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Synthetic peptides from conserved regions of the *Plasmodium falciparum* early transcribed membrane and ring exported proteins bind specifically to red blood cell proteins

Jeison Garcia[a,b], Hernando Curtidor[a,b], Ana Z. Obando-Martinez[a,b], Carolina Vizcaíno[a,b], Martha Pinto[a,b], Nora L. Martinez[a,b], Manuel A. Patarroyo[a,b], Manuel E. Patarroyo[a,c,*]

[a] *Fundación Instituto de Inmunología de Colombia FIDIC, Carrera 50 No. 26-20, Bogotá, Colombia*
[b] *Universidad del Rosario, Calle 63 D No. 24-31, Bogotá, Colombia*
[c] *Universidad Nacional de Colombia, Carrera 45 No. 26-85, Bogotá, Colombia*

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ABSTRACT

Severe malaria pathology is directly associated with cytoadherence of infected red blood cells (iRBCs) to healthy RBCs and/or endothelial cells occurring during the intraerythrocytic development of *Plasmodium falciparum*. We synthesized, as 20-mer long peptides, the members of the ring exported (REX) protein family encoded in chromosome 9, as well as the early transcribed membrane proteins (E-TRAMP) 10.2 and 4, to identify specific RBC binding regions in these proteins. Twelve binding peptides were identified (designated as HABPs): three were identified in REX1, two in REX2, one in REX3, two in REX4 and four in E-TRAMP 10.2. The majority of these HABPs was conserved among different *P. falciparum* strains, according to sequence analysis. No HABPs were found in E-TRAMP 4. Bindings of HABPs were saturable and sensitive to the enzymatic treatment of RBCs and HABPs had different structural features, according to circular dichroism studies. Our results suggest that the REX and E-TRAMP families participate in relevant interactions with RBC membrane proteins, which highlight these proteins as potential targets for the development of fully effective immunoprophylactic methods.

1. Introduction

The life cycle of *Plasmodium falciparum*, the most lethal malarial pathogen which affects 3.2 billion people living in endemic areas, causing 500 million cases and more than 1 million deaths per year [1,2], comprises several processes of vital importance for disease development. The most severe complications associated with malaria (anemia and cerebral, renal, vascular and placental malaria, among others) are caused during the parasite's intraerythrocytic development as a result of multiple receptor-ligand interactions of infected red blood cells (iRBCs) with healthy RBCs and capillary vessels (cytoadherence), which induces aggregation of these cells, phenomena known as rosetting and clumping, respectively [3–6].

In consequence, *Plasmodium* proteins exported to the host cell membrane such as the members of the *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1) family and the knob-associated histidine-rich protein (KAHRP) are attractive immunological targets due to their role in these phenomena [7,8]. Interestingly, monoclonal and polyclonal anti-KAHRP antibodies are known to disrupt rosette formation, even though this protein is located in the inner face of iRBC knobs [9]. Previous reports have related protection against cerebral malaria with the activity of anti-rosetting antibodies, highlighting the importance of this intracellular protein as an attractive antimalarial immunoprophylactic target [9–12] and supporting the need of studying ALL parasite proteins, directly or indirectly involved in host–pathogens interactions, to find novel and fully effective immunoprophylactic methods.

The analysis of the *P. falciparum* genome has revealed that deletions on chromosome 9 are associated with lack of cytoadherence to CD36 endothelial cell receptors, reduction of gametocytogenesis and strain-specific agglutination by hyperimmune sera [13], indicating that genes located on this chromosome and their expression products can be related with these events, all of which are associated with symptoms of severe malaria and parasite survival.

One of these important gene families encoded by the right arm of the *P. falciparum* chromosome 9 is the ring exported (REX) protein family. This family comprises four transcripts named REX1, REX2, REX3 and REX4, which are present in all stages of the intraerythro-

* Corresponding author at: Fundación Instituto de Inmunología de Colombia, Carrera 50 No. 26-00, Bogotá, Colombia. Tel.: +57 1 4815219; fax: +57 1 4815269.
E-mail address: mepatarr@mail.com (M.E. Patarroyo).

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cytic development, and whose genes are expressed mainly within the first 20 h after RBC invasion occurs (early ring stage) [13–15].

REX1 is an 83 kDa protein comprising 713 amino acids that is located at the Maurer's clefts [14], which are organelles involved in the trafficking of many proteins to RBC membrane, including *Pf*EMP protein family members, KAHRP and other important virulence-associated molecules [6,16–18]. The *rex1* gene consists of a 189-bp exon followed by a 183-bp intron and a second larger exon (1953 bp). The protein contains a set of degenerate repeats in its second half, a coiled-coil domain between amino acids 181 and 302, a transmembrane domain (aa 37–59), three low complexity regions and a region rich in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST sequence) located at the N-terminus and related with targeting of proteins to their site of processing [3,14].

Same as REX1, the 10-kDa REX2 protein is also located at the Maurer's clefts, whereas REX3 (38 kDa) and REX4 (26 kDa) are soluble proteins exported into the host cell cytoplasm [15]. Low complexity regions and a single transmembrane domain are common features of all REX proteins, except for REX4. Additionally, REX3 and REX4 contain a *Plasmodium* export element (PEXEL) motif, which is a short amino acid sequence necessary for the export of parasite proteins into the host cell cytoplasm [15,19,20]. Nevertheless, a clear function has not been yet described for this protein family.

It is important to mention that all REX proteins are encoded by genes consisting of two exons separated by an intron, being one exon larger than the other one [15], which is a feature commonly observed in important protein families exported to the membrane of infected RBCs, such as *Pf*EMPs, histidine-rich proteins (HRPs), the repetitive interspersed family (RIF), subtelomeric variable open reading frame proteins (STEVOR) and surface-associated interspersed (SURFIN) proteins, among others [20–23].

Together with the REX protein family, another set of 13 proteins, named early transcribed membrane proteins (E-TRAMP), is expressed during early ring stages and trafficked across the parasitophorous vacuole membrane (PVM). This family of highly cationic proteins shares a common structure consisting of a signal peptide followed by a short (~20 aa) cationic domain, a transmembrane (Tm) region and a highly charged C-terminal domain. Several E-TRAMPs contain coiled-coil domains flanking the Tm, as in the case of E-TRAMP 10.2 [24].

Different studies have demonstrated that E-TRAMPs are located at the PVM, a structure derived from host cell membrane taken by the parasite during RBC invasion. The PVM has been described to play a key role in the survival and propagation of intracellular *Apicomplexa* pathogens such as *Toxoplasma*, *Eimeria*, *Theileria* and *Plasmodium*, among others, being at the same time a barrier and a gate for the passage of nutrients, waste products and signals [24–28]. Some studies suggest that the PVM surrounds each individual merozoite during the schizont stage, either partially or completely, being the PVM apparently integrated into the cluster of newly formed merozoites [29]. Furthermore, it has been demonstrated that the cysteine protease inhibitor E64 prevents PVM rupture, producing PVM-enclosed merozoite structures (PEMS), which are also detected in untreated control cultures. These results suggest that merozoites are enclosed within the PVM during host cell rupture, from which they rapidly escape by a proteolysis-dependent mechanism [30].

