Evidence for Two-stage Binding by the 175-kD Erythrocyte Binding Antigen of *Plasmodium falciparum*

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Summary

*Plasmodium falciparum* malaria merozoites invade human erythrocytes bearing sialic acid in a multistage process involving the sialic acid–dependent binding of a malaria molecule, the 175-kD erythrocyte binding antigen (EBA-175). We show here that after the initial interaction of EBA-175 with its sialic acid–containing erythrocyte determinant, endogenous proteases can cleave EBA-175 to 65-kD fragment(s), whose binding to erythrocytes is sialic acid independent. A 65-kD fragment was immunoprecipitated by antibodies against peptides between residues 354 and 1061 but not beyond residue 1062. Binding experiments utilizing combinations of native protein, expression-PCR–synthesized EBA-175 polypeptides, peptide synthesis, and antibodies, demonstrated that sialic acid–independent binding could be further mapped to a small (about 40-amino acid) homologous part of the dimorphic allelic region of EBA-175, residues 898–938 (Camp strain numbering). These data support a two-step binding hypothesis and are discussed in relation to the formation of a junction between the merozoite and the erythrocyte, and the finding that after the interaction of some viruses with specific cellular receptors, they undergo conformational changes or cleavage permitting membrane fusion with the host cell.

Current *Plasmodium falciparum* vaccine strategies include the development of a multistage subunit vaccine consisting of antigens from different stages of the parasite life cycle (1, 2). As the primary pathophysiologic events of falciparum malaria occur during the erythrocytic or blood stages, merozoite antigens would represent important components of such a vaccine. Among the best candidates for inclusion into a blood stage vaccine are merozoite receptors for erythrocyte ligands through which host immune responses might block erythrocyte binding and invasion. Camus and Hadley (3) described a 175-kD *P. falciparum* antigen (erythrocyte binding antigen [EBA-175]) that appears to be involved in the attachment of the merozoite to the erythrocyte. EBA-175 binds to a sialic acid containing glycoporphin-associated ligand on RBCs in a manner that correlates with invasion. The gene encoding EBA-175 has been cloned and sequenced from two strains of *P. falciparum* (Camp and FCR3) that show restriction fragment length polymorphism (4, 5). The EBA-175 gene is largely conserved in these strains except for a 423-bp region found only in FCR3 (F seg) and a 345-bp segment found only in Camp C (C seg). These allelic regions are conserved in all cultured and wild isolates examined to date (5, 6). Polyclonal antibodies generated to a conserved region of EBA-175 immediately downstream from these allelic regions have been shown to inhibit invasion of RBCs by homologous merozoites in vitro (4, 7). However, the molecular basis of the interaction of EBA-175 with uninfected RBCs is unknown. Understanding the molecular interactions of *P. falciparum* erythrocyte binding proteins with URBCs may help define the sequence of events resulting in merozoite invasion and may identify functional domains of parasite proteins that would be important to evaluate for inclusion in a subunit vaccine. The available techniques to map functional domains of large molecular weight proteins are dependent on cloning, sequencing, and protein purification. These approaches suffer from inflexibility and require an extensive allocation of time and resources. We recently developed a rapid noncloning in vitro gene expression system, expression-PCR (E-PCR), ideally suited for structure-function analysis of proteins (8). In this study we examine the molecular basis of erythrocyte binding by EBA-175 utilizing E-PCR in combination with...
studies of the native protein. We show that upon binding to RBCs in vitro, native EBA-175 is cleaved to a 65-kD fragment that remains bound to the erythrocyte. The binding of this 65-kD fragment appears to involve a RBC determinant distinct from that of full-length EBA-175. These observations may further elucidate the sequence of molecular interactions required for erythrocyte invasion by P. falciparum merozoites.

