Specific erythrocyte binding capacity and biological activity of *Plasmodium falciparum* erythrocyte binding ligand 1 (EBL-1)-derived peptides

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Abstract

Erythrocyte binding ligand 1 (EBL-1) is a member of the *eb1* multigene family involved in *Plasmodium falciparum* invasion of erythrocytes. We found that five EBL-1 high-activity binding peptides (HABPs) bound specifically to erythrocytes: 29895 (⁴¹HKKKSGELNNKSGILRSTY⁶⁰), 29903 (²⁰¹LYECGK-KIKEMKWICTDNQF²²⁰), 29923 (⁶⁰¹CNAILGSYADIGDIVRGLDV⁶²⁰), 29924 (⁶²¹WRDINTNKLSEK-FQKIFMGGY⁶⁴⁰), and 30018 (²⁴⁸¹LEDIINLSKKKKKSINDTSFY²⁵⁰⁰). We also show that binding was saturable, not sialic acid-dependent, and that all peptides specifically bound to a 36-kDa protein on the erythrocyte membrane. The five HABPs inhibited in vitro merozoite invasion depending on the peptide concentration used, suggesting their possible role in the invasion process.

Keywords: malaria protein; erythrocyte binding ligand-1; peptides; *Plasmodium falciparum*

Plasmodium falciparum is the causative agent of malaria in humans and is responsible for more than two million deaths per year. Because of this parasite's increasing resistance to the commonly used antimalarial drugs, an urgent need for developing a vaccine has emerged (Miller et al. 2002; Richie and Saul 2002). Merozoite proteins involved in the invasion process are good potential vaccine candidates, since merozoites become exposed to the host immune system before they invade erythrocytes (Carvalho et al. 2002). Although the molecular basis of erythrocyte invasion by merozoites is still not completely understood, different merozoite antigens and multiple erythrocyte receptors for merozoite invasion have now been described (Dolan et al. 1994; Chitnis and Blackman 2000; Cowman et al. 2002; Goel et al. 2003).

Members of the erythrocyte binding-like (*eb1*) family represent some of those merozoite proteins involved in the

merozoite's invasion of the erythrocyte; they bind with high affinity to glycoproteins on the surface of the erythrocyte. Erythrocyte binding antigen-175 (EBA-175) binds to glycophorin A and mediates an invasion pathway for merozoite entry into erythrocytes (Camus and Hadley 1985; Orlandi et al. 1992; Sim et al. 1994; Duraisingh et al. 2003). EBA-140 (BAEBL) binds to glycophorin C and functions in a pathway for merozoite invasion (Mayer et al. 2001; Lobo et al. 2003; Maier et al. 2003); EBA-181 (JESEBL) binds to the surface of erythrocytes in a sialic acid-dependent manner to a trypsin-resistant/chymotrypsin-sensitive receptor (Gilberger et al. 2003a).

However, in the case of EBL-1, its interaction with the erythrocyte has not been studied; its receptor on the erythrocyte surface also remains unknown. EBL-1 is a putative erythrocyte binding protein which is encoded by the *eb1-1* gene (Peterson et al. 1995; Peterson and Willems 2000).

eb1-1 has been identified as a second *eb1* family member in *P. falciparum* on the basis of consensus family characteristics: a single-copy gene encoding two Cys-rich domains, one Duffy-binding-like (DBL) domain, and a C-Cys domain (Peterson and Willems 2000; Adams et al. 2001). EBL-1 has only four conserved cysteine residues, compared

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to the other *eb1* products, which have eight. The DBL domain mediates erythrocyte binding activity in *eb1* products (Smith et al. 2000). *eb1-1* is also transcribed in late schizonts and is linked to a rapid proliferation phenotype (Adams et al. 2001).

The *eb1-1* gene presents characteristics similar to those of *P. falciparum eba-175* and *Plasmodium vivax* DAP genes (Peterson and Willems 2000; Michon et al. 2002). It has been determined that these genes do participate in the merozoite invasion of erythrocytes (Chitnis et al. 1996; Gilberger 2003b), and it has thus been suggested that EBL-1 is probably involved in erythrocyte receptor recognition, playing a synergistic or an alternative role in the invasion process (Peterson et al. 1995; Peterson and Willems 2000; Adams et al. 2001).

