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Research paper

Identification of peptides with high red blood cell and hepatocyte binding activity in the *Plasmodium falciparum* multi-stage invasion proteins: *Pf*SPATR and MCP-1

Hernando Curtidor [a,b], Jeison García [a], Magnolia Vanegas [a,b], Fabian Puentes [a], Martha Forero [a], Manuel Elkin Patarroyo [a,c,*]

[a] *Fundación Instituto de Inmunología de Colombia—FIDIC, Bogotá, Colombia*
[b] *Universidad del Rosario, Bogotá, Colombia*
[c] *Universidad Nacional de Colombia, Bogotá, Colombia*

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Abstract

Plasmodium falciparum multi-stage proteins are involved in vital processes for parasite survival, which turns them into attractive targets for studies aimed at developing a fully effective antimalarial vaccine. MCP-1 and *Pf*SPATR are both found in sporozoite and merozoite forms, and have been associated respectively with invasion of hepatocytes and red blood cells (RBCs). Binding assays with synthetic peptides derived from these two important proteins have enabled identifying those sequences binding with high specific activity (named High activity binding peptides—HABPs) to hepatoma-derived HepG2 cells and human RBCs. Twelve RBC HABPs were identified within the MCP-1 amino acid sequence, most of them in the C-terminal region. The MCP-1 HABPs 33387 and 33397 also presented high activity binding to HepG2 cells. *Pf*SPATR presented four RBC HABPs and two HepG2 HABPs, but only one (32686) could bind to both cell types. RBC binding assays evidenced that binding of all HABPs was saturable and differentially affected by the enzymatic treatment of target cells. Moreover, all HABPs inhibited *in vitro* invasion of merozoites at 200 μM and had particular structural features when analyzed by circular dichroism. The results suggest that these synthetic peptides capable of binding to the two *P. falciparum* target cells could be potentially included in the design of a multi-stage, subunit-based, chemically synthesized antimalarial vaccine.

Keywords: *Plasmodium falciparum*; Multi-stage proteins; MCP-1; *Pf*SPATR; High activity binding peptides

1. Introduction

Every year more than 1 million people die of malaria, children under the age of 5 being the most vulnerable population. Among species causing malaria in humans, *Plasmodium falciparum* stands out as the most lethal one as it accounts for 500 million annual cases worldwide [1]. Control measures to stop the spread of this disease are thus urgently needed; however, they should deal with the complexity of the malaria parasite life cycle, which involves the expression of multiple proteins during each of the distinctive stages [2,3]. It has been observed that an immune response directed against sporozoite antigens could inhibit sporozoite invasion of hepatocytes, while one directed against blood-stage antigens could prevent merozoite invasion of red blood cells (RBCs). Similarly, immunization against gametocyte antigens (parasite sexual forms) could prevent fertilization within the mosquito and therefore prevent transmission of malaria [4–6]. It has thus been proposed that a fully effective antimalarial vaccine must include a yet undefined number of molecules from the different parasite stages (i.e. it must be a multi-epitope and multi-stage vaccine).

* Corresponding author. Fundación Instituto de Inmunología de Colombia, Carrera 50 No. 26-00, Bogotá, Colombia. Tel.: +57 1 481 5219; fax: +57 1 481 5269.
E-mail address: mepatarr@mail.com (M.E. Patarroyo).

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Up to the moment, only a few *P. falciparum* protein antigens have been identified as being expressed during multiple stages of the parasite life cycle [2,7,8], among which it is worth mentioning: *P. falciparum* apical membrane antigen 1 (AMA-1) [9,10], membrane antigen/erythrocyte binding-like (MAEBL) protein [11], secreted protein with altered thrombospondin repeat (*Pf*SPATR) [12] and merozoite capping protein 1 (MCP-1) [13,14]. These multi-stage antigens are highly relevant for inducing a protective immune response against all parasite life cycle stages.

The *P. falciparum* MCP-1 is a ~60 kDa protein that has a cap-like distribution pattern along the merozoite membrane and appears in late schizogony during segmentation and subsequent formation of merozoites [13]. MCP-1 is located at the parasite's apical end and migrates around the merozoite surface as invasion proceeds until finally reaching the posterior pole, suggesting a key role in RBC invasion. There is no variability in the molecular mass of MCP-1 among *P. falciparum* FCR-3, Camp and 7G8 strains [13], suggesting that this molecule is not subjected to molecular processing during merozoite invasion of RBCs.

Three regions define the structure of MCP-1. An N-terminal region containing a 52-amino-acid long domain, highly conserved among a large number of bacterial and eukaryotic proteins. Based on the known function of two of these proteins and its conserved amino acid pattern, an oxidoreductase activity is predicted [15]. The two remaining regions of MCP-1 have no homology with the above proteins and may be associated to an MCP-1 specific function during target cell invasion. The central domain is negatively charged and glutamate-enriched, while the positively charged C-terminal domain is asparagine and lysine-enriched. By virtue of its positive charge, the C-terminal domain shows some similarity to cytoskeleton-associated proteins, and may be mediating *Plasmodium* MCP-1 interaction with cytoskeleton proteins [15].