Materials and Methods

Parasites and Labeled Culture Supernatants. Cloned Camp (Malaysia) and FCR3 (Africa) strains of P. falciparum were cultured and synchronized as previously described (9). Schizont-infected erythrocytes were Percoll enriched (10) and metabolically labeled at 1.5 x 10^7/ml with 50 µCi/ml [3H]sulfenyl in isoleucine-deficient RPMI-1640 culture medium containing 10% human serum (3, 11). The parasites were cultured for 16 h at 37°C to allow maturation of the schizonts and release of merozoites. The supernatants were collected by centrifugation at 300 g·min⁻¹·m⁻¹ (9) and stored at −80°C.

Erythrocytes and Enzyme Pretreatments. Human (huE) and Rhesus erythrocytes (rhE) were collected in acid citrate dextrose solution and stored at 4°C. Washed erythrocytes were treated with 50 U/ml Vibrio cholerae neuraminidase (Gibco BRL, Rockville, MD), 1.0 mg/mlTrypsin (Sigma Chemical Co., St. Louis, MO), or 0.2 mg/ml chymotrypsin (Sigma) as previously described (3, 12). The treated erythrocytes were extensively washed before being used in binding studies.

Binding and Elution from Erythrocytes. Affinity-purified EBA-175 was prepared using a modification of the method of Haynes et al. (13). Briefly, washed erythrocytes (100 µl) and culture supernatant (200 µl) were mixed for 30 min at room temperature. The suspension was layered over silicon oil (GE versilube FS0) and centrifuged at 37°C for 60 min, spun through oil, and eluted with 1 M NaCl as above. The RBC pellet was then resuspended in 100 µl of serum-free media, incubated at 37°C for 60 min, spun through oil, and eluted with 1 M NaCl as above. Eluates were diluted to 100 µl with complete media (RPMI-1640 media plus 10% human serum) and dialyzed for 60 min against complete media. This dialyzed fraction was then rebound to untreated and enzyme-treated rhE or huE (in the presence of PMSF) as described above. Rebound EBP were then either eluted with salt or sequentially eluted, first with 0.5 M NaCl, then with 1 M NaCl.

These eluted fractions were heated with an equal volume of 2× SDS sample buffer, separated by SDS-PAGE and processed for fluorography (14).

Immunoprecipitation. Immunoprecipitation of radiolabeled culture supernatants was performed with polyclonal monospecific antisera raised in mice and rabbits against the following peptides of the Camp strain of EBA-175 (4): peptide N1 (residues 354–377); peptide P2 (residues 939–969); peptide P5 (residues 970–1014); peptide P6 (residues 1015–1061); and peptide P4 (residues 1062–1103). Immunoprecipitations were performed as previously described (15) and precipitated antigens were detected by fluorography.

In Vitro Synthesis of EBA-175 using E-PCR. The gene encoding EBA-175 was derived from genomic DNA of the Camp and FCR3 strains of P. falciparum. Overlapping polypeptides of EBA-175 were synthesized using E-PCR as previously described (8). Gene fragments of EBA-175 (see Fig. 3) were amplified from genomic DNA in an automated thermocycler (Perkin-Elmer Cetus Corp., Norwalk, CT). 100 ng of genomic DNA was added to a 100-µl amplification mixture containing 50 pmol each of an appropriate 5' and 3' primer (see Table 1) derived from the published sequences of EBA-175 from Camp and FCR3 strains of P. falciparum (4, 5) and either 2.5 U of Taq (Perkin-Elmer Cetus Corp.) or 5 U of Vent polymerase (New England Biolabs Inc., Beverly, MA). The reaction mixture was initially denatured at 95°C for 5 min followed by 25 cycles of amplification (94°C for 30 s, 50–55°C for 30 s, and 72°C for 2–6 min) followed by an extension of 72°C for 7 min. PCR products were separated on a 2% low melt agarose gel, and the DNA bands were excised and stored at 4°C.

