Primary Structure of the 175K *Plasmodium falciparum* Erythrocyte Binding Antigen and Identification of a Peptide Which Elicits Antibodies That Inhibit Malaria Merozoite Invasion

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Abstract. The *Plasmodium falciparum* gene encoding erythrocyte binding antigen-175 (EBA-175), a putative receptor for red cell invasion (Camus, D., and T. J. Hadley. 1985. *Science (Wash. DC).* 230:553-556.), has been isolated and characterized. DNA sequencing demonstrated a single open reading frame encoding a translation product of 1,435 amino acid residues. Peptides corresponding to regions on the deduced amino acid sequence predicted to be B cell epitopes were assessed for immunogenicity. Immunization of mice and rabbits with EBA-peptide 4, a synthetic peptide encompassing amino acid residues 1,062–1,103, produced antibodies that recognized *P. falciparum* merozoites in an indirect fluorescent antibody assay. When compared to sera from rabbits immunized with the same adjuvant and carrier protein, sera from rabbits immunized with EBA-peptide 4 inhibited merozoite invasion of erythrocytes *in vitro* by 80% at a 1:5 dilution. Furthermore, these sera inhibited the binding of purified, authentic EBA-175 to erythrocytes, suggesting that their activity in inhibiting merozoite invasion of erythrocytes is mediated by blocking the binding of EBA-175 to erythrocytes. Since the nucleotide sequence of EBA-peptide 4 is conserved among seven strains of *P. falciparum* from throughout the world (Sim, B. K. L. 1990. *Mol. Biochem. Parasitol.* 41:293–296.), these data identify a region of the protein that should be a focus of vaccine development efforts.

MALARIA disease, which is estimated to directly account for more than one million deaths per year worldwide (Sturchler, 1989), is caused by the erythrocytic stages of parasites of the genus *Plasmodium.* Conventional approaches to control malaria using insecticides and antimalarial drugs have met with major problems due to the resurgence of resistant strains of both the mosquito vector and the parasite. In recent years there have been considerable efforts to develop vaccines against malaria (Miller et al., 1986). Within the human host, the malarial parasite is intracellular for the major part of its life cycle. Exceptions to this are the sporozoite stage, which is introduced by the mosquito vector and invades liver hepatocytes; and the merozoite stage, which invades erythrocytes. Both the sporozoite and the merozoite are briefly extracellular, and must invade their respective cells within minutes to ensure further development. Attempts at achieving protection to this parasite have focused on trying to attain immunity to the extracellular stages because it is during this short period that the parasite should be most vulnerable to immune attack. One approach to malaria vaccine development is to induce immune responses that prevent interaction of merozoite receptors with complementary erythrocyte ligands.

Merozoite recognition and invasion of erythrocytes is probably a multi-step process and depends on the ability of the merozoite to recognize ligands on the erythrocyte membrane (Miller et al., 1977; Pasvol and Jungery, 1984; Perkins, 1984; Okoye and Bennet, 1985; Hadley and Miller, 1988). Several studies of *P. falciparum* merozoite–erythrocyte interaction have identified erythrocyte membrane glycophorins and sialic acid as ligands important for invasion (Miller et al., 1977; Pasvol et al., 1982; Pasvol and Jungery, 1984; Hadley et al., 1988). A potential sialic acid binding antigen is the 175-kD erythrocyte binding antigen (EBA-175) (Camus and Hadley, 1985). This antigen was isolated by using intact erythrocytes as an affinity substrate. EBA-175 binds to erythrocytes susceptible to invasion, with a clear correlation between invasion efficiency and the ability for EBA-175 to bind (Camus and Hadley, 1985; Orlandi et al., 1990). Evidence obtained so far suggests that EBA-175 rec-

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ognizes sialic acid residues on the *O*-linked tetrasaccharides of the erythrocyte glycophorins (Camus and Hadley, 1985; Orlandi et al., in preparation). EBA-175 also binds to merozoites. EBA-175 is distinct from the 155-kD antigen (Camus and Hadley, 1985) and the merozoite surface antigen Pf200 (gp195) (Orlandi et al., 1990). On the basis of the binding specificities and binding to merozoites, EBA-175 was proposed to be a parasite receptor acting as a "bridge" between the merozoite and the erythrocyte.

Since EBA-175 was thought to be involved in the attachment of the merozoite to the erythrocyte, our approach was to isolate the gene encoding EBA-175, and then determine whether antibodies against B cell epitopes on the protein encoded by this gene could interfere with merozoite invasion of erythrocytes. We present data on the characterization of the gene encoding EBA-175, and show that antibodies against a synthetic peptide comprising amino acid residues 1,062–1,103 (EBA-peptide 4) block the binding of EBA-175 to erythrocytes and also inhibit merozoite invasion of erythrocytes in vitro.

Materials and Methods

Cultivation of P. falciparum and Isolation of EBA-175

Cloned Camp strain (Malaysia) *P. falciparum* were cultured in vitro and synchronized using conventional protocols (Vernes et al., 1984). Affinity-purified EBA-175 was prepared by using human erythrocytes as an affinity substrate to bind EBA-175 harvested from culture supernatants, and then eluted off with a high salt buffer (Haynes et al., 1988; Orlandi et al., 1990).

Specific Antibodies against EBA-175

Polyclonal monospecific antibodies against EBA-175 were isolated from the sera of immune Aotus monkeys immunized against the Camp strain of *P. falciparum* by affinity purification against EBA-175 adsorbed to nitrocellulose (Orlandi et al., 1990).

Immunoblotting

Antigens were fractionated by SDS-PAGE (Towbin et al., 1979), and immunoblotted as described (Harn et al., 1984). Diluent for antisera was TBS-0.05% Tween 20. Reactions were detected with alkaline phosphatase–linked anti-Aotus IgG (gift from Dr. A. Thomas, Department of Immunology, Walter Reed Army Institute of Research, Washington, D.C.) or anti–mouse IgG (Promega Biotec, Madison, WI) and developed with 50 mg/ml nitro blue tetrazolium and 50 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Promega Biotec) by method of Blake et al. (1984).

Library Screening

Initially, affinity-purified monospecific antibodies to EBA-175 were used to screen a Camp strain genomic mung bean nuclease expression library in lambda gt11 (Lyon, et al., 1986). Nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) were saturated with 10 mM isopropyl-beta-thio-D-galactopyranoside (IPTG) (Sigma Chemical Company, St. Louis, MO), and duplicate plaque lifts of ∼300,000 plaques plated at a density of 30,000 plaques per 150-mm Petri dish were made (Young and Davis, 1983). The filters were blocked in 0.3% Tween 20 in 50 mM Tris, pH 8.0, 150 mM NaCl TBS for 1 h at room temperature, and rinsed in 0.05% Tween 20 in TBS before air drying. The filters were next rehydrated in 0.05% Tween 20 in TBS for 20 min and probed with affinity-purified monospecific antibodies to EBA-175 (Orlandi et al., 1990), overnight at 4°C. These filters were then rinsed in 0.05% Tween 20 in TBS, probed with alkaline phosphatase–conjugated anti-Aotus IgG for 90 min and developed following the same protocols as in immunoblotting. One positive plaque EBA1.8/Y1090 with a 1.8-kb insert was isolated and plaque purified through four cycles of subcloning and plating until all plaques tested positive.

