washing twice with HBS solution (pH 7.4), and radiolabeled binding peptide was measured in a gamma counter. Assays were done in triplicate.

Enzyme treatment

HABPs' specific binding to enzyme-treated and untreated RBCs was compared in RBC binding assays. In brief, RBCs suspensions (60% hematocrit) were incubated with 150 μ U/mL neuraminidase (ICN 9001-67-6) in HBS buffered solution (pH 7.4), 1 mg/mL trypsin (Sigma T-1005), or 1 mg/mL chymotrypsin (Sigma C-4129), for 60 min at 37°C. Following incubation, enzyme-treated RBCs were washed twice and used in typical binding assays with HABPs. Untreated erythrocytes were used as positive binding control.

Cross-linking assays

¹²⁵I-radiolabeled HABPs were cross-linked to erythrocytes for identifying erythrocyte-binding molecules. The binding test was performed by using a final 1.5% cell concentration and following incubation with the radiolabeled peptide in the presence or absence of 35 μ M unlabeled peptide for 60 min at room temperature. After incubation, cells were washed with HBS and the bound peptide was cross-linked with 10 μ M bis(sulfo-succinimidyl suberate) (BS³, Pierce) for 15 min at 4°C. The reaction was stopped with 20 mM Tris-HCl (pH 7.4) and washed again with HBS. The cells were treated with lysis buffer (5 mM Tris-HCl, 7 mM NaCl, 1 mM EDTA, and 0.1 mM PMSF) and then spun at $15,000 \times g$. Membrane proteins were recovered, solubilized in Laemmli buffer, and size-separated in 12% (w/v) SDS-PAGE. Cross-linked proteins with labeled peptides were visualized on Imaging Screen K (BioRad Quantity One, Quantitation Software). The apparent molecular weights were determined by using molecular weight markers (New England Bio Labs).

Circular dichroism (CD) spectroscopy

CD analysis was used for obtaining general information regarding HAPB structure and folding. Spectra were acquired on Jasco J-810 equipment using a 1-cm path length quartz cell at 20°C in aqueous TFE (30% [v/v]) solution. Peptide concentration was ~0.1 mM. Each spectrum was obtained from averaging three scans taken at a 20-nm/min scan rate with 1 nm spectral bandwidth and corrected for baseline. Deconvolution of the CD data was performed by using SELCON3, CDSSTR, or CONTINILL software (Compton and Johnson Jr. 1986; Sreerama et al. 1999).

Invasion inhibition assays

HABPs 30577, 30583, and B70 sera were tested for their ability to inhibit *P. falciparum* FCB-2 strain merozoite in vitro invasion of erythrocytes in culture. The pre-immune sera (B-70PI) and the low binding activity peptide 31120 were used as control. A synchronized schizont stage culture (Trager and Jensen 1976) was seeded in 96-well plates with noninfected erythrocytes with 100 or 200 µM peptide concentrations at 1.5% final hematocrit and 1.25% final parasitemia. The supernatant was recovered after 18 h incubation at 37°C in 5% O₂, 5% CO₂, and 90% N₂ atmosphere. The medium was removed, and the cells were stained with 15 µg/mL hydroethylin for 30 min at 37°C and washed three times with PBS. The suspension was analyzed using a FACsort in Log FL2 data mode using CellQuest software (Becton Dickinson immunocytometry system) (Wyatt et al. 1991). The assays were performed in triplicate.

PCR amplification

Two microliters of genomic DNA from the *P. falciparum* FCB-2, FVO, and PAS-2 strains were used as template for amplifying the regions encoding peptides 30577 and 30583 from protein Pf25-IMP. The primers used were designed using the Gene Runner v3.05 software, based on the available 3D7 genomic sequence (*MAL8P1.3*). The primers obtained were DIR1 (5'-TTGGTTATTGATCTTTTCTGTG-3') and REV1 (5'-TTTC AACTTCAGTGATCATATC-3') for peptide 30577 and DIR2 (5'-GATTTTATTATGGACGATTTAGC-3') and REV2 (5'-GAAC CATTACAAGCTCCTAC-3') for peptide 30583.

The reaction was carried out in a 25-µL final volume, under the same above-described RT-PCR conditions. Thermocycling conditions were as follows: initial denaturing at 95°C for 5 min, followed by 35 cycles consisting of 1 min annealing at 58°C for DIR1/REV1 or 60°C for DIR2/REV2, a 1 min extension step at 72°C, and 1 min denaturing at 95°C. A final extension cycle was performed at 72°C for 5 min.