E-PCR was performed by mixing equal molar concentrations of the universal promoter (UP) and the desired amplified P. falciparum gene fragment (after melting at 60°C), together with 1.3 U of Taq polymerase or 2.5 U of Vent polymerase (without primers) for 15 cycles, (each consisting of 30 s at 94°C, 30 s at 25°C, and 2–6 min at 72°C). Samples were then amplified for an additional 25 cycles (30 s at 94°C, 30 s at 55°C, and 2 min at 72°C) after adding 50 pmol of both primer H3T7 and the 3' gene-specific primer (Table 1) and 1.3 U of Taq polymerase or 2.5 U of Vent polymerase. E-PCR products were extracted once with chloroform, precipitated with ethanol, and resuspended in 10 µl of RNase-free water.

In Vitro Translation. DNA templates produced by E-PCR were added to a 50-µl transcription reaction containing 40 mM Tris-HCl, pH 8.0, 8 mM MgCl2, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol (DTT), 40 U of RNasin (Promega, Madison, WI), 500 µM each of rATP, rCTP, rGTP, and rUTP, and 25 U of T7 RNA polymerase (Promega). The reaction was incubated at 37°C for 60 min. The DNA template was digested with 1 U of RQ1 DNase (Promega) at 37°C for 10 min followed by phenol extraction, ethanol precipitation, and resuspension in 10 µl of RNase-free water.

In Vitro Translation. EBA-175 mRNA produced in the T7 transcription reaction was translated in a wheat germ or rabbit reticulocyte cell-free system in the presence of [3H]methionine, according to the manufacturers' specifications (Promega). The efficiency of protein synthesis was determined by analyzing 2-µl samples of the reaction for TCA-precipitable radioactivity, as measured by liquid scintillation counting. The [3H]- or [35S]-labeled proteins produced by cell-free translation were analyzed by SDS-PAGE and fluorography. Functional activity of synthesized proteins was assessed by immunoprecipitation and erythrocyte binding studies as described above. Equivalent TCA-precipitable counts of synthesized proteins were used in all binding and immunoprecipitation studies.

Immunization with E-PCR Synthesized Protein. In vitro translation was performed as described above but without the addition of isotope and with equal molar concentrations of amino acids. BALB/c mice were immunized directly with cold E-PCR synthesized proteins. 100 µl (100–200 ng) of synthesized protein in a translation mixture was injected subcutaneously in CFA. Mice were boosted twice at 2-wk intervals with 100 µl of protein in IFA. Control mice were similarly immunized by using translation mixture to which no EBA mRNA had been added.

Production of Antisera to Peptides. Synthetic peptides with an
added NH2-terminal cysteine (peptides P2, P5, P6, P4, and N1) or lysine (peptide CP-1) residue were generated on a protein synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA). Analytical HPLC and quantitative amino acid analysis confirmed the identity and purity of the products. Peptides were conjugated to KLH with glutaraldehyde (16). Conjugated proteins were dialyzed with two changes of 4 liters of PBS for 48 h (4°C). Rabbits (peptides P4 and CP-1) and mice (peptides P2, P5, P6, and N1) were immunized with conjugated proteins: primary immunizations were emulsified in CFA and boosting immunizations were emulsified in IPA.

Binding Inhibition Assays. EBA-175 and the 65-kD fragment, generated by binding to huE, were salt eluted, dialyzed, and rebound to untreated huE as described above, but rebinding was performed in the presence of increasing concentrations of peptide CP-1 (1, 10, and 100 mM peptide CP-1) or anti-CP-1 sera in complete media. Rebinding of EBA-175 and the 65-kD fragment was also examined in the presence of a 100-mM concentration of control peptide 4. Rebound samples were then salt eluted and analyzed by SDS-PAGE and fluorography.