In the present study, we attempted to delineate specific erythrocyte binding capacity and biological activity of *P. falciparum*-derived EBL-1 peptides. The results show that five peptides bound specifically and not sialic acid-dependently to erythrocytes: 29895 (⁴¹HKKKSGELNNNKS GILRSTY⁶⁰) toward the N-terminal region, 29903 (²⁰¹LY ECGKKIKEMKWICTDNQF²²⁰) located in the DBL/F1 region, 29923 (⁶⁰¹CNAILGSYADIGDIVRGLDV⁶²⁰), 29924 (⁶²¹WRDINTNKLSEKFQKIFMGGY⁶⁴⁰) located in the DBL/F2 region, and 30018 (²⁴⁸¹LEDIINLSKKKKK SINDTSFY²⁵⁰⁰) located toward the C-terminal region of EBL-1. Interestingly, all five of these high-activity binding peptides (HABPs) specifically bound to a 36-kDa protein on erythrocyte membrane and inhibited in vitro merozoite invasion depending on the peptide concentration used.

Results

EBL-1 peptides bind specifically to human erythrocytes

Specific binding was calculated as being the difference between total and nonspecific binding. Peptide binding activity, defined as being the amount (pmol) of peptide that bound specifically to erythrocyte per added peptide (pmol), corresponded to the slope of the specific binding curve. HABPs were defined as being those peptides showing activity $\geq 2\%$, according to criteria established earlier (Urquiza et al. 1996; Rodriguez et al. 2000). The specific erythrocyte binding activity for 133 synthetic peptides covering the total length of the *P. falciparum* 3D7 strain EBL-1 protein (GenBank accession no. CAD52344) was determined by using binding assays. Five erythrocyte HABPs were found in EBL-1-peptides: 29895 (⁴¹HKKKSGELN NKS GILRSTY⁶⁰), 29903 (²⁰¹LY ECGKKIKEMKWIC TDNQF²²⁰), 29923 (⁶⁰¹CNAILGSYADIGDIVRGLDV⁶²⁰), 29924 (⁶²¹WRDINTNKLSEKFQKIFMGGY⁶⁴⁰), and 30018 (²⁴⁸¹LEDIINLSKKKKKSINDTSFY²⁵⁰⁰).

The results show that the HABPs were located in different EBL-1 protein regions: peptide 29895 toward the N-terminal region, peptide 29903 was located in the DBL/F1 region; peptides 29923 and 29924 were located in the DBL/F2 region, and peptide 30018 toward the EBL-1 C-terminal region (Fig. 1). No HABPs were found in the central region.

Binding assay with HABP jumbled-peptide

Analog peptides containing a jumbled sequence from each HABP were synthesized and tested in binding assays to determine whether HABP binding to erythrocytes was due to the amino acid composition of the HABPs or just specific sequences.

Figure 2 shows that the jumbled peptides presented specific binding activity lower than that for native HABPs. HABP 29923 analog jumbled peptide 32257 presented the highest specific binding activity (1.2); HABP 29923 happened to be the peptide having the highest specific binding activity (2.8). The results thus indicated that HABP binding activity was due to their specific sequences.

Binding constants for high-binding peptides to erythrocytes

In order to determine the binding constants for human erythrocyte interaction with HABPs, saturation binding assays were performed with each HABP. Saturation curves and Hill analysis (Fig. 3) allowed calculation of affinity constants (K_d) and Hill coefficients (n_H) and the approximate number of binding sites per cell (Attie and Raines 1995).

The affinity constants (K_d) were between 245 and 513 nM and Hill coefficients between 1.0 and 1.5, suggesting positive cooperativity. The number of binding sites per cell was found to be between 5500 and 10,500.

Cross-competition assays

Cross-competition assays were carried out for each of the HABPs by inhibiting them with unlabeled native peptide or other unlabeled HABPs to determine whether they were able to displace the radiolabeled peptide. The cross-competition assays showed that ¹²⁵I-labeled HABPs were inhibited, in some cases, by other nonlabeled HABPs (Table 1). For example, it can be seen that radiolabeled peptide 29895 was inhibited by nonlabeled 29903 and 30018 peptides. Radiolabeled peptide 29924 was inhibited by all the other nonradiolabeled HABPs, mainly for peptide 29903 and 29923 peptides; in contrast, radiolabeled peptide 29923 was not inhibited by any of the other nonradiolabeled HABPs. The majority of HABPs were not mutually inhibiting.

