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Conserved regions of the *Plasmodium falciparum* rhoptry-associated protein 3 mediate specific host-pathogen interactions during invasion of red blood cells

Jeison García[a,b], Hernando Curtidor[a,b], Magnolia Vanegas[a,b], Gabriela Arévalo-Pinzon[a,b], Manuel A. Patarroyo[a,b], Manuel E. Patarroyo[a,c,*]

[a] *Fundación Instituto de Inmunología de Colombia FIDIC, Carrera 50 No. 26-20, Bogotá, Colombia*
[b] *Universidad del Rosario, Calle 14 No. 6-25, Bogotá, Colombia*
[c] *Universidad Nacional de Colombia, Carrera 45 No. 26-85, Bogotá, Colombia*

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ABSTRACT

Invasion of red blood cells (RBCs) by the *Plasmodium falciparum* malaria merozoite is mediated by parasite surface molecules and proteins contained within apical organelles that are capable of recognizing receptors on the membrane of RBCs. The identification and characterization of these *P. falciparum* invasion-associated proteins is the first step for unveiling potential new drug and vaccine target molecules to eradicate this deadly disease. Among the exclusive set of malarial vaccine candidates, the members of the rhoptry-associated protein (RAP) family have been associated with the parasite's binding to and invasion of RBCs. Remarkably, the third member of this family (named RAP-3) has been recently detected on the surface of non-infected RBCs exposed to free merozoites, therefore suggesting the participation of this protein during RBC infection. In this study, the sequence of RAP-3 was finely mapped using synthetic peptides in order to identify which are the specific binding regions involved in RAP3–RBC interactions. Two high-activity binding peptides (HABPs) established high affinity interactions with RBC surface molecules of about 27–90 kDa, which were differentially affected by different enzymatic treatments. RAP-1 and RAP-2 HABPs inhibited binding of RAP-3 HABPs to different extents, thus suggesting the recognition of similar binding sites on RBC membrane, as well as ability of RAP-3 HABPs to inhibit *P. falciparum* infection *in vitro*. Altogether, these functional analyses of RAP-3 HABPs strongly suggest a potential role for this protein in RBC invasion, and highlight its HABPs as potential targets to develop a fully protective minimal subunit-based malarial vaccine.

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1. Introduction

More than 3.2 billion people live at risk of being infected with any of the *Plasmodium* species that cause malaria in humans: *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium knowlesi* and *Plasmodium falciparum*. Of these five species, *P. falciparum* causes the most lethal form of malaria, accounting for ~250 million cases and nearly 1 million deaths each year [46–48]. Malaria is characterized by periodic cycles of acute and febrile illness occurring every 48 or 72 h, which are accompanied by anemia, headache, muscle pain, nausea and lassitude. In addition to these symptoms, severe complications such as cerebral and placental malaria occur due to the sequestration (rosetting and cytoadherence) of trophozoite- and schizont-infected red blood cells (RBCs) to endothelial cells of deep vascular beds in vital organs [4,30].

Successful infection of RBCs by *P. falciparum* merozoites requires of complex and specific host–pathogen interactions, which are mediated by merozoite surface molecules and proteins contained within the apical organelles that recognize and bind to different receptors on the host cell surface [10,13]. Genome, transcriptome and proteome analyses of *P. falciparum* erythrocytic stages indicate that around 58–90 proteins are associated with RBC invasion [5,7,8], and that the parasite employs multiple alternate routes of invasion, formation of macrocomplexes, high sequence variability and differential expression of invasins as highly efficient strategies to evade the host's immune response [3,9,14,36].

These observations clearly show that the development of a fully effective control strategy against malaria requires a deep understanding of the molecular mechanisms underlying *P. falciparum* invasion and need to be supported by a comprehensive analysis of most of the parasite invasion-associated proteins (if not all) and their role during RBC infection [28,33]. Among the parasite's exquisite invasion machinery, proteins contained within the invasion-specialized apical organelles (micronemes, rhoptries and dense granules) of *P. falciparum* merozoites are some of the most

* Corresponding author at: Fundación Instituto de Inmunología de Colombia FIDIC, Carrera 50 No. 26-20, Bogotá, Colombia. Tel.: +57 1 4815219, fax: +57 1 4815269.
E-mail address: mepatarr@mail.com (M.E. Patarroyo).

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interesting targets. Among these proteins, two rhoptry-associated protein complexes are considered attractive antimalarial targets: the high molecular weight (HMW or RhopH complex) and the low molecular weight (LMW) complexes; the first one containing the RhopH-CLAG proteins and the second one consisting of the rhoptry-associated proteins (RAP)-1, RAP-2 and RAP-3 [21,22]. The HMW and LMW complexes participate during *P. falciparum* invasion of host cells together with some of most the widely studied vaccine candidates: the families of merozoite surface proteins (MSPs), erythrocyte binding antigens (EBAs) and reticulocyte-binding homologues (RHs or RBPs) [10,20], and therefore have been interesting targets to induce a direct a protective immune response against blood stage malaria parasites.

