Immunogenicity of Duffy Binding-Like Domains That Bind Chondroitin Sulfate A and Protection against Pregnancy-Associated Malaria

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Sequestration of Plasmodium falciparum-infected erythrocytes in the placenta is implicated in pathological outcomes of pregnancy-associated malaria (PAM). P. falciparum isolates that sequester in the placenta primarily bind chondroitin sulfate A (CSA). Following exposure to malaria during pregnancy, women in areas of endemicity develop immunity, and so multigravid women are less susceptible to PAM than primigravidae. Protective immunity to PAM is associated with the development of antibodies that recognize diverse CSA-binding, placental P. falciparum isolates. The epitopes recognized by such protective antibodies have not been identified but are likely to lie in conserved Duffy binding-like (DBL) domains, encoded by var genes, that bind CSA. Immunization of mice with the CSA-binding DBL3γ domain encoded by var1CSA elicits cross-reactive antibodies that recognize diverse CSA-binding P. falciparum isolates and block their binding to placental cryosections under flow. However, CSA-binding isolates primarily express var2CSA, which does not encode any DBLγ domains. Here, we demonstrate that antibodies raised against DBL3γ encoded by var1CSA cross-react with one of the CSA-binding domains, DBL3X, encoded by var2CSA. This explains the paradoxical observation made here and earlier that anti-rDBL3γ sera recognize CSA-binding isolates and provides evidence for the presence of conserved, cross-reactive epitopes in diverse CSA-binding DBL domains. Such cross-reactive epitopes within CSA-binding DBL domains can form the basis for a vaccine that provides protection against PAM.

Following repeated exposure to Plasmodium falciparum infections, adults in areas of endemicity develop immunity to clinical malaria (42, 46). However, women in areas of endemicity are uniquely susceptible to P. falciparum malaria during pregnancy (7, 36). Infection with P. falciparum is an important cause of maternal anemia and increases the risk of abortion, premature delivery, low birth weight, neonatal mortality, and infant anemia, especially in primigravidae (8, 28, 31, 35, 52). P. falciparum infections during pregnancy are frequently characterized by the sequestration of infected erythrocytes (IEs) in placental blood spaces (35), which can lead to inflammatory responses (54), deposition of fibrinoid material (57), and reduced blood flow to the fetus (18).

There has been considerable interest in understanding the molecular mechanisms that mediate placental sequestration of IEs and the reasons for the apparent lack of immunity to P. falciparum malaria in primigravidae residing in areas of endemicity. Multigravid women appear to be protected against the deleterious effects of P. falciparum infection during pregnancy (20, 36), suggesting that strain-transcending immunity develops rapidly following exposure to placental P. falciparum isolates. The mechanisms that mediate protective immunity against pregnancy-associated malaria (PAM) are not completely understood.

Adhesion studies have revealed that IEs derived from placentas predominantly bind chondroitin sulfate A (CSA) (1, 16, 24, 43). Binding to hyaluronic acid and normal immunoglobulins (Igs) may also play a minor role in placental sequestration (5, 6, 16, 23). In contrast, IEs derived from peripheral blood of P. falciparum-infected pregnant women or from that of non-pregnant donors, including children and adult men, commonly bind other endothelial receptors, such as CD36 (24). These findings suggest the possibility that the placenta may select for rare CSA-binding P. falciparum variants that are not commonly found in infected children or nonpregnant adults.

The cytoadherence of IEs to the host endothelium is mediated by variant surface proteins that belong to the P. falciparum erythrocyte membrane protein-1 (PfEMP-1) family (13). The P. falciparum genome contains ~60 var genes that encode diverse PfEMP-1 variants (3, 48, 49, 53). Expression of PfEMP-1 undergoes antigenic variation due to the switching of var gene expression during blood-stage growth (48). Immune adults residing in areas of endemicity acquire antibodies that recognize diverse PfEMP-1 variants and agglutinate diverse P. falciparum isolates (33). Antibodies directed against PfEMP-1 are thought to be important components of naturally acquired immunity to P. falciparum malaria (10). While sera from immune adult men and primigravid women residing in areas of endemicity recognize a wide range of peripheral P. falciparum isolates, they exhibit poor recognition of placental P. falciparum isolates (4, 25) and CSA-binding laboratory strains (41, 51). Following P. falciparum infection during pregnancy, women develop antibodies that show improved recognition of a wide range of placental isolates and CSA-binding laboratory strains (4, 25, 41, 51). The levels of antibodies recognizing placental isolates or CSA-binding laboratory strains are significantly correlated with parity (4, 25, 41, 51). This indicates the development of
antibodies that recognize conserved epitopes on the IE sur-
faces of diverse placental and CSA-binding isolates. The iden-
tity of such conserved epitopes has not yet been defined, but
they are likely to lie within PiEMP-1 variants that mediate
adhesion to CSA. The PiEMP-1 variants that were initially
implicated in CSA binding include var1CSA from P. falci-
parum FCR3CSA (9) and CS2var from P. falciparum CS2 (39,
40). Adhesion to CSA is mediated by the DBL3y domain of
var1CSA (9) and CS2var (39, 40). Monoclonal antibodies
raised against CHO cells expressing DBL3y of var1CSA and
antisera raised against recombinant DBL3y expressed in insect
cells recognize a wide range of placental P. falciparum isolates,
suggesting that DBL3y contains conserved, cross-reactive epitopes
shared by diverse CSA-binding placental isolates (15,
32). However, although var1CSA was initially implicated as the
var gene responsible for CSA binding in P. falciparum FCR3CSA,
subsequent studies demonstrated that the expression of another
var gene, var2CSA, and not that of var1CSA, is upregulated in
diverse CSA-binding parasite lines and placental
isolates (19, 21, 30, 44, 56). The var2CSA gene implicated in
CSA binding does not encode any DBLy domains. The re-
ported reactivity of anti-rDBL3y sera with placental CSA-
binding P. falciparum isolates is thus paradoxical.

