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Identification of *Plasmodium falciparum* var1CSA and var2CSA domains that bind IgM natural antibodies

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Abstract

Malaria in pregnancy is responsible for maternal anaemia, low-birth-weight babies and infant deaths. *Plasmodium falciparum* infected erythrocytes are thought to cause placental pathology by adhering to host receptors such as chondroitin sulphate A (CSA). CSA binding infected erythrocytes also bind IgM natural antibodies from normal human serum, a process that may facilitate placental adhesion or promote immune evasion. The parasite ligands that mediate placental adhesion are thought to be members of the variant erythrocyte surface antigen family *P. falciparum* erythrocyte membrane protein one (PfEMP1), encoded by the var genes. Two var gene sub-families, var1CSA and var2CSA, have been identified as parasite CSA binding ligands and are leading candidates for a vaccine to prevent pregnancy-associated malaria. We investigated whether these two var gene subfamilies implicated in CSA binding are also the molecules responsible for IgM natural antibody binding. By heterologous expression of domains in COS-7 cells, we found that both var1CSA and var2CSA PfEMP1 variants bound IgM, and in both cases the binding region was a DBL epsilon domain occurring proximal to the membrane. None of the domains from a control non-IgM-binding parasite (R29) bound IgM when expressed in COS-7 cells. These results show that PfEMP1 is a parasite ligand for non-immune IgM and are the first demonstration of a specific adhesive function for PfEMP1 epsilon type domains.

Keywords

*Plasmodium falciparum; PfEMP1; IgM; Natural antibody*

1. Introduction

During pregnancy-associated malaria, *Plasmodium falciparum* infected red blood cells are sequestered in the placenta causing low-birth-weight, foetal and infant death and maternal anaemia [1,2]. In the placenta, adhesion seems to occur between the host receptor chondroitin sulphate A (CSA) and *P. falciparum* erythrocyte membrane protein one (PfEMP1) [3-5]. PfEMP1 is a variant surface antigen encoded by the var gene family [6-8]. Every parasite contains 50–60 var genes in its genome, but only one is expressed at the surface of the infected erythrocyte [7,9]. PfEMP1 molecules are composed of Duffy
binding-like (DBL) domains classified into six types (alpha, beta, gamma, delta, epsilon and type X) and cysteine-rich interdomain region domains (CIDR) classified into three types (alpha, beta and gamma) [10,11]. Var genes differ from one other by the number and the type of these domains. PfEMP1 is involved in adhesion of the infected erythrocyte to various host receptors such as CD36 and ICAM-1 [12] during cytoadhesion to endothelium, and complement receptor 1 (CR1) in the case of rosetting parasites [13]. The well-conserved varICS A and var2CS A sub-families are the main var gene candidates described so far to be involved in pregnancy-associated malaria [3,14]. Heterologous expression experiments have shown that the DBL3 gamma domain of varICS A binds CSA [3], while several var2CS A domains (DBL2X, DBL3X and DBL6ε) bound CSA [15].

Along with CSA adhesion, it has been shown that infected erythrocytes implicated in placental adhesion are able to bind natural non-specific immunoglobulin M (IgM) antibodies [16], a phenomenon previously observed on rosetting parasites [17,18]. PfEMP1 mediates IgM binding on rosetting parasites [19] and may also be involved in IgM binding on CSA binding parasites [16]. The function of these IgM natural antibodies on infected erythrocytes is not understood. In the case of rosetting it has been suggested that IgM could act as a bridge between infected and uninfected erythrocytes to strengthen the rosettes [18]. Another hypothesis is that parasites allow binding of natural, non-specific IgM antibodies to block the binding of specific immunoglobulins and therefore avoid clearance by the immune system [17].

To further characterise CSA binding var gene candidates, we expressed each DBL and CIDR domain of the PfEMP1 variants encoded by varICS A and var2CS A to determine if they bind IgM natural antibodies and to identify which domain or domains mediate IgM binding.

2. Materials and methods

2.1. Parasite culture and var gene transcription

FCR3CS A parasites were cultured in complete RPMI as described previously [17]. Transcription of var2CS A in FCR3CS A was examined by northern blot as described previously [20,21] using as a probe the exon 2 of the 3D7var2CS A allele. The blot was hybridised and washed at low stringency (50 °C, 0.5X SSC wash).

