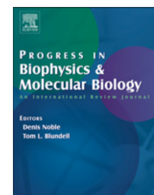
Progress in Biophysics and Molecular Biology 102 (2010) 38–44

Contents lists available at ScienceDirect

Progress in Biophysics and Molecular Biology

journal homepage: www.elsevier.com/locate/pbiomolbio



Review

Atomic fidelity of subunit-based chemically-synthesized antimalarial vaccine components

Manuel E. Patarroyo [a,b,*], Gladys Cifuentes [a,c], Nora L. Martínez [a,c], Manuel A. Patarroyo [a,c]

[a] Fundación Instituto de Inmunología de Colombia (FIDIC), Bogotá D.C., Colombia
[b] Universidad Nacional de Colombia, Ciudad Universitaria, Bogotá D.C., Colombia
[c] Universidad del Rosario, Bogotá D.C., Colombia

A R T I C L E I N F O

Article history:
Available online 2 December 2009

Keywords:
Malaria
Chemically-synthesized vaccines
Plasmodium falciparum
MHC II-peptide-TCR complex
HLA-DRβ1* molecules

A B S T R A C T

The tri-dimensional (3D) structure determined by NMR of functionally relevant High Activity Binding Peptides (HABPs) of chemically-synthesized malarial proteins, involved in invasion to target cells, is practically identical, at the atomic level, to their corresponding recombinantly produced proteins, determined by X-ray crystallography. Both recombinant proteins as well as these chemically-synthesized HABPs bind to host-cell receptors through channels or troughs formation, stabilized by hydrogen bonding; most of them are located on distant segments to the highly polymorphic, highly antigenic, strain specific amino acid sequences the parasite uses to evade immune pressure. When these immunologically silent conserved HABPs are specifically modified, they become highly immunogenic and capable of inducing protective immune responses, supporting the specifically modified minimal subunit-based, multiepitopic, chemically-synthesized vaccines concept.

© 2009 Elsevier Ltd. All rights reserved.

Contents

1. Introduction . . . 38
2. Atomic fidelity of conserved HABPs . . . 39
   2.1. Erythrocyte binding antigen 175 (EBA-175) . . . 39
   2.2. Duffy Binding Like (DBL) . . . 39
   2.3. Apical Merozoite Antigen-1 (AMA-1) . . . 39
   2.4. Merozoite Surface Protein 1 (MSP1) . . . 39
   2.5. Thrombospondin Related Associated Protein (TRAP) . . . 39
3. Immune evasion mechanisms . . . 41
4. Specific modifications to induce immunogenicity and protection . . . 42
5. Conclusion . . . 43
   Acknowledgements . . . 43
   References . . . 43

1. Introduction

We chose malaria caused by the *Plasmodium falciparum* parasite as our model disease for vaccine development due to several reasons: its tremendous impact on public health worldwide, inducing over 300 million annual cases and 3 million deaths (Snow et al., 2005); because the infectious parasite particle to red blood cells (RBCs), named merozoite, can be isolated, quantified with high accuracy and clearly defined regarding its biological and immunological activities; because of the tremendous genetic polymorphism of malarial parasite proteins (as has been shown in several molecular studies), which allows identifying the multiple mechanisms this parasite uses to evade the immune system's pressure and finally, because of the availability of a unique experimental model in the Amazon basin: the *Aotus* monkey, which can be infected with

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

0079-6107/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.pbiomolbio.2009.10.006

malaria parasites and other human microbes exhibiting a similar course of infection and has an immune system almost identical to that of humans, as shown by molecular biology studies.

With this purpose in mind, most of the merozoite proteins involved in RBC invasion (Rodriguez et al., 2008) and sporozoite proteins participating in invasion to hepatic cells (Garcia et al., 2006) were entirely synthesized as 20-amino-acid long fragments in order to identify their HABPs through the use of a highly robust, sensitive and specific methodology (Rodriguez et al., 2008).

2. Atomic fidelity of conserved HABPs

2.1. Erythrocyte binding antigen 175 (EBA-175)

A clear example of surface exposed HABPs is observed in the crystal structure of the recombinant dimer fragment II from the *P. falciparum* Erythrocyte Binding Antigen 175 (EBA-175 RII) described by Tolia et al., (2005). Fig. 1a (Connolly surface), b (ribbon diagram), c (ribbon enlarged detail) show F1 domains (N terminus or head) of molecules 1 and 2 shaded in darker colors (gold and blue) while F2 domains (C terminus or tail) are shaded in lighter colors (yellow and pale blue), both of which contain the amino acid sequences N355-I355 (in pink), corresponding to our HABP 1779 and H436-K455 which corresponds to our HABP 1783, shown in red (Rodriguez et al., 2008; Tolia et al., 2005). These monomers are stabilized by two “β fingers”, where D30 of F1 “β finger” forms a salt bridge with R446 of the F2 cavity and vice versa. EBA-175 RII dimerizes head–tail through H bonds established between HN from Val 435 of monomer 1 with O from Asn 433 of monomer 2 and vice versa, via two antiparallel β sheets of identical F2 residues (↓N433 to H436 and ↑N433 to H436) (Fig. 1c), which leads to the formation of a channel having a ~15 Å diameter in its narrowest section (Fig. 1a; asterisks) through which the proteic portion of the erythrocyte's Glycophorin A has been suggested to go through (Tolia et al., 2005). R446E/D mutations on this fragment containing HABP 1783, completely abolishes binding of RBCs to COS cells expressing the mutated genes. Similarly, Neu5Acl of Glycan 1 (shown as a green Connolly structure) forms H bonds with K439 and D442 of HABP 1783 that mediate and stabilize binding of this protein to the glycosidic portion of Glycophorin A, being RBC binding abolished when the K439A mutant is expressed by COS cells.

