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Plasmodium falciparum rhoptry neck protein 5 peptides bind to human red blood cells and inhibit parasite invasion

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ABSTRACT

Plasmodium falciparum malaria parasite invasion of erythrocytes is an essential step in host infection and the proteins involved in such invasion are the main target in developing an antimalarial vaccine. Secretory organelle-derived proteins (micronemal AMA1 protein and the RON2, 4, and 5 rhoptry neck proteins) have been recently described as components of moving junction complex formation allowing merozoites to move into a newly created parasitophorous vacuole. This study led to identifying RON5 regions involved in binding to human erythrocytes by using a highly robust, sensitive and specific receptor–ligand interaction assay; it is further shown that the RON5 protein remains highly conserved throughout different parasite strains. It is shown that the binding peptide–erythrocyte interaction is saturable and sensitive to chymotrypsin and trypsin. Invasion inhibition assays using erythrocyte binding peptides showed that the RON5–erythrocyte interaction could be critical for merozoite invasion of erythrocytes. This work provides evidence (for the first time) suggesting a fundamental role for RON5 in erythrocyte invasion.

1. Introduction

P. falciparum induces more than 200 million cases of malaria and more than 1 million deaths a year [25,52]. It has a complex lifecycle involving different parasitic forms named sporozoites (infecting hepatic cells in mammals and salivary glands in the mosquito vector), merozoites (invading red blood cells (RBC)) and gametocytes invading a mosquito's midgut cells. Merozoite invasion begins with low affinity and reversible binding interaction, followed by reorientation to juxtapose the merozoite apical end onto RBC membrane [14], thereby establishing an irreversible tight junction (TJ). A merozoite pulls itself through a TJ by using its actin–myosin motor machinery to form the parasitophorous vacuole (PV) [14].

TJ is defined as an electron-dense structure formed between merozoite and RBC plasma membranes [6], involving rhoptry neck proteins (RON) 2, 4 and 5 and apical membrane antigen 1 (AMA1) [2,3,7,11,42]. It has been suggested that RON proteins are released and translocated onto RBC membrane acting as AMA1 receptors which become localized in the micronemes after synthesis and translocated to the merozoite membrane [2,3,7,11,42]. AMA1-RON complex formation is essential for invasion to take place, since monoclonal antibody (mAb) 4G2 and the R1 peptide, both binding to the AMA-1 hydrophobic trough, do not allow AMA1–RON interaction, thereby blocking parasite invasion [11,37]. Functional analysis has demonstrated that RON2 is directly implicated in AMA1–RON interaction [18,40,46]; however, despite RON2 importance in AMA1–RON interaction for TJ formation and its association with RON4 and RON5 being clear, the role of other RON proteins (*e.g.* RON4 and RON5) has not been clearly established.

PfRON5 (MAL8P1.73), a ~110 kDa molecular mass protein, identified as being a member of the TJ complex [11], is a highly-conserved protein among apicomplexan parasites and is expressed in RBC invasion-related blood stages: schizont and merozoite [15]. The present work has confirmed PfRON5 localization in the rhoptry neck by immunoelectron microscopy (IEM) and found that PfRON5 high activity binding peptides (HABPs) inhibited merozoite invasion *in vitro*. This would suggest an important role for PfRON5 in invasion and highlights the potential use of its HABPs (when properly modified) as components of a new anti-malarial vaccine.

2. Materials and methods

2.1. Immunofluorescent antibody assay (IFA)

Aotus sera against modified peptides 24148 (HABP **5501** derived from merozoite surface protein 1 (MSP-1)) [48], 36340

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(HABP **33460** derived from rhoptry associated membrane antigen (RAMA)) [32] were assayed by double immunofluorescent staining of *P. falciparum* (FCB-2 strain)-infected RBC cultured *in vitro*, as described previously *Aotus* serum raised against modified peptide 38556 (KIRNYRDKESSFTIL**ST**FM**Q**, HABP **37742** (Sections 2.4 and 3.3)) was used for detecting *Pf*RON5. Analog 38556 was designed according to a previously described methodology and rules [30,31]. Rhodamine isothiocyanate (RITC)-labeled anti-*Aotus* IgG (MSP-1 and RAMA) and fluorescein-labeled anti-*Aotus* IgG (FITC) (Vector Laboratories) (RON5) were used as secondary antibodies for protein recognition. After 60 min incubation, three phosphate buffer solution (PBS) washes were performed. Parasite nuclei were stained with a 2 μg/mL solution of 4′,6-diamidino-2-phenylindole (DAPI) for 20 min at room temperature; fluorescence was visualized on a fluorescence microscope (Olympus BX51) using an Olympus DP2 camera and Volocity software (Perkin Elmer).