The first member of the RAP family, RAP-1, is a 782-amino acids long protein which is proteolytically processed from an 84-kDa polypeptide. RAP-1 has been shown to be target of invasion inhibitory monoclonal antibodies *in vitro* and to induce protection in *Saimiri* monkeys against *P. falciparum* challenge [31,32,38]. Interestingly, this protective ability has been associated with the formation of a rhoptry-associated complex consisting of at least three polypeptides of 80 kDa, 65 kDa and 42 kDa [18]. The first two molecules correspond to processed forms of RAP-1, whereas the last one is consistent with the 40–42 kDa mature form of RAP-2 [37]. Altogether, these immunogenic and functional characterization studies of the LMW members [2,26,43], together with the importance of RAP-1 and RAP-2 for binding of *P. falciparum* parasites to RBCs [41], highlight the potential of the members of the RAP family as asexual antimalarial vaccine candidates.

Given the shown relevance of the RAP family members in parasite invasion and the host's immune response, the third member of this family could also be an interesting antimalarial target. RAP-3 is expressed as a 45 kDa polypeptide which shares 68% of similarity and 44% of identity with its homologous protein, RAP-2 [2]. In contrast with RAP-1 and RAP-2, defining a concrete functional role for RAP-3 during parasite invasion to RBCs had remained elusive since no conclusive evidence supporting binding of RAP-3 to host cells has been reported and its importance in mutated parasites was only found until recently [2,41]. However, in addition to the localization of RAP-3 in detergent-resistant membranes of mature schizonts together with important parasite invasins [35], it has been described that anti-RAP-3 antibodies recognize molecules on the surface of non-parasitized RBCs in a parasitemia-dependent manner after merozoite release *in vitro* (a phenomenon directly associated with the establishment of a RAP-3–RBC interaction) [1]. Together, these suggest RAP-3 as a potential target to prevent merozoite invasion and arise interesting questions regarding its role during the exquisite complex host–pathogen interactions mediating parasite invasion into RBCs.

On the basis of the possible involvement of RAP-3 in merozoite infection of RBCs, we performed ligand receptor assays with synthetic peptides spanning the entire sequence length of this protein to map the specific regions of RAP-3 involved in establishing high affinity interactions with RBC surface receptor(s). Conserved RAP-3 high-activity binding peptides (HABPs) able to bind specifically to RBCs and inhibit the *P. falciparum* invasion to RBCs *in vitro* could be attractive targets for immunological and structural characterization studies conducted as part of a rational and logical methodology to completely protective malaria vaccine [28,33],

2. Materials and methods

2.1. Synthesis and radiolabeling of RAP-3 peptides

The complete sequence of RAP-3 (PlasmoDB: PFE0075c, 3D7 strain) was synthetically reproduced using 20-mer-long non-overlapping peptides, according to the *t*-Boc solid-phase multiple peptide synthesis strategy [19,25]. The so obtained peptide products were then purified by reverse-phase high performance liquid chromatography (RP-HPLC) and characterized by matrix-assisted laser desorption time-of-flight (MALDI-ToF) mass spectrometry before being radiolabeled. Radiolabeling of tyrosine-free peptides was enabled by adding such residue to the peptide's C-terminal.

Subsequently, RAP-3 purified peptides were radiolabeled with ^{125}I. Briefly, 5 μL of peptide solution in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline (HBS; 1 mg/mL) were added to 0.3 μmol of chloramine T (2.75 μg/μL) and 5 μL of Na^{125}I (100 mCi/mL, MB Biomedicals). After incubation for 15 min at room temperature, the reaction was stopped by adding 0.18 μmol of sodium metabisulfite. Radiolabeled peptides were recovered from the mixture using size exclusion chromatography on a Sephadex G10 column (100 mm × 5 mm, Pharmacia, Uppsala, Sweden) and quantified on a gamma counter (Auto Gamma Counter Cobra II Packard) [15,34].

2.2. RAP-3–RBCs binding assays

To identify the specific binding regions of RAP-3, 1 × 10^8 human RBCs were incubated for 90 min at room temperature with increasing concentrations of each radiolabeled peptide (0–560 nM) in the absence (total binding) or presence (non-specific binding) of unlabeled peptide (4 nmol). Cells were then washed with HBS buffer and the RBC-associated radioactivity was measured in a gamma counter. All assays were carried out in triplicate. For each RAP3 HABP, the specific binding activity (specific binding = total binding − unspecific binding) was determined at four increasing concentrations bearing in mind that peptides with a specific binding curve (amount of radiolabeled peptide bound specifically to RBCs versus added radiolabeled peptide) with a slope of ≥0.02 (2% binding) were considered as HABPs [33,34,45]. In addition, a scrambled HABP (i.e., a peptide with the same amino acid composition to that of RAP-3 HABP 33860 but with a random sequence) denoted as 36098 was synthesized (Fig. 1) and also tested as described above.

To determine the kinetic constants associated with the bindings interactions of RAP-3 HABP, a modified binding assay was performed to determine binding saturation. Briefly, 7.5 × 10^7 RBCs were incubated with a wider range of concentrations of each radiolabeled HABP (0–1800 nM), following the scheme of the binding assays described above.

2.3. Polymorphism analysis of RAP-3: PCR amplification and cloning

RBCs parasitized with the *P. falciparum* FCB-2 (Colombian), FVO (Vietnamese) and PAS-2 (unknown origin) strains were obtained from asynchronous cultures, which were maintained as described elsewhere [23,44]. Genomic DNA (gDNA) was extracted from 200 μL aliquots of each strain's culture (30% parasitemia) using 0.2% saponin and then purified using UltraClean DNA Blood Isolation kit (MO BIO, Carlsbad, CA). Two microliters of gDNA was used to amplify the region encoding the RAP-3 HABPs 33860 and 33873 in the *P. falciparum* FCB-2, FVO and PAS-2 strains by PCR. Amplification primers (RAP-3-f 5′-AAGAAAGCATTGATAGATATTG-3′ and RAP-3-r 5′-TAATGATCTGCTGCACGA-3′) were designed using Gene Runner v3.05 software based on the *P. falciparum* 3D7 genomic sequence (PlasmoDB ID PFE0075c). DIR1 and REV1 primers amplifying the genome region encoding HABP 33577 of the *P. falciparum* integral membrane protein Pf25-IMP were included as positive amplification control [11].

