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A pre-PEXEL histidine-rich protein II erythrocyte binding peptide as a new way for anti-malarial vaccine development

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Abstract

The *Plasmodium falciparum* malaria parasite produces several proteins characterised by an unusually high histidine content in infected red blood cells (iRBC). The histidine-rich protein II (HRP-II) is synthesised throughout the parasite's asexual and gametocyte stages, transported through the parasitophorous vacuole (PV) to iRBC cytosol and membrane and released to the bloodstream via a PEXEL motif. Immunogenicity and protection-inducing studies were begun with an RBC high activity binding peptide (HABP) from this protein named 6800 (preceding the PEXEL motif) in the experimental *Aotus* monkey model. Modifying critical residues (determined by glycine scanning in this HABP) induced immunogenicity and protection against experimental challenge. Native 6800 did not bind to any HLA-DRβ_1* molecule, but these modified HABPs acquired the ability to specifically bind to HLA-DRβ_1*0701. ^{1}H NMR studies revealed that whilst 6800 had a random structure, modified immunogenic and protection-inducing 24230 displayed very short α-helical segments allowing appropriate binding to the MHCII-pep-TCR complex. Modifications in conserved HABPs preceding PEXEL motifs thus open up new avenues for subunit-based, multi-component synthetic anti-malarial vaccine development.

Keywords: Malaria; HRP-II protein; PEXEL motif; HLA-DRβ_1* molecule

Plasmodium falciparum causes the most aggressive form of human malaria. Blood-stage parasites infecting mature erythrocytes are responsible for most of the disease's symptoms and pathologies [1]. A series of receptor–ligand interactions having different affinities are begun during merozoite invasion of red blood cells (RBC), starting with rolling, apical pole reorientation towards the RBC, penetration and formation of the parasitophorous vacuole (PV) which invaginates the parasite within the PV membrane (PVM) separating the merozoite from erythrocyte cytoplasm to allow its replication [2]. A series of communication pathways become established during this process between the parasite and RBC cytosol [3] with the formation of a series of vesicles and membranes such as the tubovesicular network (TVN), loop-like membranous extensions (LM), Maurer's Clefts (MC) [4], etc., thereby facilitating parasite nutrition with external elements or present in erythrocytes and enabling it to communicate with the exterior [5].

The erythrocyte surface becomes dramatically modified during this process, increasing its size several times, changing its flexibility and forming a series of small, plate-shaped, electron-dense protuberances called knobs (K) in which knob-associated histidine-rich protein (KAHRP) [6], histidine-rich protein II (HRP-II) [7], and *P. falciparum* erythrocyte membrane protein Pf.EMP-1 accumulate on the cytoplasmatic side of these structures. These knobs and these proteins have been involved in forming clumps with non-infected RBC called rosettes [8]. Membrane-exposed

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proteins are responsible for many disease pathologies like cerebral malaria and abortions [9].

The HRP-I, HRP-II, and MAHRP-1 histidine-rich proteins have a vacuolar transport system (VTS) [10] or host targeting (HT) signal, also named plasmodium exporting element (PEXEL) [11], within their ~100 residues downstream from the endoplasmic-reticulum (ER)-type cleavage signal system (SS). PEXELs are present in these three secreted HRPs as well as in a large number of known and hypothetical proteins. These motifs are required to export the protein from the parasite's ER to the lumen of the parasitophorous vacuole (PV), to the erythrocyte cytosol and from there being secreted to the *milieu* or exposed on iRBC membrane [12].

The most clearly defined PEXEL motif carries the RxLxE/Q sequence where the first x mainly consists of hydrophobic amino acids (L and I) and the second x is less stringent. Replacing R, L, E by A or truncating this motif blocks HRP-II export to the PV and RBC cytoplasm, indicating the existence of a signal for a receptor/transport system putatively named "transportome" or "secretome" [11,12]. PEXEL motifs are therefore required for soluble or membrane-bound *P. falciparum* protein export.

PfHRP-II is synthesised as a 60–100 kDa precursor by the intracellular asexual parasite throughout its replication cycle, exported through the erythrocyte cytoplasm, packed into the MC and knobs or released to the milieu [13].

