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A single amino acid change in the *Plasmodium falciparum* RH5 (P*f*RH5) human RBC binding sequence modifies its structure and determines species-specific binding activity

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ABSTRACT

Identifying the ligands or regions derived from them which parasites use to invade their target cells has proved to be an excellent strategy for identifying targets for vaccine development. Members of the reticulocyte-binding homologue family (P*f*RH), including RH5, have been implicated in invasion as adhesins binding to specific receptors on erythrocyte surface. The regions mediating P*f*RH5-RBC specific interactions have been identified here by fine mapping the whole P*f*RH5 protein sequence. These regions, called high activity binding peptides (HABPs), bind to a receptor which is sensitive to trypsin treatment and inhibit merozoite invasion of RBCs by up to 80%, as has been found for HABP 36727. Our results show that a single amino acid change in the HABP 36727 sequence modifies a peptide's 3D structure, thereby resulting in a loss of specific binding to human RBCs and its inhibition ability, while binding to *Aotus* RBC remains unmodified. Such invasion differences and binding ability produced by replacing a single amino acid in an essential molecule, such as P*f*RH5, highlight the inherent difficulties associated with developing a fully effective vaccine against malaria.

1. Introduction

Plasmodium falciparum is the protozoan parasite that causes the most lethal form of malaria in humans, infecting ~300 million people and producing about one million deaths annually [1]. This parasite displays a sophisticated molecular machinery enabling merozoites (the parasite forms invading red blood cells – RBC) to evade a host's immune response. Evasion mechanisms include antigenic polymorphism, immune suppression, molecular mimicry, inducing blocking antibodies, immunological silence and alternative RBC invasion pathways [2,3].

The last-mentioned evasion mechanism confers extraordinary plasticity on the parasite to invade RBCs, depending on receptors available on host cell membrane, and is mediated by at least two protein families acting as ligands: Duffy binding-like proteins (DBL), also named erythrocyte binding ligands (EBL), and the reticulocyte-binding protein homologues (RBL or RHs) [4–6]. Genetic disruption studies of some members of these two families [7–9] have shown that although these proteins are not essential (since their functions overlap) they are associated with host cell receptor recognition and the subsequent formation of an irreversible tight junction (TJ) [7]. This TJ is characterised by increased electro-dense thickening under the RBC membrane at the contact site between merozoites and RBC [10].

235 kDa *P. yoelii* rhoptry proteins were the first members of the RBL family to be identified [11] and, from then on, they have been proposed as being mediators of host cell selection throughout RBC maturation stages [12]. Additional members have been identified in *P. vivax*, such as reticulocyte-binding proteins 1 and 2 (RBP-1 and RBP-2) [13], and in *P. falciparum*, such as RH1, RH2a, RH2b, RH3 (pseudogene), RH4 and RH5 [5,14–17]. Despite amino acid sequence similarity amongst all members being low and as they lack a characteristic structural domain (such as the DBL superfamily's cysteine-rich region), they share stretches of semi-conserved amino acid blocks [18]. A structural characterisation of the Py01365 protein (member of the Py235 family) RBC binding region, named EBD1-194, has been recently carried out. Comparison of the EDB structure to other characterised *Plasmodium* receptor binding domains suggests that there might be an overall structural conservation [19].

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RH family characterisation and the partial knowledge gathered regarding their receptors have led to identifying alternative parasite ligand–host receptor combinations used during RBC invasion [20–22]. Previous studies have shown different transcription and expression patterns for RH genes in several *P. falciparum* parasite strains and isolates [15,21,23]. For instance, the FCB-1 strain expresses P*f*RH1, but not P*f*RH2a or P*f*RH2b; by contrast, the 3D7 strain expresses P*f*RH2a and P*f*RH2b, but P*f*RH1 is expressed at a lower rate [15]. Likewise, 42 isolates taken from malaria patients in Kenya were divided into three major groups; EBA-175 was expressed as the main protein involved in RBC invasion in the first while RH4 was most abundant in the second group and P*f*RH2b in the third one [23]. In addition to variations in phenotype, it has been found that different parasite lines exhibit sequence variation amongst family members themselves, ranging from the substitutions of a few residues to large deletions [15,24]. The parasite thus not only uses different invasion pathways by turning gene expression on and off, but polymorphic variations also enable it to interact with the different receptors available on host cell membrane. It has thus been suggested that a fully effective anti-malarial vaccine should include all antigens associated with alternative invasion routes, this being a scientific approach which has been fiercely defended by our group [3,25].

A new level of complexity in designing a fully effective, anti-malarial vaccine has been encountered in preliminary studies carried out by Hayton et al. [26] showing that point mutations in the recently described *Pfrh*5 gene mediate not only changes in protein binding ability regarding different receptors on RBC membrane (as has been proposed for EBA-140 [27]) but are also involved in specific invasion and infection of different hosts *in vivo*.

P*f*RH5 ($a \sim 63$ kDa molecular mass protein) has been identified and characterised as being the fifth member of the P*f*RH family [17,28] located at the TJ and being a component of the macromolecular complex formed with apical merozoite antigen-1 (AMA-1) and rhoptry neck protein 4 (RON4). This protein seems to be non-covalently associated within the complex due to a lack of transmembrane domains or a glycosylphosphatidylinositol (GPI) anchor in its sequence. Several unsuccessful attempts have been made to disrupt P*f*RH5 in strains encompassing the broad spectrum of alternative RBC invasion routes (i.e. 3D7, W2mef, HB3 and D10 strains), as opposed to results obtained for the rest of the P*f*RH and EBL family members [7]. Agreeing with these reports, it has been found that P*f*RH5 does not change its expression levels in *P. falciparum* strains using different invasion routes and it is recognised by sera from patients suffering natural malarial infection [17], suggesting it as an excellent candidate to be included as a component in a fully effective anti-malarial vaccine.

Reports published during the last two years [17,26,28] have shown that P*f*RH5 protein fragments bind to receptors on RBC membrane. However, results are contradictory regarding the P*f*RH5 region which interacts with RBCs, as well as receptor sensitivity to different enzyme treatments and anti-P*f*RH5 sera's inhibitory ability. Rodriguez et al. [28] have suggested that a 143 amino acid region (N^{31}-V^{174}) having great similarity amongst RH members, contains an interaction site with $a \sim 32$ kDa receptor which is sensitive to high trypsin concentrations but is neuraminidase- and chymotrypsin-resistant. However, antibodies directed against this recombinant fragment have exerted no significant inhibitory effect on parasite invasion of RBC [28]. On the other hand, Baum et al. [17] have shown that most RH5 in parasite supernatant is processed to a 45 kDa fragment which is part of the carboxy-terminal portion of the protein [17]. This 45 kDa fragment binds to an RBC trypsin, chymotrypsin and neuraminidase treatment-resistant receptor with greater affinity than the complete protein; this is glycosylated in nature but has yet to be characterised [17].

