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

Lev M. Kats [a,1], Nicholas I. Proellocks [a,1], Donna W. Buckingham [a], Lionel Blanc [b], John Hale [b], Xinhua Guo [b], Xinhong Pei [b], Susann Herrmann [a], Eric G. Hanssen [c], Ross L. Coppel [a], Narla Mohandas [b], Xiuli An [b], Brian M. Cooke [a,*]

[a] Department of Microbiology, Monash University, Victoria 3800, Australia
[b] New York Blood Center, New York, NY 10021, USA
[c] Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Victoria 3010, Australia

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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

* Corresponding author. Tel.: +61 3 9902 9146; fax: +61 3 9902 9222.
E-mail address: brian.cooke@monash.edu (B.M. Cooke).
[1] These authors contributed equally to this paper.

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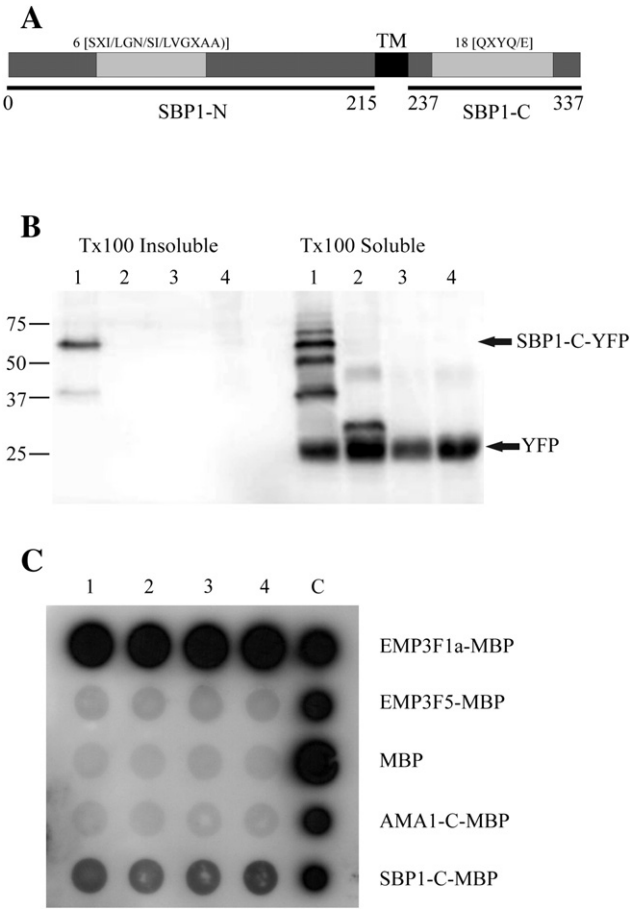

Fig. 1. Association of the C-terminal domain of SBP1 with the RBC membrane skeleton. A. Schematic representation of SBP1. Repeat regions (light grey) and the trans-membrane domain (TM) are indicated. B. Binding of the SBP1 C-terminal domain (SBP1-C) to the RBC skeleton. RBCs resealed with recombinant 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.

2.6. Protein–protein interaction assays

2.6.1. Plate-based interaction assays

Plate-based protein–protein interaction assays using both purified proteins and inside-out vesicles (IOVs) were performed as previously described [28]. Briefly, IOVs (RBCs that have been 'flipped' inside-out by osmotic lysis to generate vesicles that possess a properly conformed RBC membrane skeleton exposed to the external surface) or purified spectrin were used to coat 96-well plates. After washing the coated wells, recombinant SBP1 proteins were then added and incubated overnight at 4 °C. Wells were then washed three times and bound proteins striped from the plates with SDS sample buffer. Samples were then analysed by conventional dot-blotting on nitrocellulose membrane.

