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Journal of Cellular Biochemistry 110:882–892 (2010)

Journal of Cellular Biochemistry

Conserved Regions From *Plasmodium falciparum* MSP11 Specifically Interact With Host Cells and Have a Potential Role During Merozoite Invasion of Red Blood Cells

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ABSTRACT

Despite significant global efforts, a completely effective vaccine against *Plasmodium falciparum*, the species responsible for the most serious form of malaria, has not been yet obtained. One of the most promising approaches consists in combining chemically synthesized minimal subunits of parasite proteins involved in host cell invasion, which has led to the identification of peptides with high binding activity (named HABPs) to hepatocyte and red blood cell (RBC) surface receptors in a large number of sporozoite and merozoite proteins, respectively. Among these proteins is the merozoite surface protein 11 (MSP11), which shares important structural and immunological features with the antimalarial vaccine candidates MSP1, MSP3, and MSP6. In this study, 20-mer-long synthetic peptides spanning the complete sequence of MSP11 were assessed for their ability to bind specifically to RBCs. Two HABPs with high ability to inhibit invasion of RBCs in vitro were identified (namely HABPs 33595 and 33606). HABP-RBC bindings were characterized by means of saturation assays and Hill analysis, finding cooperative interactions of high affinity for both HABPs (n_H of 1.5 and 1.2, K_d of 800 and 600 nM for HABPs 33595 and 33606, respectively). The nature of the possible RBC receptors for MSP11 HABPs was studied in binding assays to enzyme-treated RBCs and cross-linking assays, finding that both HABPs use mainly a sialic acid-dependent receptor. An analysis of the immunological, structural and polymorphic characteristics of MSP11 HABPs supports including these peptides in further studies with the aim of designing a fully effective protection-inducing vaccine against malaria. J. Cell. Biochem. 110: 882–892, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: MEROZOITE SURFACE PROTEIN 11; H103; *Plasmodium falciparum*; MEROZOITE INVASION OF RED BLOOD CELLS; MOLECULAR INTERACTIONS HOST–PATHOGEN; ANTIMALARIAL VACCINE; SYNTHETIC PEPTIDES

The *Plasmodium falciparum* parasite causes the most serious form of malaria, which is considered as one of the most important infectious diseases worldwide since it annually affects around 250 million people causing the death of 1 million of them [WHO, 2008]. Control of malaria has been significantly hampered by the emergence of drug-resistant parasites and the spread of insecticide-resistant mosquito vectors [Mita et al., 2008; Munhenga et al., 2008]. A potent vaccine has been therefore considered as the most cost-effective measure to help eradicate this dreadful disease.

In particular, our approach to develop a fully effective antimalarial vaccine has consisted in including multiple conserved epitopes of functionally relevant proteins from the different parasite life-cycle stages, especially those involved in invasion of hepatocytes and red blood cells (RBCs) [Patarroyo and Patarroyo, 2008]. With such purpose in mind, we have worked in the identification of amino acid sequences from proteins expressed during the asexual life cycle that have a potential role in parasite invasion of RBCs and therefore could be included as components of a multi-epitopic, multistage, minimal subunit-based, chemically synthesized antimalarial vaccine [Patarroyo and Patarroyo, 2008].

The malaria parasite *P. falciparum* uses different pathways to invade RBCs that are mediated by receptor–ligand molecular interactions between merozoite proteins and RBC surface receptors. A large number of these merozoite invasion proteins have been

Additional Supporting Information may be found in the online version of this article.

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Received 26 November 2009; Accepted 1 March 2010 • DOI 10.1002/jcb.22600 • © 2010 Wiley-Liss, Inc.
Published online 14 April 2010 in Wiley InterScience (www.interscience.wiley.com).

identified in the apical organelles (rhoptries, micronemes, and dense granules) and the merozoite surface, and their roles have been recently reviewed [Rodriguez et al., 2008]. Some of these proteins have been reported to participate in the formation of the moving junction and the parasitophorous vacuole, while micronemes and merozoite surface proteins (MSPs) have been shown to be important for the initial attachment of the merozoite to the RBC and the reorientation of the parasite's apical pole toward the RBC membrane [Rodriguez et al., 2008].

The transcriptome analysis of the *P. falciparum* intraerythrocytic developmental cycle suggests that around 58–90 merozoite proteins might be involved in RBC invasion [Bozdech et al., 2003]. Therefore, a fully effective antimalarial vaccine should contain RBC binding regions of the majority of these proteins in order to block all possible parasite invasion pathways. Bearing in mind this idea, 20-mer-long non-overlapping peptides covering the entire amino acid sequences of a large number of these proteins have been chemically synthesized and screened using a highly sensitive, specific and robust methodology in order to identify high activity binding peptides (HABPs), that is, peptides that have high affinity and specificity for RBC receptors [Rodriguez et al., 2008].

P. falciparum MSPs have been extensively studied as potential vaccine candidates because they are most likely to have a role in RBC invasion processes, are accessible to host's antibodies and have been associated with clinical immunity against malaria [Eisenhut, 2007]. MSP-1, -2, -4, -5, and -10 are attached to parasite membrane via glycosylphosphatidylinositol (GPI) anchors [Gaur et al., 2004; Sanders et al., 2006], whereas MSP-3, -6, -7, and -9 (also known as acid basic repeat antigen (ABRA)) are soluble and weakly bound to merozoite surface possibly via their association with other membrane proteins [Gaur et al., 2004]. HABPs identified in MSPs, for example, MSP1, MSP3, and MSP6, have dissociation constants (K_d) in the nanomolar range as expected for high affinity ligands, and their role in parasite binding to RBCs has been suggested based on their ability to inhibit merozoite invasion of RBCs [Urquiza et al., 1996; Rodriguez et al., 2008].

Additionally, antibodies against MSP3 have been found in sera from naturally infected people and have been shown to inhibit parasite growth in vitro and in vivo. Moreover, anti-MSP3 antibodies have been reported to induce antimalarial protection in monkeys and humans [Oeuvray et al., 1994; Hisaeda et al., 2002; Carvalho et al., 2004]. Similarly, its paralogue MSP6, has been shown to induce production of protective antibodies [Singh et al., 2005]. Subsequent studies identified three MSP3 orthologues containing alanine-rich heptad repeats in *Plasmodium vivax* that are not present in MSP6, therefore prompting the search for other MSP3 paralogues in *P. falciparum*.

