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Conserved high activity binding peptides from the *Plasmodium falciparum* Pf34 rhoptry protein inhibit merozoites *in vitro* invasion of red blood cells

Gabriela Arévalo-Pinzón[a,b], Hernando Curtidor[a,b], Magnolia Vanegas[a,b], Carolina Vizcaíno[a,b], Manuel A. Patarroyo[a,b], Manuel E. Patarroyo[a,c,*]

[a] *Fundación Instituto de Inmunología de Colombia FIDIC, Carrera 50 # 26-20, Bogotá, Colombia*
[b] *Universidad del Rosario, Carrera 24 # 63C-69, Bogotá, Colombia*
[c] *Universidad Nacional de Colombia, Carrera 45 # 26-85, Bogotá, Colombia*

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ABSTRACT

Rhoptries are specialized secretory organelles found in all members of the genus *Plasmodium* whose proteins have been considered as promising vaccine candidates due to their involvement in cell invasion and the formation of the parasitophorous vacuole (PV). The *Plasmodium falciparum* Pf34 protein was recently identified as a rhoptry-neck protein located in detergent-resistant microdomains (DRMs) that is expressed in mature intraerythrocytic parasite stages, but its biological function is still unknown. Receptor–ligand assays carried out in this study found that peptides 36,051 (^{101}DKKFSESLKAHMDHLKILNN^{120}Y), 36,053 (^{141}KKYIIKEIQNNKYLNKEKKS^{160}), 36,055 (^{181}WLESVNNIEEKSNILKNIKS^{200}Y) and 36,056 (^{201}QLLNNIASLNHTLSEEIKNI^{220}Y), located in the central portion of Pf34, were found to establish protease-sensitive interactions of high affinity and specificity with receptors on the surface of red blood cell (RBCs). *In vitro* assays showed that Pf34 high activity binding peptides (HABPs) inhibit invasion of RBCs by *P. falciparum* merozoites, therefore suggesting that Pf34 could act as an adhesin during invasion and supporting the inclusion of Pf34 HABPs in further studies to develop antimalarial control methods.

1. Introduction

Invasion of red blood cells (RBCs) by *Plasmodium falciparum* merozoites, the causative agent of the most severe form of human malaria, comprises a series of coordinated receptor–ligand interactions between an array of different *P. falciparum* proteins and RBC surface receptors. A large number of proteins contained in three sets of morphologically distinct organelles located at the apical end of malarial merozoites (rhoptries, micronemes and dense granules) are sequentially secreted during RBC invasion to mediate the attachment, recognition and internalization of parasites into the host cells. Among these apical organelle proteins, a large number have emerged as important vaccine candidates [29].

P. falciparum rhoptry proteins have important and relevant roles for the parasite's attachment to host cells, RBC invasion, forming a strong ring-like bond in the intimate contact between the parasite and the host cell known as a moving junction (MJ), and the biogenesis and maintenance of the parasitophorous vacuole (PV) [5,21,29,34]. Several proteins that are subcompartmentalized within the neck or bulb of the rhoptries have been identified and characterized. Among these proteins are the high molecular weight (HMW) rhoptry complex proteins RhopH1, RhopH2 and RhopH3; the rhoptry-associated proteins RAP-1, RAP-2 and RAP-3 from the low molecular weight (LMW) rhoptry complex; the rhoptry-associated membrane antigen (RAMA), the reticulocyte binding-like (RBL) protein family [21] and the recently identified rhoptry-neck proteins RON2, RON4 and RON6 [1,8,23].

The role of these rhoptry proteins during RBC invasion and their importance as promissory vaccine candidates have been established by studies reporting the partial inhibition of *P. falciparum* growth *in vitro* and *in vivo* by antibodies targeted against the HMW complex [12,38], *in vitro* inhibition of RBC invasion by monoclonal antibodies raised against RAP-1 and RAP-2 [18,27], lower densities of *P. falciparum* parasitemia associated to anti-RAMA antibodies [24], and protection of *Saimiri boliviensis* monkeys against experimental challenge with *P. falciparum* conferred by immunization with recombinant RAP-1 and RAP-2 [9]. Additionally, the importance of the *pfrhoph3* gene in parasite growth and the implication of some RBL proteins in different pathways of RBC invasion have been described [19,44]. Peptide sequences derived from these rhoptry proteins have been shown to bind specifically and with high affinity to RBCs, and such binding interaction has resulted in a moderate to high inhibition of merozoite invasion to RBCs *in vivo* [33].

* Corresponding author. Tel.: +57 1 4815219; fax: +57 1 4815269.
E-mail address: mepatarr@mail.com (M.E. Patarroyo).