The PCR amplification mixtures (50 μL) contained: 1U Taq polymerase (Bioline, Taunton, MA), 1× Taq polymerase reaction buffer,

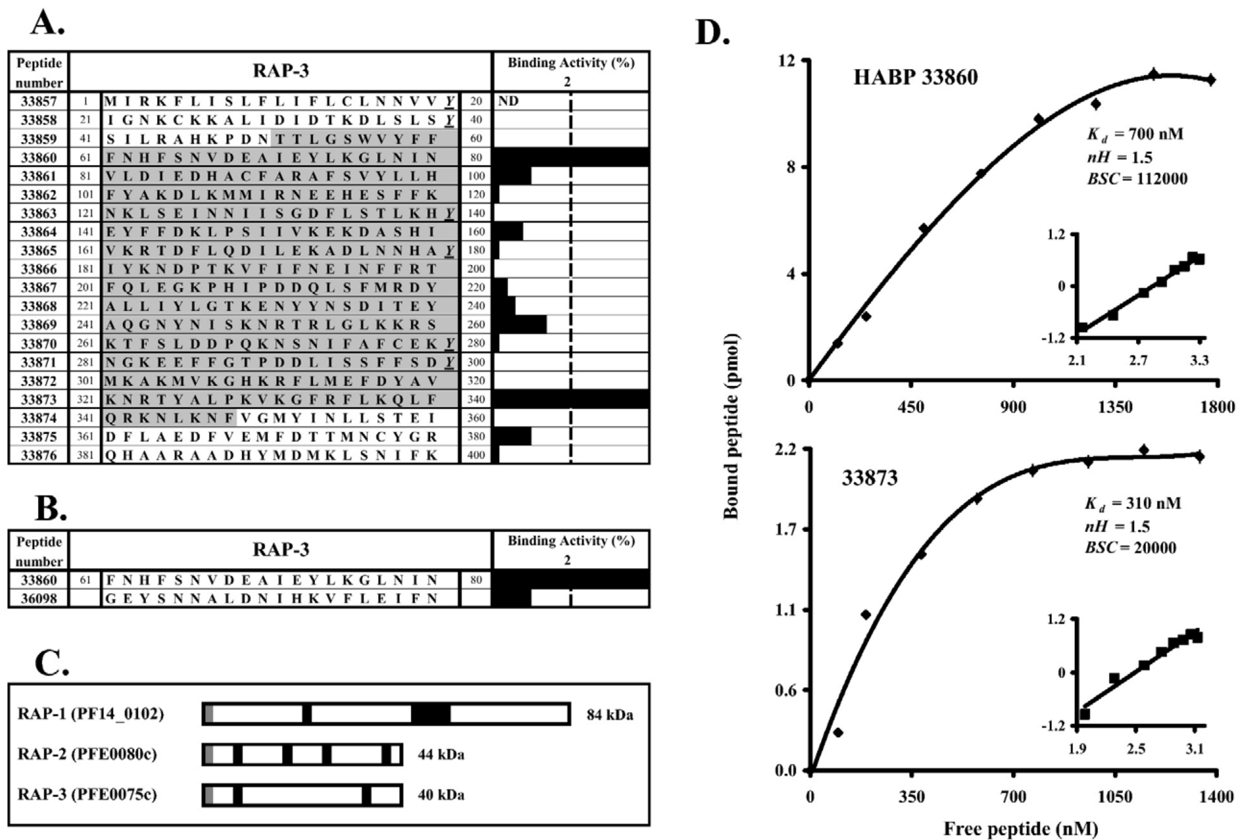

Fig. 1. Specific binding features of RAP-3 synthetic peptides. (A) Binding profile of RAP-3 peptides. Peptides are numbered according to our institute's serial system. The length of black horizontal bars indicates the specific binding activity of each RAP-3 sequence. HABPs 33860 and 33873 were the only ones able to overcome the 2% cut-off point. The localization of each sequence within RAP-3 is also shown as superscripts, while region highlighted in gray is highly conserved between different *P. falciparum* strains. (B) Specific binding activity of RAP-3 HABP 33860 and its corresponding scrambled peptide 36098. The sequence of the scrambled peptide was designed using the Shuffle protein server [42]. (C) Schematic representations of RAP family members, showing their PlasmoDB IDs and molecular weights. Signal sequences are indicated in gray, while the localization of each HABP within RAP-3 is showed in black. (D) Saturation assays for RAP-3 HABPs. *Inset*: the dissociation constant (K_d), the Hill coefficient (n_H) and the number of binding sites per cell (*BSC*) found for each HABP are also shown. In the Hill analysis plot (*insert*), the abscissa is Log *F* and the ordinate is $\log(B/B_{max} - B)$, where *F* corresponds to free peptide, *B* to the amount of bound peptide and B_{max} is the maximum amount of bound peptide.