As a result, two genes denominated as *H101* and *H103* were found to encode proteins similar to MSP3 in the *P. falciparum* 3D7 strain; however, same as in MSP6, such proteins lack of alanine-rich heptad repeats [Pearce et al., 2005]. Immunofluorescence and immunoblotting studies using anti-H103 antibodies co-localized H103 with MSP4 on the surface of merozoites, and recognized a protein of about 65 kDa in schizont and merozoite lysates, later renamed as MSP11 (PlasmoDB accession number PF10_0352) [Pearce et al., 2005]. Interestingly, gene disruption of MSP11 has no effect on parasite viability and growth, which has lead to suggesting a nonessential role for this protein in the parasite's asexual life cycle. However, the functional relevance of MSP11 was not completely discarded in that study since it is possible that the MSP11 truncated product had retained some functionality and for that reason MSP11 truncation has not resulted in a different invasion phenotype in the transfected line [Pearce et al., 2005].

Additionally, the same study suggests that MSP11 and its paralogues, MSP6 and MSP3, could be utilized in alternative RBC invasion pathways, as has been reported for erythrocyte binding ligands (EBLs), reticulocyte-binding protein homologues (PfRhs) and the MSP protein family in which some proteins have been shown to be dispensable but still functional given that they are redundant proteins for the parasite that can bind different RBC receptors or the same receptor but with different affinities, as a mechanism to have a large repertoire of invasion pathways available [Baum et al., 2005; Pearce et al., 2005; Sanders et al., 2006; Rodriguez et al., 2008]. In the present study, we focused only on MSP11 (H103) and not on H101 because expression of H101 on merozoite surface has not been demonstrated up to the moment, whereas neither the functionality of MSP11 nor its involvement in alternative pathways of RBC invasion have been discarded [Pearce et al., 2005].

MSP11 is a 405-amino-acid long protein with no sites for GPI anchorage or transmembrane domains (Figs. 1 and 2). It contains a signal peptide at its N-terminus, an acidic region that is likely to interact with positively charged molecules and a leucine-zipper-like sequence known to mediate protein–protein interactions. These features support a possible interaction of soluble MSP11 with either RBC receptors during invasion or other merozoite surface proteins to form a co-ligand complex, as has been proposed for MSP3, 6, 7 and 9 [McBride and Heidrich, 1987; Li et al., 2004].

MSP11 shares some sequence similarities with its paralogues MSP3 and MSP6, as for instance the glycine-rich motif gWEfGGGaP (Fig. 1). Particularly in MSP3, this motif lies inside the MSP3b peptide comprising amino acids 211–237, which has been reported to be target of antibodies produced during a natural infection and to inhibit

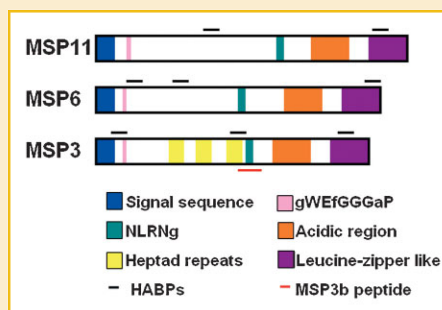

Fig. 1. Schematic representation of MSP11, MSP6, and MSP3. NLRNg: motif with this amino acid sequence. gWEfGGGaP: glycine rich motif. Uppercase letters correspond to amino acids that are conserved in MSP11, MSP6, and MSP3 at the indicated position and lowercase letters represent amino acids that are present only in two of the three proteins at such position. The position of the MSP11 HABPs found in this study as well as of HABPs previously identified in MSP6 and MSP3 [Rodriguez et al., 2005; Lopez et al., 2006] is indicated by the black horizontal bars shown above each protein.

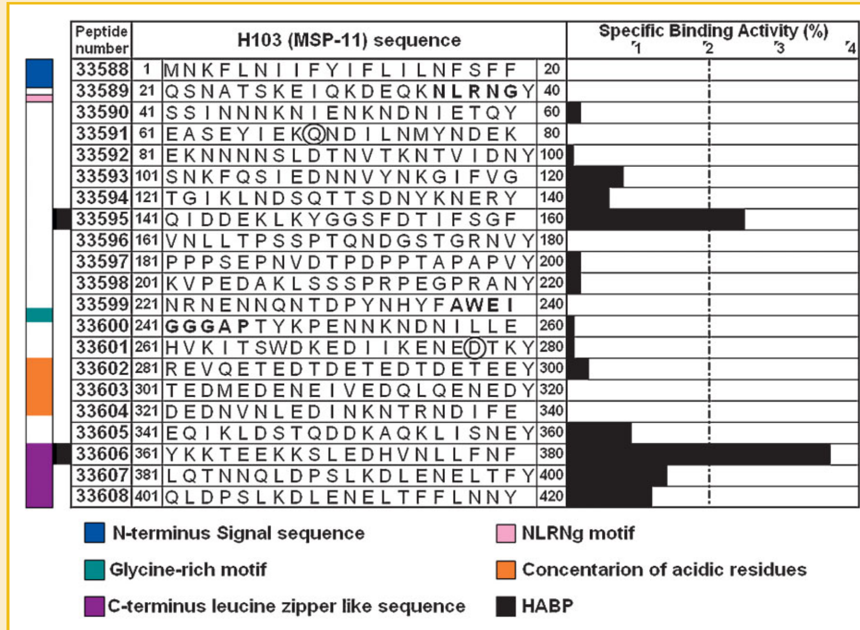

Fig. 2. Screening of MSP11 peptides. The percentage of specific binding activity of each MSP11 peptide is represented by the length of the horizontal black bar shown in front of its corresponding amino acid sequence. Peptides are named according to our institute's serial numbering system. The position of each protein domain in MSP11 is indicated in the vertical bar shown at the left according to the color key shown in the bottom of the image. Bold types correspond to amino acids of the NLRNg and Glycine-rich motifs. The two amino acid positions where synonymous nucleotide substitutions were found by polymorphism studies are encircled.

