Common Surface-Antigen var Genes of Limited Diversity Expressed by Plasmodium falciparum Placental Isolates Separated by Time and Space

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Plasmodium falciparum placental parasites from Cameroon have been shown to express surface variant var genes encoding Duffy binding–like (DBL)–γ domains that bind chondroitin sulfate A. All 5 domains exhibited sequences with 39%–55% amino acid (aa) identities and appear sufficiently conserved to function in receptor binding. Transcripts of 2 samples showed complete conservation over 4 kb, demonstrating for the first time distinct conserved placental var genes. Four placental isolates from Gabon collected 4 years later expressed DBL-γ sequences with 85%–99% aa identities to those from Cameroon, confirming the conserved nature of placental variants separated by time and location. Five peripheral parasites from children also displayed DBL-γ sequences with 75%–97% homologies. From this, it can be concluded that P. falciparum parasites expressing unique var DBL-γ genes can cause placental malaria, referred to as varPAM genes. This demonstration of structurally/functionally constrained DBL-γ chondroitin sulfate A–binding domains is relevant to understanding pregnancy-associated malaria pathogenesis and to vaccine development.

Pregnancy-associated malaria (PAM) is a major cause of infant morbidity and mortality and anemia and death in the mother [1]. Although adults in areas where malaria is endemic are normally clinically immune to the disease, women experiencing their first pregnancies are peculiarly more vulnerable to infection and can present clinical manifestations of the disease. Plasmodium falciparum–infected erythrocytes (IEs) are preferentially sequestered in the placenta by interacting with a glycosaminoglycan, chondroitin sulfate A (CSA), on syncytiotrophoblast cells lining the placenta [2]. CSA acts as a receptor molecule for P. falciparum erythrocyte membrane protein 1 (PfEMP-1), which is expressed by the parasites on the surface of IEs [3–5]. Other receptor molecules such as hyaluronic acid and IgG also have been implicated in the adhesion of IEs in placentas [6, 7]. Antibodies from secundigravid and multigravid women living in areas where malaria is endemic can block the binding of placental parasites to CSA [8] and are thought to be generated in a strain-independent manner, with the capacity of recognizing parasites from different geographic locations. The panreactive anti-CSA binding antibodies also react with the surface of IEs in a sex-specific manner, such that serum samples from malaria-exposed men do not show any reactivities.
In several independent experiments, a polypeptide domain of the var-encoded protein PEMP-1, the Duffy binding–like (DBL–γ) domain, was shown to be associated with the cytoadherence phenomenon involving CSA [3–5]. The CSA-binding DBL–γ domains characterized in our laboratory from clinical isolates from malaria-infected placentas exhibited 39%–55% aa sequence identities with each other, and this unique region is thought to maintain functional integrity through a conserved conformational structure [5]. Of interest, we also found DBL–γ sequences from 2 different parasite isolates, 485 and 701, to be nearly identical to each other, as were 2 other isolates, 720 and 734. In part, these results provide the molecular basis to support the immunoepidemiological data that suggest that the CSA-binding ligand is antigenically conserved and that women who are infected with parasites expressing this varPAM variant during pregnancies can gain lifelong immunity against PAM.

Recently, a unique regulatory element located at the 5′ untranslated region of the varCSA gene was correlated with the expression of this gene involved in placental malaria. This finding provided a plausible explanation for the relative conservation of the varCSA gene in genetically diverse parasites [10]. Because one of the central issues of PAM research concerns structure, function, and amino acid sequence conservation in DBL–γ mediating the cytoadherence of IEs to the placenta, we have extended the scope of our earlier study of Cameroonian placental parasites to look specifically at the expression of this var gene segment in placental parasites collected from a different study site (Gabon). There was a 4-year gap between our 2 studies. The present study also focused on DBL–γ domains amplified from placental, as well as peripheral, parasites, to examine the occurrence in different parasite populations of the var gene fragments encoding DBL–γ domains that bind CSA and cause malaria pathogenesis in pregnant women.

SUBJECTS, MATERIALS, AND METHODS

Study site and participants. Study participants were residents of Lambaréné, which is situated on the equator in a typical Central African rainforest area in Gabon. The prevalence of plasmodial infection shows a hyperendemic pattern, and transmission is intense and perennial [11]. Placental blood samples that tested positive for \textit{P. falciparum} in thick blood smears were collected from women who presented at the maternity ward of the Albert Schweitzer Hospital for delivery, and 5 samples were randomly chosen for the study (parasitemia counts of 1000–140,000 parasites/μL). These individuals were asymptomatic carriers, and parity varied from 1 to 6. Another group of participants included schoolchildren who presented at the Medical Research Unit of the Albert Schweitzer Hospital with uncomplicated malaria—their parasite counts were 10,000–30,000 parasites/μL. All patients were treated with quinine.

