Expression of the Erythrocyte-Binding Antigen 175 in Sporozoites and in Liver Stages of *Plasmodium falciparum*

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Screening of a *Plasmodium falciparum* genomic expression library for antigens expressed at the pre-erythrocytic stages resulted in the isolation of a recombinant phage (DG249) whose insert corresponded to regions II and III of a 175-kDa erythrocyte-binding antigen (EBA-175). EBA-175 is a parasite ligand implicated in red blood cell invasion. Reverse-transcriptase polymerase chain reaction, indirect immunofluorescent antibody test, and Western blot analysis confirmed that EBA-175 is expressed not only in blood-stage parasites but also in infected hepatocytes and on the sporozoite surface. The presence of EBA-175 on pre-erythrocytic parasites enhances the vaccine potential of this antigen by adding another target to the immune responses elicited by immunization.

The 175-kDa erythrocyte-binding antigen (EBA-175) of *Plasmodium falciparum* was the first parasite ligand involved in red blood cell (RBC) invasion to be identified [1]. This protein is found in the supernate of cultured blood-stage parasites and specifically binds uninfected RBCs. The RBC ligand of EBA-175 has been identified as glycophorin A [2], and binding occurs via the recognition of the sialic acids and the peptide backbone of the ligand [2]. The domain of EBA-175 implicated in RBC attachment is called region II. This consists of a cysteine-rich 616-aa domain that has strong homology with a motif present in the Duffy-binding proteins of *P. vivax* and *P. knowlesi* [3]. The invasion of fresh RBCs is inhibited in the presence of antibodies raised against EBA-175 [4], thus making it an attractive malaria blood-stage vaccine candidate. Although some *P. falciparum* parasites can adopt alternative invasion pathways that are independent of the sialic acid residues of glycophorin A [5], anti–region II antibodies are effective in blocking the erythrocyte invasion, regardless of the pathway adopted [6].

In the course of our current research on pre-erythrocytic stage vaccines, we focused on a promising recombinant polypeptide derived from a single clone (DG249) isolated from a set of 120 clones selected by use of a strategy designed to identify genes expressed in the pre-erythrocytic stages [7]. This recombinant corresponded to a fragment of EBA-175.

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Materials and Methods

Parasites. *P. falciparum* salivary gland sporozoites were obtained from laboratory-maintained strain NF54 and from mosquitoes fed in vitro on blood obtained from 10 Thai patients [8]. *P. falciparum* liver schizonts were obtained from liver biopsy specimens of a *Cebus apella* on day 5 after infection by sporozoites derived from a patient isolate [9] and from a chimpanzee *(Pan troglodytes)* on day 6 (i.e., mature schizonts) after infection with NF54 strain sporozoites [10]. Intraerythrocytic parasites (NF54 strain) were cultivated in vitro, as described elsewhere [11]. *P. yoelii yoelii* salivary gland sporozoites were obtained from laboratory-maintained clone 1.1.

Sequence analysis. The DG249 insert was amplified from the recombinant phage by using primers 21D (CCTGGAGCCCGTCAGTATCGGCGG) and 26D (GGTAGCGACCGGCGCTCAGCTGG) flanking the *Eco*R1 cloning site and was subcloned in the pTrc His_6-tagged vector (Invitrogen). The sequence of the insert was obtained by dideoxy sequencing from 4 separate phages.

Reverse-transcriptase polymerase chain reaction (RT-PCR). Total RNA was purified from *P. falciparum* NF54 sporozoites and blood stages [12]. RT-PCR (with the OneStep RT-PCR kit; Qiagen) was performed with the sense primer (GTGACCATATGATTCATGAAGAAATCCCA) and the antisense primer (CCTCATGGTATTCAGAAAAATCG; denoted 1, figure 1) by use of 30 cycles of 1 min of denaturation at 94°C, 2 min of annealing at 50°C, and 1 min of elongation at 72°C. We used 1 μL of this PCR product as template in a second reaction (35 cycles, same conditions as above) with a primer pair encompassing the last 2 introns (denoted 2, figure 1) with the same antisense primer as above but with a different sense primer (GCCATATTATGCAGGAGCAGGTGTGT). PCR products were purified and cloned in the Topo vector (Invitrogen). We did sequence homology searches by use of the