2.6.2. Quantification of protein–protein interactions by surface plasmon resonance

A well-established surface plasmon resonance-based technique was used to quantify protein–protein interactions using a BIAcore 3000 (BIAcore, Piscataway, NJ, USA). The 16 kDa domain of protein 4.1 or the α4 repeat region of α-spectrin was covalently immobilised onto a CM-5 sensor chip using amino coupling (BIAcore). A series of binding assays were performed by passing increasing concentrations of SBP1-C over the same regenerated chip surface multiple times. Binding reactions were performed at pH 7.4 in HEPES-buffered saline (150 mM NaCl, 20 mM HEPES) containing 3 mM EDTA and 0.05% (v/v) P20 surfactant. The surface was regenerated using 0.05% SDS before each new injection of SBP1. Resulting sensograms (response units *versus* time) were analysed using BIAeval v3.0 software (BIAcore). Affinity constants were estimated by curve fitting using a 1:1 binding model.

2.6.3. Protein pull-down assays

To assess binding of spectrin or protein 4.1R to recombinant SPB1 fragments, GST-tagged SPB1-C was coupled to glutathione beads at room temperature for 30 min. Beads were pelleted by centrifugation and then washed. Spectrin (1 μM) or protein 4.1R (1 μM) was added to the GST–SPB1-conjugated beads in a total volume of 100 μl. The mixture was incubated at room temperature for 1 h. The pellet was analysed by SDS-PAGE, transferred to nitrocellulose then probed with anti-spectrin or anti-protein 4.1R-specific antibodies. The reverse was also tested using GST-tagged spectrin or protein 4.1R fragments as the bait and His-tagged SPB1 as the prey and the interaction detected using anti-His-specific antibodies.

2.6.4. ELISA-based protein interaction inhibition assays

To examine the inhibition of SPB1–protein 4.1R interactions by the 16 kDa domain of protein 4.1R, purified, native protein 4.1R (200 ng in 100 μl) was coated onto 96-well plates overnight at 4 °C. Plates were then washed and blocked with 1% BSA in PBS containing 0.05% tween-20 for 1 h at room temperature. His-tagged SPB1 (pre-incubated with increasing concentrations of 16 kDa 4.1R) was added to protein 4.1R-coated plates and incubated for 30 min. Plates were then washed and SPB1 binding to protein 4.1R was detected using anti-His antibody/HRP-conjugated anti-rabbit IgG. The colour was developed by adding TMB microwell peroxidase substrate and plates were read in an ELISA plate reader at 450 nm. Similar experiments were performed to examine the effect of the α4 repeat region of α-spectrin on SPB1–spectrin interactions using, in this case, native, spectrin dimer purified from normal human RBCs to coat 96-well plates.

2.7. Quantitative immunofluorescence assays

RBCs from parasite cultures were smeared onto glass slides and fixed in 9:1 acetone/methanol for 10 min on ice before being used for quantitative immunofluorescence assays. Primary antibodies were either polyclonal mouse anti-spectrin (1:500), polyclonal rabbit anti-glycophorin A (1:2000) or polyclonal rabbit anti-SBP1 (1:500). Alexa Fluor 488- or Alexa Fluor 568-conjugated anti-rabbit or anti-mouse immunoglobulin (1:3000; Molecular Probes Inc.) was used as secondary antibodies. Immuno-labelled smears were examined by wide-field or confocal fluorescence microscopy and digital images captured for analysis and quantitation using ImageJ [29]. To quantify co-localisation of MCs (SBP1) with spectrin in RBCs infected with either normal (3D7) or transgenic SBP1–AMA1-C parasites, fluorescence images from 10 individual, randomly selected PRBCs for each parasite line were analysed and co-localisation quantified using the Manders' co-localisation coefficient [30].

2.8. PRBC adhesion assay

The adhesive properties of PRBCs were analysed and quantified using an *in vitro* microslide flow-based adhesion assay with monolayers of human, activated platelets (CD36) as the adhesive target as previously described in detail [31,32]. Assays were performed on highly synchronised parasite cultures in which the majority of PRBCs contained mature, pigmented trophozoites with parasitaemia ranging between 3 and 5%. In all cases, adhesion was quantified at a physiologically-relevant wall shear stress of 0.1 Pa.

