INFECTION AND IMMUNITY, Sept. 1997, p. 3631–3637 Vol. 65, No. 9
0019-9567/97/$04.00+0

Baculovirus-Mediated Expression of *Plasmodium falciparum* Erythrocyte Binding Antigen 175 Polypeptides and Their Recognition by Human Antibodies

JON R. DAUGHERTY,[1] CHERYL I. MURPHY,[2] LINDA A. DOROS-RICHERT,[2] ARNOLDO BARBOSA,[3] L. OSCAR KASHALA,[3] W. RIPLEY BALLOU,[1] NORMA J. SNELLINGS,[4] CHRISTIAN F. OCKENHOUSE,[1] AND DAVID E. LANAR[1]*

Departments of Immunology[1] and Bacterial Diseases,[4] Walter Reed Army Institute of Research, Washington, D.C. 20703, and Departments of Molecular Biology[2] and Molecular Pathogenesis,[3] Aquila Biopharmaceuticals, Inc., Worcester, Massachusetts 01605

Received 3 January 1997/Returned for modification 7 March 1997/Accepted 9 June 1997

The erythrocyte binding antigen EBA-175 is a 175-kDa *Plasmodium falciparum* protein which mediates merozoite invasion of erythrocytes in a sialic acid-dependent manner. The purpose of this study was to produce recombinant EBA-175 polypeptide domains which have previously been identified as being involved in the interaction of EBA-175 with erythrocytes and to determine whether these polypeptides are recognized by malaria-specific antibodies. The *eba-175* gene was cloned by PCR from genomic DNA isolated from the 3D7 strain of *P. falciparum*. The predicted protein sequence was highly conserved with that predicted from the published *eba-175* gene sequences from the Camp and FCR-3 strains of *P. falciparum* and contained the F segment divergent region. Purified recombinant EBA-175 polypeptide fragments, expressed as glutathione *S*-transferase fusion proteins in insect cells by using the baculovirus system, were recognized by antibodies present in serum from a drug-cured, malaria-immune *Aotus nancymai* monkey. The fusion proteins were also recognized by antibodies present in sera from individuals residing in areas where malaria is endemic. In both cases the antibodies specifically recognized the EBA-175 polypeptide portion of the fusion proteins. Antibodies raised in rabbits immunized with the recombinant fusion proteins recognized parasite proteins present in schizont-infected erythrocytes. Our results suggest that these regions of the EBA-175 protein are targets for the immune response against malaria and support their further study as possible vaccine components.

Malaria is one of the most serious diseases affecting much of the world. Its prevalence is rising as the result of increased resistance of both the vector and parasite to chemical treatment. Of the four species that cause human malaria, *Plasmodium falciparum* accounts for approximately one million to three million deaths and hundreds of millions of additional clinical cases per year (18). Because of this increase in drug resistance, much attention is being focused on the development of vaccines based on either single or multiple parasite-derived antigens. One of these proteins, the 175-kDa erythrocyte binding antigen, EBA-175, is a parasite receptor involved in sialic acid-dependent binding to and invasion of human erythrocytes by the merozoite stage of *P. falciparum* (6). EBA-175 mediates this process by interacting with the terminal NeuAc(α2-3)Gal- moieties of glycophorin A (23, 31). The gene encoding EBA-175 has been cloned from the Camp and FCR-3 strains of *P. falciparum*, and the deduced primary structures of the gene products are highly homologous (37, 42). The major difference between the two dimorphic proteins is the presence of a small defined set of amino acid residues. The Camp EBA-175 protein contains a 113-amino-acid-residue domain called the C segment, and the FCR-3 EBA-175 protein contains a 139-residue domain designated the F segment (42). Although these domains do not possess significant sequence homology, when expressed in vitro, they both bind to erythrocytes in a sialic acid-independent manner and contain cross-reactive epitopes (20).

* Corresponding author. Mailing address: Department of Immunology, Walter Reed Army Institute of Research, Washington, DC 20307-5100. Phone: (202) 782-3331. Fax: (202) 782-0748.

A second domain of EBA-175 has also been shown to be involved in erythrocyte binding (36). This domain, called region II (RII), consists of subdomains F1 and F2 and was first proposed to be involved in erythrocyte binding based on predicted primary sequence homology to the cysteine residue-rich erythrocyte binding domains of the *Plasmodium vivax* and *Plasmodium knowlesi* Duffy binding proteins (1). RII and its subdomain F2, when expressed in a COS cell surface expression system, bind to human erythrocytes in a sialic acid-dependent manner, and this binding is inhibited by soluble glycophorin A (36). An additional C-terminal cysteine-rich stretch of amino acids, region VI, has been reported to have high homology with a corresponding region of the Duffy binding proteins of *P. vivax* and *P. knowlesi* but does not bind to erythrocytes when expressed in the COS system (1, 36).

While these studies have yielded important insights into the function of the EBA-175 protein, the difficulty of isolating sufficient amounts of purified, native EBA-175 has precluded rigorous investigation of two aspects of EBA-175-mediated invasion of erythrocytes by merozoites. The first is determination of the types of molecular interactions involved in EBA-175 protein binding to its erythrocyte ligand. Structural studies require milligram to gram quantities of protein, an amount not practically obtainable from parasite culture supernatants. The second is delineation of the domains of EBA-175 which are both immunogenic and recognized by naturally acquired malaria-specific antibodies. The purpose of this study was twofold: first, to utilize the invertebrate baculovirus expression system to express in insect cells regions of EBA-175 which have been either shown or suggested to be important for erythrocyte binding and, second, to determine if the recombinant polypep-