It has been reported that the UIS16 gene, which encodes the *P. berghei* ortholog of *P. falciparum* MCP-1, is expressed both in salivary gland-sporozoites and blood-stage schizonts [14]. This suggests that MCP-1 could be performing a similar role during merozoite invasion of RBCs and sporozoite invasion of hepatocytes, such as facilitating attachment or movement of the tight junction along the parasite's cytoskeleton network; however, the precise role of MCP-1 in hepatocytes and RBCs invasion is still not clear [13,14,16,17].

The secreted protein with altered thrombospondin repeat (*Pf*SPATR), having an apparent 30 kDa mass, is expressed during sporozoite, merozoite and gametocyte stages [12]. The *Pf*SPATR protein amino acid sequence contains a WSXW motif (where X could be replaced by any amino acid) within an altered thrombospondin Type I repeat (TSR) domain, and a cysteine-rich signature which could represent a type II EGF-like domain [12,18]. The TSR domain has been found in several *P. falciparum* surface proteins, some of which have been involved in ookinete and sporozoite motility, as well as in binding and invasion of host cells, thereby making them excellent antimalarial vaccine candidates [18–22].

*Pf*SPATR and its ortholog in *P. knowlesi* have been located on the surface of sporozoites. This protein binds to hepatoma cells such as HepG2, and antibodies directed against it are capable of inhibiting *P. falciparum* sporozoite invasion of liver cells [12,23]. Seemingly, *Pf*SPATR has been detected around the rhoptries and in a lesser extent in the membrane of infected RBCs during asexual intra-erythrocytic stages. It has been reported that *Pf*SPATR also binds to human RBCs and to an anopheline mosquito larvae pluripotent cell-line (Chattopadhyay et al., unpublished results), suggesting that the same protein expressed during different parasite stages can recognize different receptors present on different host cell types [23].

This work reports the fine mapping of *Pf*SPATR and MCP-1 binding regions to HepG2 cells and human RBCs as well as their role during merozoite *in vitro* invasion of RBCs. We have reported in previous studies a highly robust, specific and sensitive methodology suitable for recognizing high activity binding peptides (HABPs), which can be used as an efficient tool for designing new peptides in the development of a multi-antigenic, multi-stage, subunit-based, chemically synthesized antimalarial vaccine [24–27].

2. Experimental procedures

2.1. Synthetic peptides

Peptides spanning the entire MCP-1 (PF10_0268) [15] and *Pf*SPATR (PFB0570w) amino acid sequence [12] were synthesized using *t*-Boc amino acids and following solid-phase methodology. Peptides were cleaved using low–high HF techniques [28–30], purified by reversed-phase HPLC and characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). The resulting synthetic peptides were 18–20 amino acids long and had a Tyr residue added to the C-terminal end (Fig. 1) whenever they did not contain this residue in their sequence to enable ^{125}I-radiolabeling. In MCP-1, the Cys residue in position 56 (peptide 33382) was replaced by Thr due to problems in its synthesis.

2.2. Radiolabeling

Peptides were radiolabeled according to Urquiza et al. [27,31]. In brief, 2 nmol of purified peptides were incubated with 5 μl Na^{125}I (100 mCi/ml, MP Biomedicals) and 15 μl chloramine-T (2.75 mg/ml) at a final volume of 25 μl for 15 min at room temperature. The reaction was stopped with 15 μl sodium metabisulfite (2.25 mg/ml) [27,32]. The resulting ^{125}I-peptides were purified on a Sephadex G-10 column and had a 50–400 μCi/nmol specific activity.

2.3. Cell culture

Hepatoma derived HepG2 cells [33,34] were kept in RPMI 1640 supplemented with 10% fetal bovine serum (ICN), penicillin (100 IU/ml, ICN), streptomycin (100 μg/ml, ICN), amphotericin B (0.25 μg/ml, ICN), vitamin (ICN) and nonessential amino acid solution (Gibco). Cells were grown as monolayers in 75–150 cm^2