2.9. Analysis of surface PfEMP1 expression on PRBCs

Exposure of PfEMP1 on the surface of PRBCs was quantified using the indirect trypsin cleavage assay exactly as previously described [10]. In essence, PRBCs were purified using Percoll from synchronised cultures following gelatin flotation. After trypsin treatment of intact PRBCs, membrane surface-exposed proteins (including PfEMP1) were extracted in 1% Triton-X 100 followed by solubilisation in SDS. Samples were then resolved using SDS-PAGE (6%) then transferred to PVDF membrane for western blot analysis.

2.10. Scanning electron microscopy

RBCs from synchronised parasite cultures (mature trophozoites) were prepared using standard methods, sputter-coated with gold, and then examined using a Quanta 200 FEG Environmental Scanning Electron Microscope. Sixty images of randomly selected PRBCs (30 for each parasite line, 3D7 and SBP1–AMA1-C) were used to quantify the number of knobs on the surface of PRBCs by counting the number of knobs on a flat, randomly-selected $1/\mu m^2$ area of PRBC membrane.

3. Results

3.1. The C-terminal domain of SBP1 (SBP1-C) binds to the RBC skeleton

We used multiple approaches to demonstrate that SBP1-C interacts directly with the RBC membrane skeleton. Firstly, we introduced a recombinant protein comprising only the C-terminal domain of SBP1 appended to YFP (SBP1-C–YFP) into intact, hypotonically-resealed RBCs then subsequently solubilised a lysate of these cells in the presence of the non-ionic detergent Tx100. We showed that SBP1-C–YFP partitioned exclusively into the Tx100-insoluble fraction. In contrast, under identical experimental conditions, recombinant YFP alone was found only in the Tx100 soluble fraction (Fig. 1B). Secondly, we confirmed that this interaction of SBP1-C with the RBC membrane skeleton was specifically with components of the membrane skeleton itself and not with other components of the membrane since neither SBP1-C–YFP nor YFP showed any detectable interaction with the RBC when these proteins were incubated with intact, normal human RBCs (Fig. 1B). Together, these data indicate that SBP1-C interacts specifically with the RBC membrane skeleton that lines the inner (cytoplasmic) face of the RBC. Thirdly, we used an *in vitro* plate-based assay to demonstrate a direct binding interaction between SBP1-C and immobilised vesicles of RBCs that had been flipped inside-out (IOVs) to expose the

membrane skeleton on their outer surface (Fig. 1C). Consistent with the resealing experiments, a recombinant fusion protein comprising SBP1-C fused to maltose-binding protein (SBP1-C–MBP) bound to IOVs whereas MBP alone showed no detectable binding under identical conditions. A fragment of a previously characterised RBC membrane-interacting malaria protein, PfEMP3 [28] was used as a positive binding control in these experiments and bound as expected to IOVs (Fig. 1C). Thus, in three different assays, SBP1-C displayed properties characteristic of a RBC membrane skeleton binding protein. Importantly, we ruled out the possibility that this interaction with the RBC membrane skeleton was a universal feature of the C-terminal domains of *P. falciparum* proteins by demonstrating that a recombinant MBP-fusion protein comprising the C-terminal domain of apical merozoite antigen 1 (AMA1) [33] (similar in length to SBP1-C but unrelated in sequence) failed to interact with IOVs under identical conditions (Fig. 1C).

