Identification of an Erythrocyte Binding Peptide from the Erythrocyte Binding Antigen, EBA-175, Which Blocks Parasite Multiplication and Induces Peptide-Blocking Antibodies

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A biotylated peptide covering a sequence of 21 amino acids (aa) from the erythrocyte binding antigen (EBA-175) of Plasmodium falciparum bound to human glycophorin A, an erythrocyte receptor for merozoites, as demonstrated by enzyme-linked immunosorbent assay (ELISA) and to erythrocytes as demonstrated by flow cytometry analysis. The peptide, EBA(aa1076–96), also bound to desialylated glycophorin A and glycophorin B when tested by ELISA. The peptide blocked parasite multiplication in vitro. The glycophorin A binding sequence was further delineated to a 12-aa sequence, EBA(aa1085–96), by testing the binding of a range of truncated peptides to immobilized glycophorin A. Our data indicate that EBA(aa1085–96) is part of a ligand on the merozoite for binding to erythrocyte receptors. This binding suggests that the EBA(aa1085–96) peptide is involved in a second binding step, independent of sialic acid. Antibody recognition of this peptide sequence may protect against merozoite invasion, but only a small proportion of sera from adults from different areas of malaria transmission showed antibody reactivities to the EBA(aa1076–96) peptide, indicating that this sequence is only weakly immunogenic during P. falciparum infections in humans. However, Tanzanian children with acute clinical malaria showed high immunoglobulin G reactivity to the EBA(aa1076–96) peptide compared to children with asymptomatic P. falciparum infections. The EBA(aa1076–96) peptide sequence from EBA-175 induced antibody formation in mice after conjugation of the peptide with purified protein derivative. These murine sera inhibited EBA(aa1076–96) peptide binding to glycophorin A.

Several Plasmodium falciparum proteins play a role in merozoite invasion of erythrocytes (2, 15). Among these, the proteins that participate in the sequence of events leading to invasion include MSP-1, which possibly mediates initial contact between merozoites and erythrocytes, and EBA-175, a microfilarial protein, which binds to erythrocytes and may be involved in junction formation.

EBA-175 may bind to erythrocytes via two mechanisms: an initial binding, which is dependent on sialic acid, and a secondary binding, which is not dependent on sialic acid. A conserved region of 42 aa of EBA-175, EBA-peptide 4(1062–1103), has been implicated in the binding to the erythrocyte (16), although it is not essential for the initial sialic acid-dependent binding (17). We have synthesized peptides from this putative erythrocyte binding region of EBA-175 and used them for identification of the minimum peptide sequence mediating attachment to erythrocytes. This peptide binding is not dependent on sialic acid. We also report that the erythrocyte binding sequence is recognized by IgG antibodies of children with acute malaria but not by IgG antibodies of children with asymptomatic infections nor by IgG antibodies of adults living in regions of malaria transmission. Antibodies to EBA (aa1076–96) can be induced in mice by immunization.

Abbreviations used in this paper: aa, amino acids; EBA, erythrocyte binding antigen; ELISA, enzyme-linked immunosorbent assay; Fmoc, fluorenylmethoxycarbonyl; HOBt, hydroxybenzotriazol; HPLC, high-pressure liquid chromatography; Ig, immunoglobulin; MBHA, methylbenzhydrylamine; MSP-1, merozoite surface protein 1; NMM, N-methylmorpholine; NMP, N-methylpyrrolidone; OD, optical density; PBS, phosphate-buffered saline; PPD, purified protein derivative; SD, standard deviation; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

Synthetic peptides. The sequences of the synthetic peptides synthesized are as follows. PMSP-1 peptide has the sequence YSLFQKEKMVL, a sequence included in the malaria vaccine SPf66. The EBA-175 peptides contain overlapping amino acid sequences from the EBA-175 region 1062 to 1104: EBA(aa1076–96) peptide; TLTKEYEDIVL; EBA(aa1085–96) peptide; YEDIVLKSHMNRESDD; EBA(aa1086–96) peptide; DI EBA(aa1087–96) peptide; VLKSHMNRESDD; and EBA(aa1088–96) peptide; VLKSHMNRESDD.

MATERIALS AND METHODS

The sequences of the synthetic peptides synthesized are as follows. PMSP-1 peptide has the sequence YSLFQKEKMVL, a sequence included in the malaria vaccine SPf66. The EBA-175 peptides contain overlapping amino acid sequences from the EBA-175 region 1062 to 1104: EBA(aa1076–96) peptide; TLTKEYEDIVL; EBA(aa1085–96) peptide; YEDIVLKSHMNRESDD; EBA(aa1086–96) peptide; DI EBA(aa1087–96) peptide; VLKSHMNRESDD; and EBA(aa1088–96) peptide; VLKSHMNRESDD.