2.2. Live cell IFA with FCR3CS A parasites grown in FCS

To determine if bovine IgM binds to FCR3CS A infected erythrocytes in a similar way to human IgM, the parasites were cultured for four cycles in complete RPMI containing 10% FCS instead of human serum. A live cell IFA was performed as described previously [16] using a mouse monoclonal antibody to bovine IgM (Sigma, clone BM-23) at 1/1000 dilution, followed by a 1/500 dilution of highly cross-absorbed Alexa Fluor™ 488 labelled goat anti-mouse IgG (Molecular Probes, Leiden, The Netherlands). Slides were viewed with a Leica fluorescence microscope.

2.3. Cloning of varICS A, var2CS A and R29var1 DBL and CIDR domains in the pRE4 vector

Each domain of varICS A from the FCR3/IT P. falciparum strain (GenBank Accession no. AJ133811) was amplified by polymerase chain reaction (PCR). For DBL1α, CIDRα, DBL2β, DBL3γ and DBL7ε the primers used were as described by Buffet et al. [3]. The amino-acid boundaries of the constructs amplified for the remaining domains were as follows: DBL4ε (1610–2097), DBL5γ (2100–2389) and DBL6β (2533–2829). The following domains were used for var2CS A from the FCR3/IT parasite strain (GenBank Accession no. AAQ73926): DBLIX (13–437), DBL2X (410–897), IDR (inter-domain...
region: 862–1187), DBL3X (1166–1566), DBL4ε (1534–1968), DBL5ε (1939–2308) and DBL6ε (2278–2600). As a negative control, the PfEMP1 domains from parasite clone R29, a rosetting non-IgM binding parasite [17] expressing the R29var1 [13] variant were also studied (GenBank Accession no. Y13402). The R29 constructs were those used by Rowe et al. [13], except DBL4δ (1605–2039) and CIDR2β (2058–2454). All domains were cloned into the pRE4 expression vector as described [22,23]. The pRE4 vector contains the herpes simplex glycoprotein D signal sequence fused to the protein of interest and a transmembrane signal sequence targeting the fusion protein to the surface of the transfected COS-7 cells [23]. After PCR amplification and sequencing of each domain, we found several differences in var1CSA, var2CSA and R29var1 compared to the Genbank sequences. For var1CSA, nucleotides 1968 (G instead of A changing amino-acid I into a M), 1977 (A instead of G is silent), 4586 and 4587 (AA instead of TT changing amino-acid V into a E), between 4608 and 4609 (insertion of AAA, amino-acid K), 6970 and 6971 (AA instead of TT, changing amino-acid L into a K) and finally nucleotide 7015 (G instead of A changing amino-acid N into D). For var2CSA, nucleotides 204 (G instead of A) and 2082 (T instead of C) are silent changes. In contrast nucleotides 3575 (A into G), 5182 (G into A), 5627 (G into A) and 6722 (G into A) changed amino-acids N into S, D into N, G into D and R into Q, respectively. For R29var1, changes were in nucleotide 5849 (G into A, changing G into E) and nucleotide 7170 (T into A, changing V into D). These modifications were confirmed by at least two different PCR amplifications and sequencing of four different clones of each PCR.

2.4. Transient transfection of COS-7 cells

For transfection experiments, COS-7 cells were grown overnight on 12-mm coverslips in 6-well plates in DMEM (Invitrogen, Paisley, United Kingdom) containing 10% heat-inactivated foetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (complete DMEM). COS-7 cells were washed and transfected in antibiotic-and serum-free DMEM with 1.5 μg DNA using “Lipofectamine reagent” (Invitrogen). After 5 h, an equal volume of antibiotic-free DMEM containing 20% FCS was added to the transfection solution. The medium was replaced with fresh complete DMEM the following day. To investigate whether bovine IgM might influence the results by blocking human IgM binding, control experiments were performed using serum-free media (VP-SFM, Invitrogen) after transfection.