1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.4 μM of each primer. Thermocycling parameters were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of 1 min annealing at 56 °C for RAP-3-f/RAP-3-r or at 58 °C for DIR1/REV1, 1 min extension at 72 °C and 1 min denaturing at 95 °C. Final extension cycle was carried out at 72 °C for 5 min. As negative control, the same reaction conditions were followed using water instead of gDNA. Amplification products were visualized in 1% agarose gels stained with SYBR® safe (Invitrogen, Eugene, OR).

After amplification, PCR products were purified using the Wizard PCR preps kit (Promega, Madison, WI) and cloned into the pGEM-T vector (Promega, Madison, WI). The cloned plasmid was then used to transform *Escherichia coli* JM109 (Promega, Madison, WI), checking the correct insertion of the cloned fragment by colony PCR using the vector-annealing primers T7 and SP6. Plasmid DNA was then extracted from recombinant colonies using the Miniprep purification kit (Promega, Madison, WI). Five recombinant clones of each strain positively confirmed by PCR were sequenced in an automatic sequencer (ABI PRISM 310 Genetic Analyzer, PE Applied Biosystems, Foster City, CA).

2.4. Cross-competition assays between LMW complex HABPs

Considering that the RAP protein family is part of the LMW complex, its protein member could be associated with cooperative and/or competitive interactions with RBCs. To analyze this statement, a cross-competition preliminary assay was performed between RAP-3 HABPs 33860 and 33873 and RAP-1 HABPs 26188, 26202 and 26203 [12] or RAP-2 HABPs 26225, 26229 and 26235 [24]. 24 nM of ^{125}I-labeled-HABP (33860 or 33783) were incubated with 1×10^8 RBCs as described above in binding assays (Section 2.2) in triplicate, in the absence or presence of unlabeled RAP-1 and RAP-2 HABPs (8–80 μM) and using RAP-3 original HABPs specific binding as control. Then, the amount of cell-bound radiolabeled peptide was determined in an automatic gamma counter for all samples.

2.5. Binding assays with enzyme-treated RBCs and cross-linking assays

The effect of different enzymatic treatments on the RBC surface receptor(s) for the RAP-3 HABPs was also tested. Enzyme-treated RBCs were obtained by incubation (3.0×10^8) with one of the following enzymes: neuraminidase (150 μU/mL, ICN 9001-67-6), trypsin (1 mg/mL, Sigma T-1005) or chymotrypsin (1 mg/mL, Sigma C-4129) enzymes 1 h at 37 °C. After remove the enzyme excess by continuous washes with HBS, radiolabeled HABPs were assessed in binding assays (Section 2.2) with each type of treated cells, using untreated RBCs as positive control according to previously reported methodologies [15,29]. This same treatment was applied to RAP-1 HABPs 26202 [12] and RAP-2 HABP 26225 [24].

Additionally, the apparent molecular weights of the RBC membrane receptors of each RAP-3 HABP were assessed in cross-linking assays. In brief, radiolabeled HABPs were cross-linked to human

RBCs (2.1×10^7) using 50 μL (1 mg/mL) of BS3 bis sulfosuccinimidyl suberate (Sigma–Aldrich). Then, cross-linked proteins were extracted with lysis buffer (5 mM Tris–HCl buffer, 7 mM NaCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and exposed to a photographic film for two weeks [15,29]. The apparent molecular weights were determined using molecular weight reference patterns (Prestained Protein Marker, Broad Range-BioLabs).

2.6. Assessing HABPs ability to inhibit merozoite invasion

Sorbitol-synchronized *P. falciparum* (FCB-2 strain) cultures were incubated until late schizogony in RPMI 1640 + 10% O_2 + plasma (0.5% parasitemia and 5% hematocrit). Cultures were seeded in triplicate in 96-well cell culture plates (Nunc, Denmark) together with 200 μM of RAP-3 HABPs 33860 and 33873 and a 1/1 of mixture HABPs. Suspensions were analyzed by flow cytometry using a FACsort in Log FL2 data mode and CellQuest software (Becton Dickinson immunocytometry system, San Jose, CA) [23,49]. Infected and uninfected RBCs treated with 500 μM ethylene glycol tetraacetic acid (EGTA) and 285 μM of chloroquine were used as positive invasion inhibition controls. The RAP-3 low activity binding peptides (LABPs) 33862 and 33864 and untreated infected RBCs were used as negative controls.

2.7. Secondary structure determination of RAP-3 HABPs

With the aim of establishing the main structural characteristics of RAP-3 HABPs, these peptides were analyzed by circular dichroism (CD) in 30% (v/v) trifluoroethanol (TFE)/H_2O, using a 1 cm path length rectangular quartz cell thermostated at 20 °C. TFE is used as cosolvente, which has been shown to stabilize the nascent structure. All spectra were recorded in a nitrogen flushed Jasco J-810 spectropolarimeter at a wavelength interval of 190–260 nm, processed with Spectra Manager and further analyzed by deconvolution software (SELCON, CONTINLL and CDSSTR) [39,40].

3. Results

3.1. RAP-3 peptides interact specifically with RBCs

Synthetic peptides spanning the complete sequence of RAP-3 were assessed to determine their capacity to interact specifically with human RBCs. The results showed that the RAP-3 peptides 33860 and 33873 establish high affinity specific interactions with RBCs (Fig. 1A). The specificity of such binding interactions was also supported by the dramatic decrease in the ability of HABP 33860 to bind to RBCs when its sequence was randomized (Fig. 1B), thus indicating that the binding ability of RAP-3 HABPs depends not only on the amino acid composition but also on the sequence order (which is directly associated with peptide structural features). The HABP 33860 is located toward the N-terminal region of RAP-3, while HABP 33873 lies in C-terminal region (Fig. 1A and C).