Results

Erythrocyte Binding by EBA-175 and its 65-kD Processed Product. Erythrocyte binding by EBA-175 was studied using an in vitro RBC-binding assay. In these assays, metabolically labeled EBP are identified by affinity purification on erythrocytes (12). Under these conditions, native EBA-175 binds to a sialic acid-dependent ligand and is cleaved to a 65-kD fragment recognized by monospecific antisera to EBA-175 (Figs. 1, A and B, and 2). Cleavage is inhibited in the presence of protease inhibitors and enhanced cleavage is observed when EBA-175 is experimentally bound to rhE. To characterize the binding of EBA-175 and its processed products to erythrocytes, rebinding and sequential elution studies were performed with both rhE and huE. In these experiments, EBPs were affinity purified on rhE or huE, eluted with salt, dialyzed, and then rebound to rhE or huE. Rebinding was performed in the presence of PMSF to prevent additional cleavage of full-length EBA-175. With this approach, it was possible to assess the binding characteristics of the isolated 65-kD fragment independent from the binding requirements of the full-length EBA-175 molecule. When EBPs were rebound and eluted with salt from rhE, both full-length EBA-175 and a doublet of ~65 kD were recovered (Fig. 1 A, lane 1). When rebound to rhE and sequentially eluted, first with 2-3 sialyllactose (Fig. 1 A, lane 2) followed by 1 M NaCl (lane 3), EBA-175 and the 65-kD fragment were differentially recovered. Full-length EBA-175 was eluted almost completely with sialyllactose, however, the 65-kD doublet was recovered primarily with 1 M NaCl. No detectable EBA-175 or 65 kD remained on these erythrocytes after sequential elution, as determined by SDS-PAGE of the RBC pellet (data not shown).

To enhance the production of the 65-kD fragment, EBPs initially generated in the absence of protease inhibitors were also used for re-binding experiments (Fig. 1 A, lane 5). Sequential elution studies performed using EBPs prepared in this fashion, also showed differential elution of EBA-175 and the 65-kD fragment (Fig. 1 A, lanes 7 and 8). To ensure that sufficient quantities of both full-length EBA-175 and the 65-kD fragments were present for the subsequent studies, equal volumes of eluted EBPs, initially prepared in the presence of protease inhibitors were also used for re-binding experiments (as in Fig. 1 A, lane 1) and in the absence of protease inhibitors (as in Fig. 1 A, lane 5),

| Table 1. Oligonucleotides (5' to 3') |
|------------------------------------|
| Oligo | Position | Sequence |
|-------|----------|----------|
| 1     | 1-21†    | ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| 2     | 63-84    | ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| 3     | 1092-1110| ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| 4     | 1623-1647| ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| 5     | 2277-2298| ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| 6     | 2607-2625| ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| 7     | 2928-2946| ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| 8     | 2952-2973| ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| 9     | 3458-3476| ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| 10    | 4333-4306| ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| F1    | 2332-2353‡ | ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| F2    | 2727-2748‡ | ATGGCAGCTGATGAAATGTAATATTAGTATA† |
| H3T7  | —        | ATGGCAGCTGATGAAATGTAATATTAGTATA† |

† Position in Camp strain of *P. falciparum.*
‡ Sequence includes splice region for E-PCR.
§ Antisense oligonucleotide.
¶ Position in FCR3 strain of *P. falciparum.*

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Figure 1. (A) Rebinding and sequential elution studies of EBA-175 and its 65-kD processed fragment utilizing rhE. EBPs (175 and 65 kD) used in the following experiments were affinity purified in rhE binding assays (in the presence [lanes 1-4] or absence [lanes 5, 7, 8] of 2 mM PMSF), eluted with 1 M NaCl and dialyzed before being used in rebinding experiments. EBPs were then rebound to untreated or treated rhE (in the presence of PMSF), eluted with 1 M NaCl or 2-3 sialyllactose, and analyzed by 7.5% SDS-PAGE and fluorography. (Lane 1) Rebinding by EBA-175. Salt eluate of rebound EBPs. (Lanes 2 and 3) Sequential elution of rebound EBPs, first with 50 mM 2-3 sialyllactose (lane 2), then with 1 M NaCl (lane 3). (Lane 5) Salt eluate of rebound EBPs. (Lane 6) Salt eluate of rebound EBPs, initially prepared in the presence (as in lane 1) and absence of PMSF (as in lane 5), were mixed in equal proportions, rebound, and salt eluted from untreated rhE. (Lane 6) Rebinding of mixed EBPs (prepared as in lane 4) to neuraminidase-treated rhE. (Lanes 7 and 8) Sequential elution of rebound EBPs, first with 2-3 sialyllactose, then with 1 M NaCl. In these experiments, only a single 65-kD band of the 65-kD doublet recovered upon elution with salt. Its binding characteristics were the same as those described for the lower band of the 65-kD doublet recovered from huE (Fig. 1 B). In contrast to full-length EBA-175, the 65-kD fragment eluted from huE bound to neuraminidase-treated huE (Fig. 1 B, lane 3) and was not eluted off huE with sialyllactose (Fig. 1 B, lanes 5 and 6). Therefore, using both rhE and huE, full-length EBA-175 and the 65-kD fragment appeared to be binding to different erythrocyte determinants. In addition, in these experiments, a 140-kD sialic acid-independent erythrocyte binding protein first described by Dolan et al. (17) is observed (Fig. 1 A, lane 10). Antibodies to peptide N1 precipitated only full-length EBA-175 (Fig. 1 A, lane 10).