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The advance of -omic sciences has enabled the identification of new apical organelle proteins. Recently, Gilson et al. reported the proteome of *P. falciparum* glycosylphosphatidylinositol (GPI)-anchored membrane proteins [17], which includes merozoite surface proteins (MSPs) 1, 2, 4, 5 and 10; RAMA, apical sushi protein (ASP), Pf92, Pf38, Pf12, and a novel 34-kDa protein named Pf34 which represents ~2 to 3% of the GPI-anchored proteome at the late schizont stage. Pf34 is a 325-amino-acid-long protein specifically recognized by immune sera from people living in malaria-endemic areas of Papua New Guinea and Vietnam [30]. It is located at the neck of schizonts and merozoites rhoptries, and is exported to the nascent PV during RBC invasion [30], which has led to suggesting that, the same as other rhoptry proteins, Pf34 could be involved in RBC invasion and early steps of PV formation [21,43]. The primary structure of Pf34 is characterized by the presence of an N-terminal signal sequence containing 24 amino acids and a cleavage site between amino acids 24 and 25 (CEC-NN) [17]. Similar to RAMA, Pf34 contains a GPI-anchor preceded by a di-serine motif that is often found in this type of proteins [17,30,43]. Additionally, the region spanning from residues 140 to 249 of Pf34 is highly conserved among *Plasmodium* species and has not been described in other parasite genera [30], therefore suggesting important and unique functions for Pf34 and its orthologs.

Similar to other GPI-anchored membrane proteins, Pf34 has been isolated from detergent-resistant microdomains (DRMs), also known as lipid rafts [30]. DRMs act as platforms where specific protein–protein binding interactions take place and have been implicated in sorting of intracellular membrane proteins and signal transduction processes of different eukaryotic organisms [7]. In *P. falciparum*, DRM integrity has been shown to be essential for RBC infection [35]. So far, 62 proteins of *P. falciparum* have been identified in DRMs, including RAMA, RhopH3, and MSP-1, -2 and -4, among others [36], some of which have been studied and included in clinical trials as potential antimalarial vaccine candidates [37].

Fractionation patterns to study Pf34 and RAMA have suggested the existence of multiple rhoptry DRM subpopulations, presumably involved in the subcompartmentalization of these proteins into the rhoptries' neck or bulb [30,43]. Indeed, it has been shown that the GPI-anchored RAMA, which appears to participate in both rhoptry biogenesis [43] and RBC invasion through high activity binding sequences [28], could be also acting as escorter for the LMW complex via its direct association with the N-terminus of RAP-1 [32]. This is the first evidence showing that a GPI-anchored rhoptry membrane protein participates in sorting of proteins to the different rhoptry compartments and protein recruitment inside different DMRs. This evidence, added to Pf34's recruitment inside DRMs, its conservation among *Plasmodium* species and peak transcription toward the end of the erythrocytic cycle suggest that Pf34 could be an excellent malarial antigen to study.

In this study, the complete amino acid sequence of Pf34 was synthesized as 20-mer-long peptides and assessed using a robust, specific and sensitive receptor–ligand assay to identify high activity binding peptides (HABPs) and determine the possible role of such Pf34 HABPs in invasion of RBCs by *P. falciparum* merozoites.

2. Experimental

2.1. Chemical synthesis and binding assays

Seventeen, non-overlapping, 20-mer-long peptide spanning the complete sequence of Pf34 (PlasmoDB Id: PFD0955w), whose sequences are shown in Fig. 1A, were synthesized and purified according to a previously reported protocol [42]. Synthetic peptide (7 μl) was radiolabeled with $Na^{125}I$ according to a previously described methodology, and assessed by binding assays in triplicate [33]. Briefly, 2×10^7 O+ human RBCs were incubated at room temperature with different concentrations of each radiolabeled peptide (0–800 nM) in absence (total binding) or presence (non-specific binding) of the same unlabeled peptide (20 μM) dissolved in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline (HBS; final volume: 200 μl). After 90 min of incubation, cells were washed twice with HBS and the amount of cell-bound radiolabeled peptide was quantified in an automatic gamma counter.

2.2. Saturation assays

Modified binding assays were used to determine dissociation constants (K_d), Hill coefficients (n_H) and binding sites per cell for each Pf34 HABP. Briefly, 1.5×10^7 RBCs were incubated with increasing concentrations of radiolabeled peptide ranging from 0 to 2500 nM for HABP 36,051 and from 0 to 2200 nM for HABP 36,053 in the absence or presence of unlabeled peptide (20 μM), using a final volume of 255 μl. Cell-bound radioactivity was quantified as in binding assays.

2.3. Enzymatic treatment

RBC suspensions (60% hematocrit) were incubated at 37 °C for 60 min with either neuraminidase (150 μU/ml; ICN 9001-67-6), trypsin (1 mg/ml; Sigma T-1005) or chymotrypsin (1 mg/ml; Sigma C-4129). RBCs were then washed twice by centrifugation with HBS at 2500 *g* for 5 min and assessed as described above in binding assays. Binding to untreated RBCs was used as positive control (100% binding).