TABLE 1. Synthetic oligonucleotides used in this study

Oligonucleotide	Positions[a]	Sequence (5′ to 3′)[b]
JRD2	373–396	CCTATTAACGCTGTACGTGTCT
JRD4	3136–3160	TTCCTTCATCCAAGCTACTAGAAGG
JRD5	527–552	ACGATCAGGAATACATACATAGTTGC
JRD8	1–17	ATGCGGATCCATGAAATGTAATATTAG
JRD39	2962–2983	GAACAAGGGGATAATATTTCCG
JRD40	4245–4263	ATATGCGGCCGCTCATGAAAAAGCCTCCTTTCTG
JRD61	1318–1339	ATGCGGTACCTGGTAACGACAATACAATTAAGG
JRD62	2305–2325	ATATGCGGCCGCTCACGTTCTGTGTGCTATTTCAGT
JRD91	2326–2344	ATGCGGATCCGAAACTCGTACGGATGAAC
JRD92	2729–2748	ATATGAATTCTCACTCTGTATCAGAACTTCCAC
JRD96	1381–1401	ATGCGGATCCGGATGTGATAAAAATTCCGTT
JRD97	2239–2259	ATATGAATTCTCATATTATAGAAATTGGTACATC
JRD111	3913–3939	ATGCGGATCCTCTAATAATAATAATTTTAATAATATT
JRD112	4245–4263	ATATGAATTCTCATGAAAAAGCCTCCTTTCTG

[a] Nucleic acid residue numbering is based on the 3D7 strain *eba-175* gene sequence.
[b] Nucleic acid residues added for cloning and translation termination are underlined.

tides are recognized by antibodies in sera obtained from regions of the world where malaria is endemic. The results of this study will facilitate identification of the domains of this important parasite ligand which will be the best candidates for inclusion in a malaria vaccine.

(A preliminary account of this work has been reported previously [10].)

MATERIALS AND METHODS

Parasite culture and genomic DNA isolation. *P. falciparum* 3D7 parasites were maintained in continuous synchronous culture by the method of Haynes et al. (17). Genomic DNA was isolated from late schizonts as previously described (42).

Bacterial growth and transformation and plasmid DNA preparation. *Escherichia coli* DH5α cells were grown and transformed as recommended by the supplier (Gibco BRL, Gaithersburg, Md.). Minipreparations of plasmid DNA were prepared by using standard methods (2). Large preparations of plasmid DNA were prepared with plasmid preparation kits according to the manufacturer's instructions (Qiagen, Inc., Chatsworth, Calif.).

Enzymes and chemicals. Restriction endonuclease and DNA modification enzymes were obtained from Gibco BRL (Bethesda, Md.). Deoxynucleoside triphosphates were purchased from Boehringer Mannheim Corp. (Indianapolis, Ind.). High-fidelity *Pfu* DNA polymerase was obtained from Stratagene (La Jolla, Calif.). Sequenase enzyme (version 2.0) was obtained from United States Biochemicals (Cleveland, Ohio). Ampli*Taq* FS enzyme was purchased from Perkin-Elmer (Foster City, Calif.).

Peptide synthesis and rabbit immunizations. Peptides derived from the predicted primary sequence of the EBA-175 protein were produced on an Applied Biosystems, Inc. (ABI) 430A peptide synthesizer by solid phase synthesis using the 9-fluorenylmethoxycarbonyl procedure (14). One of these peptides, P3 (ETRTDERKNQEPANKDLKNPC), encompassed amino acid residues 776 to 795 of the F segment domain. A second peptide, P7 (CGSNLNFEDEFKEELH SDYKNKGCC), encompassed amino acid residues 719 to 738 of the RII(F2) domain. The amino-terminal cysteine and glycine and the carboxy-terminal glycine and cysteine residues of peptide P7 were added during synthesis of the peptide to allow for circularization of the peptide. The peptides were conjugated with keyhole limpet hemocyanin, and aliquots of 200 μg of peptide-keyhole limpet hemocyanin were mixed with 50 μg of the saponin adjuvant QS-21 (22, 24). New Zealand White rabbits were injected intramuscularly at two sites at 2-week intervals for a total of 6 weeks.

Synthetic oligonucleotides. Oligonucleotide primers were synthesized with an ABI DNA-RNA synthesizer (Perkin-Elmer), deprotected, and then purified by using a PD-10 column (Pharmacia Biotech, Inc., Piscataway, N.J.).

PCR amplification, cloning, and sequencing of parasite DNA. Amplification of the *eba-175* gene was done directly from *P. falciparum* 3D7 genomic DNA that had been fingerprint typed as described previously (13). Oligonucleotide primers (Table 1) were synthesized with restriction enzyme-compatible ends for cloning. PCR amplification and cloning of DNA fragments were performed by standard methods (2). The nucleotide sequences of the cloned gene fragments were determined by the dideoxy chain terminal sequencing method of Sanger with either the Sequenase enzyme and [α-^{35}S]dATP or Ampli*Taq* FS enzyme, dye terminators, and an ABI 377 DNA sequencer (25, 35, 40). The predicted amino acid sequence for the EBA-175 protein was analyzed with the Genetics Computer Group (University of Wisconsin) sequence analysis package (11).

Cloning of the 3D7 *eba-175* gene and construction of recombinant baculovirus transfer plasmids. Molecular cloning of parasite DNA fragments was performed by standard methods (2). The entire DNA fragment encoding the predicted extracellular domain of EBA-175, exon I, was amplified by the PCR in three fragments with the oligonucleotide primer pairs JRD5-JRD8, JRD2-JRD4, and JRD39-JRD40 (Table 1). The fragments were then sequentially ligated together into the plasmid pBluescript SK$^+$ (Stratagene) by using the unique *Xba*I and *Eco*RI restriction endonuclease sites present in the *eba-175* gene.

The plasmid transfer vectors used for construction of recombinant baculovirus have been described previously (27, 41). The vector used for expression and secretion of the EBA-175 polypeptides as glutathione *S*-transferase (GST) fusions was plasmid pAcSG2T (Pharmingen, San Diego, Calif.) (38). PCR-amplified *eba-175* gene fragments were cloned into the unique *Bam*HI and *Eco*RI recognition sites in plasmid pAcSG2T. This plasmid contains a nucleotide sequence that encodes the secretion signal sequence from the baculoviral P67 protein (43). The oligonucleotide primers used for amplification of the gene fragments are shown in Table 1. Primer pair JRD96-JRD97 was used to amplify the DNA encoding the RII(F2) region (plasmid pAcJD15; amino acid residues 461 to 753), primer pair JRD91-JRD92 was used for amplification of the F segment domain (plasmid pAcJD10; amino acid residues 776 to 916), and primer pair JRD111-JRD112 was used for amplification of the 3′ Cys region (plasmid pAcJD25; amino acid residues 1305 to 1421) (region VI in reference 1).