HABP 1779 (residues N355-I375, in pink) (Tolia et al., 2005) displayed on the lateral portion of the channel lies very close to binding sites of glycans 5 and 6, but is not involved directly in erythrocyte binding (Tolia et al., 2005) (Fig. 1a–c) in spite of being the most surface exposed HABP in this protein.

2.2. Duffy Binding Like (DBL)

In the structure of the Duffy Binding Like (DBL) protein of *Plasmodium knowlesi*/*Plasmodium vivax* described by Singh et al., (2006), HABPs 1629 (residues K89-G107 in yellow) and 1639 (residues L277-R296 in purple) (Ocampo et al., 2002) are located within sub-domain 2. This sub-domain conforms a trough (Fig. 1d, arrow) stabilized by an H bond between HZ2 from Lys 100 (HABP 1629) and Oε1 from Glu 280 (HABP 1639) and to which the 1–60 N-terminal sequence of the Duffy Antigen Receptor for Chemokines (DARC) binds (Hadley and Peiper, 1997). Both the fully conserved HABP 1639 and the semi conserved 1629 containing the critical binding residues Y94, N95, K96, F98, I102 and R103 (Fig. 1e, red) that interact with the DARC sulfated Y41 (Hadley and Peiper, 1997) are exposed on the surface of this channel.

2.3. Apical Merozoite Antigen-1 (AMA-1)

Another example of surface exposed HABPs can be seen in the 3D structure of recombinant *P. falciparum* Apical Merozoite Antigen-1 (AMA-1) published by Bai et al., (2005), which includes domains I (Fig. 1g–i; fuchsia) and II (gray). The model shows the structural proximity of conserved HABP 4313 (residues D134-G153 in yellow) located in domain I with HABP 4325 (residues M374-H393 in red) localized in domain II, (Bai et al., 2005) and the trough (Fig. 1g, arrow) formed by the H bonds established between HH21 from Arg 143 with O from Tyr390; Oδ2 from Asp 134 with HZ2 from Lys 391 and Hδ2 from Asp 134 with NZ from Lys 391, inside which a still not characterized RBC receptor is suggested to bind. Eight out of the nine conserved residues (V169, F183, M190, M224, Y251, I252, L357 and F367) forming a recently described hydrophobic trough (Fig. 1g, h; green) to which PfRON4, a protein of the parasite's mobility machinery considered a promising vaccine candidate, binds; shows that these are discontinuous sequences (Bai et al., 2005).

2.4. Merozoite Surface Protein 1 (MSP1)

In the recombinant the *P. falciparum* Merozoite Surface Protein 1 (MSP1) 19 kDa-protein fragment described by Morgan et al., (1999), which is the only protein fragment remaining anchored to merozoite membrane through a glycosylphosphatidylinositol (GPI) tail and entering recently invaded RBCs, where it mediates formation of the Parasitophorous Vacuole (PV) inside which the parasite will reproduce (Dluzewski et al., 2008); the random structure of conserved HABP 5501 (residues N1 to S16 (Fig. 1j–l, brown) is in complete agreement with our NMR studies) and shows that it is located in domain I (gray) towards the N terminus, while domain II containing two Epidermal Growth Factor (EGF)-like domains (in green) is towards the C terminus (Morgan et al., 1999).

2.5. Thrombospondin Related Associated Protein (TRAP)

In the *P. falciparum* sporozoite (the hepatocyte infectious particule) Thrombospondin Related Associated Protein (TRAP) structure described by Tossavainen (Tossavainen et al., 2006) (Fig. 1m–o) and containing our HABP 3287/3289 (Garcia et al., 2006) (residues A3 to K26, in gold), positively charged residues (Fig. 1n, red) form a 20 Å-long trough which makes contact with 3 subunits of negatively charged heparin sulfate proteoglycans (HSPGs) present on hepatocyte membrane. Fig. 1o shows the ten H bonds formed between amino acids W (green), R (pink) and S (red) allowing the staggering and establishment of the 3 antiparallel β-sheets A, B, (where our HABP 3287/89 is located) and C in this recombinant protein (Tossavainen et al., 2006).

It can be observed in Fig. 2a that the EBA-175 RII 436–455 amino acid fragment (red) superimposes very well with the 3D structure of our conserved HABP 1783 (blue) determined by ^{1}H NMR studies (0.89 Å rmsd) (Cifuentes et al., 2003; Tolia et al., 2005). The structure of HABP 1779 (insoluble for NMR structure) displays in EBA-175 (Fig. 2b, pink) a distorted α helix, which is in complete agreement with our circular dichroism data (Rodriguez et al., 2008). Fig. 2c shows the *P. vivax*/*P. knowlesi* DBL 3D structure (in yellow) superimposed with that of our expanded 1629 peptide (in gray) determined by ^{1}H NMR (Garcia et al., 2006; Singh et al., 2006), which as shown in this manuscript have a rmsd 0.93. In Fig. 2d, the only short α-helical structure in the AMA-1 recombinant fragment 3D structure located between residue 384 to 387 (red) and completely containing our conserved HABP 4325 (green) superimposes with that of our conserved HABP between residues 13–16 with a 0.99 rmsd