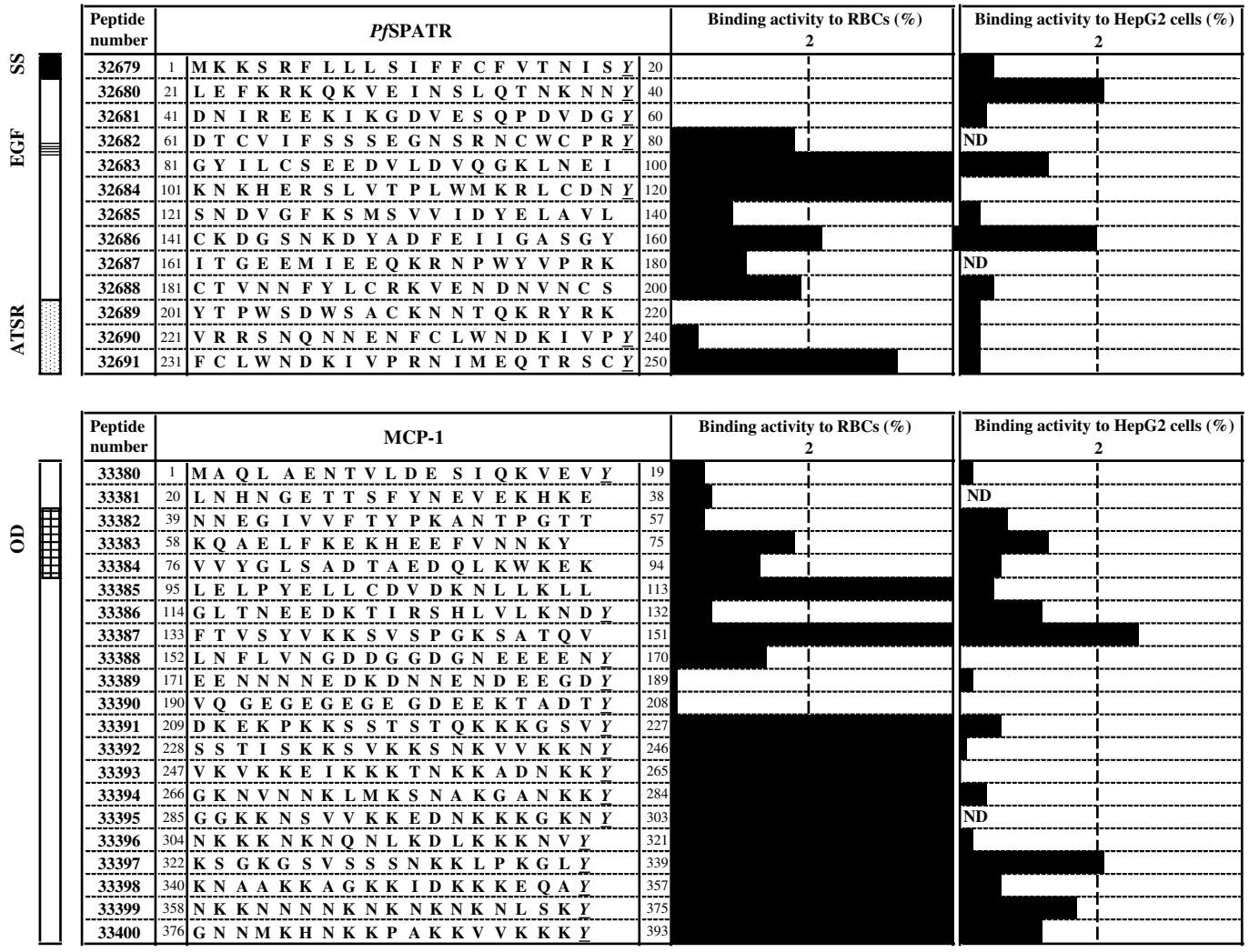

Fig. 1. Binding assays to RBCs and HepG2 cells of peptides covering the complete *Pf*SPATR and MCP-1 sequences. Peptides are numbered according to our institute's serial system. The peptide amino acid sequence and position within each protein are also shown. Black bars represent slope values obtained from the specific RBC or HepG2 cell binding curves of each peptide, while the vertical bar at the left side of the binding profile illustrates each protein's domains. SS, signal sequence; EGF, epidermal growth factor-like domain; ATSR, altered thrombospondin Type I repeat; OD, oxidoreductase domain.

culture flasks coated with a 2% collagen solution (Nunc and Falcon) at 37 °C in a 5% CO_2 atmosphere. The monolayers were removed using a 0.25% trypsin, 0.1% EDTA solution after 4 days had elapsed. The culture was then expanded and kept in the above-described conditions. Cells were harvested by adding PBS–EDTA and centrifuging, washed five times with PBS and counted in a Neubauer chamber before being used in binding assays.

2.4. Cell binding assays

Binding assays to hepatoma cells were performed according to Garcia et al. [35] with a few modifications. In brief, HepG2 cells (1.2×10^6 cells) were incubated with increasing concentrations of ^{125}I-peptide in the presence (140 excess) or absence of unlabeled peptide in a 120 μl final volume for 1 h at 4 °C. The reaction mixture was passed through a 60:40 dioctyl phthalate/dibutyl phthalate cushion (1.015 g/ml density), spun at 15,000 × *g* for 1.5 min and the cell-associated radioactivity was quantified in an automatic gamma counter (Gamma Counter Cobra II).

For RBC binding assays, 2×10^7 cells were incubated with varying concentrations of radiolabeled peptide in the presence or absence of unlabeled peptide to a 200 μl final volume for 1 h at room temperature. Cells were then washed twice with HEPES buffer saline (HBS) and the cell-associated radioactivity was quantified as before. Binding assays were performed by triplicate. Peptide binding activity was defined as the slope of amount (pmol) of peptide specifically bound to RBC per added peptide (pmol) at four logarithmic increasing concentrations. High activity binding peptides (HABPs) were defined as being those peptides showing an activity greater than or equal to 2%, since these peptides recognize more than 200 specific binding sites per cell at low concentrations of radiolabeled peptide [27,32].

2.5. Saturation assays

Modified binding assays were performed to determine binding constants of MCP-1 and *Pf*SPATR HABPs to RBCs; the

following modifications were introduced: for RBC saturation assays 1.5×10^7 cells were used at a 250 µl final volume, radiolabeled peptide concentrations ranged between 0 and 1800 nM and the unlabeled peptide was 24 µM. The curves obtained were analyzed by Hill equation [36,37].