Results of rebinding and sequential elution using huE were identical except that, in these experiments, only a single 65-kD fragment was recovered upon elution with salt. Its binding characteristics were the same as those described for the lower band of the 65-kD doublet recovered from huE (Fig. 1 B). In contrast to full-length EBA-175, the 65-kD fragment eluted from huE bound to neuraminidase-treated huE (Fig. 1 B, lane 3) and was not eluted off huE with sialyllactose (Fig. 1 B, lanes 5 and 6). Therefore, using both rhE and huE, full-length EBA-175 and the 65-kD fragment appeared to be binding to different erythrocyte determinants. In addition, in these experiments, a 140-kD sialic acid-independent erythrocyte binding protein first described by Dolan et al. (17) is observed (Fig. 1 B, lanes 3 and 6). The binding characteristics of this treated huE. (Lanes 5 and 6) Sequential elution of rebound EBPs (prepared in the absence of PMSF), first with 2-3 sialyllactose, then with 1 M NaCl. (Lane 7) Immunoprecipitation of mixed rebound EBPs (prepared as in lanes 3 and 4) with antisera to peptide 4 (residues 1062 to 1103 in Camp EBA-175).
protein are similar to the 65-kD fragment. Both of these EBP;
bind to neuraminidase-treated huE (Fig. 1 B, lane 3) and both
are eluted from huE with 1 M NaCl but not with sialyllac-
tose (Fig. 1 B, lanes 5 and 6). These observations provide
additional evidence that erythrocyte binding by the 65-kD
fragment is sialic acid independent.

To epitope map the location of the 65-kD fragment within
EBA-175, immunoprecipitations with monospecific antisera
generated to synthetic peptides of Camp EBA-175 were per-
formed (Fig. 2). The 65-kD fragment was precipitated by
antisera to peptides N1 (residues 354-377), P2 (residues
939-969), P5 (residues 970-1014), and P6 (residues 1015-1061),
but not by antisera to P4, suggesting that the 65-kD frag-
ment lies in the amino and middle region of EBA and does
not contain the peptide 4 region of EBA-175 (residues
1062-1103).

E-PCR Synthesis and Functional Analysis of EBA-175 Constructs.
To further explore the binding characteristics of EBA
and its cleavage products, we synthesized radiolabeled poly-
peptides of EBA-175 using E-PCR. Overlapping constructs of
EBA-175 (Fig. 3, top) were synthesized by E-PCR, trans-
lated in vitro, and assessed for binding to huE (Fig. 3, bottom).
Fragments containing, but not exceeding, residues 869-982
bound to huE. Residues 869-982, a region called C seg, cor-
respond to one of the two described alleles of EBA-175. A
second allele of EBA (5, 6) found in the strain FCR3 (F seg),
was also expressed by E-PCR, and analyzed for huE binding
(Fig. 4). Like the 65-kD fragment, both F seg and C seg
bound to untreated (Fig. 4, lanes 4, 5, and 8) and neur-
aminidase-treated huE (data shown for C seg, Fig. 4, lane 6).
In addition, 2-3 sialyllactose would not elute them off huE.