The vector used for expression of the EBA-175 RII(F2) polypeptide lacking a GST tag was plasmid pJD7, a derivative of plasmid pVLBS1*egt* (27). Plasmid pJD7 was constructed from pVLBS1*egt* by filling in the *Eco*RI site at nucleotide 2900 and inserting the polylinker 5′-*Kpn*I *Pst*I *Xba*I *Bam*HI *Eco*RI *Nco*I *Not*I-3′ at the *Kpn*I site of the plasmid such that the 3′ *Kpn*I site was destroyed. The polylinker fragment consisted of annealed oligonucleotides JRD25 (5′-CCTGCAGTCTAGAGGATCCGAATTCCCATGGGCGGCCGCGTAC-3′) and JRD26 (5′-GCGGCCGCCCATGGGAATTCGGATCCTCTAGACTGC AGGGTAC-3′). Plasmid pJD7 contained a nucleotide sequence that encodes the secretion signal sequence from the baculoviral ecdysteroid UDP-glucosyl transferase (Egt) protein (30). The DNA fragment encoding the RII(F2) domain (amino acid residues 440 to 775) was amplified by PCR with the primer pair JRD61-JRD62 and cloned into the *Kpn*I and *Not*I sites in the polylinker of plasmid pJD7. The resulting construct was called plasmid pJD15.

Insect cell transfection and recombinant baculovirus construction. Cotransfections of plasmid and baculoviral DNA were performed with BacPAK6 baculoviral DNA (Clontech Laboratories, Palo Alto, Calif.) by standard methods (2).

Expression and purification of recombinant EBA-175 polypeptides produced in insect cells. Small-scale expression of recombinant EBA-175 polypeptides was performed by standard techniques (2). Modifications were made for large-scale expression of the recombinant polypeptides. Ten-liter batches of insect cells were maintained at pH 6.2 by using a controlled bioreactor system at 27°C with a constant agitation rate of 60 rpm. The dissolved oxygen level was maintained at 50% air saturation throughout the course of the recombinant viral infection cycle. The bioreactor vessel contained a serum-free medium (Cyto-Sf9; Kemp Biotechnologies, Inc., Frederick, Md.) and was inoculated with Sf21 cells at an initial density of 2.5×10^5/ml. The viability of the inoculum was >90% by the trypan blue exclusion test. When the cell density reached a level of approximately 1.5×10^6/ml, a high-titer stock of the appropriate virus produced in Cyto-Sf9 containing 10% fetal bovine serum (Hyclone, Logan, Utah) was added at a multiplicity of infection of 1. The culture was harvested at 72 h postinfection by using centrifugation at $800 \times g$ for 5 min. The supernatant fluid was collected, and the protease inhibitor phenylmethylsulfonyl fluoride was added to a final concentration of 10 mM. The supernatant fluid was stored frozen at −20°C prior to purification.

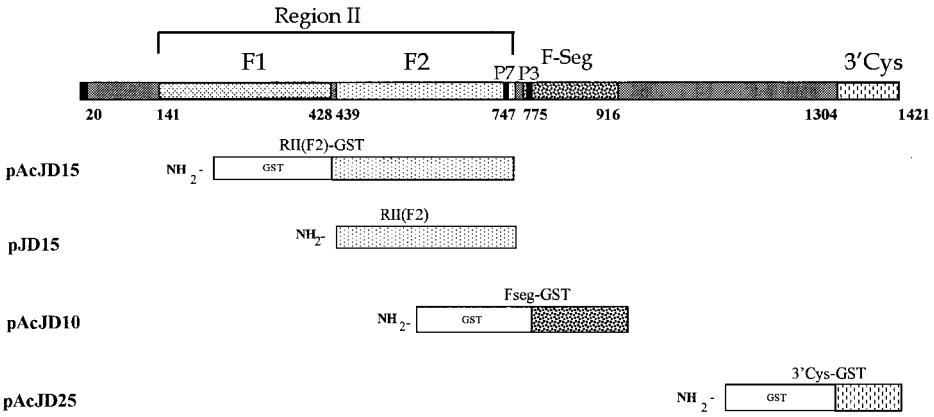

FIG. 1. Schematic diagram of the exon I-encoded EBA-175 extracellular domain and the polypeptides studied in this work. The names of the recombinant expression constructs are shown on the left. F-seg or Fseg, F segment.

Purification of recombinant proteins. The recombinant 3′ Cys-GST and GST were both purified in single passages over glutathione-Sepharose 4B resin chromatography columns (Pharmacia) (38). The RII(F2)-GST and F segment-GST proteins, however, did not initially bind directly to the glutathione-Sepharose. Purification of the F segment-GST protein was achieved with a combination of Q Sepharose Fast Flow (Pharmacia), G-75 Sepharose (Pharmacia), and glutathione-Sepharose 4B chromatography. Purification of the RII(F2)-GST fusion protein required Microprep S (Bio-Rad Laboratories, Hercules, Calif.), G-75 Sepharose, and glutathione-Sepharose 4B chromatography. Details of the column chromatography procedures are available upon request. Protein concentrations were determined by the method of Bradford (3). Purity of the recombinant proteins was approximated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining of 15 μg of each purified recombinant EBA-GST fusion protein.

***Aotus* and human sera.** Malaria-immune serum was obtained from an *Aotus nancymai* monkey that had been experimentally infected three times with the FVO strain of *P. falciparum*. Human serum samples were obtained from adults living in areas where *P. falciparum* is highly endemic and were pooled for use in Western blotting and enzyme-linked immunosorbent assays (ELISA).