When designing a rational, logical approach towards chemically synthesised malarial vaccines, HRP-II was totally synthesised in 20 mer-long, non-overlapping peptides (the HRP-II complete amino acid sequence is given in Fig. 1A) which were radio-labelled and used in a highly specific and robust test which led to identifying HABPs in binding to RBC [14,15]. A single HABP was found, named 6800, located between residues 24–43 [16] (Fig. 1A green amino acid sequence) preceding the canonical PEXEL $\underline{R}_L\underline{L}_H\underline{E}_T$ (Fig. 1A in red). A diagram of HRP-II and the location of preceding 6800 HABP and the PEXEL motif are given in Fig. 1B.

The affinity constant for 6800 HABP was 200 nM, suggesting a strong interaction with its binding sites on RBC and it had a 2.45-nH Hill coefficient, suggesting positive cooperativity in this conserved HABP's binding to RBC. Likewise, the number of receptor sites per cell was calculated to be 6000 [16]. Inhibiting HABP 6800 binding to RBCs by anti-glycophorin A and B was 35 + 5% whilst it was ~100% with anti-glycophorin C, clearly suggesting that HABP 6800 preferentially bound to glycophorin C. Cross-linking this radio-labelled peptide with intact RBC displayed an interaction with a 32-kDa erythrocyte band, by auto radiography [16].

Due to the enormous potential for inducing a protective immune response directed against iRBC, immunogenicity studies were begun with peptide 6800 in the experimental

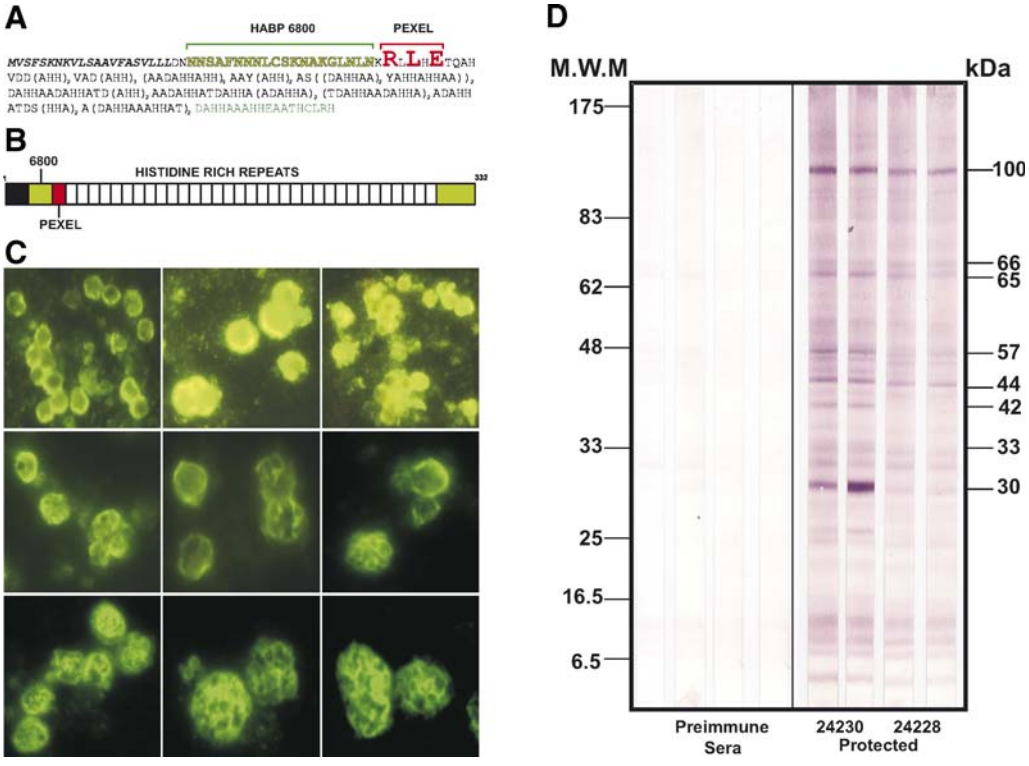

Fig. 1. (A) Identifying a PEXEL motif in *P. falciparum* HRP-II protein. HRP-II sequence, in which the signal peptide is shown in cursive and bold, HABP 6800 highlighted in green and conserved PEXEL motif in red. (B) Schematic representation of HRP-II containing the signal sequence (ss) in black, 6800 in green, the PEXEL motif in red, the histidine repeat region and the conserved C-terminal region histidine-rich repeats. (C) HRP-II detected by IFA antibodies induced by modified immunogenic HABPs on PV, iRBC cytosol, MC and merozoite membrane. (D) Western blot analysis of solubilised antigens obtained from late stage *P. falciparum* schizonts using sera from *Aotus* monkeys immunised with peptide 24230 and 24228.