2.4. Circular dichroism (CD) analysis

The secondary structure elements of Pf34 HABPs were examined by CD spectroscopy. CD data of HABPs (5 μM) were acquired at room temperature in a nitrogen-flushed Jasco J-810 spectrometer using a 1-cm light-pass length quartz cell thermostated at 20 °C and 30% (v/v) 2,2,2-trifluroethanol (TFE) as cosolvent, which stabilizes nascent structures. Spectra were obtained by averaging three sweeps from 260 to 190 nm at a scan rate of 20 nm/min and 1-nm bandwidth. CD results were compared to a secondary structure prediction of the entire Pf34 protein obtained using the NPS@ Self-Optimized Prediction Method from Alignment (SOPMA) server [15].

2.5. Extraction and purification of P. falciparum genomic DNA

RBCs parasitized with the FCB-2 (Colombian), FVO (Vietnamese) and PAS-2 (unknown origin) strains of *P. falciparum* were obtained from asynchronous cultures maintained as described elsewhere [22]. Genomic DNA from 200 μl samples of each parasite culture (30% parasitemia) were extracted using 0.2% saponin and then purified using the UltraClean DNA Blood Isolation kit (MO BIO, Carlsbad, CA, USA).

2.6. PCR amplification

The regions encoding Pf34 HABPs 36,051, 36,053, 36,055 and 36,056 in the *P. falciparum* FCB-2, FVO and PAS-2 strains were amplified from 2 μl of each strain's genomic DNA by PCR, as previously described elsewhere [11]. The Pf34-f (5′-ATCATCACCACGTTAATAATG-3′) and Pf34-r (5′-TTAAGAGATAATCTGATGAGG-3′) specific primers were designed based on the available *P. falciparum* 3D7 genomic sequence using Gene Runner v3.05 software. The DIR1 and REV1 specific primers amplifying the region encoding HABP 33,577 from the *P. falciparum* integral membrane protein Pf25-IMP were included as PCR control