3.2. SBP1-C binds to spectrin and protein 4.1

We next sought to identify the specific component(s) of the RBC membrane skeleton with which SBP1-C interacts. Given that we have previously described in detail the interactions of numerous exported malaria proteins with protein 4.1 and/or spectrin [34] we purposely selected these proteins as initial potential targets. Using either protein 4.1 or spectrin that had been purified from human RBCs and GST-tagged recombinant versions of both SBP1-C and the entire N-terminal domain of SBP1 (SBP1-N) in multiple protein pull-down experiments, SBP1-C but not SBP1-N nor GST alone was able to consistently pull-down both protein 4.1 (Fig. 2A) and spectrin (Fig. 3A). Further, identical pull-down assays using multiple non-overlapping sub-fragments of either protein 4.1 (Fig. 2B) or α-spectrin (Fig. 3B) revealed that the interaction of SBP1-C with protein 4.1 was confined predominantly to the 16 kDa fragment of protein 4.1, although there was also a weak interaction with the 10 kDa fragment (Fig. 2C). For spectrin, binding was confined exclusively to within the first five N-terminal repeats of α-spectrin (αN1-5) (Fig. 3B), specifically to the α4 repeat (Fig. 3C).

To further confirm the specificity of these interactions, we used the 16 kDa fragment of protein 4.1 and the α4 repeat region of α-spectrin in a competitive binding assay (Figs. 2D and 3D). In the presence of increasing concentrations of either the 16 kDa fragment of protein 4.1 or the α4 repeat region of α-spectrin, the binding of SBP1-C to either native protein 4.1 or spectrin respectively was progressively inhibited in a concentration-dependent manner.

3.3. Kinetic analysis of the interaction between SBP1-C, protein 4.1 and spectrin

We used surface plasmon resonance to quantify the interaction between SBP1-C and protein 4.1 or spectrin. Analysis of quantitative binding data, obtained from four separate experiments, revealed that SBP1-C bound to the 16 kDa fragment of protein 4.1 or the α4 repeat region of α-spectrin with very high affinity ($K_D \approx 0.06$ μM and 0.02 μM respectively; Table 1). These dissociation constants reflect higher affinity interactions than are commonly seen for malaria proteins at the RBC skeleton but are not unprecedented [3].

3.4. Protein 4.1R is necessary for efficient localisation of Maurer's clefts at the RBC membrane skeleton

To determine whether the interaction between SBP1-C and the RBC membrane skeleton was of functional relevance *in vivo*, we determined and quantified, by IFA and confocal microscopy, the localisation of MCs in parasite-infected human RBC that were genetically deficient in protein 4.1R. Notably, while the appearance and number of MCs by IFA was similar in both normal and 4.1R-deficient infected RBCs, the proportion of MCs that were located at the RBC membrane skeleton (as measured by the co-localisation of SBP1 with the integral RBC membrane-skeleton protein, glycophorin A) was significantly lower ($P < 0.0001$; Mann–Whitney *U* test) in 4.1R-deficient RBCs (mean co-localisation = 62.8 ± 2.4%; n = 73 *versus* 42.2 ± 1.9%; n = 102 for normal and 4.1R-deficient RBCs respectively) (Fig. 4). This data strongly supports that the interaction between SBP1-C and protein 4.1R plays a significant role in localising MCs to their final destination in the PRBC, tethered to the RBC membrane skeleton [35].

