International Journal for Parasitology 39 (2009) 1195–1204

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Chondroitin sulphate A (CSA)-binding of single recombinant Duffy-binding-like domains is not restricted to *Plasmodium falciparum* Erythrocyte Membrane Protein 1 expressed by CSA-binding parasites

Mafalda Resende [a,1], Sisse B. Ditlev [a,1], Morten A. Nielsen [a], Sabrina Bodevin [b], Silas Bruun [a], Vera V. Pinto [a], Henrik Clausen [c], Louise Turner [a], Thor G. Theander [a], Ali Salanti [a], Madeleine Dahlbäck [a,*]

[a] *Centre for Medical Parasitology, Department of International Health, Immunology and Microbiology, University of Copenhagen and Department of Infectious Diseases, Copenhagen University Hospital (Rigshospitalet), CSS, Øster Farimagsgade 5 A, DK-1014 Copenhagen K, Denmark*

[b] *Department of Medical Biochemistry and Microbiology, The Biomedical Center, Uppsala University, SE-751 23 Uppsala, Sweden*

[c] *Department of Cellular and Molecular Medicine, University of Copenhagen, Panum Institute, Blegdamsvej 3, 2200 Copenhagen N, Denmark*

ARTICLE INFO

Article history:
Received 2 December 2008
Received in revised form 21 January 2009
Accepted 17 February 2009

Keywords:
VAR2CSA
Chondroitin sulphate A
Placental malaria
PfEMP1
Vaccine

ABSTRACT

Individuals living in areas with high *Plasmodium falciparum* transmission acquire immunity to malaria over time and adults have a markedly reduced risk of contracting severe disease. However, pregnant women constitute an important exception. Pregnancy-associated malaria is a major cause of mother and offspring morbidity, such as severe maternal anaemia and low birth-weight, and is characterised by selective accumulation of parasite-infected erythrocytes (IE) in the placenta. A *P. falciparum* protein named VAR2CSA, which belongs to the large *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) family, enables the IE to bind chondroitin sulphate A (CSA) in the placenta. Knock-out studies have demonstrated the exclusive capacity of VAR2CSA to mediate IE binding to CSA, and it has been shown that four of the six Duffy-binding-like (DBL) domains of VAR2CSA have the ability to bind CSA in vitro. In this study, we confirm the CSA-binding of these DBL domains, however, the analysis of a number of DBL domains of a non-VAR2CSA origin shows that CSA-binding is not exclusively restricted to VAR2CSA DBL domains. Furthermore, we show that the VAR2CSA DBL domains as well as other DBL domains also bind heparan sulphate. These data explain a number of publications describing CSA-binding domains derived from PfEMP1 antigens not involved in placental adhesion. The data suggest that the ability of single domains to bind CSA does not predict the functional capacity of the whole PfEMP1 and raises doubt whether the CSA-binding domains of native VAR2CSA have been correctly identified.

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1. Introduction

In malaria endemic regions individuals acquire clinical immunity during childhood and adults are in general protected against malaria infections (Christophers, 1924). The malaria parasite *Plasmodium falciparum* expresses proteins on the surface of parasite-infected erythrocytes (IE) which enables the IE to adhere to human endothelial receptors. By this process the parasite avoids being filtered through the spleen where it potentially could be cleared from the bloodstream and destroyed (David et al., 1983). *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) has been shown to be expressed on the surface of IE and to mediate binding to various receptors in the host endothelium (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). The PfEMP1 family constitutes high-molecular weight proteins of 200–350 kDa, which are encoded by the polymorphic *var* gene family containing 50–60 variants per parasite genome (Gardner et al., 2002). The *var* gene products are expressed in a mutually exclusive manner and clonal switching determines the adhesive phenotype of the parasites. Antibodies against PfEMP1 can interfere with parasite binding and the acquisition of a broad range of PfEMP1-specific antibodies is important for the development of immunity (Bull et al., 1998). However, women who have acquired immunity during childhood become susceptible to malaria during pregnancy due to the developing placenta which provides a new environment for sequestration of parasites. In the placental intervillous space the IE adhere to an unusually low-sulphated form of chondroitin sulphate A (CSA) (Fried and Duffy, 1996; Achur et al., 2000). CSA is present in the placenta as glycosaminoglycan (GAG) sidechains covalently attached to a core protein and this chondroitin sulphate proteogly-

* Corresponding author. Tel.: +45 35327684; fax: +45 35327851.
E-mail address: dahlback@sund.ku.dk (M. Dahlbäck).
[1] These two authors contributed equally to the work.