Taken together, these observations indicate that proteins associated with the PVM, despite not being directly involved with RBC invasion, could be interesting targets for developing therapeutic and immunoprophylactic methods. Some E-TRAMPs localized at the PVM define distinct domains for the distribution of the *P. falciparum* exported protein-1 (EXP-1), which has been shown in PVM oligomeric arrangements together with E-TRAMP 2, E-TRAMP 10.1 and E-TRAMP 4 [31].

Among the E-TRAMP family members, E-TRAMP 4 and E-TRAMP 10.2 are transcribed throughout the *P. falciparum* intraerythrocytic cycle [24], suggesting an important role in parasite survival. The importance of studying ALL molecules involved, either directly or indirectly, in parasite survival has been evidenced by the discovery of new vaccine candidates against malaria, such as the intracellular *P. falciparum* ribosomal phosphoprotein P0 (PfP0), which induces production of polyclonal antibodies that block merozoite invasion [32], as well as protection against experimental challenge with *Plasmodium yoelii* in mice [33,34]. These facts, together the recognition of E-TRAMPs by sera of naturally infected patients [24], point at these proteins as possible antimalarial targets.

In the continuous search for new candidates to be included in a minimal subunit-based, multi-stage, multi-epitopic chemically synthesized vaccine against malaria, our institute has developed a highly robust, specific and sensitive methodology that allows identifying the regions of different pathogen proteins that specifically interact with their corresponding host cell (*Mycobacterium tuberculosis*, Epstein-Barr virus, Hepatitis C virus, *Plasmodium* spp., among others) [35–41]. Herein, we have identified and characterized amino acid sequences derived from the E-TRAMP and REX proteins having high specific binding activity to RBCs. The peptides so identified could be potential new candidates for the development of new and more effective antimalarial vaccines and therapeutic alternatives against this worldwide public health problem.

2. Materials and methods

2.1. Peptide synthesis and radiolabeling

The entire amino acid sequences of *P. falciparum* (3D7 strain) REX1 (**PFI1735c**), REX2 (**PFI1740c**), REX3 (**PFI1755c**), REX4 (**PFI1760w**), E-TRAMP 10.2 (**PF10_0323**) and E-TRAMP 4 (**PFD1120c**) were synthesized as 20-mer long peptides by the solid-phase multiple peptide system [42,43], using MBHA resin (0.5 meq/g), *t*-Boc amino acids (Bachem) and low-high HF cleavage techniques [44]. Peptides were purified by RP-HPLC and then analyzed by MALDI-TOF mass spectrometry (Autoflex, Bruker Daltonics). A Tyr residue was added to the C-terminus of peptides that did not contain this residue in their sequence to enable radiolabeling. The REX1 peptides spanning the repeat regions 460–480 and 500–580 were not synthesized since their sequences were identical to peptides 33935 and 33936. Radiolabeling of synthetic peptides was carried out according to previously described techniques [45–47], adding 5 μL $Na^{125}I$ (100 mCi/mL, MP Biomedicals) and 15 μL chloramine T (2.75 mg/mL) to 5 μL of peptide (1 mg/mL). After 15 min, the reaction was stopped by adding 15 μL sodium metabisulfite (2.25 mg/mL). A Sephadex G-10 column (80 mm × 5.0 mm, Pharmacia, Uppsala, Sweden) was used to isolate radiolabeled peptides.

2.2. Cell binding assays

RBCs were obtained by venipuncture from healthy donors, washed several times with isotonic HEPES buffered saline (HBS), spun at 1000 × *g* for 5 min (at room temperature) and taken to a final hematocrit of 20%. Cells were incubated in the absence (total binding) or presence (non-specific binding) of an excess of unlabeled peptide (20 μM) to determine the slope of the specific binding activity curve. Increasing amounts of radiolabeled peptide (0–560 nM) were tested, according to a methodology previously described by us [21,22]. After 90 min, cells were washed twice with isotonic HBS. All assays were carried out in triplicate. The amount of radiolabeled peptide bound to RBCs was determined using a

Cobra II auto-gamma counter (Packard). Peptides showing a ratio of radiolabeled peptide specifically bound to RBCs/added radiolabeled peptide greater than or equal to 2% (0.02 pmol of bound peptide per mol of added peptide) were considered as HABPs, according to a previously established criteria [38,46,48].

2.3. Extraction, purification and PCR amplification of P. falciparum genomic DNA

RBCs infected with *P. falciparum* FCB-2 (Colombian), FVO (Vietnamese) and PAS-2 (unknown origin) strains were obtained from an asynchronous culture and maintained as described elsewhere [49,50]. Genomic DNA (gDNA) was extracted from 200 μL cultures of each parasite strain at 30% parasitemia using 0.2% saponin. gDNA was then purified using the UltraClean DNA Blood Isolation kit (MO BIO, Carlsbad, CA).

The genes encoding REX 1, 2, 3, 4 and E-TRAMP 10.2 in the *P. falciparum* 3D7 strain (**PFI1735c** (REX1), **PFI1740c** (REX2), **PFI1755c** (REX3), **PFI1760w** (REX4) and **PF10_0323** (E-TRAMP 10.2)) were analyzed using Gene Runner v3.05 in order to design specific primer for the region encoding HABPs. The sequences of the five primer sets were (1) *rex1*-f (5′-AAAGGAGAATGCCAACTCG-3′) and *rex1*-r (5′-TCATATTTTGTTCATCTTGTTC-3′), for the region encoding HABPs 33919, 33925 and 36069. (2) *rex2*-f (5′-GATACATTATACATTTTTAAACC-3′) and *rex2*-r (5′-ATGTTCCTAATGTTGTTGTTG-3′), for the region encoding HABPs 33781 and 33782. (3) *rex3*-f (5′-CTTTTCTTTTTTATTTTTGTATTC-3′) and *rex3*-r (5′-GAGGTGGTATATTTATAGTTG-3′) for the region encoding HABP 33945. (4) *rex4*-f (5′-CATGCTTGCGAAGAATTGC-3′) and *rex4*-r (5′-AACTTTTCCATTCTTCATCAC-3′) for the region encoding HABPs 33965 and 33969. (5) *e-tramp10.2*-f (5′-CCTTGTAGTATGCCACTTC-3′) and *e-tramp10.2*-r (5′-TTATTCTTTTCTGACTCTTGG-3′) for the region encoding HABPs 33882, 33885, 33891 and 33897. The *rex2*-f and *rex3*-f were designed 45 and 85 bp upstream the initiation codon, respectively. DIR1 and REV1 primers amplifying the region encoding HABP 33577 of the *P. falciparum* integral membrane protein *Pf*25-IMP were included as positive PCR control [51].

gDNA from *P. falciparum* FCB-2, FVO and PAS-2 strains (2 μL) was used as template for PCR amplification. PCR mixtures (50 μL) contained: 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 initial denaturation at 95 °C for 5 min followed by 35 cycles of 1 min annealing at 56 °C for *rex1*-f/*rex1*-r, *rex2*-f/*rex2*-r, *rex3*-f/*rex3*-r, *rex4*-f/*rex4*-r and *e-tramp10.2*-f-*e-tramp10.2*-r or 58 °C for DIR1/REV1, 1-min elongation at 72 °C and 1-min denaturing at 95 °C; final elongation was carried out at 72 °C for 5 min. Distilled water was used instead of DNA as reaction control. Amplification products 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.