Immunoblot analysis of secreted EBA-175 polypeptides. Cloned antigens were investigated by immunoblotting for reactivity with specific antibodies (2, 5). Either supernatant from cultures of recombinant virus-infected Sf9 cells or purified protein was subjected to SDS-PAGE under reducing conditions. Proteins were electrophoretically transferred to polyvinylidene difluoride membrane, and the membrane was blocked overnight in blocking buffer (10% nonfat milk in 1× phosphate-buffered saline). Diluted serum or horseradish peroxidase (HRP)-conjugated secondary antibody was incubated with the membrane for 1 h, and washed proteins were detected by enhanced chemiluminescence (Pierce Chemical Co., Rockford, Ill.). All reactions and washes were done at room temperature. Molecular mass standards were obtained from either Amersham or Gibco BRL.

Immunofluorescence assay (IFA). New Zealand White rabbits were immunized with 200 μg of recombinant proteins mixed with 50 μg of the adjuvant QS-21, and sera were obtained. Each serum sample was incubated at a 1:200 dilution with methanol-fixed 3D7 schizont-infected erythrocytes for 1 h at room temperature. The infected erythrocytes were washed and then incubated with fluorescein isothiocyanate-labeled secondary antibody for 1 h at room temperature, washed, and examined with a fluorescence microscope.

ELISA. Microtiter plates were incubated overnight with 2 μg of recombinant proteins/ml at 4°C and then blocked in Blocker casein in Tris-buffered saline (Pierce) plus 0.1% Tween 20 for 1 h at room temperature. Serum specimens diluted 1:100 in casein buffer were incubated with the plates for 1 h at room temperature. Plates were washed and incubated for 1 h with an alkaline phosphatase-labeled goat anti-human immunoglobulin G (IgG) antibody. The plates were washed, and the absorbance at 405 nm was measured by kinetic ELISA every 2 min for 1 h. The results were recorded as milli-optical density per minute. All samples were run in triplicate.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in the GenBank database under accession no. U32207.

RESULTS

Analysis of the predicted amino acid sequence of EBA-175 from the 3D7 strain and comparison with the homologous proteins from the FCR-3 and Camp strains of *P. falciparum*.

Sequencing of the cloned exon I of the 3D7 strain *eba-175* gene (Fig. 1) demonstrated that it contained the F segment-encoding region and that the predicted protein sequence differed from that of the FCR-3 strain at only three positions, one located in the cysteine-rich RII domain and the other two located carboxy terminal to the RII domain (Table 2). Besides the C or F segment, the predicted 3D7 EBA-175 protein sequence differed from the Camp amino acid sequence by nine residues. Six of the nine substitutions were located in the RII domain, and the other three were situated carboxy terminal to the RII domain. The 3D7 EBA-175 protein sequence contained 19 putative N-linked glycosylation sites; however, since *P. falciparum* has been reported to be unable to N glycosylate proteins, the significance of these sequences, if any, is unknown (12).

Immunoreactivity of baculovirus-expressed EBA-175 polypeptides. The RII(F2)-GST, F segment-GST, 3′ Cys-GST, and GST were expressed in insect cells, as was the RII(F2) region without a GST tag. The predicted molecular masses of the secreted recombinant fusion polypeptides are as follows: F segment-GST, 42 kDa; RII(F2)-GST, 62 kDa; RII(F2), 41 kDa; and 3′ Cys-GST, 40 kDa. The EBA-GST proteins were all recognized by polyclonal anti-GST antibodies by immunoblotting (Fig. 2A). Neither the GST fusion proteins nor GST alone was present in the supernatant from an uninfected Sf9

TABLE 2. Amino acid residue substitutions in the predicted primary sequences of the EBA-175 protein from the 3D7, FCR-3, and Camp strains of *P. falciparum*

Residue[a]	Strain[b]		
	3D7	FCR-3	Camp
286	K	**E**	**E**
478	K	K	**N**
481	K	K	**I**
577	N	N	**K**
584	Q	Q	**K**
664	R	R	**S**
768	S	S	**N**
932	K	K	**E**
1058	E	**V**	E
1100	G	**D**	**D**

[a] Numbering is based on the 3D7 EBA-175 sequence.
[b] Boldface type denotes differences in the predicted Camp and FCR-3 EBA-175 amino acid sequences compared to that of EBA-175 from strain 3D7.

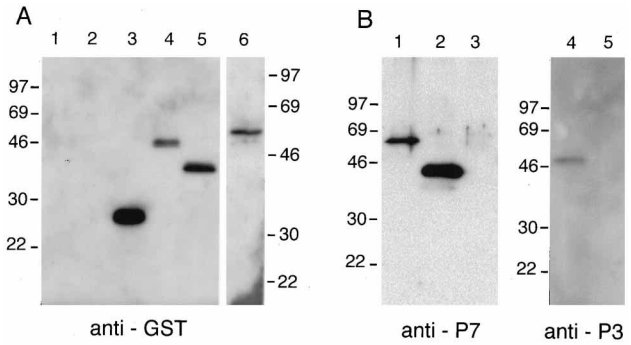

FIG. 2. Immunoreactivity of baculovirus-expressed recombinant EBA-GST fusion proteins with polyclonal anti-GST and anti-EBA-175 peptide antibodies as shown by immunoblotting. Ten microliters of insect cell culture supernatant containing secreted recombinant protein (5 μl for GST-containing supernatant) was analyzed. (A) Lane 1, uninfected insect cells; lane 2, insect cells infected with wild-type baculovirus; lane 3, baculovirus expressing GST alone; lane 4, baculovirus-expressed F segment-GST; lane 5, baculovirus-expressed 3′ Cys-GST; lane 6, baculovirus-expressed RII(F2)-GST. The supernatant containing RII(F2)-GST in lane 6 was electrophoresed through a 10% Tris-glycine gel in a separate experiment. The primary antibody was goat anti-GST at a 1:100 dilution, while the secondary antibody was rabbit anti-goat IgG HRP at a 1:2,000 dilution. (B) Lane 1, RII(F2)-GST; lane 2, RII(F2); lane 3, GST; lane 4, F segment-GST; lane 5, GST. The primary antibody was either rabbit anti-P7 at a 1:200 dilution (lanes 1 to 3) or rabbit anti-P3 at a 1:200 dilution (lanes 4 and 5). The secondary antibody was goat anti-rabbit IgG-HRP at a dilution of 1:5,000. Molecular masses are shown in kilodaltons.