To determine if C seg and F seg might be contained within
the 65-kD fragments of Camp and FCR3, respectively, mice
were directly immunized with E-PCR-generated C seg and
F seg. Interestingly, antisera generated to F or C seg precipi-
tated the 65-kD fragment of Camp (Fig. 2), indicating that
C seg is within the 65-kD fragment of Camp and that cross-
reactive epitopes exist between F and C seg despite their se-
quence divergence. Overall F seg and C seg share only ~15%
nucleotide or amino acid homology (5). However, when their
primary amino acid sequences are aligned (GCG Software;
University of Wisconsin), a series of residues are conserved
between these domains (Fig. 5). 17 (71%) of 24 residues in
one region of F seg and C seg are either identical (33%) or
conservative substitutions (38%).

To define the smallest region of C seg capable of binding
to huE, overlapping polypeptides of C seg were synthesized by
E-PCR (Fig. 6 B) and analyzed for huE binding (Fig. 6
A). Interestingly, C seg fragments, C-Hinp and C-22, con-
taining most of the region of homology between F and C
allelic regions, bound to huE (Fig. 6 A, and lanes 1 and 2),
but fragment C-23, devoid of this segment (Fig. 6 A, lane
3), did not. A peptide (CP-1: KATVSESSSNTGLSIDD-
DRNGDTFVRQTDANTED) corresponding to the smal-
lest binding domain of C seg was chemically synthesized and
used in competitive binding experiments with native EBA-
Figure 4. Binding analysis of E-PCR-synthesized C seg (Camp EBA-175) and F seg (FCR3 EBA-175). E-PCR-synthesized proteins were incubated with huE, spun through silicon oil, eluted with 1 M NaCl, and analyzed by 10% SDS-PAGE and fluorography. (Lane 1) Salt eluate from neuraminidase-treated huE incubated with native Camp EBA-175. (Lane 2) Salt eluate from untreated huE incubated with native Camp EBA-175. (Lane 3) E-PCR-synthesized C seg (50,000 counts/lane). (Lane 4) Salt eluate from untreated huE incubated with C seg (50,000 counts/lane). (Lane 5) Salt eluate from neuraminidase-treated huE incubated with C seg (50,000 counts/lane). (Lane 6) Salt eluate from untreated huE incubated with C seg (75,000 counts/lane). (Lane 7) E-PCR-synthesized F seg (50,000 counts/lane). (Lane 8) Salt eluate from untreated huE incubated with F seg (50,000 counts/lane).

Figure 6. (A) Functional mapping of C seg by E-PCR. Analysis of E-PCR-synthesized overlapping fragments of C seg (113 amino acids corresponding to residues 869-982 in Camp EBA-175) bound and eluted from huE and analyzed by 17.5% SDS-PAGE and fluorography. (Lane 1) Salt eluate from huE incubated with construct C-Hinp (residues 869-953; 50,000 counts/lane). (Lane 2) Salt eluate from huE incubated with construct C-22 (residues 899-982; 50,000 counts/lane). (Lane 3) Salt eluate from huE incubated with construct C-23 (residues 939-982; 50,000 counts/lane). (Lane 4) E-PCR-synthesized C seg (50,000 counts/lane). (Lane 5) E-PCR-synthesized F seg (50,000 counts/lane).

Figure 5. Alignment of FCR3 and Camp EBA-175 amino acid sequences beginning at residue 900 in FCR3 and 898 in Camp. Gaps (−) have been introduced to optimize alignments; (I) identical residues, and (:) conserved residues between Camp EBA-175 in the region of C seg (bottom) and FCR3 EBA-175 in the region of F seg (top).

N-acetylneuraminic acid (α2-3)-Gal-determinants on O-linked carbohydrates of glycophorin A on the RBC membrane (12). Several lines of evidence indicate that EBA-175 is involved in the erythrocyte invasion process and suggest that it may be involved in the infection process.

