MAJOR ARTICLE

Effects of Sex, Parity, and Sequence Variation on Seroreactivity to Candidate Pregnancy Malaria Vaccine Antigens

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Background. *Plasmodium falciparum*–infected erythrocytes adhere to chondroitin sulfate A (CSA) to sequester in the human placenta, and pregnancy malaria (PM) is associated with the development of disease in and the death of both mother and child. A PM vaccine appears to be feasible, because women become protected as they develop antibodies against placental infected erythrocytes (IEs). Two IE surface molecules, VAR1CSA and VAR2CSA, bind CSA in vitro and are potential vaccine candidates.

Methods. We expressed all domains of VAR1CSA and VAR2CSA as mammalian cell surface proteins, using a novel approach that allows rapid purification, immobilization, and quantification of target antigen. For serum samples from East Africa, we measured reactivity to all domains, and we examined the effects of host sex and parity, as well as the effects of parasite antigenic variation.

Results. Serum samples obtained from multigravid women had a higher reactivity to all VAR2CSA domains than did those obtained from primigravid women or from men. Conversely, serum samples obtained from men had consistently higher reactivity to VAR1CSA domains than did those obtained from gravid women. Seroreactivity was strongly influenced by antigenic variation of VAR2CSA Duffy binding–like domains.

Conclusions. Women acquire antibodies to VAR2CSA over successive pregnancies, but they lose reactivity to VAR1CSA. Serum reactivity to VAR2CSA is variant specific, and future studies should examine the degree to which functional antibodies, such as binding-inhibition antibodies, are variant specific.

Plasmodium falciparum parasites sequester in the human placenta [1], and pregnancy malaria (PM) is associated with the development of disease in and the death of both mother and child [2–5]. Previous studies identified chondroitin sulfate A (CSA) as a major receptor molecule for sequestration of infected erythrocytes (IEs) in the placenta [6]. Malaria parasites variably express antigens on the IE surface that bind a variety of endothelial receptors [7, 8], including CSA. PfEMP1 is a variant surface antigen family encoded by ~60 *var* genes per malaria parasite genome [9], and these proteins have been implicated in a number of binding interactions. The sequences of *var* genes vary substantially within and between genomes. PfEMP1 forms are expressed in a mutually exclusive manner [10], creating extensive antigenic variation and the potential for multiple adhesion profiles. This variation is a major obstacle to the development of a PfEMP1-based antimalarial vaccine.

Resistance to PM increases over successive pregnancies [3] as women acquire antibodies against placental parasites. Serum samples obtained from immune multigravid women, but not those from men, can inhibit binding of placental IEs to CSA [11], even IEs collected in distant geographic regions. This serum activity is related to protection from infection and disease during pregnancy [12, 13]. Two PfEMP1 molecules, VAR1CSA and VAR2CSA, have been implicated in PM and are potential vaccine candidates (reviewed in [14]). Both are large molecules of >350 kDa with 7 and 6 distinct

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Duffy binding–like (DBL) domains, respectively, and each is extensively cross-linked by disulfide bonds.

To study the role of these molecules in protective immunity, we expressed all domains of VAR1CSA and VAR2CSA on the surface of mammalian cells as green fluorescent protein (GFP) fusion proteins, by use of a novel vector that allowed rapid purification, immobilization, and quantification of antigen. We prepared arrays of individual VAR1CSA and VAR2CSA domains from laboratory strains and field isolates, and we tested their immunoreactivity by use of serum samples obtained from East African donors, to determine the effects of host sex and parity, as well as the effects of parasite antigenic variation, on antibody recognition.

MATERIALS AND METHODS

Vector for the expression of malaria antigens on the surface of mammalian cells. The DNA sequence encoding enhanced GFP (EGFP) was excised from pEGFP-N1 (Clontech) by means of *Xho*I/*Not*I digestion. The sequence encoding the transmembrane and cytoplasmic (TMC) domains of the rat surface receptor megalin [15] was amplified by use of reverse-transcription polymerase chain reaction (PCR) performed using forward and reverse primers with *Eco*RI and *Xho*I sites at their 5′ ends, respectively (forward primer: 5′-TTTGAATTCCTCCAGGGACGACAATGGCTGTT-3′; reverse primer: 5′-TTTCTCGAGTACGTCGGATCTTCTTTAACGAG-3′). The sequence then was digested with *Eco*RI and *Xho*I. Plasmid vector pSecTag2C (Invitrogen) was digested with *Bam*HI and *Eco*RI and then was ligated to a double-stranded (ds) oligonucleotide adaptor (AdEx) with a multicloning site created by annealing 2 single-stranded (ss) oligonucleotides: 5′-GATCCTTAAGTCCGGAGGCGCCTCTAGACTTAACGG-3′ and 5′-AATTCCGTTAACTCTAGAGGCGCCTCCGGACTTAAG-3′. The resulting vector was digested with *Eco*RI and *Xho*I and then was ligated to the megalin TMC fragment described above. This construct, in turn, was digested with *Xho*I and *Bsp*120I and was ligated to the EGFP fragment. The resulting vector was digested with *Xho*I and *Age*I to remove double-digestion sites, and it then was ligated to a ds oligonucleotide adaptor (created by annealing the following 2 ss oligonucleotides: 5′-TCGAGCTGAAGCTTCGAATCCTGCAGTCGACGGTACCGCGGGCCCGGGACCCA-3′ and 5′-CCGGTGGGTCCCGGGCCCGCGGTACCGTCGACTGCAGGATTCGAAGCTTCAGC-3′) that introduced point mutations to eliminate unwanted restriction sites. The resulting vector, known as "pAdEx," was used to clone and express the *P. falciparum* antigens described in the present study (figure 1). The integrity of the construct was verified by restriction digestion and sequencing.