2.4. Cloning

Purified PCR products of *e-tramp 10.2* were cloned into the pGEM-T vector (Promega, Madison, WI) and used to transform *E. coli* JM109 (Promega, Madison, WI). Insertion of the cloned fragment was verified by colony PCR using the vector-annealing primers T7 and SP6. Plasmid DNA was then extracted from recombinant colonies using the Miniprep purification kit (Promega, Madison, WI).

Five recombinant clones positively confirmed by PCR were sequenced in an automatic sequencer (ABI PRISM 310 Genetic Analyzer, PE Applied Biosystems, Foster City, CA). The nucleotide and amino acid sequences of the regions analyzed in FCB-2, FVO and PAS-2 (the three strains included in this study) were aligned with the sequence reported in the 3D7, HB3 and Dd2 strains using Clustal W software [52].

2.5. Determining binding constants

According to previously described methods [45–47], saturation assays were carried out for all HABPs. RBCs (1.5×10^7 cells) were incubated with increasing concentrations of radiolabeled peptide (0–1600 nM) for 90 min at 18 °C, in the presence or absence of 24 μM unlabeled peptide. After incubation, unbound peptide was rinse using isotonic HBS. Same as before, each assay was performed in triplicate and cell-associated radiation was quantified using a gamma counter. The curves obtained were analyzed by saturation analysis and Hill equation [47,48].

2.6. Cross-linking assays with radiolabeled HABPs

Cross-linking experiments were performed to recognize the receptor molecules for some HABPs by incubating 4.2×10^6 RBCs with radiolabeled peptide for 90 min at room temperature. Bound peptide was cross-linked by incubating cell with 50 μL bis-(sulfosuccinimidyl) suberate (BS^3, 1 mg/mL) for 1 h at 4 °C. Tris–HCl buffer was added to stop the reaction, samples were centrifuged at $1000 \times g$ by 5 min and the supernatant was discarded. Membrane proteins cross-linked with radiolabeled peptides were solubilized in 15 μL of 5 mM Tris–HCl buffer (containing 7 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) and 15 μL Laemmli buffer, and then spun at $15{,}000 \times g$ for 15 min. The supernatant was separated in 12% SDS-PAGE and exposed on radiation-sensible films during 15 days at −70 °C. Apparent molecular weights were determined by using molecular weight markers ranging from 6.5 to 175 kDa (New England-BioLabs) [51].

2.7. RBCs enzymatic treatment and binding assays

RBCs (60% hematocrit) suspended in HBS were enzymatically treated with either neuraminidase (ICN 9001-67-6) at ∼150 μU/mL, trypsin (Sigma T-1005) or chymotrypsin (Sigma C-4129) at 1 mg/mL for 60 min at 37 °C. HABP binding assays with enzyme-treated RBCs (2.0×10^7 cells/μL) were performed in triplicate. Binding to untreated RBCs considered as 100% binding control [53].

2.8. Invasion inhibition in vitro assay

P. falciparum intra-RBC schizont-stages (FCB-2 strain) obtained from a synchronized parasite culture (5% final parasitemia and 5% hematocrit) [50] were incubated with 200, 100 and 50 μM concentrations of HABPs. Each peptide was analyzed by triplicate. Following incubation for 18 h at 37 °C in a 5% O_2, 5% CO_2 and 90% N_2 atmosphere, the supernatant was discarded and cells were labeled by incubation with 15 μg/mL hydroethidine for 30 min at 37 °C. Excess of hydroethidine was washed with PBS and cells were analyzed in FacsCalibur flow cytometer (FACsort, FL2 channel) equipped with CellQuest software, using infected RBCs and uninfected RBCs treated with EGTA and chloroquine as controls [54].

2.9. Circular dichroism (CD) analysis

CD was performed to determine HABP's secondary structure features. Aqueous solution of 5 μM peptides in 30% v/v trifluoroethanol (TFE) were analyzed on a 1-cm optical path length quartz

cell thermostated at 20 °C. Spectra were obtained by averaging three sweeps taken at a 20 nm/min scan rate, on nitrogen-flushed on Jasco J-810 equipment using a 2-nm bandwidth. Data were processed using Spectra Manager software [55,56]. A computational analysis was performed for each CD spectra employing SELCON3, CONTINLL and CDSSTR deconvolution programs [56,57].

3. Results

3.1. REX and E-TRAMP families screening

The sequences of REX1, REX2, REX3 and REX4 were synthesized as 20-mer non-overlapping peptides in order to identify peptides binding with high activity to *P. falciparum* host cell (Fig. 1). After determining the specific binding activity of these sequences at 4 different logarithmic concentrations of radiolabeled peptide, those having a ratio of specifically bound ^{125}I-peptide/added ^{125}I-peptide larger than 2% were considered HABPs, according to previously established criteria [46,48,58]. Fig. 1 shows the sequences of all peptides and their localization in each protein, where the ratio of specifically bound ^{125}I-peptide/added ^{125}I-peptide express as percentage is represented by the length of the black bar in front of its sequence and the 2% cut-off point (0.02 specific binding) is presented as a discontinuous line.

The following are the names (which were assigned according to our institute's serial numbering system) and sequences of the HABPs identified in REX proteins. In REX1, peptides 33919 ^{101}IEGNKSGNGEEHNKHKRRQV^{120}; 33925 ^{221}LHKKLQEQLQRLKEQEKKKV^{240} and 36069 ^{281}RDLQVQLRHIQQRIQSLKST^{300}, corresponded to HABPs (Fig. 1a). Two HABPs were identified in REX2, named 33781 ^{1}MKMYLAEIFSSGKESLLSLK^{20} and 33782 ^{21}DTLGSSNFSPLKPCEGLECL^{40} (Fig. 1b), while REX3 contained only HABP 33945 ^{1}MQTRKYNKMLSKVETKQFIY^{20} (Fig. 1c), and REX4 presented HABPs 33965 ^{61}REININKNIPSYPVKFSKLE^{80} and 33969 ^{141}INEQKMKFKNFLHKLKEKRI^{160} (Fig. 1d).