Cloning

PCR products were purified using a Wizard PCR preps kit (Promega) and cloned into the pGEM-T vector (Promega). The *E. coli* DH5α strain (Invitrogen) was then used for the transformation. A colony PCR was also performed using vector-annealing primers T7 and SP6 to ascertain the presence of the cloned fragment. Plasmid DNA was then extracted from the recombinant colonies using a Miniprep purification kit (Promega). Three recombinant clones confirmed by PCR and digestion from each amplified region were sequenced in an automatic

sequencer (ABI PRISM 310 Genetic Analyzer, PE Applied Biosystems).

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References

- Aruna, K., Chakraborty, T., Nambeesan, S., Mannan, A.B., Sehgal, A., Balachandara, S.R., and Sharma, S. 2004. Identification of a hypothetical membrane protein interactor of ribosomal phosphoprotein P0. *J. Biosci.* **29**: 33–43.
- Bahl, A., Brunk, B., Crabtree, J., Fraunholz, M.J., Gajria, B., Grant, G.R., Ginsburg, H., Gupta, D., Kissinger, J.C., Labo, P., et al. 2003. PlasmoDB: The *Plasmodium* genome resource. A database integrating experimental and computational data. *Nucleic Acids Res.* **31**: 212–215.
- Baum, J., Maier, A.G., Good, R.T., Simpson, K.M., and Cowman, A.F. 2005. Invasion by *P. falciparum* merozoites suggests a hierarchy of molecular interactions. *PLoS Pathog.* **1**: e37. doi: 10.1371/journal.ppat.0010037.
- Bendtsen, J.D., Nielsen, H., von Heijne, G., and Brunak, S. 2004. Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* **340**: 783–795.
- Black, C.G., Wu, T., Wang, L., Hibbs, A.R., and Coppel, R.L. 2001. Merozoite surface protein 8 of *Plasmodium falciparum* contains two epidermal growth factor-like domains. *Mol. Biochem. Parasitol.* **114**: 217–226.
- Black, C.G., Wang, L., Wu, T., and Coppel, R.L. 2003. Apical location of a novel EGF-like domain-containing protein of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **127**: 59–68.
- Cifuentes, G., Guzman, F., Alba, M.P., Salazar, L.M., and Patarroyo, M.E. 2003. Analysis of a *Plasmodium falciparum* EBA-175 peptide with high binding capacity to erythrocytes and their analogues using 1H NMR. *J. Struct. Biol.* **141**: 115–121.
- Combet, C., Blanchet, C., Geourjon, C., and Deleage, G. 2000. NPS@: Network protein sequence analysis. *Trends Biochem. Sci.* **25**: 147–150.
- Compton, L.A. and Johnson Jr., W.C. 1986. Analysis of protein circular dichroism spectra for secondary structure using a simple matrix multiplication. *Anal. Biochem.* **155**: 155–167.
- Cooke, B.M., Lingelbach, K., Bannister, L.H., and Tilley, L. 2004. Protein trafficking in *Plasmodium falciparum*-infected red blood cells. *Trends Parasitol.* **20**: 581–589.
- Cowman, A.F., Baldi, D.L., Duraisingh, M., Healer, J., Mills, K.E., O'Donnell, R.A., Thompson, J., Triglia, T., Wickham, M.E., and Crabb, B.S. 2002. Functional analysis of *Plasmodium falciparum* merozoite antigens: Implications for erythrocyte invasion and vaccine development. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **357**: 25–33.
- Curtidor, H., Urquiza, M., Suarez, J.E., Rodriguez, L.E., Ocampo, M., Puentes, A., Garcia, J.E., Vera, R., Lopez, R., Ramirez, L.E., et al. 2001. *Plasmodium falciparum* acid basic repeat antigen (ABRA) peptides: Erythrocyte binding and biological activity. *Vaccine* **19**: 4496–4504.
- Dolan, S.A., Proctor, J.L., Alling, D.W., Okubo, Y., Welles, T.E., and Miller, L.H. 1994. Glycophorin B as an EBA-175 independent *Plasmodium falciparum* receptor of human erythrocytes. *Mol. Biochem. Parasitol.* **64**: 55–63.
- Duraisingh, M.T., Triglia, T., Ralph, S.A., Rayner, J.C., Barnwell, J.W., McFadden, G.I., and Cowman, A.F. 2003. Phenotypic variation of *Plasmodium falciparum* merozoite proteins directs receptor targeting for invasion of human erythrocytes. *EMBO J.* **22**: 1047–1057.
- Escalante, A.A., Lal, A.A., and Ayala, F.J. 1998. Genetic polymorphism and natural selection in the malaria parasite *Plasmodium falciparum*. *Genetics* **149**: 189–202.
- Espejo, F., Bermudez, A., Torres, E., Urquiza, M., Rodriguez, R., Lopez, Y., and Patarroyo, M.E. 2004. Shortening and modifying the 1513 MSP-1 peptide's α-helical region induces protection against malaria. *Biochem. Biophys. Res. Commun.* **315**: 418–427.
- Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S., et al. 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**: 498–511.