the development of *P. falciparum* asexual stages through an antibody-dependent cellular inhibition mechanism (ADCI) [Oeuvray et al., 1994]. Interestingly, human antibodies purified using MSP3b peptide have been shown to cross-react with MSP11 and MSP6, but not with H101 in which the glycine-rich motif is less conserved compared to the other two proteins [Pearce et al., 2005]. It has been recently demonstrated that the C-terminal portion of MSP11 (in such study denoted as MSP3.7) is strongly antigenic and that antibodies purified against this portion from hyperimmune sera of patients living in malaria-endemic areas crossreact with MSP3 and four of its paralogues similar as has been reported for anti-MSP3 antibodies [Oeuvray et al., 1994; Pearce et al., 2005; Singh et al., 2009]. This shows that the homology existing between these two proteins might be responsible for such crossreactivity and that MSP11 is target of antibodies produced in response to malaria infection.

This study provides evidence of the involvement of MSP11 in RBC invasion based on the identification of protein regions binding with high specificity to RBCs (namely HABPs 33595 and 33606) that show ability to inhibit merozoite invasion in vitro and appear to bind to glycosylated RBC receptors. The potential inclusion of these two MSP11 HABPs in further studies for the design of an antimalarial vaccine is discussed based on their binding, polymorphism and structure conformational features.

MATERIALS AND METHODS

PEPTIDE SYNTHESIS

Twenty-one 20-mer-long non-overlapping peptides were synthesized based on the MSP11 amino acid sequence reported in the genome of the *P. falciparum* 3D7 strain (PlasmoDB accession number PF10_0352), according to the solid-phase multiple peptide system [Merrifield, 1963], using MBHA resin (0.5 milliequivalents of free NH_2 groups per gram of resin (mEq/g)) and *t*-Boc amino acids (Bachem). Peptides were cleaved using the low-high hydrogen fluoride (HF) technique [Tam et al., 1983] and analyzed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (ToF proteins, Bruker). During synthesis, a Tyrosine (Y) residue was added to the C-terminus of those peptides not containing such residue to enable ^{125}I-radiolabeling. The amino acid sequences of the so synthesized peptides, which were named according to our institute's serial numbering system, are shown in Figure 2.

RADIOLABELING

Peptides were ^{125}I-radiolabeled according to previously described techniques [Yamamura et al., 1978; Hulme, 1993]. Briefly, 7 µl (2.6 mM) of peptide in isotonic 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline (HBS), pH 7.4, reacted for 15 min with 15 µl (2.75 mg/ml) of Chloramine T and 4 µl (4.0 nM) of Na^{125}I (100 mCi/ml specific activity; MB Biomedicals). The reaction was stopped by adding 15 µl of sodium metabisulfite (2.25 mg/ml). Radiolabeled peptides were purified using a Sephadex G-10 column (10 m × 5 mm; Pharmacia) and quantified on a gamma counter (Auto Gamma Counter Cobra II, Packard).

PEPTIDE SCREENING

RBCs obtained from healthy donors were washed several times with HBS and centrifuged at 1,500*g* for 5 min. RBCs (1×10^8) were incubated with increasing amounts of ^{125}I-radiolabeled peptide (0–570 nM) in absence (total binding) or presence

(unspecific binding) of an excess of unlabeled peptide (20 μM) in order to determine specific binding (total binding − unspecific binding). Peptides were assessed in triplicate in a final volume of 200 μl. Unbound peptide was removed after 90 min by washing RBCs twice with isotonic HBS. Binding of radiolabeled peptides to RBCs was quantified in a gamma counter (Auto Gamma Counter Cobra II, Packard). Specific binding activity is defined as the amount (%) of peptide (pmol) that binds specifically to RBCs per added peptide (pmol), and is calculated from the slope of the specific binding curve. Those peptides having a slope greater than or equal to 0.02 in the specific binding curve are defined HABPs. In other words, HABPs have a binding activity equal to or greater than 2%, taking into account that the binding activity is related to the ratio between specifically bound radiolabeled peptide and added radiolabeled peptide. The same criteria have been used in previous studies with several merozoite proteins [Urquiza et al., 1996; Curtidor et al., 2008; Rodriguez et al., 2008]. Additionally, one scrambled-sequence peptide (with sequence: GKDFKQDTFLIDEIGSFGSY) was synthesized based on the sequence of HABP 33595 and screened as described above in order to determine whether peptide binding was associated to the amino acid composition rather than to the amino acid sequence.

HABP SATURATION CURVES

MSP11 HABPs were evaluated in saturation binding assays according to previously described methods [Yamamura et al., 1978; Hulme, 1993]. Briefly, RBCs (7.5×10^7) were incubated with increasing concentrations of ^{125}I-labeled peptide for 90 min in the presence (unspecific binding) or absence (total binding) of 24 μM unlabeled peptide in order to determine the specific binding. After incubation, cells were washed twice with isotonic HBS to remove unbound ligand. Assays were carried out in triplicate. Saturation curves of each HABP were plotted based on the cell-bound radioactivity data acquired on a gamma counter and analyzed by the law of mass action to obtain maximum number of binding sites (B_{max}), dissociation constants (K_d) and Hill coefficients (n_H) [Urquiza et al., 1996; Curtidor et al., 2008].

ENZYMATIC TREATMENT

RBC suspensions (60% hematocrit) in HBS were incubated with ~150 μU/ml neuraminidase (ICN 9001-67-6) or 1 mg/ml of either trypsin (Sigma T-1005) or chymotrypsin (Sigma C-4129) for 60 min at 37°C. Binding of HABPs to enzymatically treated RBCs (1×10^8 cells) was evaluated in triplicate as described in peptide screening [Urquiza et al., 1996; Curtidor et al., 2008]. Binding to untreated RBCs was considered as 100% binding.