Here, we have produced recombinant DBL3y (rDBL3y)
of var1CSA in its functional form and examined its immunogene-
icity. We demonstrate that immunization with rDBL3y does
elicit sera that cross-react with a wide range of placental iso-
lates and block their binding to placental cryosections under
static as well as physiologically relevant flow conditions. Im-
portantly, we show that anti-rDBL3y sera cross-react with one
of two CSA-binding DBL domains, namely, the DBL3x do-
main of var2CSA. This observation suggests that the CSA-
binding DBL domains DBL3y and DBL3x share conserved
B-cell epitopes and explains the paradoxical observations
that anti-rDBL3y sera recognize CSA-binding P. falciparum
isolates even though these parasites express var2CSA that
does not encode any DBLy domains. Such conserved
epitopes within diverse CSA-binding DBL domains may
provide the basis for the development of vaccines that elicit
cross-reactive antibodies against CSA-binding isolates and
protect against PAM.

MATERIALS AND METHODS

Production and characterization of functional rDBL3y, DNA encoding the
DBL3y domain of var1CSA from P. falciparum FCR3 fused to a C-terminal
six-histidine tag was amplified by PCR using primers 5′ ACT TGC CCA TGG
GAA AAC GAT GAA AAA C 3′ and 5′ ACG AGT GCG GCC GCT
CAG TGA TGG TGA TGG TGA TGT CTC TTC AAG TAA TCT GTG G 3′
and a cloned fragment of the FCR3 var1CSA gene (kindly provided by Artur
Scherf, Institut Pasteur, Paris, France) (9) as the template. The PCR product
was cloned as a Ncol-NsiI fragment downstream of the T7 promoter in expression
vector pET28a+ (Novagen) and transformed into Escherichia coli BL21(DE3)
(Novagen). Expression of rDBL3y with a C-terminal six-histidine tag was
induced by the addition of 1 mM isopropyl-1-thio-B-D-galactopyranoside (IPTG)
to cultures of E. coli BL21(DE3)pET28a+rDBL3y grown to mid-logarithmic
phase. Induced cultures were harvested after 4 h of growth at 37°C and lysed by
sonication. Fraction bodies were collected by centrifugation and solubilized in 6
M guanidine-hydrochloride. His-tagged rDBL3y was purified under denaturing
conditions by metal affinity chromatography using a Ni-nitrilotriacetic acid col-
umn (QIAagen), refolded by the method of rapid dilution, and purified further
to homogeneity by ion-exchange chromatography using SP-Sepharose (Pharma-
cia) and gel filtration chromatography using Superdex 75 as described earlier
for other DBL domains (37, 47).

Refolded and purified DBL3y was separated by sodium dodecyl sulfate-poly-
acrylamide gel electrophoresis (SDS-PAGE) before and after reduction with 5
mM dithiothreitol and detected by silver staining. The homogeneity of rDBL3y
was analyzed by reverse-phase chromatography on a C4 column as previously
reported (37, 47). The presence of free thiols was detected by Ellman’s method
with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (22, 37, 47).