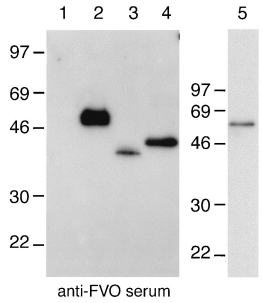

FIG. 3. Recognition of EBA-GST fusion proteins by serum antibodies produced by an *A. nancymai* monkey infected with the FVO strain of *P. falciparum*. Serum was used at a 1:100 dilution, and the secondary antibody was rabbit anti-*Aotus* IgG-HRP at a 1:500 dilution. Lane 1, 10 μl of culture supernatant containing GST alone; lane 2, 10 μl of culture supernatant containing F segment-GST; lane 3, 10 μl of culture supernatant containing 3′ Cys-GST; lane 4, 10 μl of culture supernatant containing RII(F2); lane 5, 0.5 μg of purified RII(F2)-GST. Molecular masses are shown in kilodaltons.

cell culture or from insect cells infected by wild-type baculovirus. Serum antibodies produced by a rabbit immunized with a synthetic peptide, P7, derived from the RII(F2) region recognized both the RII(F2)-GST and RII(F2) proteins but did not recognize the GST protein alone (Fig. 2B). Serum antibodies obtained from a rabbit immunized with peptide P8, the linear counterpart of peptide P7, showed similar reactivity by immunoblotting (data not shown). The F segment-GST fusion protein was recognized by antibodies present in serum from a rabbit immunized with peptide P3, which was derived from the F segment region of 3D7 EBA-175; GST protein alone was not (Fig. 2B).

Recognition of recombinant EBA-175 polypeptides by *Aotus* antibodies. As shown by immunoblotting, secreted F segment-GST, 3′ Cys-GST, RII(F2)-GST, and nontagged RII(F2) polypeptides present in infected insect cell supernatants were all recognized by serum antibodies obtained from an *A. nancymai* monkey infected with the FVO strain of *P. falciparum* and drug cured. As expected, GST control protein was not recognized by this antiserum (Fig. 3). Serum obtained from a malaria-naive *A. nancymai* monkey did not recognize the fusion proteins (data not shown). PCR and DNA fingerprint analyses have demonstrated that EBA-175 from all strains of *P. falciparum* tested to date contain either the F segment or the C segment dimorphic domain (42). The observation that the F segment was recognized by the FVO-immune serum antibodies suggested that the F segment domain was present in the EBA-175 protein derived from the FVO strain. Presence of the F segment domain was confirmed by PCR analysis (data not shown).

Recognition of recombinant EBA-175 polypeptides by human antibodies. Immunoblot analysis demonstrated that the 3′ Cys-GST, F segment-GST, RII(F2)-GST, and nontagged RII (F2) proteins present in culture supernatants were all recognized by antibodies present in pooled sera obtained from people residing in areas of Kenya and Indonesia where malaria is endemic, while GST alone was not (Fig. 4). No specific reactivity between malaria-naive human serum antibodies and any of the recombinant EBA-175 proteins was detected (data not shown). The recombinant proteins were purified by column chromatography such that each product was more than 90% restricted to a single predicted molecular mass band as described in Materials and Methods (Fig. 5). An ELISA was performed to determine whether the purified recombinant EBA-GST polypeptides would react under native conditions with antibodies in human serum (Fig. 6). The F segment-GST, RII(F2)-GST, 3′ Cys-GST, and GST proteins all reacted to antibodies present in sera from persons residing in regions where malaria is endemic, although the reactivity with GST was in all cases lower than that seen with the EBA-GST fusion proteins. A pattern of reactivity similar to that for the RII(F2)-GST was seen with purified RII(F2) which lacked the GST moiety (data not shown).

Recognition of parasite proteins by rabbit antibodies raised against recombinant EBA-175 polypeptides. Sera collected from rabbits immunized with recombinant EBA-GST fusion proteins were assayed by IFA for the presence of antibodies which were able to recognize malarial parasite proteins (Table 3). Serum antibodies from animals immunized with either RII(F2)-GST, F segment-GST, or 3′ Cys-GST recombinant fusion proteins recognized parasite proteins in methanol-fixed smears of infected erythrocytes, while serum obtained from an

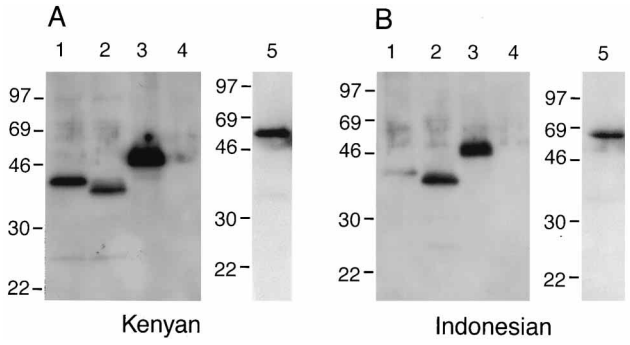

FIG. 4. Recognition of EBA-GST fusion proteins by human serum antibodies. Either 10 μl of supernatant containing fusion protein or 0.5 μg of pure fusion protein (lanes 5) was analyzed. Human sera were pooled samples from individual volunteers and were used at a 1:100 dilution. The secondary antibody was rabbit anti-human IgG-HRP at a 1:10,000 dilution. Lanes 1, RII(F2); lanes 2, 3′ Cys-GST; lanes 3, F segment-GST; lanes 4, GST; lanes 5, RII(F2)-GST. Molecular masses are shown in kilodaltons.