CROSS-LINKING ASSAYS

Radiolabeled HABPs were incubated with 5×10^7 RBCs with and without of non-radiolabeled peptide for 1 h at room temperature in order to assess unspecific and total binding, respectively. Bound peptides were cross-linked by incubation with 100 μl of bis(sulfosuccinimidyl) suberate (BS^3, 1 mg/ml) for 1 h at room temperature. Tris–HCl buffer was then added to stop the reaction. RBCs were separated by centrifugation at 1,500*g* for 5 min and then lysed adding 15 μl of lysis buffer (5 mM Tris–HCl, 7 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) and 15 μl of Laemmli buffer. After 1 h of incubation, membranes were separated by centrifugation at 15,000*g* for 15 min and the supernatant (proteic fraction) was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels were exposed to Kodak film (X-OMAT) for 5 days. Apparent molecular weights were estimated by comparing migration distances with the distances of prestained molecular weight markers (New England BioLabs) [Urquiza et al., 1996; Curtidor et al., 2008].

MEROZOITE INVASION INHIBITION ASSAYS

P. falciparum schizonts (FCB-2 strain) obtained from sorbitol-synchronized parasite cultures (5% hematocrit and 5% final parasitemia) were seeded on 96-well cell culture plates. Cultures were incubated with 50, 100 and 200 μM concentrations of unlabeled HABPs in triplicate for 18 h at 37°C under a 5% O_2, 5% CO_2 and 90% N_2 atmosphere. The supernatant was then discarded and RBCs were labeled by incubation with 15 μg/ml hydroethidine for 30 min at 37°C. RBCs were washed and analyzed in a FacsCalibur flow cytometer (FACsort, FL2 channel) equipped with CellQuest software [Wyatt et al., 1991]. Infected and uninfected RBCs treated with ethylene glycol tetraacetic acid and chloroquine were used as positive controls. MSP11 low activity binding peptides 33600, 33601, and 33604 were included in this assay as negative controls.

POLYMORPHISM ANALYSIS ON MSP11 HABPs

Asynchronous cultures of the *P. falciparum* FCB-2 (Colombia), FVO (Vietnam) and PAS-2 (unknown origin) strains maintained according to previously described techniques [Trager and Jenson, 1978] were used as source of genomic DNA (gDNA). In brief, gDNA of each parasite strain was extracted from 200 μl culture samples (30% parasitemia) using 0.2% saponin and purified using the UltraClean DNA Blood Isolation kit (MO BIO, Carlsbad, CA). The region encoding MSP11 HABPs 33595 and 33606 in the three *P. falciparum* strains was amplified by PCR using 2 μl of each strain's gDNA as template and the MSP11-f (5′-GTACTGGGATAAAATTAAATGA-3′) and MSP11-r (5′-AAAGTTAACTCATTTTCTAAATC-3′) primers, which were designed using Gene Runner v3.05 software based on the *P. falciparum* 3D7 genome sequence (PF10_0352). The DIR1 and REV1 primers amplifying the region encoding HABP 33577 of the *P. falciparum* integral membrane protein Pf25-IMP were included as positive PCR control [Curtidor et al., 2008].

PCR amplification was carried out in 50-μl reactions containing 1 U Taq polymerase (Bioline, Taunton, MA), 1× Taq polymerase reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.4 μM of each primer. Thermocycling conditions were as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of 1 min annealing at 56°C for MSP11-f/MSP11-r or 58°C for DIR1/REV1, 1 min extension at 72°C and 1 min denaturing at 95°C; followed by a final 5 min extension at 72°C. Distilled water was used instead of DNA as negative PCR control.

PCR products were separated in 1% agarose gels, visualized by SYBR® safe staining (Invitrogen, Eugene, OR) and purified using Wizard PCR preps kit (Promega, Madison, WI). The purified amplicons were sequenced using MSP11-f and MSP11-r primers

and the so obtained nucleotide sequences were aligned to sequences of the 3D7 (The Netherlands), Dd2 (Indochina), and HB3 (Honduras) reference strains (PF10_0352, PFDG_00167, PFHG_02498, respectively), using Clustal W software [Thompson et al., 1994].

Additionally, sequences of MSP11 HABPs were individually aligned to the HABPs identified in MSP1, MSP4, MSP7, MSP8, and MSP10, as well as to those identified in MSP3 and MSP6 (which are MSP11 paralogues) using Clustal W software (28 HABPs in total) [Thompson et al., 1994].

CIRCULAR DICHROISM (CD) ANALYSIS

The secondary structure elements of MSP11 HABPs were determined by analyzing their CD spectra. CD spectra of 5 μM peptide solutions in 30% (v/v) trifluoroethanol (TFE) were measured in a Jasco J810 CD spectrometer using a nitrogen-flushed 1-cm path-length cuvette. Each spectrum was obtained by averaging three scans taken at room temperature over a wavelength range of 190–260 nM at a scan-rate of 20 nm/min and 1 nm spectral bandwidth (corrected for baseline values). Data were processed using the Spectra Manager software, which is equipped with the SELCON3, CONTINLL and CDSSTR deconvolution programs [Compton and Johnson, 1986; Sreerama et al., 1999]. Additionally, a secondary structure prediction for MSP11was obtained using the self-optimized prediction for MSP11 method from alignment (SOPMA) software [Geourjon and Deleage, 1995].

RESULTS

HIGH ACTIVITY BINDING PEPTIDES OF MSP11

The screening of the MSP11 peptides with binding assays identified two HABPs, namely 33595 and 33606, whose amino acid sequences were ^{141}QIDDEKLKYGGSFDTIFSGF^{160} and ^{361}YKKTEEKKSLEDHVN LLFNF^{380}, respectively (Fig. 2). Both peptides showed binding activities to RBC surface receptors greater than 2%, as indicated by the slope of their specific binding plots (data not shown). As it can be observed in Figure 2, HABP 33595 was located toward the central portion of MSP11 whereas HABP 33606 was located closer to the protein's C-terminus inside a leucine-zipper like sequence. On the contrary, the scrambled-sequence peptide that was synthesized based on the sequence of HABP 33595 showed low binding activity (0.8% specific binding); therefore confirming that the specific binding ability of HABPs depends on the amino acid sequence and not on the amino acid composition, as previously shown with HABPs of other MSPs [Rodriguez et al., 2005].

