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Well-Defined Regions of the *Plasmodium falciparum* Reticulocyte Binding Protein Homologue 4 Mediate Interaction with Red Blood Cell Membrane

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Two widely studied parasite protein families are considered attractive targets for developing a fully effective antimalarial vaccine: the erythrocyte binding antigen (EBA) family defining a sialic acid-dependent invasion pathway, and reticulocyte-binding homologue (RH) proteins associated with sialic acid-independent red blood cell (RBC) invasion. In this study, the micronemal invasive *Pf*RH4 protein was finely mapped using 20-mer-long synthetic peptides spanning the entire protein length to identify protein regions that establish high affinity interactions with human RBCs. Twenty conserved, mainly α-helical high-activity binding peptides (HABPs) with nanomolar dissociation constants and recognizing 32, 25, 22, and 20 kDa RBC membrane molecules in a chymotrypsin and/or trypsin-sensitive manner were identified in this protein. Anti-*Pf*RH4 rabbit sera and *Pf*RH4 HABPs inhibited merozoite invasion in vitro, therefore suggesting the implication of these HABPs in *Plasmodium falciparum* invasion and supporting their inclusion in further structural and immunological studies to design potential components of a minimal subunit-based, multiantigenic, chemically synthesized antimalarial vaccine.

Introduction

Invasion to human red blood cells (RBCs) is an essential process for the survival and development of *Plasmodium falciparum*, the parasite associated with the most severe form of malaria. New and more effective treatments and vaccines are urgently needed to achieve control and eradication of this disease, as it remains one of the most serious problems for public health systems worldwide,[1,2] causing more than 500 million cases and 1 million deaths per year.[3,4] Several parasite membrane proteins and those contained inside specialized organelles (micronemes, rhoptries, and dense granules) are involved in complex receptor−ligand interactions taking place during *P. falciparum* recognition, reorientation, junction formation, and invagination into RBCs,[5,6] all these molecules being promissory candidates for the development of a multiantigenic antimalarial vaccine.

It has been demonstrated that *P. falciparum* parasites use alternative receptors on the RBC surface to overcome receptor polymorphism and evade the host's immune system, thus defining alternative invasion pathways for the erythrocyte binding-like (EBL[a]) proteins such as the "classical" sialic acid-dependent pathway (associated with RBC membrane glycoproteins named glycophorins), which includes the use of erythrocyte binding antigen-175 (EBA-175), EBA-181/JESEBL, EBA-140/BAEBL, and MAEBL.[6−8] Among these adhesins, the EBA-175-glycophorin A association was the first malarial host−pathogen interaction to be described and has been the main and more deeply studied merozoite invasion pathway in *P. falciparum*, thus pointing at this parasite protein as a highly promising immunological target.[6,9−12]

Other invasion-related proteins include the reticulocyte binding-like (RBL) protein family, which comprises important targets for the design of a fully protective vaccine that have been identified to be key for merozoite recognition and binding to host cells in several *Plasmodium* species. This family includes the *Plasmodium vivax* reticulocyte binding proteins (*Pv*RBP-1 and *Pv*RBP-2) and the *Plasmodium yoelii* 235 kDa rhoptry protein (*Py*235), which play essential roles in reticulocyte and mice RBC invasion, respectively.[13−15] In *P. falciparum*, six orthologues of these proteins are part of the normocyte binding protein (NBP) or reticulocyte binding protein homologues (RHs) family: *Pf*RH1, *Pf*RH2a, *Pf*RH2b, the untranslated *Pf*RH3, *Pf*RH4, and the recently described *Pf*RH5 proteins.[16−20]

*Pf*RH proteins are members of the parasite's exquisite protein machinery of alternative invasion pathways, where *Pf*RH1 binds to a putative sialic acid-dependent trypsin-resistant receptor on RBC membrane named "Y" receptor,[17,21] while *Pf*RH2b interacts with the chymotrypsin-sensitive putative receptor "Z",[22] these host cell receptors being independent of other known RBC membrane receptors for merozoite infection. In addition, the recently described *Pf*RH5 has been reported to act as ligand for an unrecognized *P. falciparum* invasion pathway.[18]

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[a] Abbreviations: BSC, binding sites per cell; EBA, erythrocyte binding antigen; EBL, erythrocyte binding-like; HABPs, high-activity binding peptides; HBS, HEPES buffer saline; K_d, dissociation constant; MBHA, 4-methylbenzhydrylamine hydrochloride; n_H, hill coefficient; RBL, reticulocyte binding-like; RBP, reticulocyte binding protein; RH, reticulocyte binding protein homologue; TFE, trifluoroethanol.

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The high infective efficiency of *P. falciparum* has been related to a switch in the expression of the *ebl* and *rh* genes, associated with the differential use of sialic acid-dependent and -independent host-cell invasion routes, principally involving the protein pairs *Pf*RH1 versus *Pf*RH2b, EBA-175 versus *Pf*RH2b, and EBA-175 versus *Pf*RH4.[23,24] In addition to these observations, it has been described that variations in the sequence, transcription, and expression of the *Pf*RH1, *Pf*RH2a, and *Pf*RH2b proteins among different *P. falciparum* strains may determine the parasite's preference for alternative infection routes, this behavior being connected with gene number redundancy and sequence similarity in *Pf*RH proteins, as well as with transcriptional and expression divergence.[25] These studies, and the analysis of the *P. falciparum* genome indicate that a fully effective antimalarial vaccine must include ALL proteins (or their functional fragments) employed by the parasite as ligands for the different invasion pathways.[26−28]

In 2002, Kaneko et al. described a gene located on *P. falciparum* chromosome 4 encoding the fourth member of the RH family, named *P. falciparum* reticulocyte binding protein homolog 4 (*Pf*RH4), which is a micronemal 220 kDa protein consisting of 1716 amino acids. The *pfrh4* gene contains two exons; the first exon encodes a putative secretory signal peptide (amino acids 1−26), while the second exon consists of a putative extracellular sequence followed by transmembrane (amino acids 1627−1649) and cytoplasmic domains, which are structural features observed in all members of the *Pf*RH family (Figure 1).[16] Two variable repeat regions are present in *Pf*RH4: the first comprises amino acids 1494−1543 [H(T/A)NEKNI(N/Y)(N/Y)E × 5] of the extracellular domain, and the second one spans amino acids 1686−1712 [(D/N/V)E × 13] from the cytoplasmic domain.[16] In addition, point modifications are observed between different *P. falciparum* strains in *Pf*RH4, two amino acid (aa) substitutions at positions 12 and 1482, one insertion within aa 839−840, and three deletions at aa 1076−1079, 1696−1699, and 1702−1703, indicating that the *Pf*RH4 extracellular region is highly conserved among parasite strains from different geographical regions.[16,29] Furthermore, a sequence target for the rhomboid proteases *Pf*ROM-1 and *Pf*ROM-4 has been described in all members of the *Pf*RH family, including *Pf*RH4, thus indicating that the protein is cleaved before parasite entry into the host cell.[30]