The EBA-175 peptides covered a sequence reported to be involved in the malaria vaccine SPf66. The EBA-175 peptides contain overlapping amino acid sequences from the EBA-175 region 1062 to 1104: EBA(aa1076–96) peptide; SN NEYKVNREDERTLTKEYEDIVL; EBA(aa1079–96) peptide; TLTKEYEDIVL; EBA(aa1080–96) peptide; YEDIVLKSHMNRESDD; EBA(aa1081–96) peptide; YEDIVLKSHMNRESDD; EBA(aa1082–96) peptide; YEDIVLKSHMNRESDD; EBA(aa1083–96) peptide; YEDIVLKSHMNRESDD; EBA(aa1084–96) peptide; YEDIVLKSHMNRESDD; EBA(aa1085–96) peptide; YEDIVLKSHMNRESDD; EBA(aa1086–96) peptide; YEDIVLKSHMNRESDD; and EBA(aa1087–96) peptide; YEDIVLKSHMNRESDD. The EBA-175 peptides covered a sequence reported to be involved in erythrocyte binding, while the PMSP-1 peptide is included in the SPf66 vaccine (14) and has been reported to bind to erythrocytes (1).

Peptides were synthesized automatically on MBHA resins (Novabiochem; 0.1 to 0.5 mg/g) with a Rink-A (42, 44-dimethoxyphenyl-Fmoc-amino methyl) phenoxacycatriamido link. Automatic syntheses were performed on a Mark-III machine under continuous-flow conditions with conductivity monitoring (Schafer-N). Amino acids were amineprotected with Fmoc and activated for 10 min just before coupling by using the free acids with TBTU-HOBt-NMM (1:1:2 equivalents compared to amino acid; NMM used at 0.4 M in NMP). The peptides were biotinylated, after removal of the last Fmoc group, by using biotin-TBTU-HOBt-NMM in NMP with the molecular amounts given above until a negative ninhydrin test (9) was obtained.

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The finished peptide was cleaved by treatment of the solid-phase-coupled peptide with 95% trifluoroacetic acid in water (5 ml) for 10 mg of resin. Incubation took place in a closed container with shaking at room temperature for 30 min; then another 5 ml was added, and the mixture was incubated for 30 min. This was repeated six times. The resin was washed five times with 20 volumes of hexane, five times with 20 volumes of cold methanol, and five times with 20 volumes of water. The liberated peptide was precipitated from the trifluoroacetic acid-water mixture with cold diethyl ether, filtered through a 0.45-μm-pore-size filter, dissolved in water, freeze-dried, and analyzed by C30 reverse-phase HPLC with diode-array detection by using Varian Star Chromatography software data handling for calculation of within-peak purity factors and total purity at 220 nm. To reproduce the results, an additional batch of peptides was synthesized in the same way. The peptides were subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry on a Fisons VG Tofspec E apparatus with associated hardware, with α-cyano-4-hydroxycinnamic acid as the matrix to confirm masses. Peptide concentrations were determined by quantitative HPLC with full-length EBA(aa1076–96) peptide as the standard.

**Evaluation of synthetic peptides.** Peptide purities were ascertained through HPLC purification and within-peak evaluation with diode array detection. The peaks were >95% pure, and all had good purity factors. Mass spectrometry confirmed the predicted molecular mass within 3 Da.

**EBAbinding to erythrocytes.** Biotinylated EBA(aa1076–96) peptide showed a strong concentration-dependent binding to glycophorin A immobilized on polystyrene plates compared to biotinylated peptides EBA(aa1086–1104) and EBA(aa1062–86) as well as the PIMSP-1 peptide (Fig. 1a). Similar binding of biotinylated EBA(aa1076–96) peptide to human erythrocyte ghosts was detectable in four different experiments (data not shown). Saturation of peptide binding to glycophorin A was obtained with concentrations exceeding 8 μM biotinylated EBA(aa1076–96) peptide. Figure 1b shows that native EBA-175 inhibited the binding of biotinylated EBA(aa1076–96) peptide to glycophorin A; 9% of the peptide binding was not blocked by native EBA-175 and appears to be nonspecific background binding. To test the specificity of the peptide binding to glycophorin A, we also tested peptide binding to desialylated glycophorin A and to glycophorin B immobilized on polystyrene plates in addition to glycophorin A. EBA(aa1076–96) peptide showed similar binding to all glycophorin preparations tested (data not shown), indicating that peptide binding is not specific for glycophorin A and is not dependent on sialic acid.