SATURATION CURVES AND HILL ANALYSIS OF MSP11 HABPs

Binding of HABPs 33595 and 33606 showed a clear tendency toward saturation (Fig. 3). Data analysis indicated that each HABP recognized about 96,400 and 160,600 binding sites per RBC and showed dissociation constants of 800 and 600 nM, respectively. Hill coefficients were greater than 1 in both cases, therefore indicating positive cooperativity for both binding interactions (1.5 for HABP 33595 and 1.2 for HABP 33606).

BINDING TO ENZYME-TREATED RBCs

Binding of MSP11 HABPs to RBCs treated with three different enzymes that are known to cleave RBC surface receptors differentially depending on their chemical nature was assessed and compared to binding of the same HABP to untreated RBCs (considered as 100% binding). Binding of HABP 33595 decreased by 37% upon treatment with neuraminidase and by 95% upon treatment with chymotrypsin (Table I). Binding of HABP 33606 was sensitive to all enzymatic treatments but was most notably affected by neuraminidase and trypsin, both of which reduced binding by more than 50% (Table I).

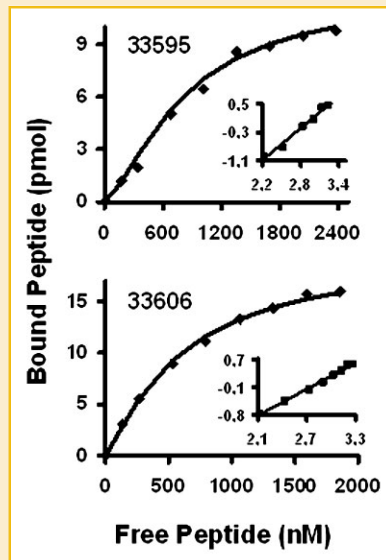

Fig. 3. Saturation and Hill plots for MSP11 HABPs 33595 and 33606. Saturation curves (outer plot) were obtained by incubating increasing amounts of radiolabeled peptides, with and without unlabeled peptide, to obtain the specific binding. In the Hill plot (inner plot), the abscissa corresponds to log F and the ordinate to $\log(B/(B_{max} - B))$, where F is the amount of free peptide, B the amount of bound peptide and Bmax the maximum amount of bound peptide.

MOLECULAR MASSES OF THE RBC RECEPTORS FOR MSP11 HABPs

Crosslinking assays under total and unspecific binding conditions were carried out to determine the apparent molecular weights of the RBC receptors for HABPs 33595 and 33606. As depicted in Figure 4, three bands of approximately 73, 62, and 26 kDa were identified under total binding conditions with HABP 33595 (Fig. 4, lane 1); whereas only a 73 kDa band was recognized when HABP 33606 was assessed (Fig. 4, lane 3). In both cases, these bands were no longer detected under unspecific binding conditions (Fig. 4, lanes 2 and 4).

TABLE I. Percentage of Specific Binding of MSP11 HABPs to Enzyme-Treated RBCs

	Specific binding (%)[a]	
	33595	33606
Control	100	100
Neuraminidase	63	16
Chymotrypsin	5	55
Trypsin	175	22

[a]Standard deviations were below to 6% in all cases.

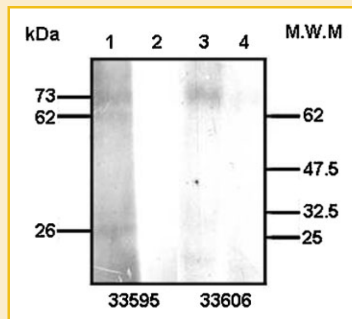

Fig. 4. Autoradiogram of RBC surface proteins cross-linked with MSP11 HABPs 33595 and 33606. Lanes 1 and 3 correspond to assays carried out under total binding conditions, while lanes 2 and 4 correspond to assays carried out under unspecific binding assay conditions. Molecular weight markers (MWM) are shown to the left, while apparent molecular weight of each band are shown to the right of the figure.

SECONDARY STRUCTURE OF HABPs

CD analyses of MSP11 HABPs 33595 and 33606 showed one molar ellipticity maximum at 192 nm and two molar ellipticity minima at 205 and 220 nm (Fig. 5A), respectively, therefore suggesting a predominant α-helical structure with a minor content of other structural features in these peptides, as indicated by the slight variation of their maximum and minima compared to typical α-helical spectra. In addition, deconvolution studies indicated a major percentage of α-helical structure for both HABPs, which ranged between 72.3% and 98.4% in HABP 33595 and between 66.5% and 99.7% in HABP 33066.

The secondary structure obtained based on the amino acid sequence of MSP11 using SOPMA (Fig. 5B) was in agreement with the CD structural features obtained here for MSP11 HABPs because it predicted a major α-helical structure (blue bars) between residues 141 and 160 (corresponding to HABP 33595), and a completely α-helical structure for the region comprising residues 361 and 380 (which corresponds to HABP 33606).

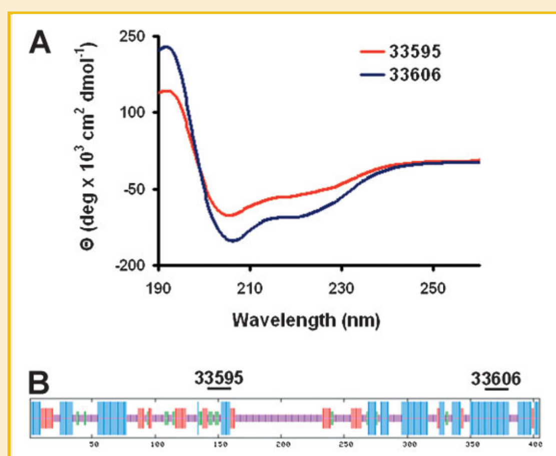

Fig. 5. Secondary structure analysis of MSP11 HABPs. A: Circular dichroism spectra for HABPs 33595 and 33606 showing a clear tendency of α-helical structure in both HABPs. B: SOPMA secondary structure prediction of MSP11. Predicted structures are represented by colored bars according to the following code: blue: helixes, red: sheets, purple: coils, and green: turns. The position of the two MSP11 HABPs is indicated by the horizontal black bars shown above the schematic representation of the secondary structure of MSP11.