The interaction of RAP-3 HABPs with human RBCs was characterized by dissociation constants (K_d) within the nanomolar range, as indicated by K_d values of 700 nM for HABP 33860 and 310 nM for HABP 33873. A Hill analysis of the saturation curves evidenced a cooperative effect on the RBC–HABP binding interaction, with hill coefficients (n_H) of 1.5 (Fig. 1D). A significant difference was also observed in the number of binding sites that recognized by each HABP per cell (binding sites per cell (*BSC*)); HABP 33860 showed a larger number of receptors (112 000 BSC) in comparison with HABP 33873 (20 000 BSC) (Fig. 1D).

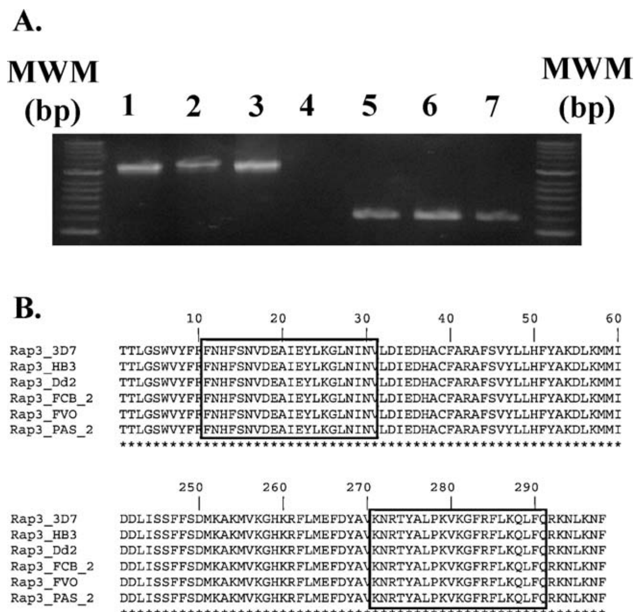

Fig. 2. Polymorphism analysis of RAP-3. (A) PCR amplification of the region encoding the RAP-3 HABPs 33860 and 33873. Lanes 1, 2 and 3 correspond to PCR products amplified from gDNA of the FCB-2, FVO and PAS-2 parasite strains, respectively. Lane 4: negative control. Lanes 5, 6 and 7: positive amplification control: amplification of the Pf25-IMP HABP 33577 in the FCB-2, FVO and PAS-2 strains, respectively. MWM: 100 bp molecular weight marker. (B) Amino acid alignment of the RAP-3 regions comprising HABPs 33860 (upper box) and 33870 (bottom box) in the *P. falciparum* 3D7, HB3, Dd2, FCB-2, PAS-2 and FVO strains.

3.2. HABPs are highly conserved between different P. falciparum strains

The gDNA of the FVO, FCB-2, and PAS-2 *P. falciparum* strains was used as template to amplify the region encoding the RAP-3 HABPs 33860 and 33873 by using the primer set detailed in Section 2.3. A single band of ~1094 bp was observed when the amplification products were visualized on agarose gels, while the expected band of ~438 bp was observed for the positive control (Fig. 2A). A multiple alignment of the amino acid sequences of RAP-3 HABPs found in the three strains herein analyzed and the sequences reported for the 3D7 (The Netherlands), HB3 (Honduras), and Dd2 (Indochina) reference strains clearly showed a 100% of amino acid sequence identity. No substitutions were observed among nucleotide sequences either, therefore ruling out the presence of synonymous substitutions in the studied region (data not shown).

3.3. RAP family members recognize similar molecules on RBC surface

The results of cross-competition preliminary assays between radiolabeled RAP-3 HABPs and three increasing concentrations of unlabeled HABPs of RAP-2 and RAP-1 (8 μM, 20 μM and 80 μM) are shown in Fig. 3C. Binding of RAP-3 HABP 33860 was strongly inhibited in more than 50% by RAP-1 HABP 26202 and RAP-2 HABPs 26225 and 26229. In contrast, binding of RAP-3 HABP 36873 was more drastically inhibited when RAP-1 HABPs 26188, 26202 and 26203 were used as unlabeled competitors.

3.4. Bindings of RAP-3 HABPs to 27–90 kDa receptors are differentially affected by enzymatic treatments

The nature of the RBC receptor(s) for RAP-3 HABPs was analyzed performing binding assays with enzyme-treated human RBCs and