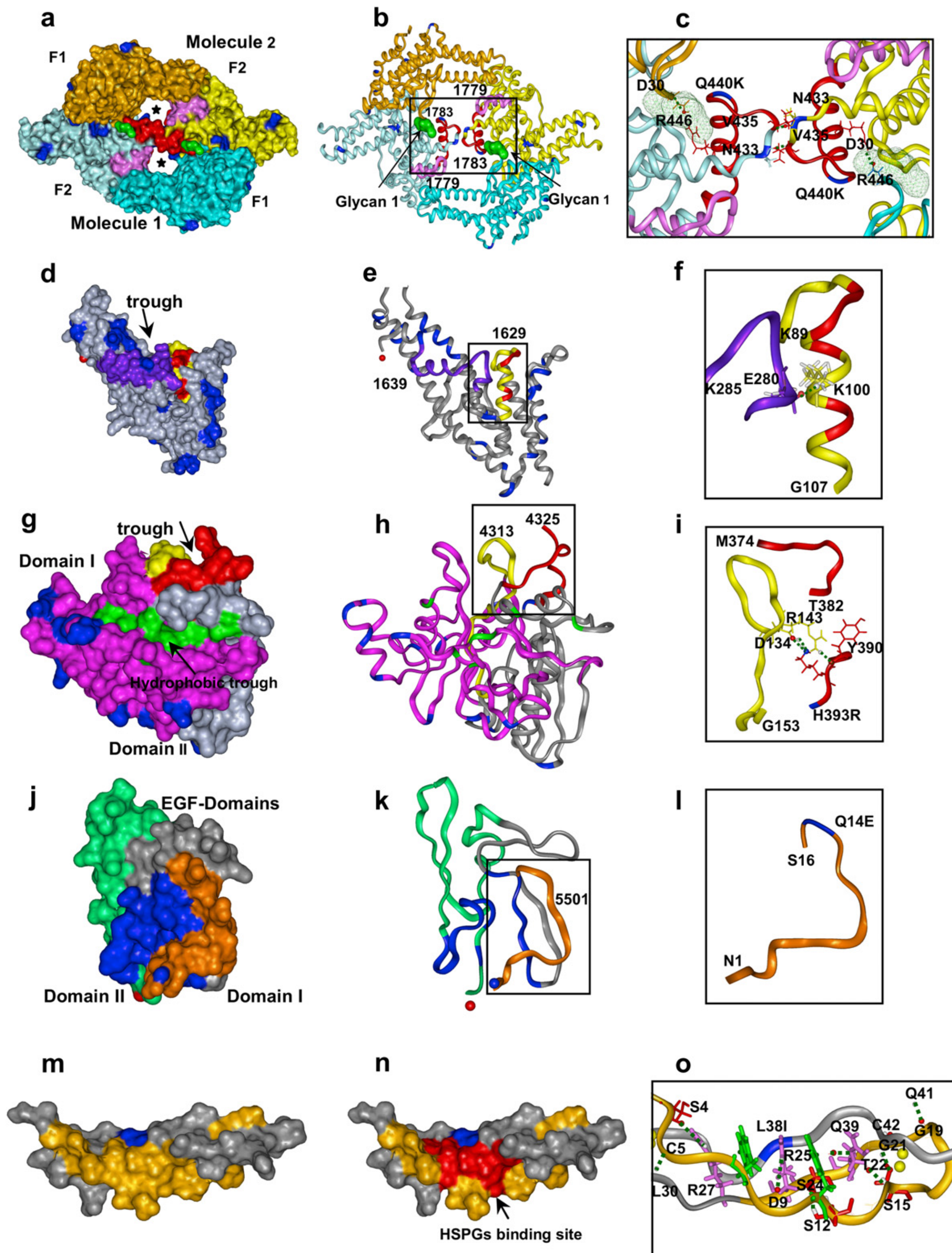


Fig. 1. X-ray crystallography 3D structures displaying the localization of the different HABPs (see text) and the channels (asterisks) or troughs (black arrows) formed by them shown as Connolly surfaces (a, d, g, j, m, and n), ribbon (b, e, h, k and n) and as enlarged structural details (c, f, i, l and o, boxed) highlighting the H bonds formed between the different HABPs, a–c. The dimeric *P. falciparum* merozoite EBA-175 RII (Tolia et al., 2005). (PDB accession 1ZRO). d–f. The P. *vivax* merozoite DBL fragment (Singh et al., 2006). (PDB accession 2C6J), g–i. AMA-1 protein domains I (fuchsia) and II (white) (Bai et al., 2005). (PDB accession 1Z40) The hydrophobic trough is in green. j–l. The recombinant *P. falciparum* merozoite MSP-1 protein 19 kDa fragment (Morgan et al., 1999). (PDB accession 1cei, r1cejmr). m-o. 3D structure of *P. falciparum* TRAP sporozoite protein determined by ^{1}H NMR (Tossavainen et al., 2006). (PDB accession 2BBX). Amino acids displaying polymorphic or blocking antibody activity induction are shown in dark blue.

(Bai et al., 2005; Cubillos et al., 2002). The 3D structure of the recombinant MSP1-19 kDa fragment containing our HABP 5501 (brown) shows that this segment is totally random, as confirmed by our NMR data (Fig. 2e) (Morgan et al., 1999; Torres et al., 2003). The same holds true for liver-stage proteins (Fig. 2f), where the ^{1}H NMR resolved 3D structure of the recombinant TRAP sporozoite protein is compared to the one of our hepatocyte binding HABP 3287/89. Pink residues G19 to T22 superimpose

with residues G8 to T11 (blue) of our HABP 3287/89 with an rmsd of 1.5 (Patarroyo et al., 2008; Tossavainen et al., 2006).