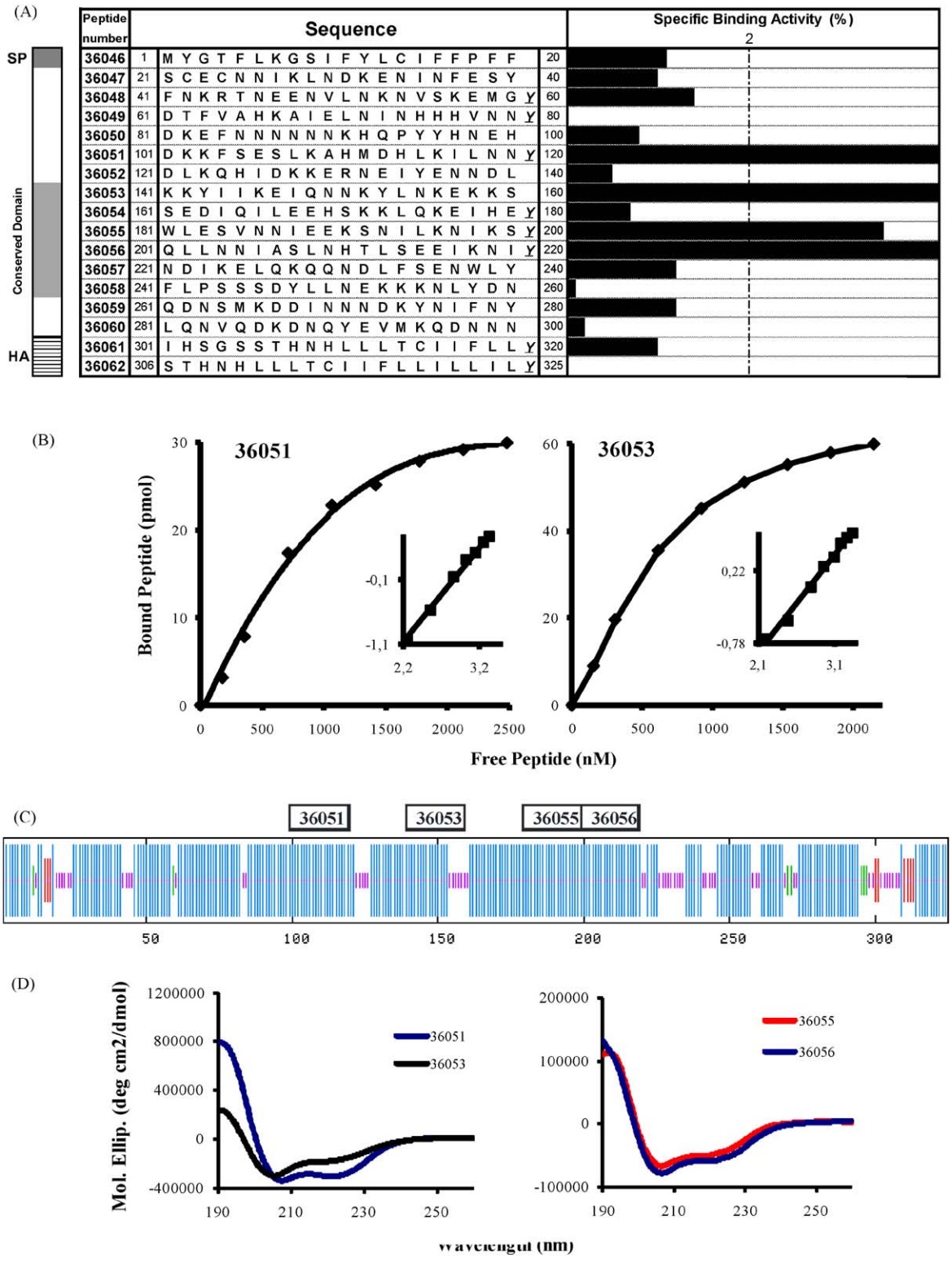

Fig. 1. Binding profiles of HABPs identified in the rhoptry-neck protein Pf34 and secondary structure CD profiles. (A) Binding profile of synthetic peptides from Pf34 to RBCs. Black bars in front of each HABP represent the percentage of binding activity, which is defined as the amount of peptide (pmol) specifically bound per added peptide (pmol). A tyrosine residue was added to the C-terminus of those peptides which did not contain this amino acid in its sequence to enable ^{125}I-labeling. The schematic representation of Pf34 shown to the left of the binding profile indicates the position of the signal peptide (*SP*), the central domain conserved among *P. vivax*, *P. knowlesi* and *P. falciparum* (residues 140–249) and the hydrophobic anchor (*HA*). (B) Saturation curves for HABPs 36,051 and 36,053. Increasing amounts of radiolabeled peptide were added in the presence or absence of the same unlabeled peptide. Curves represent specific binding. In the Hill plot (inset), the *x*-axis represents log F = free peptide and the *y*-axis log $(B/B_{max}-B)$, where B is the amount of bound peptide and B_{max} the maximum amount of bound peptide. (C) SOPMA secondary structure prediction of Pf34. The positions of HABPs 36,051, 36,053, 36,055 and 36,056 are indicated on top of the schematic representation. Blue bars: α-helix; red bars: β-sheet; violet bars: random coil; green bars: β-turn. (D) CD profile of Pf34 HABPs. Spectra were obtained by averaging three scans taken in aqueous 30% (*v*/*v*) TFE solution. The results were expressed as mean residue ellipticity [Θ], the units being degrees square centimeter per decimol according to the $[\Theta] = \Theta\lambda/(100 \times l \times c \times n)$ function, where $\Theta\lambda$ represents measured ellipticity, l the optical path length, c the peptide concentration, and n the number of amino acid residues in the sequence [40].

[11]. A PCR mix containing water instead of DNA was processed as negative control. Amplified products were visualized by gel electrophoresis in SYBR® safe-stained 1% agarose (Invitrogen, Eugene, OR, USA), purified using the Wizard PCR preps kit (Promega, Madison, WI, USA), and sequenced using the Pf34-f and Pf34-r primers.

2.7. Invasion inhibition assays

P. falciparum late schizonts (FCB-2 strain) obtained from synchronized parasite cultures (5% parasitemia and 5% hematocrit) [22] were incubated at 37 °C for 18 h in triplicate with 50, 100 and 200 μM concentrations of each HABP or mixtures of HABP 36,051 with 36,053 (m1) or 36,055 with 36,056 (m2) at 100 μM concentration for each HABP in a 5% O_2, 5% CO_2 and 90% N_2 atmosphere. Culture supernatants were removed and cells were labeled by additional incubation with 15 μg/ml hydroethidine at 37 °C for 30 min. Cell suspensions were washed thrice with phosphate buffered saline (PBS) and analyzed in a FacsCalibur flow cytometer (FACsort, FL2 channel) equipped with CellQuest software [46]. Parasitized RBCs and uninfected RBCs treated with EGTA/chloroquine were used as controls. A low activity binding peptide (LABP) was used as negative binding control.