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doi:10.1016/j.ijpara.2009.02.022

can (CSPG) complex is a major component of the extracellular matrix. The adhesion of IE to placental tissue is highly specific for CSA and is not inhibited by other GAGs (Alkhalil et al., 2000). CSA is not exclusively localised in the placenta but rather commonly expressed, although at lower levels in the extracellular matrix of other endothelial tissues. However, the unique sulphation pattern of placental CSA is thought to be an important factor supporting the selective accumulation of IE in the placenta.

The parasite antigen mediating the sequestration in the placenta has been shown to be a uniquely structured molecule belonging to the PfEMP1 family, named VAR2CSA (Salanti et al., 2003). The *var2csa* gene was found to be the only *var* gene markedly up-regulated in *P. falciparum* parasites selected in vitro for CSA-binding (Salanti et al., 2003), and the corresponding protein (VAR2CSA) has been shown to be expressed on the surface of these laboratory parasites (Salanti et al., 2004; Barfod et al., 2006; Avril et al., 2006; Nielsen et al., 2007; Oleinikov et al., 2008; Fernandez et al., 2008) as well as parasites isolated directly from the placenta (Magistrado et al., 2008). Furthermore, it has been demonstrated that targeted disruption of the *var2csa* gene results in the loss of the ability of parasites to adhere to CSA (Viebig et al., 2005; Duffy et al., 2006). VAR2CSA is a large protein with an estimated molecular weight of 350 kDa consisting of six Duffy-binding-like (DBL) domains. Due to its size, the major challenge for vaccine development is to define which parts of the molecule bind CSA and design a recombinant vaccine, which in turn should induce antibodies blocking parasite adhesion in the placenta.

Prior to the identification of VAR2CSA, several PfEMP1 proteins were proposed to be the parasite ligand mediating CSA adhesion. In particular two *var* gene products have been extensively studied, *FCR3varCSA* (Buffet et al., 1999) and *CS2var* (Reeder et al., 1999). These two genes were identified by reverse transcription-PCR using degenerate *var* gene primers on parasite lines selected for CSA-binding in vitro and appeared to be the predominantly transcribed *var* genes (Buffet et al., 1999; Reeder et al., 1999). The DBL3γ domain of *FCR3varCSA*, also named *var1CSA* or *varCOMMON,* has been shown to bind to CSA in vitro in several independent studies (Buffet et al., 1999; Gamain et al., 2004; Bir et al., 2006) as well as other DBLγ domains (Khattab et al., 2001; Badaut et al., 2007), and two domains of the *CS2var* gene, the cysteine-rich interdomain region (CIDRβ) and DBL3γ have also been demonstrated to interact with CSA (Reeder et al., 1999, 2000). Based on these binding assays using recombinant proteins, these two genes were considered as potential pregnancy-associated malaria (PAM) vaccine candidates. In an additional study, a number of recombinant CIDR proteins, which had previously been shown to bind to CD36, were proposed to also bind CSA in vitro (Degen et al., 2000). Despite these reports showing CSA-binding of various recombinant domains, VAR2CSA seems to be the only *var* gene product responsible for the CSA adhesion of the laboratory *P. falciparum* isolates 3D7CSA and FCR3CSA (Salanti et al., 2003; Viebig et al., 2005; Ralph et al., 2005; Duffy et al., 2006). Recent studies indicate that at least four VAR2CSA domains (DBL2X, DBL3X, DBL5ε, DBL6ε) bind specifically to CSA (Gamain et al., 2005; Avril et al., 2006; Bir et al., 2006). However, the same DBL domains from different VAR2CSA variants show varying binding affinities. The following recombinant proteins of VAR2CSA have been shown to bind CSA: DBL2X-3D7 (Gamain et al., 2005; Avril et al., 2006; Bir et al., 2006), DBL2X-FCR3 (Gamain et al., 2005), DBL3X-FCR3 (Gamain et al., 2005; Dahlbäck et al., 2006; Higgins, 2008), DBL3X-3D7 (Bir et al., 2006; Dahlbäck et al., 2006), DBL5ε-3D7 (Avril et al., 2006) and DBL6ε-3D7 (Gamain et al., 2005; Avril et al., 2006).