Aotus monkey model which is highly susceptible to human malaria [17] and has an immune system which is almost identical to that of humans [18,19]. However, conserved HABPs are not immunogenic or very weakly so. Peptide analogues were thus synthesised replacing amino acids which are critical in binding to RBC (previously identified by glycine analogue scanning) [16] by others having similar mass, volume and surface but different polarity. They were used in these monkeys in studies of immunogenicity and protective ability against experimental challenge with an *Aotus*-adapted *P. falciparum* strain (FVO) 100% infective for these monkeys [17].

These peptides' secondary and three-dimensional structure was determined when seeking an association between their structure and immunological function by means of circular dichroism (CD) and ^{1}H nuclear magnetic resonance (^{1}H NMR) studies.

Compelling evidence has shown that these conserved HABPs' lack of immunogenicity is associated with their inappropriate fit into major histocompatibility complex Class II (MHC Class II) molecules, particularly HLA-DR molecules. This is why these native and modified HABPs' binding ability was determined.

Several modified HABPs (6800 analogues) were highly immunogenic and two of them induced protection against experimental challenge, suggesting a new route for controlling the parasite by inducing a protective immune response against modified conserved HABPs close to the PEXEL motifs and exposed on iRBC surface, finding a correlation with their 3D structure and ability to bind to HLA Class II molecules.

Materials and methods

Synthetic peptides. The peptides were synthesised by solid-phase *t*-Boc chemistry [20] numbered according to our Institute's sequential numbering system (Table 1), their purity analysed by HPLC and their molecular masses determined in a Bruker MALDITOF spectrometer (MS). Peptide polymers for immunisation studies were obtained after Cys and Gly were added at the N- and C-termini after cysteine oxidation as mentioned [21].

Animals and immunisation. Aotus monkeys were immunised three times with synthesised polymeric peptide analogues mixed with Freund's Adjuvant (FA) to induce humoral immune responses and protection against experimental challenge with the *P. falciparum* malaria parasite as reported [21]. Blood was drawn for immunological analysis on days 0 and 20 days after each immunisation (II_{20} and III_{20}). Controls only received FA in water.

Challenge and parasitaemia assessment. Immunised and control *Aotus nancymaae* monkeys were infected with 200,000 *P. falciparum* FVO-strain infected RBC for challenge 20 days after the last immunisation [17]. Protection was defined as being the total absence of parasites as assessed by Acridine Orange staining in blood during the 15 days of the experiment. Non-protected monkeys developed patent parasitaemia from day 5 or 6, reaching $\geqslant$6% levels between days 8 and 10 which required treatment.

IFA and Western Blot. Synchronised late-stage schizonts from a continuous *P. falciparum* culture (FCB-2 strain) method were washed and treated as before [17]. 20% late parasitaemia RBCs were washed with PBS (pH 7.2) and lysed with 0.2% saponine (Merck) for Western blotting.

HLA-DR molecule affinity purification. Human molecules were purified from DR1 WT100BIS (DRβ_1*0101), DR3 COX (DRβ_1*0301), DR4 BSM (DRβ_1*0401), DR7 EKR (DRβ_1*0701), and DR11 BM21 (DRβ_1*1101) homozygous EBV-B cell lysates by affinity chromatography using anti-HLA-DR mAb L-243 cross-linked to protein-A Sepharose CL-4B (Amersham, Pharmacia Biotech, AB) as affinity support [22].

Competition binding assays. Peptide binding competition assays measured unlabelled peptide's ability to compete with biotinylated indicator peptides for binding to purified HLA-DR molecules, as previously described [22]. Relative binding affinities were determined for peptide binding by competition assay, where a good competitor peptide was able to inhibit more than 50% of indicator peptide binding to any HLA molecule being tested.

Circular dichroism measurement. Circular dichroism (CD) assays were performed at room temperature using a Jasco J-810 spectropolarimeter. Results were expressed as mean residue ellipticity [Θ] units.