INVASION INHIBITION ASSAYS

To determine the involvement of MSP11 HABPs in *P. falciparum* invasion of RBCs, each HABP was incubated with RBC suspensions (5% parasitemia) and the percentage of invasion inhibition was determined by flow cytometry. HABPs 33595 and 33606 inhibited invasion by a large percentage and such percentage of invasion inhibition increased in a concentration dependent-manner (Table II). On the contrary, no significant reduction was observed in merozoite invasion with low activity binding peptides of MSP11 (Table II).

POLYMORPHISM ANALYSIS OF HABPs 33595 AND 33606

The PCR amplification of the region encoding MSP11 HABPs 33595 and 33606 in the FCB-2, FVO and PAS-2 strains yielded a single band of ~841 bp (Fig. 6A). An alignment of the amino acid sequences encoding MSP11 HABPs in the FCB-2, FVO and PAS-2 strains, and with the MSP11 sequences reported for the 3D7, Dd2, and HB3 reference strains showed 100% identity within the studied region and in the rest of the protein (Fig. 6B).

Only two synonymous substitutions were detected within the MSP11 gene; however, both substitutions were located outside the region encoding MSP11 HABPs. The first substitution corresponded to nucleotide position 834 where the cytosine in 3D7, Dd2, and HB3 was substituted by thymine in the FCB-2, FVO and PAS-2 strains in the codon encoding for aspartic acid (Fig. 2, residue 278 in peptide 33601). The second substitution corresponded to nucleotide position 207 where adenine in the 3D7 strain was substituted by a guanine in the Dd2 and HB3 strains in the codon encoding for glutamine (Fig. 2, residue 69 in peptide 33591). However, the presence of this latter substitution in the other strains (FCB-2, FVO and PAS-2) could not be confirmed since only the region encoding MSP11 HABPs was sequenced for these stains. According to this alignment (either from

TABLE II. Percentage of Merozoite Invasion Inhibition by MSP11 Peptides at Different Concentrations

	Invasion inhibition (%)[a]		
Peptide	50 μM	100 μM	200 μM
33595[b]	49	87	94
33606[b]	68	90	95
33600[c]	2	9	45
33601[c]	3	13	48
33604[c]	1	7	41

Controls	0.93 mg/ml	1.85 mg/ml
Chloroquine	90	91
EGTA	ND	68

ND: no data available.
[a]Standard deviations were below to 5% in all cases.
[b]High activity binding peptides.
[c]Low activity binding peptides.

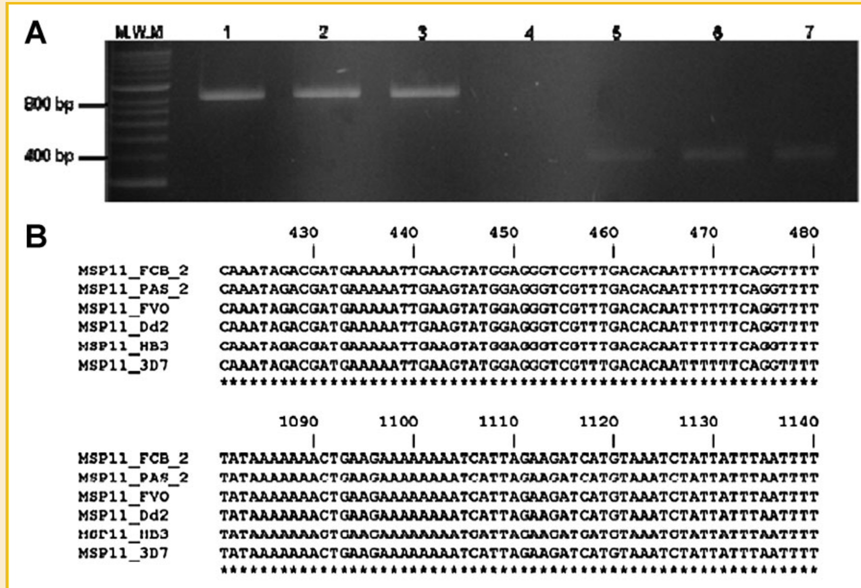

Fig. 6. Polymorphism analysis of the region encoding MSP11 HABPs. A: PCR amplification of the region encoding MSP11 HABPs 33595 and 33606 using gDNA isolated from the FCB-2, FVO and PAS-2 strains (lanes 1, 2, and 3, respectively). Lane 4: negative control (distilled water). Lanes 5, 6, and 7: Positive control (amplification of a Pf25-IMP HABP in the aforementioned strains, respectively). MWM: 100-bp molecular weight marker. B: Clustal W alignment of MSP11 HABPs in FCB-2, PAS-2, FVO, Dd2 HB3 and 3D7 showing the conservation of these HABPs among these P. falciparum strains. Upper panel: HABP 33595 comprising nucleotides 301–480. Lower panel: HABP 33606 comprising nucleotides 1,081–1,140.

the amplified region or from the complete gene), no amino acid substitutions were observed within MSP11.

On the other hand, an alignment of each of the MSP11 HABPs with each of the HABPs identified in MSP 1, 3, 4, 6, 7, 8, and 10 showed identity and similarity values below 50% in all cases, even in the case of the HABPs located inside the leucine zipper-like sequence of MSP3, MSP6 and MSP11. In all alignments, identity values were below 20% except in four cases in which the identity was no larger than 50% (alignments are provided as Supplementary Material).

DISCUSSION

Merozoite surface proteins of *P. falciparum* have been extensively studied as potential vaccine candidates due to their participation as ligands in host–pathogen interactions that take place during merozoite invasion of RBCs. Among these proteins, MSP1, the leading asexual-stage vaccine candidate, is proteolytically cleaved into various fragments that associate between each other and with other MSPs to form a large macromolecular membrane complex [Holder and Freeman, 1984; McBride and Heidrich, 1987]. Several studies have shown that MSP1 is involved in RBC invasion and have demonstrated the ability of this protein to induce protective immunity in monkeys [Espejo et al., 2004]. In fact, the first synthetic antimalarial vaccine, named SPf66, which provided 30–60% protection in large human trials carried out in different parts of the world, contained a semi-conserved sequence, derived from the 83 kDa cleavage fragment of MSP1, which is included inside MSP1 HABP 1513 [Patarroyo et al., 1987; Valero et al., 1993; Alonso et al., 1994; Sempertegui et al., 1994; Urquiza et al., 1996].