Discussion
Merozoite attachment and invasion of uninfected erythrocytes is a multistep process, involving a sequence of adhesive events mediated by specific receptor–ligand interactions (13, 18–25). EBA-175 is a P. falciparum antigen released into culture supernatants that binds with receptor-like specificity to huE. In contrast, peptide 4 inhibited binding of full-length EBA-175 but had no effect on binding of the 65-kD fragment. Of note, this putative binding domain represented by CP-1, incorporates 71% of the region of homology between F seg and C seg.

Figure 7. Inhibition of EBA-175 and the 65-kD fragment binding to erythrocytes by peptide CP-1 and anti-CP-1 sera. Samples were bound to huE, salt eluted, and analyzed by 7.5% SDS-PAGE and fluorography. (A) Erythrocyte binding by EBA-175 and its 65-kD fragment in the presence of 1- (lane 1), 10- (lane 2), and 100-mM (lane 3) peptide CP-1, or 100-mM peptide 4 (lane 4). (B) Erythrocyte binding by EBA-175 and its 65-kD fragment in the presence of 1/5,000 (lane 1), 1/1,000 (lane 2), 1/100 (lane 3), or 1/10 (lane 4) dilutions of rabbit anti-CP-1 sera.
be a vaccine candidate antigen (3, 4). It has recently been proposed that EBA-175 may be a member of a family of malaria erythrocyte binding proteins based on the presence of conserved cysteine-rich regions present in the Duffy binding proteins of P. vivax and P. knowlesi and the EBA-175 of P. falciparum. It is further postulated that the erythrocyte binding domain(s) of these molecules may reside in these conserved cysteine-rich regions (26). However, the molecular basis of the binding of infected RBCs by EBA-175 is unknown. In this study, we provide evidence for a two-stage binding process by EBA-175.

Upon binding to huE or rhE in vitro, EBA-175 is cleaved to a 65-kD fragment that remains bound to the erythrocyte. Although it is not known if EBA-175 is proteolytically processed once bound to huE in vivo, the observation that the 65-kD fragment remains bound to the erythrocyte after proteolysis clearly suggests that an erythrocyte binding domain for an unknown erythrocyte ligand is contained within this region of EBA-175. To characterize this putative binding domain within the 65-kD fragment, we first generated this fragment by binding native full-length EBA-175 to the rhE or huE, similar to the original experiments describing EBA-175 performed by Camus and Hadley (3). However, unlike Camus and Hadley, we than eluted the bound 65-kD product from RBCs, dialyzed it, and then examined its binding characteristics using rebinding and sequential elution studies. Only in this way was it possible to examine the binding of the 65-kD fragment independent from the binding requirements of the intact EBA-175 molecule. With this approach we found, like others (3, 11), that full-length EBA-175 will not bind to sialic acid–deficient erythrocytes and can be eluted from untreated erythrocytes with soluble sialyllactose (Fig. 1, A and B) indicating that its initial recognition and binding is dependent upon a sialic acid–containing ligand on the erythrocyte surface. However, in contrast to full-length EBA-175, we demonstrated that the 65-kD fragment was not significantly eluted from erythrocytes with sialyllactose and bound, albeit in reduced amounts, to neuraminidase-treated huE and are not eluted from huE with 2-3 sialyllactose. Antisera generated to F and C seg, obtained by directly immunizing mice with E-PCR–synthesized protein, will precipitate their respective 65-kD fragments, indicating they are contained within the 65-kD fragment of each strain. It is intriguing that antibodies against F seg will also precipitate the 65-kD fragment of Camp, indicating the presence of a conserved immunogenic epitope between F seg and the Camp 65-kD fragment. Whereas there is little overall amino acid or base pair homology between the allelic regions of EBA-175, a series of amino acids and their primary structure are relatively conserved between F seg and C seg (Fig. 5). In addition, binding analysis of overlapping fragments of C seg (Fig. 6) define a putative binding domain of ~40 amino acids (residues 903 to 943) that encompasses most of this region of homology between F seg and C seg. Finally, a synthesized peptide (peptide CP-1) corresponding to this region selectively inhibits binding of the 65-kD fragment to huE, as does antisera generated to it. In contrast, peptide 4 inhibits the binding of full-length EBA-175 but not its 65-kD fragment.