The high relevance of *Pf*RH4 in RBC invasion has been associated with its expression profile, since this protein is up-regulated in the absence of EBA-175[24] or when the RBC membrane receptor for EBA-175 is absent.[31] For instance, it has been described that up-regulation of *Pf*RH4 results in a switch from a sialic acid-dependent to a sialic acid-independent invasion route, hence allowing infection of RBCs and favoring *P. falciparum* survival. Additionally, the differential inhibition of *P. falciparum* strains varying in their use of specific EBL and *Pf*RH proteins points to these ligand families as major targets of inhibitory antibodies.[32] The foregoing studies once more highlight the importance of generating a broad inhibitory response against multiple ligands to induce a fully effective immune response.[27,28,32,33]

Recently, Gaur et al. demonstrated that a 30 kDa recombinant fragment of *Pf*RH4 (r*Pf*RH4, aa 328−588) binds to RBCs in a neuraminidase-resistant manner, in the same way that the native protein does, therefore supporting the importance of *Pf*RH4 as a *P. falciparum* ligand. Additionally, the same authors demonstrated that anti-r*Pf*RH4 antibodies inhibit binding of native *Pf*RH4 but do not inhibit invasion to RBCs, thus suggesting that although *Pf*RH4 is required for invasion, this region is not accessible to invasion-blocking antibodies.[34]

On the basis of the importance of *Pf*RH4 in the multiple pathways for *P. falciparum* infection to host cells, we have finely mapped its entire protein sequence by using synthetic peptides because of their feasible production in high quantities and purity, with the aim of identifying sequences with high specific binding activity to human RBCs by means of a highly robust and sensitive receptor−ligand methodology thoroughly described by us for *Plasmodium* spp. and other important pathogens.[28,35−39] Additionally, the degree of polymorphism in *Pf*RH4 was analyzed in the so identified sequences and their interactions with RBCs were partially characterized by means of saturation, enzymatic treatment, cross-linking, and invasion inhibition assays, together with circular dichroism studies to determine their main structural features. The results indicate that *Pf*RH4 interacts with human RBCs through well-defined regions and support suggesting these regions as excellent candidates to be included in further studies for the design of a multiantigenic, multistage, fully effective, minimal subunit-based synthetic vaccine against *P. falciparum* malaria.

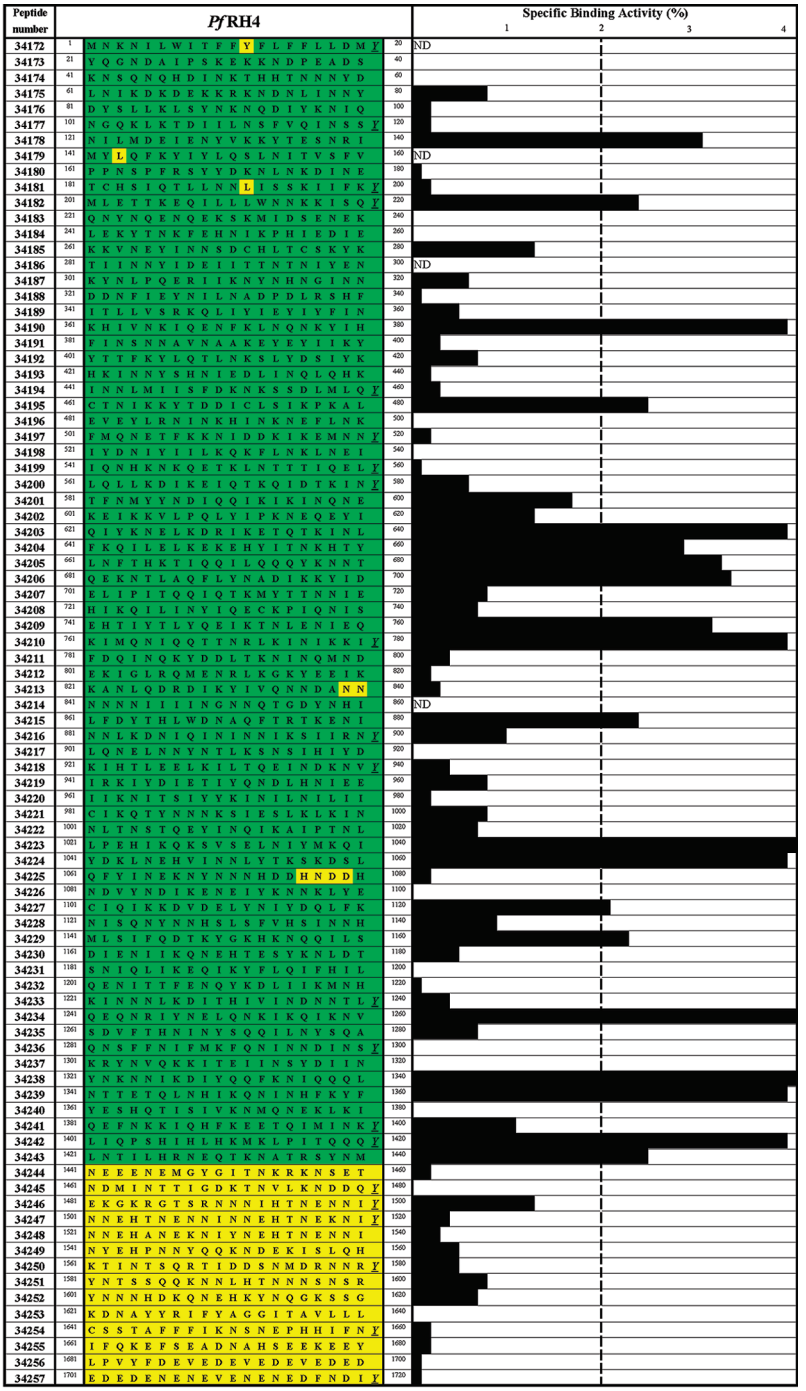
Results

***Pf*RH4 Peptides Specifically Bind to RBCs.** Binding assays to human RBCs were carried out with the 82 synthetic peptides spanning the entire *Pf*RH4 sequence in order to identify regions interacting specifically with host cells. A total of 20 HABPs were identified (Figure 1), based on a previously described criteria defining a ratio of specifically bound peptide/added peptide equal to or greater than 0.02 (2% binding) for HABPs, which corresponds to the recognition of more than 2000 receptor sites per cell.[28] HABPs 34178 ^{121}NILMDEIENYVKKYTESNRI^{140} and 34182 ^{201}MLETTKEQILLLWNNKKISQY^{220} were located toward the *Pf*RH4 N-terminal region, while the other HABPs were distributed mainly throughout the conserved central region of *Pf*RH4, all HABPs being located in the noncytoplasmic portion. Binding assays performed with scrambled peptides, i.e., having the same amino acid composition of HABPs but different sequence, showed that altering the order of amino acids in HABPs resulted in the lost of their high specific binding activity (Figure 2), thus indicating that HABPs' interactions are directly related to the sequence order and therefore dependent on their structure.

Saturation assays and Hill analysis were performed with some *Pf*RH4 HABPs, finding dissociation constants (K_d) within the nanomolar range (500−1200 nM), Hill coefficients (n_H) of > 1, and about 40000−400000 binding sites per cell (Table 1). Only HABPs 34178 and 34206 were not saturable under the assay conditions followed in this study.