2.2. Immunoelectron microscopy (IEM)

P. falciparum schizonts from a synchronized culture [47] were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde solution. The pellet was dehydrated in graded ethanol series (50–70–80–90–100%) and embedded in LR-white resin (SPI supplies) using a specific cold polymerization accelerator. Ultrathin sections were fixed on 60-mesh nickel grids coated with collodion support. Grids were blocked with 5% bovine serum albumin and 1% Tween-20 and incubated at 4 °C with a 1:20 primary antibody dilution (rabbit serum against PfRON5 peptides) [15]. Grids were then incubated with anti-rabbit antibody coupled to 10-nm colloidal gold particles for 1 h at room temperature; 6% uranyl acetate was added to enhance image contrast. Samples were examined by transmission electron microscope (Hitachi Hu-12A).

2.3. Peptide synthesis and radio-labeling

PfRON5 sequence (MAL8P1.73) was synthesized in sequential peptides (20-mer) using the t-Boc amino-acid strategy and p-methylbenzhydrylamine resin (0.5 mequiv./g, Bachem)), following solid-phase multiple peptide synthesis methodology [19,27]. Peptides were cleaved by the low–high hydrogen fluoride technique and analysed by RP-HPLC (LaChrom) and MALDI-TOF mass spectrometry (Bruker Daltonics). Peptides were radiolabelled using 3 μL ^{125}I-Na (100 mCi/mL; ARC) and 15 μL chloramine T (2.75 mg/mL, Sigma). The reaction was stopped after 15 min by adding 15 μL sodium metabisulphite (2.3 mg/mL, Sigma) [38]. Radiolabelled peptides were separate by eluting the reaction mixture on a Sephadex G-10 column (Pharmacia). Each eluted fraction was then analysed by gamma counter (Packard Cobra II).

2.4. RBC binding assay

RBC (2×10^7, type O+) from healthy donors were incubated with increasing radiolabelled peptide concentrations (0, 200, 400, 600 and 800 nM) in the absence (total binding) or presence (non-specific binding) of excess unlabelled peptide (20 μM). Peptide samples were incubated at room temperature for 90 min and washed twice with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Merck) buffered saline (HBS) (0.01 M HEPES, 0.15 M NaCl, pH 7.4) before measuring cell-associated radioactivity by gamma counter (assays performed in triplicate) [38].

A modified RBC binding assay ascertained saturation with HABPs; 1.5×10^7 cells, 0–3200 nM radiolabelled peptide and 40 μM unlabeled peptide were used. Cells were washed with HBS and cell-bound radiolabelled peptide measured by gamma counter [38].

Some HABPs' glycine analogs were synthesized to identify amino acids directly involved in cell binding (critical residues). A competition binding assay was then carried out with 100 nM radiolabelled HABP in the presence or absence of non-radiolabelled HABP or each of their analogs (12, 40 and 160 μM) [33,38].

2.5. PCR amplification

cDNA was obtained from the PAS-2 strain, as described elsewhere [15]. The PAS-2 strain sequence was obtained by direct sequencing of PCR products using primers F6 (5-ATGTTGAAATACACTTTGCTCAT-3) and F10 (5-AGGTATTCTAGTGT-GTACAATAA-3). cDNA was amplified in 25 μL reaction mixture containing 1X KAPA HiFi HotStart Ready Mix, and 0.3 μM of each primer. The thermocycling profile consisted of an initial denaturing step at 95 °C for 5 min, followed by 35 cycles consisting of 30 s denaturing at 98 °C, 20 s annealing at 58 °C and 3 min extension at 72 °C, followed by a final extension step at 72 °C for 5 min.

2.6. Binding assays with enzyme-treated RBCs

HABP's specific binding activity was evaluated in a binding assay with enzyme-treated RBCs. RBCs (60%) were independently incubated with 150 U/mL neuraminidase (ICN 9001-67-6) in HBS buffered solution, 1 mg/mL trypsin (Sigma T-1005) or 1 mg/mL chymotrypsin (Sigma C-4129) for 2 h at 37 °C. Excess enzyme was removed following incubation by washing twice with HBS buffer; a typical binding assay was then carried out using untreated RBCs as binding control.

2.7. Circular dichroism (CD) spectroscopy

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 and 0.1 mM each peptide. Each spectrum was obtained by averaging three sweeps taken 260–190 nm at 20 nm/min scan rate with 1 nm spectral bandwidth and corrected for baseline. CD data was deconvoluted using SELCON3, CDSSTR and CONTINLL software [13,39].

2.8. Merozoite invasion inhibition assays

P. falciparum (FCB-2 strain) schizonts obtained from a sorbitol synchronized culture in RBC (O+) (5% haematocrite and 5% final parasitemia) [47] were incubated with each HABP (50, 100 and 200 mM), 18 h at 37 °C in a 5% O_2, 5% CO_2 and 90% N_2 atmosphere. Cells were washed thrice with PBS and incubated with 15 mg/mL hydroethidine for 30 min at 37 °C, washed again and analysed on a FacsCalibur flow cytometer (FACsort, FL2 channel) equipped with CellQuest software [53]; EGTA- or chloroquine-treated infected RBC were used as controls.