Assessment of binding of rDBL3y to biotinylated CSA immobilized on
streptavidin-coated wells by ELISA. CSA, Csb, and Csc (Calbiochem) were
biotinylated using a biotinylation kit (Pierce) as described by the manufacturer.
Biotinylated CSA (Bio-CSA), Bio-CSB, and Bio-CSC (100 μl of 5 μg ml−1) were
immobilized in streptavidin-coated wells (S treptawell-96; Roche). Residual free
sites were saturated with 3% bovine serum albumin (BSA) in phosphate-buffered
saline (PBS) with 0.05% Tween 20 (PBST) for 1 h at 37°C. rDBL3y (5 μg ml−1)
was added to wells and allowed to bind overnight at 4°C. Unbound rDBL3y was
removed by washing with PBST four times. A mouse monoclonal antibody
against penta-histidine (penta-His) (QIAGEN) was added to each well at a
1:5,000 dilution and incubated at 37°C for 1 h to detect bound rDBL3y. Bound
mouse anti-penta-His monoclonal antibodies were detected with goat anti-
mouse IgG peroxidase conjugate (Sigma) (1:5,000 dilution) and O-phenylene-
diamine (OPD) (Sigma). The optical density at 490 nm was measured as an
enzyme-linked immunosorbent assay (ELISA) plate reader ( Molecular Devices)
to estimate relative binding efficiencies. Biotinylated hyaluronic acid (Bio-HA)
and the recombinant receptor for complement component C1q, also known as
hyaluronic acid-binding protein 1 (gC1qR/HABP1) (17) (kindly provided by
Anup Biswas and Kasturi Datta, Jawaharlal Nehru University, New Delhi, India)
were used as controls.

Immunization of BALB/c mice. BALB/c mice were immunized intraperitone-
ally with 25 μg of rDBL3y formulated in 250 μl Freund’s complete adjuvant
and subsequently given three booster immunizations with 25 μg of rDBL3y formu-
lated with Freund’s incomplete adjuvant on days 21, 42, and 73. Sera were
collected on days 14, 35, 56, 70, and 87 and stored at −80°C until use.

Inhibition of binding of CSA to rDBL3y by anti-rDBL3y sera raised in mice.
rDBL3y (100 μl of 5 μg ml−1 per well) was coated in 96-well polystyrene
microtiter plates (Nunc) by incubation overnight at 4°C followed by blocking
with 3% BSA. After blocking, wells were washed with PBST. After two washes,
plates were incubated with 1:5,000 dilutions of mouse sera raised against rDBL3y
or human sera from areas of

endemicity. Pooled preimmune mice sera were used as controls. One hundred
microliters of Bio-CSA (5 μg ml−1) was added to each well and allowed to bind
for 1 h at 37°C. Following four washes with PBST, bound Bio-CSA was detected
with Extravidin peroxidase and OPD (Sigma). The optical densities at 490 nm
were used to estimate the relative binding efficiencies to CSA in the presence
of test sera compared to those of controls.

Recognition of P. falciparum IEs by anti-rDBL3y mouse sera using FACS.
Recognition of P. falciparum IEs by mouse anti-rDBL3y sera was evaluated by
fluorescence-assisted cell sorting (FACS) as described earlier (50). Briefly,
5 × 105 erythrocytes from asynchronous P. falciparum cultures were washed twice
with RPMI medium, pH 6.8, and incubated for 30 min at 37°C with ethidium
bromide (5 μg ml−1). After two washes, cells were incubated with anti-mouse
IgG (heavy plus light chains) chicken at a dilution of 1:200 for 30 min on ice.
Erythrocytes were washed twice with RPMI medium and incubated with a goat anti-chicken IgG conjugated to Alexa-488 (Molecular Probes)
at a dilution of 1:200 for 30 min on ice. The recognition of intact IEs by antibodies
was quantified with a Coulter EPICS flow cytometer and expressed as the mean
Alexafluor intensity of ethidium bromide-gated IEs. Mouse sera raised against
rDBL3y were tested at a 1:20 dilution by both FACS and liquid immunofluores-
cence assay (L-IFA). Preimmune mouse sera were used as controls.

Inhibition of cytocoaghesis of IE under static conditions by anti-rDBL3y sera.
Static cytoadherence assays were performed as described previously (26).
Briefly, Saimiri monkey brain endothelial C2 (SBEC C2) cells were grown to confluence
on 12-well slides (Bio-Rad). Trophozoite- and schizont-stage IEs (20 μl of 105
IEs ml−1) were added to each well to allow binding. Heat-inactivated (56°C)
anti-rDBL3y mouse sera (dilution of 1:20) and soluble CSA (100 μg ml−1)
(Calbiochem) were tested for inhibition of binding. Cytoadherence medium and
mouse preimmune serum served as controls. In another experiment, SBEC C2 cells
were preincubated with rDBL3y (200 μg ml−1) prior to the addition of IEs.
Adherent IEs were detected by Giemsa staining and scored by light microscopy.