Cloning malaria antigen genes into the pAdEx vector. DNA encoding each antigen was amplified by PCR from strain FCR3 and strain 3D7 *P. falciparum* genomic DNA or from cloned placental parasite sample 661 cDNA (see below), by use of PCR performed using primer pairs with appropriate restriction enzyme sites (table 1). After PCR was performed, amplified DNA fragments and the pAdEx vector were digested, ligated, and cloned. The integrity of each construct was verified by sequencing.

Cloning and sequencing of* var2csa *from placental parasite sample 661. Clinical placental parasite sample 661 was a placental intervillous blood sample obtained, after delivery, from a woman at Muheza Designated District Hospital (Muheza, Tanzania) who was participating in the Mother-Offspring Malaria Studies (MOMS) Project (described in [16]). Parasite samples were stored in RNALater (Ambion) at −20°C. RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Purified RNA was treated with DNA-free reagent (Ambion) to remove genomic DNA. RNA was then reverse-transcribed using Superscript III and random hexamers (Invitrogen) for 2 h at 42°C. DBL6 forward primer 5′-AAGAACATTGTTCTAAATGTC-3′ and reverse primer 5′-TGTAAATATTGTTCAATAAAATCC-3′ were designed by aligning PFL0030c sequences from strains 3D7 and ITG (GenBank accession no. AY372123) to identify conserved sequences that flank the DBL6 domain. The PCR product was cloned into pCR2.1 vector by use of the TOPO TA Cloning System (Invitrogen) and was sequenced in both directions.

Preparation of quantitative protein arrays with malaria antigens. COS-7 cells (50%–70% confluent) were transfected with various constructs by use of Fugene transfection reagent (Roche) according to the manufacturer's protocol. Cells from

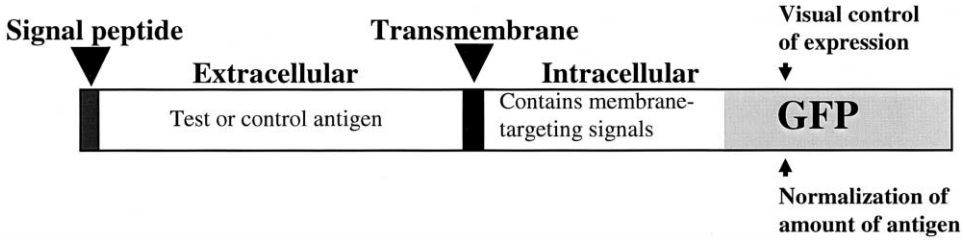

Figure 1. Hybrid protein for expression of *Plasmodium falciparum* antigens on the surface of mammalian cells. GFP, green fluorescent protein.

Table 1. Polymerase chain reaction primers for the amplification of antigen domains.

Domain (nucleotide positions)[a]	Forward primer[b]	Reverse primer[b]
VAR1CSA		
DBL1α-CIDR (271–2280)	CCCGGATCCAGGATCATAAGGAACATACTAATTTACGG	CCCGAATTCCATTTTTAGTGGGTTGCGTGCCTCCACG
DBL2β (2440–3402)	CCCCCTTAAGTCTAATCGTAATCTTGGTTTTTCAAATG	CCCGAATTCCAGACATTTGTGCTTGTTCATGTAATTC
DBL3γ (3802–4698)	TTCGGATCCTTAAAGAAAACGATGGAAAGAAAC	TTTGAATTCCATAGTCTGTAACCATTACACCAATG
DBL4ε (4855–5805)	CCCGGATCCAGGAAAATGACGACAAATATACTAACATT	CCCGAATTCCCTCGGAATATATTTTGTCTTTATTCTC
DBL5γ (5968–7146)	CCCGGATCCAGGACGATGAACCAAAAGAAGTTGAAGG	CCCGAATTCCATCCTTATACTTTTTGCCATCTTTATC
DBL6β (7594–8436)	CCCGGATCCAGGATAAATATATAGGAAGAAGAAACCC	CCCGAATTCCAGATTTCCATTTAAGAACAAAATTTTT
DBL7ε (8761–9540)	CCCGGATCCAGAAGGAATTACAAACTTTTACCTTCTG	CCCGAATTCCTTTATTGTCTATATTACCTGAAGATTG
VAR2CSA		
DBL1X (1–1347)	CCCCTCCGGAATGGATAAATCAAGTATTGCTAAC	CCCGAATTCCGATACATGTTTTATTCGACGACGG
DBL2X (1534–2586)	CCCCTCCGGATCTAGTTCTAATGGTAGTTGTAATAAC	CCCGAATTCCATTTGTAGTACTACTTGGGCCACAAT
DBL3X (3580–4557)	CCCGGATCCAGAAGGAAAATGAAAGTACCAATAATAAAA	CCCGAATTCCATCACTCGCAGATTTTCCTACATATTTA
DBL4ε (4708–5643)	CCCGGATCCAGGAGAAAAAAAATAATAAATCTCTTTG	CCCGAATTCCAGGTTCCATAATCATTGAATAATCTTT
DBL5ε (5944–7008)	CCCGGATCCAGTTAGATAGATGTTTTGACGACAAG	CCCGAATTCCTTTATTACAAATATAATCATTACC
DBL6ε (6973–7761)	CCCGGATCCAGGAGTATGATAAAGGTAATGATTATATTT	CCCGAATTCCTTTTTCTGCTTTGGTTTCTTTATAATTC
AMA-1 (70–1629)	CCCGGATCCAGGGACAGAATTATTGGGAACATCC	CCCTCTAGAATCATAAGTTGGTTTATGTTCAGG
MSP-1 19-kDa CTD (4588–5160)	CCCGGATCCAGATTGTTGAAAAAGATGAAGCACATG	CCCGAATTCCAATGAAACTGTATAATATTAACATG
661-VAR2CSA-DBL6ε	CCCGGATCCAGGAGTATGATAAAGGTAATGATTATATTT	CCCGAATTCCATTACCATTTTGGTTTTTAAATTTAGC

NOTE. AMA-1, apical membrane antigen–1; CIDR, cysteine-rich interdomain region; CTD, C-terminal domain; DBL, Duffy binding–like domain; MSP-1, merozoite surface protein–1.