3. Results

3.1. PfRON5 was located at Plasmodium merozoite rhoptry neck

Anti-24148-MSP-1 (HABP **5501**)-derived *Aotus* antiserum (red membrane fluorescence) and anti-36340-RAMA (HABP **33460**)-derived *Aotus* antiserum (red rhoptry fluorescence) were used to further characterize RON-5 subcellular localization. IFA showed that punctuate green fluorescence, suggestive of rhoptry localization [15], was different from membrane fluorescence and nuclear fluorescence displayed by blue emission DAPI fluorochrome (Fig. 1A). RON5 localization was close to that of RAMA which is localized in the rhoptries' bulb. A previous study using rabbit anti-PfRON5 polyclonal antibodies and mature schizonts gave a

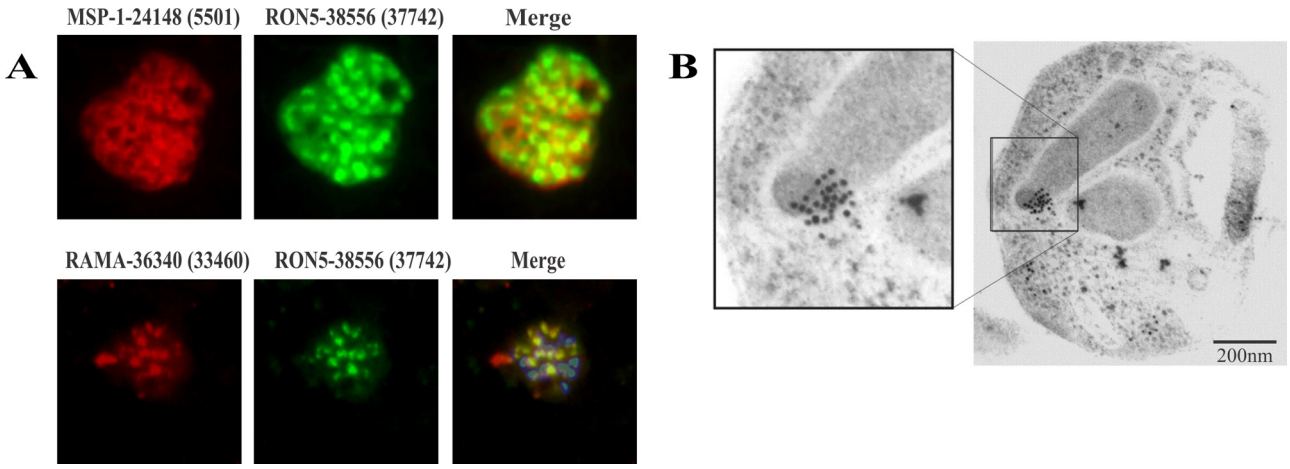

Fig. 1. PfRON5 localizes to the rhoptry neck. (A) *Aotus* antibodies against MSP-1 peptide 24148 (5501) displaying the membrane, and against RAMA peptide 36340 (33460), showing the rhoptries (red fluorescence), together with RON5 peptide 38556 (37742) (green fluorescence) and DAPI (blue fluorescence, merged at the bottom), confirming RON5 localization in the rhoptries but differently to that of RAMA. (B) Immuno-electron microscopy with rabbit antibodies against RON5 peptides and protein A-gold showed specific labeling in the *P. falciparum* merozoite rhoptry neck. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

punctate fluorescent pattern characteristic of rhoptry proteins [15]. PfRON5 was detected in pear-shaped rhoptry neck portion in segmented schizonts when using rabbit polyclonal antibodies for IEM assay (Fig. 1B).

3.2. PfRON5 peptides specifically interacted with RBCs

Binding assays determined PfRON5 peptides binding to RBCs. Each peptide's specific binding plot was obtained from the difference between total binding (binding in the absence of non-radiolabelled peptide) and unspecific binding (binding in the presence of non-radiolabelled peptide) plots. Peptides having ≥0.02 slope specific binding plot (≥2% specific binding activity) were considered to be HABPs [38]; PfRON5 protein had 16 HABPs distributed along its sequence (Fig. 2).

Dissociation constants (*Kd*), Hill coefficients (n_H) and number of binding sites per cell (NSC) were calculated from saturation curves and Hill analysis for HABPs 37727, 37736 and 37744 (Fig. 3A) [4]. *Kd* were around 1000 nM, n_H were > 1.0, suggesting positive cooperativity, and NSC ranged from 60,000 to 265,000 (Fig. 3A).

3.3. Critical amino acids in RBC binding

Competition binding assays between labeled HABPs and their glycine analogs were performed to identify whether HABPs 37672, 37736, 37740 and 37744 had critical residues for RBC binding. Critical binding residues were defined as being those whose specific binding decreased ≥50% when replaced by glycine in the conditions used: 12, 40 and 160 μM unlabelled peptide [49]. HABPs 37672 (^{411}YLDVSSISTDDEF**NLI**NKTI^{430}) (Fig. 3B) and 37742 (^{851}KIRNYRDKESSFTIL**CP**FM**E**^{870}) had three critical binding residues (in bold and underlined) located near the C-terminal region, while 37736, 37740 and 37744 had none (data not shown).