To further identify the glycophorin binding sequence, we produced 11 truncated EBA(aa1076–96) peptide variants containing between 20 and 10 aa (see Materials and Methods). Biotinylated truncated peptides EBA(aa1077–96) to EBA(aa1085–96) showed strong binding to glycophorin A, while truncated peptides (EBA(aa1086–96) and EBA(aa1087–96) showed no or little binding activity. For simplicity, Fig. 2 shows the results obtained with peptides EBA(aa1085–96), EBA(aa1086–96), and EBA(aa1087–96) only. Our data indicate that the peptide sequence VLKSHMNRESDDD encompasses the glycophorin A binding sequence.

**EBAbinding to erythrocytes in flow cytometry analysis.** EBA(aa1076–96) peptide bound to erythrocytes (Fig. 3). Maximum binding was 85% of the erythrocytes at peptide concentrations above 1 mg/ml. The binding decreased to 65%...
at peptide concentrations of 0.5 mg/ml. EBA(aa1086–1104) peptide did not bind to erythrocytes (data not shown).

**Inhibition of parasite multiplication in vitro by EBA peptide.** EBA(aa1076–96) peptide showed a concentration-dependent blocking of parasite growth in vitro (Fig. 4). At 8 μM EBA(aa1076–96), peptide almost completely blocked parasite multiplication. Peptide EBA(aa1086–1104) showed no blocking of parasite multiplication.

**Reactivities of human sera to EBA peptides in ELISA.** Serum reactivity with peptide was defined as being positive if the OD of the serum sample was higher than the mean plus 3 times the standard deviation of OD values obtained with 10 Danish controls. When tested at a dilution of 1:100, only a proportion of the sera tested reacted with EBA(aa1076–96) peptide. Of 44 tested Sudanese serum samples, 5 were reactive with the peptide, whereas 6 of 33 Indonesian sera and 2 of 20 Nigerian sera were reactive.

Seven Tanzanian children with clinical episodes of malaria had higher reactivities of IgG in serum to EBA(aa1076–96) peptide (median, 2.11 ELISA units; 25 and 75% quartiles, 1.09 and 4.06 ELISA units) than did 101 children with asymptomatic *P. falciparum* infections (median, 1.00 ELISA unit; 25 and 75% quartiles, 0.67 and 1.51 ELISA units) \((P = 0.02)\).

**Immunization of mice with EBA peptides.** BCG-primed mice immunized with PPD-conjugated EBA(aa1076–96) peptide in the absence of Freund’s complete adjuvant produced antibodies against this peptide (Fig. 5). The antibody reactivity increased with each immunization, and intraperitoneal immunization was superior to subcutaneous immunization. Sera from mice immunized three times inhibited EBA(aa1076–96)
peptide binding to glycoporphin A in the solid-phase assay (Table 1), while sera from the Indonesian, Nigerian, and Sudanese donors did not block peptide binding to glycoporphin A whether they were reactive with the EBA peptide or not.

**DISCUSSION**

The main finding in this study is the identification of a 12-aa peptide sequence of the malaria vaccine candidate EBA-175 which binds to glycoporphin and may be involved in the invasion process of merozoites into erythrocytes. The 12-aa sequence is contained within a 43-aa conserved sequence originally identified as the putative erythrocyte binding region, since rabbit antisera against this region block parasite multiplication and EBA-175 binding to erythrocytes (13, 19).

When merozoites attach to and invade erythrocytes, a sequence of events takes place in which the first step is a lectin-like binding of merozoites to erythrocytes, followed by reorientation of merozoites, bringing the apical pole in contact with the erythrocyte and leading to junction formation (7). Two different proteins, EBA-175 and MSP-1, have been implicated in mediating the initial binding of merozoites to the erythrocyte. EBA-175 is a protein, located in micronemes (18) and released into culture supernatants (2), that binds N-acetyl-neuraminic acid, α,2-3-Gal determinants on O-linked carbohydrates of glycoporphin A on the erythrocyte membrane (12). EBA-175 may mediate a two-step invasion procedure, an initial lectin-like binding followed by a second, possibly hydrophobic binding, triggering internalization of the merozoite. A cysteine-rich region of EBA, the F2 fragment, mediates the lectin-like binding to glycoporphin A (17), while a 65-kDa processing fragment of EBA was reported to bind to an erythrocyte determinant in a sialic acid-independent manner (8). This fragment does not contain the 43-aa sequence, and the relative role of the 65-kDa fragment and the fragment containing the 43-aa sequence in any secondary binding steps remains unknown.