DNA Amplification and *Pf*RH4 Strain-Specific Polymorphism Analysis. The region encoding *Pf*RH4 HABPs was amplified by PCR from genomic DNA of the FVO, FCB-2, and PAS-2 *P. falciparum* strains by using the five primer sets detailed in Experimental Section and visualized on agarose gels. Single bands of about 474, 590, 994, 949, and 527 bp were amplified with each primer set, while control primers amplified a single band of about 438 bp (Supporting Information Figure 1). The amino acid sequences of *Pf*RH4 HABPs in the three strains herein analyzed were aligned to the reported sequences of the 3D7 (The Netherlands), HB3 (Honduras), and Dd2 (Indochina) reference strains using ClustalW software.[40] As

a.



b.

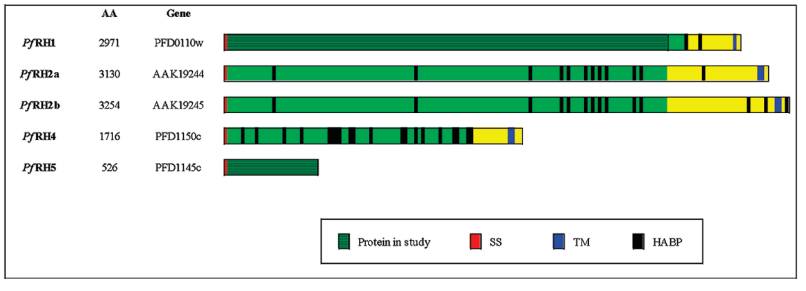


Figure 1. (a) Binding profile of *Pf*RH4 synthetic peptides. Superscript numbers indicate the peptide's localization in the protein sequence. ND = not determined. (b) Structural representations of *Pf*RH family members, showing the number of amino acids (AA) and the GenBank accession number (*Pf*RH2a and *Pf*RH2b) or the PlasmoDB ID (*Pf*RH1, *Pf*RH4, and *Pf*RH5) of each protein. Signal sequence (SS), transmembrane domain (TM), and HABPs are also located within *Pf*RHs. The C-terminal region of the *Pf*RH1 protein and the whole *Pf*RH5 sequence are currently under study. Conserved regions are shown in green and variable regions in yellow, according to the results of this and previous studies.[16,17,20,25,29,49]

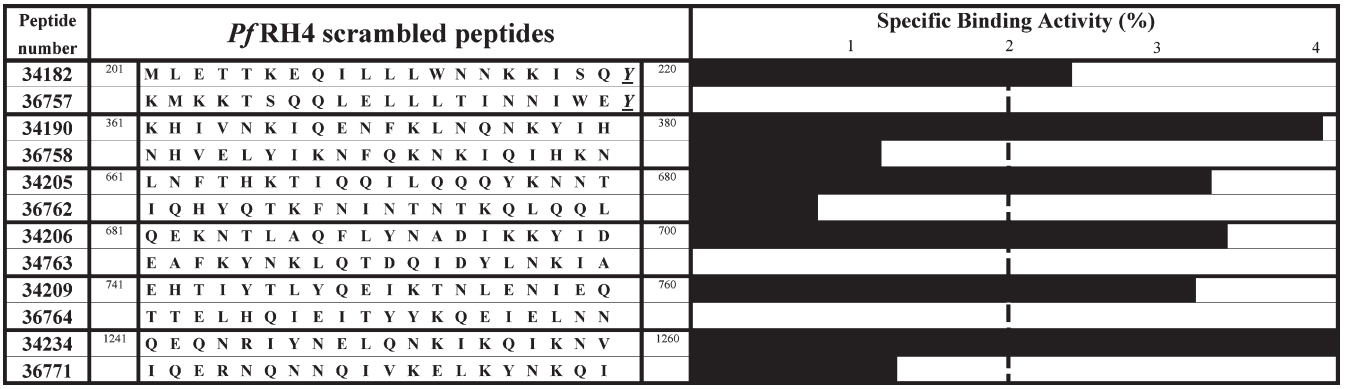


Figure 2. *Pf*RH4 scrambled peptides' binding assays. The specific binding activity of some *Pf*RH4 HABPs and its corresponding scrambled peptide is shown. Scrambled sequences were designed using the Shuffle protein server.[72]

Table 1. *Pf*RH4 HABPs' Binding Constants

	binding constants[a]		
peptide	$K_d \times 10^2$	BSC $\times 10^5$	n_H
34190	5.9	1.8	1.7
34195	8.7	4.0	2.0
34203	5.9	0.9	1.7
34205	8.5	1.1	1.8
34210	7.5	1.5	1.7
34215	12	1.5	2.4
34224	4.8	0.6	1.8
34234	8.8	0.6	1.7
34238	4.0	0.4	2.0
34243	8.5	0.9	1.7

[a] K_d: dissociation constant. BSC: binding sites per cell. n_H: Hill coefficient. All standard deviations were below 4%.

shown in Supporting Information Figure 2, HABPs in these strains shared 100% amino acid sequence identity. No substitutions were observed among nucleotide sequences either, therefore ruling out the presence of synonymous substitutions in the studied regions (data not shown).

The following strain-specific polymorphisms were found outside the regions of interest: (1) one substitution from T to A in nucleotide position 427 of 3D7, corresponding to residue 143, where TTA encodes for leucine (L) in 3D7, Dd2, and HB3 while triplet ATA encodes for isoleucine (I) in FCB-2, FVO, and PAS-2; (2) one substitution from C to G in nucleotide position 574 of 3D7 corresponding to residue 192, where triplet CTA encodes for leucine (L) in 3D7, Dd2, and HB3 while triplet GTA encodes for valine (V) in the other strains (Supporting Information Figure 2A). (3) Nucleotides encoding for three residues (asparagine, isoleucine, asparagine (NIN)) in the FCB-2, PAS-2, Dd2, and HB3 strains were not found in the FVO strain or in the 3D7 strain (Supporting Information Figure 2C). (4) Finally, the Dd2 strain showed a deletion of residues DDHN, which were present in the remaining strains (Supporting Information Figure 2D).

***Pf*RH4 HABPs Bind to a 32 kDa Protein in a Neuraminidase-Resistant Manner.** In order to identify the apparent molecular weight of the possible RBC membrane receptor(s) interacting with *Pf*RH4 HABPs, radiolabeled peptides were cross-linked to RBC membranes and separated by SDS−PAGE. The autoradiograms of HABPs 34195, 34203, 34206, 34227, 34234, and 34238 shown in Figure 3 indicate the recognition of four bands having apparent molecular weights of 32, 25, 22, and 20 kDa, such recognitions being highly specific because these bands' intensities diminished in the presence of unlabeled peptide.