2.6. Cross-linking assays

Some HABPs were cross-linked to RBCs for identifying RBC binding sites. The binding test was performed incubating a final 4% cell concentration with radiolabeled peptide in the presence or absence of 6 nmol unlabeled peptide for 1 h at room temperature. After incubation, cells were washed with HBS and the bound peptide was cross-linked with 50 µl 1 mg/ml bis(sulfosuccinimidyl suberate), BS^3 (Pierce), for 90 min at 4 °C. The reaction was stopped with 20 nM Tris–HCl (pH 7.4) and washed again with HBS. Cells were then treated with lysis buffer (Tris–HCl 5 mM, NaCl 7 mM, EDTA 1 mM, PMSF 0.1 mM). The obtained membrane proteins were solubilized in Laemmli buffer, before being size-separated by 12% SDS–PAGE and stained with Coomassie blue. Proteins cross-linked to radiolabeled peptides were exposed on BioRad Imaging Screen K (Bio-Rad Molecular Imager FX) for 5 days and the apparent molecular weight was determined by using molecular weight standards (Fermentas Life Sciences) [38].

2.7. Enzyme treatment and binding assays

RBCs (60% hematocrite) suspended in HBS buffer were treated with 150 µU/ml neuraminidase (ICN 9001-67-6) at 37 °C for 1 h, washed thrice with HBS buffer, and centrifuged at 1000 × *g* for 5 min. Similarly, RBCs (5%) were treated with trypsin (Sigma T-1005) or chymotrypsin (Sigma C-4129) at a final 1 mg/ml concentration, incubated at 37 °C for 1 h and washed thrice with HBS buffer. After enzyme treatment, these RBCs were tested in a binding assay with HABPs as previously described [32,39]. Binding to non-treated human RBCs was considered as positive control (100%).

2.8. Merozoite invasion inhibition assay

Sorbitol-synchronized *P. falciparum* (FCB-2 strain) cultures were incubated until parasites were at late schizogony (0.8% parasitaemia and 5.0% hematocrit) in RPMI 1640 + 10% O_2 + plasma [40,41]. The culture was then seeded in 96-well cell-culture plates (Nunc, Denmark), in the presence of 50 and 200 µM of the peptides to be tested. Each sample was tested by triplicate. The supernatant was removed after 18 h of incubation at 37 °C in 5% O_2, 5% CO_2 and 90% N_2 atmosphere. Cells were then stained with 15 µg/ml hydroethidine, further incubated at 37 °C for 30 min and finally washed thrice with PBS. The suspensions were analyzed in a FACsort, in Log FL2 data mode, using CellQuest software (Becton Dickinson). Infected RBCs were used as infection controls, while infected RBCs treated with EGTA and chloroquine served as invasion inhibition controls and uninfected RBCs as blank controls [42].

2.9. Circular dichroism analysis

HABP secondary structure folding was studied by circular dichroism (CD). Peptides (5 µM) in 30% v/v TFE aqueous solution were placed in a 1-cm optical pass quartz cell and their spectra were taken by averaging three sweeps at 20 nm/min (with 2 nm bandwidth) in a Jasco J-810 apparatus. Data were processed with Spectra Manager software [43,44].

3. Results

3.1. MCP-1 and PfSPATR peptides specifically bound to HepG2 cells and human RBCs

Binding assays were used for determining the specific RBC and HepG2 binding activity of 21 synthetic peptides covering the total length of the MCP-1 amino acid sequence [15] and 13 synthetic peptides covering the total length of the *Pf*SPATR protein [12].

Twelve RBC HABPs were found among MCP-1-peptides: two of them, the 33385 ^{95}LELPYELLTDVDKNLLKLL^{113} and 33387 ^{133}FTVSYVKKSVSPGKSATQV^{151} peptides, were located in the N-terminal region (Fig. 1). The remaining 10 HABPs were found in the protein's central and C-terminal region which is a lysine- (residues 210–393) and asparagine- (residues 258–393) rich region (Fig. 1). The highly degenerated XKKX motif, where X could be replaced by any amino acid, is repeated 23 times within this region (Fig. 1). Two RBC MCP-1 HABPs bound also with high activity to HepG2; 33387 ^{133}FTVSYVKKSVSPGKSATQV^{151} and 33397 ^{322}KSGKGSVSSSNKKLPKGLY^{339}, both having only one degenerated XKKX motif.

Four RBC HABPs were found among *Pf*SPATR-peptides: 32683 ^{81}GYILCSEEDVLDVQGKLNEI^{100}, 32684 ^{101}KNKHERSLVTPL WMKRLCDN^{120}, 32686 ^{141}CKDGSNKDYADFEIIGASGY^{160} and 32691 ^{231}FCLWNDKIVPRNNIMEQTRSC^{250}. The first three HABPs were localized towards the central region whereas the latter one was located at the protein's C-terminal region (Fig. 1). No HABPs were found within *Pf*SPATR's N-terminal region. Part of the HABP 32683 sequence ^{81}GYILCSEEDVLDVQGKLNEI^{100}, which displayed the highest specific binding, corresponded to a short segment of an EGF-like domain (shown underlined) [12]. The HABP 32691 sequence is totally included within the altered TSR domain (Fig. 1).