2.5. Transfected COS-7 cell IgM binding and immunofluorescence assay (IFA)

IFAs were carried out as follows. Forty-eight hours after transfection, COS-7 cells were incubated for one and a half hours at 37 °C in a medium containing 10% normal pooled human sera. Five different lots of pooled human sera were tested, each containing serum from at least two donors. For inhibition assays, the cells were pre-incubated for 1 h with 0.01, 0.1 or 1 mg/ml CSA before addition of human serum. Cells were washed in phosphate-buffered saline (PBS), fixed for 10 min in PBS/2% formaldehde and washed again in PBS. Coverslips were incubated for 1 h either with 1D3 antibody (directed against the pRE4 glycoprotein D), [23], or with mouse monoclonal antibodies (IgG) to human IgM (Serotec, Oxford, United Kingdom), both diluted 1:1000 in PBS/0.1% BSA. After washing for 10 min in PBS/0.1% BSA, COS-7 cells were incubated 45 min in a 1:6000 dilution of highly cross-absorbed Alexa Fluor™ 488 labelled goat anti-mouse IgG (Molecular Probes, Leiden, The Netherlands). Cells were finally washed for 10 min in PBS/0.1% BSA before viewing under the fluorescence microscope. The entire coverslips were scanned with a 20× objective to look for positive cells, and the percentage of COS-7 cells that were positive for surface fluorescence was counted using the 40× objective. A control experiment was performed with secondary antibody only for each domain. Transfection efficiency and IgM binding are
given as the percentage of positive cells using the control 1D3 antibody and the mouse anti-human IgM antibody, respectively.

3. Results and discussion

The FCR3CSA parasite line binds to both CSA and human IgM [16]. This parasite transcribes var1CSA at the trophozoite stage [3,20], and var2CSA at ring stage (supplementary Fig. 1). In order to characterise the possible role of these 2 variants in IgM binding, we tested every domain of var1CSA and var2CSA for the capacity to bind human IgM natural antibodies. Domains of the R29var1 gene [13] were used as negative controls, as live parasites expressing this var gene have been shown not to bind human IgM [17].

All domains of var1CSA were expressed at the surface of the COS-7 cells. Only the DBL7\(\varepsilon\) domain showed IgM binding by IFA with a mouse mAb to human IgM, with 10–15% of the COS-7 cells showing bright surface fluorescence (Table 1, Fig. 1A). No IgM binding was detected with any of the other domains including the highly expressed domain DBL5\(\gamma\) (Table 1, Fig. 1B). Identical results were obtained with an affinity-purified goat polyclonal anti-human IgM reagent (Rockland, Gilbertsville, PA, USA, data not shown). For the var2CSA gene, each domain was also expressed at the surface of the COS-7 cells. Again, only one construct, the DBL6\(\varepsilon\) domain, gave a positive result for human IgM binding (Table 1 and Fig. 1C and D). The fluorescent signal was less intense than the signal obtained with the construct var1CSA DBL7\(\varepsilon\) (Fig. 1A). It is unclear whether this corresponds to a real difference between the two domains in affinity for human IgM, or merely reflects an artefact of the in vitro expression system. With both var1CSA and var2CSA, no fluorescent signal was detected on controls without primary antibody (Fig. 1A–D). In the control experiments with R29var1, a PfEMP1 variant expressed by a non-IgM binding parasite [13,17], each domain was expressed and none of them bound IgM, including the highly expressed epsilon domain, DBL3\(\varepsilon\) (Table 1). The absence of IgM binding to the negative control R29var1 suggests that the COS cell transfection system is a reliable method for assessment of IgM binding function.

Five different pools of serum, each composed of 2–4 donors, were used in the above experiments, and each pool gave identical results. In addition, IgM purified from normal human serum (Rockland, Gilbertsville, PA, USA) was tested and also bound only to var1CSA DBL7\(\varepsilon\) and var2CSA DBL6\(\varepsilon\), and not to any other domain.

In the 3D7 parasite clone, the var2CSA DBL6\(\varepsilon\) domain has been shown to have CSA binding activity [15]. We therefore investigated whether CSA might inhibit IgM binding to the FCR3 var2CSA DBL6\(\varepsilon\). We found that IgM binding to var2CSA DBL6\(\varepsilon\) transfected COS-7 cells was not inhibited by pre-incubation with CSA in the range 0.01–1 mg/ml. This suggests that the binding sites for CSA and IgM are likely to be distinct, although it remains to be confirmed that the FCR3 var2CSA DBL6\(\varepsilon\) domain tested here also has CSA binding activity.