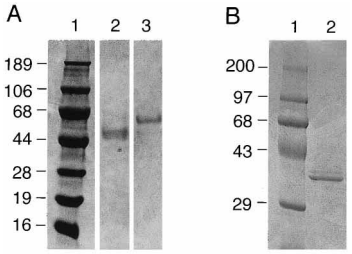

FIG. 5. Purification of EBA-GST fusion proteins. The EBA-GST fusion proteins were purified by column chromatography as described in Materials and Methods. Aliquots of the eluate from the glutathione-Sepharose columns were analyzed by SDS-PAGE and Coomassie blue staining. (A) Lane 1, molecular mass markers (in kilodaltons); lane 2, F segment-GST; lane 3, RII(F2)-GST. (B) Lane 1, molecular mass markers (in kilodaltons); lane 2, 3′ Cys-GST.

TABLE 3. Anti-recombinant EBA-175 antibody recognition of parasite proteins detected by IFA

Immunogen	Parasite recognition[a]	
	Preimmune[b]	Postdose
RII(F2)-GST	−	+
F segment-GST	−	+
3′ Cys-GST	−	+
GST	−	−

[a] +, fluorescent late-schizont-stage parasites present; −, no fluorescent parasites present.

[b] Rabbits were bled prior to the first immunization with recombinant protein and again 2 weeks after the third immunization.

animal which was immunized with GST alone was nonreactive. In addition, sera from animals immunized with recombinant EBA-GST proteins recognized a parasite protein with an approximate molecular mass of 175 kDa by immunoblotting, consistent with the known size of EBA-175 (data not shown).

DISCUSSION

Invasion of erythrocytes by malaria merozoites is a process that is mediated on the molecular level by multiple ligand-receptor interactions. Several proteins have been identified as being involved in this process, both on the parasite (MSP1, EBA-175, SERA, AMA-1, and a group of rhoptry proteins known as RHOP-H) and on the erythrocyte (6, 16, 32–34). Once the molecules involved have been identified, studies can be performed to investigate the effects on erythrocyte binding and invasion by interrupting these receptor-ligand interactions. Synthesis and purification of *Plasmodium* proteins by recombinant methods has proved difficult, but recent advances in bacterial, yeast, and baculovirus expression systems and the addition of purification tags have yielded several of these proteins in forms pure enough for immunization studies. Regions of MSP1 have been produced in bacteria, yeast, and baculovirus (4, 7, 21, 28). An N-terminal fragment of the SERA protein was made in yeast, and recently fragments of the SERA protein have been made in bacteria by using synthetic genes designed to mimic *E. coli* codon usage (19, 39). AMA-1 has been synthesized in and purified from baculovirus-infected insect cells (29). All of these recombinant proteins are now in vaccine trials in an attempt to induce antibodies that will block merozoite recognition of erythrocyte ligands and the subsequent invasion process through the erythrocyte membrane. Unlike these malaria antigens, to date, EBA-175 has not been produced in sufficient quantity or purity to permit detailed immunogenicity studies.

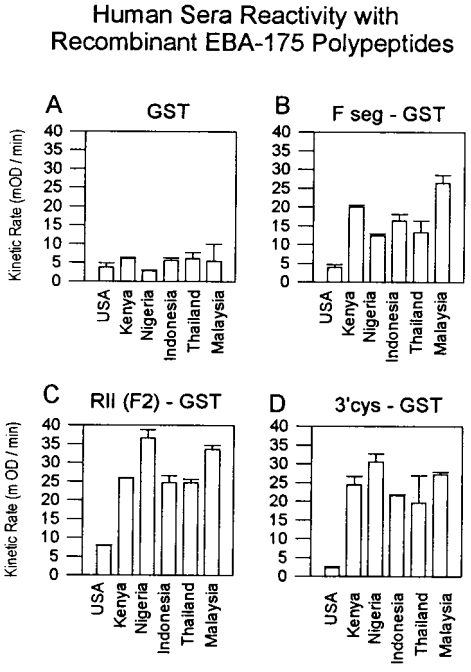

FIG. 6. Kinetic ELISA data showing recognition of native, purified EBA-GST fusion proteins by antibodies present in sera from humans residing in areas where malaria is endemic. All sera were pooled samples obtained from individual volunteers. The pooled sera from Kenya and Indonesia were the same samples used for the immunoblots shown in Fig. 4. ELISA was performed as described in Materials and Methods. F seg, F segment.

The gene encoding EBA-175 was cloned from the 3D7 strain, which is used in human malaria vaccine trials due to the strain's ability to be easily maintained in culture, its infectivity for mosquitoes, and its sensitivity to chloroquine and mefloquine (9). DNA sequence analysis demonstrated that the predicted sequence of the 3D7 protein was highly homologous to its counterpart from the FCR-3 strain of *P. falciparum*. Most of the amino acid substitutions consisted of nonconserved amino acids.

The reported roles of the cysteine-rich RII, F segment, and C segment domains in binding the EBA-175 protein to human erythrocytes suggests that these regions might be targets of the immune response to infection with *P. falciparum*. The results of the present study demonstrate that these domains contain B-cell epitopes which are accessible to the immune system, as shown by their reactivity with *Aotus* and human malaria-immune sera. While the 3′ cysteine-rich region does not bind to erythrocytes in the COS system, our results demonstrated that this region was recognized by antibodies present in the malaria-immune sera. Expression in COS cells is transient, with a transfection efficiency of 0.5 to 10%, and not all COS7 cells that express recombinant proteins bind erythrocytes (8). Therefore, it is still possible that the 3′ Cys domain binds erythrocytes but that the affinity of binding was too low to be detected in the COS expression system. Erythrocyte binding studies using the purified 3′ Cys-GST protein produced in this study will be required to answer this question. It is also possible that this domain has a function other than erythrocyte binding which is not detectable in the COS system, such as signal transduction. Cysteine-rich domains have been reported to be involved in receptor-mediated signaling (15, 26). Deletion and

site-directed mutagenesis studies will help clarify this possible role in vivo.