Inhibition of cytoadhesion of IEs to placental cryosections with anti-rDBL3y
sera under flow conditions. The cytoadherence of IEs on placental cryo-
secions was tested under flow conditions as described earlier (2, 26). Briefly,
follows: for DBL1X, amino acids (aa) 46 to 343; for DBL2X, aa 535 to 934; for DBL3X, aa 1214 to 1562; for DBL4ε, aa 1581 to 1931; for DBL5ε, aa 1983 to 2333; for DBL6ε, aa 2333 to 2617. Briefly, the DBL domains were fused to the signal sequence and transmembrane domain of herpes simplex virus glycoprotein D (HSV gD) to allow targeting to the mammalian cell surface as described previously (12, 34, 38). The plasmid pRE4 (kindly provided by Roselyn Eisenberg and Gary Cohen), which contains the gene for HSV gD (14), was digested with PvuII and Apal to excise the central region encoding amino acids 33 to 248 of HSV gD. DNA fragments encoding DBL domains of 3D7 var2CSA were amplified by PCR using Pyrococcus furiosus DNA polymerase (Stratagene) by use of var2CSA-specific primers and cloned into PvuII and Apal sites in the vector pRE4 as previously described (12) to yield plasmids pRE4-DBL1X, pRE4-DBL2X, pRE4-DBL3X, pRE4-DBL4ε, pRE4-DBL5ε, and pRE4-DBL6ε.

The DNA sequence of the insert in each expression plasmid was confirmed by sequencing with an ABI310 automated DNA sequencer. The sequence of each insert was identical to that reported for 3D7 var2CSA (PFL0030c; GenBank accession no. NP_701371). Mammalian 293T cells were transfected as described below to express the DBL domains (DBL1X to DBL6ε) on the surface. Transfected cells were tested for binding to CSA as described previously (34). CSA-

slides of four cryosections from each of two different placentas were mounted in a cell adhesion flow chamber (CAF-10; Immunechens) held in place by vacuum. The system was connected to an infusion/withdrawal pump (model 210P; KD Scientific) to control the flow of IE suspension or cell-free medium through the perfusion chamber. Reservoirs containing the IE suspension or cytoadhesion medium (RPMI medium, pH 6.8) were connected to the outlet of the perfusion chamber through a three-way valve. The cryosections were perfused with IEs at 5% parasitemia (mature stages) and 25% hematocrit in RPMI medium for 10 min at a shear stress of 0.05 Pa. The chamber was then flushed with RPMI medium to remove nonadherent IEs. Adherent IEs were observed with an inverted microscope (Eclipse TE 200; Nikon) with a PlanFluor 40/0.60 objective (Nikon). Bound IEs were counted on 10 randomly distributed fields with an area of 0.081 mm². Assays were performed in the presence of anti-rDBL3ε mouse serum (1:20 dilution) to test the ability of the IEs to block adhesion. Results were expressed as percent inhibition of binding with respect to binding in presence of preimmune sera.

Parasites and cells. P. falciparum laboratory strains as well as field isolates were cultured in O+ erythrocytes in RPMI 1640 (Sigma, France) in candle jars as described previously (55). P. falciparum laboratory strains FCR3CSA (9) and BC-1-CSA (kindly provided by Artur Scherf, Institut Pasteur, Paris, France) and P. falciparum placental isolates 24-CSA, 42-CSA, 42DJ-CSA, 193-CSA, 938-CSA, and 939-CSA (29), all of which bind CSA, were used for the study. In addition, P. falciparum strains FCR3CD36 (9), D10, and T996 (kindly provided by David Walliker, University of Edinburgh) and peripheral isolates RAJ-68, RAJ-104, and JDP8 (collected from regions of India where malaria is endemic and kindly provided by C. R. Pillai, Malaria Research Centre, Delhi, India), which do not bind CSA, were used as controls. The CSA-binding ability of placental isolates and CSA-binding laboratory strains was maintained by periodic panning on Sc17 cells (29).

Placental cryosections. Four cryosections from each of two different normal placentas collected in France were mounted sideways in the centers of 76- by 25-mm superfrost glass slides (Menzel-Glaser, Germany) for cytoadherence analysis under flow conditions. Placental cryosections (7 μm) were cut with a cryotome (CM 3050; Leica, Germany), air dried, fixed in 2.5% paraformaldehyde in PBS, pH 7.2, for 30 min, washed with PBS, dried, and preserved in an airtight box at −80°C until use. Before being used in adhesion assays, the slides were air dried quickly to prevent the condensation of water. Informed consent was obtained from all the participants. Biological material was sampled in strict accordance with the Mattei Law 666-8.

