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ORIGINAL ARTICLE

Protective immunity provided by a new modified SERA protein peptide: its immunogenetic characteristics and correlation with 3D structure

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Abstract The serine repeat antigen (SERA) protein is a leading candidate molecule for inclusion as a component in a multi-antigen, multi-stage, minimal subunit-based, chemically synthesised anti-malarial vaccine. Peptides having high red blood cell binding affinity (known as HABPs) have been identified in this protein. The 6733 HABP was located in the C-terminal portion of the 47-kDa fragment while HABP 6754 was located in the C-terminal region of the 56-kDa fragment. These conserved HABPs failed to induce an immune response. Critical red blood cell binding residues and/or their neighbours (assessed by glycine-analogue scanning) were replaced by others having the same mass, volume and surface but different polarity, rendering some of them highly immunogenic when assessed by antibody production against the parasite or its proteins and protection-inducers against experimental challenge with a highly infectious *Aotus* monkey-adapted *Plasmodium falciparum* strain. This manuscript presents some modified HABPs as vaccine candidate components for enriching our tailor-made anti-malarial vaccine repertoire, as well as their 3D structure obtained by ^{1}H-NMR displaying a short-structured region, differently from the native ones having random structures.

Keywords SERA 5 · NMR · Structure · Malaria vaccine

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Introduction

The *Plasmodium falciparum* serine repeat antigen (SERA) protein family consists of a group of six closely related proteins (SERA 1–6); including the SERA-5 molecule (synthesised as a 111-kDa precursor) which has been studied in depth and which has been considered as a potential erythrocyte-stage vaccine candidate. SERA proteins are expressed during late trophozoite and schizont maturation stages where they undergo proteolytic processing by the *Pf* subtilisin 1 (*Pf*SUB-1) enzyme prior to the merozoite release and invasion of red blood cells (RBCs). Such proteolytic processing gives rise to a 47-kDa N-terminal fragment, a soluble 56-kDa inner domain fragment having a significant active serine-protease homologous region and an 18-kDa C-terminal portion. The 47- and 18-kDa fragments contain cysteine-rich domains, both remaining associated by disulphide bridges and forming a soluble 73-kDa hybrid protein fragment in non-reducing conditions (Sato et al. 2005). The 47-kDa fragment N-terminal is further processed into two 25-kDa fragments; one of these is attached to the 18-kDa fragment by a disulphide bridge (Fig. 1a), remaining bound to the merozoite membrane. The 56-kDa fragment is further processed in its C-terminal region and a 6-kDa peptide is removed to yield a 50-kDa fragment having putative serine-like activity. Although its exact function is still not very clear, SERA is the target of in vivo parasite antibodies before and after merozoite release; its recognition leads to merozoite agglutination and their subsequent dispersion obstruction. Such evidence strongly supports considering SERA as a good candidate to be included in a multi-antigenic anti-malarial vaccine.

In previous studies (Puentes et al. 2000), 49 non-overlapping 20 residue-long peptides encompassing the whole SERA protein were synthesised; six native peptides showed high binding capacity to RBCs, named high-activity binding

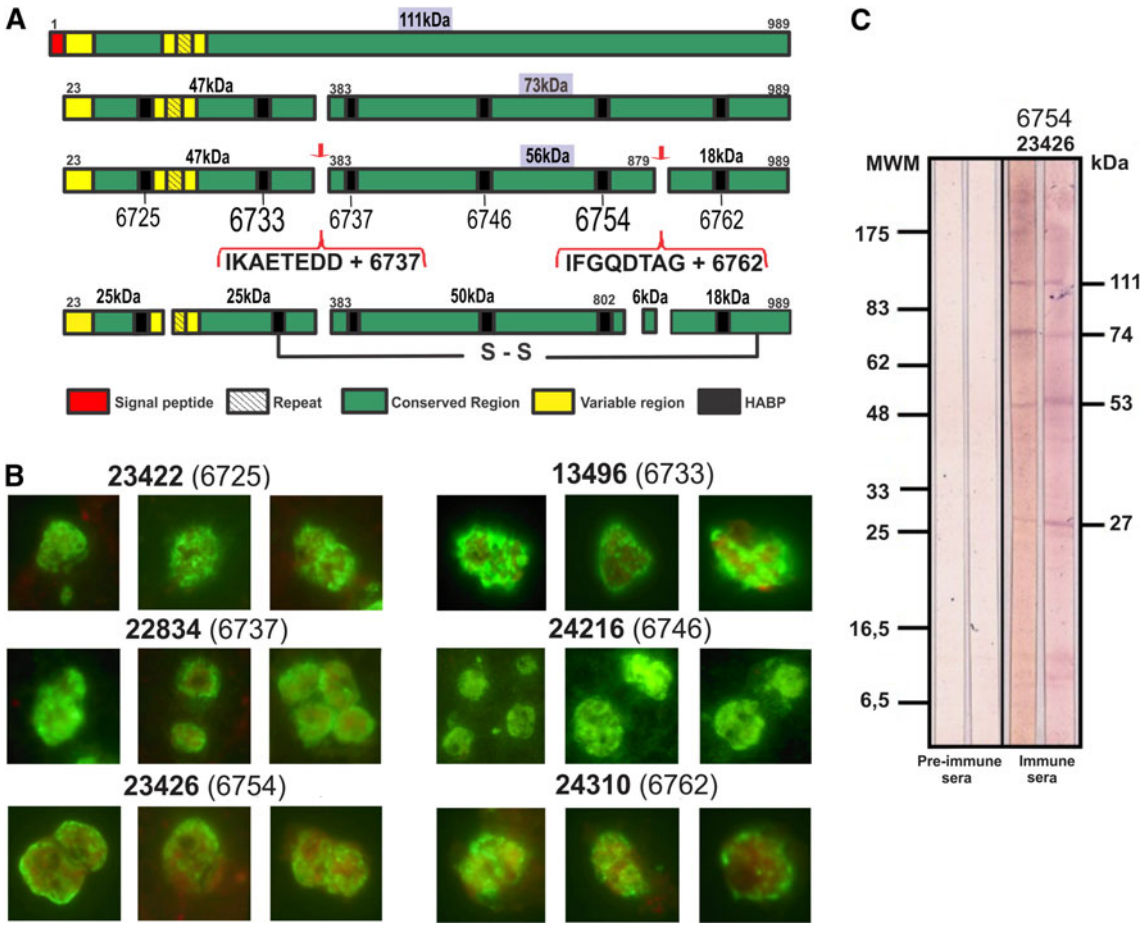

Fig. 1 **a** Schematic representation of the *P. falciparum* SERA protein involved in RBC invasion. The cleavage sites and their corresponding amino acid sequences have been indicated by *red arrows* together with HABPs 6725, 6733, 6737, 6746, 6754 and 6762 localizations, represented by *black vertical bars* (peptides identified as HABPs are indicated by our Institute's serial numbering system). *Bar length* shows approximate molecular weights and putative cleavage places and fragments. *Green fragments* show conserved amino acid sequences while *yellow regions* show variable amino acid sequences. The signal peptide is shown in *red*. **b** Immunofluorescence patterns shown by sera from protected *Aotus* monkeys, immunised with SERA protein-derived HABPs. These modified analogues (shown in *bold*) were derived from previously reported conserved HABPs (shown *inside parenthesis*) and the ones included in this manuscript. **c** Western blot analysis of sera from *Aotus* monkeys immunised with modified analogue **23426,** derived from conserved HABP 6754. The recognition of protein bands agreed with proteins' theoretical weight from which their amino acid sequences or their cleavage products were derived. Molecular weight markers are shown to the *left* in kDa, while the molecular weights of recognised bands are shown to the *right*