Two HepG2 HABPs were found among *Pf*SPATR peptides: 32680 ^{21}LEFKRKQKVEINSLQTNKNN^{40} and 32686 ^{141}CKD GSNKDYADFEIIGASGY^{160}, the first located at the N-terminal region of *Pf*SPATR, immediately after the signal sequence (SS) and the second one in the protein's central region. Interestingly, HABP 32686 bound with high specificity to both cell types.

3.2. HABP binding constants

In order to determine the binding constants of HABP interaction with human RBCs, saturation binding assays were performed for each HABP. Saturation curves and Hill analysis (Fig. 2) allowed calculating dissociation constants (K_d), Hill

coefficients (nH) and the approximate number of binding sites per cell (BSC) [31,32,36,45]. K_d values ranged between 400 and 800 nM, and Hill coefficients between 1.1 and 1.7, suggesting a high affinity HABP–receptor interaction with positive cooperativity. The number of binding sites per cell was found to range between 78,000 and 572,000 (Table 2).

3.3. Enzymatic treatment

The effect of enzymatic treatment on HABP–RBC interaction was determined in binding assays with enzyme-treated human RBCs. Enzymatic treatment affected the binding of each peptide differently (Table 1). *Pf*SPATR HABPs 32683 and 32684 binding was not affected by neuraminidase treatment. Both chymotrypsin and trypsin treatment increased 32683 binding, while HABP 32684 binding decreased by almost 80% when RBCs were treated with these same enzymes. *Pf*SPATR HABP 32686 binding activity only decreased when RBCs were treated with chymotrypsin, suggesting a receptor of proteic nature and a sialic acid-independent binding for this HABP, as has been observed for the "Z" putative receptor [46].

The bindings of MCP-1 HABP 33387 and *Pf*SPATR HABP 32691 were sensitive to all enzymatic treatments (15–70%), suggesting both sialic acid-dependent glycoproteic or proteic receptors on the RBC surface.

It is worth noting that mainly the HABPs located in the Lys- and Asn-rich C-terminal region of MCP-1 were affected by the enzymatic treatment, which also contains 22 copies of the XKKX motif. The binding of HABPs 33393, 33394, 33395, 33396, 33397 and 33400 decreased >61% when RBCs were treated with trypsin, possibly indicating the interaction of these HABPs with receptors of proteic nature, whereas neuraminidase and chymotrypsin treatment increased their binding percentages, possibly because these treatments exposed cryptic epitopes or improved accessibility to specific receptor sites for

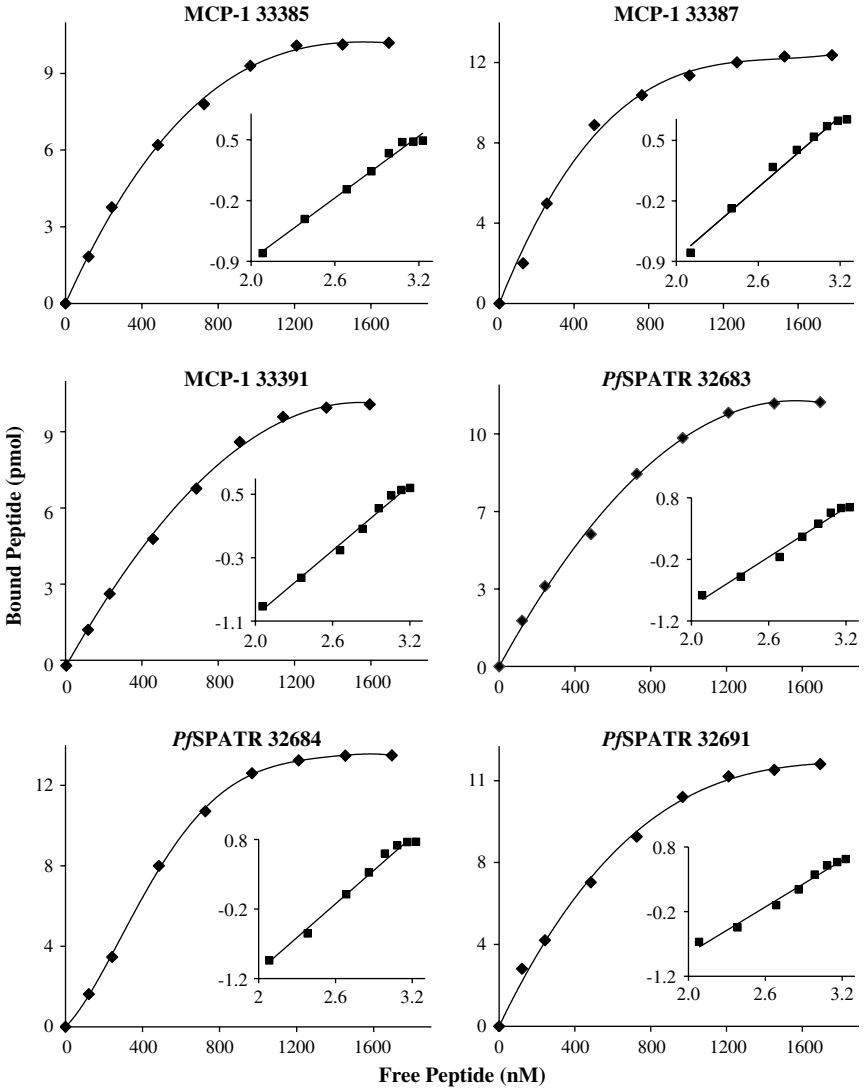

Fig. 2. Saturation curves for MCP-1 HABPs 33385, 33387 and 33391; and the *Pf*SPATR HABPs 32683, 32684 and 32691. Increasing quantities of labeled peptide were added in the presence or absence of unlabeled peptide. Curves represent each peptide's specific binding. In the Hill plot (inset), the abscissa is log F and the ordinate is $\log(B/(B_{max} - B))$, where F stands for the amount of free peptide, B the amount of bound peptide and B_{max} the maximum amount of bound peptide.