Collection of parasites. Placental blood was extracted by squeezing a piece of placenta tissue into 0.9% NaCl that contained 10 U/mL heparin [12] and was examined for the presence of malaria parasites in thick blood smears using the Lambaréné method [13]. The parasites collected in this way were also shown to have the CSA-binding phenotype. Packed erythrocytes (100-μL aliquots) were collected from infected blood by centrifugation through ficoll-hypaque to separate the cell pellet from the plasma, stored at −80°C, and subsequently used for DNA preparation, polymerase chain reaction (PCR) amplification, and genotyping experiments. Peripheral blood was collected in a monovette, parasite densities were determined, and packed erythrocytes were collected as described above.

PCR cloning of expressed varPAM genes. PCR amplifications were carried out to amplify longer \textit{var} gene sequences that partially corresponded to 2 identical DBL–γ sequences previously isolated by reverse-transcriptase (RT)–PCR on parasite isolates from Cameroonian patients (nos. 720 and 734) [5]. The \textit{var} sequences amplified were ~4 kb in length. Genomic DNA from patients 720 and 734 were isolated from 100 μL of packed erythrocytes, using the blood DNA mini kit (Qiagen) according to the manufacturer’s instructions. DNA was eluted in 50 μL of 10 mM Tris buffer (pH 8.5), and 5 μL was used in a PCR in the presence of the expand amplification enzyme (Roche Molecular Biology). Primer AF served as a forward primer and has been shown elsewhere [14] to universally amplify DBL–α sequences; 720/734-R (5′-ATC ATT ATC TGC TGT GTG TGT TGC) was used as a reverse primer, designed around a highly variable region on the 720/734 DBL–γ sequences. The resulting PCR products were cloned into pCR-XL-TOPO plasmid from the TOPO XL PCR cloning kit (Invitrogen). Positive clones were verified by DNA sequencing.

RT-PCR amplification of DBL–γ domains. Total RNA was prepared from placental parasites Gb21 and Gb35 using the QIAamp RNA blood mini kit (Qiagen), as described elsewhere [5, 12]. Placental packed erythrocytes (100 μL) were mixed with 500 μL of the RLT buffer provided. The extraction steps thereafter were conducted according to the manufacturer’s instructions. The RNA was pretreated on column with RNase-free DNase. After first-strand cDNA synthesis with random primers (Roche first-strand cDNA kit) and RT-PCR amplification using degenerate primers UNIEBP5 and UNIEBP3′ [16], the products were excised from the agarose gels and sequenced directly or cloned into the TA cloning vector pCR2.1 (Invitrogen).

Preparation and amplification of genomic DNA. Parasite DNA was extracted from pellets of packed erythrocytes collected from the peripheral blood of \textit{P. falciparum}–infected children.
produced amino acid sequence analysis demonstrated that the 2
sequences (GenBank accession nos. AF547158 and AF547159, respectively) were isolated and sequenced. The deduced amino acid sequence of sequence 734-related sequences (GenBank accession nos. AF547158 and AF547159) was 96% identical to sequence 734 at the nucleotide and deduced amino acid sequence levels. The analysis of these 2 expressed var genes was extended into the 5′ upstream sequences encoding the DBL-α/cysteine-rich interspersed domain region (CIDR) head structure regions. Clones of 4 kb each of the 720- and 734-related sequences (GenBank accession nos. AF547158 and AF547159, respectively) were isolated and sequenced. The deduced amino acid sequence analysis demonstrated that the 2 var genes extending from the DBL-α to the DBL-γ regions shared almost complete conservation with each other, on the order of 96%. The fact that parasites 720 and 734 analyzed in the present study have different genotypes (data not shown) argues against the possibility that the 2 individuals from whom the parasites have been isolated had been infected with the same strain. The results presented here provide clear evidence for the existence of var genes of genetically diverse placental isolates that are almost identical, at least in their N-terminal–deduced amino acid sequences.

**Comparison of varPAM sequences of placental isolates and laboratory clones.** To determine the extent of similarities between well-characterized var genes (FCR3varCSA, CS2varCSA, and 3D7ch5var) known to bind CSA and those placental parasites that cause disease during pregnancy, we aligned each of the sequences in the form of a diagram (figure 1). The alignment of ~4 kb of nucleotides covers the N-terminal half of the var domains, namely DBL-α, CIDR-α, DBL-β, and DBL-γ. The levels of amino acid conservation in each of these blocks are shown as percentages in figure 1. For simplification, the comparison was made on sequence 720.