3. Results

3.1. Most of Pf34 HABPs located inside the conserved central domain of Pf34 bind with high affinity to human RBCs

Specific binding was defined as the amount (%) of peptide (pmol) that binds specifically to RBCs per amount of added peptide (pmol). The binding curve was obtained by calculating the difference between total binding (binding in the absence of non-radiolabeled peptide) and unspecific binding (binding in the presence of non-radiolabeled peptide). Based on these data, peptides having a slope of ≥0.02 in the specific binding curve were considered to be HABPs, according to previously reported criteria establishing that a HABP recognizes more than 2 000 binding sites per cell [33]. As a result, the Pf34 peptides 36,051 (^{101}DKKFSESLKAHMDHLKILNN^{120}Y), 36,053 (^{141}KKYIIKEIQNNKYLNKEKKS^{160}), 36,055 (^{181}WLESVNNIEEKSNILKNIKS^{200}Y) and 36,056 (^{201}QLLNNIASLNHTLSEEIKNI^{220}Y) were identified as HABPs given that they exhibited specific binding activities to RBCs larger than 2% (Fig. 1A). The majority of Pf34 HABPs were found in the protein's central region but only HABPs 36,053, 36,055 and 36,056 were located inside the conserved central domain of Pf34 which has been suggested to be functionally important and unique among the genus *Plasmodium* [30].

Dissociation constants (K_d), Hill coefficients (n_H) and binding sites per cell (BSC) that characterize the interactions of Pf34 HABPs with RBC surface receptors were determined by analyzing their saturation curves and Hill plots. K_d values of 600 and 780 nM were determined for HABPs 36,053 and 36,051, respectively, and a n_H of 1.4 was found for both HABPs (Fig. 1B). There were ~297,000 binding sites per cell for HABP 36,051 and ~562,000 for HABP 36,053, whereas RBC receptors for HABPs 36,055 and 36,056 were not saturable in the assessed conditions and therefore and estimate could not be obtained.

A secondary structure of Pf34 was predicted using the Self-Optimized Prediction Method with Alignment (SOPMA) [15]. The analysis showed a high content of α-helical structures along the entire protein, but especially in the regions where Pf34 HABPs had been identified (Fig. 1C). CD analysis showed ordered structures with predominant α-helical tendency in all Pf34 HABPs, as indicated by two minima found at 208 and 220 nm, respectively, and one ellipticity maximum at 190 nm (Fig. 1D).

Table 1
Inhibiting parasite invasion of RBCs by Pf34 HABPs.

	% of invasion inhibition[a]
Peptides (200 μM)	
36,051	32 ± 1
36,053	31 ± 1
M1: 36,051–36,053	52
36,055	37 ± 1
36,056	41 ± 1
M2: 36,055–36,056	47
36,057	3 ± 2
36,058	5 ± 3
Controls	
Parasites	0 ± 2
Chloroquine (1.85 mg/ml)	90 ± 1
EGTA (1.9 mg/ml)	68 ± 1

[a] Mean standard deviation of three experiments.

3.2. Pf34 HABP specific bindings is affected by different types of enzymatic treatment

RBCs were treated with different enzymes that cleave specific surface protein motifs to identify the nature of RBC receptors for Pf34 HABPs; RBCs were then incubated with each peptide as in the binding assays. Results showed that HABP 36,055 specific binding was reduced to 40 and 55% of its former binding power when RBCs were pre-treated with chymotrypsin and trypsin, respectively. HABP 36,053 specific binding fell to 31% when RBCs were treated with chymotrypsin but only dropped to 90% specific binding when RBCs were treated with trypsin. On the other hand, HABP 36,056 specific binding was only slightly reduced (25%) when RBCs were pre-treated with neuraminidase. HABP 36,051 binding to RBCs treated with neuraminidase, trypsin or chymotrypsin was higher than that displayed by the control (untreated RBCs), reaching 165, 333 and 123% specific binding, respectively (Fig. 2A).

3.3. Pf34 HABPs are highly conserved among P. falciparum strains

PCR amplification of the region encoding HABPs 36,051, 36,053, 36,055 and 36,056 in the three analyzed strains resulted in ~531 bp specific band (Fig. 2B), whose sequences were obtained and aligned to the sequence reported for the 3D7 (isolated in The Netherlands) using Clustal W software [10]. As it can be seen in Fig. 2C, amino acid sequences shared a 100% of identity. No nucleotide substitutions were found within the studied region, therefore ruling out the presence of synonymous substitutions (data not shown).

3.4. Pf34 HABPs block invasion of RBCs by P. falciparum merozoites

Considering Pf34 HABP interaction with receptors on RBC surface, invasion assays were carried out to determine whether Pf34 HABPs affected RBC invasion. Each HABP was incubated with *P. falciparum* late schizonts. As shown in Table 1, all Pf34 HABPs inhibited invasion of RBCs by 31–41% compared to low activity binding peptides (LABPs), which inhibited invasion by 5%. 52 and 47% invasion inhibition was also observed when HABPs were used in mixtures (Table 1).