3.4. PfRON5 was a conserved protein

PfRON5 nucleotide and amino acid sequences obtained from the PAS-2 strain were aligned with FCB-2 and 3D7 strains using Clustal W software [12]. Four substitutions regarding 3D7 were found in the PAS-2 and FCB-2 sequences and one change (Q848R) was observed in PAS-2 when compared to FCB-2 and 3D7 strains (Supplementary Table 1, Fig. 2).

3.5. RBC receptors for PfRON5 HABPs were susceptible to enzyme treatment

RBC were enzymatically treated (chymotrypsin, trypsin and neuraminidase) prior to RBC-binding assays to determine whether HABPs (37672, 37736, 37740 and 37744) directly interacted with a particular RBC membrane's receptor. Specific binding to enzyme-treated RBC was determined using untreated RBC as control (100% specific binding). The HABPs evaluated had similar enzyme-associated binding patterns; HABP 37736 and 37744 binding were affected by chymotrypsin (98% and 99% reduction, respectively) and trypsin treatment (78% and 82% reduction, respectively) (Fig. 4), while neuraminidase treatment affected a maximum of 15% RBC binding, as occurred with HABP 37744 (Fig. 4).

3.6. CD secondary structure

CD analysis was used for obtaining information regarding HABP's secondary structure since merozoite HABPs' functional compartmentalisation has been proved to be associated with their secondary structure [35]. CD spectra and data deconvolution for HABPs 37672, 37673, 37677, 37727, 37730, 37734 and 37735 revealed ≥98% α-helical content in these HABP structures (Supplementary Figure 1) [54]. HABP 37665, 37736, 37742 and 37745 deconvolution data and CD spectra showed that these peptides had different structural elements, for example, HABP 37665 displayed α-helical and random structural elements and HABPs 37736 and 37742 displayed β-strands and random structures, respectively (Supplementary Figure 1).

3.7. HABPs inhibited invasion

PfRON5 HABPs' functional role was evaluated by invasion inhibition assay. All HABPs inhibited RBC invasion by merozoites (Table 1) while low-activity binding peptide (LABP) did not. HABPs 37730 and 37742 had the highest inhibition, which became increased when HABP mixtures were used (Table 1).

4. Discussion

Proteins involved in *Plasmodia* parasite invasion are considered antimalarial vaccine candidates since this is an obligatory step in a parasite's lifecycle [21]. The rhoptries discharge their