Puzzled by the published data showing that non-VAR2CSA proteins bind CSA in vitro, we raised the question whether the binding data using VAR2CSA recombinant proteins reflect native VAR2CSA binding to CSA. In this study, we test a large panel of VAR2CSA DBL domains and a number of non-VAR2CSA domains including DBL3γ of *FCR3varCSA* for the ability to bind CSA, chondroitin sulphate C (CSC) and heparan sulphate (HS).

2. Materials and methods

2.1. Materials

CSPG-decorin (D8428), heparan sulphate proteoglycan (H4777), heparan sulphate (H7640), bovine tracheal CSA (C9819), chondroitinase ABC (C2905) and HIS-Select Nickel Affinity Gel (H8286) were obtained from Sigma–Aldrich. Shark cartilage CSC (400670) was from Seikagaku, Japan. BSA-50, immunoglobulin and protease free) was purchased from Rockland, USA, and 96 well flat-bottom plates (Falcon 351172) from BD Biosciences, USA. The horseradish peroxidase (HRP)-conjugated anti-V5 antibody (R96125) was from Invitrogen, USA. *o*-Phenylenediamine (OPD) tablets were obtained from DAKO, Denmark, and the *Baculovirus* vector (pAcGP67-A) and Bakpak6 *Baculovirus* DNA were purchased from BD Biosciences.

2.2. Parasites and parasite adhesion assay

The parasite culture was grown as previously described (Nielsen et al., 2007). To select for VAR2CSA expression, IE were repeatedly panned on BeWo-cells (Haase et al., 2006) and the parasite adhesion assay was performed as described previously (Nielsen et al., 2007). Briefly, 2×10^5 tritium-labelled late-stage FCR3-BeWo parasites in a total volume of 100 μl were added in triplicates to wells coated with 20 or 1 μg/ml of GAG (CSPG, chondroitinase treated CSPG, bovine tracheal CSA, CSC or HS) and incubated for 30 min at 37 °C. CSPG (0.06–8 μg/ml) and CSA (1.6–800 μg/ml) were further titrated in separate assays to compare the parasite binding efficiency. Unbound IE were washed away by re-suspension performed by a pipetting robot. The proportion of adhering IE was determined by liquid scintillation counting on a Topcount NXT.

2.3. Chondroitinase treatment of CSPG

CSPG of 2 mg/ml was treated with chondroitinase ABC (0.3 U/mg CSPG) in 0.05 M sodium acetate buffer, containing Tris (50 mM), overnight at 37 °C with gentle shaking.

2.4. Expression of PfEMP1 domains

All six domains of VAR2CSA (DBL1X, DBL2X, DBL3X, DBL4ε, DBL5ε and DBL6ε) were cloned from native *var2csa* amplified from genomic DNA of the *P. falciparum* strains FCR3, 3D7, HB3 and DD2 (domain boundaries can be seen in Supplementary Table S1). Gene fragments were cloned into the *Baculovirus* vector, pAcGP67-A, which has been modified to contain a V5 epitope upstream of a histidine tag in the C-terminal end of the constructs. Linearized Bakpak6 *Baculovirus* DNA was co-transfected with pAcGP67-A into Sf9 insect cells for generation of recombinant virus particles. Recombinant protein was purified on HIS-Select Nickel Affinity Gel as secreted histidine-tagged proteins from the supernatant of virus-infected High-Five insect cells using an ÄKTA-express purification system. The non-VAR2CSA proteins were cloned from FCR3 (DBL3γ *var1CSA*) or 3D7 (all others) and produced as described above. The corresponding protein IDs and PlasmoDB accession numbers for the control domains are as follow: CIDR1α (PFC0005w), DBL4ζ(a) (PFL0020w), DBL2β (PF08_0141), CIDR1γ (PFF1580c), DBL2C2 (PFF1580c), DBL6ε (PFF1580c) and DBL4ζ(b) (PFF0010w).

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