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Interactions between *Plasmodium falciparum* skeleton-binding protein 1 and the membrane skeleton of malaria-infected red blood cells

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ABSTRACT

During development inside red blood cells (RBCs), *Plasmodium falciparum* malaria parasites export proteins that associate with the RBC membrane skeleton. These interactions cause profound changes to the biophysical properties of RBCs that underpin the often severe and fatal clinical manifestations of falciparum malaria. *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is one such exported parasite protein that plays a major role in malaria pathogenesis since its exposure on the parasitised RBC surface mediates their adhesion to vascular endothelium and placental syncytioblasts. En route to the RBC membrane skeleton, PfEMP1 transiently associates with Maurer's clefts (MCs), parasite-derived membranous structures in the RBC cytoplasm. We have previously shown that a resident MC protein, skeleton-binding protein 1 (SBP1), is essential for the placement of PfEMP1 onto the RBC surface and hypothesised that the function of SBP1 may be to target MCs to the RBC membrane. Since this would require additional protein interactions, we set out to identify binding partners for SBP1. Using a combination of approaches, we have defined the region of SBP1 that binds specifically to defined subdomains of two major components of the RBC membrane skeleton, protein 4.1R and spectrin. We show that these interactions serve as one mechanism to anchor MCs to the RBC membrane skeleton, however, while they appear to be necessary, they are not sufficient for the translocation of PfEMP1 onto the RBC surface. The N-terminal domain of SBP1 that resides within the lumen of MCs clearly plays an essential, but presently unknown role in this process.

1. Introduction

Although malaria mortality rates have fallen by more than 40% over the last decade or so, this parasitic disease continues to exert significant, yet avoidable health, social and economic burdens on society, particularly in resource-poor, low and middle income countries. Each year, about 200 million people become infected with malaria parasites and more than half a million (predominantly young children in Africa) die as a result of their infection [1,2]; almost always due to *Plasmodium falciparum*. The extreme virulence of this parasite, compared to other species of *Plasmodium* that infect humans and its propensity to cause severe, often fatal disease, is underpinned by its ability to make the red blood cell (RBC) in which it resides abnormally adhesive for a number of other cell types including vascular endothelial cells, placental syncytiotrophoblasts, platelets, and other infected or non-infected RBCs. Consequently, RBCs infected with mature stages of *P. falciparum* cease to circulate and accumulate in multiple organs including the brain and placenta with subsequent severe pathological consequences (see [3–5] for reviews).

The altered adhesive properties of parasitised RBCs (PRBCs) is mediated by a family of high molecular weight, antigenically-diverse, parasite-encoded proteins collectively called *P. falciparum* erythrocyte protein 1 (PfEMP1) that are transcribed from the *var* multi-gene family and presented on the surface of RBCs infected with mature-stage parasites. Different variants of PfEMP1 can bind to a number of host receptors, principally CD36 and intracellular adhesion molecule-1 (ICAM-1), expressed on the surface of vascular endothelial cells, and chondroitin sulphate A (CSA) in the placenta [3].

The ability of PfEMP1 to mediate adhesion is dependent on its correct presentation on the PRBC surface [6–9]. We and others have previously shown that a parasite-encoded protein, skeleton-binding

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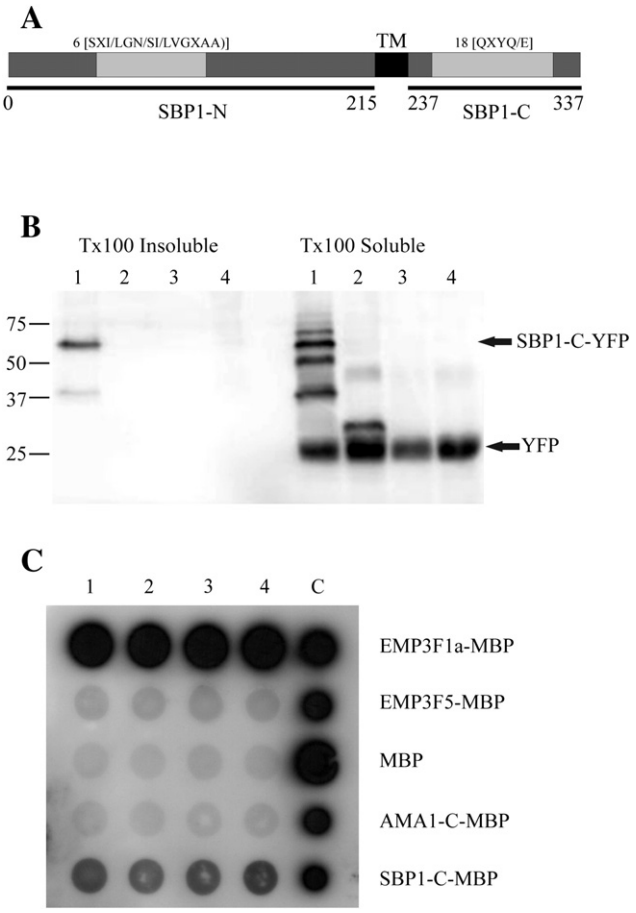