Figure 1. (Legend on next page)

Peptide number	SEQUENCE	Binding Activity (%)
29895	⁴¹ H K K K S G E L N N N K S G I L R S T Y ⁶⁰	2
32253	K K G L N K G L S Y H K S E N N S I R T	
29903	²⁰¹ L Y E C G K K I K E M K W I C T D N Q F ²²⁰	
32256	Q D C W M K K G E L F N T I K E I K C Y	
29923	⁶⁰¹ C N A I L G S Y A D I G D I V R G L D V ⁶²⁰	
32257	N I G Y D G I R L V D G V D I A S L A C	
29924	⁶²¹ W R D I N T N K L S E K F Q K I F M G G Y ⁶⁴⁰	
32258	E L F Q T I I M R G W G D F N N K N K K S ^Y	
30018	²⁴⁸¹ L E D I I N L S K K K K K S I N D T S F Y ²⁵⁰⁰	
32261	K S I N K S D I K L S F K E D T K L I N Y	

Figure 2. HABP jumbled-peptide erythrocyte binding profile. The jumbled peptides' binding activities were lower than those for native HABPs.

Cross-linking assay

All HABPs were identified as being able to bind specifically to one erythrocyte membrane protein having an apparent molecular weight of 36 kDa, when erythrocyte membranes and HABPs were cross-linked with Bis sulfosuccinimidyl suberate (BS³) followed by separation in SDS/PAGE. The radiolabeled peptide interaction with this protein was inhibited when the binding was performed in the presence of unlabeled peptide, indicating that it was a specific interaction (Fig. 4).

Enzymatic treatment

The effect of enzymatic treatment on HABP-erythrocyte interaction was determined in binding assays with enzyme-treated human erythrocytes. Each HABP's binding to non-treated human erythrocytes was considered as positive control (100%). Table 2 shows that enzymatic treatment of erythrocytes similarly affected the binding of peptides 29924 and 30018, becoming lessened when they were treated with chymotrypsin or trypsin and increasing when this was done with neuraminidase. Similarly, the binding of peptides 29903 and 29923 lessened when erythrocytes were treated with chymotrypsin, but this was not affected when they were treated with trypsin or neuraminidase. Peptide 29895 presented behavior different from that of the other HABPs—binding to erythrocytes treated with trypsin diminished, and was not affected by treatment with neuraminidase; this was the only HABP whose binding was not affected when erythrocytes were treated with chymotrypsin (on the contrary, it increased it).

Merozoite invasion inhibition assays

The HABPs were added to in vitro cultures at the schizont stage before the merozoites were liberated from infected

erythrocytes to determine EBL-1 HABPs' possible role in merozoite invasion. The results (Table 3) show that all peptides inhibited merozoite invasion by more than 70%. It can also be seen that invasion inhibition depended on peptide concentration.

Discussion

Plasmodium merozoite invasion of erythrocytes is a complex process involving ligand-receptor interactions between invading parasite and host cell (Cowman et al 2002; Miller et al. 2002). Some erythrocyte receptors have been identified using different approximations: binding assays, mutant red blood cells, and enzyme treatment (Hadley et al. 1987; Dolan et al. 1994; Sim et al. 1994; Reed et al. 2000; Mayer et al. 2001; Thompson et al. 2001; Lobo et al. 2003). Different molecules have also been identified on the merozoite surface and in its apical organelles which could be important mediators of the invasion process (Perkins 1992; Sim et al. 1992; Chitnis and Blackman 2000).

Among those merozoite ligands involved in the invasion are products belongs to a family of genes (*eb1*) encoding proteins involved in the specific recognition of host cell receptors, including the *P. vivax* and *P. knowlesi* Duffy-binding proteins (Adams et al. 2001). Each *eb1* appears as a single-copy gene not having cross-hybridization to any other locus in the *P. falciparum* genome, and all have similar exon-intron structure with conserved splicing boundaries, indicating a common evolutionary origin (Adams et al. 1992, 2001; Michon et al. 2002).