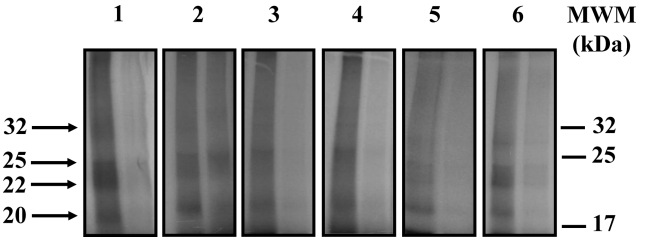


Figure 3. Autoradiograms of *Pf*RH4 HABPs. In each autoradiogram, the right lane corresponds to total binding and the left lane to binding in the presence of unlabeled peptide. HABPs being analyzed were (1) 34195, (2) 34203, (3) 34206, (4) 34227, (5) 34234, and (6) 34238.

Table 2. Effect of Different Enzymatic Treatments in HABPs Binding to RBCs

	binding (%)[a]		
HABP	neuraminidase	chymotrypsin	trypsin
34178	94.3	169	220
34190	115	132	94.1
34195	101	**52.8**	172
34203	128	288	**0.03**
34205	94.9	79.7	**43.0**
34206	110	**0.01**	**1.01**
34210	129	**60.1**	**15.2**
34215	166	**31.4**	**24.9**
34224	312	**26.3**	**16.7**
34227	80.7	**53.3**	75.2
34229	200	93.2	93.0
34234	93.0	102	**32.0**
34238	153	114	87.0
34239	89.5	135	**55.5**
34242	152	209	79.4
34243	92.2	**22.5**	**43.9**

[a] Binding to untreated RBCs was used as 100% binding control. All standard deviations were below 12% except for trypsin treatment of HABP 34178 (25% standard deviation). Binding values ≤ 50% are shown in bold.

The nature of RBC surface receptor(s) for *Pf*RH4 HABPs was examined in binding assays with neuraminidase-, chymotrypsin-, or trypsin-treated cells. Bindings of all HABPs were resistant to treatment with neuraminidase, indicating sialic acid-independent interaction of the *Pf*RH4 HABPs with RBC membrane proteins. In contrast, binding of *Pf*RH4 HABPs was differentially sensitive to chymotrypsin and trypsin, depending on the proteic nature of the RBC membrane receptor(s) (Table 2).

***Pf*RH4 Is Recognized by Rabbit Sera.** Specific anti-*Pf*RH4 sera obtained by immunization in New Zealand rabbits were

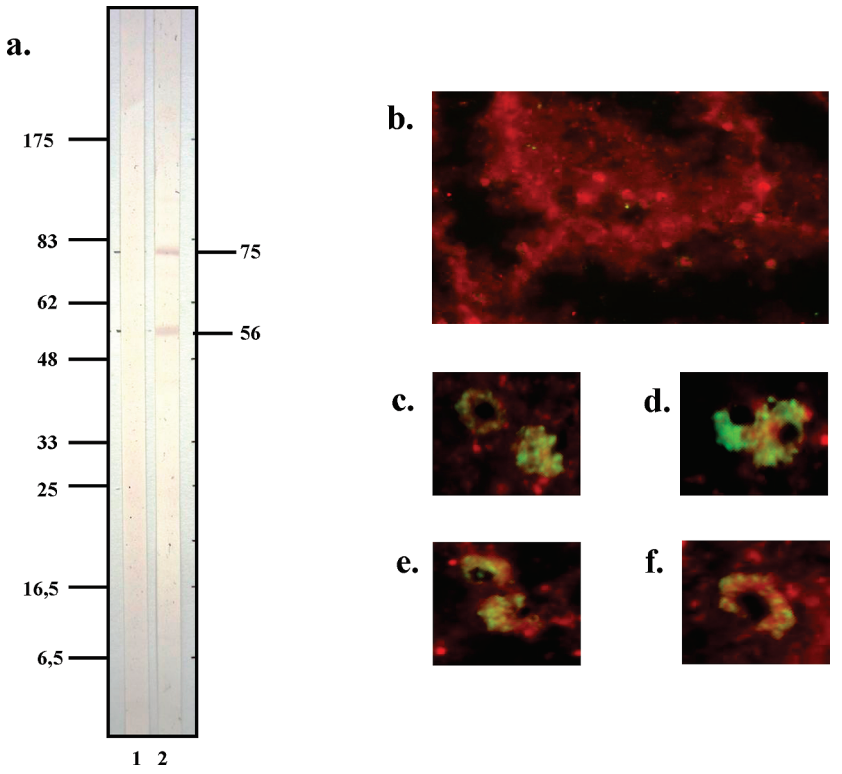


Figure 4. (a) Western blot analysis of *P. falciparum* (FCB2 strain) schizont lysate with preimmune rabbit serum (lane 1) or immune serum (lane 2). (b−f) Immunofluorescence assays. (b) Fixed infected RBCs incubated with preimmune rabbit serum showing no immunoreactivity. (c−f) Fixed infected RBCs incubated with immune serum showing a punctuate-like immunofluorescence pattern characteristic of merozoite apical organelle proteins in late schizont stages, in addition to the detection of *Pf*RH4 in parasite membrane.

used to perform recognition, localization, and invasion inhibition assays. It is important to mention that from the eight rabbits being used in this study, only the sera raised against the mixture of HABPs 34178 (^{121}NIL**MDEIENYVKKYTESNRI**^{140}), 34195 (^{461}CTNIKKYT**DDICLSIKPK**AL^{480}), and 34210 (^{761}KIMQNIQQTTNRLKINIKKIY^{780}) (amino acids in bold correspond to predicted B epitopes) detected the native protein at a low dilution by IFA (1:2) and Western blot (1:20). The high antisera concentration is associated with the widely described poor immunogenic capacity of conserved HABPs derived from several parasite adhesins and highlights the need of modifying conserved sequences to induce high and protective antibody titers.[27,33,41]

The same preadsorbed immune rabbit sera detected two bands of 75 and 56 kDa by Western blotting in a mature parasite's lysate (Figure 4a). Although the expected mass for *Pf*RH4 is ~195 kDa, according to its processing by the *Plasmodium* rhomboid proteases *Pf*ROM-1 and *Pf*ROM-4,[30] the bands detected may correspond to proteolytic fragments from this protein, since no recognition was observed with preimmune sera (Figure 4a). Additionally, anti-*Pf*RH4 antiserum recognized the native form of *Pf*RH4 in mature-schizonts, as indicated by the punctuated fluorescence pattern characteristic of apically localized proteins[16] detected by IFA (Figure 4c−f).

***Pf*RH4 Acts as Ligand in RBC Invasion by Means of Well-Defined Regions.** To evaluate the biological relevance of *Pf*RH4 HABPs, their ability to inhibit RBC invasion by *P. falciparum* merozoites was assessed in vitro. All HABPs showed a moderate to high inhibitory effect (Table 3), which was not affected when RBCs were pretreated with neuraminidase, HABPs 34209, 34234, and 34239 being the ones

Table 3. Invasion Inhibition Ability of *Pf*RH4 HABPs

	invasion inhibition (%)[a]	
	RBCs	RBCs treated
*Pf*RH4 HABPs[b]		
34178	46	49
34190	**51**	**51**
34195	**52**	47
34203	47	49
34204	46	47
34205	45	47
34206	38	**53**
34209	**72**	**71**
34210	**54**	46
34215	**50**	**58**
34223	41	42
34224	43	39
34227	46	**50**
34229	**52**	42
34234	**85**	**88**
34238	46	49
34239	**97**	**97**
34242	48	43
34243	47	46
LABPs[b]		
34174	37	3
34183	39	37
34244	43	40
anti-*Pf*RH4 serum[c]	**95** (1:2) to **85** (1:4)	
chloroquine 1/27 (1.85 mg/mL)	97	
chloroquine 1/54 (0.93 mg/mL)	96	
EGTA (1.9 mg/mL)	90	

[a] Invasion inhibition values of ≥ 50% are shown in bold. All standard deviations were below 3%. [b] Peptides were assessed at a 200 μM. [c] 1:2 and 1:4 anti-*Pf*RH4 serum dilutions were studied.