Fig. 1. Association of the C-terminal domain of SBP1 with the RBC membrane skeleton. A. Schematic representation of SBP1. Repeat regions (light grey) and the trans-membrane domain (TM) are indicated. B. Binding of the SBP1 C-terminal domain (SBP1-C) to the RBC skeleton. RBCs resealed with recombinant SBP1-C appended to YFP (SBP1-C–YFP; lane 1), RBCs resealed with YFP alone (YFP; lane 2), intact RBCs incubated with SBP1-C–YFP then washed (lane 3) or untreated RBCs (lane 4) were solubilised in TX-100. The TX-100 soluble and insoluble fractions were resolved by SDS-PAGE and immuno-blotted using an anti-YFP antibody. C. IOV binding assay with EMP3F1a as a positive control and EMP3F5 and MBP as negative controls.

protein 1 (SBP1), is essential for trafficking and translocation of PfEMP1 onto the RBC surface and consequently for adhesion of PRBCs to the vascular endothelium [10,11]. SBP1 is a trans-membrane protein, located in parasite-induced membranous structures within the PRBC cytoplasm known as Maurer's clefts (MCs) [12,13]. The topology of SBP1 is such that its entire N-terminal domain (SBP1-N; Fig. 1) is enclosed within in the lumen of the MC while its relatively shorter C-terminal tail (SBP1-C; Fig. 1) is exposed on the outside of the cleft, facing into the RBC cytosol [14]. Interestingly, disruption of the gene encoding SBP1 in *P. falciparum* appears to alter the cellular distribution of MCs, such that in RBCs infected with transgenic parasites lacking SBP1 expression, MCs are located further from the RBC membrane skeleton than in RBCs infected with wild-type parasites [10]. We therefore hypothesised that SBP1-C or domains within it bind specifically to protein components of the RBC membrane skeleton and mediate transfer of PfEMP1 from MCs onto the PRBC surface. To test this hypothesis, we have used a combination of molecular, cellular and biophysical approaches to identify the proteins (and sub-domains within them) that partake in this pathophysiologically-important interaction. Our studies provide a better understanding of the function of the C-terminal domain of SBP1, its role in the association MCs with the RBC membrane skeleton and the placement of PfEMP1 onto the surface of PRBCs.

2. Materials and methods

2.1. Malaria parasites

P. falciparum (3D7) was cultured *in vitro* in Albumax II-supplemented RPMI1640 as previously described [15] in either normal or protein 4.1R-deficient RBCs [16]. Cultures were selected for the expression of membrane knobs once per week using gelatin [17]. Asynchronous or synchronous parasite extracts were prepared by either Percoll gradient purification [18] or saponin lysis [19].

2.2. Generation of transgenic SBP1–AMA1-C *P. falciparum* clones

RBCs infected with young, ring-stage 3D7 parasites were transfected with approximately 150 μg of plasmid DNA as previously described [20,21]. The transforming plasmid was generated in the pCC1 vector [22] in order to generate a double crossover event replacing the entire C-terminal domain of SBP1 (amino acids 239–338) with that of the C-terminal domain of AMA1 (amino acids 566–622). Parasites were cultured in the presence of 2.5 nM WR99210 (Sigma-Aldrich) until parasites were observed (~6 weeks). Four clonal parasite lines, derived from two independent transfection events, were obtained by limiting dilution. DNA from 3D7 parasites and all transgenic parasite lines was purified using Nucleon BACC2 (GE Healthcare Life Sciences).

2.3. Expression and purification of proteins

Native spectrin (α/β-dimer) or protein 4.1R was purified from normal human RBCs as previously described [23]. Various recombinant GST-tagged sub-domains of spectrin and protein 4.1R were expressed in *E. coli* and purified as previously described [24,25]. Recombinant proteins for SBP1 were expressed as either the N-terminal region consisting of amino acids 1–215 (SBP1-N) or the C-terminal domain consisting of amino acids 239–338 (SBP1-C). Recombinant proteins for 6xHIS-yellow fluorescent protein (YFP) and SBP1-C–YFP were expressed from the pET24a vector (Novagen), SBP1-C–GST and SBP1-N–GST were cloned from the pGEX-KG vector [26] and SBP1-C–MBP and AMA1-C–MBP were expressed from the pMAL vector (New England Biolabs). All Proteins were expressed in *E. coli* BL21 DE3 and purified on TALON metal affinity resin (Clontech Laboratories) or amylose resin (for MPB-fusion proteins) (New England Biolabs) or glutathione resin (for GST-fusion proteins) (GE Healthcare) according to the manufacturer's instructions.

2.4. SDS-PAGE and immunoblotting

All samples of parasite lysates were resolved by SDS-PAGE using either 12% (w/v) or 8% (w/v) polyacrylamide gels and stained with Coomassie blue or transferred to polyvinylidene fluoride (PVDF) membranes (NEN) for western blot analysis. Anti-rabbit and anti-mouse immunoglobulins conjugated to horseradish peroxidise (Silenus) were used as secondary detection antibodies.

2.5. Resealed RBC interaction assay

To determine whether SBP1-C could bind directly to the RBC membrane skeleton, either SBP1-C–YFP or YFP recombinant proteins were resealed inside RBCs then solubilised using the non-ionic detergent TX-100 as previously described [27]. As a control, SBP1-C–YFP was also incubated with intact, normal human RBCs, washed, and then solubilised in the same way.