To further characterize the erythrocyte binding sequence, we synthesized three overlapping peptides covering the 43-aa region. We showed that EBA(aa1076–96) bound strongly to erythrocytes and more specifically to glycoporphin A when tested in a solid phase binding assay. We also showed that soluble EBA(aa1076–96) bound to intact erythrocytes. The binding of EBA(aa1076–96) to erythrocytes does not appear to be dependent on sialic acid, since the peptide bound to desialylated glycoporphin A. We hypothesized that EBA(aa1076–96) is involved in the second step of a two-step binding process which may resemble HIV-1 gp160 binding to lymphocytes, where both gp120 and gp41 processing fragments remain attached to the lymphocytes through different binding sites. Virus entry is facilitated by an envelope-mediated fusion of the viral and target cell membranes. After formation of gp120 binding to CD4 as well as processing of gp160 to gp120 and gp41 fragments, the mobility of the envelope protein is afforded by the noncovalent nature of the gp120-gp41 bond, which may allow efficient exposure of the lymphocyte membrane to the hydrophobic gp41 regions that mediate the fusion process (10). Multiple regions of gp41 are involved in the invasion process by interaction with CD4 and other cellular receptors, as well as being involved in conformational changes of gp41 (21). Likewise, we hypothesized that the initial and specific cystein-rich fragment binding to sialic acid on glycoporphin A induces a conformational change in EBA-175 which may expose the EBA(aa1076–96) peptide fragment to subsequent erythrocyte binding. Both the EBA(aa1076–96) peptide-containing fragment and the 65-kDa fragment reported by Kain et al. (8) may participate in the
secondary binding to the erythrocyte, which is independent of sialic acid and may be more nonspecific. The two regions play different roles, since an EBA-175 peptide of 42 aa comprising the EBA(aa1076–96) peptide blocks binding of the full-length EBA-175 whereas the peptide from the 65-kDa EBA-175 fragment blocks the binding only of the 65-kDa fragment but not of full-length EBA-175 (8, 19). To further characterize the binding sequence of the EBA(aa1076–96) peptide, we showed that the amino acid sequence, VLKSHMNRESDD, at positions 1085 to 1096 of EBA-175 contained the binding sequence when the binding of 11 truncated peptide variants, EBA(aa1077–96) to EBA(aa1087–96), to glyco phorin A immobilized on polystyrene plates was tested. To substantiate the evidence that EBA(aa1076–96) contains the erythrocyte binding site, we tested the ability of EBA(aa1076–96) peptide to block parasite multiplication. The peptide showed a strong parasite-blocking activity. EBA(aa1076–96) peptide may compete with merozoites for binding to erythrocytes.

Antibodies against erythrocyte binding domains of EBA-175 may block the ability of merozoites to invade erythrocytes. Such antibodies may be responsible for the achievement of clinical immunity against malaria. The immune response to EBA-175 among humans living in regions of endemic infection remains poorly characterized. However, lymphocyte proliferation responses to EBA(aa1086–1104) but not to EBA(aa1076–96) among Ghanaian donors have been reported (4). None of the peptides were recognized by IgG antibodies from the Ghanaian donors. In this study, we selected sera from donors living in Indonesia, Nigeria, and Sudan with long exposure to malaria; the sera were highly reactive with a recombinant RAP-1 (5). The majority of these sera had low or negligible IgG reactivities of sera collected from children with asymptomatic infections. Whether these antibodies play a harmful role needs further investigations. Our data indicate that the amino acid sequence, VLKSHMNRESDD, at positions 1085 to 1096 of EBA-175 contained the binding sequence when the binding of 11 truncated peptide variants, EBA(aa1077–96) to EBA(aa1087–96), to glyco phorin A immobilized on polystyrene plates was tested. To substantiate the evidence that EBA(aa1076–96) contains the erythrocyte binding site, we tested the ability of EBA(aa1076–96) peptide to block parasite multiplication. The peptide showed a strong parasite-blocking activity. EBA(aa1076–96) peptide may compete with merozoites for binding to erythrocytes.

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References