NMR analysis. ^{1}H NMR experiment samples were prepared by dissolving 10 mg peptide in 500 µl 2,2,2-trifluoroethanol-d_3 (Cambridge Isotope, 99.94%)/H_2O mixture (30/70, v/v) for the structure analysis. ^{1}H spectra were run in a BRUKER DRX-500 spectrometer. Proton spectra were assigned by using double quantum filter correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY) and nuclear overhauser enhancement spectroscopy (NOESY) experiments. 2D NMR data were processed with XWIN-NMR software. The NOESY spectra recorded at different temperatures (285–315 K) were used for obtaining amide temperature coefficients ($-\Delta\delta HN/\Delta T$). Spin coupling constants ($^3J_{NH-C\alpha H}$) on DQF-COSY spectra were measured as described [21–23].

Structure calculations. Peptide structure was determined by Accelrys software. NOE peaks, selected from 300 ms NOESY data sets, were integrated and converted into distance restraints. These restraints were grouped as strong, medium and weak corresponding to 1.8–2.5 Å, 2.5–3.5 Å, and 3.5–5.0 Å distance restraints, respectively. Hydrogen bond constraints were introduced for the slow exchange rate peptide NH, distance ranges involving these likely NH···O hydrogen bonds were set at 1.8–2.5 Å. The ϕ angle constraints derived from $^3J_{NH-C\alpha H}$ were restricted to $-70° \pm 30°$ if $^3J_{NH-C\alpha H} < 6$ Hz and to $-120° \pm 30°$ if $9\text{ Hz} > {}^3J_{NH-C\alpha H} > 8$ Hz. Distance Geometry (DGII) software was used for producing 50 starting structures.

Results and discussion

Peptide characterisation

Analytical chromatography results showed that peptide purity (following semi-preparative HPLC) was sufficiently high to be analysed by NMR. Experimental masses were the same as theoretical masses (data not shown). Their secondary structure as determined by CD is shown in Fig. 2A.

The polymers used for immunisation had molecular weights in the 8–24 kDa range as assessed by size exclusion chromatography (SEC). Their structural conformation was similar to their monomeric counterparts as determined by CD (data not shown).

Immunological studies

Immunising *Aotus* monkeys with native HABP 6800 did not induce antibodies against the parasite as detected by IFA or protection against experimental challenge, confirming that conserved HABPs are not immunogenic or protection-inducers (like other modifications made to critical residues), suggesting that highly specific changes must be made to break these conserved HABPs immunological code of silence. Only those modifications made to peptides 24228 and 24230 (replacing critical amino acids by others

Table 1
Native and modified HABPs' (in bold) amino acid sequences, with critical residues underlined

Peptide	Peptide sequence																						IFA			Prot	% of HLA-DRB1* molecule binding				
						P_1			P_4		P_6			p_9										PI	II_{20}	III_{20}		0101	0301	1101	0401	0701
6800	Y	N	N	S	A	F	N	N	N	L	C	S	K	N	A	K	G	L	N	L	N			0	0	0	0/5	3	10	15	16	40
24230a				S	A	F	D	D	N	L	T	A	A	N	A	M	G	L	I	L	N	K	R	0	2(320)	ND	2/8	0	37	6	27	**50**
24230b				S	A	F	D	D	N	L	T	A	A	N	A	M	G	L	I	L	N	K	R	0	1(320)	ND	1/10	0	37	6	27	**50**
24228a				S	A	F	D	D	N	L	T	A	M	N	A	M	G	L	I	L	N	K	R	0	1(320)	ND	1/8	21	29	16	-2	**48**
24228b				S	A	F	D	D	N	L	T	A	M	N	A	M	G	L	I	L	N	K	R	0	1(320)	ND	1/7	21	29	16	-2	**48**
11880		N	N	S	A	F	N	N	N	L	S	S	K	N	A	M	G	L	V	L	N			0	1(640)	0	0/5	3	**50**	7	26	ND
11882		N	V	S	A	F	N	N	N	L	S	S	K	N	A	M	G	L	V	L	N			0	1(320)	0	0/5	4	**62**	10	11	7
8540		N	N	S	A	F	N	N	N	L	C	S	K	N	A	G	G	L	N	L	N			0	0	0	0/5			*		
8541		N	N	S	A	F	N	N	N	L	C	S	K	N	A	K	G	G	N	L	N			0	0	0	0/5			*		
8542		N	N	S	A	F	N	N	N	L	C	S	K	N	A	K	G	L	G	L	N			0	0	0	0/5			*		
8543			N	S	A	F	N	N	N	L	C	S	K	N	A	G	G	L	N	L	N			0	0	0	0/5			*		
8544		N	N	S	A	F	N	N	N	L	C	S	K	N	A	G	G	L	G	L	N			0	0	0	0/5			*		
9944		N	N	S	A	F	N	N	N	L	S	S	K	N	A	M	G	L	R	L	N			0	0	0	0/4			*		
9946		N	N	S	A	F	N	N	N	L	S	S	K	N	A	M	G	L	M	L	N			0	0	0	0/4			*		
10006		N	N	S	A	F	N	N	N	L	S	S	K	N	A	M	G	L	L	L	N			0	0	0	0/3			*		
11348		N	M	S	A		N	N	N	L	S	S	K	N	A	M	G	L	I	L	N			0	0	0	0/3			*		
12692		N	V	S	A	F	N	N	N	L	C	S	K	N	A	M	G	L	V	L	N			0	0	0	0/4			*		
13856		N	I	S	A	F	I	I	N	L	S	S	K	N	A	M	G	L	I	L	N			0	0	0	0/5			*		
13858		N	I	S	A	F	I	I	N	L	S	S	K	N	A	M	G	L	D	L	N			0	0	0	0/5			*		
23022				S	A	F	N	N	N	L	V	S	K	N	A	M	G	L	I	L	N	K	R	0	0	0	0/10			*		
23430						F	N	N	N	L	T	G	M	N	A	M	G	L	I	L	N	K	R	0	0	0	0/9			*		