- Goel, V.K., Li, X., Chen, H., Liu, S.C., Chishti, A.H., and Oh, S.S. 2003. Band 3 is a host receptor binding merozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes. *Proc. Natl. Acad. Sci.* **100**: 5164–5169.
- Hulme, E.C. 1993. *Receptor–ligand interactions. A practical approach*. IRL Press, Oxford, UK.
- Kyhse-Andersen, J. 1984. Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* **10**: 203–209.
- Lambros, C. and Vanderberg, J.P. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol.* **65**: 418–420.
- Mahanty, S., Saul, A., and Miller, L.H. 2003. Progress in the development of recombinant and synthetic blood-stage malaria vaccines. *J. Exp. Biol.* **206**: 3781–3788.
- Mayer, D.C., Kaneko, O., Hudson-Taylor, D.E., Reid, M.E., and Miller, L.H. 2001. Characterization of a *Plasmodium falciparum* erythrocyte-binding protein paralogous to EBA-175. *Proc. Natl. Acad. Sci.* **98**: 5222–5227.
- Merrifield, R.B. 1963. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* **85**: 2149–2154.
- Mitchell, G.H., Hadley, T.J., McGinniss, M.H., Klotz, F.W., and Miller, L.H. 1986. Invasion of erythrocytes by *Plasmodium falciparum* malaria parasites: Evidence for receptor heterogeneity and two receptors. *Blood* **67**: 1519–1521.
- Nacer, A., Berry, L., Slomianny, C., and Mattei, D. 2001. *Plasmodium falciparum* signal sequences: Simply sequences or special signals? *Int. J. Parasitol.* **31**: 1371–1379.
- Okoyeh, J.N., Pillai, C.R., and Chitnis, C.E. 1999. *Plasmodium falciparum* field isolates commonly use erythrocyte invasion pathways that are independent of sialic acid residues of glycophorin A. *Infect. Immun.* **67**: 5784–5791.
- Pasvol, G. 1984. Receptors on red cells for *Plasmodium falciparum* and their interaction with merozoites. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **307**: 189–200.
- Patarroyo, M.E., Romero, P., Torres, M.L., Clavijo, P., Moreno, A., Martinez, A., Rodriguez, R., Guzman, F., and Cabezas, E. 1987. Induction of protective immunity against experimental infection with malaria using synthetic peptides. *Nature* **328**: 629–632.
- Patarroyo, M.E., Amador, R., Clavijo, P., Moreno, A., Guzman, F., Romero, P., Tascon, R., Franco, A., Murillo, L.A., Ponton, G., et al. 1988. A synthetic vaccine protects humans against challenge with asexual blood stages of *Plasmodium falciparum* malaria. *Nature* **332**: 158–161.
- Perkins, M.E. and Holt, E.H. 1988. Erythrocyte receptor recognition varies in *Plasmodium falciparum* isolates. *Mol. Biochem. Parasitol.* **27**: 23–34.
- Puentes, A., Ocampo, M., Rodriguez, L.E., Vera, R., Valbuena, J., Curtidor, H., Garcia, J., Lopez, R., Tovar, D., Cortes, J., et al. 2005. Identifying *Plasmodium falciparum* merozoite surface protein-10 human erythrocyte specific binding regions. *Biochimie* **87**: 461–472.
- Rayner, J.C., Vargas-Serrato, E., Huber, C.S., Galinski, M.R., and Barnwell, J.W. 2001. A *Plasmodium falciparum* homologue of *Plasmodium vivax* reticulocyte binding protein (PvRBP1) defines a trypsin-resistant erythrocyte invasion pathway. *J. Exp. Med.* **194**: 1571–1581.
- Richie, T.L. and Saul, A. 2002. Progress and challenges for malaria vaccines. *Nature* **415**: 694–701.