In E-TRAMP 10.2, HABPs 33882 ^{41}EQDLQQKKNRKRNLILYSLG^{60}, 33885 ^{101}EKPAEKKKTTVKIVSKRVPVY^{120}, located in N-terminal region were found together with HABPs 33891 ^{221}TPTESSHGISDGKKDTSTNDY^{240} and 33897 ^{331}LRDSSGRSSGRSTTPRVRKEY^{350}, which are located in the central and C-terminal regions, respectively (Fig. 1e). There were no HABPs in E-TRAMP 4, suggesting that this protein could be fulfilling a role in parasite survival that does not involve its interaction with RBC membrane receptors.

3.2. DNA amplification and strain-specific polymorphism analysis

Amplification products of the region encoding the REX1, 2, 4, and E-TRAMP 10.2 HABPs in the three *P. falciparum* strains were visualized on agarose gels, detecting single bands of about (1) 791 bp, (2) 432 bp, (4) 471 bp and (5) 1033 bp, respectively, which corresponded to the expected sizes. It was not possible to amplify the region encoding REX3 HABP and therefore such sequences could not be analyzed. Control primers amplified a single band of about 438 bp, which corresponded also to the sizes expected for *Pf*25-IMP (Fig. 2).

The amino acid alignment of the sequences encoding HABPs in the FCB-2, FVO and PAS-2 strains and the 3D7, HB3 and Dd2 reference strains showed the following substitutions (see supplementary material for a full alignment):

Four non-synonymous substitutions were found in REX1, two of which lie outside the region encoding REX1 HABPs. These substitutions were (1) one mutation from C to T in nucleotide position 500 in 3D7 where the TCT triplet corresponding to residue 106 encodes for a serine (S) in 3D7, FVO, PAS-2, HB3 and Dd2 strains, while triplet TTT encodes for a phenylalanine (F) in the FCB-2 strain.

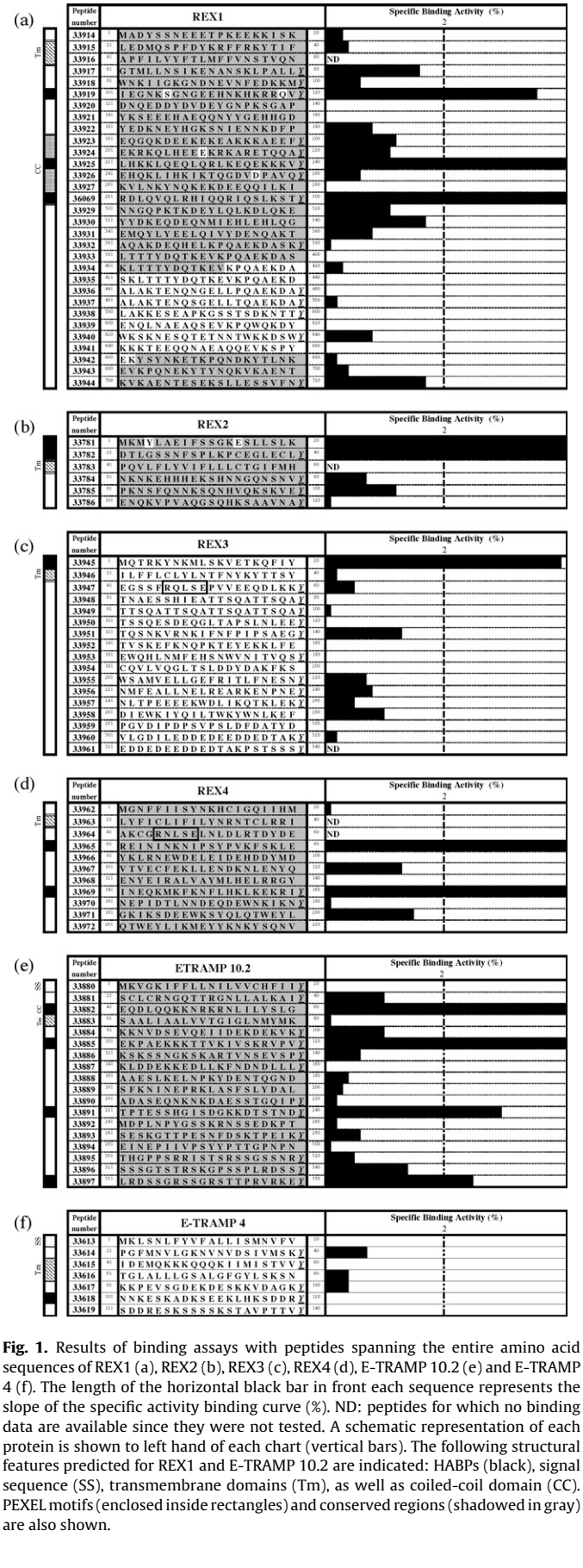

Fig. 1. Results of binding assays with peptides spanning the entire amino acid sequences of REX1 (a), REX2 (b), REX3 (c), REX4 (d), E-TRAMP 10.2 (e) and E-TRAMP 4 (f). The length of the horizontal black bar in front each sequence represents the slope of the specific activity binding curve (%). ND: peptides for which no binding data are available since they were not tested. A schematic representation of each protein is shown to left hand of each chart (vertical bars). The following structural features predicted for REX1 and E-TRAMP 10.2 are indicated: HABPs (black), signal sequence (SS), transmembrane domains (Tm), as well as coiled-coil domain (CC). PEXEL motifs (enclosed inside rectangles) and conserved regions (shadowed in gray) are also shown.

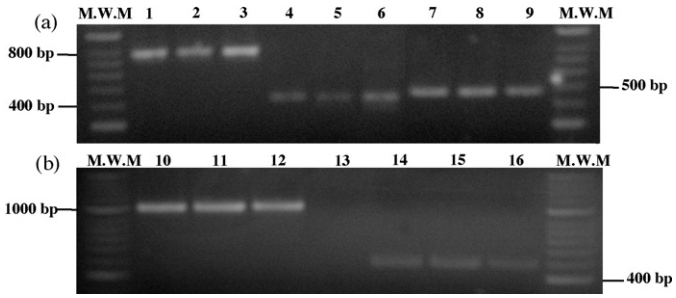

Fig. 2. PCR amplification of the regions encoding HABPs in REX1, 2, 4 and E-TRAMP 10.2. (a) Lanes 1–3: genomic region encoding REX1 HABPs 33919, 33925 and 36069 in the *P. falciparum* FCB-2, FVO and PAS-2 strains, respectively. All PCR products were loaded into the gel maintaining the same strain order. Lanes 4–6: region encoding HABPs 33781 and 33782 of REX2. Lanes 7–9: region encoding HABPs 33965 and 33969 in REX4. (b) Lanes 10–12: region encoding HABPs 33882, 33885, 33891 and 33897 in E-TRAMP 10.2. Lane 13: negative control. Lanes 14–16: positive control (Pf25-IMP) for the FCB-2, FVO and PAS-2 strains, respectively. MWM: 100-bp molecular weight marker.