Taken together, these data suggest that there are two distinct binding domains within EBA-175. We propose that EBA-175 may bind to erythrocytes by a two-stage process that includes a sialic acid–independent second step. During invasion, the initial binding of EBA-175 is dependent on the presence of a sialic-acid–containing determinant. The data presented here supports the hypothesis that after this initial binding, a second binding step occurs as a result of either a receptor/ligand-induced conformational change or the limited proteolysis of bound EBA-175 exhibited in vitro in this study. This second binding step is independent of the presence of sialic acid and maps to allelic regions within the 65-kD fragment. Conserved residues (Fig. 5) within these allelic regions may constitute the contact residues with an invariant but unknown RBC binding site.

It is unknown if EBA-175 cleavage is a feature of erythrocyte invasion in vivo, or an artifact of our in vitro preparation of this protein. Nonetheless, it is meaningful observation suggesting that a conformational change occurring upon the initial binding of EBA-175 might expose regions involved
in sialic acid–independent binding and in addition, render EBA-175 more susceptible to cleavage.

The concept that merozoites might bind by a two-step/site model was first proposed by Pasvol et al. (20) and later by Breuer (27). According to their scheme, the binding of a merozoite component to oligosaccharides on glycophorin A, exposed another domain, proximal to, or buried within the lipid bilayer. The second binding event would trigger internalization of the merozoite. Our hypothesis that the sialic acid–dependent binding of EBA-175 initiates a conformational change or limited proteolysis permitting the next sequential event in invasion, is also supported by observations with polio and influenza viruses. The proteolysis of polio capsid proteins is a prerequisite step to render virus particles competent to attach to specific cellular receptors. Upon binding to its cellular receptor, the polio virion undergoes irreversible conformational changes, exposing an amphipathic helix that may contribute to viral entry into the target cell (28). Similarly, after binding to its cellular receptor, cleavage of the influenza hemagglutinin (HA) is necessary for subsequent steps of viral infectivity, including membrane fusion and viral entry. Strains of influenza in which the HA are more readily cleaved display increased pathogenicity. Cleavage of the HA permits a subsequent conformational change to expose a hydrophobic fusion peptide contributing to membrane fusion (29). It is therefore conceivable that our proposed receptor ligand–mediated conformational change or cleavage of EBA-175 exposes similar hydrophobic regions resulting in membrane junction between the merozoite and erythrocyte, a necessary prerequisite for invasion (22).

In our model, peptide 4 may form part of the rim of the binding pocket and may contribute to the first sialic acid–dependent stage of binding. Antisera generated to this peptide has been shown to inhibit parasite invasion in vitro and to block the sialic acid–dependent binding of full-length EBA-175 (4). Similarly, antibodies generated to the putative erythrocyte binding domain identified in C seg may inhibit the second sialic acid–independent binding step. It is interesting to speculate that such an immune response may, in combination with antisera to peptide 4, inhibit parasite invasion more efficiently than antipeptide 4 antisera alone by inhibiting successive invasion steps. Even if this proves to be true, however, several strains of P. falciparum can invade erythrocytes through sialic acid independent pathways (18, 30, 31), therefore, an effective vaccine would also require the identification and inhibition of receptor–ligand interactions involved in these alternate invasion pathways.

The potential importance of F seg and C seg is further supported by the observations that these allelic regions appear to be uniquely and absolutely conserved. An analysis of 12 different laboratory clones and over 50 geographically diverse wild isolates revealed no isolate of P. falciparum that did not contain either F or C seg (5, 6).

In summary, based on studies of a native protein and on E-PCR synthesized polypeptides, we provide evidence to support a two-stage binding process by EBA-175 that includes a sialic acid–independent step. This sialic acid–independent binding domain resides within the 65-kD processed fragment of EBA-175 and appears to involve a homologous part of the dimorphic allelic region of EBA-175.

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