Gene Isolation

The 1.8-kb insert was labeled by nick translation (Nick Translation System, Bethesda Research Laboratories, Gaithersburg, MD) with ^{32}P-alpha-dTTP (3,000 Ci/mmol; New England Nuclear, Boston, MA) and used as a probe to identify a single 5.5-kb fragment on a restriction endonuclease Xba I genomic Southern blot (Southern, 1975). To isolate this 5.5-kb fragment which appeared to contain further unidentified EBA-175 gene sequences, a size selected (4.4–6.6 kb) Xba I genomic library was constructed and screened with labeled 1.8-kb fragment. A 200-bp sequence from the 5′ end of this 5.5-kb fragment thus isolated, was amplified using oligonucleotide primers by the polymerase chain reaction (Saiki et al., 1988), labeled, and used as a probe to isolate a clone (EBA2.5/Y1090) containing a 2.5-kb fragment from the initial lambda gt11 library. Subsequent to this, a 250-bp sequence was amplified from the 5′ end of the 2.5-kb fragment, labeled, and used as a probe to identify a 3.5-kb fragment on an Xba I genomic Southern blot. To isolate this 3.5-kb Xba I fragment which appeared to contain the rest of the gene encoding EBA-175, another size-selected (2.3–4.4 kb) Xba I genomic library was constructed and screened using the same 250-bp fragment as a probe.

Expression and Analysis of Product

Purified DNA from EBA1.8/Y1090 was transfected into the expression host cell *Escherichia coli* Y1089 and expressed by standard procedures as a polypeptide fused to beta-galactosidase (Young and Davis, 1983). The lysate from clone 1.8/Y1089, before and after induction, was analyzed in duplicate by SDS-PAGE and immunoblotted (Harn et al., 1984). One blot was probed with affinity-purified monospecific antibodies to EBA-175, and the other with antibodies against beta-galactosidase (gift from Dr. D. Lanar). The expressed protein of clone 1.8/Y1089 was used to affinity purify antibodies as previously described (Orlandi et al., 1990) from immune Aotus monkeys. These affinity-purified antibodies were then used to probe blots of purified authentic EBA-175. Similarly, nitrocellulose filters saturated with 10 mM IPTG were used to make lifts of confluent plates (Lyon et al., 1986) of phage EBA1.8/1090. Affinity purification of Aotus monkey antibodies specific for the expressed protein on these filters was performed as described (Orlandi et al., 1990) and these antibodies were used to probe preparations of authentic EBA-175, as mentioned above.

Sequencing

All clones to be analyzed were subcloned into the bidirectional sequencing vector pGEM4Z (Promega Biotec). The clones containing the 1.8- and 5.5-kb inserts, respectively, were prepared for sequencing by constructing progressive exonuclease III deletion subclones from the 3′ end with the erase-a-base kit (Promega Biotec), following the manufacturer's specifications. Sequencing of the entire 1.8-kb insert as well as relevant parts of the 5.5-, 2.5-, and 3.5-kb inserts was performed with a modified T7 polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH). Sequencing was performed in both orientations for all fragments analyzed.

Sequence Analysis

The DNA and deduced amino acid sequence of EBA-175 was compared to the Swiss Protein Data bank (release 10.0) through BIONET using the FASTA program (Pearson and Lipman, 1988); as well as to the GENEMBL data base using the GCG program (Devereux et al., 1984). Antigenic determinants were predicted for the deduced amino acid sequence of EBA-175 using an average group length of six amino acid residues to include the areas of highest hydrophilicity indices (Hopp and Woods, 1981). Studies of the electrical charge as a function of the pH was analyzed using the PCGENE software program CHARGEPRO.

Antisera

Synthetic peptides with an added NH_2-terminal cysteine residue were generated on a protein synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA). Analytical HPLC and quantitative amino acid analyses confirmed identity and purity of the products. Peptides were conjugated separately with glutaraldehyde (Wirtz et al., 1987) to the carrier molecule keyhole limpet hemocyanin (KLH)[1] and dialyzed with 2 × 2 L PBS before use. BALB/c mice and rabbits were injected subcutaneously with an estimated 100 and 150 μg each of peptide in Freund's complete adjuvant, respectively. These animals were boosted twice at 3-wk intervals with 100 μg of peptide in Freund's incomplete adjuvant. Antibodies to unconjugated peptides were measured by ELISA (Wirtz et al., 1989).

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[1] *Abbreviation used in this paper*: KLH, keyhole limpet hemocyanin.

Immunoprecipitation

Immunoprecipitation of unlabeled or radiolabeled culture supernatants containing EBA-175 were performed with antibodies against EBA-peptide 4 as previously described (Lyon et al., 1987). After SDS-PAGE, precipitated antigens were detected either by immunoblot analysis or fluorography with EN^{3}HANCE (New England Nuclear, Boston, MA).

Immunofluorescence Assays

Reactivity of sera from immunized animals with *P. falciparum* blood stage parasites was determined by a two-color immunofluorescence assay as previously described (Klotz et al., 1989). mAb 3B10.1 (Lyon et al., 1987) against the merozoite surface antigen gp195 of blood stage *P. falciparum* parasites was used as a control.

Blocking of Erythrocyte Binding Assay

Serum from rabbits immunized with three doses of EBA-peptide 4 conjugated to KLH or rabbits immunized with three doses of KLH alone were preabsorbed twice on human erythrocytes by incubating 1 vol packed erythrocytes to 2 vol of serum, and gently rocking at 4°C for 1 h. Sera were heat inactivated at 56°C for 30 min before use. Reaction mixtures of 100 μl final volume included 50 μl of EBA-175 containing supernatants from 3H-isoleucine-labeled cultures (Camus and Hadley, 1985; Orlandi et al., 1990), 0, 5, or 20 μl sera from rabbits that had been immunized with EBA-peptide 4 conjugated to KLH, or with 0, 5, or 20 μl sera from rabbits that had been immunized with KLH alone, and culture medium sufficient to bring the final volume to 100 μl. After incubation overnight at room temperature, the reaction mixtures were incubated for 30 min with 100 μl of packed erythrocytes and eluates were obtained as described (Orlandi et al., 1990). Eluates were separated by SDS-PAGE and detected by fluorography. Quantitation of EBA-175 binding in the presence of anti-EBA peptide 4 sera was performed by scanning laser densitometry (LKB 2022 Ultrascan Laser densitometer) of the resulting autoradiograph, and expressed as a percentage of KLH-control samples in which labeled culture supernatants without immune serum were incubated with erythrocytes.

Inhibition Assay

The inhibition of merozoite invasion assay was a modification of that previously described (Chulay et al., 1981). Percoll-purified, synchronized (Vernes et al., 1984) Camp strain schizonts (5×10^5) and fresh erythrocytes (5×10^6) were cultured with the respective erythrocyte-preadsorbed, heat-inactivated sera in triplicate 100-μl samples. After 18 h, thin blood films were prepared and the number of infected erythrocytes per 1,000 erythrocytes determined. Percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

"Control" is the number of parasites which invaded 1,000 erythrocytes in the presence of serum from rabbits immunized with KLH alone, and "Test" is the number of parasites which invaded 1,000 erythrocytes in the presence of serum from rabbits immunized with EBA-peptide 4 conjugated to KLH.