Identification of CSA-binding DBL domains of var2CSA by expression on the surface of mammalian cells. Plasmids were constructed to express the DBL domains (DBL1X to DBL6ε) of var2CSA of P. falciparum 3D7 (PFL0030c) on the surface of mammalian cells as described previously for other DBL domains (12, 34, 38). Amino acid boundaries of each DBL domain construct were as follows: for DBL1X, amino acids (aa) 46 to 343; for DBL2X, aa 535 to 934; for DBL3X, aa 1214 to 1562; for DBL4ε, aa 1581 to 1931; for DBL5ε, aa 1983 to 2333; for DBL6ε, aa 2333 to 2617. Briefly, the DBL domains were fused to the signal sequence and transmembrane domain of herpes simplex virus glycoprotein D (HSV gD) to allow targeting to the mammalian cell surface as described previously (12, 34, 38). The plasmid pRE4 (kindly provided by Roselyn Eisenberg and Gary Cohen), which contains the gene for HSV gD (14), was digested with PvuII and Apal to excise the central region encoding amino acids 33 to 248 of HSV gD. DNA fragments encoding DBL domains of 3D7 var2CSA were amplified by PCR using Pyrococcus furiosus DNA polymerase (Stratagene) by use of var2CSA-specific primers and cloned into PvuII and Apal sites in the vector pRE4 as previously described (12) to yield plasmids pRE4-DBL1X, pRE4-DBL2X, pRE4-DBL3X, pRE4-DBL4ε, pRE4-DBL5ε, and pRE4-DBL6ε. The DNA sequence of the insert in each expression plasmid was confirmed by sequencing with an ABI310 automated DNA sequencer. The sequence of each insert was identical to that reported for 3D7 var2CSA (PFL0030c; GenBank accession no. NP_701371). Mammalian 293T cells were transfected as described below to express the DBL domains (DBL1X to DBL6ε) on the surface. Transfected cells were tested for binding to CSA as described previously (34). CSA-
TABLE 1. Recognition of *P. falciparum* IEs by anti-DBL3γ mouse sera by L-IFA and FACS and inhibition of adhesion of *P. falciparum* IEs to SBEC C2 cells by CSA and recombinant DBL3γ

<table>
<thead>
<tr>
<th>Parasite strain*</th>
<th>Source</th>
<th>% Inhibition of binding to SBEC C2 cells under static conditions (avg. ± SD)by:</th>
<th>% Recognition by:</th>
<th>rDBL3γ</th>
<th>CSA</th>
<th>L-IFA</th>
<th>FACS</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCR3CSA</td>
<td>Lab strain</td>
<td>94 ± 2</td>
<td>96 ± 1</td>
<td>65</td>
<td>46.1</td>
<td></td>
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<tr>
<td>BC-1-CSA</td>
<td>Lab strain</td>
<td>89 ± 2</td>
<td>94 ± 1</td>
<td>32</td>
<td>24.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D10</td>
<td>Lab strain</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>2.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T996</td>
<td>Lab strain</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FCR3CD36</td>
<td>Lab strain</td>
<td>NT</td>
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<td>0</td>
<td>3.57</td>
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<td></td>
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<tr>
<td>24-CSA</td>
<td>Placental isolate</td>
<td>84 ± 3</td>
<td>92 ± 2</td>
<td>59</td>
<td>44.2</td>
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<tr>
<td>42-CSA</td>
<td>Placental isolate</td>
<td>87 ± 5</td>
<td>90 ± 3</td>
<td>87</td>
<td>72.9</td>
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<tr>
<td>42DJ-CSA</td>
<td>Placental isolate</td>
<td>83 ± 3</td>
<td>92 ± 2</td>
<td>39</td>
<td>44.1</td>
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<tr>
<td>193-CSA</td>
<td>Placental isolate</td>
<td>90 ± 2</td>
<td>95 ± 1</td>
<td>94</td>
<td>87.1</td>
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<tr>
<td>938-CSA</td>
<td>Placental isolate</td>
<td>88 ± 3</td>
<td>93 ± 3</td>
<td>42</td>
<td>21.9</td>
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<tr>
<td>939-CSA</td>
<td>Placental isolate</td>
<td>86 ± 2</td>
<td>94 ± 2</td>
<td>51</td>
<td>32.2</td>
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<tr>
<td>RAJ-68</td>
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* P. falciparum* peripheral and placental field isolates as well as *P. falciparum* laboratory strains were tested for binding to SBEC C2 cells. *P. falciparum* laboratory (Lab) strains FCR3CD36, D10, and T996 and field isolates RAJ-68, RAJ-104, and JD8 collected from peripheral blood of nonpregnant donors do not bind SBEC C2 cells and were not tested (NT) for inhibition of binding with CSA and rDBL3γ.

a Number of IEs bound to SBEC C2 cells in control wells was in the range of 90 to 125 bound IEs per field. Results represent inhibition efficiencies (average ± standard deviation) determined from three independent experiments. Each assay was performed in duplicate wells in each experiment.

b Mouse sera raised against rDBL3γ of FCR3 var1CSA were tested for recognition of *P. falciparum* laboratory strains and field isolates by L-IFA and FACS. The percentages of IEs that reacted with anti-rDBL3γ sera by L-IFA and FACS are reported.

binding assays were performed in the presence of anti-rDBL3γ mouse sera and preimmune sera to test the abilities of sera to block binding.