peptides (HABPs): 6725 (Alba et al. 2004), 6733 (this paper), 6737 (Cubillos et al. 2003), 6746 (Alba et al. 2003), 6754 (this paper) and 6762 (Salazar et al. 2008). They were numbered according to our institute's peptide coding system, meaning that their localisation (Fig. 1a, black bars) in the protein's amino acid sequence has been written in small superscript numbers. Native HABP numbers will be shown throughout this document in normal script whilst their modified analogues will be shown in bold (the effect of modified HABPs being the main emphasis of this paper). Immunological and structural studies from some of these native peptides and their modified HABPs have been previously reported, with the exception of peptides 6733 (^{321}YALGS**D**IPEKCDT**L**AS**NC**FLS^{340}) and 6754 (Y^{749}K**K**V**V**QNLCG**D**DTADHAVNI**V**G^{768}) along with their critical RBC-binding residues (assessed by glycine-analogue scanning, shown in bold and underlined above). These have been localised in the 47- and 56-kDa fragments, respectively, and form the focus of this manuscript. These native HABPs have been used in the attempt to induce antibodies against the *P. falciparum* parasite, as well as protective immunity against experimental challenge with this parasite but giving negative results, as has happened with previous experimental data for all conserved HABPs derived from most merozoite proteins studied so far (Patarroyo and Patarroyo 2008; Patarroyo et al. 2011). Critical binding residues and some of their neighbours were thus replaced by others having the same mass, volume and surface, but opposite polarity, as

thoroughly described beforehand (Cifuentes et al. 2008). This rendered some of these modified HABPs highly immunogenic, as assessed by antibodies production against the parasite or its proteins, and protection induction against challenge with a highly infectious *P. falciparum* strain adapted to *Aotus* monkeys (a primate species highly susceptible to human malaria).

Our previous studies have also identified modified peptides derived from other SERA-conserved HABPs [6725 (Alba et al. 2004), 6737 (Cubillos et al. 2003), 6746 (Alba et al. 2003) and 6762 (Salazar et al. 2008)] which were able to induce high immune responses and protective immunity, highlighting them as strong vaccine candidates. The present manuscript has concentrated on studying native peptides 6733 and 6754 (which displayed random configuration 3D structures as determined by CD and ^{1}H-NMR studies) known to be involved in RBC invasion due to the aforementioned studies. This paper highlights the importance of HABP **23426** (KK**V**QNLTG**D**DTADLATNI**V**G) which was modified from native peptide 6754; the modified HABP displayed a type V (β-turn structure) as evidenced by ^{1}H-NMR and the modifications made were T7C, L14H and T16V. Since the immune response against malaria is genetically controlled by the major histocompatibility complex region II (MHC II), it was found that this peptide bound with higher affinity to HLA DRβ1*0401 molecules than to the other HLA molecules involved in this study.

A very relevant aspect concerns the fact that HABP **23426** induced high antibody titres and protected *Aotus* monkeys against experimental challenge. Molecular modelling studies of this peptide in the HLA DRβ1*0403 molecule showed that 12 H-bonds were established between **23426** backbone and MHCII molecule lateral chains atoms, suggesting that these atomic features would be relevant in an immune response generated by this modified peptide in *Aotus* monkeys. This HABP has thus been considered a good candidate for being a component in an antimalarial vaccine.

Materials and methods

Solid-phase peptide synthesis

Native peptides and their corresponding modified analogues (shown in bold type throughout the paper) 6733 (**13496**) and 6754 (**22892**, **23426**) were synthesised by the standard solid-phase peptide synthesis method, (Merrifield 1963), purified by reverse-phase HPLC and their molecular masses were determined by MALDI-TOF mass spectrometry (Autoflex Bruker Daltonics). Glycine-cysteine (GC) were added to the C- and N-terminals of all peptides used for immunisation studies to polymerise them by an oxidation reaction; this established disulphide bonds amongst them and guaranteed the formation of high-molecular-weight polymers (8–24 kDa) for immunisation purposes.

Animals

Naïve, spleen-intact *Aotus* monkeys from the Colombian Amazon basin, which had been kept in our monkey colony in Leticia, were used for this trial; this non-human primate has proved to be very susceptible to experimental infection with the highly infective *Aotus*-adapted *P. falciparum* FVO strain (Rodriguez et al. 1990). The animals were housed in strict accordance with the Colombian Institute of Health's (INS) animal guidelines and the Colombian Ministry of Health laws (84/1989). They were supervised by expert biologists and veterinarians from Colombian wild-life authorities (CORPOAMAZONIA) and by FIDIC's Primate Station Ethics Committee. Monkey sera were tested by immunofluorescence assay (IFA) for the presence of anti-*P. falciparum* parasite antibodies at 1:20 dilution; monkeys seen to have positive sera at this point were returned to the jungle without further manipulation.

Immunisation and challenge

Groups of 5–9 *Aotus* monkeys (depending on availability) were immunised with 125 μg peptide, as described in previous work (Bermudez et al. 2007). Blood samples were obtained before each immunisation (day 0, 20, 40) and 20 days after the third immunisation for immunological studies. Immunised and control *Aotus* monkeys were intravenously infected with 100,000 *P. falciparum* FVO-strain infected RBCs which had been freshly obtained from another infected monkey. This dose is known to be 100% infective for these monkeys (Rodriguez et al. 1990).

Parasitaemia assessment

Blood parasitaemia levels were monitored daily for 15 days using Acridine Orange staining to reveal parasitaemia levels. Protection was defined as the total absence of parasites in blood during these 15 days. Non-protected monkeys developed evident parasitaemia on day 5 or 6, $\geq$5% levels being reached between days 8 and 10. The infected monkeys received treatment with anti-malarial drugs; they were kept in quarantine until complete cure had been ensured and then released back into the jungle, directly supervised by CORPOAMAZONIA officials.

Immunofluorescence antibody (IFA) testing

Synchronised late-stage schizonts from a continuous *P. falciparum* culture (FCB-2 strain) were washed and

treated as described earlier (Rodriguez et al. 1990). The slides on which the dry parasites had been mounted were blocked for 10 min with 1% non-fat milk and incubated for 30 min with increasing dilutions of monkey sera for antibody analysis, starting at 1:40 dilution. Reactivity was observed by fluorescence microscopy using the F(ab′)2 fragment from a 1:100 diluted goat affinity purified IgG anti-monkey IgG–FITC conjugate. Pre-immune sera from all monkeys were used as negative controls.