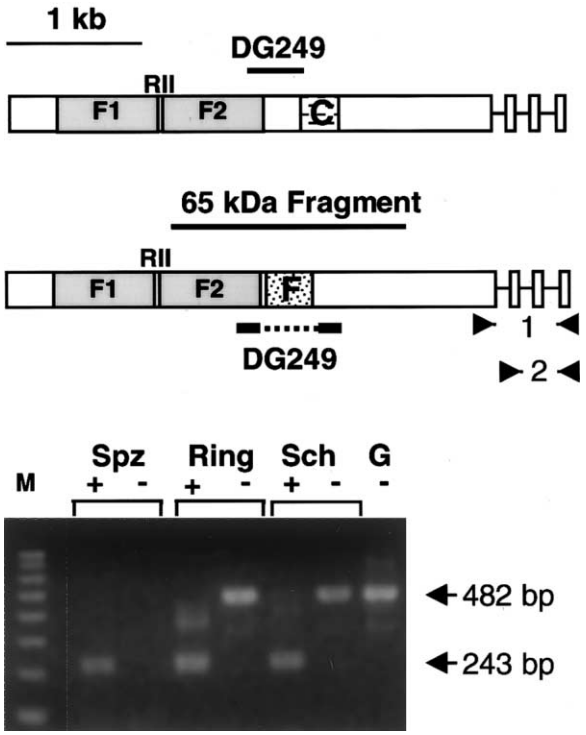

Figure 1. *Top,* Schematic presentation of *eba-175* gene: 2 allelic variants of erythrocyte-binding antigen 175 (EBA-175) [28] are characterized by insertion of either a 114-aa segment in Camp-like strains (C-segment) or a 141-aa segment in FCR-3–like strains (F-segment); the rest of the molecule is highly conserved. The F-segment is inserted 91 aa upstream of the C-segment insertion site; however, in EBA-175 of the FCR-3 allelic variant, the DG249 insert would span the F-segment insertion site and thus would be interrupted by this stretch. EBA-175–specific IgG1 monoclonal antibody R217 is directed against the RII region (F1 + F2). *1,* Reverse-transcriptase polymerase chain reaction (RT-PCR) primers; *2,* second PCR primers. *Bottom,* RT-PCR product resulting from last amplification (primer pair 2) by using total RNA from sporozoites (Spz), blood-stage ring forms (Ring), and schizonts (Sch); +, reactions done with RT; −, reactions done without RT. *Lane G,* product obtained with *Plasmodium falciparum* genomic DNA. Lower 243-bp band corresponds to completely spliced mRNA; 482-bp band was observed for nonspliced or genomic DNA. Contamination of RNA purified from blood-stage rings and schizont forms with small amounts of parasite genomic DNA resulted in 482-bp bands in negative tracks corresponding to these samples. Amplification from this minor contaminant will be outcompeted by cDNA resulting from RT reaction in positive samples. Intermediate-size band seen in the Ring-positive track probably corresponds to incompletely spliced product. *Lane M,* Molecular weight markers.

BLAST server [13] (available at http://www.ncbi.nlm.nih.gov/BLAST/) against the *P. falciparum* genome databases (accessed May 2000) and the available genomic sequences from *P. yoelii* (TIGR database; available at http://www.tigr.org/tdb; accessed November 2000).

Recombinant protein expression and purification of specific antibodies. The His_6-recombinant protein (His_6-249) was purified by affinity chromatography on a 1-mL Hi-Trap chelating column linked with nickel chloride, followed by anion exchange chromatography on Hi-Trap Q columns (Pharmacia). Immunopurification of specific human antibodies contained in pooled African hyperimmune serum samples was done on ELISA plates [14] coated with His_6-249 after depletion of antibodies reacting nonspecifically with untransformed *Escherichia coli* extracts from the serum samples. Antibodies against His_6-249 were also obtained by immunization of Balb/C mice. The specificity of the purified antibodies to the His_6-249 protein was confirmed by the lack of staining of the following molecules blotted on nitrocellulose paper: $(NANP)_{50}$ peptide corresponding to the repeat unit of the circumsporozoite (CS) protein of *P. falciparum* and recombinant proteins, similarly derived from the same library, that correspond to different fragments of LSA3 and SALSA. Specific recognition of EBA-175 was further confirmed by comparison with the proteins recognized with an IgG1 mouse monoclonal antibody, R217, raised against the EBA-175 region II (provided by D. Narum, EntreMed, Rockville, MD) and used at a 1:100 dilution.