Mammalian cell culture, transfection, and immunofluorescence assays. 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% heat-inactivated fetal calf serum in a humidified CO₂ (5%) incubator at 37°C. Fresh monolayers of 40 to 60% confluent 293T cells growing in 35-mm-diameter wells were transfected with 2 μg of plasmid DNA using Lipofectamine Plus reagent (Invitrogen) as indicated by the manufacturer. Transfected cells were used for immunofluorescence and binding assays 36 to 40 h after transfection (34). Immuno-fluorescence assays using mouse monoclonal antibody DL6 (kindly provided by Roselyn Eisenberg and Gary Cohen), which reacts with amino acids 272 to 279 (14) of HSV gD, or anti-rDBL3γ mouse sera were performed as described earlier (12) to detect the expression of the fusion proteins. The binding of Bio-CSA to transfected 293T cells was tested as previously described (34).

Binding of refolded and purified rDBL3γ to biotinylated CSA immobilized on streptavidin-coated microwells by ELISA. One hundred microliters of Bio-CSA, Bio-CSB, or Bio-CSC was immobilized on each well of streptavidin-coated microwell plate (Sutrwell-96; Roche Applied Science) as indicated by the manufacturer. Five hundred nanograms of refolded and purified rDBL3γ was incubated overnight at 4°C, and unbound rDBL3γ was removed by washing four times with PBST. Residual free sites in the wells were saturated with 3% BSA in PBST for 1 h at 37°C, and the wells were washed four times with PBST. One hundred microliters of anti-penta-His antibody (Sigma) diluted to 1:5,000 was added to each well, and the wells were incubated at 37°C for 1 h, followed by four washes with PBST. One hundred microliters of Extravidin peroxidase (Sigma) (1:5,000 dilution) was incubated in each well for 1 h at 37°C. Bound immunocomplexes were detected with OPD (Sigma). Absorbance was measured at 490 nm using an ELISA reader (Molecular Devices).

Inhibition of binding of rDBL3γ-CSA binding by anti-rDBL3γ sera raised in mice. Anti-rDBL3γ mouse sera were tested for inhibition of the rDBL3γ-CSA interaction. Microtiter plate wells were coated with 100 μl of rDBL3γ (5 μg ml⁻¹). Plates were incubated at 4°C overnight and blocked with 3% BSA as described above. Microtiter plate wells were incubated with different dilutions of mouse antibodies raised against rDBL3γ. Preimmune sera were used as controls. Five hundred nanograms of Bio-CSA was incubated in each well at 37°C for 1 h. After being washed with PBST four times, the well was incubated with Extravidin peroxidase (Sigma) for 1 h at 37°C. Results expressed as the percentages of binding observed with respect to the values for controls.

RESULTS

Purity, homogeneity, and functional activity of rDBL3γ. The DBL3γ domain of var1CSA derived from *P. falciparum* FCR3 was expressed in *E. coli*, purified from inclusion bodies under denaturing conditions by metal affinity chromatography, refolded by rapid dilution, and purified to homogeneity by ion-exchange and gel filtration chromatography. Refolded and purified rDBL3γ was analyzed for purity, homogeneity, and functional activity. A single band of the expected size (~38 kDa) was detected on silver-stained SDS-PAGE gels (Fig. 1). rDBL3γ migrated slower on SDS-PAGE gels after reduction with dithiothreitol, indicating that the refolded protein contained disulfide linkages (Fig. 1). The free thiol content in rDBL3γ, which contains 10 cysteines, was measured using Ellman’s method (22). The detection limit for free thiols by this assay was 30 μM. No free thiols were detected in rDBL3γ at a protein concentration of 50 μM, indicating that >94% of cysteines were disulfide linked. The homogeneity of refolded rDBL3γ was analyzed by reverse-phase chromatography. rDBL3γ eluted from a C₈ column as a single symmetric peak, suggesting that refolded rDBL3γ was conformationally homogeneous (Fig. 1).

The functional activity of rDBL3γ was examined by testing the binding of rDBL3γ with its receptor, CSA. Biotinylated...
receptors, namely, Bio-CSA, Bio-CSB, Bio-CSC, and Bio-HA, were immobilized on streptavidin-coated ELISA plate wells and incubated with refolded rDBL3γ to allow binding. Bound rDBL3γ was detected using a mouse monoclonal antibody against the C-terminal penta-histidine tag. Binding of rDBL3γ was observed only for wells coated with Bio-CSA (Fig. 2). The lack of binding to CSB, CSC, and HA indicates that rDBL3γ bound CSA with specificity. The human endothelial receptor gC1qR/HABP1, which binds the globular head of complement component C1q as well as HA (17), and the receptor-binding domain, region II (PvRII), of P. vivax Duffy binding protein (12), were used as control ligands. PvRII did not bind CSA, CSB, or HA (Fig. 2). Recombinant gC1qR/HABP1 bound HA as expected but not CSA, CSB, or CSC (Fig. 2).