Western blotting

Late-stage schizonts from continuous *P. falciparum* cultures, exhibiting 20% parasitaemia, were collected, washed in sterile PBS and RBCs lysed with 0.2% saponine solution with vigorous vortexing for 45 s. The pellet was washed twice with large volumes of PBS to remove haemoglobin and erythrocyte debris. The enriched schizont pellet was further lysed with Laemmli's buffer and 5% SDS. The soluble proteins were separated in a discontinuous SDS-PAGE system using 7.5–15% acrylamide (w/v) gradient, transferred to nitrocellulose membranes and then blocked with TBS-T (0.02 M Tris–HCl, pH 7.5, 0.05 M NaCl, 1% Tween-20) and 5% skimmed milk (blocking solution) for 1 h and cut into strips. Each strip was individually incubated with monkey sera diluted 1:200 in blocking solution, washed several times with TBS-T and then incubated with goat anti *Aotus* IgG, alkaline phosphatase (AP) conjugated at 1:1,000 dilution and developed with NBT/BCIP (Blake et al. 1984).

HLA-DR molecule affinity purification

Purified human molecules were obtained from WT100BIS (DRβ1*0101), COX (DRβ1*0301), BSM (DRβ1*0401), EKR (DRβ1*0701) and DR11 BM21 (DRβ1*1101) homozygous EBV-transformed 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.

Peptide-binding competition assays

Peptide-binding competition assays measured unlabelled peptides' ability to compete with biotinylated indicator peptides in binding to purified HLA-DR molecules, as previously described (Sinigaglia et al. 1991; Vargas et al. 2003). Biotinylated-labelled haemagglutinin HA peptide 306-318 (PKYVKQNTLKLAT) was used as control peptide for DRβ1*0101, DRβ1*0301, DRβ1*0401 and Gly-Phe-Lys-$(Ala)_7$ ($GFKA_7$) for DRβ1*1101 and DRβ1*0701.

Purified HLA-DR molecules were diluted in freshly prepared binding buffer containing 100 mM citrate/phosphate buffer (pH 7), 0.15 mM NaCl, 4 mM EDTA, 4% NP-40, 4 mM PMSF and 40 μg/ml for each of the following: soybean trypsin inhibitor, antipain, leupeptin and chymostatin. About 90 μl of HLA-DR molecule (0.1 μM) was added to Eppendorf tubes, together with 30 μl biotinylated-labelled peptide (5 μM) in DMSO:PBS (1:4) for direct binding assay; an additional 250 μM was added for the competition assay. After 24 h of incubation at room temperature, the peptide/class II complexes were transferred to ELISA well-plates (NuncImmuno Modules Maxisorp Loose Brand product, Denmark) which had been coated with a 10-μg/ml anti-HLA-DR mAb-L-243 solution and subsequently blocked with PBS containing 5% bovine serum albumin. Plates were washed with PBS, 0.05% Tween-20 after 2 h incubation at room temperature. After incubation with alkaline phosphatase-labelled streptavidine (Vector Laboratories, Burlingame, CA), labelled peptide/HLA-DR complexes were revealed with 4-nitrophenyl-phosphate substrate (Kirkegaard and Perry Laboratories, MD, USA). A Titertek MC Multi-scan ELISA reader (Labsystems, Franklin, Mass) with 405 nm filter was used for determining peptide binding to HLA-DR molecules by measuring optical density (OD). Relative binding affinity for other peptides was determined by competition assay; according to this assay, a good competitor was a peptide which was able to inhibit indicator peptide binding to the HLA molecule being tested by more than 50%.

Circular dichroism (CD)

A JASCO J-810 spectropolarimeter was used to take spectra for native HABP 6733 and 6754, their modified and corresponding monomers and polymers; the spectra were smoothed using JASCO software. The peptide sample was analysed in 500 μl TFE-water mixtures (30:70, v/v) using a 1-mm path-length rectangular cell (Greenfield 1996). Measurements were taken at 20°C and expressed in terms of mean residue elipticity (deg $cm^2/dmol^{-1}$). The spectra were measured between 190 and 250 nm using 0.2 nm spectra bandwidth and 10 nm/min scan speed.

NMR analysis and structural calculations

Seven to ten milligram of HPLC purified peptides was dissolved in 600 μl TFE-water (30/70 v/v) for NMR experiments. NMR spectra were recorded on a Bruker DRX-600 spectrometer at 295°K. Double-quantum filter correlation spectroscopy (DQF-COSY) (Rance et al. 1983), total correlation spectroscopy (TOCSY) (Bax and Davis 1985) and nuclear overhauser enhancement spectroscopy (NOESY) experiments were used for assigning spectra (Jeener et al. 1979) and data were processed on an Indy computer (Silicon Graphics) equipped with updated

TOPSPIN software (Bruker). Distance Geometry (DGII) software was used for providing a family of 50 structures. These structures were refined using a simulated annealing protocol (DISCOVER software). Structures having reasonable geometry and few distance and angle violations were selected.

Molecular modelling

The HLADRβ1*0401 human molecule (PDB code 1J8H) crystal structure was used as template for molecular modelling peptides **23426** and **22892** (6754 analogues) to ascertain their fit into this complex; in turn, this was used for analysing whether the three-dimensional structure of modified, immunogenic, protection-inducing peptide **23426** had been correctly obtained, compared with the fit of non-immunogenic, non-protection-inducing peptide **22892**. The amino acids have been written using one-letter code when they have been derived from the modified peptide and in three-letter code if they have been HLA-DRβ1* chain-derived. Replacements were made in this molecule's sequence based on the differences found in the protein-binding region (PBR), as reported in previous studies (Suarez et al. 2006; Patarroyo et al. 2010a). The amino acids replaced in the HLA-DRβ1*0403 molecule were Argβ71Lys, Gluβ74Ala and Valβ86Gly. Replacements made in haemagglutinin (HA) for modified peptide **23426** were Q4P, N5K, L6Y, T7V, G8K, D9Q, D10N, A12L, and D13K and Q4P, N5K, L6Y, S8K, D9Q, D10N, A12L, D13K and V16T for peptide **22892**.

A conjugate gradient algorithm was applied to minimise energy and build a more stable model showing atom position within the peptide-HLA-DRβ1*0403-like complex in terms of energy. Five to seven simulations using 10,000 iterations were carried out for both complexes (peptide **23426**-HLA-DRβ1*0403-like and peptide **22892**-HLA-DRβ1*0403-like) to obtain the most appropriate model using each sequence contained in the complete template. Insight II (2000) Biopolymer module software (Accelrys Software Inc., USA), run on an Indigo 2 Station (Silicon Graphics), was used for superimposing the calculated models onto the original template backbones (without further refinements).

Results and discussions

Peptide analysis

HPLC monomer analysis revealed one single peak after purification, which was pure enough for ^{1}H-NMR analysis; peptide masses were similar to theoretical masses (data not shown). The polymers used for immunisation had molecular masses ranging from 8 to 24 kDa, as assessed by size-exclusion chromatography (SEC).

Immunogenicity studies

Native conserved HABPs 6754 and 6733 were not immunogenic, given the antibody production against this protein was not induced after the third *Aotus* monkey immunisation with these polymer peptides, as assessed by IFA and Western blot. Likewise, protection against experimental challenge with the parasite was not induced (Table 1).

Immunogenicity was seen to have been induced when **13496** (peptide 6733 analogue) was modified by changing V5D, E6I, S10C, V16N and V17C, as assessed by the presence of high levels of IFA antibodies and Western blot reactivity, even though absolutely no protection was induced (Table 1). A similar thing occurred with **14536** (6733) with modifications P5D, N6I, S10C, H13L, I16N and V17C.