[a] In the sequences of *var1csa* (FCR3 strain; GenBank accession no. AJ133811), *var2csa* (3D7 strain; PlasmoDB accession no. PFL0030c), *ama1* (3D7 strain; PlasmoDB accession no. PF11_0344), and *msp1* (3D7 strain; PlasmoDB accession no. PFI1475w).

[b] Restriction enzyme sites are underlined.

each 150-mm^2 flask were lysed 48 h after transfection (transfection efficiency, >80%) with 5 mL of CellLytic reagent (Sigma). Recombinant products were confirmed on Western blots with anti-GFP monoclonal antibody (MAb) (1:500 dilution; Clontech), followed by horseradish peroxidase (HRP)–conjugated anti–mouse IgG (1:1000 dilution; Sigma). Concentrations of fusion proteins (expressed in relative fluorescence units) were measured using GFP fluorescence with the use of the Fluoroskan Ascent FL fluorometer/luminometer (Thermo Labsystems), and they then were equalized by dilution with lysate of nontransfected cells (lysate K). A total of 100 μL of diluted lysate was added to each well of 384-well white plates coated with anti-GFP antibody (Pierce), and plates were incubated at 4°C overnight. Undiluted lysate K was used as a control for nonspecific background fluorescence and chemiluminescence. Lysate prepared from cells transfected with control construct (pAdEx vector alone without malaria antigen fusion partner) was used as a negative control in each assay. After washing with washing buffer (PBS plus 0.05% Tween-20), plates were ready for immunoprofiling experiments involving serum samples from humans.

Validation of expressed merozoite surface protein–1 (MSP-1) antigen by structure-sensitive monoclonal antibody. Recombinant MSP-1_{19} or control construct product AdEx was immobilized in anti-GFP plates as described above and then was incubated with mouse MAb 12.10 (1:5000 dilution), which is reactive only to the properly folded structure of MSP-1 [17] (provided by Dr. J. A. Lyon, Walter Reed Army Institute of Research), followed by HRP-conjugated anti–mouse IgG (1:1000 dilution; Sigma). Reactivity signals were obtained (expressed in relative luminescence units) by use of 100 μL of ECL chemiluminescence substrate (Amersham Biosciences) per well and a Fluoroskan luminometer.

Serum samples. The human serum samples used in these studies were collected from East African donors, under protocols approved by relevant ethics review committees. The study participants, who provided written, informed consent before donating samples, included adult men and multigravid women from Kenya [18, 19], as well as multigravid and primigravid women from Tanzania [20]. In brief, 18- to 45-year-old multigravid women and 18- to 50-year-old men (median age, 28 and 29 years, respectively; $P = .62$) from Kenya, as well as 18- to 45-year-old gravid women from Tanzania, were included in the study. Serum samples obtained from pregnant women were collected at the time of delivery and were tested individually. The number of serum samples used in each experiment is indicated in the corresponding figure legends. Serum samples obtained from 10 randomly selected nonimmune donors in the

United States were separated from whole blood obtained from commercial sources (Valley Biomedical) and were used in a pool as a negative control.

Immunoprofiling study of malaria antigens. All serum samples were preincubated at 4°C for at least 24 h with an equal volume of 10 mg/mL goat IgG, to eliminate nonspecific reactivity against goat anti–GFP IgG bound to the wells of 384-well plates. The preincubated serum samples were further diluted 1:100 with Superblock (Pierce) and were incubated with the antigen array for 2 h at room temperature. After 3 washes with washing buffer, plates were incubated with donkey anti–human IgG (H+L) affinity-purified antibody conjugated to HRP (Jackson Immunoresearch) diluted 1:1000 in Superblock. After 1 h at room temperature, the wells were washed; 100 μL of ECL chemiluminescence substrate (Amersham Biosciences) were then added per well, and chemiluminescence and fluorescence signals were measured. The use of the chemiluminescence substrate does not affect the fluorescence measurement.

Chemiluminescence signal reflects immune reactivity, and fluorescence signal reflects the amount of immobilized antigen–GFP fusion proteins. Fluorescence signal was corrected by subtraction of background values measured in lysate K wells, and then the immunoreactivity signal (chemiluminescence) was normalized to the amount of immobilized antigen (fluorescence) in each well. Average reactivity was calculated for duplicate wells, and a final specific immunoreactivity (expressed as arbitrary units [AUs]) was calculated by subtracting the control value (defined as either the average reactivity of the same serum sample to control construct +3 SD or the reactivity of pooled nonimmune serum samples to the same antigen +3 SD, whichever was greater). Correlations were analyzed using Spearman's rank test. Differences between group reactivities were tested for significance by use of the Mann-Whitney *U* test. $P < .05$ was considered to be statistically significant. GraphPad Prizm software was used for all statistical analyses.

RESULTS AND DISCUSSION

Features and performance of quantitative protein arrays. Heterologous expression of malaria surface antigens is known to be difficult, in part because of their high AT content (up to 80%) and their highly conformational cysteine-rich structure. An expression system that provides a transmembrane protein trafficking pathway and cell-surface presentation may significantly improve the cotranslational folding of PfEMP1 surface molecules, in which each domain contains 6–9 disulfide bonds. We engineered a pAdEx vector encoding a hybrid receptor with a signal peptide (from the immunoglobulin κ chain), an extracellular domain, and individual transmembrane and cytoplasmic domains (both from the single-spanning transmembrane receptor megalin) (figure 1). The cytoplasmic domain has signals that direct this protein to the plasma membrane surface. In addition, the GFP-reporter protein is fused to the cytoplasmic domain and reports protein expression levels, which can be quantified. The multicloning site allows simple and rapid preparation of different constructs that express *Plasmodium* antigen extracellular domains on the surface of mammalian cells.