Peptides having high RBC binding activity (RBC HABP) have been identified and characterised in P*f*RH5 in this paper by a thoroughly described, highly specific, sensitive and robust methodology [25]. The protein's role as ligand during invasion has been seen by some of these HABPs' ability to inhibit merozoite invasion of RBCs. It has been found that a single change in HABP 36727 (I204K) amino acid sequence generated structural changes in the molecule which determined species-specific binding to human and *Aotus* monkey RBCs, thus highlighting that minimum amino acid sequence variation might imply enormous functional outcomes, thereby making RH5 a critical protein in malarial parasite invasion.

2. Methods

2.1. Chemical synthesis and peptide radiolabelling

The P*f*RH5 sequence in the 7G8 strain (ACB87908.1) was synthesised in sequential 20 non-overlapping amino acid peptides (Fig. 1) using the t-Boc amino acid strategy (Bachem) and p-methylbenzhydrylamine (MBHA) resin (0.5 meq/g), following the solid-phase, multiple peptide synthesis methodology [29]. Peptides were cleaved by the low–high hydrogen fluoride technique and analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF). A tyrosine residue was added to the carboxyl-terminus of peptides lacking this residue in their sequence to enable $Na^{125}I$-radiolabelling [25]. Briefly, peptides were individually radio-labelled using 5 μL $Na^{125}I$ (100 mCi/mL; MP Biomedicals) and 15 μL chloramine-T (2.8 mg/mL) as oxidising agent. The reaction was stopped after 15 min by adding 15 μL sodium metabisulphite (2.3 mg/mL). Radiolabelled peptides were separated by eluting the reaction mixture through a Sephadex G-25 column (Pharmacia). Each eluted fraction was then analysed by gamma counter (Auto Gamma Cobra II Packard).

The same methodology was used for synthesising two P*f*RH5-derived polymer peptides (37171 (CG^{118}DGMLLNEKNDVKNNEDYKNV^{137}GC) and 37173 (CG^{273}EVEDSIQDTDSNHTPSNKKK^{292}GC) which had been predicted to be B-cell epitopes through analysis using Antheprot 3D software, available at http://antheprot-pbil.ibcp.fr/ [30]. Shuffle Protein software [31] was used for designing random peptides (having the same amino acid composition but different sequences) from the identified HABP sequences which were then used as control in cell binding assays.

2.2. Cell binding assays

Total blood, obtained from healthy donors, was washed several times with 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid buffered saline (HBS) (pH 7.2) until RBCs were completely clean. RBCs (2×10^7) were then incubated with increasing quantities of radio-labelled peptide (0, 200, 400, 600, and 800 nM) in the absence (total binding) or presence (non-specific binding) of an excess of unlabelled peptide (20 μM). Peptide samples were incubated at room temperature for 90 min and washed twice with HBS before measuring cell-associated radioactivity by gamma counter. *Aotus spp* RBCs obtained according to the conditions established by Corpoamazonía (00066 resolution, September 13th, 2006) were washed with HBS and used (2×10^7) for binding assays, as described for human RBC.

HABPs 36727 and 36740 were tested in a HeLa cell binding assay. HeLa cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco) and antibiotic/antimycotic mixture (Gibco) and then incubated at 37 °C in a 5% CO_2 atmosphere. After