No antibodies were produced by modified peptide **22892** (6754 analogue) when replacing V7C, S8G, L14H, as assessed by IFA and Western blot reactivity, and no protection against experimental challenge was induced (Table 1). Similar results were obtained when other modifications were made, such as changing critical residues in other analogous peptides synthesised. On the contrary, immunogenicity and protection were induced in *Aotus* monkeys inoculated with modified HABP **23426** where changes were made to residues T7C, L14H and T16V (Table 1), categorically confirming that critical binding residues or their neighbours have to be changed for others having similar mass and volume but apposite polarity (Cifuentes et al. 2008; Patarroyo et al. 2008, 2011).

IFA and Western blot analysis

Immunofluorescence assay analysis (Fig. 1b) revealed that antibodies against SERA-derived modified HABPs displayed a diffuse intracytoplasmic fluorescence pattern in mature schizonts when the sera from *Aotus* monkeys immunised with previously reported modified HABPs **23422** (6725), **13496**(6733), **22834** (6737), **24216** (6746), **23426** (6754) and **24310** (6762) were used (Fig. 1b). Western blot analysis revealed strong reactivity between sera from protected monkeys immunised with HABP **23426** (6754) with a 111-kDa molecule, corresponding to the complete SERA protein precursor and its 74 kDa complex cleavage fragment (Fig. 1c). *Aotus* sera immunised with this HABP also recognised 53 and 27 kDa fragments corresponding to cleavage and processing products (being quite similar to this protein's 56 and 25 kDa fragments) (Fig. 1a, red arrows and corresponding cleavage sites' amino acids sequences and Western blot analysis shown in Fig. 1c).

Table 1 Humoral immune response and protective efficacy induced by native peptides 6733 and 6754 and their modified analogues and structural features of conserved HABP 6754 and its modified analogue **23426,** as determined by ^{1}H-NMR. This peptide's percentage binding to different purified HLA-DRβ1* haplotype molecules (shown in bold, showing ≥50% binding affinity)

A

Peptide No.	Sequence																				PI	post I	post II	Prot.
						P1			P4		P6			P9											
*6733	Y	A	L	G	S	**D**	I	P	E	K	C	D	T	**L**	A	**S**	**N**	**C**	F	L	S	0	0	0	0/4
***13496**		A	L	G	S	**V**	**E**	P	E	K	S	D	T	L	A	S	**V**	**V**	F	L	S	0	3(1280)	2(640)	0/6
14536		A	L	G	S	**P**	**N**	P	E	K	S	D	T	**H**	A	S	**I**	**V**	F	L	S	0	5(640)	1(320)	0/4
13498		A	L	G	S	**V**	**E**	P	E	K	S	D	T	L	A	S	**M**	**S**	F	L	S	0	0	0	0/4
13778		A	L	G	S	**V**	**E**	P	E	K	S	D	T	**H**	A	S	**V**	**V**	F	L	S	0	0	0	0/4
13500		A	L	G	S	**P**	**K**	P	E	K	S	D	T	**L**	A	S	**M**	**S**	F	L	S	0	0	0	0/4
12746		A	L	G	S	**P**	**E**	P	E	K	S	D	T	**H**	A	S	**V**	**S**	F	L	S	0	0	0	0/4
14534		A	L	G	S	**P**	**N**	P	E	K	S	D	T	**H**	A	S	N	**V**	F	L	S	0	0	0	0/4
10142		A	L	G	S	D	I	P	E	K	S	D	T	**H**	A	S	**S**	**S**	F	L	S	0	0	0	0/4
10144		A	L	G	S	D	I	P	E	K	**S**	D	T	**A**	A	S	**R**	**S**	F	L	S	0	0	0	0/4
10146		A	L	G	S	D	**I**	P	E	K	S	D	T	**R**	A	S	A	S	F	L	S	0	0	0	0/4
*6754	Y	K	K	**V**	Q	N	L	**C**	**G**	**D**	D	T	A	**D**	H	A	**V**	N	I	**V**	G	0	0	0	0
***23426**		K	K	V	Q	N	L	**T**	G	D	D	T	A	D	**L**	A	T	N	I	V	G	0	3(320)	ND	**1/9**
24220		K	K	V	Q	N	L	**T**	G	D	D	T	A	**I**	**L**	A	T	N	I	V	G	0	3(320)	ND	0/7
***22892**		K	K	V	Q	N	L	**V**	**S**	D	D	T	A	D	**L**	A	V	N	I	V	G	0	0	0	0/9
13848		K	K	**T**	Q	N	L	**S**	**A**	**P**	D	T	A	D	H	A	T	N	I	V	G	0	0	0	0/5
22434					Q	N	L	**S**	G	D	D	T	A	D	**L**	A	V	N	I	**T**	G	0	0	0	0/6

B

						Haplotypes				
						DR1	DR52		DR53	
						% Binding HLA-DRβ1* alleles				
Peptide	Structure	NOEs Used	RMSD	Distance Å	Distance #	0101	0301	1101	0401	0701
6754	Random	ND	ND	-	ND	0	15	5	8	5
23426	Type V β turn V3 to L6	212	0.39	24.31	23	0	16	13	**56**	19

Peptide amino acid sequences used for immunising *Aotus* monkeys are shown in one-letter code (numbered according to our Institute's serial system)

The number outside the bracket shows the total number of *Aotus* monkeys presenting these antibody titres

IFA reciprocal antibody titres (shown in brackets) determined from serum samples taken 20 days after the 1st and 2nd immunisations

Sequences from peptides are aligned according to the HLA-DRβ1 molecule's pockets 1, 4, 6, and 9 (*shadowed*) to peptide's bound and are assigned according to their corresponding binding motifs and binding registers

Prot. total number of *Aotus* protected against experimental challenge from those presenting the antibody titres, *ND* Not determined

* Structures determined by NMR

Peptide binding to purified HLA-DRβ1* molecules

Native HABP 6754 did not bind to any of the molecules studied here (representative of the main HLA-DR1, DR52 and DR53 haplotypes); however, HABP **23426** (6754) did bind to HLA-DRβ1*0401 (Table 1). This suggested that the above modifications allowed this modified peptide's better fit into this HLA-DRβ1* molecule, thereby allowing more stable MHC II-Peptide–TCR complex formation and, therefore, a better immune response to be induced.

Binding motifs and reading registers

Binding profiles were not determined for modified HABPs **13496** and **14536** (6733) due to limitations in the availability of purified HLA-DR molecules for performing these assays and because they did not induce protection. However, they did induce high antibody titres, similar to HABP **24220** (6754) which only induced high antibody titres with the first immunisation and which disappeared later on (Table 1). Such phenomena have been previously described by our group and

named, respectively, non-protective, long-lasting antibody induction (Patarroyo et al. 2006) and short-lived antibody induction (Patarroyo et al. 2005) which we have thoroughly analysed at the 3D structural level, finding different structural features and residue orientation in such molecules which have induced these non-protective immune responses.

HABPs **13496** and **13498** (6733) also displayed a binding register characteristic of the HLA-DRβ1*0401 molecule (data not shown) according to Rammensse's classifications (Rammensee et al. 1995). No HLA-DRβ1 binding studies were performed with these and the other modified HABPs shown in Table 1 due to their low relevance in immunological activity.