(2) A mutation from A to C in nucleotide position 538 in 3D7 where triplet AAA corresponding to residue 119 encodes for a lysine (K) in FCB-2, FVO, PAS-2, HB3 and Dd2 strains while triplet CAA encodes for a glutamine (Q) in the 3D7 strain. (3) A mutation from G to A in nucleotide position 811 in 3D7, where triplet GAA corresponding to the residue 210 encodes for a glutamic acid (E) in the 3D7, FVO, PAS-2, HB3 and Dd2 strains, while triplet AAA encodes for a lysine (K) in the FCB-2 strain. (4) The same type of substitution was found also in position 949, where the GAT triplet corresponding to residue 256 encodes for an aspartic acid (D) in the 3D7, FVO, PAS-2, HB3 and Dd2 strains while AAT triplet encodes for an asparagine (N) in the FCB-2 strain. The first and second non-synonymous substitutions corresponded to residues 6 and 19 of HABP 33919.

Two substitutions were found in REX2. The first one was a mutation from T to A in nucleotide position 10 where triplet TAT corresponding to the residue 4 in 3D7 encodes for a tyrosine (Y) in 3D7, FCB-2 and PAS-2 while triplet AAT encodes for an asparagine

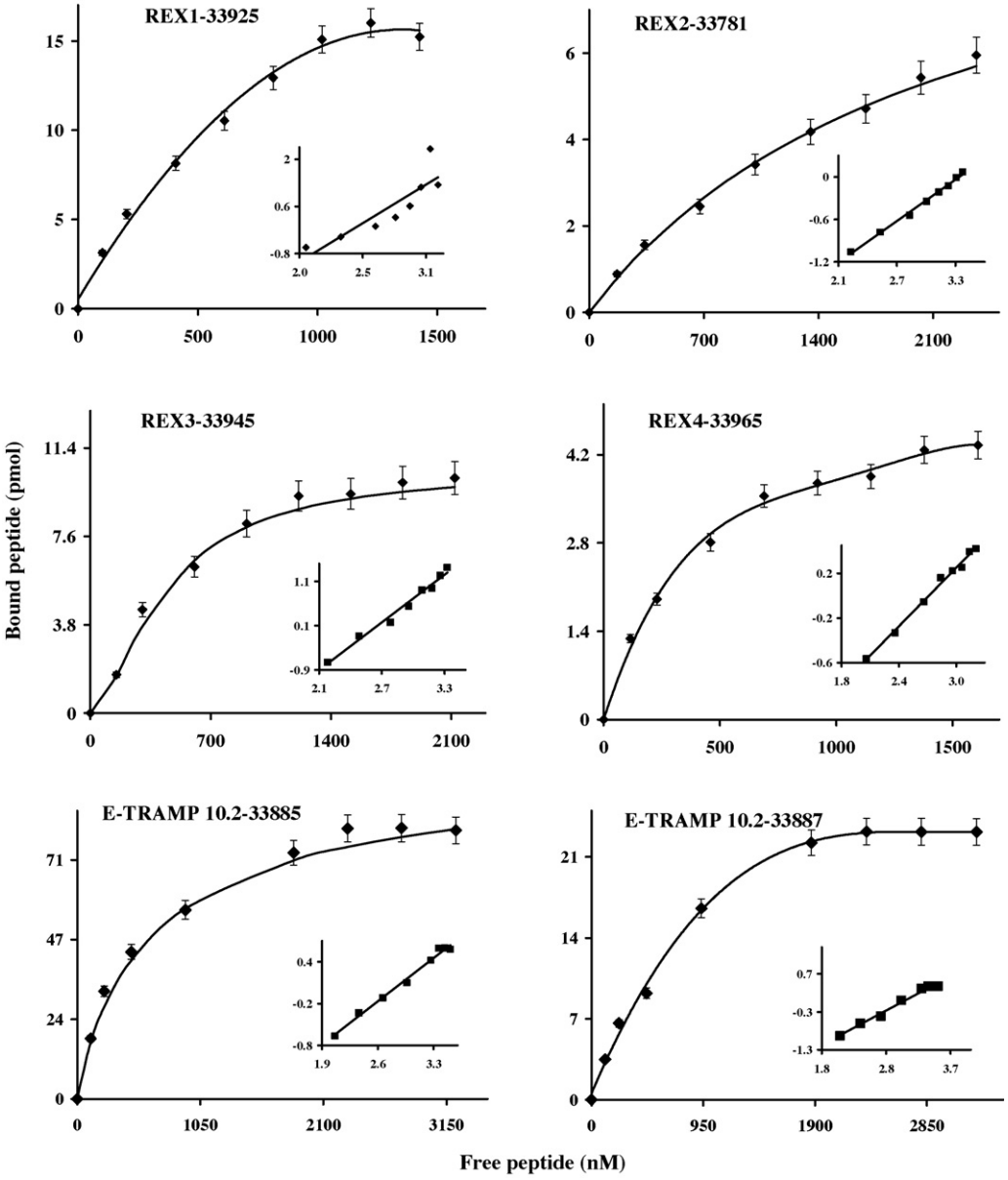

Fig. 3. Saturation assays with REX and E-TRAMP 10.2 HABPs. Each curve represents specific binding. In the Hill graph (inset), the abscissa is $\log F$ and the ordinate $\log (B/B_{max} - B)$, being F the amount of free peptide, B the amount of bound peptide and B_{max} the maximum amount of bound peptide. Standard deviations were below 15% in all cases.

Table 1
Binding constants of the HABPs found in the E-TRAMP and REX protein families. Dissociation constants (K_d), Hill coefficients (nH) and binding sites per cell (BSC) were obtained from saturation assays.

	HABP	Binding constants		
		K_d (nM)	n_H	BSC
REX1	33919	880	1.5	160,610
	33925	340	2.1	129,300
	36069	290	1.9	48,900
REX3	33945	440	1.6	84,320
REX4	33965	500	0.8	48,190
	33969	600	1.3	224,860
E-TRAMP 10.2	33882	900	1.0	465,780
	33885	700	0.9	803,070
	33897	900	0.7	265,010

(N) in the FVO and HB3 strains. The second one, a mutation from A to C in nucleotide position 41, where triplet GAG corresponding to the residue 14 in 3D7 encodes for a glutamic acid (E) in 3D7 strain while triplet GCG encodes for an alanine (A) in the FVO, HB3, PAS-2 and FCB-2 strains.

Regarding REX4 and E-TRAMP 10.2, the amino acid sequence alignment of the region encoding HABPs in these two proteins showed a 100% identity among the different *P. falciparum* strains. Nucleotide sequences were also 100% identical, indicating that there are no synonymous substitutions within the studied region of these strains (data not shown).

3.3. Binding constants of HABPs

Saturation assays were performed to determine the dissociation constant (K_d), Hill coefficient (n_H) and total binding sites per cell (BSC) of REX and E-TRAMP 10.2 HABPs. K_d values of all HABPs were within nanomolar range, nH ranged from 0.7 to 2.1 and BSC between 40,000 and 800,000 (Fig. 3 and Table 1). REX2 HABPs 33781 (Fig. 3) and 33782 (data not shown) were not saturable under the assay conditions.