Being these the ONLY structural data on *P. falciparum* or *P. knowlesi/P. vivax* vaccine candidates published to date they confirm that the structures of short conserved HABPs (20-mer long) of EBA-175, DBL, AMA-1, TRAP and MSP1 (α helical, β turns or random) are identical at the atomic level to that of their recombinant counterparts (regardless the methodology used for their 3D structure determination). The other two described molecules are the same fragment (DBL3x) of the *P. falciparum* erythrocyte membrane protein (PfEMP, VAR2CSA) which contains high polymorphic DBL domains, binding chondroitin sulfate A (CSA) via coordination with K1324, R1467 and K1504 very distant residues located in different sub-domains (Higgins, 2008; Singh et al., 2008), therefore they were not included. We also found that these conserved HABPs form channels or troughs via H bonding between them (Fig. 1) regardless of their position in the protein's amino acid sequence. Some of these HABPs are also located towards the protein's N terminus in sequences of high atomic segmental mobility (Morgan et al., 1999). Furthermore, binding of these minimal subunits (HABPs) to the different receptor molecules exposed on the surface of target cells (RBCs and hepatocytes) as shown by cross-linking assays and *in vitro* invasion inhibition assays, makes them functionally relevant minimal subunits in host cell invasion processes (Garcia et al., 2006; Rodriguez et al., 2008).

Therefore, an immune response directed against these HABPs can destabilize intermolecular interactions, block dimerization or channel formation, such as occurs in EBA-175; impede trough formation such as in DBL, AMA-1 and TRAP; or entry to RBCs and PV formation such as in MSP1-19 kDa, thereby blocking binding of these molecules to their corresponding receptors and therefore impeding parasite invasion to RBCs, makes these HABPs the first functionally described epitopes in *P. falciparum* malaria.

3. Immune evasion mechanisms

Interestingly, we found that most of these conserved HABPs are located outside highly polymorphic amino acid sequences triggering strain-specific immunity or outside regions inducing antibodies capable to impede or block protective immunity (all displayed here as dark blue colored amino acids). This can be clearly seen in EBA-175 RII where most of the genetic variants are located in residues far away from HABPs (Fig. 1a, b) with few exceptions like the N433K (outside 1783) and the Q440K variation located in the N terminus of HABP 1783 (Tolia et al., 2005) (Fig. 1c), suggesting that the immune system is trying to impede stabilization, dimerization and channel formation in EBA-175 by blocking this embedded HABP. This was the reason why long before we decided to delete the 4N-terminal residues from analogue peptides used in our immunization studies (Cifuentes et al., 2003). Meanwhile 1779 is well exposed on the surface and has no genetic variations, thus making this HABP (once properly modified) an excellent protective immunity-inducing antigen to be included in a multiepitopic, minimal subunit-based, chemically-synthesized antimalarial vaccine (Fig. 1a–c).

It has been elegantly shown that polymorphic residues in this *Pk/Pv* DBL fragment are located distantly or opposite to DARC binding sites (Hadley and Peiper, 1997; Singh et al., 2006; Tsuboi et al., 1994) (Fig. 1d–f), suggesting that these conserved DARC recognition sites are expressed only "just on time" during invasion so that only polymorphic regions are exposed to the immune system's attack, as suggested by Singh (Singh et al., 2006).

Another example of this frequently used genetic polymorphism evasion mechanism comes from 355 AMA to 1 sequences where >64 polymorphic sites with 27–45 haplotypes have been found

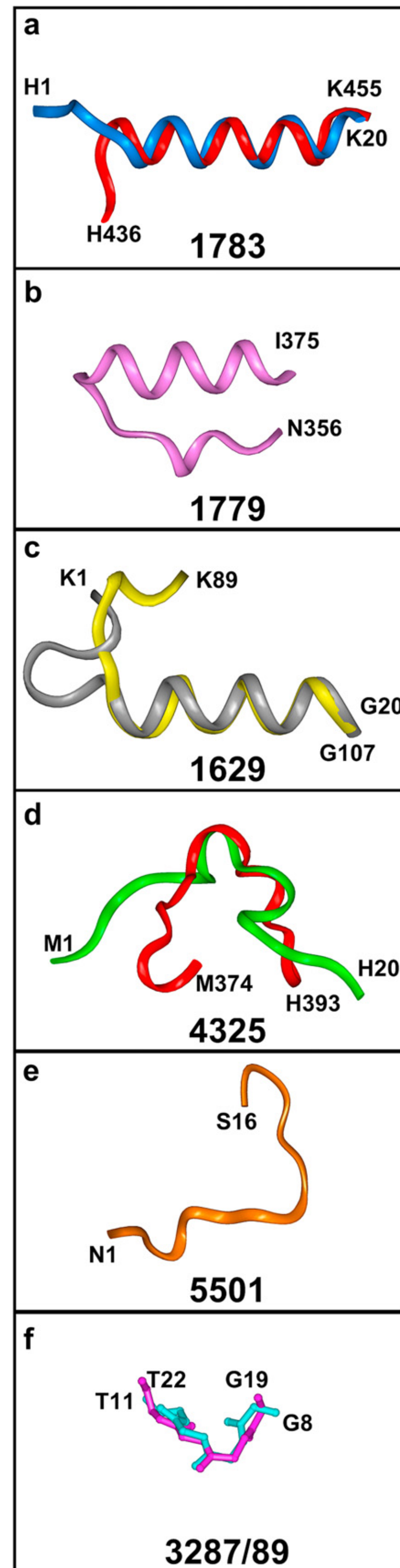


Fig. 2. Structural comparison of recombinant proteins. a. EBA-175, b. EBA-175, c. Pk/Pv DBL, d. AMA-1, e. MSP1-19 kDa fragment and f. TRAP with their corresponding superimposed a. HABPs 1783, b. 1779 (insoluble for NMR studies), c. 1629, d. 4325, e. 5501 (random) and f. 3287/89, respectively.