Using this construct, we expressed several DBL domains from *var1csa* and *var2csa* genes, in addition to other *P. falciparum* antigens (the apical membrane antigen–1 [AMA-1] and MSP-1 19-kDa carboxy-terminal fragment), as GFP fusion proteins (figure 2). All antigens were successfully expressed using the native malaria coding sequence. Cysteine-rich interdomain region (CIDR)–α domains always follow DBL-α domains, and they may act as a single functional domain [9]; therefore,

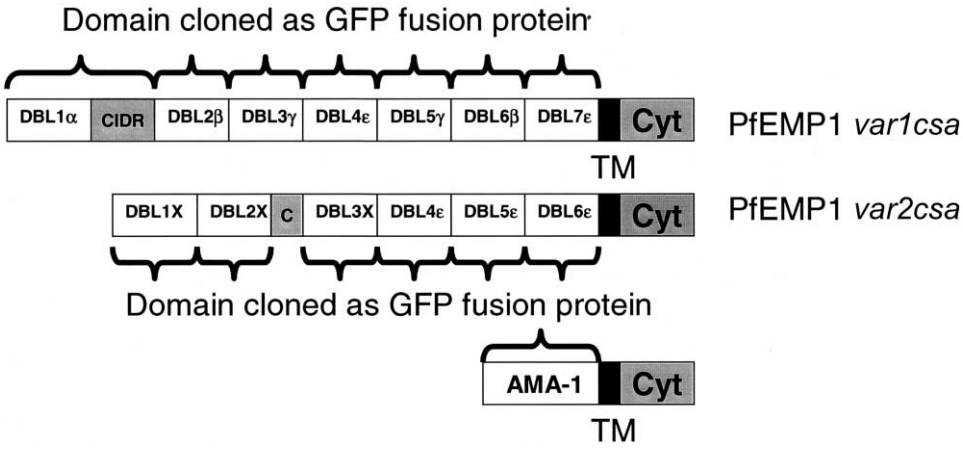

Figure 2. *Plasmodium falciparum* protein domains expressed and used for seroreactivity studies. Indicated domains were expressed as green fluorescent protein (GFP) fusion proteins in COS-7 cells and were immobilized individually for antigen arrays. As a positive control, apical membrane antigen–1 (AMA-1)–GFP fusion protein was used. Cyt, cytoplasmic domain; DBL, Duffy binding–like domain; TM, transmembrane domain.

var1csa DBL1-α domain was expressed together with CIDR1-α domain. For negative control wells, we used a GFP fusion protein (AdEx) containing an irrelevant extracellular domain of 37 aa that resulted from the translation of the multicloning site in the pAdEx DNA construct.

The integrity of fusion proteins was tested by Western blot analysis with anti-GFP antibodies (figure 3*A*). Recombinant proteins demonstrated the expected molecular weight and produced green fluorescence in cells as well as in cell lysates. Fluorescence was preserved after immobilization of fusion proteins in 384-well plates. GFP fluorescence has been shown to be a good indicator of properly folded membrane proteins when GFP is fused to the cytoplasmic tail [21]. We also tested the reactivity of the disulfide-rich MSP-1 19-kDa fusion protein by use of conformation-dependent MAb 12.10 [17], which readily recognized the antigen (figure 3*B*), thereby confirming correct folding.

Malaria antigens were organized into arrays by use of a single-step procedure performed in 384-well plates. The GFP fusion partner has a number of advantages. First, the tag can be used for immobilization and purification of antigens in a single simple step. Second, the GFP allows the amount of antigen in each lysate to be measured and equalized, thereby reducing variance. Third, the immunoreactivity of serum samples (measured by chemiluminescence) can be normalized to the amount of antigen (measured simultaneously by GFP fluorescence) in each well, which further reduces variance.

Seroreactivity to irrelevant antigens. As was observed in earlier studies [22, 23], we found that serum samples obtained from immune individuals in malaria-endemic regions frequently react to completely irrelevant proteins (data not shown), and this nonspecific reactivity corresponds to an elevated reactivity to malaria antigens. In contrast, serum samples obtained from nonimmune individuals (NISS) living in areas of nonendemicity have low nonspecific reactivity. For this reason, NISS control may not be adequate to demonstrate specific reactivity of serum samples tested in seroepidemiologic studies of malaria, because this approach may falsely identify serum samples with high levels of nonspecific reactivity as having a positive result. The use of the control construct provides the means to quantify and, therefore, correct for nonspecific reactivity of each construct in each serum sample.

Seroreactivity to VAR1CSA and VAR2CSA associated with a dichotomous pattern related to sex. We measured the seroreactivity of East African and nonimmune individuals to domains of VAR1CSA and VAR2CSA expressed as GFP fusion proteins. AMA-1 was used as a positive control because it is known to react strongly to the majority of serum samples obtained from individuals in malaria-endemic regions [24]. As expected, serum samples obtained from immune individuals uniformly showed high levels of reactivity to relatively conserved AMA-1, and seroreactivity did not differ between men and multigravid women (inset in figure 4*A*) (median for 44 serum samples obtained from men, 5289 AUs; median for 52