Human serum samples. Hyperimmune serum samples were obtained from persons residing in a highly endemic area of the Ivory Coast (100 infective bites/person/year). Serum samples also were obtained from 4 volunteers who were immunized by irradiated *P. falciparum* sporozoites and who were protected against a viable challenge (gift of D. Haynes, Walter Reed Army Institute of Research, Washington, DC, and R. Edelman, Center of Vaccine Development, University of Maryland, Baltimore). These serum samples were used at a 1:100 dilution, and their end point titers were 1:4000 and 1:6000. Finally, serum samples were collected from 4 patients who accidentally contracted *P. falciparum* malaria after receiving transfusions with infected blood. Of note, one of these patients harbored parasites for 45 days, and parasitemia had reached 26% at the time of the correct diagnosis. This serum was used at a 1:100 dilution, but the limited quantity available did not justify end-point titration.

Western blotting and ELISAs. Parasite protein extracts from sporozoite and blood stages of *P. falciparum* NF54 strain corresponding to ~10^6 parasites were subjected to 10% SDS-PAGE and were electroblotted onto nitrocellulose membranes. These were probed with His_6-249–immunopurified human antibody from the serum of one volunteer or with anti–His_6-249 mouse antiserum diluted 1:100. ELISA plates were coated with His_6-249 and were probed with serum samples from volunteers and from patients with transfusion malaria.

IFA. Surface immunofluorescence was performed on sporozoites deposited on poly-L-lysine–coated slides in a "wet" sporozoite assay [15]. For the liver stages, we used both Carnoy-fixed material and 5-μm cryosections from unfixed material stored in liquid nitrogen. Acetone-fixed thin blood smears from synchronized *P. falciparum* blood cultures in ring and schizont stages were used in the standard indirect immunofluorescent antibody test (IFAT) with a second antibody, a fluorescein-labeled anti–IgG+M (Diagnostics Pasteur) diluted 1:200 in PBS.

Results

The clone (DG249) contained a 507-bp insert that perfectly matched a 169-aa stretch of the EBA-175 protein of the Camp