With the aim of developing a second generation fully effective subunit-based antimalarial vaccine, MSPs with features similar to those of MSP1 have been attractive research targets to find HABPs that could be used for designing protection-inducing peptides capable of blocking all parasite RBC invasion pathways. In the present study, the complete sequence of MSP11 was chemically synthesized as 20-mer-long peptides, seeking to identify peptides that bind with high activity to RBCs and are able of blocking parasite invasion of RBCs.

As a result, HABPs 33595 and 33606 were identified in MSP11 (Fig. 2). However, it is worth noting that although adjacent peptides to both HABPs (peptides 33593 and 33594 located adjacent to HABP 33595; and peptides 33605, 33607 and 33608 located adjacent to HABP 33606 in Fig. 2) showed no significant binding activity so as to be considered HABPs, their binding activities were larger than the ones of the remaining MSP11 peptides; which could be indicating that MSP11 uses the two regions comprised by these peptides to bind to RBCs and that HABPs 33595 and 33606 are the ones that bind more strongly and specifically to RBCs.

Interestingly, previous studies have shown that disruption of the MSP11 gene has not effect on the parasite's invasion efficiency, which led to suggesting that MSP11 is not essential for parasite invasion of RBCs, although its involvement during RBC invasion as alternative ligand was not completely discarded [Pearce et al., 2005]. Evidence of MSP11 involvement in RBC invasion was gathered in this study by the strong invasion inhibition ability shown by both MSP11 HABPs (Table II), taking into account that parasite invasion

of RBCs was poorly inhibited by low activity binding peptides even at the highest peptide concentration used in the assay (Table II), which strongly suggests that HABPs identified by this method to RBCs are indeed binding with high specificity to RBCs. Therefore, the results of the invasion inhibition assays could be explained by the high RBC binding activity of MSP11 HABPs that prevented binding of native MSP11 to RBCs and even of other parasite proteins employing the same RBC surface receptors as MSP11.

Therefore, it is not possible to rule out the participation of MSP11 in invasion processes, whether it be by acting independently or in coordination with other merozoite proteins as part of the exquisite repertoire of invasion pathways used by *P. falciparum*, which is directed by a hierarchy of molecular interactions involving several parasite proteins to bind to alternative receptors [Duraisingh et al., 2003a; Baum et al., 2005; Triglia et al., 2005]. Indeed, it has been reported that some merozoite proteins that are not essential for parasite survival act as ligands in alternative invasion pathways [Dolan et al., 1990; Baum et al., 2005; Desimone et al., 2009]. For instance, the *P. falciparum* reticulocyte-binding-like homologues 2a and 2b (PfRh2a and PfRh2b) are dispensable but functional in the W2mef strain, which uses a sialic acid-dependent invasion pathway but can also use a sialic acid-independent one [Desimone et al., 2009].

Additionally, it has been reported that members of the invasion-related EBLs and PfRhs protein families use exclusively sialic acid-containing receptors or sialic-acid independent receptors, or even both types of receptors. In addition, no significant changes in the parasite's invasion efficiency have been observed when some of these proteins have been genetically disrupted, thus corroborating functional redundancy among invasion proteins, which allows parasites to use either the same or different invasion pathways or receptors [Duraisingh et al., 2003a; Baum et al., 2005; Triglia et al., 2005]. These evidences suggest that MSP11 and its paralogues (MSP3 and MSP6) could be also acting as ligands in alternative parasite invasion pathways by fulfilling redundant binding functions so that when any of these paralogues or its receptors are absent, defective or not functional, the other proteins compensate for the binding function possibly by shifting the invasion route and therefore allowing for the parasite to retain its invasive ability.

In order to elucidate the invasion pathway used by MSP11 HABPs, we assessed the binding interactions of HABPs to RBCs treated with different enzymes (Table I). According to the results, HABPs 33595 and 33606 appear to be using both sialic acid dependent and independent pathways, since neuraminidase and chymotrypsin treatments reduced binding of both HABPs whereas trypsin reduced binding of HABP 33606 but had no effect on the binding of HABP 33595 (Table I). Thus, the binding susceptibility profile of HABP 33595 indicates that it interacts with glycosylated RBC surface receptors. Glycophorin B and the unknown receptor 'E' of EBA 181 are examples of RBC receptors susceptible to cleavage by neuraminidase and chymotrypsin but resistant to trypsin [Baum et al., 2005]. On the other hand, the susceptibility of the binding of HABP 33606 to all enzymatic treatments indicates that this HABP could bind to glycosylated and non-glycosylated proteins, since a receptor susceptible to all enzymatic treatments has not been reported to date [Baum et al., 2005]. However, glycophorins C and A are known to be susceptible to neuraminidase and trypsin [Baum et al., 2005], the two enzymes that had the larger effect on the binding of HABP 33606 (up to 78% reduction, as shown in Table I).

Cross-linking assays were carried under both total and unspecific binding conditions to gather additional information about the nature of the RBC surface receptors for MSP11 HABPs. According to these assays, HABP 33595 interacts with three RBC membrane proteins (Fig. 4), one of which corresponds to a 26 kDa protein that is only recognized by this peptide and whose apparent molecular weight is close to the one reported for glycophorin B (23 kDa) [Baum et al., 2005]. On the other hand, HABP 33606 appears to be interacting with a glycosylated protein of about 73 kDa that is susceptible to all enzymatic treatments tested in this study. Further studies are however needed in order to completely elucidate the identity of the RBC surface receptors for both MSP11 HABPs.

It is worth noting that same as other HABPs previously identified in MSP3 and MSP6 [Rodriguez et al., 2005; Lopez et al., 2006], MSP11 HABPs are not located inside a particular protein region, except for one HABP that was found inside the leucine zipper-like sequence of each of these three proteins (Fig. 1). These observations suggest the involvement of the leucine zipper-like sequence in molecular interactions between these three MSPs and RBC surface receptors, but probably not in molecular interactions with other merozoite proteins as has been suggested by other authors [Pearce et al., 2005]. On the other hand, such differential distribution of HABPs inside the three proteins give additional support to the notion that these paralogues could be binding to the same or to different RBC surface receptors by using different sequences, as a mechanism for the parasite to use the diverse invasion pathways mentioned in this study.