To date, six *eb1* proteins have been identified in the *P. falciparum* genome: EBA-175 (Camus and Hadley 1985; Orlandi et al. 1992; Sim et al. 1994; Duraisingh et al. 2003), EBA-140 (Thompson et al. 2001; Mayer et al. 2003; Lobo et al. 2003; Maier et al. 2003), EBA-165 (Triglia et al. 2001), EBA-181 (Gilberger et al. 2003a), MAEBL (Ghai et

Figure 1. Erythrocyte binding assays using EBL-1 peptides. Each one of the black bars represents the slope of the specific binding graph, which is called the specific binding activity. Peptides having specific binding activity $\geq 2\%$ were considered as having high specific erythrocyte binding (HABPs). A schematic representation of the EBL-1 protein is at the right, showing SP (signal peptide), F1 and F2 (DBL domains), TR (transmembrane region), and Cys (cysteine-rich region) (Adams et al. 2001; Michon et al. 2002).

Figure 3. Saturation curves for (top) 29895, 29903, (middle) 29923, 29924, and (bottom) 30018 HABPs. Increasing quantities of labeled peptide were added in the presence or absence of unlabeled peptide. The curve represents the specific binding. The affinity constants (K_d) were 513, 481, 415, 450, and 245 nM; Hill coefficients were 1.1, 1.4, 1.0, 1.5, and 1.0; and the number of binding sites per cell was 5500, 5700, 10,500, 7000, and 8000, respectively. In the Hill plot (*inset*) the abscissa is $\log F$ and the ordinate is $\log (B/B_{\max} - B)$, where F is free peptide, B is bound peptide, and $B_{\max}$ is the maximum amount of bound peptide.

al. 2002), and EBL-1 (Peterson et al. 1995; Peterson and Willems 2000). The receptor has been identified for some of these proteins which bind to the RBC membrane; its participation in the invasion process has been determined (Gilberger et al. 2003a,b; Lobo et al. 2003). The interaction between EBL-1 protein and the erythrocyte has not been

characterized to date, meaning that it remains unknown whether EBL-1 binds to erythrocytes and thus which is its receptor.

However, the similarity of the *ebf-1* gene's characteristics to those of the rest of the members of the *ebf* family, their transcription in late schizonts, their relationship with a rapid

Table 1. Cross-competition assays

Unlabeled peptide	Radiolabeled peptide				
	29895	29903	29923	29924	30018
29895	100 ^{a,b}	103	14	150	95
29903	79	100	23	180	92
29923	0	87	100	200	83
29924	0	62	0	100	61
30018	82	104	17	130	100

The binding of each radiolabeled HABP was inhibited by all the other nonradiolabeled HABPs.

^a The data are presented as specific binding percentages (%) related to original HABP.

^b Mean of three experiments. The S.D. was minor of 10% in all cases.

proliferation phenotype, and the EBL-1 protein's high homology with other *ebf* products suggests that EBL-1 is probably involved in some of the merozoite-erythrocyte interactions in the invasion process (Peterson et al. 1995; Peterson and Willems 2000; Adams et al. 2001).

This work focused on analyzing EBL-1 erythrocyte binding sequences as EBL-1 (or a region derived from it) could be directly acting on erythrocyte receptor recognition, merozoite attachment, or synergistic invasion of erythrocytes in an alternative role in the invasion process.

The binding assays showed that five *P. falciparum* EBL-1 derived peptides specifically bound to erythrocytes (Fig. 1) and that HABP binding depends on each specific sequence in particular and not on amino acid composition (Fig. 2). The high affinity (K_d values ranging from 245–513 nM) and positive cooperativity (n_H values of 1.0–1.5) indicate strong HABP interaction with erythrocytes (Fig. 3). Lesser or similar K_d values have been reported for HABPs derived from EBA-175 (Rodriguez et al. 2000) and EBA-140 (Rodriguez et al. 2003) proteins in the *ebf* family.

The cross-competition assays showed that nonlabeled HABPs inhibited radiolabeled HABP binding to different

Table 2. Binding of EBL-1 peptides to enzyme-treated erythrocytes

Peptide	Control (%) ^a	Neuraminidase	Chymotrypsin	Trypsin
29895	100 ± 9 ^b	103 ± 9	177 ± 8	45 ± 8
29903	100 ± 8	131 ± 9	15 ± 10	110 ± 6
29923	100 ± 7	104 ± 7	57 ± 8	109 ± 6
29924	100 ± 5	160 ± 7	15 ± 7	26 ± 5
30018	100 ± 1	234 ± 3	35 ± 7	8 ± 6

Peptide binding was compared between enzyme-treated erythrocytes and untreated erythrocytes.

^a The data are presented as specific binding percentages (%) related to untreated erythrocytes.