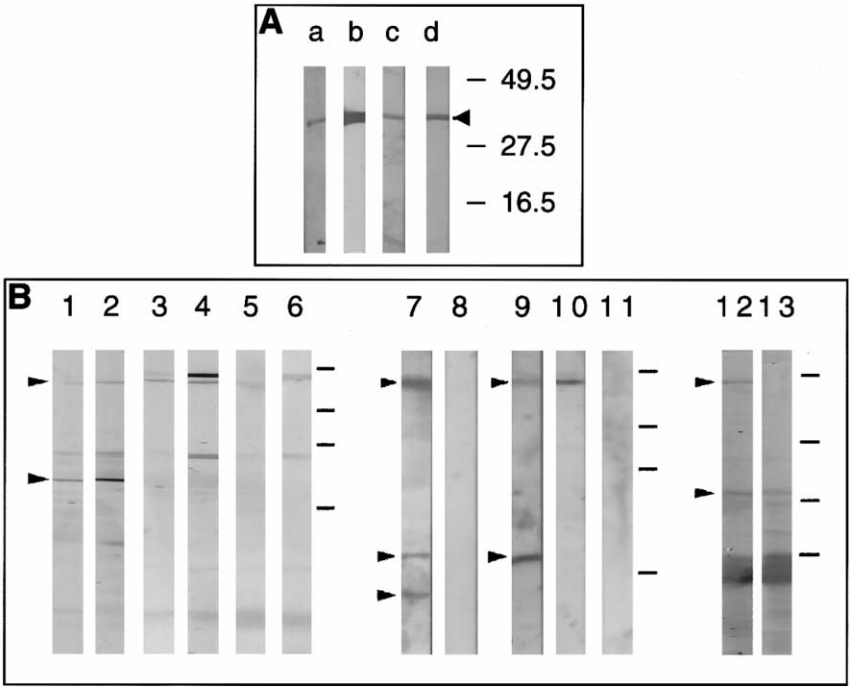

Figure 2. *A,* Western blot of purified recombinant His_6-249 protein, blotted with the serum used to screen the library [7] from which clone DG249 was obtained *(a),* anti–mouse His_6-249 serum raised against it and used throughout the study *(b),* serum from an irradiated sporozoite-immunized volunteer *(c),* and serum from transfusion malaria case patient *(d)*. Position and relative molecular mass (in kilodaltons) of molecular markers are indicated at right. *B,* Recognition of erythrocyte-binding antigen 175 (EBA-175) by specific antibodies. Specificity of anti–His_6-249 mouse serum *(lanes 1–6):* Western blot extracts from sporozoite *(lanes 1* and *2),* mature trophozoite *(lanes 3* and *4),* and uninfected red blood cells (RBCs; *lanes 5* and *6*) probed side-by-side with EBA-175–specific monoclonal antibody R217 *(lanes 1, 3,* and *5)* and anti–His_6-249 mouse serum *(lanes 2, 4,* and *6)*. Recognition of EBA-175 in parasites of different stages. Protein extracts (isolated independently from those used in *lanes 1–6*) probed with anti–His_6-249 mouse serum: sporozoites *(lane 7),* uninfected *Anopheles gambiae* salivary glands *(lane 8),* early ring forms and mature schizonts from blood-stage parasites *(lanes 9* and *10,* respectively), and uninfected RBCs *(lane 11)*. *Lane 12,* Recognition of EBA-175 on Western blotted sporozoite extracts by His_6-249–immunopurified human antibodies from serum of a protected irradiated sporozoite-immunized volunteer; *lane 13,* polypeptides recognized by antibodies immunopurified from this serum on $(NANP)_{50}$, a circumsporozoite protein immunodominant repeat region peptide. Positions of molecular weight markers are at right of each set, in decreasing order of relative molecular masses: 209, 120, 78, and 47 kDa *(lanes 1–6);* 205, 116, 80, and 49 kDa *(lanes 7–11);* and 175, 83, 62, and 47.5 kDa *(lanes 12* and *13)*.

strain of *P. falciparum* (residues 688–857), stopping short of the C-segment insertion site (figure 1). Since this clone was selected as a putative pre-erythrocytic stage antigen, we did a number of experiments, to establish the actual expression of EBA-175 in sporozoites and liver stage parasites.

RT-PCR analysis of total RNA isolated from *P. falciparum* sporozoites or blood stages confirmed the genetic expression of the *eba-175* gene in the sporozoite. The primers used were designed to span the last 2 of the 3 introns identified at the 3′ end of the gene [3] (figure 1). The product obtained by RT-PCR from sporozoite RNA was similar in size to that obtained from ring or schizont RNA. This size corresponded to that of the predicted correctly spliced mRNA. Sequencing of all the products confirmed the putative splice sites of the last 2 introns and further demonstrated that these splice sites were the same in sporozoites and blood stages.

Antibodies specific to His_6-249 were used to probe material in Western blots and IFAT from pre-erythrocytic parasites. The homogeneity of the purified fusion protein used for raising antisera or affinity purification of human antibodies was established by acrylamide gel electrophoresis (data not shown) and was further confirmed when the Western blotted preparation was probed with serum samples from mice immunized with the His_6-249, the serum used to screen the library from which the DG249 clone was obtained, serum from a volunteer immunized from irradiated sporozoites, and serum from a person who accidentally acquired a malaria infection through blood transfusion (figure 2*A*).

Two types of antibodies specific to the His_6-249 recombinant protein were obtained: those resulting from immunization of mice with the His_6-249 recombinant and those affinity purified on this recombinant from serum samples of malaria-exposed persons and thus representing the antibodies elicited by natural infection. The specificity of these antibodies to EBA-175 was established in preliminary experiments. No cross-reactivity was observed with non–EBA-175 dot-blotted recombinant parasite

proteins or untransformed *E. coli* extracts. The polypeptides recognized by the anti–His_6-249 antibodies raised in mice (predominantly IgG2A) on Western blotted sporozoite and blood-stage parasite extracts (figure 2*B, lanes 1–6*) were compared with those reacting with an EBA-175–specific mouse monoclonal antibody (R217, an IgG1 antibody raised against region II of EBA-175; gift of D. Narum, EntreMed). EBA-175 is synthesized as a 190-kDa protein that is then converted to the 175-kDa polypeptide [16]. The doublet (~190 and 175 kDa) observed for blood-stage parasites with R217 is observed also with the mouse anti–His_6-249 antibodies. The pattern observed with sporozoite extracts is similar for the 2 antibodies, and the 65-kDa polypeptide is clearly labeled.