HABP **23426** (6754) had 56% binding to purified HLA-DRβ1*0401 molecule, this being much more higher than its ability to bind to the other purified HLA-DRβ1* molecules. It also displayed characteristic HLA-DRβ1*0401 binding motifs, such as L6 in pocket 1, D9 in pocket 4, T11 in pocket 6 and L14 in pocket 9 (Table 1), just like HABP **22892** which displayed similar characteristic HLA-DRβ1*0401 binding motifs and reading registers in its sequence (data not shown). The same HLA-DRβ1*0401 binding motifs were observed when comparing HABPs **23426** and **22892** which had been modified in T7/V (pocket 2) G8/S (pocket 3) and T16/V, suggesting a different orientation for the contact of these residues' side chains with TCR residues. Modifications made to **23426** could thus be playing a critical role in the induction of protective immune responses, thereby altering polarity in T7C (pocket 2) and T16V to allow better contact with the TCR, besides allowing a perfect fit for L14H in HLA-DRβ1*0401 pocket 9.

CD determination of secondary structure for peptides 6733 and 6754 and their analogues

The secondary structures of monomer and polymer peptides 6754 and 6733 and their analogues, determined by circular dichroism (CD) in 30% TFE and 70% water, had similar spectrums and structural features. The native peptides had random conformation and modified peptides showed distorted structural features due to the displacement and change in spectrum shape and the minima present in the spectrum. The immunogenic modified peptides seemed to be more structured than the native peptides from which they had been derived according to spectrum distortion and minimum shifting patterns (close to 225 nm), as shown by deconvolution analysis using CONTINLL, SELCON and CDSSTR software (Fig. 2a).

3D structural analysis by ^{1}H-NMR

The peptides selected for ^{1}H-NMR 3D structure determination were representative of important immunological functions, such as immunogenicity and protection induction, as seen with **23426** (6754); those which had no specific immunological reactivity were used for comparing both structural conformations. ^{1}H-NMR analysis found the peptides' structural conformation and correlated them regarding immunogenicity and protection-induced response. Figure 2b shows all NOE connectivities observed.

Native peptides 6733 and 6754 only presented $d_{\alpha N}(i, i+1)$ and $d_{NN}(i, i+1)$ sequential signals in NOESY spectra (Fig. 2b), indicating that these peptides did not present any conformational preferences and suggesting a random coil conformation throughout the whole peptide. These observations confirmed the previous CD data obtained in deconvolution analysis.

NMR analysis of **13496** (6733-derived peptide immunogenic but non-protective) displayed d_{NN} $(i, i+1)$, $d_{\alpha\beta}$ $(i, i+3)$ and $d_{\alpha N}$ $(i, i+4)$ short- and medium-range interactions (Fig. 2b). NOE connectivities and low-temperature coefficients for amide proton chemical displacement revealed the presence of an α-helix conformation. A set of 50 structures was calculated for this HABP using 195 distance restraints and one hydrogen bond restraint. A family of 32 low-energy structures having 0.25 Å root-mean-square deviation (rmsd) allowed superimposing backbone atoms from residues E8 to L13 onto the structure having the lowest energy conformer; each structure did not have an angle-constraint violation larger than 1.10° or distance constraint violation larger than 0.23 Å. The NOE connectivity pattern, together with low-temperature coefficients, determined the presence of an α-helical structure between E8 and L13 (Fig. 3a).

Peptide **22892** (6754-derived, non-immunogenic and non-protective) had a distorted type III' β-turn between Q4 and V7; the ideal values for this type of structure are $\Phi i+1 = 60$, $\Psi i+1 = 30$, $\Phi i+2 = 60$, $\Psi i+2 = 30$, and the values observed for this peptide were 55.88, 56.50, 70.56 and 55.70, respectively (Table 1; Fig. 3b).

A set of 50 independently produced structures were obtained for **23426** (6754) satisfying experimental constraints when using 173 NOEs derived from distance restraints which had been previously classified according to signal strength (including 18 dihedral restraints). The structural calculations led to obtaining a family of 19 low-energy conformers having no distance violation larger than 0.35 Å. **23426** (immunogenic and fully protective for some monkeys) showed a type V β-turn structure between V3 and L6. The ideal values for this turn are $\Phi i+1 = -80$, $\Psi i+1 = 80$, $\Phi i+2 = 80$, $\Psi i+2 = -80$; the values observed for **23426** were −92.23, 54.30, 68.86 and −73.03, respectively.

Three-dimensional structure results for all monomer peptides analysed by NMR in this article were consistent