4. Discussion

Several apical organelle proteins are secreted into the extracellular space or translocated to the surface of merozoites during invasion of human RBCs, which leaves them particularly vulner-

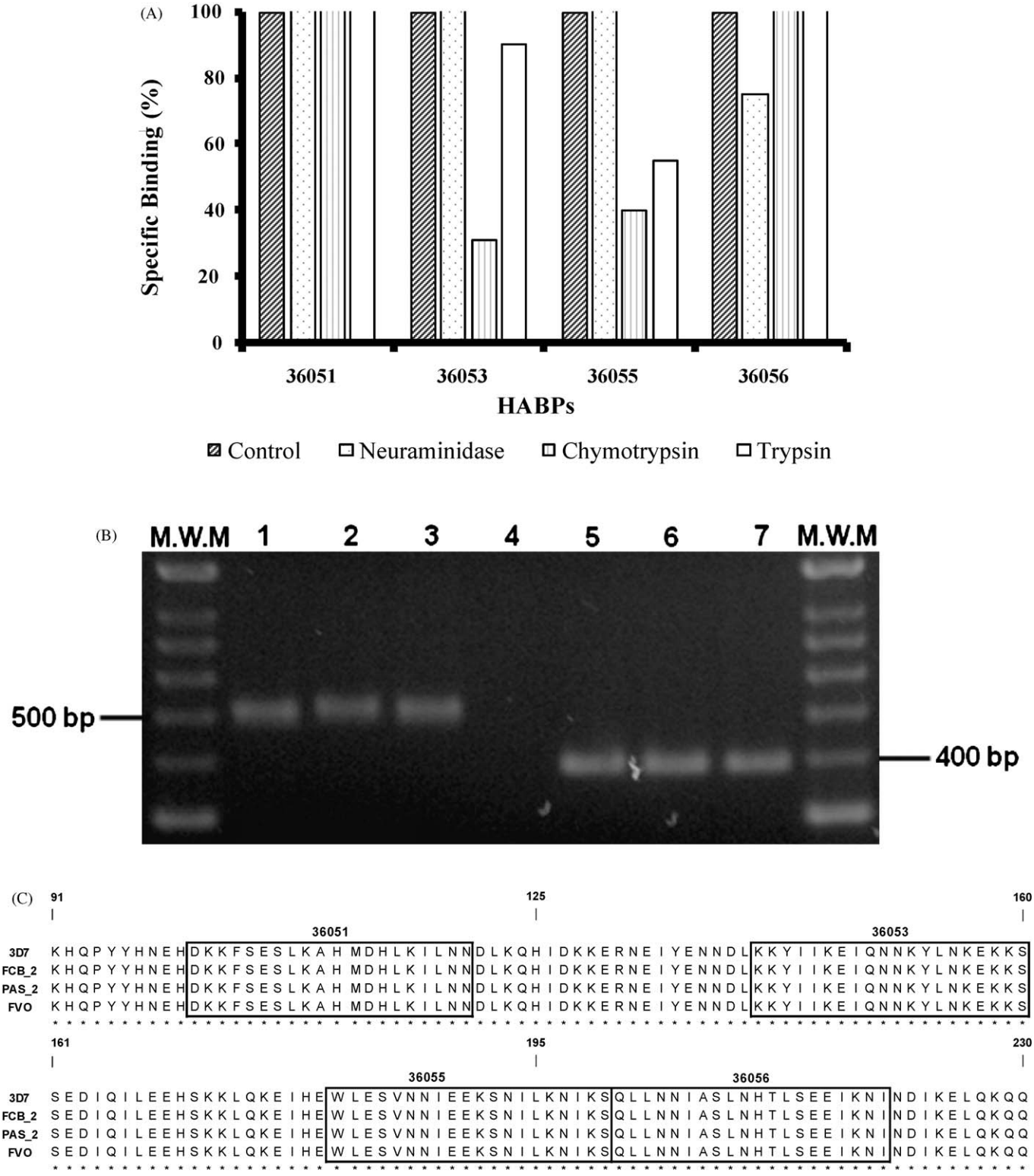

Fig. 2. Analysis of the nature of RBC membrane receptors and polymorphism of region encoding Pf34 HABPs. (A) Binding assays using RBCs treated with neuraminidase, chymotrypsin and trypsin as well as non-enzyme-treated RBCs (100% binding). (B) PCR amplification products of the region encoding Pf34 HABPs 36,051, 36,053, 36,055 and 36,056 obtained from genomic DNA of the *P. falciparum* strains FCB-2 (lane 1), FVO (lane 2) and PAS-2 (lane 3). Lane 4: negative control. Lanes 5, 6 and 7: positive control (Pf25-IMP) for the FCB-2, FVO and PAS-2 strains, respectively. M.W.M: 100-bp ladder. (C) Amino acid sequence alignment of the Pf34 region studied in the 3D7, FCB-2, FVO and PAS-2 strains.

able to the attack of the host's immune system. Some of these proteins are transcribed during the intraerythrocytic cycle, have adhesin domains involved in receptor–ligand interactions, and possess classical secretory signal sequences and GPI-anchor sequences or transmembrane domains [3,6].