Table 1
*Pf*SPATR and MCP-1 HABPs binding to neuraminidase-, chymotrypsin- and trypsin-treated RBCs

	HABP	Neuraminidase	Chymotrypsin	Trypsin
*Pf*SPATR	32683	100	191	142
	32684	102	**33**	**18**
	32686	150	61	130
	32691	76	**31**	**49**
MCP-1	33385	85	**41**	86
	33387	59	**32**	64
	33393	113	143	**46**
	33394	130	147	**48**
	33395	148	135	61
	33396	114	143	56
	33397	128	125	53
	33400	129	120	**23**

Standard deviations were less than 10%. Numbers in bold represent a reduction >50% in HABP binding with respect to control (HABP binding to untreated cells).

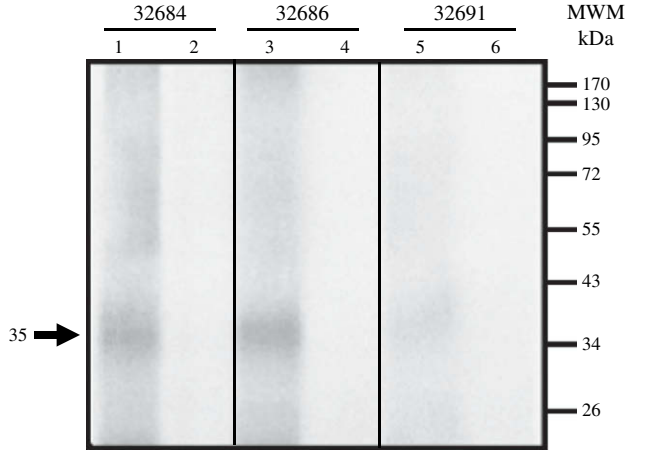

Fig. 3. Cross-linking assays. RBC membrane proteins were cross-linked to radiolabeled *Pf*SPATR HABPs prior SDS–PAGE separation. Lanes 1, 3 and 5 show total binding (i.e., cross-linking in the absence of unlabeled peptide) and lanes 2, 4 and 6 show inhibited binding (i.e., cross-linking in the presence of unlabeled peptide).

each HABP (i.e. neuraminidase removes sialic acid residues on glycoproteins, leaving the proteic backbone exposed).

3.4. Cross-linking assays

The molecular weight of the RBC receptor(s) was determined by cross-linking MCP-1 and *Pf*SPATR HABPs to RBC membranes. *Pf*SPATR HABPs 32684, 32686 and 32691 bound specifically to a RBC membrane protein with an apparent molecular weight of 35 kDa (only recognized slightly by HABP 32691). The interaction of this RBC receptor with radiolabeled peptides was inhibited when binding assays were performed in the presence of unlabeled peptides, thus indicating a specific interaction (Fig. 3). This finding, together with enzymatic treatment results, indicates that HABPs can be recognizing different receptor sites on the same molecule.

3.5. Inhibition of merozoite invasion to human RBCs

The HABPs were added to *in vitro* schizont-stage cultures prior to merozoite's release from infected RBCs, to determine *Pf*SPATR and MCP-1 HABPs' possible role in merozoite invasion. Most of *Pf*SPATR and MCP-1 HABPs inhibited moderately merozoite invasion at 200 μM. The MCP-1 HABP 33385 presented the highest percentage of invasion inhibition (nearly 60%) (Table 3), whereas low activity binding peptides did not inhibit merozoite invasion of RBCs (data not shown).

3.6. CD spectroscopy

Circular dichroism studies provided general information about the HABP secondary structure and folding. CD profiles of *Pf*SPATR HABP 32684 and MCP-1 HABP 33385 in 30% TFE/water clearly indicated an ordered α-helical structure, with a characteristic maximum at 190 and double minima at 208 and 220 (Fig. 4) [43,44]. *Pf*SPATR HABPs 32683, 32686 and 32691 presented little evidence of α-helical features (Fig. 4), while MCP-1 HABPs 33387 and 33397 displayed a random coil-type structure. These results were in total concordance with the secondary structures obtained by self-optimized prediction methods from alignment (SOPMA) [47].