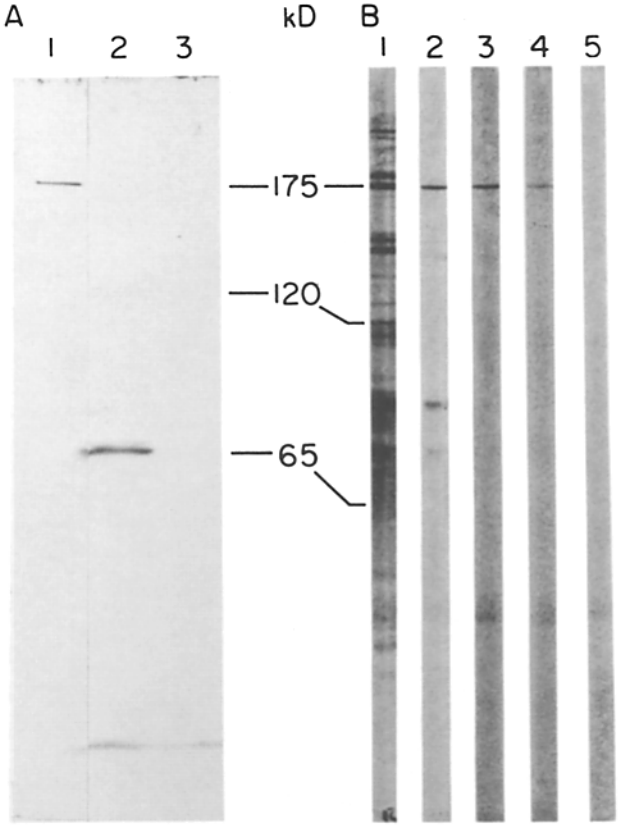


Figure 1. Evaluation of a 1.8-kb gene fragment identified by affinity-purified monospecific antibodies against EBA-175 by immunoblot analysis. (*A*) Blots were probed with antibodies against EBA-175 affinity purified from immune monkey serum using purified EBA-175 as the affinity substrate as described in Materials and Methods. Immunoblot of purified EBA-175 as described in Materials and Methods (lane *1*); proteins from EBA1.8/Y1089 grown with IPTG to induce beta-galactosidase (lane *2*); or EBA1.8/Y1089 grown without IPTG (lane *3*). The expressed protein was rapidly broken down from being fused to beta-galactosidase (total size ~120 kD) into a 65-kD fragment of expressed protein without beta-galactosidase. (*B*) Immunoblot of total parasite culture supernatant antigens. Strips were probed with: total immune monkey serum (lane *1*), antibodies against EBA-175 affinity purified from immune monkey serum (lane *2*), antibodies selected from immune monkey serum by affinity-purification with the 65-kD expression protein (lane *3*), antibodies selected from immune monkey serum by affinity purification with filter lifts of confluent plaques of EBA1.8/Y1089 (lane *4*), or normal monkey serum (lane *5*).

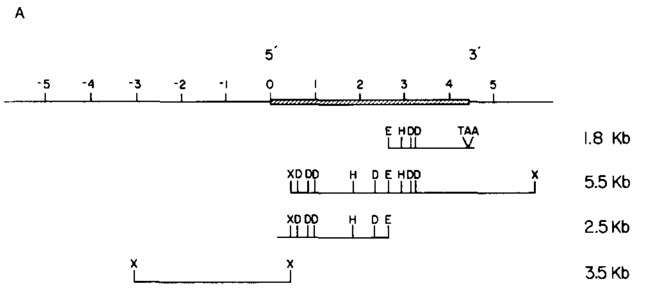
Results

Evaluation of Gene Fragment Identified by Affinity-purified Monospecific Antibodies against EBA-175

Preparations of EBA-175 (Camus and Hadley, 1985; Orlandi et al., 1990) were used to obtain affinity-purified monospecific polyclonal antibodies against EBA-175 (Orlandi et al., 1990) and subsequently used to identify the initial clone (EBA1.8/Y1090) from the library. This clone contained a 1.8-kb insert which when subcloned into the expression vector Y1089 expressed a beta-galactosidase fusion protein that was recognized in immunoblot analysis by the affinity-purified antibodies to EBA-175. The fusion protein was unstable and rapidly degraded to a 65-kD protein (Fig. 1 *A*). Both the 65-kD expressed protein and nitrocellulose filter lifts from confluent plates of clone EBA1.8/Y1090 were used to affinity purify antibodies from immune monkey serum containing antibodies against EBA-175. Such affinity-purified antibodies recognized the authentic EBA-175 in immunoblots (Fig. 1 *B*).

Nucleotide and Deduced Amino Acid Sequence of EBA-175 Gene

The 1.8-kb insert contained a 1.7-kb-long open reading frame that is in-frame with beta-galactosidase, followed by a highly AT-rich region with multiple stop codons at the 3′ end. The 1.8-kb insert was used as a probe to isolate a 5.5-kb Xba I fragment that contained an additional 2.2-kb open reading