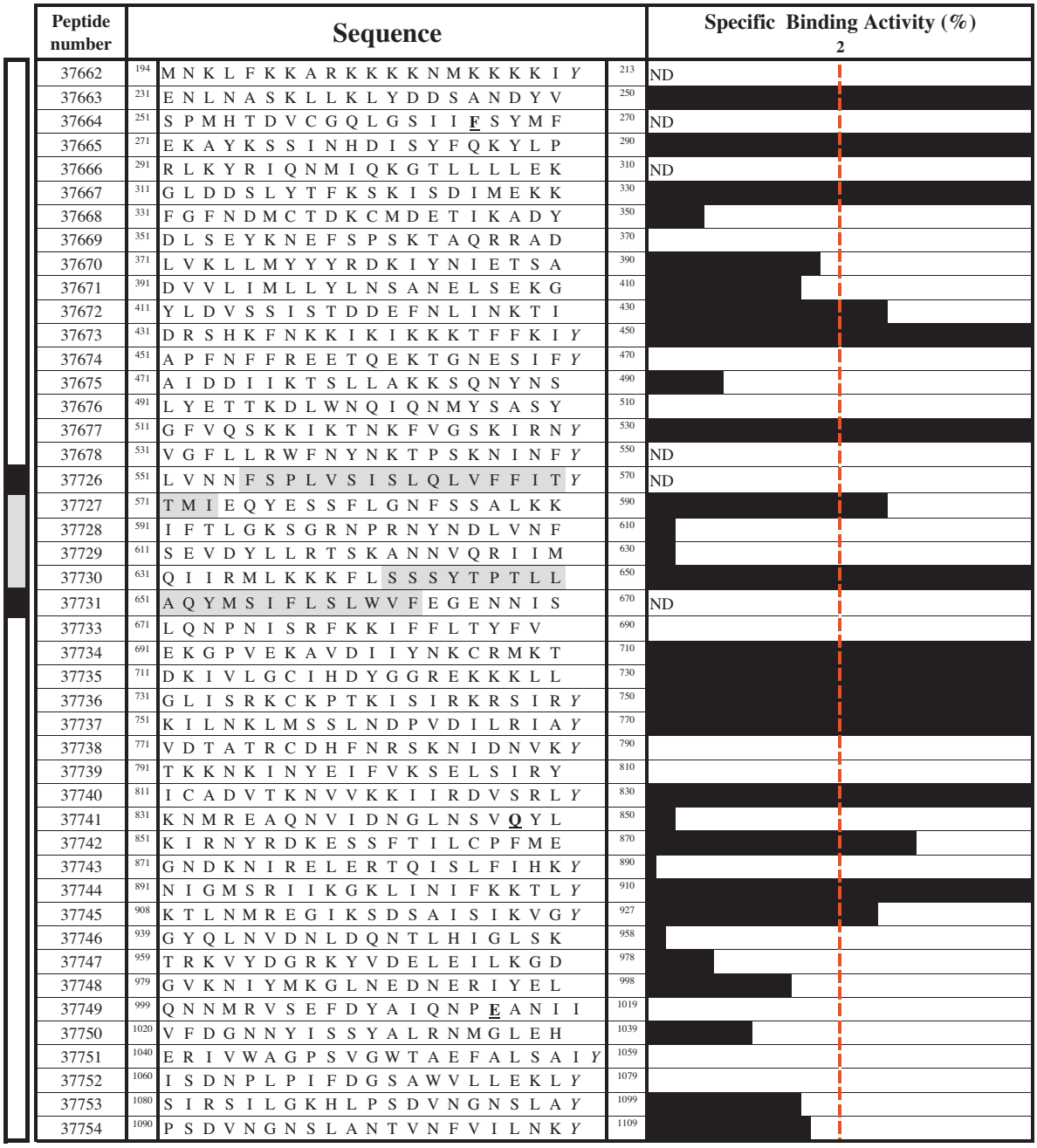

Fig. 2. RBC binding profile. The PfRON5 protein sequence was analysed with 20-mer non-overlapping long peptides. The figure shows each HABP sequence and localization within the protein. A tyrosine residue was added at the carboxyl-terminus of peptides which did not contain this residue within their sequence to enable $Na^{125}I$-radiolabelling. The black bars represent specific binding activity (%) and those having ≥2% specific binding (red line) were considered HABPs [38]. Peptides have been named according to our institute's serial numbering system. Polymorphic residues are underlined and shown in bold. On the left, a schematic representation of PfRON5 extracellular and intracellular regions according to consensus based on previous bioinformatics studies [15]; gray shows transmembrane regions. ND: not determined.

contents during invasion [5,21] in accordance with rhoptry proteins' expression during schizont stage [8,9]. The rhoptry proteins characterized to date include the reticulocyte binding-like (RBL) family, *i.e.* PfRH proteins in *P. falciparum* (PfRH1-2a-2b-4 and PfRH5) implicated in host cell selection and antigenic variation [44], the high molecular weight protein complex (RhopH1-2 and 3) and the low molecular weight protein complex (RAP1-2 and 3) [21]. Both proteins establish complexes around the rhoptry-associated membrane antigen (RAMA) and MSP1 GPI-anchored proteins (Fig. 5) [34,37,41], some of these complexes being transferred to the PV after invasion [36,50].

PfRON2, 4, and 5 proteins are the other rhoptry protein family, being associated with AMA1 in the moving junction (MJ) complex (Fig. 5), playing a critical role in merozoite invasion of RBC [11,20,37,40]. RON2 has been the most studied protein since it interacts directly with AMA1 in TJ formation [23,40]; however, other RON proteins' role in invasion should also be clarified. PfRON5 would thus seem to be a protein of great interest for evaluation as it is expressed during the schizont stage, is highly conserved and forms part of the TJ complex.

A previous study has shown PfRON5 localization in mature schizont rhoptries [15]; attempts to localize PfRON5 at sub-cellular level using IEM have been unsuccessful [37]. Our IFA results have shown that RON5 localization is different to that of MSP-1 which is on the membrane and that of RAMA which is localized in the rhoptry bulb (Fig. 1A). Interestingly, PfRON5 rhoptry neck