3.4. Binding of HABPs to enzyme-treated RBCs

Human RBCs were treated with neuraminidase, chymotrypsin and trypsin to evaluate how such treatments affected the binding of HABPs (Table 2). Non-treated RBCs were used as control of total binding (100%). Regarding REXs-derived peptides, incubated of RBCs with neuraminidase affected binding of HABP 33782, binding of HABP 33919 to trypsin-treated RBCs was reduced compared to the control, while HABPs 33781 and 36069 binding was sensible to treatments with trypsin and chymotrypsin [59]. The interaction of HABPs 33925 and 33945 with RBC was susceptible to all enzymatic treatments.

Table 2
Effect of preincubating RBCs with neuraminidase (N), chymotrypsin (Ch) or trypsin (T) on the binding percentage of the HABPs derived from the REX and E-TRAMP proteins. Binding to non-treated RBCs (C) was considered as 100% binding, with all standard deviations being below 7%. Binding activities larger than to 50% are shown in bold.

	HABP	% Binding		
		N	Ch	T
REX1	33919	92	68	44
	33925	**27**	69	26
	36069	125	**37**	23
REX2	33781	72	**23**	**49**
	33782	**0**	116	77
REX3	33945	**25**	**29**	**38**
REX4	33965	72	**0**	**54**
	33969	60	83	**38**
E-TRAMP 10.2	33882	**58**	**66**	112
	33885	102	119	95
	33891	**0**	91	**9**
	33897	79	190	**54**

Binding of E-TRAMP 10.2 HABP 33891 was highly sensitive to treating RBCs with neuraminidase and trypsin, while HABP 33897 binding to trypsin-treated RBCs was reduced by 46% and increased when RBC were treated with chymotrypsin. In addition, binding of peptide 33882 was affected by treatment with neuraminidase and chymotrypsin. No enzymatic treatment affected binding of HABP 33885.

3.5. HABPs recognize different RBC membrane proteins

Cross-linking of HABP-RBC membrane receptor(s) and SDS-PAGE was performed to analyze the molecular weight of RBC membrane receptor (s) for some REX HABPs. The autoradiogram of cross-linking assays with REX2 HABP 33782 and REX3-HABP 33945 let us to identify three proteins of about 70, 51 and 29 kDa. REX4-HABP 33969 recognized these same proteins plus an additional band of approximately 38 kDa (Fig. 4). Recognition diminished in presence of each unlabeled peptide, indicating specific interactions with RBC membrane receptors.

3.6. Secondary structure analysis by CD

CD spectra of HABPs in presence of TFE 30% allowed determining their secondary structural elements. Spectra of some REX and E-TRAMP 10.2 HABPs presented a minimum at 201–202 nm, which is indicative of the presence of random-coil and β-turn features. HABPs 33925 and 36069 (both derived

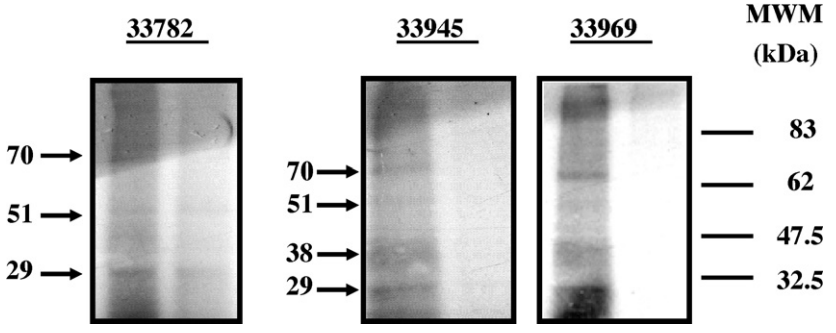

Fig. 4. Autoradiograms of REX2, REX3 and REX4 HABPs. The molecular weight marker (MWM) is shown to the right side.