In a previous publication, Creasey et al. [16] demonstrated that in addition to human IgM, some mouse IgM monoclonal antibodies were able to bind to CSA-binding infected erythrocytes. We therefore considered the possibility that bovine IgM in the FCS used to culture the COS-7 cells could have the potential to bind to PfEMP1 domains and interfere with the human IgM binding assays. To test the possibility that bovine IgM might bind to PfEMP1, we cultured FCR3CSA parasites in FCS and then carried out a live cell IFA with a monoclonal antibody to bovine IgM. We saw faint punctuate fluorescence indicating low level IgM binding on both uninfected and infected erythrocytes in the culture suspension, with no evidence for increased binding to the infected erythrocytes. We therefore conclude
that bovine IgM does not bind to PfEMP1 on infected erythrocytes, and is unlikely to interfere with the COS-cell human IgM IFAs shown here. To further exclude the possibility that bovine IgM might influence our results, we repeated the human IgM IFA experiments using transfected COS-7 cells grown in serum-free media. This resulted in greatly increased levels of background fluorescence seen with all domains (including negative controls and mock-transfected cells). The only domains showing positive fluorescence clearly above background levels in the serum-free medium experiments were var1CSA DBL7ε and var2CSA DBL6ε.

It has been suggested that parasites involved in placental adhesion via non-CSA-binding mechanisms are able to bind non-immune IgG [24]. However, CSA-binding parasites do not bind IgG or other human Ig sub-classes [16]. In order to confirm these previous findings that CSA-binding parasites do not bind IgG [16], we attempted to test IgG binding on both var1 and var2CSA domains expressed in COS-7 cells. Unfortunately, we found unacceptably high background levels in the IFA for human IgG using mock transfected COS-7 cells, therefore the COS-7 cell expression system is not suitable for studies of non-immune IgG binding.

Our results indicate that PfEMP1 is a parasite ligand for IgM natural antibodies and show that a single PfEMP1 molecule can bind both CSA and IgM. For both the PfEMP1 variants studied here (var1CSA and var2CSA), the IgM binding domain was of the epsilon type and occurred proximal to the transmembrane region. It is unclear whether the position of the IgM binding domain is important for its function, or whether the location proximal to the membrane in these two PfEMP1 variants is coincidental. The identification of epsilon-type domains with IgM binding activity is in contrast to results described previously for P. falciparum rosetting parasites, in which the CIDRa of the rosetting clone FCR3S1.2 [19] and the DBLβ of the rosetting clone TM284S2 were identified as IgM binding domains [24]. The DBLβ domain of var1CSA was well expressed and did not bind IgM in our experiments, however, the CIDRa domain had low expression (1% transfection efficiency), therefore, we cannot exclude the possibility that this domain could bind IgM but we were unable to detect it in this assay. However, careful examination of the entire coverslip in the var1CSA CIDRa transfected cells revealed no IgM-positive cells, therefore, we think that this is unlikely. It may be that rosetting parasites differ from CSA binding parasites in the fine specificity of their IgM binding mechanisms, however, further data from both rosetting and CSA binding parasites are needed before drawing any general conclusions.

We have shown that IgM natural antibodies bind a DBL epsilon domain, but IgM binding was not seen to all the epsilon domains examined. Indeed, var1CSA has two epsilon domains and var2CSA has three, and in each case, only one of these domains binds IgM. The negative control R29var1 has one epsilon domain, and this did not bind IgM. Even though all epsilon domains share some common motifs, there is still a large diversity among these domains that can explain such differences in binding capacity. Comparison of the two IgM binding epsilon domains with the four non-binding ones showed four small motifs (indicated by a line) that are unique to the IgM binding domains (Fig. 2). These are SAF, DMM, SD/EKIXKI (X being K or G) and EXRKZWW (X being N or K and Z being T or K). Interestingly, these motifs, especially the last three, are in the central part of the domain. Recently, Mayor et al. [25] showed that residues of DBL domains involved in red cell invasion and cytoadherence (to CR1 and CSA) map to central regions of DBL domains. The sequence of the 3D7 var2CSA allele shows the same motifs with only one conservative substitution, “SAI” instead of “SAF”. Future work will characterise the role of these motifs in a broad range of IgM natural antibody binding domains.
Many studies on pregnancy-associated malaria are now focused on \textit{var1CSA} or \textit{var2CSA} as possible vaccine candidates. Recent publications suggest that \textit{var2CSA} is the most promising candidate [14,26], however, controversy remains over the role of \textit{var1CSA} [27]. In particular, it remains unclear whether the \textit{var1CSA} or the \textit{var2CSA} PfEMP1 variants (or both) are expressed on the surface of CSA binding infected erythrocytes. Our previous studies have shown that CSA binding and IgM natural antibody binding are linked phenotypes [16], therefore, we hoped that assessment of the IgM binding capacity of the \textit{var1CSA} and \textit{var2CSA} PfEMP1 variants might provide additional evidence to indicate which of these variants is the adhesive protein on the surface of CSA binding infected cells. Our finding that both \textit{var1CSA} and \textit{var2CSA} bind IgM natural antibodies does not help to differentiate between these two vaccine candidates, and is consistent with a role for both variants in CSA binding.