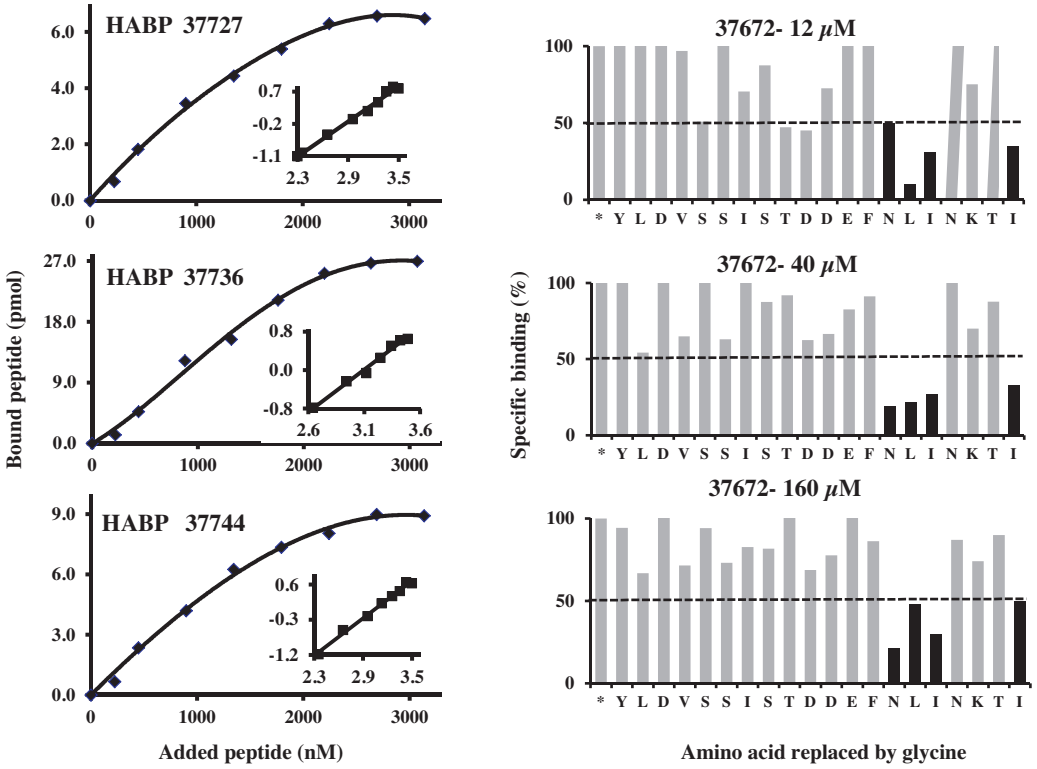

Fig. 3. Saturation and competition binding assay. (A) Saturation curves (specific binding) for HABPs 37727, 37736 and 37744. Increasing quantities of labeled peptide were added in the presence or absence of unlabelled peptide. The affinity constants (*Kd*) were 980, 1300, and 1100 nM; Hill coefficients were 1.7, 2.0 and 1.5 and the number of binding sites per cell was 66,000, 265, and 88,000, respectively. In the Hill plot (inset) the abscissa is log *F* and the ordinate is log $(B/B_{max} - B)$, where *F* is free peptide, *B* is bound peptide and B_{max} is the maximum amount of bound peptide. (B) HABP 37672 glycine scanning peptides were synthesized and tested in RBC competition binding assays between original labeled peptides and their analogs to identify critical amino acids for HABP binding. Changes affecting HABP binding by more than 50% have therefore been critical for HABP–RBC interaction and have been named critical binding residues [38]. *Represents specific original peptide binding and was taken as being 100%. Bar height represents percentage original radiolabelled peptide binding inhibited by original unlabelled peptide or unlabelled analogous peptides (12, 40 and 160 μM). The letter at the bottom represents the amino acid replaced by glycine in the analog peptide. The critical residues are shown by black bars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

localization was confirmed by IEM in the present work for the first time (Fig. 1B).

It has been shown that RON2 interacts with AMA1 in *Toxoplasma* and *Plasmodium falciparum* and, together with other RONs, is exported to host cell membrane. However; the mechanism or signaling involved in RON protein export into a host cell is still unclear, nor have the means by which they could become inserted into, or bind to the host cell membrane been clarified [7,23]. Different models for AMA1–RON complex interaction have proposed that whilst RON4/5 or RON4/5/8 complexes are exposed to host cell membrane cytoplasmic face, RON2 is a transmembrane (TM) protein whose carboxy terminal region binds to a receptor on RBC membrane and to AMA1 [7,18,23,46,51].

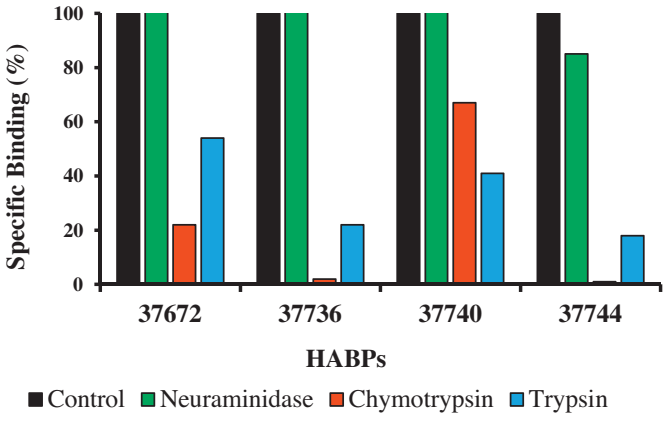

Fig. 4. PfRON5-derived HABPs binding assays with enzyme-treated RBC. HABP 37672, 37736, 37740 and 37744 binding (the highest PfRON5 binding ability) was compared between enzyme-treated and untreated RBCs. Each bar represents percentage specific binding activity shown by peptides upon incubation with neuraminidase (green), chymotrypsin (red) and trypsin (blue). Untreated RBC were used as control (black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Even though precise *Tg*RON5 and *Pf*RON5 membrane topology remains unknown, it has been suggested that *Tg*RON5 (containing one putative TM domain) is partially exposed to the cytoplasmic face of the host cell membrane [7]. Our group has proposed that *Pf*RON5 has 2 TM domains [15] and it cannot be ruled out that, similar to RON2 [18,46], some *Pf*RON5 regions bind to the RBC membrane. Our binding assays allowed the fine mapping of *Pf*RON5 regions interacting with RBC membrane, 16 HABPs distributed along the protein sequence were found in *Pf*RON5 (Fig. 2).