- Rodriguez, L.E., Urquiza, M., Ocampo, M., Suarez, J., Curtidor, H., Guzman, F., Vargas, L.E., Trivinos, M., Rosas, M., and Patarroyo, M.E. 2000. *Plasmodium falciparum* EBA-175 kDa protein peptides which bind to human red blood cells. *Parasitology* **120**: 225–235.
- Rost, B. and Sander, C. 1994. Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins* **19**: 55–72.
- Salazar, L.M., Alba, M.P., Torres, M.H., Pinto, M., Cortes, X., Torres, L., and Patarroyo, M.E. 2002. Protection against experimental malaria associated with AMA-1 peptide analogue structures. *FEBS Lett.* **527**: 95–100.
- Salazar, L.M., Alba, M.P., Curtidor, H., Bermudez, A., Luis, E.V., Rivera, Z.J., and Patarroyo, M.E. 2004. Changing ABRA protein peptide to fit into the HLA-DR β 1*0301 molecule renders it protection-inducing. *Biochem. Biophys. Res. Commun.* **322**: 119–125.
- Sim, B.K., Chitnis, C.E., Wasniowska, K., Hadley, T.J., and Miller, L.H. 1994. Receptor and ligand domains for invasion of erythrocytes by *Plasmodium falciparum*. *Science* **264**: 1941–1944.
- Sreerama, N., Venyaminov, S.Y., and Woody, R.W. 1999. Estimation of the number of α -helical and β -strand segments in proteins using circular dichroism spectroscopy. *Protein Sci.* **8**: 370–380.
- Suh, K.N., Kain, K.C., and Keystone, J.S. 2004. Malaria. *CMAJ* **170**: 1693–1702.
- Tam, J.P., Heath, W.F., and Merrifield, R.B. 1983. 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**: 57–65.
- Tonkin, C.J., van Dooren, G.G., Spurck, T.P., Struck, N.S., Good, R.T., Handman, E., Cowman, A.F., and McFadden, G.I. 2004. Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. *Mol. Biochem. Parasitol.* **137**: 13–21.
- Torres, M.H., Salazar, L.M., Vanegas, M., Guzman, F., Rodriguez, R., Silva, Y., Rosas, J., and Patarroyo, M.E. 2003. Modified merozoite surface protein-1 peptides with short α -helical regions are associated with inducing protection against malaria. *Eur. J. Biochem.* **270**: 3946–3952.
- Trager, W. and Jensen, J.B. 1976. Human malaria parasites in continuous culture. *Science* **193**: 673–675.
- Trager, W. and Jensen, J.B. 1978. Cultivation of malarial parasites. *Nature* **273**: 621–622.
- Urquiza, M., Rodriguez, L.E., Suarez, J.E., Guzman, F., Ocampo, M., Curtidor, H., Segura, C., Trujillo, E., and Patarroyo, M.E. 1996. Identification of *Plasmodium falciparum* MSP-1 peptides able to bind to human red blood cells. *Parasite Immunol.* **18**: 515–526.
- Valbuena, J.J., Bravo, R.V., Ocampo, M., Lopez, R., Rodriguez, L.E., Curtidor, H., Puentes, A., Garcia, J.E., Tovar, D., Gomez, J., et al. 2004. Identifying *Plasmodium falciparum* EBA-175 homologue sequences that specifically bind to human erythrocytes. *Biochem. Biophys. Res. Commun.* **321**: 835–844.
- Volkman, S.K., Hartl, D.L., Wirth, D.F., Nielsen, K.M., Choi, M., Batalov, S., Zhou, Y., Plouffe, D., Le Roch, K.G., Abagyan, R., et al. 2002. Excess polymorphisms in genes for membrane proteins in *Plasmodium falciparum*. *Science* **298**: 216–218.
- Wyatt, C.R., Goff, W., and Davis, W.C. 1991. A flow cytometric method for assessing viability of intraerythrocytic hemoparasites. *J. Immunol. Methods* **140**: 23–30.