To further explore this idea, the sequences of each MSP11 HABP were aligned with each of the HABPs identified previously in some MSPs and MSP11 paralogues, MSP3 and MSP6 (Supplementary Material). The results showed small identity and similarity values between MSP11 HABPs and the HABPs derived from other MSPs, which could be indicating that binding of MSPs to RBCs is mediated by different sequences in each protein, even in the case of HABPs that are located inside the leucine zipper like-domain of the paralogues MSP3, MSP6, and MSP11.

Similarly, HABPs have been identified inside the epidermal growth factor (EGF)-like domain of MSP1, MSP4, MSP8, and MSP10; however, the identity between such HABPs is not significantly high. Furthermore, such HABPs have been shown to be distributed along the EGF-like domains, suggesting that the parasite utilizes redundant proteins from the same family that contain minimal structural changes in order to avoid genetic variability in RBC receptors or the host's immune response, and therefore be able to invade RBCs [Urquiza et al., 1996; Duraisingh et al., 2003b; Baum et al., 2005; Cowman and Crabb, 2006; Sanders et al., 2006; Garcia et al., 2007; Reyes et al., 2007]. A similar event has been reported for the Duffy binding-like (DBL) domain of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) variants as well as in the EBLs, in which genetic rearrangements on DBL domains allow binding of these two protein families to RBC surface receptors as well as to receptors on other human cell lines during different processes of the pathogen's cycle: RBC invasion in the case

of EBLs, cytoadherence to blood vessels, clumping between infected RBCs, and rosetting of infected RBCs with healthy RBCs in the case of PfEMP1 [Howell et al., 2008]. This suggests the use of the same domain by different proteins to bind to RBC receptors differentially, which is another example of functional redundancy among parasite proteins and binding domains.

Interestingly, evidence of the differential usage of RBC surface receptors by the paralogues MSP3, MSP6, and MSP11 can be found by comparing the binding features of their HABPs. First, binding of only one of the three HABPs identified in MSP3 (HABP 31202) was slightly susceptible to chymotrypsin ($<30\%$ binding reduction), binding of only one of the three HABPs identified in MSP6 (HABP 31175) was affected by treating RBCs with neuraminidase, whereas binding of MSP11 HABPs was significantly affected by neuraminidase and chymotrypsin. Moreover, bindings of MSP3 and MSP6 HABPs to proteins with molecular weights different from the ones found for MSP11 HABPs' receptors provide additional evidence to support this notion [Rodriguez et al., 2005; Lopez et al., 2006].

Taking into account that a fully effective vaccine should be able to cope with the high genetic diversity and antigenic variation existing among *P. falciparum* strains, non-polymorphic proteins or their fragments are highly desirable vaccine targets [Biggs et al., 1991; Marshall et al., 1994; Patarroyo and Patarroyo, 2008]. For this reason, the degree of conservation in the gene region encoding MSP11 HABPs 33595 and 33606 among parasite's strains isolated from different geographical regions was examined in this study (Fig. 6). The alignment of the complete MSP11 gene and the sequences encoding MSP11 HABPs showed a 100% identity between the *P. falciparum* strains included in this study (Fig. 6). Thus, MSP11 seems to be one of the most conserved proteins among parasite surface proteins, which is consistent with previous sequence analyses with the 3D7, D10 (Papua New Guinea), HB3 and W2mef (Indochina) strains [Pearce et al., 2005], in contrast to its paralogues MSP3 and MSP6, and even MSP1 that have been found to be polymorphic [Pearce et al., 2005]. This high sequence conservation of MSP11 may imply an important and conserved role for this parasite protein.

Finally, the conformational shape features of MSP11 HABPs were studied based on their CD spectra and deconvolution, considering that it has been reported that induction of an effective immune response is associated with the adjustment of modified HABPs inside the binding groove of human leukocyte antigen (HLA) molecules [Patarroyo et al., 2004; Reyes et al., 2007]. CD spectra and SOPMA predictions for HABPs 33595 and 33606 showed a major percentage of α-helical structure in the protein region that corresponds to both HABPs. These same structural features have been reported for HABPs of various MSPs and other non-GPI-anchored soluble proteins that are known to bound weakly to membrane and to be involved in rolling of merozoite over RBCs [Reyes et al., 2007], for example, conserved α-helical HABPs of the MSP11 paralogues MSP3 and MSP6, as well as MSP1 that have been also shown to be immunologically silent unless being specifically modified [Patarroyo et al., 2004; Patarroyo and Patarroyo, 2008].

For this reason, it is plausible to suppose that conserved HABPs of MSP11 would not be neither immunogenic nor protection-inducing molecules and therefore studies with MSP11 HABPs specifically modified in their critical RBC binding residues should be first conducted in order to determine whether they could be included as potential vaccine components, since previous studies have demonstrated that modifications done to conserved α-helical HABPs according to previously reported rules [Espejo et al., 2004; Patarroyo and Patarroyo, 2008], can lead to improving the adjustment of these peptides inside the binding groove of HLA molecules and hence turn them into immunogenic, protection-inducing peptides [Espejo et al., 2004; Patarroyo et al., 2004]. It is worth noting that antibodies against MSP11 have been detected in the sera from naturally infected individuals and that such antibodies have been shown to crossreact with some MSP11 paralogues (MSP3, MSP6, and others), and to cause parasite killing by an ADCI mechanism [Singh et al., 2009].

In conclusion, the identification of conserved MSP11 peptides that bind with high affinity to RBC surface receptors and strongly inhibit RBC invasion of *P. falciparum* merozoites in vitro, together with the other features herein discussed, support proposing MSP11 HABPs as interesting targets for further immunogenicity studies aimed at determining their potential as components of a minimal subunit-based, multi-epitopic, multi-stage, chemically synthesized antimalarial vaccine, currently under development at our institute.

ACKNOWLEDGMENTS

Nora Martinez collaborated in the translation of this manuscript.

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