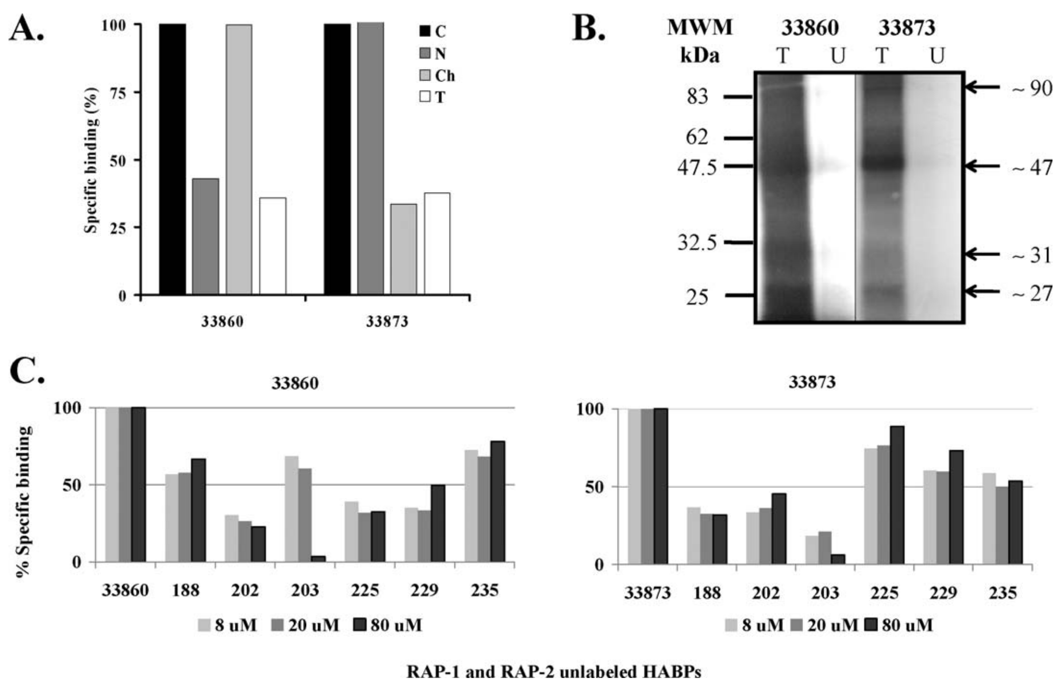

Fig. 3. Insights on the RBC membrane receptor (s) for RAP-3 HABPs. (A) Effect of different enzymatic treatments on the binding activity of RAP-3 HABPs, shown as specific binding activity to neuraminidase (N), Chymotrypsin (Ch) and Trypsin (T) treated cells. Binding control to untreated cells is also shown (C, 100% binding). (B) Autoradiograms of RAP-3 cross-linked HABPs. T: total binding, U: unspecific binding in the presence of unlabeled peptide. The pattern of the molecular weight marker (MWM) is also shown. (C) Cross-competition assay between radiolabeled RAP-3 HABPs versus three unlabeled RAP-1 HABPs (26188, 26202 and 26203) and three RAP-2 unlabeled HABPs (26225, 26229 and 26235). In the graphs, RAP-1 and RAP-2 peptides are indicated by the three last digits of their codes.

cross-linking assays. Binding of HABP 33860 was mainly affected by neuraminidase and trypsin, therefore suggestion a sialic acid-dependent interaction of this HABP with RBCs. In contrast, the HABP 33873–RBC interaction was only affected by chymotrypsin and trypsin, which indicates a receptor of proteic nature for this HABP (Fig. 3A).

On the other hand, the autoradiograms of RBC receptors cross-linked by HABPs 33860 and 33873 showed four bands at approximately 90 kDa, 47 kDa, 31 kDa and 27 kDa. These bands are the result of specific binding interactions between the RAP-3 HABPs and RBC surface molecules as their intensities clearly diminished in the presence of unlabeled peptide (Fig. 3B).

3.5. Binding sequences of RAP-3 inhibit merozoite invasion of RBCs

To evaluate a possible role of RAP-3 during *P. falciparum* invasion of RBCs, RAP-3 HABPs were assessed for their capacity to inhibit invasion of *P. falciparum* merozoites into RBCs. The two RAP-3 HABPs showed a moderate ability to inhibit RBC infection (42% for HABP 33860 and 56% for HABP 33873), whereas RAP-3 low-activity binding peptides (LABPs) cause no significant inhibition at the same assay conditions. A 1:1 mixture RAP-3 HABPs was inhibited invasion by up to 37% (Table 1).

3.6. Secondary structure features of RAP-3 HABPs

The CD spectra recorded for RAP-3 HABPs are shown in Fig. 4. According to these spectra, HABP 33860 has a high content of α-helical elements, as indicated by the characteristic minima at 209 and 222 nm, in contrast with the slightly displaced spectrum obtained for HABP 33873 (Fig. 4). These results are in total concordance with NPS@ structural prediction for RAP-3 sequence [6] (data not shown)

4. Discussion

Malaria caused by *P. falciparum* remains as one of the world's most important public health problems. *P. falciparum* growth and development inside RBCs is directly associated with the symptomatology of severe malaria. This critical stage of the parasite's life cycle begins with the specific recognition, binding and invasion of RBCs by *P. falciparum* merozoites. *P. falciparum* invasion into host cells is mediated by a series of multiple complex host–pathogen inter-

Table 1
Merozoite invasion inhibition by RAP-3 peptides *in vitro*.

	Peptide	Concentration (μM)	Invasion inhibition (%)[a]
HABPs	33860	200	42 ± 1
	33873	200	56 ± 2
LABPs	33862	200	11 ± 2
	33864	200	17 ± 6
1/1 HABP mixture		200	37 ± 4
Controls		Parasites	0
		Chloroquine 1/27 (1.85 mg/mL)	95 ± 6
		Chloroquine 1/54 (0.93 mg/mL)	96 ± 0
		EGTA (1.9 mg/mL)	58 ± 1

[a] All standard deviations were below 6%.