(Polley et al., 2003), suggesting to others the existence of a "polymorphic face" (Dutta et al., 2007; Remarque et al., 2008) where most of the Antigenic Escape Residues (AER) (Fig. 1g, dark blue residues) within a cluster of at least 13 polymorphic amino acids are in domain 1 and a second cluster of AERs is located in domain 2, all of them present in opposite or distant places from 4313 to 4325 conserved HABPs (Dutta et al., 2007); therefore, both HABPs are excellent candidates to be included in a minimal subunit-based, multigenetic antimalarial vaccine once they are properly modified.

Another evasion mechanism, elegantly analyzed by Holder and his group when with the MSP-1 19 kDa fragment (Morgan et al., 2005; Uthaipibull et al., 2001) showed that sera from individuals with multiple episodes of malaria as well as most monoclonal antibodies directed against this fragment, blocking the protective immunity induced by antibodies able to inhibit the invasion of the *P. falciparum* parasite, were directed against amino acid sequences located in the EGF-like domain 1 (Fig. 1j–l, dark blue residues) on the opposite face of the molecule to that where conserved HABP 5501 is located, or alternatively against conserved amino acid sequences of EGF-like domain 2 (Morgan et al., 1999, 2005; Uthaipibull et al., 2001). HABP 5501 only displays the Q14E variation on its C terminus, (Fig. 1l), which is why these last 3 residues were deleted in the peptide analogues used in our immunization studies (Torres et al., 2003).

Finally, the TRAP segment where our HABP 3287/89 is located displays only one amino acid variation (L38I) in the C-strand opposite to the A and B β-strands (Fig. 1m–o, dark blue) (Patarroyo et al., 2008).

All these data clearly confirm at the structural level, that parasites are exposing opposite or distant amino acid sequences to those functionally relevant regions (HABPs) as a way to protect themselves by distracting or blocking the action of inhibitory antibodies and thus escape the immune pressure. Another mechanism, not here analyzed, are the variations in the invasion pathways by switching on and off the synthesis of proteins with similar functions, as seen in erythrocyte binding antigens (EBAs) and reticulocyte-binding analogues (PfRhs) (Persson et al., 2008).

Therefore immunization with complete proteins or their recombinant fragments as well as with DNA segments, alone or inserted in different vectors, will have to cope with all these genetically polymorphic regions, segmental sequences inducing blocking antibodies and deal with on and off protein synthesis switching, approaches that have always led to very frustrating results. These are some of the reasons why we consider the use of conserved HABPs to be a most attractive approach to vaccine development.

4. Specific modifications to induce immunogenicity and protection

However, it had been thoroughly shown that conserved HABPs are neither antigenic nor immunogenic (Espejo et al., 2001). To overcome such problem, we carried out extensive studies in *Aotus* monkeys (Bermudez et al., 2003; Cifuentes et al., 2008, 2003; Cubillos et al., 2002; Espejo et al., 2001; Patarroyo et al., 2008, 2004; Patarroyo and Patarroyo, 2008; Purmova et al., 2002; Torres et al., 2003) finding after hundreds of trials with specifically modified HABPs that the immunological properties of these HABPs had to be modified by shifting the polarity of their critical host-cell binding residues, but maintaining their same mass and volume (Cifuentes et al., 2008). Such modifications rendered these conserved HABPs into peptides capable of inducing high-antibody titer against both parasites and their proteins, as assessed by indirect immunofluorescence (IFA) (Table 1) and Western blot. The IFA titers were determined before the first (P0) immunization, and 15 days after the second (II_{15}) and third (III_{15}) immunizations, and correspond to the reciprocal dilution (shown in parenthesis). The prefix corresponds to the number of monkeys developing such antibody titers. What is even more important is that these modified HABPs induced protection in immunized monkeys against experimental challenge with a highly infective *Aotus*-adapted *P. falciparum* strain (Bermudez et al., 2003; Cifuentes et al., 2008, 2003; Cubillos et al., 2002; Patarroyo et al., 2008, 2004; Patarroyo and Patarroyo, 2008; Purmova et al., 2002; Torres et al., 2003), as

Table 1
Conserved HABPs and their corresponding immunogenic, protection-inducing analogues (in bold types) with their corresponding amino acid sequences and modifications (also in bold types), ^{1}H NMR assessed structural features and distances between residues fitting into their respective HLA-DRβ1* alleles' Pocket 1 to 9 (shadowed in gray). Different experiments in *Aotus* monkeys are named a, b and c. ND = not done and Prot = number of fully protected monkeys in the experimental challenge with a highly infective dose of an *Aotus*-adapted *P. falciparum* strain. Ref = references.