Western blotting of protein extracts of NF54 *P. falciparum* sporozoites probed with the mouse antiserum confirmed the presence of the EBA-175 protein in sporozoites (figure 2*B*, *lanes 7* and *8*). In these blots, 2 polypeptides of smaller molecular mass, 65 and 45 kDa, were observed, in addition to the 175-kDa band (figure 2*B, lane 7*). Blood-stage parasite extracts were concurrently blotted and probed. In schizonts, the 175-kDa band was observed alone (figure 2*B, lane 10*), whereas, in rings, this band was faint, but the 65-kDa band was strongly labeled (figure 2*B, lane 9*). The same pattern was observed when His_6-249 affinity–purified human antibodies were used (data not shown). The origin of the 45-kDa band is not known, but the 65-kDa polypeptide most likely corresponds to the proteolytic cleavage of EBA-175, as described elsewhere [17].

Antibodies against EBA-175 were detected by ELISA in serum samples from 4 persons accidentally contaminated by blood transfusion and exposed solely to blood stages and in serum samples of 4 volunteers immunized by irradiated sporozoites and protected against a *P. falciparum* sporozoite challenge and thus exposed to sporozoites and early liver forms only. This latter immune recognition of EBA-175 further confirmed the expression of the antigen at the pre-erythrocytic stages. Further analysis of the antibodies in these serum samples showed a contrasting pattern of specific IgG isotype distribution (IgG2/IgG1 optical density ratio, 0.73 ± 0.28 in the transfusion malaria serum samples and 1.72 ± 0.16 in the volunteer serum samples; $P = .021$, Mann-Whitney U test). His_6-249 recombinant antibodies were affinity purified from one serum sample from one volunteer. These antibodies recognize smears of 175, 65, and ~45 kDa in Western blot sporozoite extracts (figure 2*B, lane 12*). Antibodies affinity purified from this serum by use of the $(NANP)_{50}$ peptide that corresponds to the immunodominant region of the CS protein also labeled a band close to, but slightly larger than, 65 kDa and the smear near the 45-kDa band but not the 175-kDa polypeptide (figure 2*B, lane 13*). It is unlikely that the 65-kDa polypeptide observed with the anti–His_6-249 in the sporozoite extracts is the CS protein, since a band of this size is observed also in blood parasites in which the CS protein is not expressed.

The IFAT consistently showed a strong fluorescence on all *P. falciparum* sporozoites derived from the NF54 strain and from 10 different Thai isolates, regardless of use of human His_6-249 affinity–purified antibodies from hyperimmune serum samples or mouse anti–His_6-249 serum samples (figure 3*A*). The wet sporozoite method used for these IFAT slides is specific to antigens at the surface of the sporozoite [15]. Thus, it is highly likely that the EBA-175 protein in sporozoites is exposed to the environment. Mature hepatic schizonts from *P. troglodytes* also were labeled with these antibodies (figure 3*B*). The IFAT pattern observed in liver stages, which differed somewhat from that previously obtained for all of the pre-erythrocytic antigens studied to date [18–21], showed a low-level diffuse reactivity with the parasite's cytoplasm and a very strong labeling of the parasitophorous vacuolar membrane (figure 3*B*). A similar pattern was observed in the less mature liver forms of *C. apella* but with a less intense peripheral label (data not shown), which suggests that the antigen synthesis and export to the parasitophorous vacuolar membrane increases as the liver parasite matures. By use of the same antibodies, a noticeably weaker IFAT labeling was obtained in the blood ring stages, compared with the mature schizonts (figure 3*C*), which also suggests that synthesis of the protein increases with parasite maturation. However, in these IFATs, the gametocytes were not labeled.