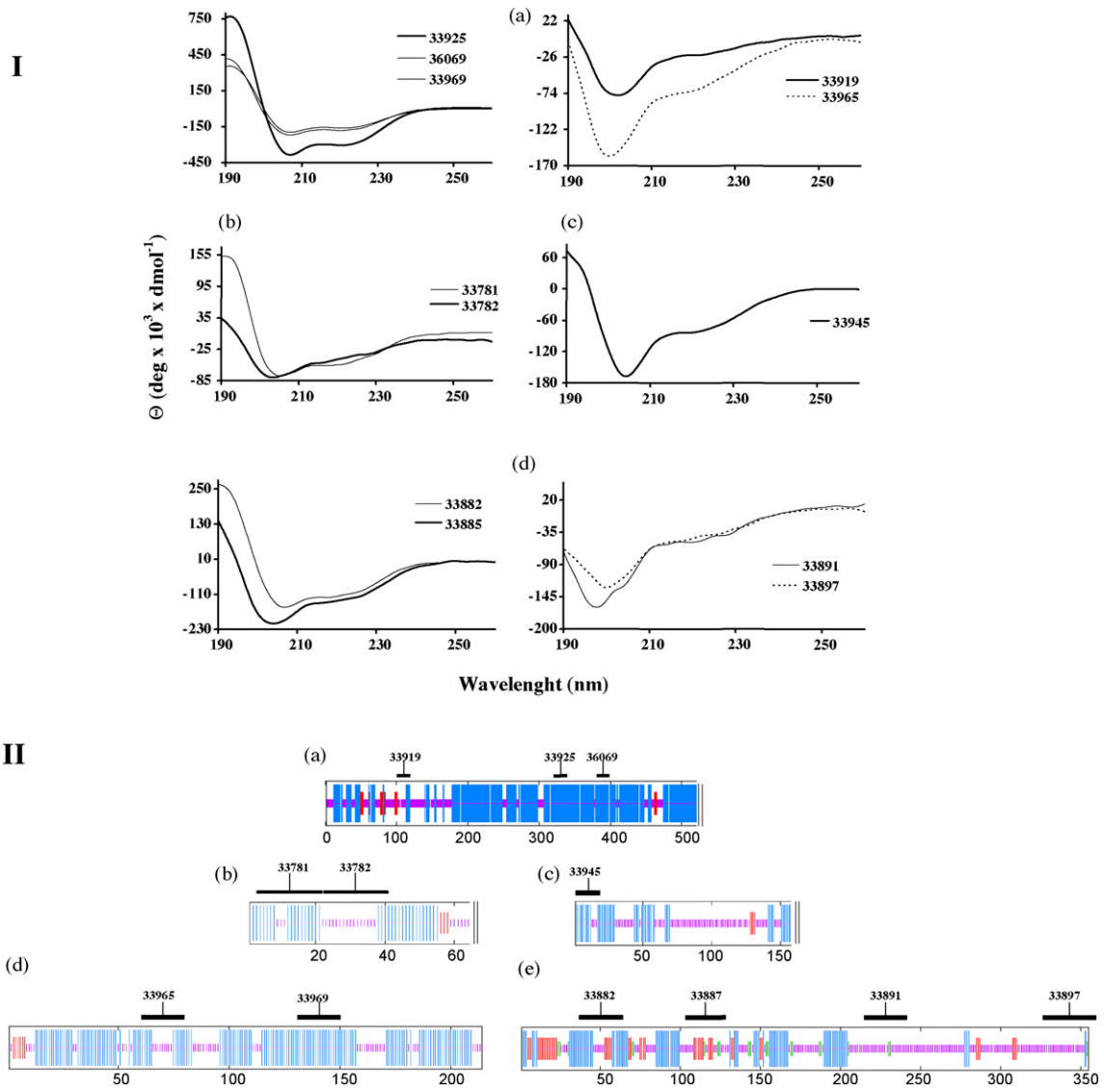

Fig. 5. (I) CD studies for HABP of REX1 and REX4 (a), REX2 (b), REX3 (c), and E-TRAMP 10.2 (d). The spectra of each HABP dissolved in 30% TFE were recorded between 260 and 190 nm. (II) Position of HABPs with respect to the secondary structure prediction obtained for REX1 (a), REX2 (b), REX3 (c), REX4 (d) and E-TRAMP 10.2 (e) using SOPMA software (http://npsa-pbil.ibcp.fr) (black horizontal bars). Vertical bars correspond to α-helical (blue), β-turn (red) and random-coil (violet) features. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

from REX1), 33781 (REX2), 33969 (REX4) and 33885 (E-TRAMP 10.2) displayed typical α-helical components, characterized by two minima at 207 and 221 nm. CD spectra are shown in Fig. 5.

3.7. *Merozoite invasion is moderately inhibited by REX and E-TRAMP HABPs*

Invasion inhibition assays were performed with some REX and E-TRAMP HABPs, finding a moderate inhibition rate (20–35%) of *P. falciparum* FCB-2 strain invasion to healthy erythrocytes, when compared to the effect of chloroquine and EGTA effect (positive inhibition controls) (Table 3). Assays carried out low activity binding peptides showed no significant inhibition activity (<5% invasion inhibition, data not shown).

4. Discussion

Cytoadherence and rosette-forming characteristics of *P. falciparum* are directly associated with severe malaria pathology caused by this species, which is responsible for the largest number of deaths caused by this disease. During the intraerythrocytic cycle (comprising ring, trophozoite and schizont-stages), export of several proteins of *P. falciparum* parasites beyond its plasma membrane (PM) and the parasitophorous vacuole membrane (PVM), leads to

Table 3
Invasion inhibition assays with REX and E-TRAMP HABPs at 200 μM concentration. Standard deviations were lesser than 3% in all cases.

	HABP	Invasion inhibition (%)
REX1	33919	23
	33925	23
REX2	33781	23
REX3	33945	25
REX4	33965	34
E-TRAMP 10.2	33882	36
	33897	36
Chloroquine (285 μM)		75
EGTA (500 μM)		65

extensive modifications of the host cell's cytoplasm and membrane [5,6,14]. It has been suggested that the apparent lethargy of *P. falciparum* parasites during ring stages might be associated with induction of modifications in the iRBC needed for the parasite to grow and evade being removed from the bloodstream circulation (sequestration), as has been observed in mature intraerythrocytic forms [14,15]. Accordingly, those proteins involved in parasite invasion and survival inside RBCs could be important targets for developing more potent and effective immunoprophylactic methods. REX proteins comprise a family of molecules whose genes are encoded on *P. falciparum* chromosome 9 and are expressed exclusively during the ring stage [14,15]. E-TRAMP proteins are expressed together with this family during early stages of the parasite's intraerythrocytic development and exported to PVM [24], where they have been suggested to fulfill important roles for parasite survival. In this article, we determined which sequences of REX proteins, E-TRAMP 4 and E-TRAMP 10.2 have high specific binding activity to RBCs, considering that these sequences could be relevant for parasite development.

The screening of the complete sequences of REX proteins by means of a robust, highly sensitive and specific binding assay identified eight HABPs; three were found in REX1, two in REX2, one in REX3 and finally two in REX4. In REX1, HABP 33919 is located after the Tm domain, while HABPs 33925 and 36069 lie inside a predicted coiled-coil domain (see amino acids 181–302 in Fig. 1), and therefore could be participating in protein–protein interactions and be involved in numerous cellular processes, including regulation of gene expression and protein signaling [60].

HABPs identified in REX2 and REX3 are located at the N-terminal region, where no signal peptides or cleavage sites are predicted for REX proteins by SignalP [61]. The two HABPs identified in REX4 are located at the central region, following a predicted Tm domain. There were no HABPs inside the PEXEL motifs located at the N-terminal regions of REX3 and REX4, neither in the entire sequence of E-TRAMP 4. E-TRAMP 10.2 contains four HABPs, one of them (33882) is localized at the protein's N-terminus before the Tm domain and inside a predicted coiled-coil domain [24], while the other three HABPs are located towards the protein's C-terminal region (Fig. 1). Binding of the majority of E-TRAMP and REX HABPs were saturable, which allowed determining that these HABPs establish high affinity interactions (as indicated by their nanomolar K_d) with around 40,000–800,000 binding sites per cell (BSC), being REX1 HABP 36069 the one showing the highest affinity (K_d 290 nM). Moreover, such binding interactions are of either negative, positive nor non-cooperative nature, as indicated by n_H values between 0.7 and 2.1 obtained from saturation assays (Fig. 3 and Table 1).

An important aspect for antimalarial vaccine development is to be able to tackle the exquisite polymorphism used by *P. falciparum* parasites to evade the host's immune system response. Our results show that the nucleotide sequence of the majority of REXs and E-TRAMP 10.2 HABPs are highly conserved among the *P. falciparum* 3D7, FCB-2, FVO, PAS-2 and HB3 strains (Fig. 2), which is a highly relevant finding considering that the immunological properties of conserved HABP (i.e. conserved HABP have been shown not to induce antigenicity, immunogenicity nor protection in *Aotus* monkeys against challenge with *P. falciparum*), are completely altered when their critical host cell binding residues are modified by other having same mass and volume but opposite polarity [12,62,63]. Only HABP 33919 (REX1) and HABP 33781 (REX2) were found to vary among the different parasite strains.

REX1 and REX2 have been described as important structural components of the Maurer's clefts, which are organelles involved in trafficking of parasite proteins to iRBC membrane [15,64,65]. Recently, Dixon et al. [64] reported that the N-terminal region plus 10 amino acids are necessary for exporting REX1 into the host cell cytoplasm, and that the predicted coiled-coil region is vital for targeting this protein to the Maurer's clefts. These findings strongly suggest that the two HABPs located in coiled-coil region of REX1could be involved in anchoring or interaction processes between Maurer's clefts and iRBC membrane, which is also in agreement with the recently described importance of REX1 in the architecture of these organelles [65]. Similar to REX-1, the Maurer's cleft-associated REX2 protein appears to be also interacting with host cell membrane proteins, probably fulfilling an important function for Maurer's cleft interactions with the membrane of infected RBCs [15].