B

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  -55                                                                                                      TATATATATATATATATATAGATAA  TAACATATAAATATATTCAATGTGCATACA
       M  K  C  N  I  S  I  Y  F  F    A  S  F  F  V  L  Y  F  A  K    A  R  N  E  Y  D  I  K  E  N    E  K  F  L  D  V  Y  K  E  K    F  N  E  L  D  K  K  K  Y  G  50
    1 ATGAAATGTAATATTAGTATATATTTTTTT  GCTTCCTTCTTTGTGTTATATTTTGCAAAA  GCTAGGAATGAATATGATATAAAAGAGAAT  GAAAAATTTTTAGACGTGTATAAAGAAAAA  TTTAATGAATTAGATAAAAAGAAATATGGA
       N  V  Q  K  T  D  K  K  I  F    T  F  I  E  N  K  L  D  I  L    N  N  S  K  F  N  K  R  W  K    S  Y  G  T  P  D  N  I  D  K    N  M  S  L  I  N  K  H  N  N  100
  151 AATGTTCAAAAAACTGATAAGAAAATATTT  ACTTTTATAGAAAATAAATTAGATATTTTA  AATAATTCAAAATTTAATAAAAGATGGAAG  AGTTATGGAACTCCAGATAATATAGATAAA  AATATGTCTTTAATAAATAAACATAATAAT
       E  E  M  F  N  N  N  Y  Q  S    F  L  S  T  S  S  L  I  K  Q    N  K  Y  V  P  I  N  A  V  R    V  S  R  I  L  S  F  L  D  S    R  I  N  N  G  R  N  T  S  S  150
  301 GAAGAAATGTTTAACAACAATTATCAATCA  TTTTTATCGACAAGTTCATTAATAAAGCAA  AATAAATATGTTCCTATTAACGCTGTACGT  GTGTCTAGGATATTAAGTTTCCTGGATTCT  AGAATTAATAATGGAAGAAATACTTCATCT
       N  N  E  V  L  S  N  C  R  E    K  R  K  G  M  K  W  D  C  K    K  K  N  D  R  S  N  Y  V  C    I  P  D  R  R  I  Q  L  C  I    V  N  L  S  I  I  K  T  Y  T  200
  451 AATAACGAAGTTTTAAGTAATTGTAGGGAA  AAAAGGAAAGGAATGAAATGGGATTGTAAA  AAGAAAAATGATAGAAGCAACTATGTATGT  ATTCCTGATCGTAGAATCCAATTATGCATT  GTTAATCTTAGCATTATTAAAACATATACA
       K  E  T  M  K  D  H  F  I  E    A  S  K  K  E  S  Q  L  L  L    K  K  N  D  N  K  Y  N  S  K    F  C  N  D  L  K  N  S  F  L    D  Y  G  H  L  A  M  G  N  D  250
  601 AAAGAGACCATGAAGGATCATTTCATTGAA  GCCTCTAAAAAAGAATCTCAACTTTTGCTT  AAAAAAAATGATAACAAATATAATTCTAAA  TTTTGTAATGATTTGAAGAATAGTTTTTTA  GATTATGGACATCTTGCTATGGGAAATGAT
       M  D  F  G  G  Y  S  T  K  A    E  N  K  I  Q  E  V  F  K  G    A  H  G  E  I  S  E  H  K  I    K  N  F  R  K  E  W  W  N  E    F  R  E  K  L  W  E  A  M  L  300
  751 ATGGATTTTGGAGGTTATTCAACTAAGGCA  GAAAACAAAATTCAAGAAGTTTTTAAAGGG  GCTCATGGGGAAATAAGTGAACATAAAATT  AAAAATTTTAGAAAAGAATGGTGGAATGAA  TTTAGAGAGAAACTTTGGGAAGCTATGTTA
       S  E  H  K  N  N  I  N  N  C    K  N  I  P  Q  E  E  L  Q  I    T  Q  W  I  K  E  W  H  G  E    F  L  L  E  R  D  N  R  S  K    L  P  K  S  K  C  K  N  N  T  350
  901 TCTGAGCATAAAAATAATATAAATAATTGT  AAAAATATTCCCCAAGAAGAATTACAAATT  ACTCAATGGATAAAAGAATGGCATGGAGAA  TTTTTGCTTGAAAGAGATAATAGATCAAAA  TTGCCAAAAAGTAAATGTAAAAATAATACA
       L  Y  E  A  C  E  K  E  C  I    D  P  C  M  K  Y  R  D  W  I    I  R  S  K  F  E  W  H  T  L    S  K  E  Y  E  T  Q  K  V  P    K  E  N  A  E  N  Y  L  I  K  400
 1051 TTATATGAAGCATGTGAGAAGGAATGTATT  GATCCATGTATGAAATATAGAGATTGGATT  ATTAGAAGTAAATTTGAATGGCATACGTTA  TCGAAAGAATATGAAACTCAAAAAGTTCCA  AAGGAAAATGCGGAAAATTATTTAATCAAA
       I  S  E  N  K  N  D  A  K  V    S  L  L  L  N  N  C  D  A  E    Y  S  K  Y  C  D  C  K  H  T    T  T  L  V  K  S  V  L  N  G    N  D  N  T  I  K  E  K  R  E  450
 1201 ATTTCAGAAAACAAGAATGATGCTAAAGTA  AGTTTATTATTGAATAATTGTGATGCTGAA  TATTCAAAATATTGTGATTGTAAACATACT  ACTACTCTCGTTAAAAGCGTTTTAAATGGT  AACGACAATACAATTAAGGAAAAGCGTGAA
       H  I  D  L  D  D  F  S  K  F    G  C  D  K  N  S  V  D  T  N    T  K  V  W  E  C  K  N  P  Y    I  L  S  T  K  D  V  C  V  P    P  R  R  Q  E  L  C  L  G  N  500
 1351 CATATTGATTTAGATGATTTTTCTAAATTT  GGATGTGATAAAAATTCCGTTGATACAAAC  ACAAAGGTGTGGGAATGTAAAAACCCTTAT  ATATTATCCACTAAAGATGTATGTGTACCT  CCGAGGAGGCAAGAATTATGTCTTGGAAAC
       I  D  R  I  Y  D  K  N  L  L    M  I  K  E  H  I  L  A  I  A    I  Y  E  S  R  I  L  K  R  K    Y  K  N  K  D  D  K  E  V  C    K  I  I  N  K  T  F  A  D  I  550
 1501 ATTGATAGAATATACGATAAAAACCTATTA  ATGATAAAAGAGCATATTCTTGCTATTGCA  ATATATGAATCAAGAATATTGAAACGAAAA  TATAAGAATAAAGATGATAAAGAAGTTTGT  AAAATCATAAATAAAACTTTCGCTGATATA
       R  D  I  I  G  G  T  D  Y  W    N  D  L  S  N  R  K  L  V  G    K  I  N  T  N  S  K  Y  V  H    R  N  K  K  N  D  K  L  F  R    D  E  W  W  K  V  I  K  K  D  600
 1651 AGAGATATTATAGGAGGTACTGATTATTGG  AATGATTTGAGCAATAGAAAATTAGTAGGA  AAAATTAACACAAATTCAAAATATGTTCAC  AGGAATAAAAAAAATGATAAGCTTTTTCGT  GATGAGTGGTGGAAAGTTATTAAAAAAGAT
       V  W  N  V  I  S  W  V  F  K    D  K  T  V  C  K  E  D  D  I    E  N  I  P  Q  F  F  R  W  F    S  E  W  G  D  D  Y  C  Q  D    K  T  K  M  I  E  T  L  K  V  650
 1801 GTATGGAATGTGATATCATGGGTATTCAAG  GATAAAACTGTTTGTAAAGAAGATGATATT  GAAAATATACCACAATTCTTCAGATGGTTT  AGTGAATGGGGTGATGATTATTGCCAGGAT  AAAACAAAAATGATAGAGACTCTGAAGGTT
       E  C  K  E  K  P  C  E  D  D    N  C  K  S  K  C  N  S  Y  K    E  W  I  S  K  K  K  E  E  Y    N  K  Q  A  K  Q  Y  Q  E  Y    Q  K  G  N  N  Y  K  M  Y  S  700
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       E  F  K  S  I  K  P  E  V  Y    L  K  K  Y  S  E  K  C  S  N    L  N  F  E  D  E  F  K  E  E    L  H  S  D  Y  K  N  K  C  T    M  C  P  E  V  K  D  V  P  I  750