^b Mean ± S.D. of three experiments.

degrees (Table 1). However, radiolabeled peptide 29923 was not inhibited by any of the other nonradiolabeled HABPs. It was also seen that some HABPs were mutually inhibiting. The results suggest that HABPs bind to different receptors on erythrocyte membrane or to a single receptor in different binding sites. The latter would be the most feasible, bearing in mind that all EBL-1 HABPs bound to a protein having an apparent 36 kDa molecular weight on erythrocyte membrane (Fig. 4).

When the binding assays were performed with enzyme-treated human erythrocytes, it was also observed that neuraminidase (cleaving terminal sialic acids from glycoproteins) did not affect the binding of any of the HABPs; on the contrary, the binding of HABPs 29924 and 30018 increased (Table 2). Chymotrypsin treatment lessened the binding of all HABPs except HABP 29895, whose binding increased. Trypsin treatment diminished the binding of HABPs 29895, 29924, and 30018. These preliminary results indicate that peptides bind to different receptor sites on the same receptor-molecule on the erythrocyte membrane, such binding not being sialic acid-dependent. This is interesting, since it has been reported that *P. falciparum* can invade erythrocytes independently of sialic acid (Narum et al. 2000; Duraing et al. 2003).

Figure 4. Autoradiograph from HABP cross-linking assays. Erythrocyte membrane proteins were cross-linked with all radiolabeled peptide HABPs. Only HABP 29903 (lanes 1,2) and 29923 (lanes 3,4) autoradiographs are shown. Lanes 1 and 3 show total binding (i.e., cross-linking in the absence of unlabeled peptide), and lanes 2 and 4 show inhibited binding (i.e., cross-linking in the presence of unlabeled peptide).

Table 3. Inhibition of parasite invasion to erythrocytes by EBL-1 peptides

Peptide	% Inhibition invasion		
	200 μ M	100 μ M	50 μ M
29895	82 ± 1 ^a	16 ± 1	1 ± 1
29903	89 ± 1	19 ± 1	10 ± 1
29923	71 ± 9	13 ± 1	0 ± 1
29924	95 ± 2	13 ± 1	2 ± 4
30018	82 ± 5	29 ± 2	6 ± 1
Control chloroquine	100 ± 1		
Control EGTA	100 ± 1		

^a Mean ± S.D. of three experiments.

It was possible to define three EBL-1 erythrocyte specific binding regions from the binding assay results. A binding region was located in the N-terminal region, comprised of HABPs 29895 and 29903. In turn, HABP 29903 was located in the DBL-F1 domain N-terminal (Fig. 1).

The second binding region corresponds to HABP 30018, located in the EBL-1 protein's C-terminal, just before the start of the C-Cys region (Figs. 1, 5B). To date, no function has been identified for the C-Cys domain, even though this is more conserved than the DBL domains (Adams et al. 2001). However, the EBA-175 protein also presents an HABP just before the start of the C-Cys region (Rodriguez et al. 2000), and EBA-140 protein also presents an HABP at the start of the C-Cys region (Fig. 5A; Rodriguez et al. 2003). Although it is not indispensable for invasion, this cysteine-rich region could be a participant in the merozoite's initial recognition of erythrocyte.

The third binding region is comprised of HABPs 29923 and 29924, 601C–640G residues. This 40-residue binding region is located toward the DBL/F2 domain's central region (Figs. 1, 5A); this fact is interesting since it was reported that DBL domains mediate erythrocyte binding activity in the *ebf* products and that the EBA-175 DBL/F2 domain binds to glycophorin-A on erythrocyte membrane (Orlandi et al. 1992; Sim et al. 1994; Sim 1998; Michon et al. 2002). Some HABPs corresponding to EBA-175 and EBA-140 proteins have been described which are included within the proteins' DBL/F2 region (Rodriguez et al. 2000, 2003).

Sequence alignment and comparison of the erythrocyte binding profiles and the EBL-1, EBA-175, and EBA-140 protein DBL/F2 domains (Fig. 5B) lead to the observation that the HABPs cover almost all of the DBL/F2 domain's

central region. This is interesting since it was noted that *P. knowlesi* and *P. vivax* invasion depend on recognition of a single receptor (e.g., Duffy blood group antigen) (Miller et al. 1979; Adams et al. 2001). On the contrary, *P. falciparum* does not depend on a single receptor for invasion; it can use alternative invasion pathways or different receptors on the erythrocyte membrane (Hadley et al. 1987; Orlandi et al. 1992; Dolan et al. 1994; Goel et al. 2003; Lobo et al. 2003; Maier et al. 2003).