Furthermore, it has been recently reported by pre-clinical trial and bioinformatics analysis that conserved sequences contained inside α-helical coiled-coil domains are promissory vaccine targets [66,67], which further highlights the importance of REX-1 HABPs 33925 and 36069, as well as of E-TRAMP 10.2 HABP 33882, all of which are predicted to lie inside such highly relevant domains.

Additionally, the possible biological relevance of the HABPs herein identified was studied by employing the BcePred server (http://www.imtech.res.in/cgibin/bcepred/bcepred.pl), which predicts B-cell epitopes based on the hydrophilicity, flexibility/mobility, accessibility, polarity, exposed surface and turns of an amino acid sequence. The results indicated that all HABPs found in E-TRAMP 10.2, HABPs 33919 and 33925 of REX1, and 33945 of REX3 contain potential B epitopes. This prediction, together with the highly specific binding ability of these HABPs to RBCs, support their inclusion in further immunogenicity and protection studies aimed at determining their protective ability against experimental challenge with malaria in an appropriate animal model such as the *Aotus* monkey.

The enzymatic treatment of RBCs impaired the binding capacity of HABPs, finding different sensitivities to neuraminidase, chymotrypsin or trypsin for each peptide (Table 2). Preincubation of RBCs with trypsin and chymotrypsin reduced binding of REX HABPs 33781, 33965 and 36069, but no effect was observed upon preincubation with neuraminidase; being this enzyme profile consistent with sialic acid-independent receptors [59]. Likewise, binding of REX1 HABP 33919 and E-TRAMP 10.2 HABP 33897 was susceptible to treatment with trypsin, suggesting a non-glycosylated receptor of proteic nature such as the unknown "X" receptor [59,68]. On the contrary, binding of the REX2 HABP 33782 was completely abolished by preincubating RBCs with neuraminidase, thus indicating a high degree of dependence for this HABP to glycosidic residues on its membrane receptor, such as has been reported for the "Y" receptor [59,69]. Neuraminidase and trypsin reduced binding of E-TRAMP 10.2 HABP 33891; a behavior that has been documented in molecules interacting with glycoproteins such as RBC glycophorins. [59]. Treatment with proteases and neuraminidase affected binding of REX HABPs 33925, 33945 and the E-TRAMP 10.2 HABP 33882, implying a possible interaction of these peptides with either glycosylated and non-glycosylated membrane proteins (Table 2). All these results suggest that the REX family and E-TRAMP 10.2 interact with different RBC membrane receptors in a sialic acid-dependent or independent way.

The results of cross-linking assays agreed with the enzymatic binding profiles of HABPs, revealing proteins of 70, 51, 38 and 29 kDa as the possible receptors for each REX HABP. REX2 HABP 33782, whose binding was sensitive to neuraminidase, recognizes RBC membrane proteins of 70, 51 and 29 kDa (Fig. 4). These results suggest the possible interaction of this peptide with glycoproteins, such as glycophorins A (52 kDa) and B (25 kDa), and with RBC membrane protein of about 70 kDa. On the other hand, REX4 HABP 33969 showed a preferential binding to trypsin-sensitive receptor(s) and interacted with neuraminidase-sensitive RBC membrane components to a lesser extent (Table 2); while REX3 HABP 33945, whose binding was sensitive to all enzymatic treatments, recognized the

same bands detected by REX2 HABP, plus an additional band of 38 kDa (Fig. 4). Altogether, these results suggest different proteins as the possible RBC membrane receptors for these peptides, such as for instance the putative "X" receptor and glycophorins A, B and C (38 kDa) [59]. However, it is important to mention that although our results strongly suggest these RBC membrane proteins as the possible receptors for REX HABPs, further studies are needed in order to identify the precise nature of the host cell membrane receptors that interact with these peptides and hence confirm such suggestions.

The majority of HABPs, i.e. REX HABPs 33919, 33782, 33965 and E-TRAMP 10.2 HABP 33882, displayed β-turn and/or random-coil structural elements, as indicated by CD studies (Fig. 5). Interestingly, HABPs 33782 (REX2) and 33945 (REX3) located at the N-terminus previous to the Tm domain of REX2 and a PEXEL motif of REX3, respectively, display β-turn or unordered structural characteristics (Fig. 5Ib and c); while HABPs 33925, 36069 (REX1), 33781 (REX2), 33969 (REX4) and 33885 (E-TRAMP 10.2) display typical α-helical features (Fig. 5Ia, d and e), being such results in total agreement with the functional and structural compartmentalization of proteins involved in invasion and survival of *P. falciparum* that was proposed by Reyes et al. [70]. CD spectra deconvolution using SELCON3, CONTINLL and CDSSTR programs confirmed the above observations, since deconvolutions indicated the presence of α-helical features in more than 70% in HABPs 36069 (REX1) and 33969 (REX4), and a high content of random-coil structures in HABPs 33782 and 33945 (∼40% random). REX2 HABP 33781 located at the N-terminus contains a 72% of α-helical features. Similar observations were drawn from the deconvolution results of E-TRAMP 10.2 HABPs. Additionally, the secondary structure predicted by using self-optimized prediction method from alignment (SOPMA) [71] (Fig. 5II, http://npsa-pbil.ibcp.fr) is in agreement with CD studies of all HABPs. The characterization of the immunogenic properties of these HABPs would provide additional hints about the structure-immunogenicity relationship between the formation of an appropriate T cell receptor-peptide-major histocompatibility complex class II (TCR-pMHC II) conjugate, which is an important aspect to consider when searching for a rational and logical vaccine development methodology [70].

Additionally, the biological role of some REX and E-TRAMP HABPs was evaluated in a merozoite invasion inhibition assay using sorbitol-synchronized *P. falciparum* cultures (Table 3). Interestingly, HABPs inhibit RBC invasion by 30–36% at a 200 μM, whereas low binding peptides had no inhibitory effect. Even though REX proteins and E-TRAMP 10.2 are mainly implicated in the intraerythrocytic development of parasites, these results suggest that HABPs derived from these proteins could be establishing with highly specific and saturable interactions with important RBC membrane receptors, which are probability mediating phenomena such as cytoadherence, rosetting, clumping, etc.

Our results complement the characterization of the E-TRAMP and REX families in *P. falciparum*, and moreover suggest that these proteins might be involved in vital processes for parasite survival, such as protein trafficking and parasite's interaction with the membrane of iRBCs. It would be therefore important to test the immunogenicity of E-TRAMP 10.2 and REX HABPs once their critical binding amino acids have been specifically modified in such a way as to generate a protective immune response against experimental challenge with *P. falciparum* in *Aotus* monkeys [12,62,63]. Such studies would allow determining the potential of these peptides as components for the design of more potent and fully effective prophylactic antimalarial methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2009.09.009.

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