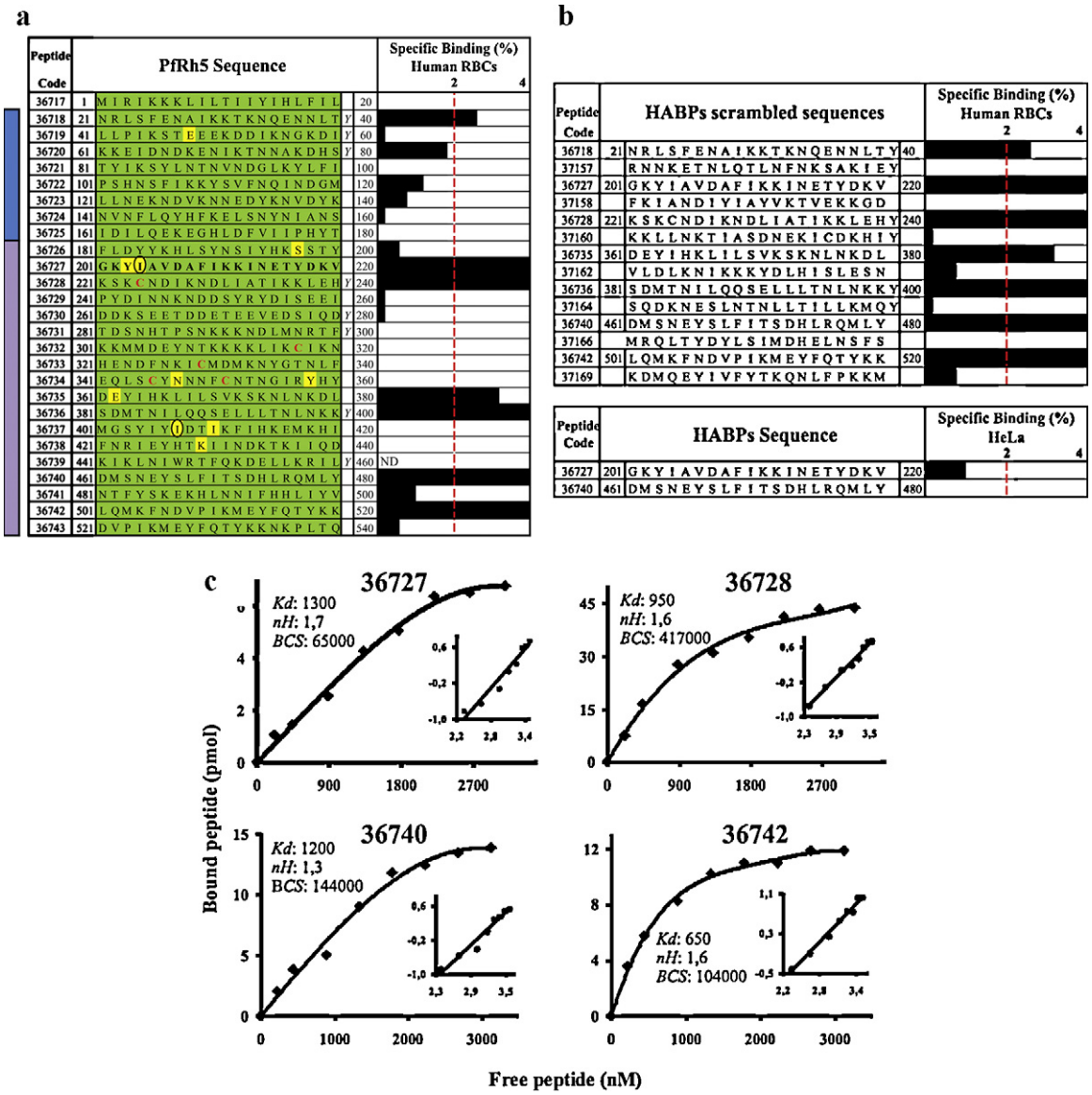

Fig. 1. Identifying and characterising P*f*RH5 peptide binding to RBCs. (a) Binding profile covering the entire P*f*RH5 protein sequence, analysed with 20-mer long peptides. The black bars represent specific binding activity (%); those having ≥2% binding were considered HABPs. Green shows the conserved residues, yellow the variable amino acids [24,26] and red the five conserved cysteines [28]. Two residues shown within circles represent polymorphic sites, suggested as being critical for converting a non-invasive strain into an invasive one [24]. On the left, a schematic representation of the RBCs binding regions postulated by Rodriguez et al. [28] (blue) and Baum et al. [17] (purple). ND (not determined). (b) Binding assays with scrambled peptides and evaluation of some HABPs' specificity in HeLa cell binding assay. (c) Determining kinetic constants for some P*f*RH5 HABPs binding to RBCs. The curves represent specific binding. In the Hill plot (inset), the *x*-axis corresponds to log *F* = free peptide and the *y*-axis to log (*B*/*Bmax* − *B*), where *B* is the amount of bound peptide and *Bmax* the maximum amount of bound peptide.

a confluent layer had been formed, cells were dissociated using 0.05% EDTA–PBS; they were then collected by adding EDTA–PBS and centrifuging, then washed with incomplete medium and their viability and concentration assessed in a Neubauer chamber using trypan blue staining. 1.2×10^6 HeLa cells were placed in 96-well cell culture plates at 120 μL final volume and incubated for 90 min at 4 °C with increasing quantities of radio-labelled peptide (0, 200, 400, 600, and 800 nM) in the presence or absence of non-radio-labelled peptide. A 100-μL aliquot of this solution was passed through a 60:40 dioctyl phthalate–dibutyl phthalate cushion (density 1.015 g/mL) and spun at 8000 × *g* for 5 min. Cell-associated radioactivity was quantified as described above. All the assays were carried out in triplicate.

A modified RBC binding assay was used for determining dissociation constants (K_d), binding cell sites (BCS) and Hill coefficient (n_H) for each RH5 HABP. 100 μL from a 15% human RBC suspension were incubated with radio-labelled peptide concentrations ranging from 0 to 3200 nM in the absence or presence of non-radio-labelled peptide (20 μM). Cells were washed with HBS and radio-labelled peptides bound to cells were quantified in an automatic gamma counter.

2.3. *P. falciparum* genomic DNA extraction and purification

200 μL of erythrocytes parasitised with *P. falciparum* FCB-2, FVO or PAS-2 strains (30% parasitaemia) were obtained from an asynchronous culture, maintained as described elsewhere [32]. Erythrocytes were lysed afterwards using 0.2% saponin and the genomic DNA (gDNA) from each strain was extracted using an UltraClean DNA Blood Isolation kit (MO BIO, Carlsbad, CA).

2.4. PCR amplification

Gene encoding the RH5 protein *P. falciparum* 3D7 reference strain (PFD1145c) was analysed for designing specific primer sets for amplifying HABP-encoding regions. Two primer sets were designed using Gene Runner v3.05. These primer sets sequences were: RH5-F1 (5′-ACCCATGAGGAATTGAGTC-3′) and RH5-R1 (5′-CGGTTTCATCATCTGTCTC-3′) amplifying the region encoding HABPs 36718, 36727 and 36728, as well as RH5-F2 (5′-TGTATAAAAAACCATGAGAATG-3′) and RH5-R2 (5′-TATTGATGCTTTGTCTAATTAG-3′) amplifying the region encoding HABPs 35735, 36736, 36740 and 36742. The region encoding *P. falciparum* integral membrane protein Pf25-IMP HABP 33577, amplified using DIR1 and REV1 primers, was included as positive PCR control.

DNA regions were amplified in 50 μL reaction mixtures containing: 1.25 U BioTaqTM DNA polymerase (Bioline, London, UK), 1× Taq polymerase reaction buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs and 1 μM of each primer. The following thermocycling profile was used for all primer sets: an initial denaturing step at 95 °C for 5 min, followed by 35 cycles consisting of: 1 min of annealing at 56 °C, 1 min of extension at 72 °C and 1 min of denaturing at 95 °C, followed by a final extension cycle at 72 °C for 5 min. The same reaction conditions were established as negative control using DNase- and RNase-free water instead of DNA. Amplification products were visualised in 1% agarose gels stained with SYBR Safe (Invitrogen, Carlsbad, CA). Products were purified using a Wizard PCR preps kit (Promega, Madison, WI) and sequenced using their corresponding forward and reverse primers.

2.5. Binding assays with enzyme-treated RBCs

The specific binding activity for each HABP was evaluated in a binding assay with enzyme-treated RBCs. Briefly, 60% RBCs were independently incubated with 150 μU/mL neuraminidase (ICN 9001-67-6) in HBS buffered solution, pH 7.4, 1 mg/mL trypsin (Sigma T-1005) or 1 mg/mL chymotrypsin (Sigma C-4129), for 60 min at 37 °C. Following incubation, enzyme-treated RBCs were washed twice and used in typical binding assays with HABPs. Untreated erythrocytes were used as positive binding control.

2.6. Cross-linking assays

Radio-labelled HABPs were crosslinked with RBC membrane proteins to identify the molecular weight of possible receptors for each HABP. The binding tests were performed using a final 7% cell concentration, following incubation with radio-labelled peptide in the presence or absence of 35 μM unlabelled peptide for 60 min at room temperature. After incubation, cells were washed with HBS and the bound peptide was cross-linked with 10 μM bis (sulosuccinimidyl suberate) (BS^3, PIERCE) for 45 min. The reaction was stopped with 20 nM Tris–HCl (pH 7.4). RBCs were washed twice with HBS and treated with lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.1 mM PMSF and 1% Triton X-100), followed by spinning at 16,000 × *g* for 15 min to extract crosslinked membrane proteins. Proteins were solubilised in Laemmli buffer and separated by 12% (w/v) SDS-PAGE. Gels were exposed to Kodak X-OMAT film for 15 days at room temperature. Apparent molecular weights were determined using molecular weight markers (New England Biolabs).