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       S  I  I  R  N  N  E  Q  T  S    Q  E  A  V  P  E  E  N  T  E    I  A  H  R  T  E  T  P  S  I    S  E  G  P  K  G  N  E  Q  K    E  R  D  D  D  S  L  S  K  I  800
 2251 TCTATAATAAGAAATAATGAACAAACTTCG  CAAGAAGCAGTTCCTGAGGAAAACACTGAA  ATAGCACACAGAACGGAAACTCCATCTATC  TCTGAAGGACCAAAAGGAAATGAACAAAAA  GAACGTGATGACGATAGTTTGAGTAAAATA
       S  V  S  P  E  N  S  R  P  E    T  D  A  K  D  T  S  N  L  L    K  L  K  G  D  V  D  I  S  M    P  K  A  V  I  G  S  S  P  N    D  N  I  N  V  T  E  Q  G  D  850
 2401 AGTGTATCACCAGAAAATTCAAGACCTGAA  ACTGATGCTAAAGATACTTCTAACTTGTTA  AAATTAAAAGGAGATGTTGATATTAGTATG  CCTAAAGCAGTTATTGGGAGCAGTCCTAAT  GATAATATAAATGTTACTGAACAAGGGGAT
       N  I  S  G  V  N  S  K  P  L    S  D  D  V  R  P  D  K  K  E    L  E  D  Q  N  S  D  E  S  E    E  T  V  V  N  H  I  S  K  S    P  S  I  N  N  G  D  D  S  G  900
 2551 AATATTTCCGGGGTGAATTCTAAACCTTTA  TCTGATGATGTACGTCCAGATAAAAAGGAA  TTAGAAGATCAAAATAGTGATGAATCGGAA  GAAACTGTAGTAAATCATATATCAAAAAGT  CCATCTATAAATAATGGAGATGATTCAGGC
       S  G  S  A  T  V  S  E  S  S    S  S  N  T  G  L  S  I  D  D    D  R  N  G  D  T  F  V  R  T    Q  D  T  A  N  T  E  D  V  I    R  K  E  N  A  D  K  D  E  D  950
 2701 AGTGGAAGTGCAACAGTGAGTGAATCTAGT  AGTTCAAATACTGGATTGTCTATTGATGAT  GATAGAAATGGTGATACATTTGTTCGAACA  CAAGATACAGCAAATACTGAAGATGTTATT  AGAAAAGAAAATGCTGACAAGGATGAAGAT
       E  K  G  A  D  E  E  R  H  S    T  S  E  S  L  S  S  P  E  E    K  M  L  T  D  N  E  G  G  N    S  L  N  H  E  E  V  K  E  H    T  S  N  S  D  N  V  Q  Q  S  1000
 2851 GAAAAAGGCGCAGATGAAGAAAGACATAGT  ACTTCTGAAAGCTTAAGTTCACCTGAAGAA  AAAATGTTAACTGATAATGAAGGAGGAAAT  AGTTTAAATCATGAAGAGGTGAAAGAACAT  ACTAGTAATTCTGATAATGTTCAACAGTCT
       G  G  I  V  N  M  N  V  E  K    E  L  K  D  T  L  E  N  P  S    S  S  L  D  E  G  K  A  H  E    E  L  S  E  P  N  L  S  S  D    Q  D  M  S  N  T  P  G  P  L  1050
 3001 GGAGGAATTGTTAATATGAATGTTGAGAAA  GAACTAAAAGATACTTTAGAAAATCCTTCT  AGTAGCTTGGATGAAGGAAAAGCACATGAA  GAATTATCAGAACCAAATCTAAGCAGTGAC  CAAGATATGTCTAATACACCTGGACCTTTG
       D  N  T  S  E  E  T  T  E  R    I  S  N  N  E  Y  K  V  N  E    R  E  D  E  R  T  L  T  K  E    Y  E  D  I  V  L  K  S  H  M    N  R  E  S  D  D  G  E  L  Y  1100
 3151 GATAACACCAGTGAAGAAACTACAGAAAGA  ATTAGTAATAATGAATATAAAGTTAACGAG  AGGGAAGATGAGAGAACGCTTACTAAGGAA  TATGAAGATATTGTTTTGAAAAGTCATATG  AATAGAGAATCAGACGATGGTGAATTATAT
       D  E  N  S  D  L  S  T  V  N    D  E  S  E  D  A  E  A  K  M    K  G  N  D  T  S  E  M  S  H    N  S  S  Q  H  I  E  S  D  Q    Q  K  N  D  M  K  T  V  G  D  1150
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       L  G  T  T  H  V  Q  N  E  I    S  V  P  V  T  G  E  I  D  E    K  L  R  E  S  K  E  S  K  I    H  K  A  E  E  E  R  L  S  H    T  D  I  H  K  I  N  P  E  D  1200
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       R  N  S  N  T  L  H  L  K  D    I  R  N  E  E  N  E  R  H  L    T  N  Q  N  I  N  I  S  Q  E    R  D  L  Q  K  H  G  F  H  T    M  N  N  L  H  G  D  G  V  S  1250
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       E  R  S  Q  I  N  H  S  H  H    G  N  R  Q  D  R  G  G  N  S    G  N  V  L  N  M  R  S  N  N    N  N  F  N  N  I  P  S  R  Y    N  L  Y  D  K  K  L  D  L  D  1300
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       L  Y  E  N  R  N  D  S  T  T    K  E  L  I  K  K  L  A  E  I    N  K  C  E  N  E  I  S  V  K    Y  C  D  H  M  I  H  E  E  I    P  L  K  T  C  T  K  E  K  T  1350
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       R  N  L  C  C  A  V  S  D  Y    C  M  S  Y  F  T  Y  D  S  E    E  Y  Y  N  C  T  K  R  E  F    D  D  P  S  Y  T  C  F  R  K    E  A  F  S  S  M  I  F  K  F  1400
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       L  I  T  N  K  I  Y  Y  Y  F    Y  T  Y  K  T  A  K  V  T  I    K  K  I  N  F  S  L  I  F  F    F  F  F  S  F  *                                              1435
 4201 TTAATAACAAATAAAATATATTATTATTTT  TATACTTACAAAACTGCAAAAGTAACAATA  AAAAAAATTAATTTCTCATTAATTTTTTTT  TTCTTTTTTTCTTTTTAGGTATGCCATATT  ATGCAGGAGCAGGTGTGTTATTTATTATAT
 4351 TGGTTATTTTAGGTGCTTCACAAGCCAAAT  ATCAAAGGTTAGAAAAAATAAATAAAAATA  AAATTGAGAAGAATGTAAATTAAATATAGA  ATTCGAGCTCGG
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Figure 2. Structure of the gene encoding EBA-175 from the Camp strain of *P. falciparum.* (*A*) Representation of the overlapping clones sequenced. The restriction sites shown are *D* (Dde I), *E* (Eco RI), *H* (Hind III), and *X* (Xba I). Cross-hatched area represents coding region. (*B*) Nucleotide and deduced amino acid sequences of EBA-175. Nucleotides are numbered on the left and amino acids are numbered on the right. EBA-peptide 4 (residues 1,062–1,103) is underlined. The hydrophobic amino-terminal signal sequence is also underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number X52524.