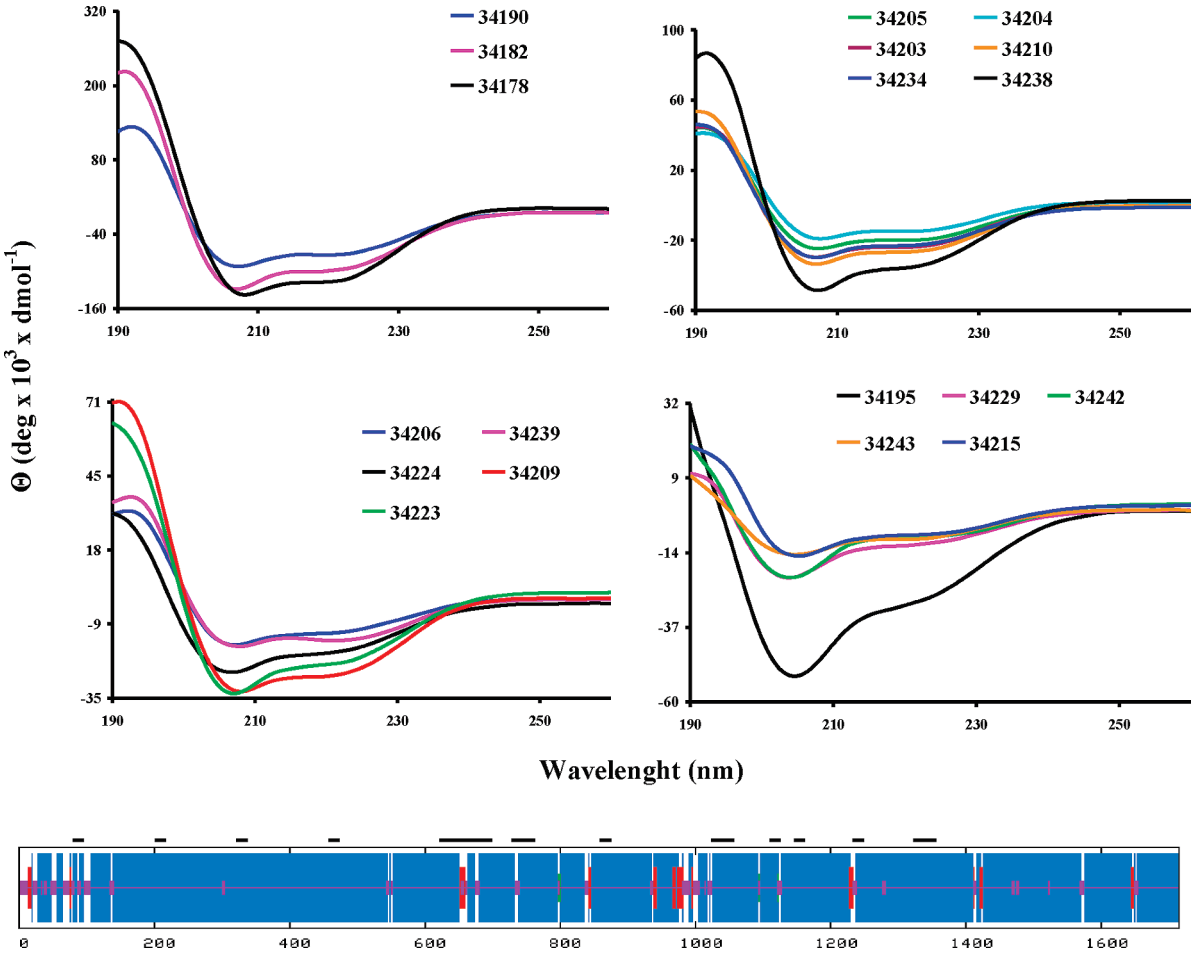


Figure 5. Circular dichroism spectra of *Pf*RH4 HABPs in 30% TFE. HABPs spectra were grouped in order to enable scale appreciation. At the bottom is the SOPMA prediction of the *Pf*RH4 secondary structure showing α-helical (blue), β-turn (red), random coil (violet) elements, and the localization of *Pf*RH4 HABPs (horizontal black bars).

showing higher inhibition values (70−100%). The *P. falciparum* FCB-2 invasion ability to neuraminidase-treated RBCs was slightly reduced in comparison to normal RBCs invasion (data not shown), suggesting that the FCB-2 strain may use both sialic acid-dependent and sialic acid-independent invasion pathways. Anti-*Pf*RH4 sera inhibited parasite infection by up to 95% (Table 3).

Secondary Structure Features of *Pf*RH4 HABPs. The secondary structures of *Pf*RH4 HABPs were studied by CD spectroscopy, finding a significant content of α-helical features in the spectra of all HABPs, which were characterized by the presence of two ellipticity minima at 206 and 221 nm and a maximum at 190 nm (Figure 5), this later being slightly displaced in some HABPs' spectra by the presence of β-turn and random coil elements. These results were consistent with deconvolution analyses using CONTINLL, SELCON, and CDSSTR programs,[42,43] predicting a 50−98% content of α-helical features in the secondary structures of *Pf*RH4 HABPs.

Discussion and Conclusions

Malaria pathology is directly related to the intraerythrocytic development of *Plasmodium* parasites, *P. falciparum* being the causing agent of the more severe form of the disease. Parasite invasion to human RBCs is a complex multistep process involving a large number of receptor−ligand interactions between *P. falciparum* proteins and RBC membrane receptors,[5,31] among which two parasite protein families are known to be actively engaged: the EBL and RH families.[44] In *P. falciparum*, the use of multiple invasion pathways is important for evading the host's immune system and controlling host-cell receptor polymorphism.[7,24] Switching from a sialic acid-dependent to a sialic acid-independent invasion route has been related to the up-regulation of the fourth member of the *Pf*RH family, the *Pf*RH4 protein, a micronemal adhesin implicated in the initial RBC recognition and an essential molecule for merozoite invasion to host cells.[6,16,24,31,45]

In our ongoing search for new candidates to design a fully effective malaria vaccine, we have studied the entire sequence of *Pf*RH4 by using a robust, sensitive, and highly specific receptor−ligand interaction assay. This led us to identify 20 peptides having high specific binding activity to human RBCs (Figure 1a), which establish high affinity interactions (K_d in nanomolar range) with about 40000−400000 binding sites per RBC (Table 1). *Pf*RH4 HABPs are distributed along the protein's noncytoplasmic domain mainly toward the central region, suggesting that these sequences are exposed and therefore are accessible to establish receptor−ligand interactions with RBC membrane proteins during merozoite invasion. Previous studies performed with other members of the *Pf*RH family (*Pf*RH1, *Pf*RH2a, and *Pf*RH2b)[46,47] have shown a similar distribution of HABPs toward the middle of the extracellular domain in these proteins (Figure 1b), strongly suggesting a relevant role for this

extracellular domain in the binding ability of *Pf*RH family members to RBCs.