2.7. Producing anti-PfRH5 rabbit sera

Two New Zealand rabbits (26 and 28), determined by western blot to be non-reactive to *P. falciparum* late schizont protein lysate were immunised on day 0 with 500 μg of a mixture of two P*f*RH5 polymer peptides, 37171 (CG ^{118}DGMLLNEKNDVKNNEDYKNV^{137}GC) and 37173 (CG^{273}EVEDSIQDTDSNHTPSNKKK^{292}GC), emulsified in Freund's complete adjuvant (FCA). The rabbits received doses of 500 μg emulsified in Freund's incomplete adjuvant (FCI) on days 20 and 40. One rabbit was immunised with phosphate buffered saline (PBS) as negative control. The rabbits were bled on day 60 and serum samples were collected for further analysis. Immunisations and bleedings were carried out according to the guidelines established by the Colombian Ministry of Health law for handling live animals with research or experimentation purposes (law 84/1989).

2.8. Sera adsorption of anti- RH5

Rabbit sera (preimmune and hyperimmune) were preabsorbed with an *Escherichia coli* and *Mycobacterium smegmatis* lysate and SPf66 synthetic vaccine to eliminate non-specific cross-reactivity, as described previously [33]. Briefly, *E. coli* and *M. smegmatis* lysate and SPf66 were collected and used individually to be coupled to CNBr-activated Sepharose AB (Pharmacia Biotech) according to the manufacturer's recommendations. Each serum (5 mL) was incubated with Sepharose-coupled resins (5 mL) and left in a gently rotating/shaking mode for 30 min at room temperature. This procedure was done twice using a new lysate coupled to a Sepharose affinity column each time. Sera were collected and stored for western blot assays and invasion inhibition.

2.9. SDS-PAGE and Western blot

P. falciparum intra-erythrocyte schizont protein lysate was separated in a discontinuous SDS-PAGE system (1 mg/mL lysate was loaded per gel) by using an acrylamide gradient (7.5–15%, w/v). Following SDS-PAGE, the proteins were transferred to a nitrocellulose membrane (Hybond 203c, Pharmacia). After being blocked with 5% skimmed milk in TBS, nitrocellulose membranes (0.4 mm) were incubated for 1 h with a 1:20 dilution of polyclonal pre-immune and hyper-immune sera (rabbit 26 or 28) in TBS-T blocking solution (1% Tween 20 and 5% skimmed milk in TBS). 1:5000 alkaline phosphatase-conjugated antigoat IgG antibody (ICN) was incubated for 1 h after five TBS-T washes. The reaction was then developed with NBT/BCIP (Promega); commercial molecular mass markers (NEB) were used for estimating the relative molecular mass.

2.10. IFA studies

Blood smears were taken from a *P. falciparum* (FCB-2 strain) synchronised culture and then fixed with 4% formaldehyde for 10 min, followed by three PBS washes for immunofluorescence assays. The blood smears were then permeabilised with 0.1% Triton X-100 for 5 min at room temperature, then washed twice and blocked for 30 min with BSA/PBS 1% at 37 °C. The plates were washed three times with filtered PBS and incubated with anti-P*f*RH5 polyclonal serum as primary antibody at 1/20 dilution for 1 h, followed by three washes with PBS. A fluorescein (FITC)-labelled anti-rabbit antibody was used as secondary antibody (Vector Laboratories) for 1 h in a dark room. Fluorescence was analysed using an Olympus BX51 microscope.

2.11. Determining PfRH5-HABP structural characteristics

Secondary structure elements in P*f*RH5 HABPs were examined by circular dichroism (CD) [34]. HABPs (5 μM) were analysed using 1-cm light pass length quartz cell thermostated at 20 °C using 30% (v/v) 2,2,2-trifluroethanol (TFE)/water as co-solvent, as this has been shown to stabilise nascent structures [35]. Spectra were obtained on a nitrogen-flushed Jasco J-810 spectrometer at room temperature by averaging three sweeps taken from 260 to 190 nm

at 20 nm/min scan rate and a 1-nm bandwidth. Data were collected using Spectra Manager Software and analysed using the SELCON3, CONTINLL, and CDSSTR software [34,36].

2.12. Invasion inhibition assays

RBCs infected with *P. falciparum* late schizonts (FCB-2 or FVO strain) obtained from a synchronised culture (5% parasitaemia and 5% haematocrit) were incubated with 100 and 200 μM concentrations of each HABP. After incubating samples for 18 h at 37 °C in a 5% O2, 5% CO2, and 90% N2 atmosphere, culture supernatants were harvested and cells were labelled by incubation with 15 μg/mL hydroethidine for 30 min at 37 °C. Cells were washed thrice with PBS and analysed in a FacsCalibur flow cytometer (FACsort, FL2 channel) equipped with CellQuest software. Infected RBCs and ethylene glycol tetra acetic acid (EGTA)/chloroquine-treated uninfected erythrocytes were used as controls. Low activity binding peptides (LABPs) and scrambled peptides were used as negative controls. Two extra peptide concentrations (50 and 25 μM) were assayed for HABP 36727 and 36740. All assays were carried out in triplicate.

2.13. NMR and structural calculations

HABP 36727 and its analogue 37959 were dissolved in 30% (v/v) TFE-d3/H_2O [8 mM] pH 4.5 and placed in a Bruker DRX600 spectrometer for standard COSY, TOCSY and NOESY experiments. Spectra were recorded at different temperatures (285–315 K) to obtain H-bonds (ΔδHN/ΔTppb/K). NOE intensity was classified as being strong, medium or weak [37]. Distance geometry (DGII) software was used for generating a family of 50 structures, later refined by a simulated annealing protocol (Discover software).

3. Results

3.1. RH5 specifically bound to human RBCs through regions distributed throughout the protein

Synthetic peptides were tested in an RBC binding assay to identify P*f*RH5 regions binding to host cells [25]. Based on this method, peptides having ≥0.02 or 2% specific binding activity were considered to be HABPs [3,28]. Seven HABPs (Fig. 1a), distributed throughout the whole RH5 sequence, were identified: 36718 (^{21}NRLSFENAIKKTKNQENNLT^{40}*Y*), 36727 (^{201}GKYIAVDAFIKKINETYDKV^{220}), 36728 (^{221}KSKCNDIKNDLIATIKKLEH^{240}*Y*), 36735 (^{361}DEYIHKLILSVKSKNLNKDL^{380}), 36736 (^{381}SDMTNILQQSELLLTNLNKK^{400}*Y*), 36740 (^{461}DMSNEYSLFITSDHLRQMLY^{480}) and 36742 (^{501}LQMKFNDVPIKMEYFQTYKK^{520}). Low, non-specific binding (less than 0.5) was detected when scrambled peptides were tested in an RBC binding assay (Fig. 1b). The same pattern was observed when HABPs 36727 and 36740 were used in a HeLa cell binding assay (Fig. 1b).