A large number of secretory proteins implicated in binding to and invasion of RBCs have been isolated from DRMs, among which are included the apical organelle proteins as well as the merozoite surface antigens [36]. For instance, multiprotein complexes such as the MSP-1/7 complex, LMW, HMW, RAMA/RhopH1

and the invasion machinery complex (gliding-associated proteins 45/50/myosin A) are known to reside in these membrane domains and to serve as platforms to a relatively large number of still uncharacterized proteins [36].

The present study focused on the novel *P. falciparum* rhoptry protein Pf34, which is expressed as a 34-kDa polypeptide in asexual parasite blood-stages and recruited into DRMs [30], and aimed at evaluating its involvement in RBC invasion. With this purpose on mind, the entire Pf34 amino acid sequence was divided into 20-mer-long synthetic peptides and the specific binding activity to RBCs of each protein fragment was assessed in order to identify HABPs. Four HABPs showed slopes above 2% in their specific binding curves and were located in the protein's central region (Fig. 1A). High affinity interactions (as indicated by K_d values within the nanomolar range) and around 297,000–562,000 BSC were found for HABPs 36,051 and 36,053 (Fig. 1B). Both peptides exhibited an n_H larger than 1, thus indicating a clear tendency to favoring binding of a second ligand (positive cooperativity). RBC receptors for HABPs 36,055 and 36,056 were not saturable in the assessed conditions, even when using fewer cells, suggesting that these HABPs interact with more than one binding site or that they have low affinity interactions.

The identification of HABPs in the rhoptry-neck protein Pf34 suggests that this protein could be participating in some stages of *P. falciparum* merozoite invasion of RBCs, and is consistent with the sequential release pattern of the rhoptries' content, where proteins located at the neck portion are the first ones to be released and thereby the first ones to interact with the host cell together with protein being released from the micronemes. Comparative proteome analyses between *P. falciparum* and *Toxoplasma gondii* have identified new rhoptry-neck proteins denoted as PfRONs, which are homologous to the TgRON proteins [1,8]. TgRONs have been implicated in MJ formation and have been suggested to be translocated to host cell membrane where they are thought to act as receptors for other parasite proteins such as TgAMA-1 [5]. These studies led to the hypothesis that *P. falciparum* proteins located within the rhoptry's neck subcompartment such as Pf34 could be participating in MJ formation, although further studies are needed in the case of Pf34 to completely elucidate its role in this process.

Strikingly, an evaluation of Pf34's secondary structure using a prediction bioinformatics tools found a high content of α-helical structures, especially in the regions where the Pf34 HABPs are located (Fig. 1C). These results are consistent with CD analysis showing ordered structures of predominant α-helical tendency in all HABPs (Fig. 1D). Previous secondary structure analyses carried out for a considerable number of HABPs using ^{1}H nuclear magnetic resonance (^{1}H NMR) have confirmed the results of CD spectroscopy determinations and have superimposed very well with the 3D structure of their corresponding portion in the *P. falciparum* recombinant protein (as assessed by X-ray crystallography) in which they were identified [26]. This evidences a correspondence between the secondary structure of HABPs, as assessed by CD spectroscopy, and the structure natively adopted in their corresponding parasite protein. Likewise, it has been reported that inducing modifications in the structure of α-helical HABPs improves their presentation in the context of the Class II major histocompatibility complex–peptide–T cell receptor complex, which results in the activation of the host's immune system and the induction of a protective immune response against *P. falciparum* challenge in *Aotus* monkeys [25].

Numerous studies with enzymatically treated RBCs have shown that *P. falciparum* uses several ligands and alternative pathways to invade RBCs [14,41]. Up to the moment, some of the RBC receptors proposed to interact with several parasite protein families include glycophorins A, B and C, band 3 protein and the yet uncharacterized receptors E, X, Y and Z, all of which display different enzyme sensitivity profiles [4,13,16,31,39]. For this reason, a fully effective antimalarial vaccine should contain antigens from all of the parasite proteins that act as ligands for RBCs in order to induce an appropriate immune response capable of inducing sterilizing immunity.

We examined the possible nature of the RBC membrane receptors for Pf34 HABPs by means of binding assays to enzyme-treated RBCs. The results showed that binding of HABP 36,055 was sensitive to treatments with chymotrypsin and trypsin (Fig. 2A). To date, RBC membrane receptors sensitive to trypsin (which removes glycophorins A and C) as well as to chymotrypsin (which acts on the extramembrane domains of band 3), but resistant to neuraminidase (a sialidase that removes sialic-acid groups of some glycophorins) have not been reported.