Interestingly, only two HABPs (34190 ^{361}KHIVNKIQENFKLNQNKYIH^{380} and 34195 ^{461}CTNIKKYTDDICLSIKPKAL^{480}) were found inside the previously described 30 kDa RBC binding region of *Pf*RH4 (amino acids 328−588), against which antibodies capable of inhibiting binding of the native *Pf*RH4 protein were raised, but such antibodies were not capable of inhibiting merozoite invasion.[34] The remaining 18 HABPs identified in this study were located outside such 30 kDa portion, therefore defining additional binding regions involved in protein−protein interactions between the native *Pf*RH4 and RBC membrane proteins. This added to the recent identification of two binding regions, named Rh4.10 (amino acids 28−340) and Rh4.11 (amino acids 233−540), spanning wider portions of *Pf*RH4 and showing a more potent invasion inhibition ability,[48] suggests that different binding regions of *Pf*RH4 must be included in order to obtain a more potent inhibitory effect, as evidenced by the ability of anti-*Pf*RH4 sera obtained by immunization with mixtures of *Pf*RH4 peptides to inhibit merozoite invasion of RBCs in vitro (Table 3). All this highlights the potential of *Pf*RH4 and more specifically of its specific binding regions to be included as candidates in the design of a fully protective vaccine against malaria.

IFA analyses of anti-*Pf*RH4 antiserum showed the recognition of an apical pole-located protein, typically observed as a punctuate pattern (Figure 4c−f). Although 75 and 56 kDa bands were recognized by this antiserum (Figure 4a), differing from the expected 195 kDa corresponding to the processed form of *Pf*RH4,[30] no recognition was observed in preimmune samples; these results indicate that bands are recognized by antibodies specifically directed against *Pf*RH4 HABPs. Taking into account that a BLAST search performed with these *Pf*RH4 inoculated HABPs did not show homology with other *Pf*RHs (data not shown), it is possible that recognized molecules correspond to proteolytic fragments of *Pf*RH4. Taking into account that the *Pf*RH4 sequence herein analyzed corresponds to the one reported for the *P. falciparum* 3D7 strain, the recognition of the native protein in a different strain indicates that peptides derived from *Pf*RH4 can act as immunogens against different parasite populations. Polymorphism studies have demonstrated that ALL extracellular regions of *Pf*RH are highly conserved among different *P. falciparum* strains except for the presence of a few sequence substitutions (Figure 1a, yellow regions),[16,17,20,25,29,49] this being an important aspect to consider given that genetic variability is part of the parasite's specialized immune evasion mechanism. Interestingly, according to our polymorphism studies with the *P. falciparum* FCB-2, FVO, PAS-2, 3D7, HB3, and Dd2 strains (Supporting Information Figures 1 and 2), ALL HABPs are located inside conserved regions of *Pf*RH4, therefore highlighting the relevance of these sequences to overcome the difficulties imposed by the parasite's polymorphism, since it has been demonstrated that specific modifications in the critical binding residues of nonimmunogenic conserved sequences can induce a protective immune response against experimental challenge with *P. falciparum* in *Aotus* monkeys.[27,33,41]

In agreement with these observations, invasion inhibition assays showed that *Pf*RH4 HABPs are able to moderately and in some cases completely prevent RBC infection by *P. falciparum* FBC-2 merozoites in vitro, HABPs 34209, 34234, and 34239 being the more potent inhibitors (Table 3). Low activity binding peptides also showed a moderate inhibition effect, which was weaker than the inhibitory effect of *Pf*RH4 HABPs. Moreover, the inhibitory ability of these HABPs was not affected when neuraminidase-treated RBCs were used in invasion assays, probably as a result of the exquisite variability of alternative invasion pathways comprising sialic acid-dependent and sialic acid-independent infection routes. Indeed, it has been reported that members of the invasion-related EBA and *Pf*RH4 protein families can use either sialic acid-containing receptors or sialic-acid independent receptors, or both receptor types, and no significant changes in the parasite's invasion efficacy have been observed when some of these proteins are genetically disrupted, thereby corroborating the functional redundancy of invasion proteins to use either the same or different invasion pathways.[6,50] Similar inhibition results were obtained with specific anti-*Pf*RH4 sera, reaching >90% inhibition rates with 1:2 and 1:4 serum dilutions, altogether highlighting the relevance of *Pf*RH4 binding regions during *P. falciparum* invasion and their potential as antimalarial targets.

As mentioned above, *Pf*RH4 participates in the multiple RBC invasion pathways of *P. falciparum* parasites as alternative ligand by defining a sialic acid-independent route of infection.[24,31] These observations were confirmed by binding assays with neuraminidase-treated RBCs where all *Pf*RH4 HABPs maintained their high binding ability after removing sialic acid groups from host-cell surface membrane proteins. In contrast, binding of *Pf*RH4 HABPs showed a differential susceptibility to chymotrypsin and trypsin treatments with respect to their binding to untreated RBCs, suggesting interaction with receptors of proteic nature (Table 2). Bindings of HABPs 34203, 34205, 34234, and 34239 were highly susceptible to trypsin treatment, in a similar way to the enzymatic susceptibility described for the putative "X" receptor,[50,51] whereas a high sensitivity to both proteases was observed for HABPs 34206, 34210, 34215, 34224, and 34243, suggesting the recognition of RBC membrane proteins sensitive to these proteases. The sensitivity of HABPs 34195 and 34227 bindings to chymotrypsin treatment indicated interaction with cell membrane proteins containing the aromatic residue targets for this enzyme (tyrosine, tryptophan, and phenylalanine), such as band 3 or the unknown "Z" receptor previously described to interact with *Pf*RH2 (Table 2).[22,50] Interestingly, neuraminidase treatment increased binding of the majority of *Pf*RH4 HABPs (Table 2), which agrees with the reported increment in the binding of recombinant *Pf*RH4 to neuraminidase-treated RBCs.[34]

Further exploration on the RBC membrane receptors by cross-linking assays indicated that some *Pf*RH4 HABPs bind to 32, 25, 22, and 20 kDa molecules on RBC surface (Figure 3). These results, analyzed in light of the binding profile to enzyme-treated RBCs, led us to suggest that although all *Pf*RH4 HABPs could be interacting with proteins of similar molecular weights, a differential behavior against the different enzymatic treatment could also be associated with binding to different regions in the same receptor (i.e., sensitive regions to both and/or each protease). An additional explanation of the cross-linking profile could be related to the possible recognition of cleavage products derived from a 32 kDa RBC membrane receptor protein by *Pf*RH4 HABPs, but more evidence is required to confirm this suggestion. Therefore, it is important to mention that the results of this study provide preliminary data regarding *Pf*RH4 receptor(s), but a complete identification would require

further studies that would allow corroborating or refuting of these suggestions.