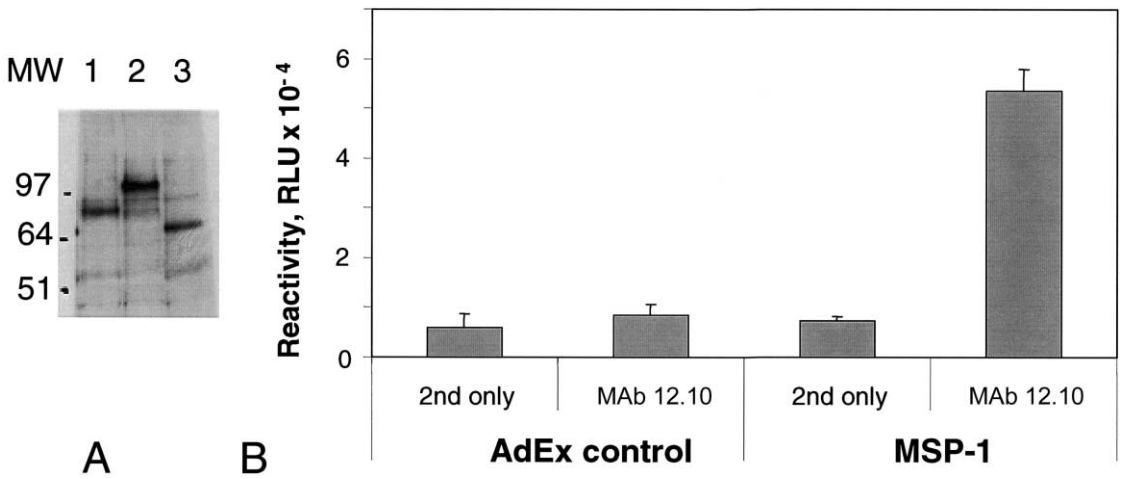

Figure 3. Characterization of malaria antigens cloned as green fluorescent protein (GFP) fusion proteins in the pAdEx vector and expressed in COS-7 cells. *A,* Western blot of expressed antigens with monoclonal anti-GFP antibody. Lane 1, Duffy binding–like (DBL) 3γ region from VAR1CSA (predicted molecular weight [MW], 85 kDa); lane 2, control construct minimegalin with extracellular domain containing the first ligand-binding domain of rat receptor megalin (nt 1–1882) [15] (predicted MW, 106 kDa); and lane 3, merozoite surface protein–1 (MSP-1) 19-kDa fragment (predicted MW, 70 kDa). *B,* Interaction of structure-sensitive anti–MSP-1 monoclonal antibody (MAb) 12.10 [17] with MSP-1 fusion protein. The control protein expressed from vector without insert (AdEx control) or MSP-1 recombinant protein was immobilized in the wells of anti-GFP plates and was tested for reactivity with monoclonal antibody (MAb) 12.10 followed by secondary anti–mouse horseradish peroxidase (HRP)–conjugated antibody (MAb 12.10) or with secondary antibody only (2nd only). Signals were measured using chemiluminescent substrate. Bars denote the average of 3 measurements, and error bars denote SEs. RLU, relative luminescence units.

serum samples obtained from multigravid women, 5872 AUs; $P = .46$).

Immune responses to PfEMP1 domains were substantially lower and more variable (figure 4) than were AMA-1 responses. Two VAR1CSA domains (DBL6β and DBL7ε) and 1 VAR2CSA domain (DBL2X) were found to be nonreactive or minimally reactive in our screening tests. Nonreactivity of VAR2CSA DBL2X was likely the result of rapid degradation of this fusion protein during and after cell lysate preparation, as detected by Western blot analysis (data not shown). The reason for nonreactivity of VAR1CSA DBL6 and DBL7 is not clear, because the proteins were stable. The results suggest that host immunoreactivity is weak against these domains, but we cannot exclude the possibility that the proteins were incorrectly folded in a way that disrupted or masked structural epitopes.

The variable response to VAR1CSA and VAR2CSA was related to the sex of the serum donors. Consistent with the findings of earlier studies from West Africa [25–27], the reactivity of all VAR2CSA domains (other than DBL-2X) was significantly higher with serum samples from multigravid women than with serum samples from men (figure 4). Of the 54 serum samples that were obtained from Kenyan multigravid women and were tested in this experiment, 10 were obtained from women with PM. Antibody levels were not significantly different (data not shown) in women with PM versus those without PM, possibly reflecting that the duration of infection is brief in the multigravid women [3, 28, 29] or that antibody levels may be maximal in this parity group by the time of delivery. Previous studies in West Africa that examined 3 DBL domains (DBL1, DBL5, and DBL6) of VAR2CSA expressed in baculovirus [25, 26] found that seroreactivity to domains 5 and 6, but not to domain 1, was significantly higher in multigravid women than in men. The increased reactivity against all VAR2CSA domains noted for serum samples obtained from multigravid women in East Africa supports the idea that this PfEMP1 molecule is preferentially expressed by PM parasites and that women acquire antibodies against this protein as they become protected. Reactivity to the DBL1X domain was significantly correlated with reactivity against 3 other domains (Spearman correlation for DBL3, $r = 0.29$ [$P = .04$]; for DBL5, $r = 0.41$ [$P = .003$];