Sequences have been aligned according to the residue corresponding to modified HABP binding motif and reading register binding to HLA-DRβ_1* in Pockets 1, 4, 6, and 9. Antibody titres were determined by IFA (shown in brackets) corresponding to sera dilution reciprocal and the number of *Aotus* which developed such antibodies on days 0 (pre-immune), 20 days after the second immunisation (II_{20}) and 20 days after the third (III_{20}). Prot = the number of monkeys which were fully protected against intravenous inoculation of 100,000 infected erythrocytes from the *P. falciparum Aotus* adapted FVO strain.

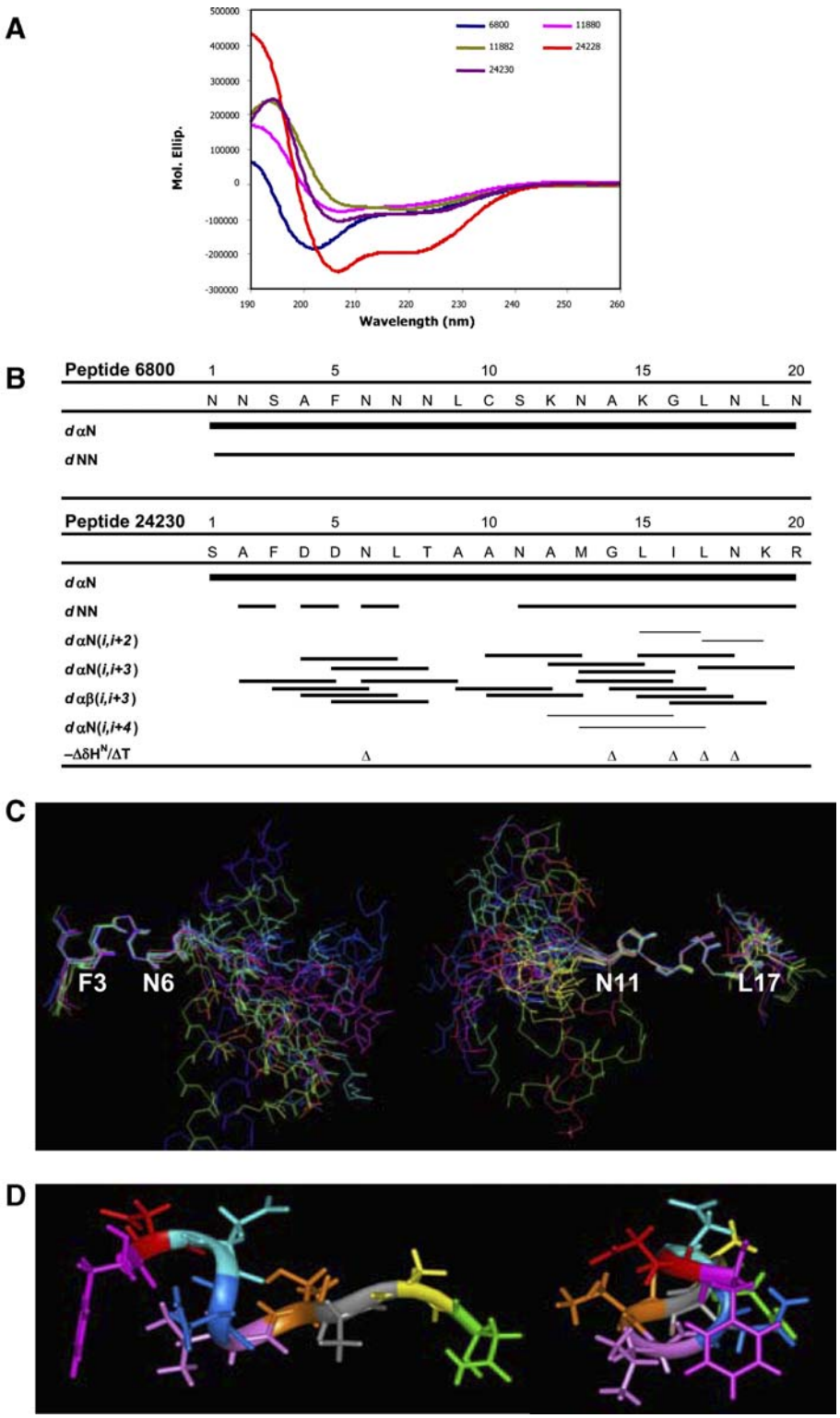

Fig. 2. Structural features for peptide 6800 and its analogues (A). CD for HABP 6800 and its analogues 11880, 11882, 24228, and 24230. (B) Summary of sequential medium range NOE connectivity (NOE intensity is represented by different line thickness). Temperature coefficient values less than 4.0 used in the calculation are indicated by Δ. (C) Representation of the two families of structures selected for peptide backbone: 224230 (immunogenic and protective). (D) Ribbon representation of the F3–N11 fragment displaying HLA-DRβ_1*0701 binding motifs and reading registers fitting into this molecules' Pockets. Colour code: fuchsia F3 (Pocket 1); red D4; pale blue D5; dark blue N6 (Pocket 4); rose L7; light-brown T8 (Pocket 6); grey A9; yellow A10; green N11 (Pocket 9).

having similar mass and volume but opposite polarity) induced reproducible high antibody titres and complete protection in 5 out of 33 (~15.6%) *Aotus* immunised with these peptides against experimental challenge (Table 1).

Such antibodies recognised PV and small granules in iRBC cytosol by immunofluorescence (IFA), suggestive of MC vesicles. The protein was the identified in entire iRBC cytosol and, on some occasions, on the membrane of merozoites present in already mature schizonts (Fig. 1C).