Mouse sera raised against rDBL3γ recognize diverse CSA-binding P. falciparum laboratory strains and placental field isolates. Pooled sera from five mice immunized with rDBL3γ detected rDBL3γ by ELISA up to a dilution of 1:100,000 and blocked the binding of rDBL3γ to CSA in an ELISA-based binding assay with 50% inhibition at a dilution of 1:500. Anti-rDBL3γ mouse sera were tested for recognition of diverse P. falciparum laboratory strains and field isolates by FACS and L-IFA. The P. falciparum laboratory strains and field isolates were first tested for binding to SBEC C2 cells, which display CSA on their surface (26). P. falciparum placental isolates bound SBEC C2 cells (Table 1). The addition of CSA (100 μg ml⁻¹) inhibited the binding of placental isolates and CSA-binding laboratory strains to SBEC C2 cells, confirming that these parasites used CSA as a receptor for adhesion to SBEC C2 cells (Table 1). Preincubation of SBEC C2 cells with rDBL3γ also inhibited the adhesion of IEs (Table 1). Anti-rDBL3γ mouse sera recognized CSA-binding P. falciparum laboratory strains and placental isolates by FACS as well as L-IFA (Fig. 3 and 4; Table 1). In contrast, anti-rDBL3γ mouse sera did not recognize P. falciparum laboratory strains or field isolates that did not bind CSA (Fig. 3; Table 1). The pattern of recognition of CSA-binding IEs by anti-rDBL3γ sera by L-IFA clearly indicated reactivity with the surface of IEs (Fig. 4). Anti-rBL3γ sera thus recognized com-

FIG. 4. Recognition of surfaces of P. falciparum IEs by anti-rDBL3γ mouse sera using L-IFA. Mouse sera raised against rDBL3γ of FCR3 var1CSA react with the IE surface of P. falciparum laboratory strain FCR3CSA and that of placental isolate 193CSA, which bind CSA, but not with that of P. falciparum laboratory strain FCR3CD36 or that of peripheral field isolate JDP8, which do not bind CSA. Parasites were stained with DNA-intercalating dye DAPI (blue) to identify IEs and with anti-rDBL3γ mouse sera followed by Alexafluor 488-conjugated chicken anti-mouse IgG (green).
mon conserved epitopes that are shared by diverse CSA-binding \textit{P. falciparum} isolates. Preimmune sera collected from mice prior to immunization with rDBL3γ showed no reactivity with any of the parasites when tested by FACS or L-IFA. While reactivity with FCR3CSA was observed by FACS when anti-mouse IgG goat serum coupled to fluorescein isothiocyanate was used for secondary staining with anti-rDBL3γ mouse sera for primary staining, no reactivity was observed when fluorescein isothiocyanate-coupled anti-mouse IgM goat serum was used for secondary staining (data not shown). This rules out the possibility that the reactivity of anti-rDBL3γ mouse sera with CSA-binding isolates was due to nonspecific binding of mouse IgM.

\textbf{Mouse sera raised against rDBL3γ block adhesion of diverse CSA-binding \textit{P. falciparum} laboratory strains and field isolates to \textit{Saimiri} monkey brain endothelial cells and placental cryosections.} Anti-rDBL3γ mouse sera and preimmune mouse sera were tested for inhibition of binding of IEs to SBEC C2 cells in static binding assays. Binding in the presence of preimmune mouse sera (1:20 dilution) was used as a control to determine inhibition efficiencies of anti-rDBL3γ sera. The number of IEs bound to SBEC C2 cells was scored in five random fields in each well by Giemsa staining. Results represent inhibition efficiencies (average ± standard deviation) determined from three independent experiments. Each assay was performed in duplicate wells in each experiment. The number of IEs bound to SBEC C2 cells in control wells was in the range of 90 to 125 bound IEs per field.

The \textbf{binding efficiency} was calculated as follows: binding efficiency (\%) = [(number of bound IEs in control wells - number of bound IEs in test wells) / number of bound IEs in control wells] × 100. Where the binding efficiency is reported as zero, no Alexafluor 488-stained IEs were seen in the entire well. Results reported are average ± standard deviation from three independent experiments.