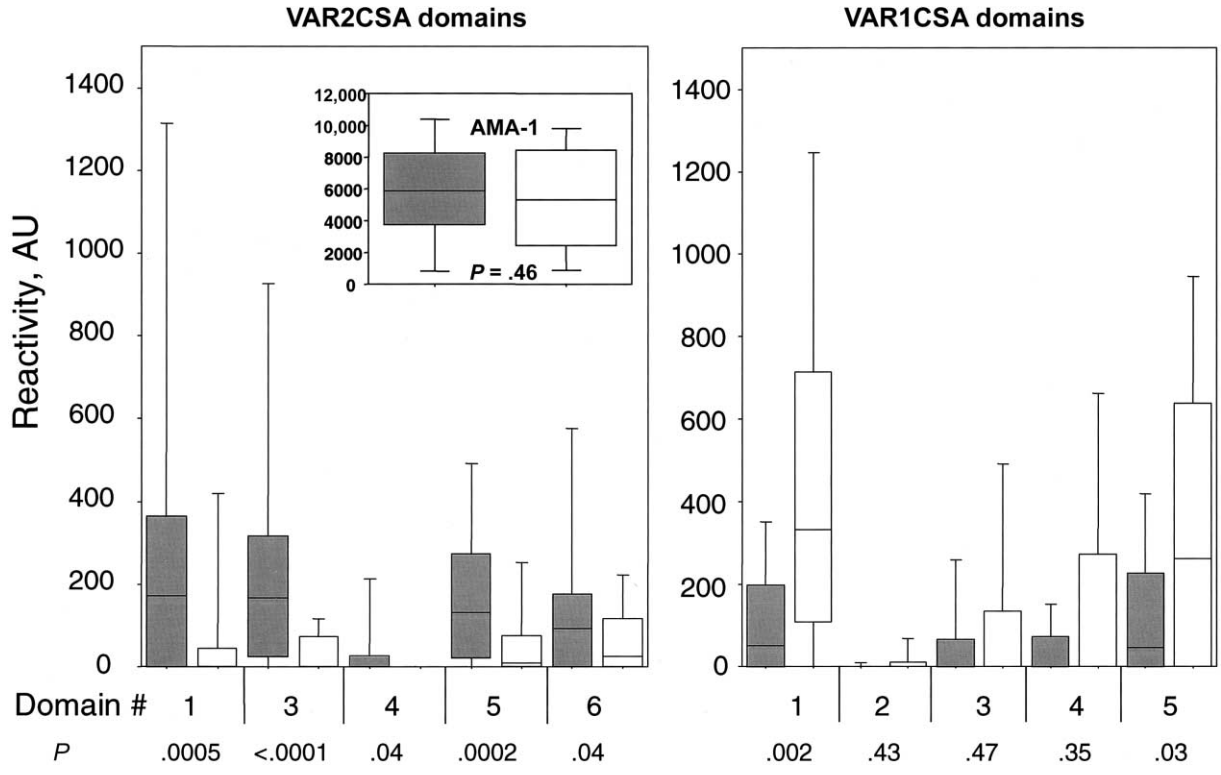

Figure 4. Preferential reaction of serum samples from multigravid women to VAR2CSA domains and preferential reaction of serum samples obtained from men to VAR1CSA domains. Seroreactivity to VAR2CSA and VAR1CSA domains (after subtraction of the control value [see Materials and Methods]) is indicated according to donor group. White bars denote serum samples obtained from men, and gray bars denote serum samples obtained from multigravid women. AU, arbitrary units. *P* values are the results of a 2-tailed Mann-Whitney *U* test (for 52 serum samples obtained from multigravid women and 44 serum samples obtained from men *[left]* and for 32 serum samples obtained from multigravid women and 32 serum samples obtained from men *[right]*). The top of the box denotes the 75th percentile, the bottom of the box denotes the 25th percentile, and the line through the middle of the box denotes the 50th percentile (i.e., the median). The whiskers denote the 10th and 90th percentiles. The inset shows the reactivity of apical membrane antigen–1 (AMA-1) for both groups.

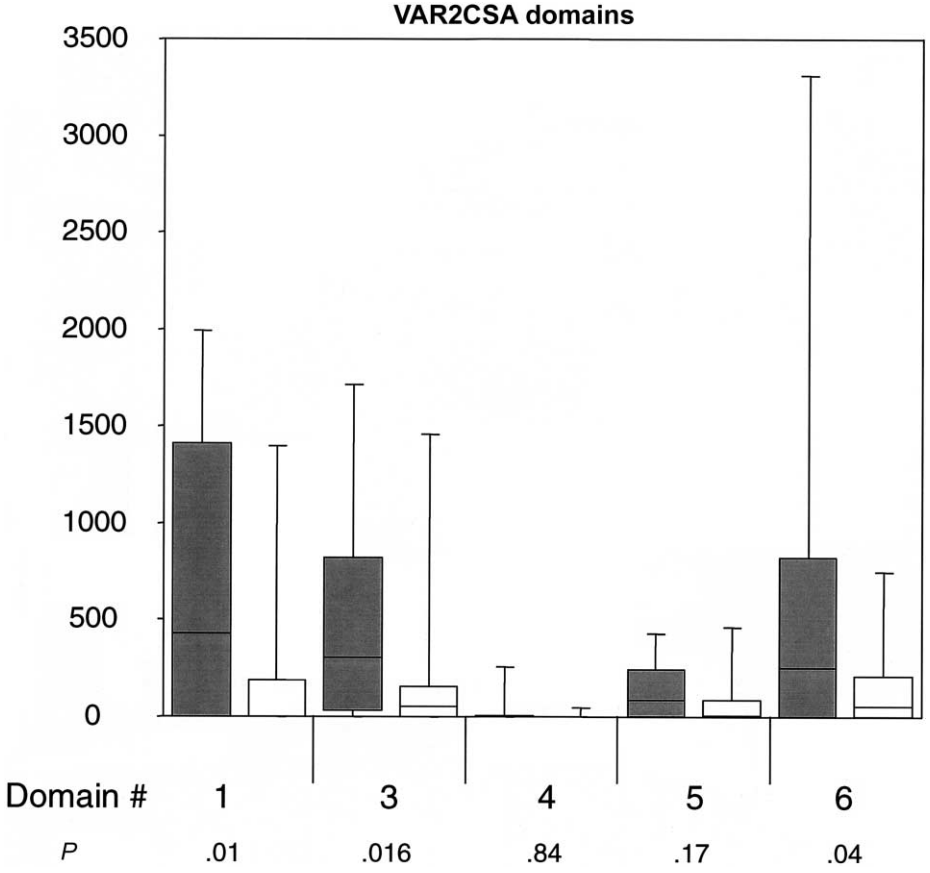

Figure 5. Increases in serum reactivity to VAR2CSA domains with gravidity. Seroreactivity to individual VAR2CSA domains is stratified by gravidity. White bars denote serum samples obtained from primigravid women, and gray bars denote serum samples obtained from multigravid women. AU, arbitrary units. *P* values are results of a 2-tailed Mann-Whitney *U* test ($n = 32$ for each group). The top of the box denotes the 75th percentile, the bottom of the box denotes the 25th percentile, and the line through the middle of the box denotes the 50th percentile (i.e., the median). The whiskers denote the 10th and 90th percentiles.

and for DBL6, $r = 0.48$ [$P = .0003$]) but not against AMA-1 antigen ($r = 0.07$; $P = .6$), suggesting that immunity to different VAR2CSA domains is acquired concordantly.