**Figure 1.** Alignment of sequences from Plasmodium falciparum placental isolates 720/734 with laboratory clones FCR3varCSA, CS2varCSA, and 3D7ch5var. The total length of the varPAM sequences being compared is ~4 kb each and spans N-terminal var domains from Duffy binding–like (DBL)–α to DBL–γ. Percentages in the boxes refer to the degree of amino acid conservation of the laboratory clones in comparison to sequence 720. Sequence 734 was erroneously labeled in a previous study [5] as being identical to 732. CIDR, cysteine-rich interspersed domain region.

(57x98)
Figure 2. Comparison of Duffy binding–like (DBL–γ) genomic sequences from placental and children’s parasites. Sequences identified in the present study are labeled varX and are preceded by the number of the clinical isolate (e.g., Gb170var5). Percentages given in each box refer to the percentage of amino acid conservation to known sequences in the database, provided in the form of their accession nos. Names of clinical isolates from Cameroon, whose DBL–γ domains have been demonstrated elsewhere to bind chondroitin sulfate A (CSA) [3–5], are included in some boxes and, for comparison purposes, also the 2 laboratory strains FCR3 varCSA and CS2 varCSA. Boxes are shaded in 3 different gray intensities. Dark gray, sequences that have at least 75% conservation to the clinical isolates; medium gray, sequences with at least 40% conservation; and light gray, sequences with similarities to the laboratory strains. White boxes, sequences that have not been implicated in CSA binding. Sequences encoding DBL–γ domains that are capable of binding CSA were detected with similar frequencies in parasites from both pregnant and nonpregnant donors. There were 9 of 28 Cameroon-specific DBL–γ sequences in the placental parasite population, vs. 9 of 29 sequences in the peripheral parasite population. Five additional placental sequences showed sequence similarities with FCR3 varCSA and CS2 varCSA, whereas 3 FCR3- and CS2-related sequences were found among the peripheral parasites.

the expressed DBL–γ CSA-binding domains were analyzed. The complete sequences from 9 clones from each isolate were available for comparison, and the results are summarized in table 1. We show here for the first time, to our knowledge, that the placental parasites collected 4 years apart originating from areas where malaria is endemic in 2 different African countries, Cameroon and Gabon, are constrained in having a limited and uniquely conserved DBL–γ repertoire. It is conceivable that distinct sets of DBL–γ sequences, such as those described here, are specifically induced and proliferate during placental sequestration. Because these are similar sequences with 39%–55% aa identities, these domains can still retain sufficient structural homology to function as CSA-binding cytoadherence molecules.

**Analysis of genomic DBL–γ sequences in placental and peripheral isolates.** To determine the array and frequency of DBL–γ–specific sequences in the natural parasite population in Gabon, their presence in the genome of *P. falciparum* placental, as well as peripheral, parasites was further investigated. A comparative analysis of a total of 57 sequences taken from parasites from the placentas of 4 women and from the peripheral blood of 5 children indicated that DBL–γ sequences were found in every tested parasite sample, with a frequency ranging from 4 to 9 different sequences, independently of the source of the isolate (figure 2). However, the possibility cannot be entirely ruled out that additional DBL–γ sequences with greater diversity could also be identified through the use of primer sets distinct from the ones selected here. Of interest, all samples, with the exception of parasites taken from one child, harbored at least 1 of 5 DBL–γ sequences previously isolated from infected placentas from Cameroonian subjects and characterized for CSA binding. The
DISCUSSION

In the present study, we have carried out a further analysis of placental *P. falciparum* parasites that have been shown elsewhere [5] to express distinct subtypes of DBL-γ domains that were capable of binding the glycosaminoglycan CSA. In considering the likelihood that *var* gene conservation extends beyond the DBL-γ domains, we analyzed the 5′ extensions of 2 expressed *var* genes of the parasites 720 and 734. The most striking result was the finding that the entire 4 kb of their deduced amino acid sequences were 96% indistinguishable from each other. Thus, contrary to the belief that *var* genes are extensively diverse, evidence is provided in the present study that some *var* genes expressed by placental parasites isolated from different individuals are unique to the extent that they are almost totally conserved in their amino acid sequences.

Using the previously described RT-PCR methodology, we analyzed more closely the DBL-γ domains of placental parasites from a different malaria-endemic area. The samples from the first study were collected in Yaounde in Cameroon, whereas those of the second study originated from Lambarené in Gabon, and the time lapse between sample collections was 4 years. The novel and significant result of our *var* gene analysis is the demonstration of a limited number of distinct highly homologous *var*DBL-γ sequences that are commonly expressed in parasites from geographically separated study areas and were collected at different time intervals.