The partial disruption of the *eba-175* in two different *P. falciparum* clones is associated with a switch toward a sialic acid-independent invasion pathway, showing that alternative parasite ligands exist (Kaneko et al. 2000; Reed et al. 2000). Additionally, distinct pathways depending on glycophorin-A, -B, and -C, and an unknown receptor (the X pathway) have been identified (Dolan et al. 1994).

It has thus been suggested that some of the receptors involved in invasion and the use of alternative invasion pathways are probably related to EBA-175, and associated with expression of different *ebf* products (Chitnis and Blackman 2000; Adams et al. 2001). The idea that EBL-1 protein or regions deriving from it presenting erythrocyte binding activity (in this case HABPs belonging to the DBL/F2 region) are involved in the invasion process thus cannot be discarded.

In fact, when we tested the HABPs on in vitro *P. falciparum* cultures, we observed that all HABPs significantly inhibited merozoite invasion (Table 3). These results suggest that the HABPs were blocking the merozoite-erythrocyte interaction, inhibiting invasion.

Further studies are still necessary to clarify EBL-1 HABPs' specific role at the time of merozoite invasion of erythrocytes. The possibility of using HABP sequences

Figure 5. (A) Schematic representation of EBA-175, EBA-140, and EBL-1 protein DBL/F2 domains. The position of each HABP is shown for each protein. The arrows show the position of conserved cysteine residues. (B) EBA-175, EBA-140, and EBL-1 protein C-Cys domain sequence alignment. Boxed amino acids indicate the HABPs for each protein: peptide 1818 in EBA-175, peptide 26170 in EBA-140, and 30018 in EBL-1 (Rodriguez et al. 2000, 2003). The arrows indicate the start of cysteine-rich regions in each protein (Adams et al. 2001).

from *P. falciparum* EBL-1 protein for designing tools for the specific inhibition of *P. falciparum* merozoite interaction with erythrocytes also needs deeper study. Taken together, the results reported in this work suggest that *P. falciparum* EBL-1 protein regions could be participating in parasite recognition or invasion of erythrocyte, in the same way as other *eb1* family members.

Materials and methods

Peptide synthesis

Sequential 20-mer peptides corresponding to the complete *P. falciparum* 3D7 strain EBL-1 protein amino acid sequence (GenBank accession no. CAD52344) were synthesized by the solid-phase multiple peptide system (Merrifield 1963; Houghten 1985); t-Boc amino acids (Bachem) and MBHA resin (0.7 meq/g) were used. Peptides were cleaved by the Low-High HF technique (Tam et al. 1983), purified by RP-HPLC, lyophilized, and analyzed by MALDI-TOF mass spectrometry. Tyrosine was added to those peptides which did not contain this amino acid in their sequences at the C-terminal to enable ^{125}I -labeling. Synthesized peptides are shown in Figure 1 in one-letter code.

Radiolabeling

The peptides were labeled with ^{125}I according to previously described methodology (Urquiza et al. 1996). Briefly, 3.2 μL Na^{125}I (100 mCi/mL) was oxidized with 12.5 μL chloramine-T (2.25 $\mu\text{g}/\mu\text{L}$) and added to 5 μg peptide for 5 min at room temperature. The reaction was stopped by adding 15 μL sodium bisulfite (2.25 $\mu\text{g}/\mu\text{L}$) and 50 μL NaI (0.16 M). The radiolabeled peptide was then separated on a Sephadex G-10 column (Pharmacia).

Binding assay

Human erythrocytes (2×10^8 cells/ μL) obtained from healthy donors were washed in PBS buffer until the buffy coat was removed and then incubated with different radiolabeled-peptide concentrations (10–200 nM), in the absence (total binding) or presence (nonspecific binding) of 40 μM unlabeled peptide. The sample reached 200 μL final volume with PBS and was incubated for 90 min at room temperature (Urquiza et al. 1996; Curtidor et al. 2001). The cells were then washed five times with PBS, and cell-bound radiolabeled peptide was quantified in an automatic gamma counter (4/200 plus ICN Biomedicals). The binding assays were performed in triplicate.