According to a proposed topological model [15], almost all HABPs were located in the PfRON5 extracellular region. HABPs 37727 and 37730 had three and nine amino acid residues, respectively, which were putatively included within transmembrane predicted domains (Fig. 2). This suggested that neighboring residues residing outside transmembrane domains were involved in RBC binding. Some HABPs' saturation analysis suggested that HABP–RBC interaction was moderate (1000 nM *Kd* values) although having positive cooperativity (>1.0 n_H values) (Fig. 3). Similar and lower affinities have been reported for RAP1-2 and 3 proteins [16,38]; however, these values were higher than those reported for EBL family proteins [38] located in the micronemes.

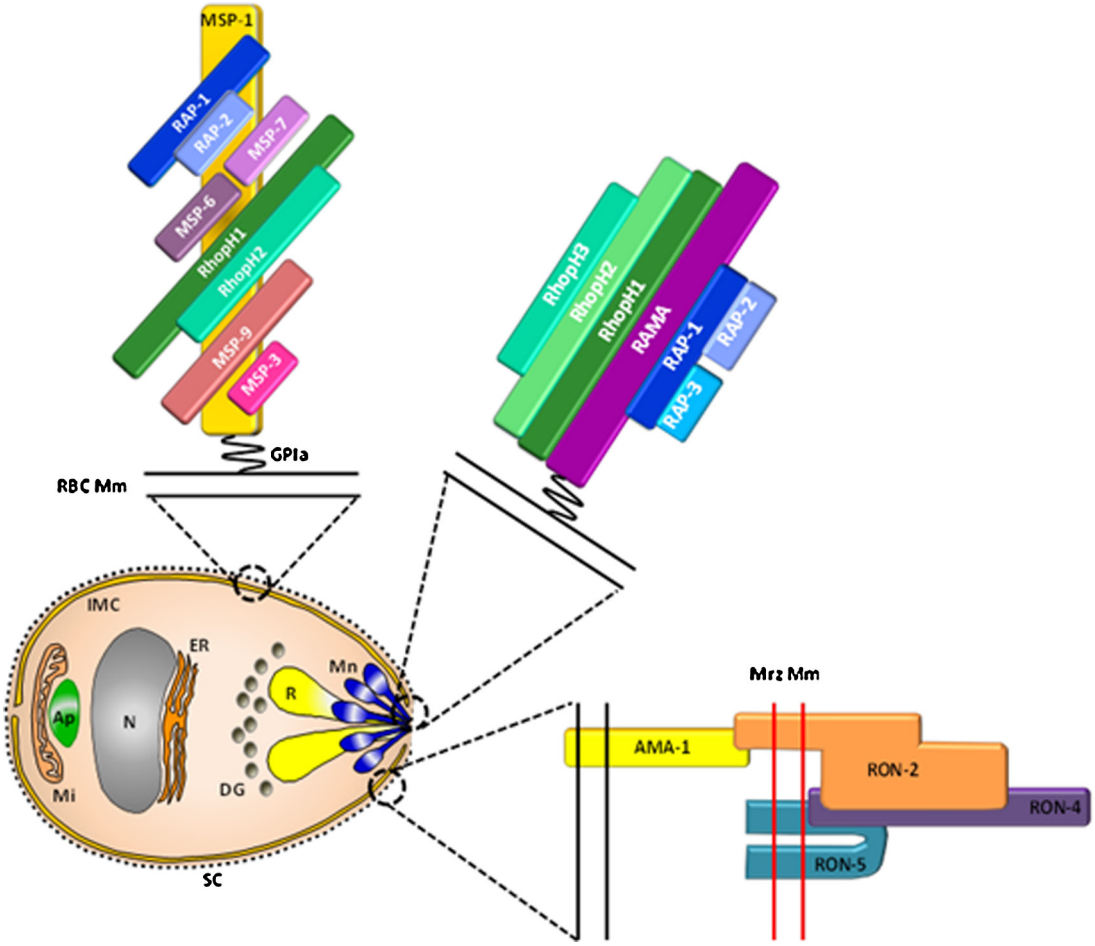

Fig. 5. *P. falciparum* merozoite protein complexes. The figure shows a model diagram of the merozoite protein complexes which it has been proposed to be formed during the invasion of RBC [22,24,34,37,41]. Ap: apicoplast, Mi: mitochondrion, N: nucleus, DG: dense granules, Mn: micronemes, R: rhoptry, IMC: inner membrane complex, SC: surface coat, ER: endoplasmatic reticulum. RBC Mm: RBC membrane, Mrz Mm: merozoite membrane.

Polymorphism analysis showed that substitutions found in PAS-2 and FCB-2 [15] strains (compared to 3D7 strain) were outside the HABP sequences, indicating HABP sequence conservation. The above agrees with elegant work by Manske et al., showing that the MAL8P1.73 gene and its encoded proteins have minimal variation in *P. falciparum* field samples [26]. This was very relevant since it has been reported that one of the main difficulties in developing an anti-malarial vaccine lies in *Plasmodium* proteins' extensive polymorphism. Many malarial proteins' sequence alterations occur in regions which are crucial to immunity, resulting in differences regarding isolates' antigenic properties [10,29].