Most interesting, the 720/734 pair of identical *var*DBL-4 genes is ∼98% homologous at the amino acid level to a 3D7 chromosome 5 *var* gene (3D7chr5var). The 3D7 DBL-γ domain was expressed in a heterologous expression system and has been shown to bind CSA [10]. Furthermore, this 3D7varCSA gene has been shown to be expressed by many placental isolates from Malawi [18], despite the fact that the N-terminal half of the gene (DBL-1e–DBL-4e) exhibits low homology to the FCR3varCSA gene, which has been implicated to have a role in placental malaria. This allows us to conclude that 3D7chr5var-like (and by extension, also 720/734-like) sequences belong to a pool of commonly expressed DBL-γ *var*DBL-4 genes set among placental isolates capable of infecting pregnant women. It is also noteworthy that other workers, using a different degenerate set of primers, were able to detect the Cameroonian-type of *var*DBL-γ sequences from placental isolates from Kenya [19]. Even though these sequences have an overall amino acid sequence homology only on the order of 39%–55%, it is very likely that this unique set of sequences is structurally and conformationally conserved in nature, which is crucial for determining an interaction with CSA. In fact, the earlier study showed that these DBL-γ domains that were expressed on the surface of COS-7L cells and capable of binding CSA were predicted to exhibit specific structural features, such as conserved α-helical stretches. Such conservation of the CSA-binding ligand can clearly explain how antibodies from

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**Figure 3.** Phylogenetic analysis of unique Duffy binding–like (DBL–γ) sequences from placental and peripheral isolates found in the natural population of Gabon. Alignment was made with the CLUSTAL program (available at http://inn-prot.weizmann.ac.il/software/ClustalX.html), and the tree was created by the CLUSTREE program (part of the CLUSTAL suite). Bootstrap values (1000 resamplings) are shown along the branch lines. Sequences are labeled as in figure 2. Sequences that show significant identities to previously published DBL–γ domains are boxed, as are those of the laboratory-selected clones.

Finding of DBL–γ–specific sequences in *var* genes from peripheral parasites would also explain why some peripheral *P. falciparum* isolates have been reported to bind CSA [17]. The presence of DBL–γ sequences in the natural population with the potential to bind CSA ensures that these tightly controlled *var*DBL–γ genes do not get lost in the population in the absence of a selection pressure, namely the placenta.

A phylogenetic analysis of the 57 DBL–γ domains together with the 2 DBL–γ sequences of the laboratory strains [3, 4] was made (figure 3). The comparison showed that sequences with highest homologies to some of the Cameroonian DBL–γ sequences (701/485 and 720/734) were found in one cluster (boxed data), whereas others with homologies with sequences 498 and 732 were found in another cluster (boxed data).
multigravid women can block the binding to CSA of placental parasites collected from different continents [8] and also how serum samples from multigravid women can react with antigens on the surface of CSA-binding parasites, whereas serum samples from adult men cannot.

The next logical question to ask is how CSA-binding var genes escape frequent recombination and rearrangement events within the parasite, processes that form the basis of clonal antigenic variation of var genes known so far. The mechanism controlling the regulation of expression of varCSA genes recently has been shown to involve a unique 1.8-kb 5′ varCSA element. The hypothesis was advanced that the FCR3varCSA gene implicated in PAM has a subtelomeric location in the chromosome and is less likely to undergo recombination and rearrangement, unlike many other centrally located var genes. Besides gene location, orientation also appeared to play a role, because the FCR3varCSA gene was found to be transcribed in the opposite sense from the more “common” var genes [10]. Moreover, the same unique, single-copy regulatory element upstream of the initiation codon was also present in the 3D7varCSA gene as well as in var genes of placental isolates [10].

It is known that the var-encoded PfEMP-1 is surface exposed and undergoes clonal variation as a result of the stress exerted on the parasite by the host immune effector mechanism. In view of the fact that the majority of the peripheral parasites bind to CD36 but not CSA, it follows that all CSA-binding variants are rapidly eliminated by the spleen. As a consequence, the parasites in the natural population that express the CSA variant are hardly exposed to the immune system, except when they infect pregnant women and accumulate in the placenta. Therefore, CSA-binding var genes, in being under less immune pressure than the CD36-binding var genes, are not as variable and can afford to be unique, not only in their conservation pattern but also in their regulatory elements. Gamain et al. [20] proposed a model that both binding domains are present on the same var gene and are mutually exclusive of each other—that is, a CSA-binding domain on a CD36-binding PfEMP-1 var gene is structurally excluded from recognition by the immune system and vice versa.

From our comparative analysis of genomic DBL-γ sequences from peripheral and placental parasites, it appears that peripheral parasites can be selected to express a specific var gene capable of binding to placenta and cause disease. Such a subset of varPAM genes expressing a limited but distinct repertoire of DBL-γ domains would be a critical property for the selection process in the placenta and, consequently, successful sequestration and infection of the organ. It is now necessary to perform a structural analysis of this common conserved varDBL-γ/CSA complex, to understand how these molecules interact with each other and to see which amino acid residues are crucial for the interaction, as first steps in the design and development of a vaccine against placental infection in pregnant women.

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