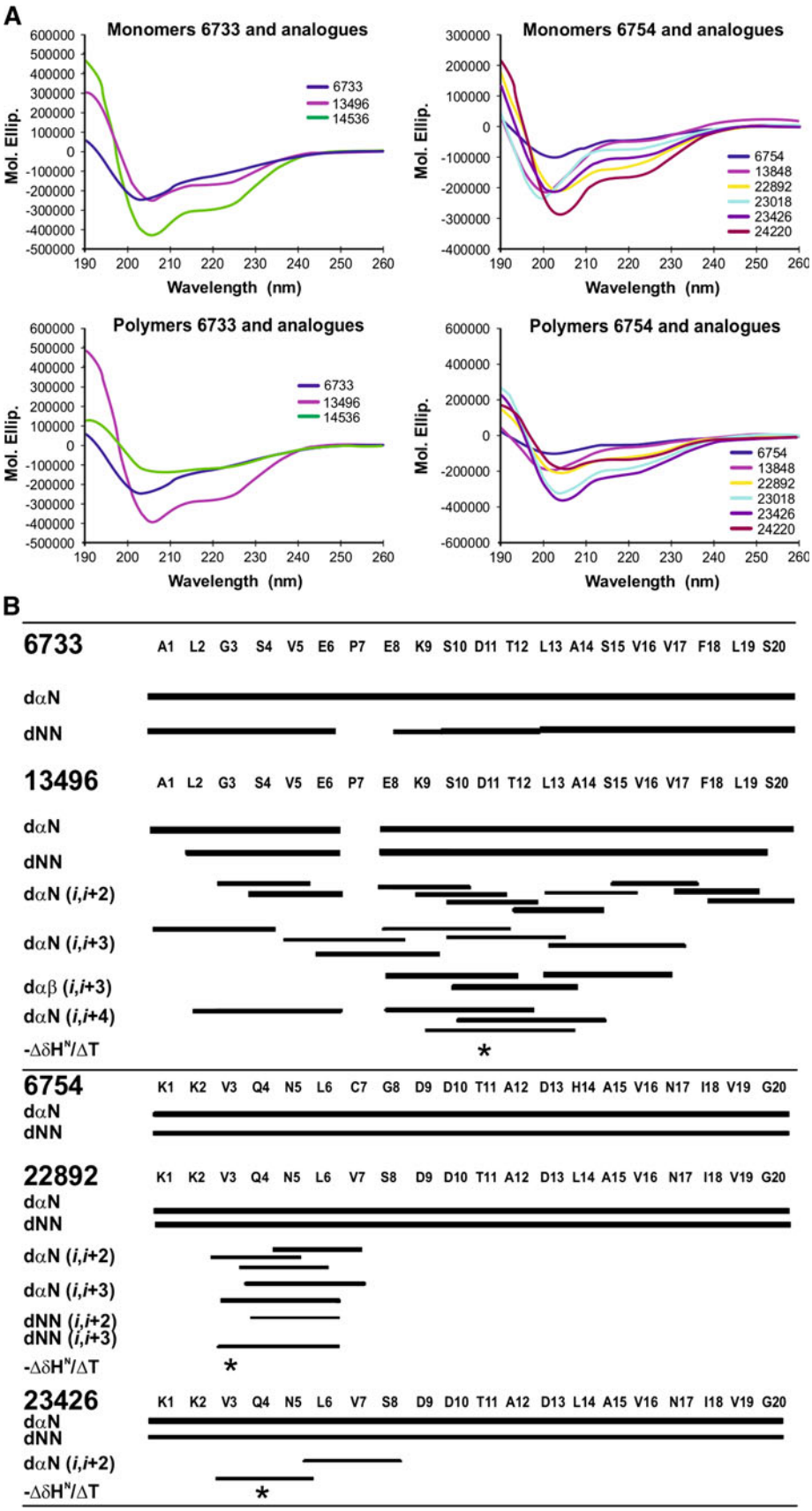

Fig. 2 **a** Circular dichroism for peptides 6733 and 6754 and their analogues in their monomer and polymer forms. **b** Summary of sequential medium-range NOE connectivity (NOE intensities are represented by *line thickness*); amide protons having low coefficients and used for structure calculations are marked with an *asterisk*

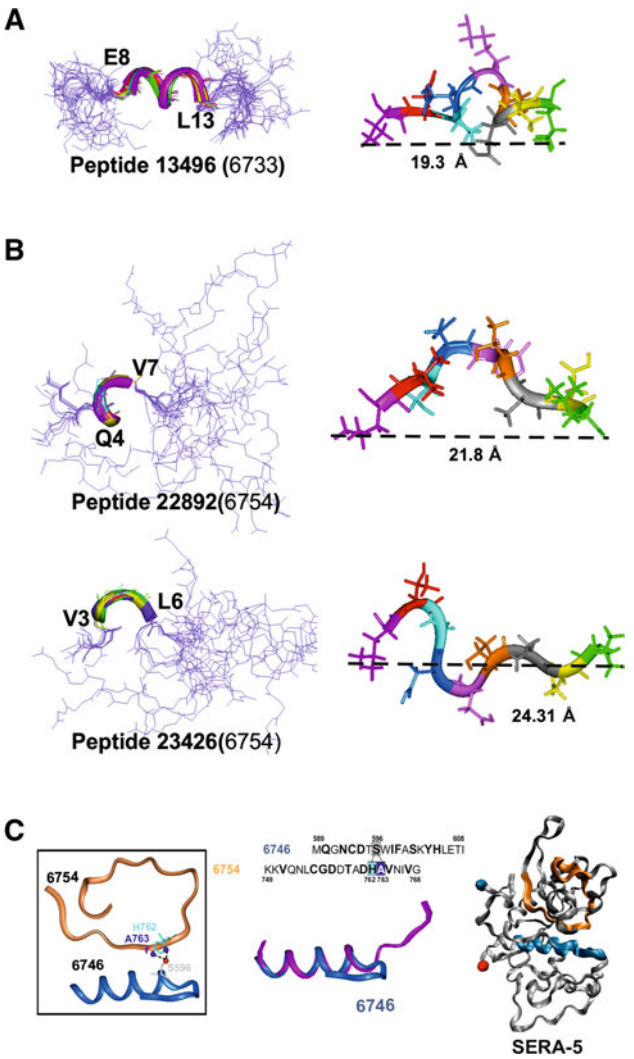

Fig. 3 Backbone and ribbon representation of SERA protein HABP solution structures. **a** and **b** *Left-hand* Front view of overlapping ^{1}H-NMR-derived structures of **13496** (6733), **22892** (6754) and **23426** (6754). *Right-hand* Front view of same structure based on the HLA-DRβ1*0401 allele binding reading register (Rammensee et al. 1995). The amino acid-colour code is based on HLA-DRβ1* binding activities, binding motifs and binding registers, as follows: pocket 1, *fuchsia*; P2, *red*; P3, *turquoise*; pocket 4, *dark blue*; P5, *rose*; pocket 6, *light brown*; P7, *grey*; P8, *yellow*; pocket 9, *green*. The distances between the farthest atoms of residues fitting inside pockets 1 and 9 were measured in Angstroms (Å). **c** *Right hand*, SERA-5 recombinant fragment 3D structure in *yellow* (PDB code 3CH2) and localisation of HABPs 6746 (*blue*) and 6754 (*orange*). In the middle, superimposition of HABP 6746 (*blue*) on the corresponding SERA-5 recombinant sequence. *Boxed*, two H-bonds from the catalytic triad present in this recombinant fragment and, in the middle, the amino acid sequences of 6746 and 6754 displaying the H-bonds established among them to form this protein's non-canonical catalytic triad

with those obtained in CD studies, thus complementing previous results and making them more robust.

Circular dichroism studies revealed that monomer and polymer structural characteristics remained unchanged, suggesting that the polymer form inoculated into *Aotus* monkeys simulated the same structure as that for the monomer form. Furthermore, unmodified native peptides did not present any type of special conformation whilst modified ones having some biological activity did so (even though having short structures, i.e. short α-helices and β-turns), thus clearly emphasising that suggest modifications must be made to conserved HABPs to render them immunogenic and protection-inducing.

Molecular modelling

Studies of **23426** and **22892** binding to HLA-DRβ1* purified molecules have shown strong **23426** binding to human HLA-DRβ1*0401 molecules (Table 1). Studies by Suarez et al. (2006) and Patarroyo et al. (2010b) have shown that the HLA-DRβ1*0403-like allele had higher frequency in the *Aotus* monkey population being studied, since immunisation and protection studies with these peptides were performed with a sequence from the *Aotus* HLA-DRβ1*0403 chain when using this non-human primate model (Suarez et al. 2006; Patarroyo et al. 2010a). It was thus decided to carry out molecular modelling analysis, making the corresponding β-chain replacements (residues highlighted in black in the turquoise ribbon shown in Fig. 4) and amino acid replacements for each peptide (designated initially in the methodology) in HLA-DRβ1*0401 3D structure. All these changes were carried out within the PDB code 1J8H template.

These studies have shown that the complex formed by the modified HLA-DRβ1*0403-like molecule and modified **23426** (6754) complex were stabilised by the spontaneous formation of 12 H-bonds (including the 11 canonical ones) (Dessen et al. 1997), compared with the six H-bonds found for peptide **22892** (data not shown), which includes five out six canonical H-bonds.