HABPs had dissociation constants (K_d) ranging from 650 to 1300 nM and greater than 1 Hill coefficients (n_H), indicating that once a HABP had bound to its receptor it facilitated the binding of the next ligand (positive cooperativity) (Fig. 1c). A variable number of binding sites per cell (BSC), ranging from ∼65,000 to ∼417,000, were determined for the HABPs evaluated in the saturation analysis, suggesting HABP interaction with different saturable binding sites or HABP interactions at different sites on the same receptor on the RBC membrane.

3.2. PfRH5 may be involved in sialic acid-independent invasion

The receptor's nature for each P*f*RH5 HABP was evaluated by treating human RBC with neuraminidase which removes sialic acid residues on RBC surface glycoproteins, or trypsin which removes RBC surface proteins (such as glycophorin A and C) or with chymotrypsin which cleaves proteins such as Band 3 and glycophorin B. The specific binding of all HABPs was completely affected by treating RBC with trypsin, differently to what was observed when RBCs were treated with neuraminidase (Table 1). On the other hand, treating RBC with chymotrypsin completely removed HABP 36735, 36736 and 36740 binding while HABP 36728, 36742, 36718 and 36727 binding became reduced by 36% to 72% (Table 1).

Table 1
Specific binding of HABPs to enzyme-treated RBCs.

HABPs	% Specific binding			
	C	Neur	Tryp	Chym
36318	100	87	0	63
36327	100	128	2	72
36728	100	139	3	38
36735	100	69	1	0
36736	100	201	3	0
36740	100	124	0	0
36742	100	180	0	36

Standard deviations were below 5%. C, Control; Neur, Neuraminidase; Tryp, Trypsin; Chym, Chymotrypsin.

Bearing in mind that HABP binding to RBCs was affected by treatment with enzymes cutting proteins on RBC membrane, preliminary crosslinking assays between HABP-RBC membranes were carried out for determining the receptors' molecular weight. Although the HABPs specifically recognised a band of around 35 kDa the signal was weak (data not shown) additional assays must be carried out to confirm these results.

3.3. PfRH5-derived HABPs were conserved in different P. falciparum strains

PCR amplification of the regions encoding RH5 HABPs in FCB-2, PAS-2 and FVO strains was carried out as described above. 1050 base pairs (bp) and 828 bp amplification products were observed, this being in agreement with their expected sizes. Control primers amplified a band of about 438 bp [33] (supplementary material).

RH5 nucleotide and amino acid sequences obtained from *P. falciparum* FCB-2, PAS-2 and FVO strains were aligned with those of the 3D7 and 7G8 reference strains using Clustal W software [38]. HABPs 36718, 36728, 35735, 36736, 36740 and 36742 showed 100% amino acid sequence identity in all five *P. falciparum* strains and no synonymous substitutions were observed in the nucleotide sequences either (data not shown). The HABP 36727-encoding sequence had a single nucleotide change from G to A in position 608 (according to 3D7 reference strain numbering) which produced a change in amino acid residue 203 from a cysteine (C) to a tyrosine (Y) in 7G8, FCB-2, PAS-2 and FVO (Fig. 2a).

3.4. RH5 chemically synthesised peptides' immunogenicity

Immunofluorescence assays were carried out using our standard *P. falciparum* FCB-2 strain using antibodies against anti-P*f*RH5 synthetic peptide mixtures to assess the presence of P*f*RH5 and its functional role. The antibodies recognised a protein having a point pattern on late-stage schizonts which is typical of apical organelles (Fig. 2b). No fluorescence was observed in *P. falciparum* schizonts incubated with pre-immune sera (Fig. 2b).

Four bands of about 53, 49, 34 and 31 kDa (Fig. 2c) were also recognised by these antisera in western blot analysis of *P. falciparum* schizont lysate, which fit with P*f*RH5 protein cleavage fragments, as reported previously with other strains [17,26].