Our results demonstrate that EBA-175 recombinant polypeptides can be efficiently produced and secreted by using a eukaryotic invertebrate expression system and that these polypeptides are recognized by antibodies in human and monkey sera following infection with *P. falciparum*. These proteins were recognized by antibodies made against synthetic peptides designed from the predicted coding sequences of the EBA-175 protein domains. Furthermore, immunization of animals with these recombinant EBA-GST fusion proteins elicited antibodies which recognized native schizont-infected erythrocytes, as shown by immunofluorescence. Therefore, we conclude that the recombinant proteins which we have produced each possess a structural conformation similar to that of the corresponding domains contained within native parasite EBA-175 protein.

Recognition of an antigen by the immune system does not necessarily translate to an ability to elicit protection against infection by the malaria parasite. However, the availability of these purified, soluble recombinant EBA-175 polypeptides will allow the in vitro and in vivo studies required to answer this question to be performed. These results add further support to the hypothesis that these regions of the EBA-175 protein will be effective immunogens, and thus they should be studied further for possible inclusion in a malaria vaccine (36).

ACKNOWLEDGMENTS

J.R.D. and C.I.M. contributed equally to this work.

Christopher W. Kemp, Kemp Biotechnologies, Inc., Frederick, Md., performed the large-scale expression and purification of the recombinant proteins described in this study under a fee-for-service contract. We thank Kathy Moch for culturing of the parasites used in this work, Bruce T. Wellde for providing the monkey and Kenyan human sera, Stephen L. Hoffman for providing the Indonesian human sera, and Brian Hansen for synthesizing the peptides used in this study. We also thank Ted Hall for the use of cell culture facilities, Victoria L. Miller for technical assistance with the DNA sequencing, and Kent Kester and Jose Stoute for their careful review of the manuscript. We thank Annabelle Wright for preparation of the figures.

This investigation received financial support in part from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR).

REFERENCES

1. **Adams, J. H., B. K. Sim, S. A. Dolan, X. Fang, D. C. Kaslow, and L. H. Miller.** 1992. A family of erythrocyte binding proteins of malaria parasites. Proc. Natl. Acad. Sci. USA **89:**7085–7089.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1993. Current protocols in molecular biology. Greene Publishing Associates and Wiley Interscience, New York, N.Y.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Burghaus, P. A., and A. A. Holder.** 1994. Expression of the 19-kilodalton carboxy-terminal fragment of the *Plasmodium falciparum* merozoite surface protein-1 in Escherichia coli as a correctly folded protein. Mol. Biochem. Parasitol. **64:**165–169. (Erratum, **67:**343.)
5. **Burnette, W. N.** 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
6. **Camus, D., and T. J. Hadley.** 1985. A *Plasmodium falciparum* antigen that binds to host erythrocytes and merozoites. Science **230:**553–556.
7. **Chang, S. P., H. L. Gibson, C. T. Lee-Ng, P. J. Barr, and G. S. Hui.** 1992. A carboxyl-terminal fragment of *Plasmodium falciparum* gp195 expressed by a recombinant baculovirus induces antibodies that completely inhibit parasite growth. J. Immunol. **149:**548–555.
8. **Chitnis, C. E., and L. H. Miller.** 1994. Identification of the erythrocyte binding domains of Plasmodium vivax and Plasmodium knowlesi proteins involved in erythrocyte invasion. J. Exp. Med. **180:**497–506.
9. **Chulay, J. D., I. Schneider, T. M. Cosgriff, S. L. Hoffman, W. R. Ballou, I. A. Quakyi, R. Carter, J. H. Trosper, and W. T. Hockmeyer.** 1986. Malaria transmitted to humans by mosquitoes infected from cultured *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. **35:**66–68.
10. **Daugherty, J. R., C. I. Murphy, L. A. Richert, A. Barbosa, L. O. Kashala, W. R. Ballou, B. T. Wellde, and D. E. Lanar.** 1996. Baculovirus expression and purification of *Plasmodium falciparum* 3D7 strain 175kD erythrocyte binding antigen (EBA-175) polypeptides, abstr. 100, p. 131. *In* Program and abstracts of the 45th Annual Meeting of the American Society of Tropical Medicine and Hygiene. American Society of Tropical Medicine and Hygiene, Northbrook, Ill.
11. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
12. **Dieckmann-Schuppert, A., S. Bender, M. Odenthal-Schnittler, E. Bause, and R. T. Schwarz.** 1992. Apparent lack of N-glycosylation in the asexual intraerythrocytic stage of *Plasmodium falciparum*. Eur. J. Biochem. **205:**815–825.
13. **Dolan, S. A., J. A. Herrfeldt, and T. E. Wellems.** 1993. Restriction polymorphisms and fingerprint patterns from an interspersed repetitive element of *Plasmodium falciparum* DNA. Mol. Biochem. Parasitol. **61:**137–142.
14. **Fields, C. G., D. H. Lloyd, R. L. Macdonald, K. M. Otteson, and R. L. Noble.** 1991. HBTU activation for automated Fmoc solid-phase peptide synthesis. Pept. Res. **4:**95–101.
15. **Ghoush, S., and R. M. Bell.** 1994. Identification of discrete segments of human Raf-1 kinase critical for high affinity binding to Ha-Ras. J. Biol. Chem. **269:**30785–30788.
16. **Hall, R., A. Osland, J. E. Hyde, D. L. Simmons, I. A. Hope, and J. G. Scaife.** 1984. Processing, polymorphism, and biological significance of P190, a major surface antigen of the erythrocytic forms of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **11:**61–80.
17. **Haynes, J. D., C. L. Diggs, F. A. Hines, and R. E. Desjardins.** 1976. Culture of human malaria parasites *Plasmodium falciparum*. Nature **263:**767–769.
18. **Hoffman, S. L., and L. H. Miller.** 1996. Perspectives on malaria vaccine development, p. 1–3. *In* S. L. Hoffman (ed.), Malaria vaccine development: a multi-immune response approach. American Society for Microbiology, Washington, D.C.
19. **Inselburg, J., D. J. Bzik, W.-B. Li, K. M. Green, J. Kansopon, B. K. Hahm, I. C. Bathurst, P. J. Barr, and R. N. Rossan.** 1991. Protective immunity induced in *Aotus* monkeys by recombinant SERA proteins of *Plasmodium falciparum*. Infect. Immun. **59:**1247–1250.
20. **Kain, K. C., P. A. Orlandi, J. D. Haynes, K. L. Sim, and D. E. Lanar.** 1993. Evidence for two-stage binding by the 175-kD erythrocyte binding antigen of *Plasmodium falciparum*. J. Exp. Med. **178:**1497–1505.
21. **Kaslow, D. C., G. Hui, and S. Kumar.** 1994. Expression and antigenicity of *Plasmodium falciparum* major merozoite surface protein (MSP1(19)) variants secreted from *Saccharomyces cerevisiae*. Mol. Biochem. Parasitol. **63:**283–289.
22. **Kensil, C. R., U. Patel, M. Lennick, and D. Marciani.** 1991. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria molina* cortex. J. Immunol. **146:**431–437.
23. **Klotz, F. W., P. A. Orlandi, G. Reuter, S. J. Cohen, J. D. Haynes, R. Schauer, R. J. Howard, P. Palese, and L. H. Miller.** 1992. Binding of *Plasmodium falciparum* 175-kilodalton erythrocyte binding antigen and invasion of murine erythrocytes requires N-acetylneuraminic acid but not its O-acetylated form. Mol. Biochem. Parasitol. **51:**49–54.
24. **Kolodny, N., and F. A. Robey.** 1990. Conjugation of synthetic peptides to proteins: quantitation from S-carboxymethylcysteine released upon acid hydrolysis. Anal. Biochem. **187:**136–140.
25. **Lee, L. G., C. R. Connell, S. L. Woo, R. D. Cheng, B. F. McArdle, C. W. Fuller, N. D. Halloran, and R. K. Wilson.** 1992. DNA sequencing with dye-labeled terminators and T7 DNA polymerase: effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments. Nucleic Acids Res. **20:**2471–2483.
26. **Mott, H. R., J. W. Carpenter, S. Zhong, S. Ghosh, R. M. Bell, and S. L. Campbell.** 1996. The solution structure of the Raf-1 cysteine-rich domain: a novel ras and phospholipid binding site. Proc. Natl. Acad. Sci. USA **93:**8312–8317.
27. **Murphy, C. I., J. R. McIntire, D. R. Davis, H. Hodgdon, J. R. Seals, and E. Young.** 1993. Enhanced expression, secretion, and large-scale purification of recombinant HIV-1 gp120 in insect cells using the baculovirus egt and p67 signal peptides. Protein Expr. Purif. **4:**349–357. (Erratum, **5:**103, 1994.)
28. **Murphy, V. F., W. C. Rowan, M. J. Page, and A. A. Holder.** 1990. Expression of hybrid malaria antigens in insect cells and their engineering for correct folding and secretion. Parasitology **100:**177–183.
29. **Narum, D. L., G. W. Welling, and A. W. Thomas.** 1993. Ion-exchange-immunoaffinity purification of a recombinant baculovirus *Plasmodium falciparum* apical membrane antigen, PF83/AMA-1. J. Chromatogr. **657:**357–363.
30. **O'Reilly, D. R., and L. K. Miller.** 1989. A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. Science **245:**1110–1112.
31. **Orlandi, P. A., F. W. Klotz, and J. D. Haynes.** 1992. A malaria invasion receptor, the 175-kilodalton erythrocyte binding antigen of *Plasmodium falciparum* recognizes the terminal Neu5Ac(alpha 2-3)Gal- sequences of glycophorin A. J. Cell Biol. **116:**901–909.