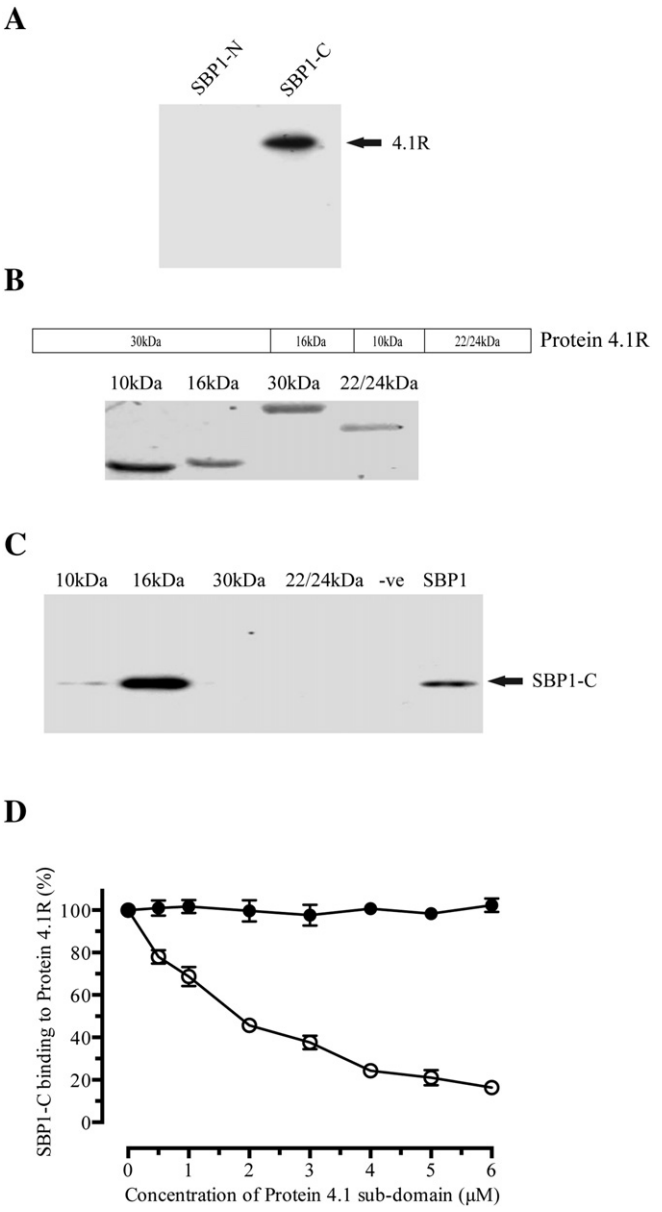

Fig. 2. Binding of SBP1 C-terminal domain to protein 4.1R. A. GST-tagged or N- or C-terminal SBP1 was used to pull down 4.1R purified from RBCs. Binding was detected by immunoblotting using specific anti-4.1R antibodies. This result is a representative example of 3 independent binding experiments all of which showed identical results. B. Schematic representation of protein 4.1R, showing the defined structural regions that were expressed as recombinant fragments as previously described [25]. The Coomassie-stained gel shows the purified recombinant proteins. C. Binding of the SBP1 C-terminal domain to protein 4.1R domains. GST-tagged 4.1R domains (expressed in pGEX-4T-2) were used to pull down His-tagged SBP1 C-term (expressed in pET24a). SBP1 binding was detected by immuno-blotting using an anti-hexahistidine antibody. D. Inhibition of binding of SBP1 C-terminal domain to protein 4.1R by the 16 kDa domain of protein 4.1. His-tagged SBP1-C was pre-incubated with increasing concentrations of either GST-tagged 16 kDa protein 4.1 domain or GST-tagged 22/24 kDa domain at room temperature for 30 min. Mixtures were then added to a 96-well plate that had been coated with 4.1R. The binding of His-tagged SBP1 C-term was detected using an anti-His antibody. Note the progressive decrease of SBP1-C binding to protein 4.1R with the increasing concentrations of 16 kDa but not with 22/24 kDa domains of protein 4.1. Data are the mean ± S.E.M. from three separate experiments.