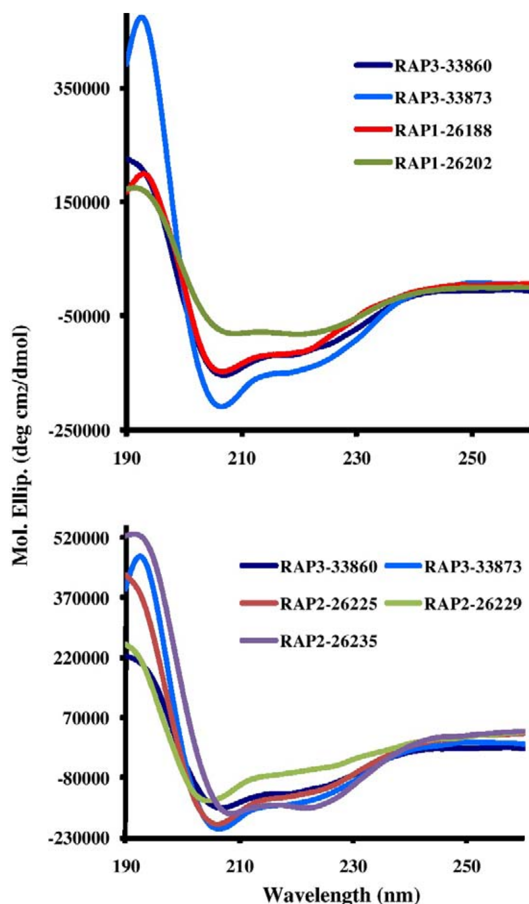

Fig. 4. Secondary structure features of RAP-1, 2 and 3 HABPs. CD spectra of HABPs 33860, 33873 (RAP-3), 26188, 26202 (RAP-1), 26225, 26229 and 26235 (RAP-2) acquired in 30% TFE between 260 and 190 nm. Deconvolution of CD spectra using SELCON, CONTINLL and CDSTR programs suggests a high content of α-helical features (83–100%) for all HABPs, excepting for HABPs 33873 and 26202 (35% and 36%, respectively).

actions between parasite invasins and RBC membrane receptors [10,13,17]. A large number of parasite surface proteins and proteins contained inside the apical organelles of merozoites play key roles in such interactions, which is why they have been regarded as potential targets for inducing a blocking and fully protective immune response against malaria.

In addition to the exquisite repertoire of malarial invasion-associated molecules, the formation of different macromolecular complexes has also been described. Members of these complexes have been associated with parasite adhesion to host cells and thus represent important mediators for the initiation of the parasite intraerythrocytic cycle [35,36]. Among these, proteins of the LMW rhoptry complex, also known as the RAP family, have been described to be targets of invasion-inhibitory antibodies and protective immune responses in animal models and humans [2,26,31,43]. The association of RAP-3 with the recognition and invasion of RBCs was only clearly established until recently thanks to studies by Awah et al. [1] elegantly showing that anti-RAP-1 and anti-RAP-3 antibodies recognize molecules on the surface of non-infected RBCs in a parasitemia-dependent manner and that such recognition mediates destruction of such RBCs through complement-associated phagocytosis or lysis mechanisms. These observations strongly suggest a possible role of RAP-3 as a ligand during parasite binding to RBCs and, in the light of its localization on the membrane surface forming macromolecular complexes with important antimalarial vaccine candidates, highlight the potential of RAP-3 as an antimalarial vaccine candidate [1,35].

In our ongoing search for a fully protective vaccine against malaria we have established a rational and logical strategy for the identification and characterization of minimal protein subunit components derived from a large number of parasite invasins that have been shown to interact specifically with RBCs [16,33]. Remarkably, once these non-immunogenic conserved antimalarial candidates are structurally analyzed and specifically modified, they induce a protective immune response in *Aotus* monkeys against experimental challenge with *P. falciparum* [27,28].

In the present study, the application of these well defined rules in RAP-3 has led us to identify the two binding regions interacting specifically with RBCs, therefore being denoted as high-activity binding peptides. These binding sequences are: the N-terminal HABP 33860 ^{61}FNHFSNVDEAIEYLKGLNIN^{80} and the C-terminal HABP 33873 ^{321}KNRTYALPKVKGFRFLKQLF^{340} (Fig. 1A), which interacted cooperatively and with high affinity with RBC membrane receptors, as shown by their nanomolar K_d values and n_H constants higher than 1 (Fig. 1D).

In the same way that the identification of specific binding regions is a critical step for the design of an antimalarial vaccine candidate, determining the conservation of such antimalarial targets is highly relevant in order for such vaccine to cope with the high variability of *P. falciparum* parasites. Remarkably, our polymorphism analyses on the sequence of RAP-3 showed a complete conservation of this protein among the parasite strains herein analyzed (Fig. 2), which strengthen the importance of the RAP-3 HABPs as multi-strain antimalarial targets with potential to overcome the parasite's specialized immune evasion mechanisms and genetic variability.