Table 2
Binding constants and number of binding sites per cell for the *Pf*SPATR and MCP-1 HABPs, obtained by saturation assays and Hill analysis

	HABP	K_d (nM)	nH	BSC
*Pf*SPATR	32683	530	1.3	83117
	32684	470	1.6	95163
	32691	520	1.3	91248
MCP-1	33385	600	1.2	83117
	33387	390	1.2	87936
	33391	580	1.4	78299
	33392	490	1.4	240920
	33398	780	1.3	433656
	33400	520	1.2	572185

Table 3
Merozoite invasion inhibition by *Pf*SPATR and MCP-1 HABPs

Protein	HABP	Invasion inhibition (%)	
		100 μM	200 μM
*Pf*SPATR	32683	13 ± 13	30 ± 7
	32684	4 ± 6	28 ± 7
	32686	0 ± 7	34 ± 25
	32691	1 ± 4	16 ± 16
MCP-1	33385	21 ± 3	59 ± 3
	33387	7 ± 1	22 ± 6
	33392	0 ± 6	18 ±
	33398	4 ± 4	21 ± 2
Control	Chloroquine		77 ± 2

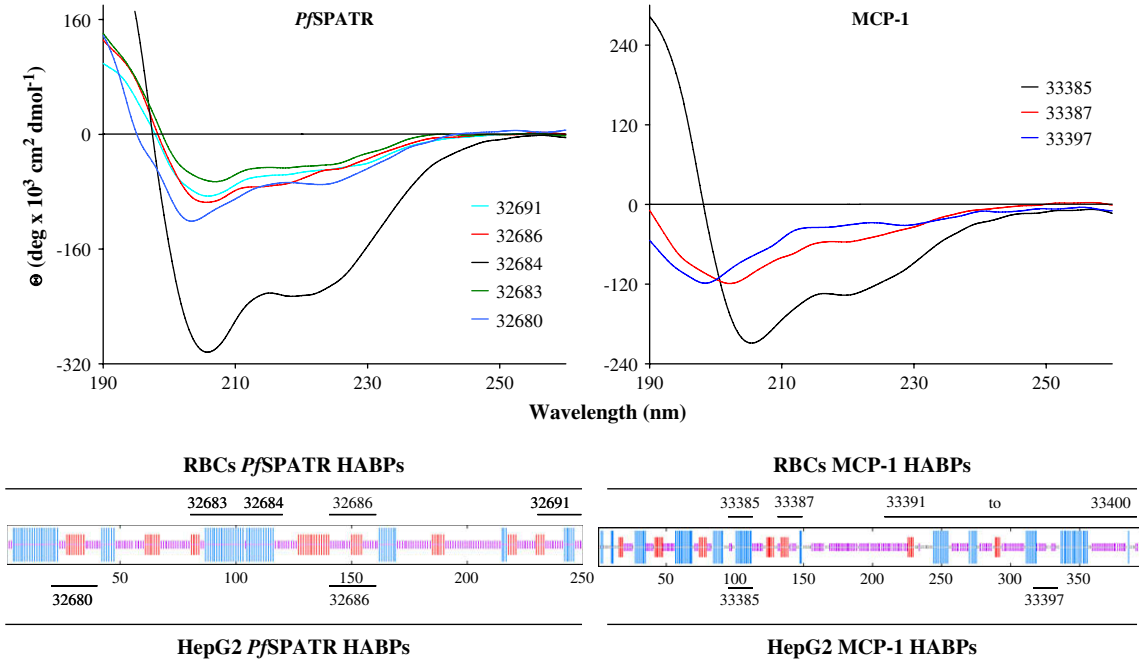

Fig. 4. Circular dichroism spectra of *Pf*SPATR HABPs 32680, 32683, 32684, 32686 and 32691; and the MCP-1 HABPs 33385, 33387 and 33397. The secondary structures predicted by self optimized prediction method from alignment (SOPMA) and relative position of *Pf*SPATR and MCP-1 HABPs are shown below. Blue bars, α-helical; red bars, b-sheet; violet bars, random coil.

4. Discussion

Proteins expressed during the different stages of *P. falciparum* are important for the complete development of the parasite's life cycle and thereby are promising candidates to be included in a multi-antigenic, multi-stage, minimal subunit-based antimalarial vaccine. Our previous works have lead us to develop a solid methodology for identifying high activity binding regions of *P. falciparum* proteins to host cells, which could be rendered into protective-immune response inducers against *P. falciparum* challenge in *Aotus* monkeys once precise modifications are done to their amino acid sequences [24–27]. This work comprises the identification of high activity binding regions derived from two important *P. falciparum* multi-stage proteins, named MCP-1 and *Pf*SPATR.

MCP-1 is a *P. falciparum* merozoite surface protein that has been associated with merozoite invasion to RBCs. A total of 12 RBC HABPs were identified within its sequence, 10 of which were localized at the C-terminal Lys- and Asn-rich region. It has been reported that the MCP-1 protein is located at the tight junction formed between merozoite and RBC, and that it migrates backwards around the merozoite during RBC invasion [13,15], even though a transmembranal domain has not be predicted in this protein. It is important to mention that the B-cell epitope predictor server BcePred (http://www.imtech.res.in/cgibin/bcepred/bcepred.pl), predicts a potential B-cell epitope in the MCP-1 C-terminal region containing HABPs 33391–33400, based on the peptide sequence's hydrophilicity, flexibility/mobility, accessibility, polarity, exposed surface and turns. However, it should be remembered that highly repetitive peptides could act as smoke screens for the immune system, as has been found with many malarial proteins such as apical membrane antigen (AMA-1), circumsporozoite (CS) protein, glutamic-rich protein (GLURP), *P. falciparum* RBC membrane protein (*Pf*EMP-1), among many others [48–51].