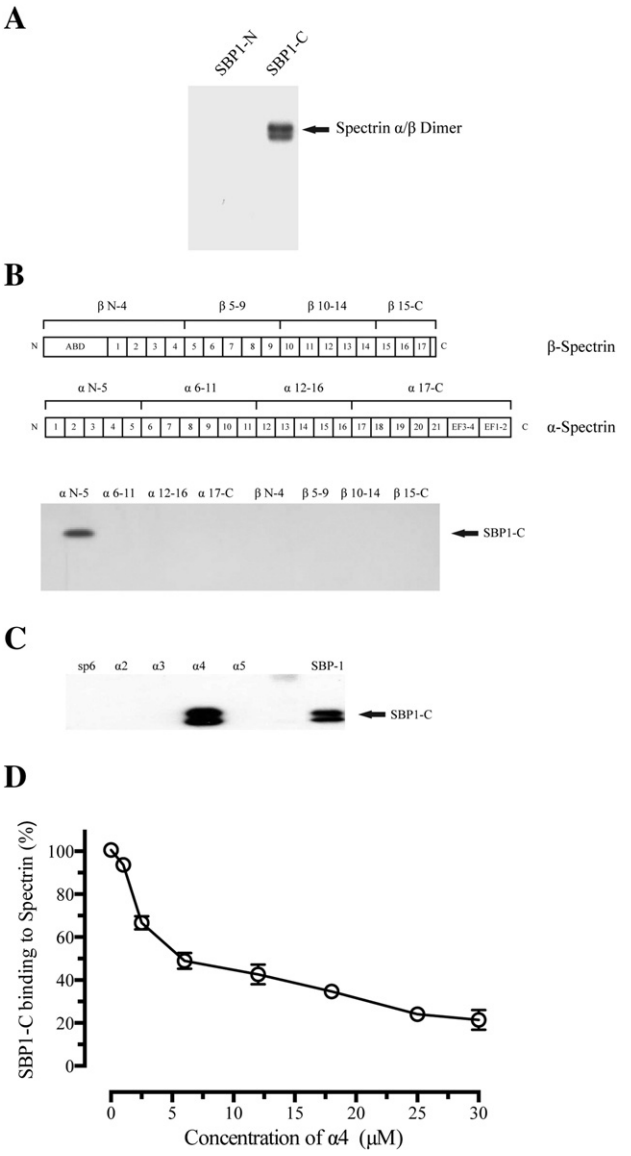

Fig. 3. Binding of SBP1 C-terminal domain to spectrin. A. GST-tagged or N- or C-terminal SBP1 was used to pull down full-length spectrin purified from RBCs. Binding was detected by immunoblotting using specific anti-spectrin antibodies. A representative example of 3 independent binding experiments is shown but all 3 experiments showed essentially identical results. B. Schematic representation of the spectrin α- and β-chains; regions expressed as recombinant fragments are indicated [24]. Western blot showing the binding of SBP1-C to spectrin fragments, specifically the α-N5 repeats. C. α-N5 repeats were divided into the individual repeats expressed as GST fusion proteins and used to pull down the hexahistidine-tagged SBP1 C. Binding was detected by western blotting using anti-hexahistidine antibody. GST was used as negative control in all experiments. SBP1 binding was detected by immuno-blotting with an anti-hexahistidine antibody. D. Inhibition of binding of SBP1-C to the α4 repeat region of α-spectrin. Competitive inhibition was measured by an enzyme-linked immunosorbent assay (ELISA). SBP1-C was coated on 96-well plates then pre-incubated with various concentrations of the α4 repeat region of α-spectrin prior to the addition of purified spectrin dimer. The binding of spectrin dimer to SBP1-C decreased with progressively increasing concentrations of the α4 repeat region of α-spectrin. Data are the mean ± S.E.M. from three separate experiments.

3.5. Functional significance of SBP1-membrane skeleton interaction in PRBCs

Based on this data we reasoned that deletion of SBP1-C would affect the localisation of MCs in PRBCs and prevent their close association with the RBC membrane skeleton. Despite multiple attempts, we were unable to derive a transgenic parasite line in which the entire SBP1-C region was deleted from the endogenous *sbp1* gene. We therefore derived 3 independent clonal lines of transgenic parasites all stably expressing a chimeric full-length SBP1 protein in which the SBP1-C domain had been replaced with a C-terminal domain of similar length from apical membrane antigen 1 (AMA1-C) (Fig. 5), a completely unrelated malaria protein. As previously stated above, when tested *in vitro*, recombinant AMA1-C showed no interaction with IOVs (Fig. 1). When analysed by IFA, parasite clones expressing chimeric SBP1 comprising the N-terminal domain of SBP1 and the C-terminal domain of AMA1 (SBP1–AMA1-C) localised this protein correctly to MCs (Fig. 5B). To our surprise however, when MCs were co-localised with spectrin (Fig. 6A) (as a measure of their association with the RBC membrane skeleton), their distribution in PRBCs was not significantly different to that found in wild type, parental 3D7 parasites (Fig. 6A).