Figure 4a shows the spontaneously formed H-bonds (very small silver balls). Interatomic distances, determined in Angstroms, were measured between Hε22 from Glnα9 and O from D9 (2.40 Å); O Serα53 and HN from L6 (1.87 Å); Hδ22 from Asnα62 and O from D9 (2.14 Å); Oδ1 from Asnα69 and HN from L14 (1.98 Å); OH from Tyrβ30 and HN from A12 (2.20 Å); HH from Tyrβ30 and O from A12 (1.85 Å); HH from Tyrβ60 and O from D13 (1.92 Å); Hε1 from Trpβ61 and O from D13 (2.06 Å); HH11 from Argβ71 and O from D10 (2.34 Å); HH21 from Argβ71 and O from D10 (2.34 Å); Oδ1 from Asnβ82 and HN from T7 (2.04 Å); Hδ22 from Asnβ82 and O from T7 (2.47 Å). Except for Tyrβ30 and Tyrβ60, all H-bonds belonged to the canonical H-bonds forming bridges.

The **23426** lateral chain orientation (obtained from molecular modelling) agreed with the 3D structure obtained by ^{1}H-NMR, highlighting residue orientation of

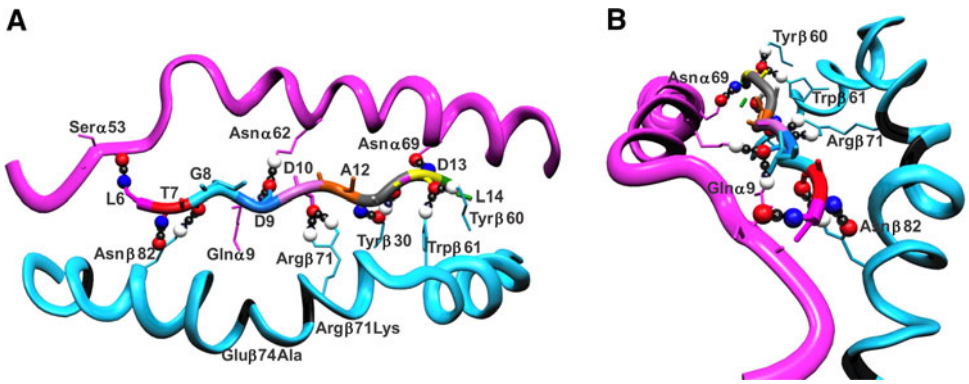

Fig. 4 **23426** interatomic interactions with HLA-DRβ1*0403. **a** Top view, **b** front view (in two directions). The orientation of some **23426** residues' lateral-chain atoms (represented as *sticks* and *balls*) is shown, as well as their positions inside MHCII molecules according to the previously established colour code in Fig. 3. The H-bonds (shown as small *silver balls*) established between **23426** backbone atoms (represented as *sticks*) and MHCII α- and β-chain residue side-chain atoms (depicted as *pink* and *blue ribbons*, respectively). Nitrogen, oxygen and hydrogen atoms have been shown as *blue*, *red* and *white balls*. *Black segments* in the β-chain show the residues that were modified according to HLA-DRβ1*0403 sequences

T7 corresponding to pocket 2 directed towards the TCR molecule (Fig. 4b), similar to that observed for A12 corresponding to pocket 7 as well as the downward orientation of L14 corresponding to the residue fitting into pocket 9. These findings confirmed the possible associations between such 3D structural features and these modified peptides in the immune response generated by this modified peptide in the *Aotus* monkeys in the study.

Atomic and immunological considerations

The best immunogenic and protection-inducing modified HABP was **23426** which bound to HLA-DRβ1*0401 and had a 24.31 Å distance between its furthest atoms, fitting into pockets 1–9 of this molecule and corresponding to residues L6 from pocket 1 and L14 from pocket 9. Non-immunogenic, non-protection-inducing modified HABP **22892** had a 21.8 Å distance between the furthest atoms (2.51 Å shorter), fitting into pockets 1–9; **22892** probably fits into HLA-DRβ1*0401 according to the register reading since no binding studies were performed with this peptide due to limitations regarding reagents. The differences in distances between these peptides showed the relevant role of polarity in the shifting of neighbouring residues, especially those spanning pocket 1–4 (T/V pocket 2, G/S pocket 3 and T/V pocket +2), regarding antibody induction and protection. This shorter distance could have led to a change in the conformation and orientation of the lateral chains pointing towards the TCR or MHC II molecules making this complex unstable for an appropriate immunological stimulation. Figure 3b shows that the lateral chains of residues fitting into pocket 2 and 7 (T7 and A12 respectively) became upwardly orientated in peptide **23426**, possibly providing better orientation for interacting with the TCR molecule in relation to modified HABP **22892** in which pocket 4, 6 and 8 fitting residue side chains were upwardly orientated toward the TCR. This was interesting, as D9 and T11 should have been downwardly orientated in **22892** to fit into MHCII molecules pockets 4 and 6, respectively; these residues' anomalous orientation could partially explain the reduced number of H-bonds (six in total established between this modified peptide **22892** and the HLA-DRβ1*0403 molecule) and the absence of immunogenicity and protective efficacy. This contrasted with **23426**, as the side chains in pockets 2 and 7 were directed upwards towards the TCR (these are critical TCR-contacting residues for this allele in the canonical system). There was also the appropriate downward orientation of D9 and T11 to fit into pockets 4 and 6 of HLA-DRβ1*0403 to allow the formation of 12 H-bonds with this MHCII molecule, thereby establishing a stable complex which could allow appropriate presentation to the TCR to induce protective immunity.

Another striking observation was that native HABPs 6737 and 6762 (Fig. 1a, red arrows) were located 20 ± 2 residues downstream of the SERA 111 kDa precursor molecule cleavage sites where SERA is processed by the *Pf*SUB1 enzyme to release the aforementioned 56/50 kDa fragment. This suggested that these conserved HABPs could be buried in the precursor molecule to be exposed later on by the *Pf*SUB1 enzyme and be relevant during invasive merozoite development and their release from erythrocytes.

On the other hand, it has been suggested for a long time that short peptides are unable to mimic native protein 3D structure, thereby casting some doubts on the minimal subunit-based, synthetic vaccine concept (Schueler-Furman et al. 2001). The 3D structures of our native conserved HABPs obtained by ^{1}H-NMR have been compared with

their corresponding segments to prove whether conserved HABPs display the same structural conformation shown in the original recombinant protein sequence from which their amino acid sequences have been derived. Only a few malarial recombinant proteins have been analysed to date by X-ray crystallography (due to production and crystallisation problems); the 3D structure of a 284 amino acid-long recombinant fragment (residues V544–N828), included in the SERA-5 protein catalytic 50-kDa cleavage product, has been published very recently (Hodder et al. 2009). Our previously described HABP 6746 (Alba et al. 2003), which was located in this fragment, has displayed the same α-helical structure as its corresponding segment in the recombinant fragment; when superimposed onto residues M589–I608 it displayed a 0.95 rmsd (Fig. 3c in dark blue and fuchsia, respectively). This suggested a complete identity, despite the two different methodologies used for their 3D structure determination (i.e. X-ray crystallography for the recombinant molecule and ^{1}H-NMR for our 6746 HABP). Furthermore, HABP 6754 (residues K749–G768, included in this manuscript) has displayed a completely random structure in the recombinant protein, totally agreeing with the structure described by our CD and ^{1}H-NMR studies for this HABP (Fig. 3c in orange).