frame (Fig. 2 *A*). Further upstream sequences were obtained from clones 2.5 and 3.5 kb. The sequences obtained coded for a total of 1,435 amino acid residues (Fig. 2 *B*). The signal sequence spans residues 1–19. The deduced amino acid sequence showed that EBA-175 is a highly charged molecule with uneven charge distribution. At physiologic pH, the NH_2-terminal portion of the molecule is positively charged (the calculated pI of residues 1–600 is 9.50), the region from amino acid residues 601–1,200 is negatively charged (pI 4.23), and the COOH-terminal portion (1,201–1,435) is again positively charged (pI 8.08).

The gene encoding EBA-175 is unique when compared with other characterized malaria antigens (Devereux et al., 1984) and does not contain repetitive sequences as are commonly found in malarial genes. However, there are two regions encompassing ∼190 amino acid residues each (179–

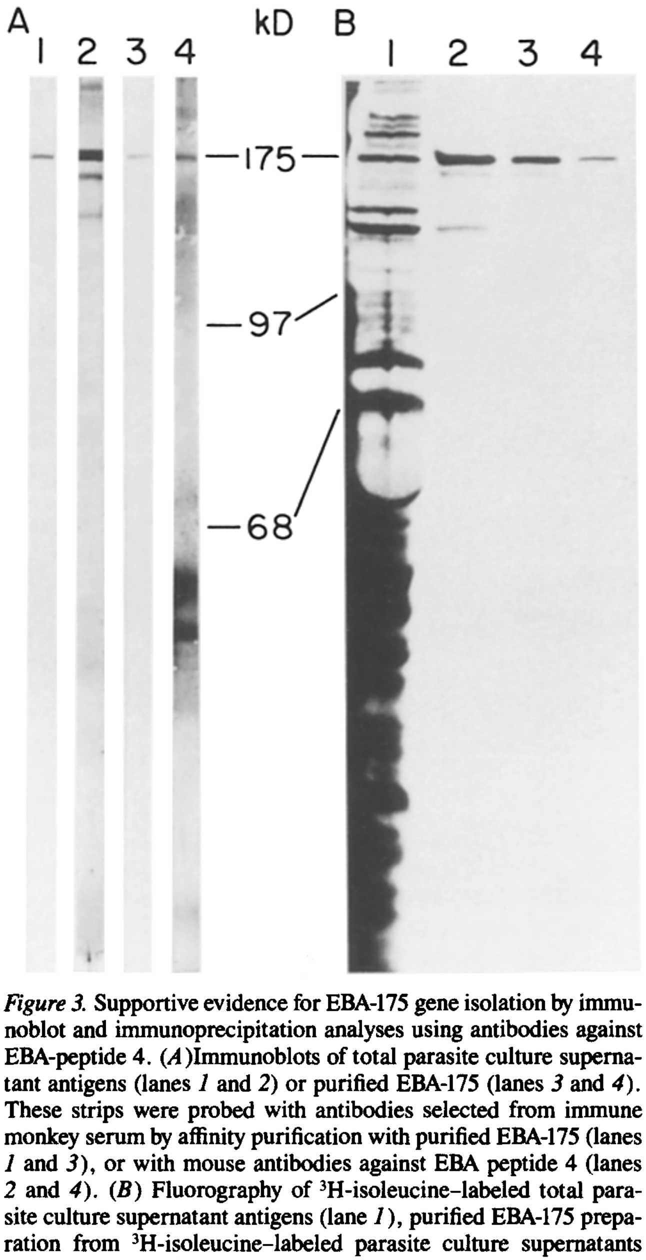

Figure 3. Supportive evidence for EBA-175 gene isolation by immunoblot and immunoprecipitation analyses using antibodies against EBA-peptide 4. (*A*)Immunoblots of total parasite culture supernatant antigens (lanes *1* and *2*) or purified EBA-175 (lanes *3* and *4*). These strips were probed with antibodies selected from immune monkey serum by affinity purification with purified EBA-175 (lanes *1* and *3*), or with mouse antibodies against EBA peptide 4 (lanes *2* and *4*). (*B*) Fluorography of ^{3}H-isoleucine–labeled total parasite culture supernatant antigens (lane *1*), purified EBA-175 preparation from ^{3}H-isoleucine–labeled parasite culture supernatants (lane 2), antigens from labeled culture supernatants immunoprecipitated by mouse antibodies against EBA-peptide 4 (lane *3*), or purified EBA-175 from labeled culture supernatants immunoprecipitated by mouse antibodies against EBA-peptide 4 (lane *4*), showing that antibodies raised against the synthetic EBA-peptide 4 recognized authentic EBA-175. Similar results were obtained when rabbit antipeptide 4 antibody was used in immunoblots (as in *A*) and in immunoprecipitation experiments (as in *B*).

370 [VCIP–RDWI] and 487–673 [VCVP–KEWI]) that have similar spacings for eight cysteine and six tryptophan residues each.

The deduced amino acid sequence of the EBA-175 gene encodes a protein with many predicted antigenic sites (Hopp and Woods, 1981). Three predicted antigenic peptides, EBA-peptide 2 (residues 939–969), EBA-peptide 5 (residues 970–1,014), and EBA-peptide 4 (residues 1,062–1,103), were synthesized based on these predictions and used to immunize mice and rabbits.

Antibodies against Synthetic Peptides, Immunoblotting, and Immunoprecipitation Analysis

ELISA antibody titers of immune sera titered against the respective unconjugated peptides, defined as the highest dilution giving an absorbance greater than two standard deviations above the mean absorbance for control sera from mice or rabbits immunized with KLH alone, were >1:50,000 for the three peptides tested. Only antibodies against EBA-peptide 4 raised in mice or rabbits identified EBA-175 in immunoblots of total parasite culture supernatant antigens, or in blots of purified EBA-175 preparations (Fig. 3 *A*). These same antibodies against EBA-peptide 4 precipitated EBA-175 from culture supernatants and preparations of purified EBA-175 (Fig. 3 *B*). Antibodies raised against EBA-peptide 2 or EBA-peptide 5 conjugated to KLH did not recognize malarial antigens in immunoblots or in immunofluorescence assays (data not shown). Controls for these experiments, performed with antibodies raised against the carrier KLH, were all negative.

Immunofluorescent Localization of EBA-175

In the immunofluorescent antibody test using sera from mice and rabbits immunized with EBA-peptide 4 conjugated to KLH, the fluorescence pattern showed staining throughout the cytoplasm of merozoites in schizonts with more intense staining near the apical end oriented away from the central pigment body (Fig. 4). These same antibodies stained free merozoites but did not stain ring-stage parasites. Two color immunofluorescence reactivities of antibodies against EBA-peptide 4 conjugated to KLH and mAb 3B10.1 against the merozoite surface antigen gp195, showed the difference between the staining patterns of these antigens. EBA-175 was localized and appeared as a dot, while in contrast, gp195 was on the surface of the entire merozoite (shown in black and white in Fig. 4). Antibodies raised against the carrier molecule KLH alone did not react with malaria parasites (data not shown).

Blocking of EBA-175 Binding to Erythrocytes by EBA-peptide 4 Antibodies

To investigate the functional relevance of antibodies raised against EBA-peptide 4, we incubated serum from a rabbit immunized with EBA-peptide 4 conjugated to KLH with culture supernatants containing EBA-175, and determined the capacity of this treated EBA-175 to bind to erythrocytes. Inhibition of binding of EBA-175 to erythrocytes was dependent on the concentration of serum raised against EBA-peptide 4 that was used; the inhibitory activity was 92% at a 1:5 dilution of the serum (Fig. 5, lane *4*). This inhibition was not seen with control serum from a rabbit that had been immunized with KLH alone (Fig. 5, lanes *5*–*7*).