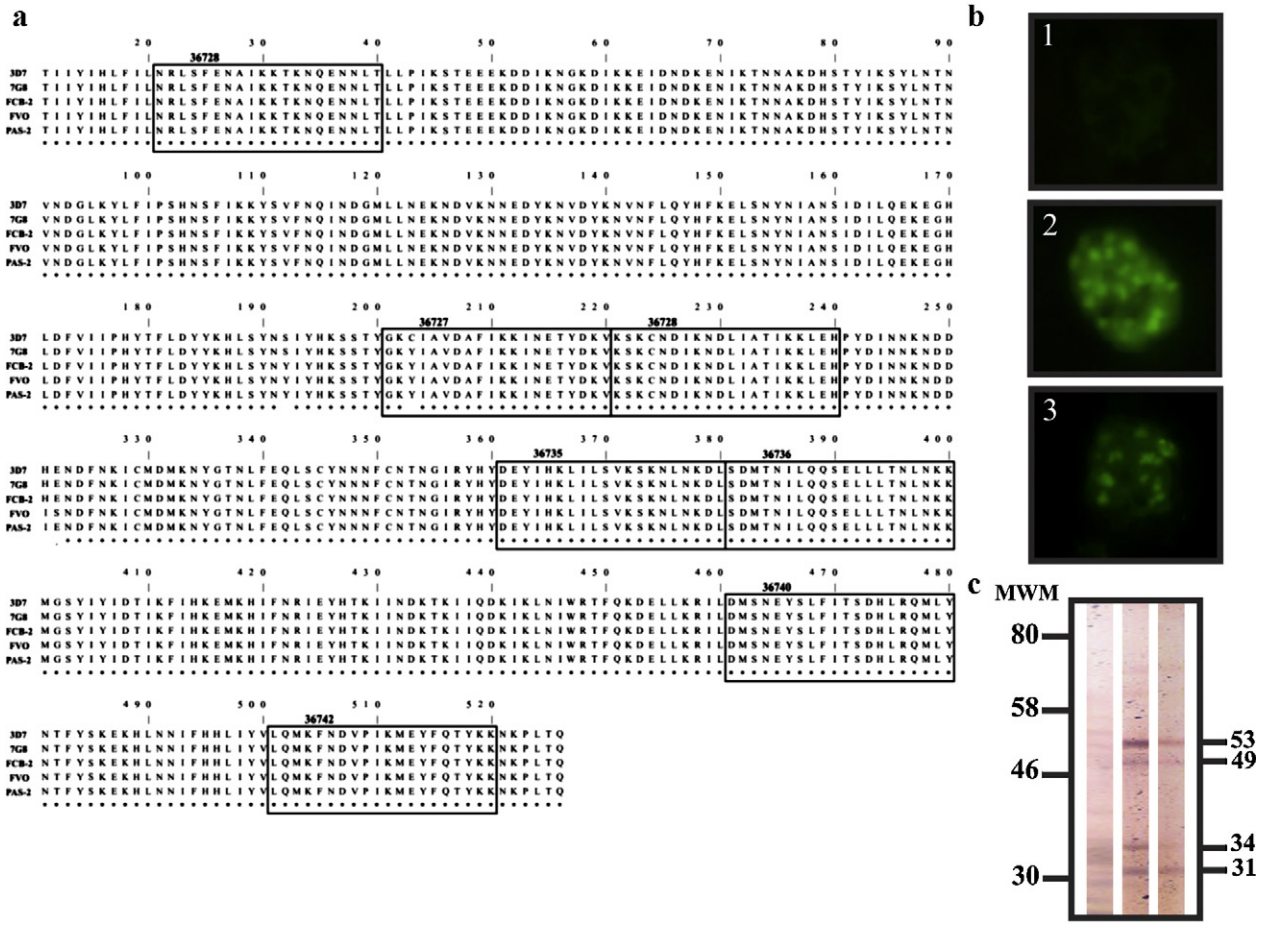

Fig. 2. P*f*RH5 biological characteristics and polymorphism results. (a) P*f*RH5 amino acid sequence alignment from different *P. falciparum* strains. FCB-2, FVO and PAS-2 strains were sequenced and compared with other strains reported in Broad Institute projects (http://www.broadinstitute.org/) as well as in the PlasmoDB database (http://plasmodb.org/plasmo/). HABPs are enclosed within black boxes. (b) Immunofluorescence detection of the RH5 protein in the *P. falciparum* FCB-2 strain using rabbit sera as primary antibody and fluorescein (FITC)-labelled anti-rabbit IgG as secondary antibody. (1) Negative control and (2) and (3) apical fluorescence. (c) Western blot analysis of *P. falciparum* schizont lysate using the same anti-P*f*RH5 rabbit antisera.

3.5. Information regarding the secondary structure of the PfRH5 protein and its HABPs

The HABPs were included in CD assays for determining their secondary structure content. A high helical content was determined for HABPs 36718, 36727, 36728, 36735 and 36740 as a 190 nm maximum and two minimums at 209 and 220 nm were observed (supplementary material). These results coincided with the deconvolution data which revealed around 75% alpha-helical structures, whereas HABP 36742 had adsorption maxima displaced to the right and around 50% helical content.

3.6. RH5 HABPs blocked merozoite invasion of RBCs

Human RBC invasion inhibition assays in *P. falciparum* synchronised cultures using FVO and FCB-2 strains showed that, regardless of the strain being assessed, HABP 36727 displayed 67–80% and 51–61% RBC invasion inhibition ability at 200 μM and 100 μM respectively (Table 2). On the other hand, HABP 36740 inhibited invasion by 49–29% (FCB-2 strain) and 48–36% (FVO-strain) at 200 μM and 100 μM, respectively (Table 2). HABP 36736 moderately inhibited invasion by 37% while the other HABPs had very low inhibition levels, these being comparable to those obtained with LABP 36734 and scrambled peptides.

The HABPs presenting greater inhibition ability at 200 μM were assayed at two additional concentrations (50 and 25 μM) for determining the minimum concentration at which the peptide would inhibit invasion by up to 10%. It was found that a 25 μM concentration for HABP 36727 and 50 μM for HABP 36740 inhibited invasion by 7% and 8%, respectively.

3.7. Changing an amino acid in 36727 HABP sequence modified its structure and determined species-specific binding

The results of a conventional human and monkey RBC binding assay carried out with HABP 36727 and an analogue peptide called 37959 (I204K) showed that parental HABP 36727 (I204) had 9.7% specific human RBC binding activity and 5.0% *Aotus* RBC binding activity (Fig. 3a), while analogue peptide 37959 (I204K) binding to human RBCs dropped by more than 80% (<2.5% binding capacity) and the same high *Aotus* RBC binding capacity was maintained (~5.0%).

The 3D structure of HABP 36727 and its 37959 analogue were determined by ^{1}H NMR [39] when trying to determine the critical role of this species-specific change at 3D structure level. HABP 36727 displayed a kinked and flexible structure, showing two α-helical structures between residues A208 to K211 and E215 to D218, a type I β-turn between residues K202 to D207 and another distorted γ type β-turn between residues K212 to N214. 37959 (I204K) analogue peptide had a completely α-helical, rigid structure between residues Y3-Y17 (it contains a large hydrophilic amino acid such as lysine) (Fig. 3).

Table 2
Invasion inhibition ability of *Pf*RH5 HABPs.

Activity binding		Invasion Inhibition (%)[a]			
		FCB2 strain		FVO strain	
		100 μM	200 μM	100 μM	200 μM
HABPs	36718	9	21		
	36727	**61**[b]	**80**	**51**	**69**
	36728	11	22		
	36735	20	28		
	36736	12	37		
	36740	29	**49**	**36**	**48**
	36742	5	13		
Analogue	37959	0	0	0	0
Scrambled	37158	9	19		
	37162	11	18		
	37169	9	18		
LABP	36734	9	28		
Parasites alone		0		0	
Chloroquine (1.85 mg/mL)		89		68	
EGTA (1.9 mg/mL)		67		55	

[a] Mean ± SD from three experiments. Standard deviations were below 10%.
[b] Values in bold indicate those peptides that induced the highest invasion inhibition percentages.