The IFAT reactivity of sporozoite liver and blood forms did

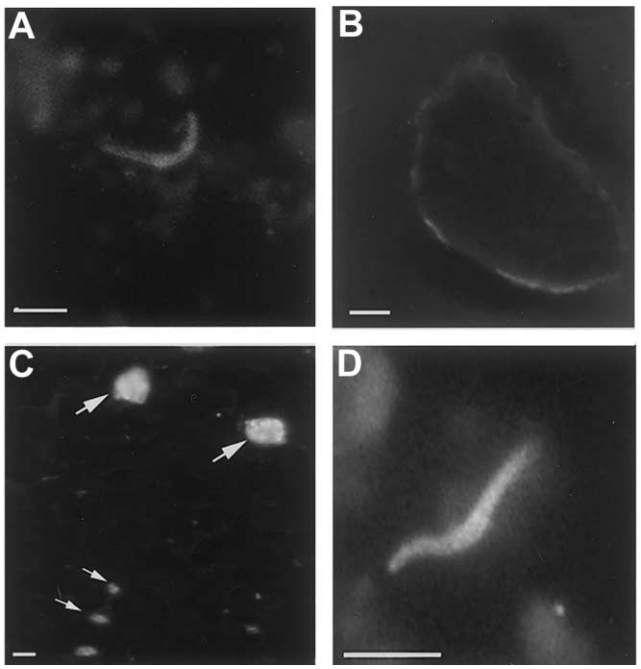

Figure 3. Indirect immunofluorescent antibody tests done with anti–mouse His_6DG249 antibodies on sporozoites of *Plasmodium falciparum* strain NF54 *(A),* mature Carnoy-fixed liver schizonts from *Pan troglodytes (B),* asynchronous *P. falciparum* blood-stage cultures *(C)* with rings *(small arrows)* and schizonts *(large arrows),* and *P. yoelii yoelii* sporozoites *(D)*. Bars in panels *A* and *C,* 5 μm; bars in panels *B* and *D,* 10 μm.

not differ when the specific anti–His_6-249 antibodies used were affinity purified from hyperimmune human serum samples. The anti–His_6-249 antibodies (human and mouse) also were tested by IFAT against *P. yoelii yoelii* and *P. berghei* sporozoites. Labeling was observed only for *P. yoelii yoelii* (figure 3*D*), and not for *P. berghei;* Western blots of sporozoites of this species revealed a single 80-kDa band (data not shown). A BLAST search of the available *P. yoelii* genome (2× coverage) on the TIGR site revealed a homology, with a cysteine-rich motif characteristic of erythrocyte-binding proteins (EBPs) [3], in a *P. yoelii* gene *(c2m2771)* coding for a putative protein of ~690 aa and currently annotated as "Duffy receptor precursor (DBP)."

Discussion

EBA-175 belongs to a well-conserved, functionally important EBP family that includes the Duffy-binding proteins of *P. vivax* and *P. knowlesi.* PCR amplification by using oligos based on the conserved 3′ cysteine-rich domain of EBA-175 revealed that a gene with a homologous domain occurs in each of the 4 rodent malaria species [22]. The *eba-175* gene has been included in the Duffy-binding–like superfamily [3, 23] because of the homology between the cysteine-rich domains (F1 and F2 in *eba-175*) in the different genes, including those of the *var* family, which code for PfEMP-1 proteins that mediate cytoadherence.

The expression of the EBA-175 antigen in the liver schizonts is not unexpected for a protein implicated in the invasion of RBCs by the merozoite and has been mentioned elsewhere in this context [24], although no published data are available. Here, we provide evidence for expression in the liver stage and show that this protein is found also on the sporozoites that invade only hepatic cells. This was demonstrated through parallel analyses of blood parasites and sporozoites at the transcriptional and the translation levels.

Sporozoites were shown to contain *eba-175* mRNA, as determined by RT-PCR. The possibility of detecting spurious messages resulting from "leaky transcription" was excluded by targeting the amplification to the introns at the 3′ end of the 4.8-kb gene. Correctly spliced *eba-175* mRNA was present in sporozoites, and the splice sites were identical in blood parasites and sporozoites. The observation that the *eba-175* gene is specifically transcribed in sporozoites strongly suggests that the protein is synthesized at this stage of the parasite's life cycle.