Likewise, sera from monkeys immunised with modified HABP 24228 recognised 100, 66, 62, 57, and 48 kDa *P. falciparum* proteins by Western blot in schizont lysate, corresponding to HRP-II molecular weight and its cleavage products. Interestingly, sera from *Aotus* immunised with modified HABP 24230 (only being differentiated in M12A) presented very strong additional reactivity with 57, 42, 33, and 30 kDa molecules, suggesting recognition of this protein in different cleavage processes which have been previously described for other molecules [21–23] (Fig. 2D).

Structural analysis. Peptide 6800 has a totally extended form due to the absence of medium-range signals; it therefore has a random coil structure. Peptides 11880, 11882 (immunogenic, non-protective), and 24228 (immunogenic and protective) were insoluble in the conditions established for ^{1}H NMR studies, which were only subjected to CD analysis. These peptides' CD spectra exhibited a minimum wavelength of around 200 nm, indicative of a random conformation (Fig. 2A). Peptide 24230 showed, $d_{NN}(i, i+3)$ $d_{\alpha\beta}(i, i+3)$, $d_{\alpha N}(i, i+3)$, $d_{\alpha N}(i, i+4)$ NOE connectivity, lowered amide proton chemical shift temperature coefficients for some of the amino acids (Fig. 2B), suggesting the presence of very short α-helical structures between residues F3–N6 and N11–L17 in 30% TFE (Fig. 2C). This is consistent with the two minima at 208 nm and 222 nm observed by CD spectra which are characteristic of α-helices. A set of 50 structures was calculated for peptide 24230 (immunogenic and protective) using 300 NOEs (214 intra-residue, 69 sequential) together with 5 hydrogen bond restraints. Thirty-four peptide 24230 conformers, best satisfying the given constraints, were then selected. These structures had no angle constraint violation larger than 1.00 (degrees) nor distance constraint violation larger than 0.32 Å. These structures had a 0.35 Å RMSD superimposition value for the backbone atoms.