CD spectra of *Pf*RH4 HABPs evidenced predominantly α-helical structural features (Figure 5), with deconvolution percentages varying between 50% and 98%. These results are in agreement with the secondary structure predicted for *Pf*RH4 by the self-optimized prediction method from alignment (SOPMA),[52] indicating the presence of α-helix elements in more than 88% of the protein's structure (Figure 5). It has been reported that soluble conserved HABPs derived from proteins involved in the initial merozoite-RBC contact, among which are included proteins from parasite membrane (merozoite surface proteins, MSPs), micronemes (EBAs), and rhoptries (such as the rhoptry-associated protein, RAP, family, and *Pf*RHs), mainly display an α-helical structure, and such association between specific functions and defined structural features appears to be part of a clear functional compartmentalization of proteins involved in RBC invasion, which is related with the formation of a stable major histocompatibility complex molecule II−peptide−T-cell receptor conjugate.[53]

In this work we have described the fine mapping of one of the most important members of the *Pf*RH family, *Pf*RH4. Twenty α-helical peptides conserved among different *P. falciparum* strains and showing neuraminidase-resistant specific binding activity were identified. The majority of these HABPs, as well as anti-*Pf*RH4 antiserum raised in rabbits, were able to inhibit merozoite invasion to RBCs in vitro. Altogether, the results highlight the importance of *Pf*RH4 during *P. falciparum* infection and point to its specific binding regions as new and promising vaccine candidates to be included in further structural and immunological studies with the aim of developing components for a new rationally designed, fully protective, multistage, multiantigenic synthetic vaccine against *P. falciparum* malaria, the raison d'être of our work.

Experimental Section

Chemical Synthesis of *Pf*RH4 Peptides and ^{125}I Radiolabeling. Eighty-six 20-mer-long nonoverlapping peptides spanning the complete sequence of *Pf*RH4 (**PFD1150c**) were synthesized by the solid-phase peptide synthesis methodology using 0.7 mequiv/g 4-methylbenzhydrylamine hydrochloride (MBHA) resin, *t*-Boc protected amino acids (Bachem), and HF-low and high cleavage techniques,[54−56] adding a tyrosine residue to the C-terminal of those peptides not containing this residue in their sequence to enable radiolabeling. Synthetic peptides were purified by RP-HPLC and characterized by MALDI-ToF mass spectrometry. *Pf*RH4 synthetic peptides were named according to our institute's serial numbering system (Figure 1) and were radiolabeled according to previously described methodologies.[57−59] Briefly, an amount of 5 μL of peptide (1 mg/mL) was incubated in HBS buffer (0.01 M HEPES, 0.15 M NaCl, pH 7.4) with 5 μL of Na^{125}I (MP Biomedicals, 100 mCi/mL) and 15 μL of 13.45 μM chloramine-T. After 15 min had elapsed, the reaction was stopped by adding 15 μL of 14 μM $NaHSO_3$; radiolabeled peptides were purified by size exclusion chromatography (Sephadex G-10 column, Pharmacia) and analyzed in a γ counter (Auto Gamma Counter Cobra II, Packard).

Binding Assays of *Pf*RH4 Peptides to RBCs. To perform binding assays, 1×10^8 RBCs obtained from healthy donors were incubated by triplicate with four increasing concentrations of each radiolabeled peptide (0−560 nM) in the presence (nonspecific binding) or absence of unlabeled peptide (total binding). RBCs were then washed with HBS buffer.[57,59,60] On the basis of cell-associated radioactivity data and previously described criteria,[28,59,60] peptides having a specific binding activity of ≥2%, defined as the slope of the specific binding curve (specific binding = total binding − nonspecific binding) between the amount of radiolabeled peptide binding specifically to RBCs and added peptide at four increasing concentrations, were denoted as high-activity binding peptides (HABPs) (Figure 1a). Once HABPs were identified, scrambled peptides conserving the same amino acid composition but having a totally random sequence order were synthesized and their binding activities were also determined by following the same methodology (Figure 2).

To determine the kinetic constants of *Pf*RH4 HABPs, 7.5×10^7 RBCs were incubated in triplicate with a wider range of peptide concentrations (0−2200 nM), in the absence or presence of 6 nmol unlabeled peptide. Same as in binding assays, cells were washed with HBS and analyzed in a γ counter.[57,61]

Extraction and Purification of *P. falciparum* Genomic DNA and PCR Amplification. With the aim of characterizing the polymorphism in *Pf*RH4 binding sequences, genomic DNA (gDNA) of *P. falciparum* FCB-2, FVO, and PAS-2 strains was isolated and analyzed by PCR and sequence analysis. In brief, parasites were cultured as described elsewhere[62,63] to obtain parasitized RBCs (30% parasitemia). Cells were lysed using 0.2% saponin, and gDNA was purified using the UltraClean DNA blood isolation kit (MO BIO, Carlsbad, CA). The following five specific primer sets were designed with Gene Runner, version 3.05, based on the *Pf*RH4 gene sequence reported in the 3D7 reference strain (**PFD1150c**): (1) *Pf*RH4-f1 (5′-ATATACAAAATGGCCAAAAGC-3′) and *Pf*RH4-r1 (5′-TATGAGGTTTGATATTATGTTC-3′) amplifying the gene segment encoding HABPs 34178 and 34182; (2) *Pf*RH4-f2 (5′-ATATAATATTCTTAATGCAGATC-3′) and *Pf*RH4-r2 (5′-CGTATATATTATTCATTTCTTTG-3′) amplifying the gene segment encoding HABPs 34190 and 34195; (3) *Pf*RH4-f3 (5′-ATACTTTTAATATGTATTATAACG-3′) and *Pf*RH4-r3 (5′-AGAGTATTATAATTGTTTAGTTC-3′) amplifying the gene segment encoding HABPs 34203, 34204, 34205, 34206, 34209, 34210, and 34215; (4) *Pf*RH4-f4 (5′-GCATCAAACAAACATATAATAAT-3′) and *Pf*RH4-r4 (5′-TCATTATTAATGTTTTGAAATTTC-3′) amplifying the region encoding HABPs 34223, 34224, 34227, 34229, and 34234; (5) *Pf*RH4-f5 (5′-GAAATTTCAAAACATTAATAATGA-3′) and *Pf*RH4-r5 (5′-TTATCATGTCATTTGTCTCAC-3′) amplifying the gene segment encoding HABPs 34237, 34238, 34239, 34242, and 34243. The DIR1/REV1 primers amplifying the gene segment encoding HABP 33577 of the *P. falciparum* integral membrane protein *Pf*25-IMP[64] were included as positive PCR control.