	Peptide Number	Peptide sequence (P_1 P_4 P_6 P_9)	Structural features	Distance(Å) P_1 to P_9	HLA-DR bind	P0	IFA titers II_{15}	III_{15}	Prot	Ref.
EBA-175	1783	HRNKKNDKLYRDEWWKVIKK	α-helix N6-K20	16.5	3,11	0	0	0	0/5	12
	22814a	------M Y--**T**---**DVW**	α-helix K3-K11	18.6	3	0	2(5120)	ND	2/10	
	22814b	------M Y--**T**---**DVW**	α-helix K3-K11	18.6	3	0	2(1280)	ND	2/10	
	1779	NIDRIYDKNLLMIKEHILAI	Distorted α-helix (CD)	-	3,11	0	0	0	0/5	29
	22812a	-**N**------**M**-**H**----**M**-----	α-helix N9-H16	18.3	11	0	1(2560)	ND	1/9	
	22812b	-**N**------**M**-**H**----**M**-----	α-helix N9-H16	18.3	11	0	1(320)	ND	1/9	
	22812c	-**N**------**M**-**H**----**M**-----	α-helix N9-H16	18.3	11	0	1(1280)	ND	1/8	
AMA-1	4313	DAEVAGTQYRLPSGKCPVF	Random	-	ND	0	0	0	0/5	30
	10022a	---------**FH**----**S**---**G**	Distorted type III′ β-turn T7-F10	25.0	7	0	0	1(5120)	1/5	
	10022b	---------**FH**----**S**---**G**	Distorted type III′ β-turn T7-F10	25.0	7	0	0	1(1280)	1/8	
	4325	MIKSAFLPTGAFKADRYKSH	β-turn and Short α-helix K13-R16	21.5	3,11	0	0	0	0/5	13
	20034a	---**A**--------**M**--------	α-helix K3-F6 and A11-R16	25.5	4,7	0	2(320)	1(160)	2/8	
	20034b	---**A**--------**M**--------	α-helix K3-F6 and A11-R16	25.5	4,7	0	1(160)	1(160)	1/8	
MSP-1	5501	MLNISQHQCVKKQCPQNSY	Random	-	7	0	0	0	0/5	14
	24148a	-----**ML**-**T**-**MMMT**--**K**	Short α-helix S5-V10	25.1	7	0	2(2560)	ND	2/8	
	24148b	-----**ML**-**T**-**MMMT**--**K**	Short α-helix S5-V10	25.1	7	0	1(640)	ND	1/8	
TRAP	3287/89	SPCSVTYGKGTRSRK	Distorted type III β-turn G8-T11		?	0	0	0	ND	15
	24246	--**T**---**V**---**AF**-**F**-**RE**	Classical type III′ β-turn T6-K9		?	0	5(1280)	2(1280)	ND	

evidenced by the complete absence of parasites in their blood during the 15 days the experiment lasted, whereas controls developed ≥5% parasitemias 6–8 days after being infected, hence requiring immediate treatment. No challenges were performed with TRAP peptides due to the lack of a reliable *Anopheles* mosquito-adapted *P. falciparum* strain for infecting *Aotus* monkeys.

The ^{1}H NMR analysis of these HABPs as well as of their analogues (written in bold types hereafter) showed that modifications performed on HABPs induce relevant structural modifications (Table 1 and Fig. 3), such as the α-helix displacement observed in 1783 compared to its modified HABP **22814** (Cifuentes et al., 2003), reorientation of residues as occurs with HABP 1779 when compared with its **22812** analogue (Bermudez et al., 2003), the acquisition of a partially structured configuration as in the random-structure 4313 HABP which acquires a distorted type III' β turn in its modified **10022** analogue (located between T7 and F10) (Purmova et al., 2002) or in the random structured 5501 peptide whose immunogenic protection-inducing analogue **24148** displays a short α-helical structure (S5 to V10) (Torres et al., 2003). These modifications can also displace the peptide's structural configuration as observed in 4325 (β turn and short α helix from K13 to R16) which changes into a more ordered structure in **20034** (Cubillos et al., 2002) acquiring an α-helical configuration between K3 to F6 and A11 to R16, or as in 3287/89 which changes from a distorted type III β turn (G8 to T11) to a classical type III β turn (T6 to K9) as in **24246** (Table 1) (Patarroyo et al., 2008).

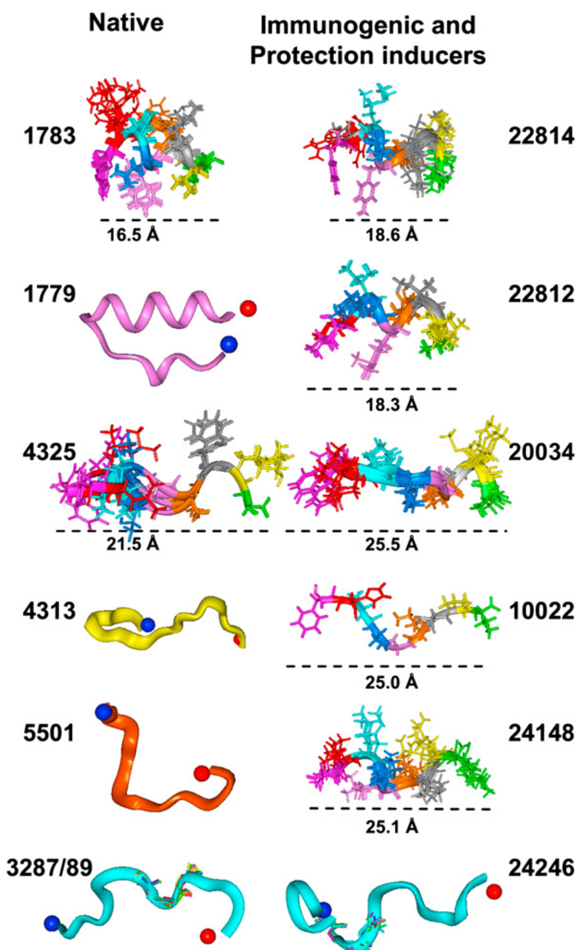


Fig. 3. Structural changes of conserved HABPs and their modified, protection-inducing immunogenic analogues. Native HABPs 4313 and 5501 display random structures and DO NOT bind to any of the HLA molecules studied here but their immunogenic protection-inducing analogues **10022** and **24148** bind to HLA-DRβ1*0701. Sporozoite HABPs 3287/89 and their highly immunogenic modified **24216** peptide DO NOT bind to any of the HLA molecules studied here, therefore, their binding motifs and reading registers could not be assigned. Color code: Pocket 1 (fuchsia), P2 (red), P3 (clear blue), Pocket 4 (dark blue), P5 (blue), P5 (pink), Pocket 6 (brown), P7 (gray), P8 (yellow) and Pocket 9 (green).