The pattern of reactivity to VAR1CSA versus VAR2CSA domains diverged markedly and was consistent against all tested domains (figure 4). Serum samples obtained from multigravid women reacted more strongly to VAR2CSA domains, whereas serum samples obtained from men reacted more strongly to VAR1CSA domains. The increased antibody levels noted in men versus those noted in multigravid women were statistically significant for 2 VAR1CSA domains (DBL1α and DBL5γ). Men and multigravid women had similar reactivity to AMA-1 (see above) and MSP-1 (data not shown), indicating that multigravid women specifically lose reactivity to VAR1CSA. Our studies of AMA-1 and MSP-1_{19} are similar to numerous earlier studies, which found that seroreactivity to various non-PfEMP1 malaria antigens did not vary with the pregnancy status or parity of the sample donors [30].

The dichotomous pattern of reactivity of men and multigravid women may be explained by mutually exclusive expression of *var* genes in *P. falciparum* [10]. PM is caused by CSA-binding parasites [6] that preferentially express *var2csa* [31], and peripheral parasites in pregnant women have features similar to those of placental parasites [16, 32]. Thus, the upregulation of *var2csa* in placental parasites may be accompanied by a down-regulation of other commonly expressed *var* genes, such as *var1csa*. Antibodies against VAR1CSA domains may therefore diminish in pregnant women, who would receive antigenic stimulation by VAR2CSA but not VAR1CSA during episodes of PM.

Gravidity-related increases in seroreactivity to VAR2CSA. We compared immunoreactivity to VAR2CSA domains in serum samples obtained from multigravid women (all 32 of whom did not have PM) and those obtained from primigravid women (8 of whom had PM and 24 of whom did not) in Tanzania. As was observed elsewhere [25, 26] (see below), VAR2CSA seroreactivity increased with the number of pregnancies (figure 5) and, consequently, with protection status.