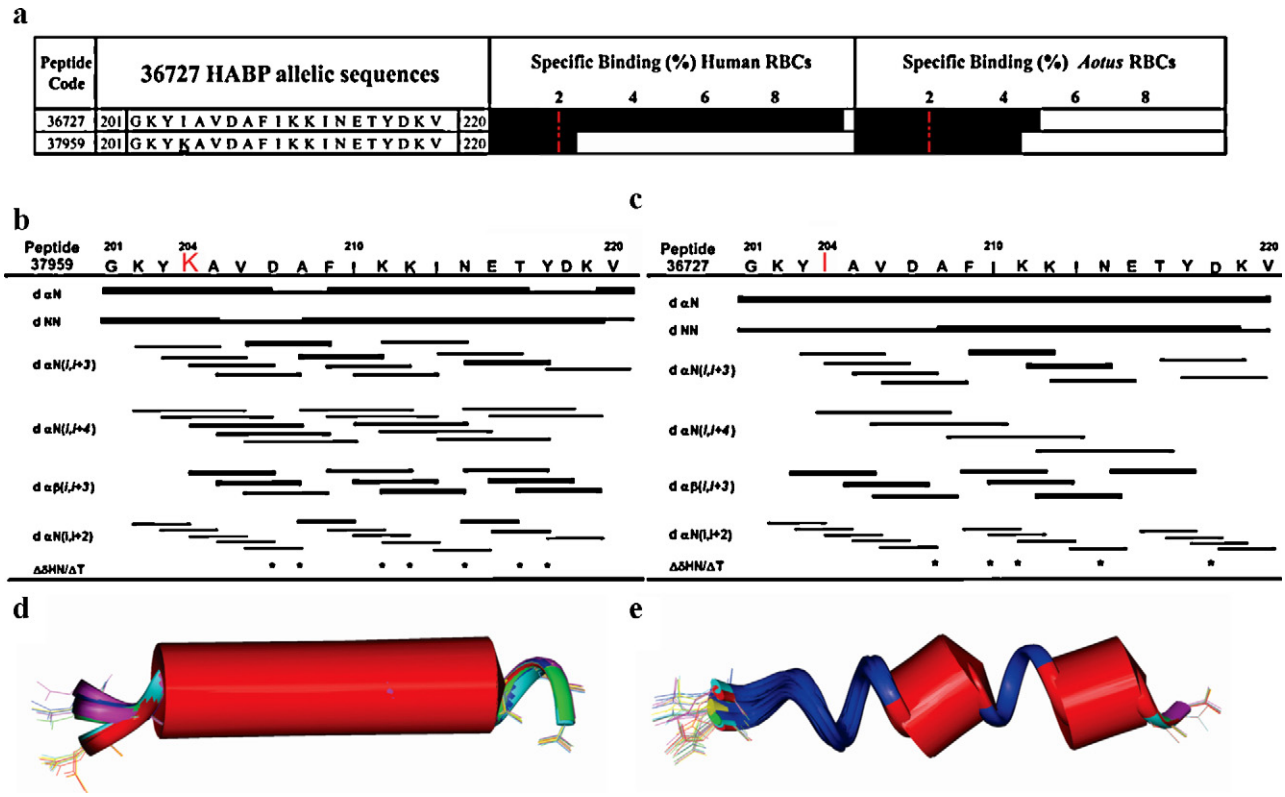

Fig. 3. Binding assays for HABP 36727 and its 37959 analogue and their respective structures. (a) Conventional human and *Aotus* RBC binding assay, testing HABP 36727 and its 37959 (I204K) analogue, (b) and (c) give a summary of sequential and medium-range NOE connectivity, represented by line thickness as *. (d) and (e) Kabash-Sander representation of 50 families of structures derived from *Aotus* RBC binding 37959 (K204I) analogue and human RBC binding 36727. The red barrels represent α-helical regions and blue ribbons show the β-turns.

4. Discussion

Transcriptome analysis of the *P. falciparum* intra-erythrocyte cycle has identified 58–90 molecules involved in RBC invasion by this malarial parasite [40]. Many of them have been grouped into families fulfilling combined or redundant functions. The merozoite surface protein (MSP) family (so far including 11 members) has been associated with initial interactions during the merozoite invasion of RBCs stage. Two more protein families (DBL and RH) have also been shown to be involved in strong and irreversible secondary interactions with RBC [25]. Molecular analysis of parasites selectively adapted by enzymatically treated RBCs or gene disruption has revealed these proteins' use of alternative invasion routes [22].

The exact mechanism through which these two families act still remains unknown; however, it has been proposed recently that

the parasite uses a combination of two ligands (one from RBL and another from EBL) and that such combination will depend on the availability of receptors on host cell surface. Merozoites will consequently tend to adopt 25 invasion routes [41]. Duraisingh et al. [21] have proposed that RBLs' initial function is to sense which RBC is most suitable to be invaded. Such interaction leads to the ligands having the greatest affinity being recruited, such as members of the EBL family, thereby leading to the micronemes' content being discharged, TJ formation and invasion. Bearing in mind that the parasite invades its target via different alternative routes using distinct proteins and different receptors available on RBC, it is thus absolutely certain that a single antigen is not enough to produce an immune response which can block parasite entry to a host cell.

Our objective has thus been to develop a peptide subunit-based, chemically synthesised vaccine derived from different parasite proteins and invasion phases. A highly sensitive, robust and specific methodology for identifying functional regions (15–20 amino acids long) has thus been designed for such approach using the proteins which have been involved in host cell invasion; these regions have been called HABPs. Regions from several important antigens such as MSP-1, apical membrane antigen 1 (AMA1), all the members of the DBL family and some from the RH family have been described to date [25].

A new protein known as P*f*RH5 has recently been identified and characterised making it the fifth member of the P*f*RH family [17,26,28]. P*f*RH5 is located in the TJ together with AMA-1 and RON4, probably in association with these proteins, due to a lack of transmembrane domains in its sequence. Several unsuccessful attempts have been made at interrupting/deleting the P*f*RH5 gene compared to those obtained for all the other members of P*f*RHs and DBLs [20–22]. Agreeing with such reports, it has been found that P*f*RH5 does not vary its expression levels in *P. falciparum* strains using different invasion routes and that it is recognised by sera from patients suffering from natural malarial infections, suggesting that P*f*RH5 could be an essential and vital antigen in parasite invasion, regardless of invasion route [17]. Such data strongly supports including the RH5 protein as a target in developing a subunit-based vaccine guaranteeing protection against a broad range of *P. falciparum* strains.

The present work has concentrated on the partial characterisation of P*f*RH5's functional role by identifying regions specifically interacting with receptors on RBC membrane. Seven HABPs have been identified which have presented high affinity and greater than 2% specific binding (Fig. 1a). Our data has confirmed and expanded Rodriguez et al. [28] and Baum et al. [17] results, showing that they are not contradictory but complementary since binding sequences may be located both towards either N-terminal or C-terminal regions. HABP 36718 is included in the binding region proposed by Rodriguez et al. [28] while a larger number of HABPs (36727, 36728, 36735, 36736, 36740 and 36742) are included in the region suggested by Baum et al. [17] (Fig. 1a). Such binding is highly selective since HABPs 36727 and 36740 have shown low and non-specific binding to HeLa cells (Fig. 1b), similar to the results obtained in scrambled peptide analysis, thereby indicating that HABP binding depends exclusively on peptide amino acid sequence (Fig. 1b).

The regions containing each HABP in different strains have been analysed, bearing in mind that antigen variation is one of the malarial parasite's major strategies for evading the host's immune response and one of the greatest obstacles to developing a completely effective vaccine. No changes in HABP nucleotides or amino acids were found, except in HABP 36727 amino-terminal where a tyrosine (Tyr) was replaced by a cysteine (Cys), both amino acids having similar polarity and charge (Fig. 2a). CD analysis of HABPs' secondary characteristics found that they presented high percentages of α-helices (supplementary material). These results are relevant as previous studies have suggested that rhoptry and microneme protein-derived HABPs display an α-helical structure and bind with high affinity to HLA-DR52 molecules [42], suggesting functional, structural and immunological compartmentalisation of each HABP from the key proteins involved in invasion.