Anti-rDBL3γ mouse sera recognize DBL3X of var2CSA and block its binding to CSA. Initial studies indicated that \textit{var1CSA} is responsible for the CSA-binding phenotype of FCR3CSA (9). However, subsequent studies demonstrated that transcription of another \textit{var} gene, \textit{var2CSA}, is upregulated in CSA-binding parasites (42, 56). Here, we have expressed the DBL domains of var2CSA from \textit{P. falciparum} 3D7 (DBL1X, DBL2X, DBL3X, DBL4ε, DBL5ε, and DBL6ε) on the surface of mammalian 293T cells and tested them for binding to Bio-CSA. In control experiments, the DBL domains were tested for binding to Bio-CSB and Bio-CSC. The DBL3γ domain of \textit{var1CSA} was used as a positive control in the binding studies. The DBL2X and DBL3X domains of var2CSA specifically bind Bio-CSA in this study (Table 3). Soluble CSA, but not CSB or CSC, inhibited the binding of DBL2X and DBL3X, and DBL3γ to Bio-CSA, confirming that these domains bound CSA with specificity (Table 4). Mouse sera raised against DBL3γ of \textit{var1CSA} recognized DBL3γ as well as DBL3X expressed on the surface of 293T cells (Table 3). Importantly, anti-rDBL3γ mouse sera completely blocked the binding of both DDB3γ and DDB3X domains with CSA at a dilution of 1:20 (Table 4). The DDB3γ domain of \textit{var1CSA} thus shares cross-reactive epitopes with the DDB3X domain of var2CSA. Antisera elicited against DDB3γ did not recognize DDB2X of var2CSA and did not block its binding to CSA (Tables 3 and 4). Anti-rDBL3γ sera also did not recognize any of the other DBL domains of var2CSA (Table 3).

\begin{table}[h!]
\centering
\begin{tabular}{|l|c|c|}
\hline
\textbf{Parasite strain} & \textbf{SBEC C2 (static)} & \textbf{Placenta (flow)} \\
\hline
\textit{P. falciparum} FCR3CSA & 86 ± 1 & 76 ± 5 \\
\textit{P. falciparum} BC1-1CSA & 90 ± 3 & 82 ± 5 \\
\textit{P. falciparum} 24-CSA & 86 ± 3 & 88 ± 2 \\
\textit{P. falciparum} 42DJ-CSA & 86 ± 4 & 79 ± 9 \\
\textit{P. falciparum} 42-CSA & 90 ± 5 & 93 ± 3 \\
\textit{P. falciparum} 192-CSA & 91 ± 1 & 93 ± 9 \\
\textit{P. falciparum} 938-CSA & 88 ± 3 & 90 ± 4 \\
\textit{P. falciparum} 939-CSA & 89 ± 3 & 83 ± 4 \\
\hline
\end{tabular}
\caption{Inhibition of adhesion of \textit{P. falciparum} isolates to SBEC C2 cells under static conditions and to placental cryosections under flow conditions by anti-rDBL3γ mouse sera.}
\end{table}

\begin{table}[h!]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
\textbf{Construct} & \textbf{Frequency of reactivity} & \textbf{Binding efficiency (\%)\textsuperscript{a}} \\
\hline
\textit{P. falciparum} DDBL3γ & 74 ± 4 & 63 ± 4 \\
\textit{P. falciparum} DDL6 & 70 ± 8 & 0 \\
\textit{P. falciparum} DDL2X & 60 ± 6 & 0 \\
\textit{P. falciparum} DDL3X & 66 ± 3 & 57 ± 3 \\
\textit{P. falciparum} DDL5ε & 58 ± 4 & 0 \\
\textit{P. falciparum} DDL6ε & 54 ± 6 & 0 \\
\textit{P. falciparum} DDL4ε & 83 ± 4 & 0 \\
\hline
\end{tabular}
\caption{Binding of DBL domains of 3D7 var2CSA to CSA and recognition by mouse sera raised against DDBL3γ of FCR3 var1CSA.}
\end{table}
placental field isolates that bind CSA (Fig. 3 and 4; Table 1). In recognizing the homologous parasite FCR3CSA as well as a wide range of anti-rBL3 sera, the DBL3X domains and explains the paradoxical observations made here and earlier (11, 15, 32) that anti-rDBL3X sera recognize CSA-binding parasites. We have demonstrated that in addition to recognizing a wide range of placental parasites, anti-rDBL3X sera block adhesion of IEs to placental cryosections under physiologically relevant flow conditions. This observation indicates that it may be possible to develop prophylactic strategies that block placental sequestration of malaria parasites.

The B-cell epitopes that are recognized by protective antibodies from sera of multigravid women have not yet been identified. Following the identification of var2CSA as the expressed var gene in CSA-binding laboratory strains and placental isolates, recent efforts to identify targets of antibodies that protect against PAM have focused on CSA-binding DBL domains of var2CSA. Here, we have demonstrated that the CSA-binding DBL domains of var2CSA, namely, DBL3X and DBL5X, respectively, share common B-cell epitopes. Such conserved epitopes that are shared by diverse CSA-binding DBL domains may serve as targets for protective antibodies and form the basis for development of a vaccine against PAM.

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