Previous work has shown that RON2, being a transmembrane protein, binds to the RBC membrane [18,46]. It has been suggested that PfRON5 might be translocated onto RBC membrane, similarly to TgRON5 [7]; however, no evidence is currently available for confirming this suggesting [37]. Nevertheless, PfRON5 has undoubtedly been associated with other RONs at the MJ [11,40]; this means that PfRON5, or regions thereof, could be interacting with RBC membrane receptors, as confirmed by the presence of the HABPs shown in this work. However, further studies are necessary to determine the precise moment during which interaction occurs or whether this is just transitory during protein passage toward RBC cytoplasmatic face.

The probable nature of PfRON5 RBC membrane receptors was evaluated in binding assays with enzyme-treated RBC. The results showed that HABP 37672, 37736, 37740 and 37744 receptors had the following pattern: neuraminidase-resistant, trypsin-sensitive and chymmotrypsin-sensitive (Fig. 4). It is worth noting that this was the same pattern as that for complement receptor 1 (CR1) which had been identified as PfRh4 protein receptor, the major ligand for invasion *via* the sialic acid-independent pathway [43,45]. Interestingly, HABP 37672, 37736 and 37740 specific binding increased with neuraminidase treatment (Fig. 4), suggesting that neuraminidase treatment promoted HABP binding to a receptor, similar to PfRh4 protein with the CR1 receptor [17]. Nevertheless, further essays should be performed to ascertain the PfRON5 HABP receptor's precise identity.

Many studies involving merozoite and sporozoite proteins have shown that HABPs having conserved sequences go unnoticed by the immune system, suggesting that they are non-antigenic, non-immunogenic and are consequently poor protection-inducing peptides; this phenomenon has been defined as "immunological silence" [29,35,38]. It has also been thoroughly described that specific replacement of critical binding residues in HABP–RBC interaction induces structural changes in such peptides, thereby enabling the immune system to recognize them. Highly immunogenic and protection-inducing analogs can thus be developed from immunologically-silent HABPs [28–30]. Competition binding assays showed that HABPs 37736, 37740 and 37744 did not have any critical binding residues, suggesting that each residue contributed equally to HABP–RBC interaction. HABP 37672 had three critical binding residues, indicating that they could be directly involved in RBC-membrane interaction (Fig. 3B) and that these particular residues must be

Table 1
Inhibition of merozoite invasion to RBC by PfRON5 peptides.

Peptide	Invasion inhibition (%)[a]		
	50 μM	100 μM	200 μM
HABP			
37663	14	17	20
37665	18	23	32
37667	14	19	26
37672	13	18	31
37673	17	22	30
37677	18	23	27
37730	12	28	54
37734	12	12	22
37736	17	19	26
37737	17	26	38
37740	17	25	33
37742	14	23	48
37745	6	16	27
HABPs mixture			
37663/37665		48	
37665/37667		51	
37672/37673		58	
37677/37727		49	
37734/37735		48	
37736/37737		50	
37740/37742		50	
37744/37745		54	
LABP			
37751	4	6	7
Chloroquine (0.93 mg/mL)		92	
EGTA (1.9 mg/mL)		78	

[a] All standard deviations were below 10%.

replaced. Ongoing assays are aimed at identifying critical residues in the other PfRON5 HABPs and thus make more modified HABPs available for performing studies in the *Aotus* monkey model.

It has been shown that changes made in HABP sequences modify peptides' 3D structure, allowing better peptide-human leukocyte antigen (HLA) interaction, therefore suggesting a very strong structure–function relationship [1,28,35]. Most PfRON5-derived HABPs had α-helical structural elements (Supplementary Figure 1), thereby agreeing with a previous report showing that rhoptry protein-derived peptides have α-helical structures and bind with high affinity to the HLA-DR52 haplotype (HLA-DRb_1*0301 and HLA-DRb_1*1101 alleles), emphasizing the structure-activity relationship [35].

PfRON5-derived HABPs were tested in an *in vitro* culture, finding that all HABPs significantly inhibited merozoite invasion in a concentration-dependent manner (Table 1). This suggested that HABPs were blocking merozoite–RBC interaction, even with not such strong affinity. Although this assay did not clarify the specific role and mechanism by which PfRON5 HABPs act, the results suggested that PfRON5 was involved in RBC invasion. Interestingly, invasion inhibition ability substantially increased when the assay was performed with HABP mixtures (Table 1), suggesting an additive effect. Rabbit polyclonal antibodies against PfRON5-derived peptides also inhibited invasion (data not shown).

The aforementioned results confirmed that PfRON5 was playing an important role during RBC invasion and reinforced the idea that this protein is a good target for inclusion in a second generation of vaccines, especially as properly modified conserved HABPs could be components of a minimal subunit-based antimalarial vaccine.

Conflict of interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2013.07.028.

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