Genomic DNA of *P. falciparum* FCB-2 (Colombian), FVO (Vietnamese), and PAS-2 (unknown origin) strains (2 μL) was used as template for PCR amplification in 50 μL PCR mixes containing 1 U of 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 initial denaturation at 95 °C for 5 min followed by 35 cycles of 1 min of annealing at 56 °C for *Pf*RH4-f1/ *Pf*RH4-r1, *Pf*RH4-f2/ *Pf*RH4-r2, *Pf*RH4-f3/ *Pf*RH4-r3, *Pf*RH4-f4/ *Pf*RH4-r4, and *Pf*RH4-f5/ *Pf*RH4-r5 or at 58 °C for DIR1/REV1; 1 min of extension step at 72 °C and 1 min of denaturing at 95 °C. Final extension was carried out at 72 °C for 5 min. Water instead of DNA was used as reaction control. Amplicons were visualized in 1% agarose gels stained with SYBR safe (Invitrogen, Eugene, OR), purified using the Wizard PCR preps kit (Promega, Madison, WI), and sequenced using their corresponding forward and reverse primers.

Binding Assays with Enzyme-Treated RBCs. To analyze the nature of the RBC membrane receptors for *Pf*RH4 HABPs, human RBCs (60% hematocrit) were independently treated with neuraminidase (150 μU/mL), chymotrypsin (1 mg/mL), or trypsin (1 mg/mL) for 1 h at 37 °C. Binding assays were carried out with these treated RBCs (20% hematocrit), same as

described before, and compared to binding results with untreated RBCs (100% binding).[57]

***Pf*RH4 HABPs Cross-Linking Assays.** Radiolabeled HABPs were incubated with 2.1×10^7 RBCs and cross-linked with 50 μL of bis(sulfosuccinimidyl)suberate (BS3, 1 mg/mL; Pierce) for 1 h at 4 °C, adding Tris-HCl buffer to stop the reaction. Cross-linked proteins were extracted with 15 μL of lysis buffer (5 mM Tris-HCl buffer, 7 mM NaCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)), diluted in 15 μL of Laemmli buffer, spun at 15000*g* for 15 min, and separated by 12% SDS−PAGE using prestained protein molecular weight markers (MWM) (New England Bio Laboratories). Autoradiograms were developed by exposing this gel to a radiation-sensitive film for 15−20 days.[57,58]

Production of Anti-*Pf*RH4 Rabbit Sera. Eight New Zealand rabbits nonreactive to *P. falciparum* lysate were subcutaneously inoculated with mixtures of the following peptides (two rabbits per mixture): 34178−34195−34210, 34182−34206−34234, 34190−34204−34224, and 34229−34239−34243, some of them containing B-cell epitopes predicted by different servers: ABC (http://www.imtech.res.in/raghava/abcpred/),[65] EMBOSS antigenic (http://bioinfo.bgu.ac.il/cgi-in/emboss.pl?_action=input&_app=antigenic),[66] BCPREDS (http://ailab.cs.iastate.edu/bcpred/index.html)[67] and BcePred (http://www.imtech.res.in/raghava/bcepred/).[68] Immunization mixtures also contained a T-helper cell determinant peptide from sperm whale myoglobin (FIS, ^{106}FISEAIIHVLHSR^{118}). Rabbits were inoculated with peptide mixtures emulsified with Freund's complete adjuvant on day 0 and incomplete adjuvant on days 20 and 40. Serum samples were collected on days 0 (preimmune), 20 (I-20), and 40 (II-20) for evaluating antibody production. A final bleeding was carried out on day 60 (III-20) for collecting anti-*Pf*RH4 serum. Immunizations as well as bleedings were done in accordance with the animal handling guidelines stipulated by the Colombian Ministry of Public Health. Rabbit sera were adsorbed with *Escherichia coli* and *Mycobacterium smegmatis* lysates and SPF66 vaccine peptides, individually coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech), as described elsewhere.[69,70] Briefly, each serum (5 mL) was added to Sepharose affinity columns (5 mL) and left in a gently rotating/shaking mode for 30 min at room temperature. This procedure was done twice using a new lysate-coupled Sepharose affinity column each time. Antisera were stored at −70 °C until use.

Indirect Immunofluorescence Assay (IFA) and Western Blot Analysis. Late-stage schizonts from a synchronized continuous culture of the *P. falciparum* FCB-2 strain (*falciparum* Colombia Bogota-2) at 10% parasitemia[62,63] were isolated and washed with sterile PBS (phosphate buffer, containing 0.015 M NaCl, pH 7.2) for IFA analysis. Infected RBCs were suspended in fetal bovine serum (FBS) and PBS (1:1, v/v) and left to dry on glass slides. Parasites were blocked for 10 min with 1% skimmed milk and incubated for 30 min with preimmune or III-20 immune rabbit sera (1:2). Reactivity was visualized by fluorescence microscopy using purified antirabbit IgG-FITC conjugate F(ab)2 fragment at a 1:5 dilution.[64] Preimmune rabbit sera were used as negative controls.

The reactivity of anti-*Pf*RH4 sera was analyzed against a lysate of mature *P. falciparum* FCB-2 trophozoites and schizonts. Briefly, an amount of 500 μg/mL of parasite lysate was separated in a discontinuous gel using a 7.5−15% (w/v) acrylamide gradient and transferred to a nitrocellulose membrane (Hybond 203c, Pharmacia) by the semidry blotting technique. The membrane was blocked with 5% skimmed milk in Tris buffered saline−Tween 0.05% (TBS-T) for 1 h and washed thrice. Preimmune and III-20 sera diluted 1:20 in blocking solution were individually incubated with independent membrane strips. After five washes with TBS-T, strips were incubated for 1 h with 1:5000 alkaline phosphatase-conjugated antigoat IgG antibody (ICN Biomedicals). The immunoreaction was developed using NBT/BCIP (KPL, Gaithersburg, MA).[64]

Merozoite Invasion Inhibition in Vitro. *Pf*RH4 HABPs' ability to inhibit invasion of *P. falciparum* merozoites (FCB-2 strain) to RBCs and neuraminidase-treated RBCs was assessed by incubating schizont-synchronized parasite cultures[62] in the presence of each HABP (100 and 200 μM) in triplicate. After incubation at 37 °C for 18 h (5% O_2, 5% CO_2, and 90% N_2 atmosphere), culture supernatants were harvested and cells were washed with PBS for hydroethidine labeling. Labeled cell suspensions were analyzed in a FacsCalibur flow cytometer (FACsort, FL2 channel). EGTA- and chloroquine-treated infected RBCs were used as inhibition controls and healthy RBCs as invasion control.[71] Similar assays were also performed using rabbit anti-*Pf*RH4 antiserum, following the same protocol.

Circular Dichroism Spectroscopy. Circular dichroism spectra of each HABP (5 μM) was determined in 30% v/v trifluoroethanol (TFE), using a 1 cm optical path length quartz cell thermostated at 20 °C. Spectra were acquired in a Jasco J-810 (JASCO Inc.) equipment by averaging three sweeps taken at 20 nm/min. Data were processed by using Spectra Manager software[42] and analyzed with CONTINLL, SELCON, and CDSSTR deconvolution programs.[42,43]

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Supporting Information Available: Polymorphism analyses of *Pf*RH4 binding regions by PCR and ClustalW alignment. This material is available free of charge via the Internet at http://pubs.acs.org.

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