HLA-DRβ$_1$ purified molecules binding*

While native HABP 6800 did not bind to any of the HLA-DRβ$_1$* molecules studied, modified HABPs 24230 and 24228 had ~50% binding to HLA-DRβ$_1$*0701 molecules, suggesting that the modifications rendered them able to bind to this molecule belonging to the HLA-DR53 haplotype. Molecule 24230 displayed classical binding motifs and reading registers: HLA-DRβ$_1$*0701 molecule F3 fitting into Pocket 1, N6 in Pocket 4, T8 in Pocket 6, and N11 in Pocket 9 [24]. Meanwhile, short-lived antibody-titres induced by non-protection-inducing modified HABPs 11880 and 11882 bound with high affinity to HLA-DR52 haplotype HLA-DRβ$_1$*0301 thereby corroborating what has been previously found that their ability to bind to molecules from another haplotype becomes shifted in these short-lived antibody, non-protection-inducing, modified HABPs [25].

En essence, native HABPs have a 3D-structure which does not allow them to be appropriately fitted to form the complex made up by molecules from the major histocompatibility complex (MHC), particularly from Class II, the peptide and the T-lymphocyte receptor (TCR) or MHCII-peptide-TCR, which is why such HABPs must be modified as described above. Ensuring an appropriate fit triggers the correct immune response.

Molecules such as merozoite surface protein-1, MSP-1, MSP-2, MSP-4, MSP-5, serine-rich protein (SERA), rhoptry-associated membrane antigen (RAMA), and some more destined to the parasite membrane are probably exported through a "classical" secretory route, while proteins destined for release within the RBC cytoplasm or iRBC membrane probably require additional machinery including the PEXEL motifs [10,11,26–28].

Some exported molecules interact with RBC cytoskeleton proteins such as mature infected erythrocyte surface antigen (MESA) which interacts with band 1 and 4.1 ring erythrocyte surface antigen (RESA) with spectrin [29], KAHRP with ankyrin and HRP-II with actin phosphatidyl-inositol, 4 biphosphate and erythrocyte ghost [30]. Some other merozoite exported proteins form part of the knob complex, such as HRP-II [7], KAHRP [6], PfEMP-1, PfEMP-3. Some others several different or unknown activities.

HRP-II has also been implicated in the formation of hemozoin, a detoxified crystalline form of ferric protoporphirin IX (Fe^{3+}–PPIX) binding 15 Fe^{3+}–PPXII molecules per 30 kDa monomer [31]. A small (~30 kDa) homologous protein named membrane associated histidine-rich protein-1 (MAHRP-1) exclusively transcribed in the early erythrocyte stages and performing a similar Fe^{3+}–PPIX binding function and with the RxMxE motif located 35 residues downstream from the SS sequence has been recently described [32]. It has been found to be located in the MC, iRBC cytosol and membrane.

Gametocytes and early trophozoites also express HRP-II as assessed by immunofluorescence, immunoelectronmicroscopy, and Western blot in iRBC cytosol during early stages of gametocyte development and internalisation in later the gametocyte form as it matures [33].

In silico analysis of the *P. falciparum* genome has suggested that ~150 proteins are exported into the iRBC cytoplasm [10,11], some of which have been involved in antigenic and structural alteration of RBC membrane, escaping immune surveillance by the spleen, mediating nutrition of the parasite from the RBC, etc. Transcriptome analysis has suggested the presence of these PEXEL motifs in sporozoites or gametocytes [34] indicating that the machinery for protein translocation across the PV membrane is equally functional in different host cell types [10,11].

Conserved HABP presence (close to the PEXELs, as happens with HRP-II, RESA, etc., staying on iRBC membrane for several hours) suggests that these modified HABPs could be excellent new targets for developing subunit-based, multi-antigenic, multistage (since some of them are present in sporozoites, merozoites, and gametocytes), chemically synthesised, anti-malarial vaccines generating a new avenue for vaccine development against this threatening disease that afflicts ~500 million people, killing ~3 million a year, mainly children below 5 in sub-Saharan Africa [35].

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