When the nature of the receptor for P*f*RH5 HABPs was being evaluated it was found that all HABPs' binding was completely resistant to neuraminidase treatment while all of them were completely susceptible to trypsin treatment, and some displayed moderate or high sensitivity to chymotrypsin (Table 1). This data suggested a sialic acid-independent invasion pathway, as has been also reported for P*f*RH2b and P*f*RH4 [21,22]. Similar results have been reported by Rodriguez et al. [28] where P*f*RH5 recombinant fragment binding was inhibited when RBCs were treated with trypsin, although at higher enzyme concentrations (10×). Preliminary HABP cross-linking assays using human RBCs membrane have suggested that the P*f*RH5 HABP receptor's molecular weight was ~35 kDa (data not shown). Taking into account that this molecular weight did not match that for glycophorine A or B, the enzymatic treatment and cross-linking results further support the hypothesis that a sialic acid-independent pathway is being used by the parasite via P*f*RH5, as has been previously suggested [28].

An invasion inhibition assay led to functionally characterising HABP-RBC membrane interaction. It was found that HABPs 36727 inhibited invasion by up to 80% (Table 2), similar to that reported for some RH4 protein HABPs [43] suggesting that 36727 is the key HABP in this protein enabling human RBC invasion. Such inhibition ability was concentration-dependent, as shown when differing peptide concentrations were assayed.

Antibodies were obtained in rabbits against synthetic peptides derived from P*f*RH5 amino acid sequences for evaluating immunogenic properties, expression time and P*f*RH5 location in Colombian FCB-2 strain schizonts. High anti-peptide reactivity was found for antibodies specifically recognising the protein in late stage schizont lysate by Western blot and a pattern typical of apical organelles (Fig. 2b and c) was revealed by immunofluorescence assays similar to that reported in other *P. falciparum* strains [17,26,28] and other RH family members [5,20,22].

Studies of P*f*RH5 protein polymorphism derived from 7G8, GB4 and 16 other parasite strains displaying varying ability to infect *Aotus* monkeys and human RBC showed about 10 non-synonymous substitutions (marked in yellow, Fig. 1a) in the *rh5* gene [26]. Transfection assays and allelic exchange experiments have shown that non-synonymous changes in two non-invasive and non-infective *Aotus* monkey 7G8 strain codons have been enough to render this genetically modified strain ($7G^{KV}$) able to bind to *Aotus* RBC and make it infectious for these monkeys when experimentally inoculated. This conversion has been mainly associated with replacing an isoleucine (I) for a lysine (K) in position 204 (I204K) [26], suggesting that this point mutation in the *Pfrh*5 gene is responsible for not only the shift in this protein's binding to different receptors as has been reported for other antigens [27], but to completely changing host specificity and infectivity *in vitro* and *in vivo*.

Interestingly, conserved residue I204 in FCB-2, PAS-2, FVO, 3D7 and 7G8 (Fig. 2a) forms part of the sequence for HABP 36727 which inhibits human RBC invasion by >80% (Table 2). Analogous peptide 37959, having the replacement I204K, was thus synthesised; it was tested in a conventional human and *Aotus spp* RBC binding assay (Fig. 3a). While parental HABP 36727 (I204) human and *Aotus* RBC binding activity did not become modified, analogous peptide 37959 (I204K) binding to human RBCs dropped by more than 80%, showing that the residue in position 204 has a fundamental role in specific binding to human RBCs.

Similar results showing that a single change in sequence is associated with a functional shift have been reported for other proteins in a different functional scenario; for example, influenza virus

polymerase regulates species-specificity replication characteristics through point changes [44]. A species-specific preference for lysine 627 in PB2 polymerase subunit has been found; this results in high viral replication levels and/or polymerase activity in both animal models and human cells [44,45]. Regarding P*f*RH5, this is the first report of a single mutation as being a determinant feature in species-specific invasion and infection.

When peptide 37959 (I204K) was used in *in vitro* assays to inhibit FVO and FCB2 strain invasion of human RBCs it had no inhibitory effect (0%) (Table 2) while its parental HABP 36727 inhibited invasion of human RBC by both strains by up to 69–80%, respectively, once more confirming the critical role in species-specific invasion. Unfortunately, experiments were not performed with these peptides due to the tremendous complexity of developing *Aotus* RBC invasion inhibition assays.

While 37959 (I204K) analogue preferential binding to *Aotus* RBC contained a large hydrophilic amino acid, such as lysine, it had a completely α-helical, rigid structure (residues 3–17, Fig. 3). Native HABP 36727 (I204), containing a hydrophobic amino acid having similar volume to lysine (both having 166 Å) but opposite polarity and charge [46], had a kinked, flexible structure with two α-helical structures between residues A208 to K211 and E215 to D218, a type I β-turn between residues K202 to D207 and another distorted γ type β-turn between residues K212 to N214. This single mutation showed that the I204K substitution had a short configurational effect on the first seven residues, as well as long range structural effects on the other residues which could partly determine its promiscuous specificity for binding to RBCs from both species and infecting both humans and *Aotus* monkeys. This data shows, for the first time, that substituting a single residue and thereby altering HABP 3D structure does modify RBC binding in a species-specific manner and their ability to infect two different species: *Aotus* and humans [26].

Previous studies have shown the crucial role of P*f*RH5 in RBC invasion, according to reports of unsuccessful genetic disruption [7,28] and our enzyme studies suggesting that P*f*RH5 might be involved in a sialic acid-independent (neuraminidase-resistant) invasion mechanism, as reported for P*f*RH2b [47]. Our results have also shown the tremendous impact a single amino acid change displays in essential malarial molecules at atom level (i.e. P*f*RH5) which could be associated with structural differences in ligands, further suggesting that there might also be species-specific differences in the receptors.

Our data supports this protein's fundamental role as a candidate for being included in a fully effective antimalarial vaccine. These results could also partly explain different infection ability in *Aotus* monkeys as shown by unrelated *P. falciparum* strains at 3D structure level [26], clearly suggesting that a single amino acid substitution can determine different binding, invasion and infection characteristics for these monkeys.

Very recently, Otsuki et al. [48] have shown that a single amino acid substitution in the *P. yoelii* EBL protein affected protein virulence and localisation. Their results, coupled with ours, show the exquisite specificity of binding, invasion, infection and virulence in a parasitic disease like malaria. These differences, demonstrated at atom level, could be more marked in other species such as mice, guinea pigs and rabbits, which could bias vaccine design and partly explain the failure of vaccine candidates developed in such experimental systems when tested in human beings.

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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.vaccine.2011.11.012.

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