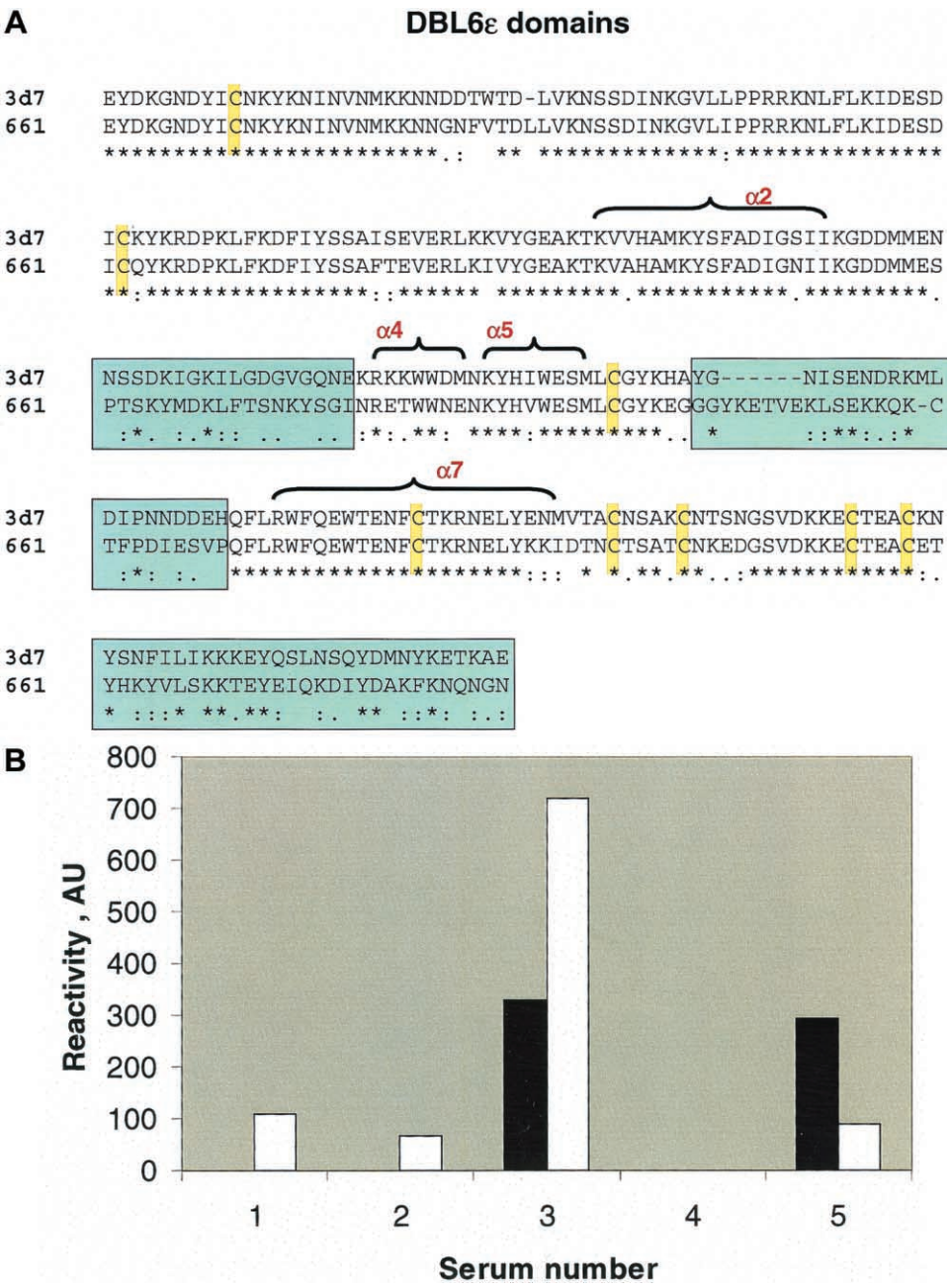

Figure 6. Antigenic variation and seroreactivity of VAR2CSA Duffy binding–like (DBL) 6ε domain. *A,* Comparison of strain 3D7 and placental parasite sample 661 DBL6ε domain sequences. Sequence alignment was performed using Clustal W at the GenomeNet Web site (available at: http://align.genome.jp). Stars denote conserved residues, and colons and dots denote more- and less-conservative substitutions, respectively. α2, α4, α5, and α7 are helical regions identifiable according to information found in [34]. Blue boxes denote regions of low homology. Yellow boxes denote cysteines conserved between these 2 variants. *B,* Reactivity of the 3D7 strain and the placental parasite sample 661 DBL6ε domains with serum samples obtained from 5 multigravid women. The 5 samples were randomly selected from the set of serum samples used in previously described experiments. Black bars denote 3D7 DBL6ε; white bars, 661 DBL6ε.

Differences in seroreactivity between groups of differing gravidities were statistically significant for 3 domains (DBL1, DBL3, and DBL6). These differences remained significant in analyses that included only serum samples from women without PM. In earlier studies, seroreactivity of the DBL5 domain [25] and 3 VAR2CSA domains (DBL1, DBL5 and DBL6) [26] correlated significantly with gravidity, but the levels of seroreactivity were not significantly different between groups of differing gravidities. Interestingly, antibody levels to VAR2CSA domains 1, 3, and 6 were significantly higher ($P < .05$, for all comparisons;

data not shown) among first-time Tanzanian mothers with PM versus those without PM in our study, suggesting specific responses to the antigen during PM. Separate studies will need to examine whether the antibodies produced by first-time mothers during malaria episodes have functional activity.

Similar studies were previously undertaken in West Africa with 2 VAR1CSA DBL domains (DBL1 and DBL2) expressed in *Escherichia coli* [33] and with varying numbers of VAR2CSA domains (2 [25], 3 [26], or 6 [27]) expressed in the baculovirus system. In those studies, for serum samples obtained from men and women, the reactivity against VAR1CSA domains did not differ significantly. Of note, *E. coli*–expressed DBL antigens may not recreate the extensive disulfide bonds and folds of the native protein [14], and expression of the DBL1 domain separate from the CIDR domain may disrupt a single functional domain and alter its conformation. In our studies, VAR1CSA domains were expressed on the surface of mammalian cells to better reproduce the native structure of these complex antigens, which may allow better discrimination of differences in seroreactivity. The earlier studies of VAR2CSA, which we discussed in detail above, generally observed a sex-specific and parity-specific pattern of reactivity, supporting the idea that VAR2CSA is preferentially expressed by placental parasites and is targeted by antibodies that correlate with immunity.

In our work, we expanded on these previous studies to incorporate all domains from each PfEMP1 protein, expressed each domain in a mammalian system to increase the probability of correct folding, and studied them together by use of serum samples from a distinct geographic region, East Africa. To our knowledge, these are the first studies to show a higher level of recognition of all immunoreactive VAR1CSA domains by serum samples obtained from men versus those obtained from multigravid women, and reactivity is highest against the first VAR1CSA domain (DBL1α plus CIDR1α). We also demonstrated that all immunoreactive VAR2CSA domains react most strongly to serum samples obtained from multigravid women, and we confirmed that this reactivity is parity specific.

Variant-specific reactivity to VAR2CSA. We compared the reactivity of immune serum samples to variant forms of domain DBL6ε representing laboratory isolate 3D7 and fresh placental parasite sample 661. These variant forms have a high level of homology throughout most of their sequence (figure 6*A*). Individual serum samples obtained from multigravid women varied substantially in their reactivity to variant forms of DBL6ε (figure 6*B*). Antigenic variation in this domain is limited to areas comprising ~30% of the domain sequence, primarily in the loops between helices α2 and α4, as well as those between helices α5 and α7 [34]. Because the remainder of the domain is largely conserved, and because the immune response against these 2 homologous domains is significantly different, we speculate that the immune response is predominantly directed toward regions of sequence variability, including the loops. This may also indicate that the most conserved parts of the domain are poorly immunogenic. We also saw a similar pattern of differential reactivity with VAR2CSA DBL1X domains (identity was ~80% between variants [data not shown]).

A previous study by Tuikue Ndam et al. [26] demonstrated no association between serum levels of anti-3D7 VAR2CSA antibodies and anti–CSA-binding antibodies in 4 of 6 placental isolates. This may have resulted from VAR2CSA sequence variation between placental samples, as the authors suggested, or it may indicate that functional antibodies are a minor subset of total antibodies. Our results regarding differential reactivity of laboratory isolate (3D7) versus placental parasite (sample 661) DBL domains do not confirm one or the other of these possibilities. If the former possibility is correct, then protective immunity in multigravid women may reflect the acquisition of antibodies against the VAR2CSA variants present in a community. A globally related pool of polymorphisms accounts for sequence variation in VAR2CSA [35], and, therefore, a limited number of variants may be adequate to elicit broadly reactive antibodies. Such a vaccine may be able to target only the loop regions, which could significantly simplify the task of developing a vaccine

Future studies will need to identify the malaria antigen, domain, or domain variant(s) and fragment(s) that are specifically targeted by protective antibodies, as well as those that elicit broadly reactive antibodies. This information could provide the basis for a PM vaccine.

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