Jumbled-peptide binding assay

The sequences of HABPs determined in the binding assay described above were used in synthesizing the same peptides but now in a jumbled order (i.e., same amino acid composition as HABPs but having random sequence) and then tested in binding assays. The assays were carried out in triplicate in conditions identical to those described above in the "Binding assay" section. Synthesized peptides are shown in Figure 2 in one-letter code.

Saturation assays

An erythrocyte binding assay was used to ascertain saturation with all HABPs; the following modifications were introduced: 1.5×10^8 cells were used at 255 μL final volume; radiolabeled peptide concentrations were between 0 and 1000 nM. The unlabeled peptide concentration was 40 μM . Cells were washed with PBS, and a gamma counter was used to measure cell-bound radiolabeled peptide (Weiland and Molinoff 1981; Enna 1984).

Cross-competition assays

The binding to erythrocytes for each HAPB was inhibited by all the other HABPs in cross-competition assays. Radiolabeled HABPs (200 nM) were incubated with 2×10^8 erythrocytes for 90 min at room temperature, in the presence or the absence of the same or other unlabeled HABPs (20 μM). After incubation, unbound peptide was removed with three 5-mL PBS washes, and cell-bound radiopeptide was measured. The cross-competition assays were done in triplicate in the same conditions.

Cross-linking assays

Radiolabeled HABPs were cross-linked to erythrocyte membranes in the presence or absence of unlabeled peptide for identifying specific erythrocyte binding sites. The cross-linking binding test was performed by using a final 1% cell concentration and following incubation with the radiolabeled peptide in the presence or absence of 40 μM unlabeled peptide for 90 min at room temperature. After incubation, cells were washed with PBS, and the bound peptide was cross-linked with 10 μM BS³. Bis (sulfosuccinimidyl suberate) (Pierce) for 20 min at 4°C. The reaction was stopped with 20 mM Tris-HCl (pH 7.4) and washed again with PBS. Then cells were then treated with lysis buffer (5 mM Tris-HCl, 7 mM NaCl, 1 mM EDTA, 0.1 mM PMSF). The obtained membrane proteins were solubilized in Laemmli buffer and separated by SDS/PAGE (12% w/v polyacrylamide gels). The gels were exposed on BioRad Imaging Screen K (BioRad Molecular Imager FX; BioRad Quantity One, Quantitation Software) for 2 d to determine which proteins had become cross-linked to the radiolabeled peptides. The apparent molecular weight was determined using molecular weight markers (NEB).

Enzymatic treatment

Erythrocytes (5%) suspended in PBS buffer were treated with 150 $\mu\text{U}/\text{mL}$ neuraminidase (ICN 9001-67-6) at 37°C for 1 h, washed five times with PBS buffer, and centrifuged at 1000g for 5 min. In the same way, erythrocytes (5%) were treated with trypsin (Sigma T-1005) or chymotrypsin (Sigma C-4129) in TBS buffer (5 mM Tris-HCl, 140 mM NaCl [pH 7.4]), at a final 0.75 g/mL concentration. After incubation at 37°C for 1 h, the samples were washed five times with PBS buffer to which 0.1 mM PMSF had been added. After enzyme-treatment, these erythrocytes were tested in a binding assay with HABPs as described (Camus and Hadley 1985; Curtidor et al. 2001).

Merozoite invasion inhibition assay

The in vitro cultures of the FCB-2 strain of *P. falciparum* were synchronized at the ring stage with sorbitol solution, and incubated until the late schizont stage. The cultures were grown in RPMI

1640 medium supplemented with 10% human plasma. (Trager and Jensen 1976; Lambros and Vanderberg 1979). The culture (0.5% final parasitemia and 5% hematocrit) was seeded in 96-well cell culture plates (Nunc) in the presence of test peptides at 200, 100 and 50 μ M concentrations. Each peptide was tested in triplicate. After incubation for 18 h at 37°C in a 5% O₂/5%CO₂/90% N₂ atmosphere, the supernatant was recovered and the cells stained with 15 μ g/mL hydroethidine, incubated at 37°C for 30 min, and washed three times with PBS. The suspensions were analyzed using a FACsort in Log FL2 data mode using CellQuest software (Becton Dickinson Immunocytometry System) (Wyatt et al. 1991). Infected and uninfected erythrocytes treated with EGTA and chloroquine were used as controls.

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