The homology between RAP-3 and its family partner RAP-2 provides some clues about the possible interrelations and/or associations between these proteins as part of the invasion-associated LMW complex. Interestingly, RAP-3 HABPs are located toward the N- and C-terminal regions of this protein, in a similar fashion to that observed with RAP-2 HABPs [24] (Fig. 1C). Indeed, an alignment between the sequences of RAP-2 and RAP-3 shows that their HABPs are located in similar regions of each protein (Supplementary Fig. 1), such that the RAP-2 HABPs 26220 and 26225 are located in the same region to that of the RAP-3 HABP 33860, while HABPs 26229 and 26235 are located toward the C-terminal region of RAP-2 same as HABP 33873 in RAP-3. Interestingly, cross-competition assays between the HABPs identified in RAP-3 and HABPs identified in other members of the LMW complex showed that binding of RAP-3 HABP 33860 could be inhibited by RAP-2 HABP 26225, both of which share a similar localization (together with RAP-2 HABP 26229 and RAP-1 HABP 26202), whereas RAP-3 HABP 33873 is mainly inhibited by RAP-1 HABPs and not by the RAP-2 HABP 26235 (Fig. 3C). These observations led to suggesting that the N-terminal RAP-3 and RAP-2 HABPs could be interacting with similar RBC membrane receptors (Supplementary Fig. 1), in contrast with the differential binding observed for the C-terminal regions of these proteins. Unfortunately, there were no samples of RAP-2 HABP 26220 available at the time this study was performed, but considering that this peptide shares high similarity with the RAP-3 HABP 33860 (Supplementary Fig. 1), it is possible to hypothesize that it has a similar behavior to that observed for RAP-2 HABP 26225 because both RAP-2 HABPs have been reported to recognize similar RBC surface receptors [24].

Binding assays performed with enzymatically treated RBCs support these observations. Binding of the RAP-3 HABP 33860 is directly associated to a sialic-acid containing receptor (Fig. 3A) in the same way that binding of the RAP-2 HABP 26225 is only affected by digestion of the RBC surface proteins with neuraminidase (decreasing up to 100%) and trypsin (diminishing up to 43%; data not shown). In contrast, the sialic acid-independent interaction of RAP-3 HABP 33873 (Fig. 3A) is only inhibited by RAP-1 HABPs,

among which binding of RAP-1 HABP 26202 (the only one able to inhibit binding of both RAP-3 HABPs, Fig. 3A) is drastically reduced up to 75% by treatment with neuraminidase and totally abolished by treatment with chymotrypsin (data not shown); a similar binding profile to the one observed for both RAP-3 HABPs. Therefore, all these observations strongly suggest that members the RAP protein family participate in parasite invasion of host cells by coordinating the use of alternate and/or redundant pathways through specific host–pathogen molecular interactions.

In addition to data on to the enzymatic profiles of the RBC receptors for RAP-3 HABPs, cross-linking assays showed the interaction of RAP-3 HABPs with 27–90 kDa RBC surface molecules. RAP-3 HABP 33860 specifically recognized molecules of about 27 kDa, 37 kDa and 47 kDa, the last one also being slightly detected in presence of unlabeled peptide (Fig. 3B). RAP-3 HABP 33873 showed a stronger interaction with the 47 kDa and 90 kDa molecules but also interacted to a lesser extent with the molecules recognized by HABP 33860. According with these apparent molecular weights and the binding behaviors observed in enzymatic treatment assays, it is possible that the RAP-3 HABP 33860 interacts specifically with glycoproteins of around 27–47 kDa, which are probably related to members of the glycophorin family [3]. On the other hand, HABP 33873 recognizes the 47 kDa molecule and an additional band of 90 kDa, which could be associated with its binding's sensibility to chymotrypsin, an enzyme that cleaves important RBC proteins such as Band 3 [3]. It is important to mention that although these results point to these receptors as the RBC molecular targets for host–pathogen interactions mediated by RAP-3, further assays are necessary to completely elucidate their exact identity.

A possible role of RAP-3 during *P. falciparum* invasion is suspected considering that the interaction of its HABPs with RBC receptors, either independent or as 1:1 mixtures (Table 1), resulted in inhibition of merozoite invasion *in vitro*, whereas only a poor inhibition is accomplished when LABPs are used (Table 1). Thus, these results highlight the biologic relevance of the functional regions of RAP-3 as potential antimalarial vaccine candidates. However, it should also be taken into account that the members of the RAP family are some of the molecules that together with some of the members of the EBA, RH and MSP protein families form part of the macromolecular complexes described in the lipid rafts [35,36]. Consequently, it would be interesting to test whether the inhibitory potential of RAP-3 HABPs, either alone or in combination, could be overcome by the parasite's "on-off" switching of multiple infection pathways [9,10,13].

Previous works have demonstrated that conserved HABPs are poor immunogens against *P. falciparum* challenge in animal models, but once their sequences are specifically modified they become highly antigenic and protective analogs due to changes in structural features critical for their adjustment within the human leukocyte antigen (HLA)–peptide–TCR complex [27,28,33]. CD spectra and deconvolution analyzes carried out in this study both show a high content of α-helical elements (>80%) in the secondary structure of HABP 33860, in contrast with the $\sim$35% of α-helix features observed in HABP 33873 (Fig. 4). It should not be discarded that the binding inhibition of RAP-3 HABPs by HABPs derived from RAP-1 y RAP-2 is associated with the structure of each one of the HABPs (Figs. 3C and 4).

The results shown here give additional support to conducting further structural and immunological characterization studies on RAP-3 HABPs to design of minimal subunit-based antimalarial vaccine candidate components.

5. Conclusions

Until recently, a role for the third member of the invasion-associated *P. falciparum* RAP family during parasite recognition, binding and invasion of RBCs was not been clearly established. Here we described two conserved specific binding regions of RAP-3 interacting specifically and with high affinity with 27–90kDa proteins in a sialic acid dependent and independent manner. In the light of the inhibitory potential of RAP-3 during merozoite invasion *in vitro*, these results support conducting a structural and immunological characterization of these RAP-3 HABPs as candidates to design potential components of a minimal sub-unit based, multi-antigenic, multi-stage and chemically synthesized antimalarial vaccine, the *Raison d'être* of our work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.peptides.2010.09.002.

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