Inhibition of Merozoite Invasion of Erythrocytes

We further investigated the effect on the parasite of antibodies against EBA-peptide 4 in an in vitro invasion inhibition assay. Similar to its inhibition of the binding of EBA-175 to erythrocytes, serum raised against EBA-peptide 4 inhibited merozoite invasion in a dose-dependent manner (Fig. 6); the inhibitory activity was 80% at a 1:5 dilution of serum. Inhi-

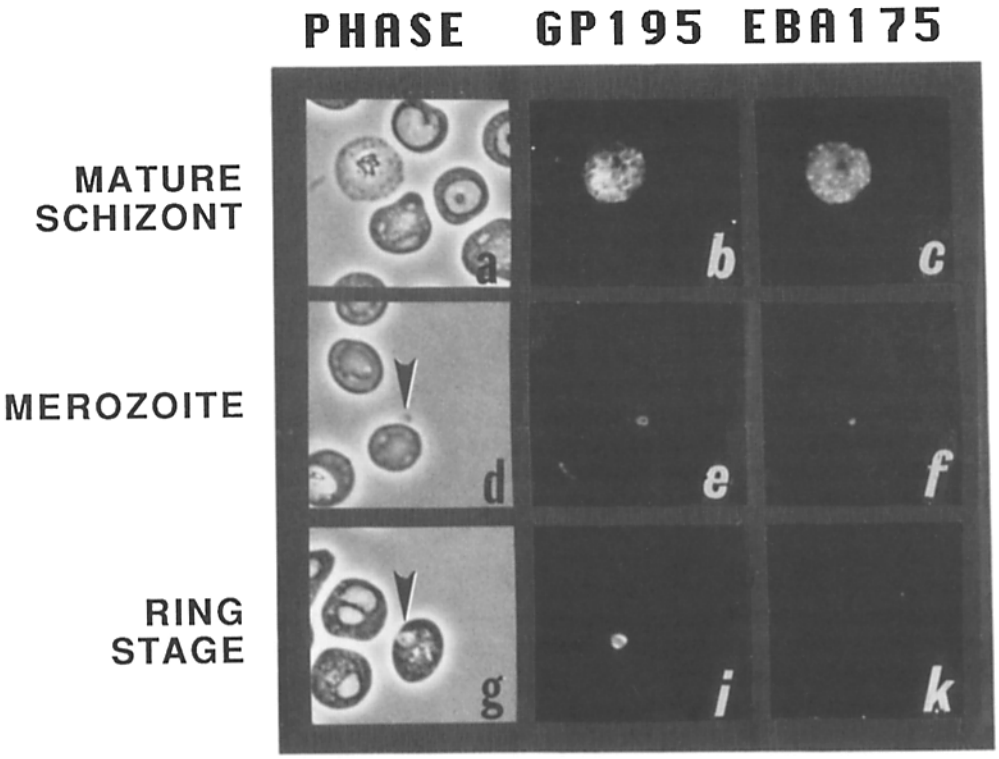

Figure 4. Reactivity with blood stage *P. falciparum* parasites of antibodies against EBA-peptide 4 and an mAb against the major merozoite surface antigen gp195 (3B10.1) by two color immunofluorescence presented in black and white. Vertical columns from left to right show phase-contrast reactivity with antibody 3B10.1 and reactivity with 1:1,000 diluted mouse anti-EBA-peptide 4 sera, respectively. Horizontal columns show various blood stages of *P. falciparum*. Arrow locates a merozoite (*d*), and a ring-stage parasite (*g*). Antibodies to EBA-peptide 4 stained the merozoite cytoplasm with more intense staining at the apical end (*c* and *f*). Magnification, 1000×.

bition was not seen with control serum from a rabbit that had been immunized with KLH alone.

Abrogation of Activity of Immune Sera

To clearly demonstrate that this activity was mediated by antibodies specific for EBA-peptide 4, we preincubated the sera from immunized animals with either EBA peptide 4 or with an unrelated peptide of similar size, and repeated the binding and invasion experiments shown in Figs. 5 and 6. Preincubation of immune sera with EBA peptide 4 (20 μg/ml) completely eliminated the capacity of the immune sera to block binding of EBA-175 to erythrocytes, and to inhibit merozoite invasion of erythrocytes. Preincubation with the unrelated peptide had no effect (data not shown).

Discussion

Since the binding of EBA-175 to intact erythrocytes correlates with the capacity of merozoites to invade these erythrocytes, and EBA-175 binds to merozoites (Camus and Hadley, 1985), it was postulated that EBA-175 may be a receptor acting as a "bridge" between erythrocytes and merozoites. We hypothesized that antibodies against this molecule would therefore inhibit the invasion of merozoites into erythrocytes, and that regions of EBA-175 would be potential vaccine candidates. In this report we describe the cloning and characterization of the gene encoding EBA-175, and show that sera from animals immunized with a synthetic peptide (EBA-peptide 4) comprising amino acid residues 1,062–1,103 block the binding of EBA-175 to erythrocytes and also inhibit parasite invasion in vitro. These results suggest that the antibodies induced by immunization with EBA-peptide 4 inhibit invasion by inhibiting binding of EBA-175 to erythrocytes. This activity is mediated by antibodies specific for EBA-peptide 4, since preincubation of anti-EBA-peptide 4 antisera with EBA-peptide 4 completely eliminated the capacity of the immune sera to mediate both these activities.

Binding of EBA-175 to erythrocytes involves sialic acid (Camus and Hadley, 1985). However, the molecular details of the interactions between merozoite, EBA-175, and erythrocyte have not been elucidated. Analysis of the deduced amino acid sequence of the gene encoding EBA-175 has led us to propose possible models for this process. The EBA-175 molecule is highly charged with uneven charge distribution. At physiologic pH, the NH_2-terminal portion of the molecule is positively charged (the calculated pI of residues 1–600 is 9.50) and the rest of the molecule is negatively charged (pI of residues 601–1,200 is 4.23) except the COOH-terminal end (residues 1,201–1,435) where it becomes positively charged again with a calculated pI of 8.08. Initial contact of the EBA-175 molecule with negatively charged sialic acids on erythrocytes could occur at the positively charged terminal portions of EBA-175. However, charge may not be the only factor important for binding to erythrocytes. There are similarities between the contact amino acid residues of the sialic acid binding site of an influenza virus hemagglutinin (Weis et al., 1988) and residues in EBA-175, occurring mainly within the negatively charged region of EBA-175, including EBA-peptide 4. Perhaps antibodies against EBA-peptide 4 interfere with an essential binding function of EBA-175 that involves sialic acid, and thereby inhibit merozoite invasion of erythrocytes.