By using DG249-specific antibodies, surface labeling of *P. falciparum* sporozoites was observed by IFAT, and labeling of Western blot sporozoite extracts revealed the protein and its 65-kDa proteolytic cleavage product. Cleavage to generate the 65-kDa polypeptide occurs when the EBA-175 isolated from culture supernate binds to uninfected RBCs [17]. This 65-kDa fragment is localized to a region of the molecule that spans the DG249 recombinant polypeptide. The presence of EBA-175 and consequently of its proteolytic cleavage products had not been detected previously in ring stages; however, this was tested only once, solely by IFAT [4], and the antibody used was specifically targeted to peptide 4, which is located at the C-terminus of the antigen and does not overlap with the DG249 protein. Our Western blots were done with extracts of washed infected RBCs devoid of proteins from the culture supernate. Thus, the presence of this fragment in ring-stage parasites suggests that the 65-kDa fragment is retained in freshly invaded RBCs, whereas its absence from the schizont extract supports the hypothesis of an extracellular cleavage of the protein.

Among the subset of 120 clones of putative pre-erythrocytic genes, the *eba-175* gene was represented by a single clone (DG249), as was the *CS* gene [7]. By contrast, several clones carrying fragments spanning different regions of the *LSA1* [19], *LSA3* [21], *SALSA* [20], and *STARP* [18] genes were found in this subset. Because these clones were selected by antibody, this might denote that, in pre-erythrocytic stages, only a restricted portion of EBA-175 is predominantly exposed to the immune system. However, the level of IFAT reactivity on the surface of sporozoites observed with antibodies affinity purified from human hyperimmune serum samples suggests that EBA-175 is not a minor component of the sporozoite surface. This was confirmed further by the detection of anti–EBA-175 antibodies in serum samples of volunteers immunized with irradiated sporozoites and therefore not exposed to blood-stage parasites. The preliminary observation of subclass difference in the specific antibodies found in the volunteers, unlike those found in 4 persons exposed only to blood-stage parasites (transfusion malaria), also leads to the interesting hypothesis that exposure of EBA-175 to the immune system by a hepatocyte that expresses major histocompatibility complex molecules contributes to substantial differences in the induced immune responses.

The fact that EBA-175, a quintessential blood-stage protein, was found in pre-erythrocytic parasites adds impetus to a search for other multistage antigens. The description of such proteins has been hampered, no doubt, by the difficulty in obtaining the relevant experimental material (i.e., sporozoites and infected hepatocytes). EBA-175 now extends the number of genes that are expressed both on the sporozoite and during blood stages, *STARP, GLURP, SALSA* [25], and *PfEMP3* [26]. The *eba-175* gene is another pre-erythrocytic stage–expressed gene that shows antigenic cross-reactivity with *P. yoelii yoelii* but not *P. berghei* [27]. The lack of anti–His_6-249 serum sample reactivity with *P. berghei* sporozoites might be due to differences in the central domain of the homologous protein in the 2 species to which the DG249 fragment might correspond. Of note, the *eba-175* gene of the *P. falciparum* NF54 line used for the present observations contains the F-segment [28] that inserts in the DG249 sequence, whereas this stretch is uninterrupted in the gene present in the T9-96 cloned line from which the DG249 clone originates (figure 1). Thus, the immunogenicity of the protein does not appear to be affected by the F-segment insertion.

The presence of EBA-175 on sporozoites might at first appear to be difficult to reconcile with its role as a ligand in the mero-

zoite invasion of an RBC, since sporozoites are not known to bind erythrocytes. This suggests that EBA-175 may require another complementary molecule on the merozoite, but absent from the sporozoite, to initiate the complex process of merozoite invasion. Alternatively, the ability of EBA-175 to bind to carbohydrates on the RBC surface might also play a role in hepatocyte recognition. Thus, one might speculate that EBA-175 serves as an initial adhesion ligand both for RBCs and for hepatocytes. This is consistent with the apparent absence in gametocytes, a noninvasive stage of the parasite life cycle.

A functional role of EBA-175 in the first invasive step of the parasite in the mammalian host and its presence in the hepatic stage present additional targets for immune responses induced by vaccines based on EBA-175, thus improving the potential of such vaccines to control the infection. This potential was demonstrated recently for the control of blood-stage parasites, in a study in which immunization of *Aotus* monkeys with different constructs corresponding to the RII region of EBA-175 resulted in lower parasitemia than in control animals and in some infections spontaneously clearing [29]. Since *Aotus* monkeys are also susceptible to *P. falciparum* sporozoites, it would be of great interest to carry out such a challenge in EBA-175–immunized animals.

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