A similar behavior was shown by HABP 36,053 whose binding decreased when RBCs were treated with chymotrypsin (Fig. 2). This behavior is consistent with the enzymatic sensitivity profile of the yet unidentified receptor Z, which has been proposed to interact with the PfRh2a/b protein [13]. On the contrary, binding of HABP 36,051 increased with all enzymatic treatments compared to binding to untreated RBCs, therefore indicating that these enzymes could be unmasking "cryptic" receptors and hence improving binding of this HABP. Interestingly, merozoite proteins like the apical membrane antigen 1 (AMA-1) bind to a putative RBC membrane receptor known as "Kx" through a process that involves exposure or modification of the RBC surface by trypsin-like enzymes [20].

On the other hand, binding of HABP 36,056 was only affected by treatment with neuraminidase (Fig. 2A). This sialic-acid dependent binding points to receptor Y (the receptor for PfRH1 [31]) as the possible receptor for this HABP. It should be however noted that the data reported in this study regarding the receptor(s) for Pf34 HABPs are only an approximation to the nature of their RBC membrane receptors and that additional studies should be performed in order to obtain a complete characterization.

Each HABP was individually incubated with *P. falciparum* late-stage schizonts to further explore Pf34 HABP interactions with host–cell membrane receptors and these HABPs possible role in merozoite invasion of RBCs. Flow cytometry data showed that merozoite invasion of uninfected RBCs was moderately inhibited by all HABPs (Table 1); however, invasion inhibition became increased when HABPs were used in mixtures, thereby strongly supporting a role in invasion for protein Pf34. Invasion inhibition for all HABP using different receptors having different affinities (see results obtained from enzyme treatment and saturation) is in agreement with the large repertoire of ligands and receptors that the parasite uses differentially to invade RBCs. It has been reported that parasite lines known to invade RBCs through a sialic-acid dependent mechanism express high levels of Rh1and tend to express low or undetectable levels of Rh2a and Rh2b, whereas parasites expressing low levels of Rh1 express higher levels of Rh2a and Rh2b, which drives invasion through a sialic-acid independent invasion pathway [13,44]. Additionally, it has been shown that the W2mef strain, which requires of sialic-acid containing proteins for invasion, has the ability to change to a sialic-acid independent pathway through clone selection by incubation with neuraminidase treated RBCs [41]. This mechanism confers parasites with an important strategy to evade the host's immune response and therefore peptide components derived from the different proteins implicated in invasion should be included to block parasite entry into RBCs effectively.

Another strategy used by the parasite to successfully complete its life cycle and one of the main obstacles for developing an effective antimalarial vaccine is the extensive genetic variability of pathogenesis-related proteins. High genetic polymorphism is probably used as a mechanism to distract and evade the host's immune response from attacking conserved protein regions that are crucial for parasite survival [2,45]. Based on this notion, the design of a subunit-based synthetic vaccine has focused on HABPs located

inside conserved amino acid regions, which is why it was relevant for this study to assess the polymorphism of each of the Pf34 HABPs among *P. falciparum* strains from different geographical regions. Sequence analyses of the region where Pf34 HABPs are Pf34 in the FCB-2, FVO and PAS-2 strains showed a 100% of identity when aligned to the sequence reported for the 3D7 strain (isolated in The Netherlands) (Fig. 2). Nevertheless, previous studies have shown that conserved HABPs are poorly immunogenic and induce poor protection against experimental challenge in *Aotus* monkeys. The immunologic characteristics of HABPs have been enhance by using the their amino sequences as template to design peptide analogs of modified structures (substituting amino acid critical binding amino acids for others with same mass but opposite polarity), which has been shown to allow a better adjustment inside the binding groove of MHC molecules and a better presentation to the T cell receptor, thus therefore inducing a protective response [25].

It would be therefore necessary for the design of an effective antimalarial vaccine to include conserved peptides from parasite proteins that interact specifically with host cells and are capable of blocking parasite invasion to host cells. Peptides with such characteristics have been identified in this study within the sequence of Pf34.

5. Conclusions

In this study, we have considered the principles defined for the development of a minimal subunit-based, multiantigenic, multistage, chemically synthesized antimalarial vaccine in order to characterize receptor–ligand interactions between peptides derived from Pf34 and RBC surface molecules. Four HABPs were identified, here denoted as 36,051 (^{101}DKKFSESLKAHMDHLKILNN^{120}Y), 36,053 (^{141}KKYIIKEIQNNKYLNKEKKS^{160}), 36,055 (^{181}WLESVNNIEEKSNILKNIKS^{200}Y) and 36,056 (^{201}QLLNNIASLNHTLSEEIKNI^{220}Y), all of which bind with high affinity and specificity to proteins on the surface of RBCs. These HABPs are completely conserved among *P. falciparum* isolated from different geographical regions, and their secondary structure contains a significant content of α helical features. Pf34 HABPs were capable of inhibiting *in vitro* invasion of human RBCs by up to 41%, which suggest they are involved in invasion.

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