We have previously shown that an increase in the distances, together with the abovementioned structural modifications resulting in changes in the residues' orientation (Fig. 3), enable these modified HABPs' residues to fit better into the major histocompatibility (MHC) class II (HLA-DRβ1*) molecules' Pockets to enable a more appropriate presentation of these modified HABPs to the T-cell receptor (TCR) by forming a stable MHC II-peptide-TCR complex and hence activate the host's immune system (Bermudez et al., 2003; Cifuentes et al., 2008; Patarroyo et al., 2004; Patarroyo and Patarroyo, 2008; Purmova et al., 2002).

As can be seen in Fig. 3, residues fitting into their corresponding HLA-DRβ1* molecules' Pockets (based on binding to purified HLA-DRβ1* molecules, binding motifs and binding registers) are downwardly orientated, while residues making contact with the TCR (mostly corresponding to the modified ones) are upwardly orientated, highlighting the importance of an appropriate orientation in these residues for inducing TCR-mediated activation of a protective immune response (Table 1).

5. Conclusion

Altogether, these structural data show that chemically-synthesized conserved HABPs are identical to their recombinant counterparts when analyzed at the atomic level; that they mediate receptor–ligand interactions by forming part of channels or troughs stabilized in these recombinant molecules by H bonds; that these conserved HABPs are located on distant or opposite sequences to the highly polymorphic regions towards which the strain-specific or blocking antibody activity is directed as a mechanism to escape the immune pressure. Furthermore being these conserved HABPs immunologically silent they must undergo specific structural modifications in order for them to fit better into the MHC II-peptide-TCR complex and elicit a highly immunogenic and protection-inducing immune response. These results give strong support to the concept of functionally relevant, minimal subunit-based (HABPs) surface-exposed, multi-antigenic, multi-stage, chemically-synthesized vaccines as a logical and rational approach to vaccine development, one of them being malaria.

Acknowledgements

This research has been supported by Colciencias contract 528-2008 special thanks to Martha Castañeda and Aurora Cortés for their patient collaboration in the writing of this manuscript.

References

Bai, T., Becker, M., Gupta, A., Strike, P., Murphy, V.J., Anders, R.F., Batchelor, A.H., 2005. Structure of AMA1 from *Plasmodium falciparum* reveals a clustering of polymorphisms that surround a conserved hydrophobic pocket. Proc. Natl. Acad. Sci. U. S. A. 102, 12736–12741.
Bermudez, A., Cifuentes, G., Guzman, F., Salazar, L.M., Patarroyo, M.E., 2003. Immunogenicity and protectivity of *Plasmodium falciparum* EBA-175 peptide and its analog is associated with alpha-helical region shortening and displacement. Biol. Chem. 384, 1443–1450.
Cifuentes, G., Bermudez, A., Rodriguez, R., Patarroyo, M.A., Patarroyo, M.E., 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.
Cifuentes, G., Guzman, F., Alba, M.P., Salazar, L.M., Patarroyo, M.E., 2003. Analysis of a *Plasmodium falciparum* EBA-175 peptide with high binding capacity to erythrocytes and their analogues using 1H NMR. J. Struct. Biol. 141, 115–121.

Cubillos, M., Salazar, L.M., Torres, L., Patarroyo, M.E., 2002. Protection against experimental *P falciparum* malaria is associated with short AMA-1 peptide analogue alpha-helical structures. Biochimie 84, 1181–1188.

Dluzewski, A.R., Ling, I.T., Hopkins, J.M., Grainger, M., Margos, G., Mitchell, G.H., Holder, A.A., Bannister, L.H., 2008. Formation of the food vacuole in *Plasmodium falciparum*: a potential role for the 19 kDa fragment of merozoite surface protein 1 (MSP1(19)). PLoS ONE 3, e3085.

Dutta, S., Lee, S.Y., Batchelor, A.H., Lanar, D.E., 2007. Structural basis of antigenic escape of a malaria vaccine candidate. Proc. Natl. Acad. Sci. U. S. A. 104, 12488–12493.

Espejo, F., Cubillos, M., Salazar, L.M., Guzman, F., Urquiza, M., Ocampo, M., Silva, Y., Rodriguez, R., Lioy, E., Patarroyo, M.E., 2001. Structure, immunogenicity, and protectivity relationship for the 1585 malarial peptide and its substitution analogues. Angew. Chem. Int. Ed. Engl. 40, 4654–4657.

Garcia, J.E., Puentes, A., Patarroyo, M.E., 2006. Developmental biology of sporozoite-host interactions in *Plasmodium falciparum* malaria: implications for vaccine design. Clin. Microbiol. Rev. 19, 686–707.

Hadley, T.J., Peiper, S.C., 1997. From malaria to chemokine receptor: the emerging physiologic role of the duffy blood group antigen. Blood 89, 3077–3091.