Several *P. falciparum* proteins including rhoptry proteins (Sam-Yellowe and Perkins, 1990), 155- and 130-kD glycophorin binding proteins (Perkins, 1984), 140-, 70-, and 35-kD (Jungery et al., 1983) glycophorin binding proteins, and the 200-kD major merozoite surface antigen (Pf200 or gp195)

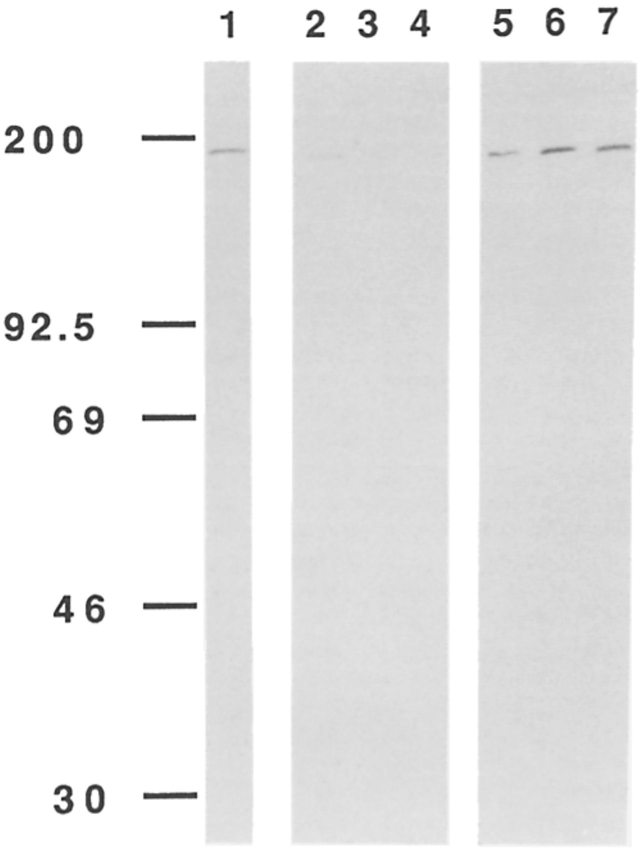

Figure 5. Blocking of EBA-175 binding to erythrocytes in vitro by antibodies against EBA-peptide 4. Lane 1 shows an autoradiograph of EBA-175 binding to erythrocytes in the absence of rabbit serum, showing EBA-175 binding under normal conditions. Lanes *2–4* depict binding in the presence of 1:20, 1:10, and 1:5 dilutions of rabbit anti-EBA-peptide 4 sera, respectively, showing decreasing amounts of EBA-175 bound to erythrocytes in the presence of increasing concentrations of serum. Lanes *5–7* show binding in the presence of 1:20, 1:10, and 1:5 dilutions of rabbit anti-KLH control sera, respectively. EBA-175 binding to erythrocytes was quantitated by scanning densitometry of the autoradiographs (LKB 2022 Ultrascan laser densitometer). Blocking of EBA-175 binding to erythrocytes in the presence of serum raised against EBA-peptide 4 is expressed as percentage blocking of EBA-175 bound to erythrocytes relative to the accompanying anti-KLH control. Values are as follows: 1:20 (23% blocking), 1:10 (85% blocking), and 1:5 (92% blocking).

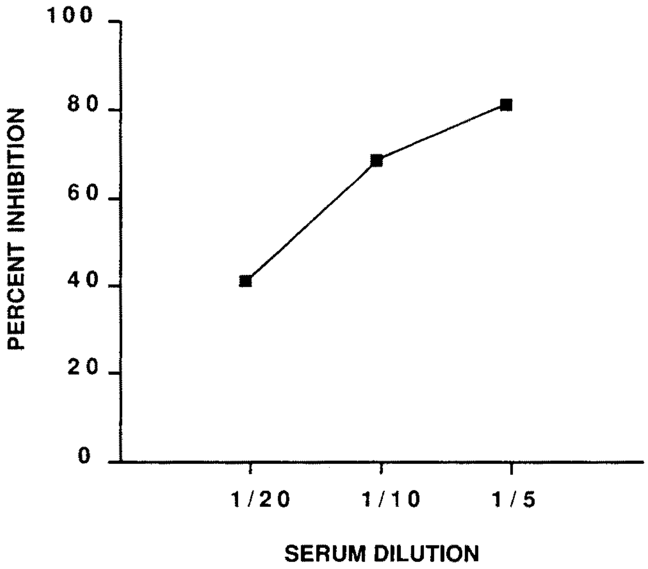

Figure 6. Inhibition of merozoite invasion into erythrocytes in vitro by rabbit serum against EBA-peptide 4 performed as described in Materials and Methods. Data are graphically expressed as the percent inhibition of merozoite invasion, calculated as described in Materials and Methods. The number of infected erythrocytes per 100 erythrocytes was significantly lower ($P < 0.005$, *t* test) in the erythrocytes exposed to anti-EBA-peptide 4 sera as compared to the control anti-KLH sera at serum dilutions of 1:5 (2.3 ± 0.33 vs 13.7 ± 0.33, mean ± SEM percentage of infected erythrocytes), 1:10 (4.3 ± 1.20 vs 13.7 ± 0.33), and 1:20 (8.3 ± 0.33 vs 14.0 ± 0.33). Inhibition of invasion was also seen in three separate experiments performed in triplicate with mouse anti-EBA-peptide 4 sera (data not shown).

(Perkins and Rocco, 1988) have also been proposed to be involved in erythrocyte invasion. Our findings demonstrate that the primary structure of EBA-175 is distinct from these proteins, as well as unique when compared to other malarial proteins. In contrast to most *Plasmodium* sp. genes that have been reported, the EBA-175 gene does not encode repeated amino acid sequences. However, analysis of the primary structure of EBA-175 shows that there is a "repeated" motif between regions comprising amino acid residues 179–370 and 487–673 in that these regions have similar spacings for eight cysteine and six tryptophan residues each. There is only limited similarity between the other residues, hydrophilicity profiles, and predicted secondary structures within these two regions; however, these regions may represent two domains diverging from a primitive gene that was duplicated some time in the distant past. The function of this cysteine and tryptophan residue "repeat" pattern is unknown.

The potential importance of EBA-peptide 4 as a vaccine candidate is also supported by the observation that this peptide is conserved at the nucleic acid level in seven strains of *P. falciparum* studied from different parts of the world (Sim, 1990). Furthermore, the structure of the EBA-175 gene among several strains of *P. falciparum* from different parts of the world appeared to be conserved when fragments of the EBA-175 gene from the Camp strain were hybridized to restriction endonuclease–digested genomic DNA from other strains. In genomic Southern blot analyses with restriction enzymes Eco RI, Dde I, Xba I, and Hind III, the 1.8-kb fragment and the 5′ Xba I–Hind III fragment (Fig. 1 *A*) showed strong hybridization to DNA from *P. falciparum* strains Camp (Malaysian), FCR3 (Gambia), and 7G8 (Brazil), plus isolates from St. Lucia, Brazil, and Honduras (data not shown). In addition, the sizes of restriction fragments recognized were virtually identical except for the FCR3 strain, which showed restriction fragment length polymorphism with restriction enzymes Dde I and Hind III. This indicates that there is a high degree of homology among different strains in regard to the gene structure and supports the observation that EBA-175 is antigenically conserved (Orlandi et al., 1990).

The putative merozoite receptor EBA-175 thus appears conserved among the *P. falciparum* strains studied. With the demonstration that antibodies to EBA-peptide 4, an invariant region of the molecule, block the binding of EBA-175 to erythrocytes and also inhibit merozoite invasion of erythrocytes, EBA-peptide 4 is clearly identified as a malaria vaccine candidate.

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