The SERA-5 recombinant fragment 3D structure has shown that our conserved HABP 6746 (residues M589–I608) established H-bonds between fundamental binding residue S596 and conserved HABP 6754 fundamental binding residues A763 and H762 (Fig. 3c, boxed), the latter being one of the close binding residues modified to render this conserved HABP highly immunogenic and protection inducing. Along with residue N787 (Hodder et al. 2009), the above have formed this molecule's non-canonical serine active catalytic triad which is deeply involved in the processing of merozoite proteins during parasite egress and invasion. Native peptide 6746 residue S596 was one of the fundamental binding residues which was modified to produce highly immunogenic and protection-inducing modified HABPs **24216**, **23230** and **24214** (Alba et al. 2003). This further confirmed our findings that residues establishing H-bonds amongst conserved HABPs in an invasion-relevant molecule are the fundamental residues which must be changed to induce a highly immunogenic and protective immune response against the *P. falciparum* parasite (Patarroyo et al. 2010b).

The pattern of these conformations has implied functionally relevant discontinuous structures bound by H-bonds (6746 and 6754 HABPs); thus, given that these HABPs mediate vital functions for parasite survival, their activities can be blocked by inducing an appropriate immune response against any one of these HABPs, thereby strongly supporting this strategy for a logical and rational approach to vaccine development.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Alba MP, Salazar LM, Puentes A, Pinto M, Torres E, Patarroyo ME (2003) 6746 SERA peptide analogues immunogenicity and protective efficacy against malaria is associated with short alpha helix formation: malaria protection associated with peptides alpha helix shortening. Peptides 24:999–1006

Alba MP, Salazar LM, Purmova J, Vanegas M, Rodriguez R, Patarroyo ME (2004) Induction and displacement of an helix in the 6725 SERA peptide analogue confers protection against *P. falciparum* malaria. Vaccine 22:1281–1289

Bax A, Davis DG (1985) MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. J Magn Resonance 65:355–360

Bermudez A, Reyes C, Guzman F, Vanegas M, Rosas J, Amador R, Rodriguez R, Patarroyo MA, Patarroyo ME (2007) Synthetic vaccine update: applying lessons learned from recent SPf66 malarial vaccine physicochemical, structural and immunological characterization. Vaccine 25:4487–4501

Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC (1984) A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal Biochem 136:175–179

Cifuentes G, Bermúdez A, Rodriguez R, Patarroyo MA, Patarroyo ME (2008) Shifting the polarity of some critical residues in malarial peptides' binding to host cells is a key factor in breaking conserved antigens' code of silence. Med Chem 4:278–292

Cubillos M, Alba MP, Bermudez A, Trujillo M, Patarroyo ME (2003) Plasmodium falciparum SERA protein peptide analogues having short helical regions induce protection against malaria. Biochimie 85:651–657

Dessen A, Lawrence CM, Cupo S, Zaller DM, Wiley DC (1997) X-ray crystal structure of HLA-DR4 (DRA*0101, DRB1*0401) complexed with a peptide from human collagen II. Immunity 7:473–481

Greenfield NJ (1996) Methods to estimate the conformation of proteins and polypeptides from circular dichroism data. Anal Biochem 235:1–10

Hodder AN, Malby RL, Clarke OB, Fairlie WD, Colman PM, Crabb BS, Smith BJ (2009) Structural insights into the protease-like antigen *Plasmodium falciparum* SERA5 and its noncanonical active-site serine. J Mol Biol 392:154–165

Jeener J, Meier B, Backman P, Ernst R (1979) Investigation of enhance processes by two dimensional NMR spectroscopy. J Chem Phys 71:4546

Merrifield RB (1963) Automated synthesis of peptides. Solid-phase peptide synthesis, a simple and rapid synthetic method, has now been automated. J am soc 85:2149

Patarroyo ME, Patarroyo MA (2008) Emerging rules for subunit-based, multiantigenic, multistage chemically synthesized vaccines. Acc Chem Res 41(3):377–386

Patarroyo ME, Alba MP, Vargas LE, Silva Y, Rosas J, Rodriguez R (2005) Peptides inducing short-lived antibody responses against *Plasmodium falciparum* malaria have shorter structures and are

read in a different MHC II functional register. Biochemistry 44:6745–6754

Patarroyo ME, Bermudez A, Salazar LM, Espejo F (2006) High non-protective, long-lasting antibody levels in malaria are associated with haplotype shifting in MHC-peptide-TCR complex formation: a new mechanism for immune evasion. Biochimie 88(7):775–784

Patarroyo MA, Bermudez A, Lopez C, Yepes G, Patarroyo ME (2010a) 3D analysis of the TCR/pMHCII complex formation in monkeys vaccinated with the first peptide inducing sterilizing immunity against human malaria. PLoS One 5:e9771

Patarroyo ME, Cifuentes G, Piraján C, Moreno-Vranich A, Vanegas M (2010b) Atomic evidence that modification of H-bonds established with amino acids critical for host-cell binding induces sterile immunity against malaria. Biochem Biophys Res Commun 394:529–535

Patarroyo ME, Bermudez A, Patarroyo MA (2011) Structural and immunological principles leading to chemically synthesized, multiantigenic, multistage, minimal subunit-based vaccine development. Chem Rev 111:3459–3507

Puentes A, Garcia J, Vera R, Lopez QR, Urquiza M, Vanegas M, Salazar LM, Patarroyo ME (2000) Serine repeat antigen peptides which bind specifically to red blood cells. Parasitol Int 49:105–117

Rammensee HG, Friede T, Stevanoviic S (1995) MHC ligands and peptide motifs: first listing. Immunogenetics 41:178–228

Rance M, Sorensen OW, Bodenhausen G, Wagner G, Ernst RR, Wuthrich K (1983) Improved spectral resolution in cosy 1H NMR spectra of proteins via double quantum filtering. Biochem Biophys Res Commun 117:479–485

Rodriguez R, Moreno A, Guzman F, Calvo M, Patarroyo ME (1990) Studies in owl monkeys leading to the development of a synthetic vaccine against the asexual blood stages of *Plasmodium falciparum*. Am J Trop Med Hyg 43:339–354

Salazar LM, Bermudez A, Patarroyo ME (2008) HLA-DR allele reading register shifting is associated with immunity induced by SERA peptide analogues. Biochem Biophys Res Commun 372:114–120

Sato D, Li J, Mitamura T, Horii T (2005) *Plasmodium falciparum* serine-repeat antigen (SERA) forms a homodimer through disulfide bond. Parasitol Int 54:261–265

Schueler-Furman O, Altuvia Y, Margalit H (2001) Examination of possible structural constraints of MHC-binding peptides by assessment of their native structure within their source proteins. Proteins 45:47–54

Sinigaglia F, Romagnoli P, Guttinger M, Takacs B, Pink JR (1991) Selection of T cell epitopes and vaccine engineering. Methods Enzymol 203:370–386

Suarez CF, Patarroyo ME, Trujillo E, Estupinan M, Baquero JE, Parra C, Rodriguez R (2006) Owl monkey MHC-DRB exon 2 reveals high similarity with several HLA-DRB lineages. Immunogenetics 58:542–558

Vargas LE, Parra CA, Salazar LM, Guzman F, Pinto M, Patarroyo ME (2003) MHC allele-specific binding of a malaria peptide makes it become promiscuous on fitting a glycine residue into pocket 6. Biochem Biophys Res Commun 307:148–156