Higgins, M.K., 2008. The structure of a chondroitin sulfate-binding domain important in placental malaria. J. Biol. Chem. 283, 21842–21846.

Morgan, W.D., Birdsall, B., Frenkiel, T.A., Gradwell, M.G., Burghaus, P.A., Syed, S.E., Uthaipibull, C., Holder, A.A., Feeney, J., 1999. Solution structure of an EGF module pair from the *Plasmodium falciparum* merozoite surface protein 1. J. Mol. Biol. 289, 113–122.

Morgan, W.D., Frenkiel, T.A., Lock, M.J., Grainger, M., Holder, A.A., 2005. Precise epitope mapping of malaria parasite inhibitory antibodies by TROSY NMR cross-saturation. Biochemistry 44, 518–523.

Ocampo, M., Vera, R., Eduardo Rodriguez, L., Curtidor, H., Urquiza, M., Suarez, J., Garcia, J., Puentes, A., Lopez, R., Trujillo, M., Torres, E., Patarroyo, M.E., 2002. *Plasmodium vivax* Duffy binding protein peptides specifically bind to reticulocytes. Peptides 23, 13–22.

Patarroyo, M.E., Cifuentes, G., Rodriguez, R., 2008. Structural characterisation of sporozoite components for a multistage, multi-epitope, anti-malarial vaccine. Int. J. Biochem. Cell Biol. 40, 543–557.

Patarroyo, M.E., Cifuentes, G., Vargas, L.E., Rosas, J., 2004. Structural modifications enable conserved peptides to fit into MHC molecules thus inducing protection against malaria. Chembiochem 5, 1588–1593.

Patarroyo, M.E., Patarroyo, M.A., 2008. Emerging rules for subunit-based, multiantigenic, multistage chemically synthesized vaccines. Acc. Chem. Res. 41, 377–386.

Persson, K.E., McCallum, F.J., Reiling, L., Lister, N.A., Stubbs, J., Cowman, A.F., Marsh, K., Beeson, J.G., 2008. Variation in use of erythrocyte invasion pathways by *Plasmodium falciparum* mediates evasion of human inhibitory antibodies. J. Clin. Invest. 118, 342–351.

Polley, S.D., Chokejindachai, W., Conway, D.J., 2003. Allele frequency-based analyses robustly map sequence sites under balancing selection in a malaria vaccine candidate antigen. Genetics 165, 555–561.

Purmova, J., Salazar, L.M., Espejo, F., Torres, M.H., Cubillos, M., Torres, E., Lopez, Y., Rodriguez, R., Patarroyo, M.E., 2002. NMR structure of *Plasmodium falciparum* malaria peptide correlates with protective immunity. Biochim. Biophys. Acta 1571, 27–33.

Remarque, E.J., Faber, B.W., Kocken, C.H., Thomas, A.W., 2008. Apical membrane antigen 1: a malaria vaccine candidate in review. Trends Parasitol. 24, 74–84.

Rodriguez, L.E., Curtidor, H., Urquiza, M., Cifuentes, G., Reyes, C., Patarroyo, M.E., 2008. Intimate molecular interactions of *P. falciparum* merozoite proteins involved in invasion of red blood cells and their implications for vaccine design. Chem. Rev. 108, 3656–3705.

Singh, K., Gittis, A.G., Nguyen, P., Gowda, D.C., Miller, L.H., Garboczi, D.N., 2008. Structure of the DBL3x domain of pregnancy-associated malaria protein VAR2-CSA complexed with chondroitin sulfate A. Nat. Struct. Mol. Biol. 15, 932–938.

Singh, S.K., Hora, R., Belrhali, H., Chitnis, C.E., Sharma, A., 2006. Structural basis for Duffy recognition by the malaria parasite Duffy-binding-like domain. Nature 439, 741–744.

Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y., Hay, S.I., 2005. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 434, 214–217.

Tolia, N.H., Enemark, E.J., Sim, B.K., Joshua-Tor, L., 2005. Structural basis for the EBA-175 erythrocyte invasion pathway of the malaria parasite *Plasmodium falciparum*. Cell 122, 183–193.

Torres, M.H., Salazar, L.M., Vanegas, M., Guzman, F., Rodriguez, R., Silva, Y., Rosas, J., Patarroyo, M.E., 2003. Modified merozoite surface protein-1 peptides with short alpha helical regions are associated with inducing protection against malaria. Eur. J. Biochem. 270, 3946–3952.

Tossavainen, H., Pihlajamaa, T., Huttunen, T.K., Raulo, E., Rauvala, H., Permi, P., Kilpelainen, I., 2006. The layered fold of the TSR domain of *P. falciparum* TRAP contains a heparin binding site. Protein Sci. 15, 1760–1768.

Tsuboi, T., Kappe, S.H., al-Yaman, F., Prickett, M.D., Alpers, M., Adams, J.H., 1994. Natural variation within the principal adhesion domain of the *Plasmodium vivax* duffy binding protein. Infect. Immun. 62, 5581–5586.

Uthaipibull, C., Aufiero, B., Syed, S.E., Hansen, B., Guevara Patino, J.A., Angov, E., Ling, I.T., Fegeding, K., Morgan, W.D., Ockenhouse, C., Birdsall, B., Feeney, J., Lyon, J.A., Holder, A.A., 2001. Inhibitory and blocking monoclonal antibody epitopes on merozoite surface protein 1 of the malaria parasite *Plasmodium falciparum*. J. Mol. Biol. 307, 1381–1394.