The results shown here demonstrate that CSA binding PfEMP1 variants also bind IgM natural antibodies. The fact that CSA and IgM binding phenotypes are co-selected [16] may indicate that natural IgM antibodies have an important role for parasite survival in the placenta. Numerous other pathogenic micro-organisms have evolved mechanisms for binding human immunoglobulins in order to facilitate immune evasion or invasion and adhesion to host cells [28-34]. Strategies to block this nonimmune immunoglobulin binding by targeting specific IgG antibodies to the pathogen’s immunoglobulin-binding protein have been suggested [32], and could be attempted for malaria parasites. Determining if IgM natural antibodies are essential for the adhesion of the parasite in the placenta and/or for protection from specific antibodies would be an important step in understanding their role in the biology of the parasite during pregnancy-associated malaria. It would be of great interest if by inhibiting IgM binding we could reduce parasite adhesion or trigger parasite clearance by the immune system.

\textbf{Acknowledgments}

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\textbf{Abbreviations}

\begin{tabular}{ll}
CIDR & cysteine-rich interdomain region \\
CSA & chondroitin sulfate A \\
DBL & Duffy-binding-like \\
IDR & interdomain region \\
FCS & foetal calf serum \\
IFA & immunofluorescence assay \\
IgM & immunoglobulin M \\
PBS & phosphate buffered saline \\
PfEMP1 & \textit{Plasmodium falciparum} erythrocyte membrane protein-1
\end{tabular}

\textbf{References}


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Fig. 1.
Immunofluorescence assay on COS-7 cells transfected with var1CSADBL7ε (A), var1CSADBL5γ (B), var2CSADBL6ε (C), var2CSADBL1X (D). COS-7 cells were incubated with 1D3 antibody to determine transfection efficiency (upper images), with secondary antibody only as a negative control (middle images) or with mouse monoclonal antibody to human IgM (lower images).
Fig. 2.
Sequence alignment of the five, var1CSA and var2CSA, epsilon domains and R29var1 DBL3e domain. Multiple sequence alignments were carried out using ClustalW software available at http://www.ebi.ac.uk/clustalw/. Amino-acids specific for the two IgM binding domains (var1CSA DBL7 and var2CSA DBL6) are shown in red, and motifs are indicated by a line. Symbols: * indicates conserved residues;: indicates conservative substitution; . indicates semi-conservative substitution.
### Table 1

Ability of expressed PfEMP1 domains to bind human IgM

<table>
<thead>
<tr>
<th>Domain</th>
<th>Transfection efficiency (%)$^{a,b}$</th>
<th>IgM binding (%)$^{b,c}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>var1CSA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBL1α</td>
<td>2–3</td>
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</tr>
<tr>
<td>CIDR1α</td>
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<td>0</td>
</tr>
<tr>
<td>DBL2β</td>
<td>2–3</td>
<td>0</td>
</tr>
<tr>
<td>DBL3γ</td>
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</tr>
<tr>
<td>DBL4ε</td>
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<td>0</td>
</tr>
<tr>
<td>DBL5γ</td>
<td>10–15</td>
<td>0</td>
</tr>
<tr>
<td>DBL6β</td>
<td>3–5</td>
<td>0</td>
</tr>
<tr>
<td>DBL7ε</td>
<td>10–20</td>
<td>10–15</td>
</tr>
<tr>
<td><strong>var2CSA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBL1X</td>
<td>20–30</td>
<td>0</td>
</tr>
<tr>
<td>DBL2X</td>
<td>10–15</td>
<td>0</td>
</tr>
<tr>
<td>IDR$^d$</td>
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</tr>
<tr>
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<tr>
<td>DBL4ε</td>
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<tr>
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<td>DBL6ε</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>CIDR2β</td>
<td>15–20</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$Transfection efficiency was calculated by IFA using mAb 1D3.

$^b$Data from at least three experiments for each domain is shown.

$^c$IgM binding was determined by IFA using a mouse anti-human IgM mAb.

$^d$Interdomain region.