32. **Perkins, M. E.** 1984. Binding of glycophorins to *Plasmodium falciparum* merozoites. Mol. Biochem. Parasitol. **10:**67–78.
33. **Perkins, M. E., and A. Ziefer.** 1994. Preferential binding of *Plasmodium falciparum* SERA and rhoptry proteins to erythrocyte membrane inner leaflet phospholipids. Infect. Immun. **62:**1207–1212.
34. **Peterson, M. G., V. M. Marshall, J. A. Smythe, P. E. Crewther, A. Lew, A. Silva, R. F. Anders, and D. J. Kemp.** 1989. Integral membrane protein located in the apical complex of *Plasmodium falciparum*. Mol. Cell. Biol. **9:**3151–3154.
35. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
36. **Sim, B. K., C. E. Chitnis, K. Wasniowska, T. J. Hadley, and L. H. Miller.** 1994. Receptor and ligand domains for invasion of erythrocytes by *Plasmodium falciparum*. Science **264:**1941–1944.
37. **Sim, B. K., P. A. Orlandi, J. D. Haynes, F. W. Klotz, J. M. Carter, D. Camus, M. E. Zegans, and J. D. Chulay.** 1990. Primary structure of the 175K *Plasmodium falciparum* erythrocyte binding antigen and identification of a peptide which elicits antibodies that inhibit malaria merozoite invasion. J. Cell Biol. **111:**1877–1884.
38. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene **67:**31–40.
39. **Sugiyama, T., K. Suzue, M. Okamoto, J. Inselburg, K. Tai, and T. Horii.** 1996. Production of recombinant SERA proteins of *Plasmodium falciparum* in Escherichia coli by using synthetic genes. Vaccine **14:**1069–1076.
40. **Tabor, S., and C. C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA **84:**4767–4771.
41. **Wang, Y.-H., A. H. Davies, and I. M. Jones.** 1995. Expression and purification of glutathione-S-transferase-tagged HIV-1 gp120: no evidence of an interaction with CD26. Virology **208:**142–146.
42. **Ware, L. A., K. C. Kain, B. K. Lee Sim, J. D. Haynes, J. K. Baird, and D. E. Lanar.** 1993. Two alleles of the 175-kilodalton *Plasmodium falciparum* erythrocyte binding antigen. Mol. Biochem. Parasitol. **60:**105–109.
43. **Whitford, M., S. Stewart, J. Kuzio, and P. Faulkner.** 1989. Identification and sequence analysis of a gene encoding gp67, an abundant envelope glycoprotein of the baculovirus *Autographa californica* nuclear polyhedrosis virus. J. Virol. **63:**1393–1399.

Editor: J. M. Mansfield