Interestingly, MCP-1 peptides 33387 and 33397 presented specific high binding activity to the two *P. falciparum* target cells: RBCs and hepatic cells. On the basis of this evidence, it has been suggested that MCP-1 fulfills similar functions during merozoite invasion of RBCs and sporozoite invasion of hepatocytes [14], a very important feature to consider in the design of a multi-antigenic, multi-stage vaccine [23].

Regarding *Pf*SPATR, four HABPs were found binding to RBCs and two more binding to hepatic cells. *Pf*SPATR RBC HABPs were located towards the middle and C-terminal regions of this protein; being HABPs 32682 and 32691 contained within the adhesive-type EGF-like domain and altered TSR [12,20,22]. In contrast, *Pf*SPATR HepG2 HABP 32680 was located towards the protein's N-terminal region, thus indicating that each host cell type is being recognized by different binding regions of this protein. This is contrary to that found for HABP 32686, which showed high specific activity binding to both target cell types, suggesting the interaction of this HABP with similar receptors on the surface of RBCs and HepG2 cells.

We have evidenced in similar studies carried out with the *P. falciparum* circumsporozoite (CS) [52] and thrombospondin-related anonymous (TRAP) proteins [53], both of them having a thrombospondin repeat domain, that HABPs were located outside the TSR domain (except for a single HABP in TRAP located towards the N-terminal region of this important adhesive domain). Fig. 5 shows the comparative localization of CS, TRAP and *Pf*SPATR HepG2 HABPs, where it can be seen that *Pf*SPATR HABPs are outside of the altered TSR domain. The presence of HABPs outside or inside TSR

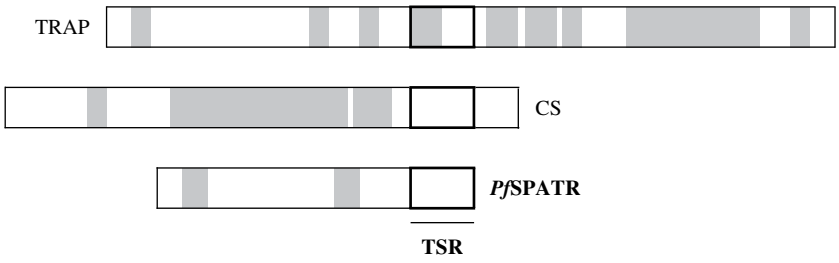

Fig. 5. Schematic representation of CS, TRAP and *Pf*SPATR. HepG2 HABPs are shown in gray and the TSR domain is enclosed inside a black box.

domains suggests the parasite's use of proteins with binding regions similar to TSR or other known adhesive domains to invade host cells.

RBC enzymatic treatment affected differently MCP-1 and *Pf*SPATR HABPs binding, indicating the recognition of different receptors on the RBC surface. Interestingly, MCP-1 HABPs 33393–33400 (located within the Lys- and Asn-rich region) presented a very similar behavior, being sensitive to trypsin treatment and showing an increased binding when RBCs were treated with neuraminidase and chymotrypsin. These results clearly indicate sialic acid-independent RBC surface receptor (or receptors) of common nature for these HABPs, such as the protein backbone of membrane glycoproteins as for instance glycophorins [46]. However, further assays are needed to identify the precise nature of these receptors.

Circular dichroism (CD) analysis provided general information of HABP secondary structure and folding. The resulting CD profiles of *Pf*SPATR HABP 32684 and MCP-1 HABP 33385 clearly indicate an α-helical ordered structure with a characteristic maximum at 190 and a double minima at 208 and 220 (Fig. 4), whereas HABPs 32683, 32686 and 32691 presented little evidence of α-helical content (Fig. 4); and MCP-1 HABPs 33387 and 33397 presented a random coil-type structure. These CD profiles were in complete agreement with the secondary structures predicted by SOPMA [47] (Fig. 4).

Invasion inhibition assays showed that all peptides inhibited moderately merozoite invasion of human RBCs *in vitro* at 200 μM. This behavior indicates a possible role in *Plasmodium* invasion of RBCs for these HABPs, which is further supported by the localization of MCP-1 in the moving junction and previous reports about *Pf*SPATR binding to RBCs and HepG2 cells (Table 3) [12–14].

The results of the present work, identifying high binding activity sequences in MCP-1 and *Pf*SPATR added to the possible role played by these multi-stage proteins in *P. falciparum* invasion of hepatocytes and RBCs, support the inclusion of the HABPs found in these two proteins in future structural modification and immunogenicity studies, aimed at assessing their potential as components of a minimal subunit-based, multi-antigenic, multi-stage, chemically synthesized antimalarial vaccine.

5. Conclusion

We have identified synthetic peptides binding with high specific activity to the two host cell types targeted by malaria parasites: RBCs and hepatocytes, in two *P. falciparum* multi-stage proteins, namely *Pf*SPATR and MCP-1. Our results not only suggest that these important proteins are involved in merozoite and sporozoite invasion processes, but also that some HABPs derived from these two proteins can bind to both host cell types and that such binding results in the inhibition of merozoite *in vitro* invasion in a dose-dependent manner. Further studies should be conducted to assess the potential inclusion of these *Pf*SPATR and MCP-1 HABPs in the design of a subunit-based, multi-stage, chemically synthesized antimalarial vaccine.

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