Furthermore, examination by scanning electron microscopy of 30 randomly selected RBCs infected with either 3D7 or SBP1–AMA1-C parasites revealed that SBP1–AMA1-C parasites formed normal membrane knobs on the PRBC surface, both morphologically (Fig. 6C) and numerically (8.72 ± 0.49 knobs/μm^2 for 3D7 *versus* 8.91 ± 0.50 knobs/μm^2 for SBP1–AMA1-C; mean ± SEM), trafficked PfEMP1 normally to the RBC surface as assessed by trypsin-cleavage analysis (Fig. 6E) and their ability to adhere to platelet-expressed CD36 under flow conditions were not significantly different to RBCs infected with wild-type 3D7 parasites (Fig. 6D). Taken together, these results indicate that the C-terminal domain of SBP1 is not required for either correct localisation of MCs at the RBC membrane skeleton, the formation of parasite-induced knobs at the RBC membrane or the delivery to and function of PfEMP1 on the PRBC surface.

Table 1
SBP1-C terminal domain interactions with proteins of the RBC skeleton. Protein–protein interactions were performed using a BIAcore 3000 (BIAcore, Piscataway, NJ, USA). Binding experiments (n = 4 for either protein fragment interacting with SBP1-C) were performed at pH 7.4 in HEPES-buffered saline (150 mM NaCl, 20 mM HEPES) containing 3 mM EDTA and 0.05% (v/v) P20 surfactant. Affinity constants were estimated by curve fitting using BIAeval v3.0 software assuming a 1:1 binding model.

SBP1-C	k_a ($\times 10^4$ m^{-1} s^{-1})	k_d ($\times 10^{-4}$ s^{-1})	K_A ($\times 10^7$ M^{-1})	K_D ($\times 10^{-8}$ M)
4.1 (16 kDa)	0.80 ± 0.55	4.59 ± 0.05	1.73 ± 0.11	5.85 ± 0.46
α-spectrin (α4)	1.61 ± 0.13	3.10 ± 0.42	5.70 ± 1.28	2.04 ± 0.42

4. Discussion

Correct trafficking and display of PfEMP1 on the surface of PRBCs is critical for the extreme virulence of *P. falciparum* malaria parasites and plays a central role in the pathogenesis of falciparum malaria. We and others have previously shown that the correct placement of PfEMP1 on the PRBC surface, clustered over membrane knobs, is dependent on the presence of SBP1, a protein resident in MCs in the cytoplasm of PRBCs [10,11]. However, the precise mechanism by which translocation of PfEMP1 onto the RBC surface occurs has, to the best of our knowledge, never been determined. Analysis of MCs during the intra-erythrocytic development of *P. falciparum* has shown that they are initially highly mobile structures during the early stages of parasite infection, but become stationary and immobilised at the RBC membrane as the parasite matures into pigmented trophozoites [36]. Furthermore, they appear to become anchored directly to the RBC skeleton, *via* an interaction involving either membrane tethers [35,37], novel actin filaments [38,39] or, most likely, a combination of the two. Since, to us, it seemed highly likely that this anchoring of MCs to the RBC membrane skeleton would be a critical requirement for the translocation of PfEMP1 onto the PRBC surface, and that the C-terminal domain of SBP1 is exposed on the surface of MCs, we set out to identify proteins of the RBC membrane skeleton with which the C-terminal domain of SBP1 could interact and whether these interactions were necessary for surface exposure of PfEMP1.

Here, we have been able to show by a number of different approaches that SBP1-C is able to specifically bind to the RBC cytoskeleton *via* both the 16 kDa domain of protein 4.1R and the α4 repeat region of

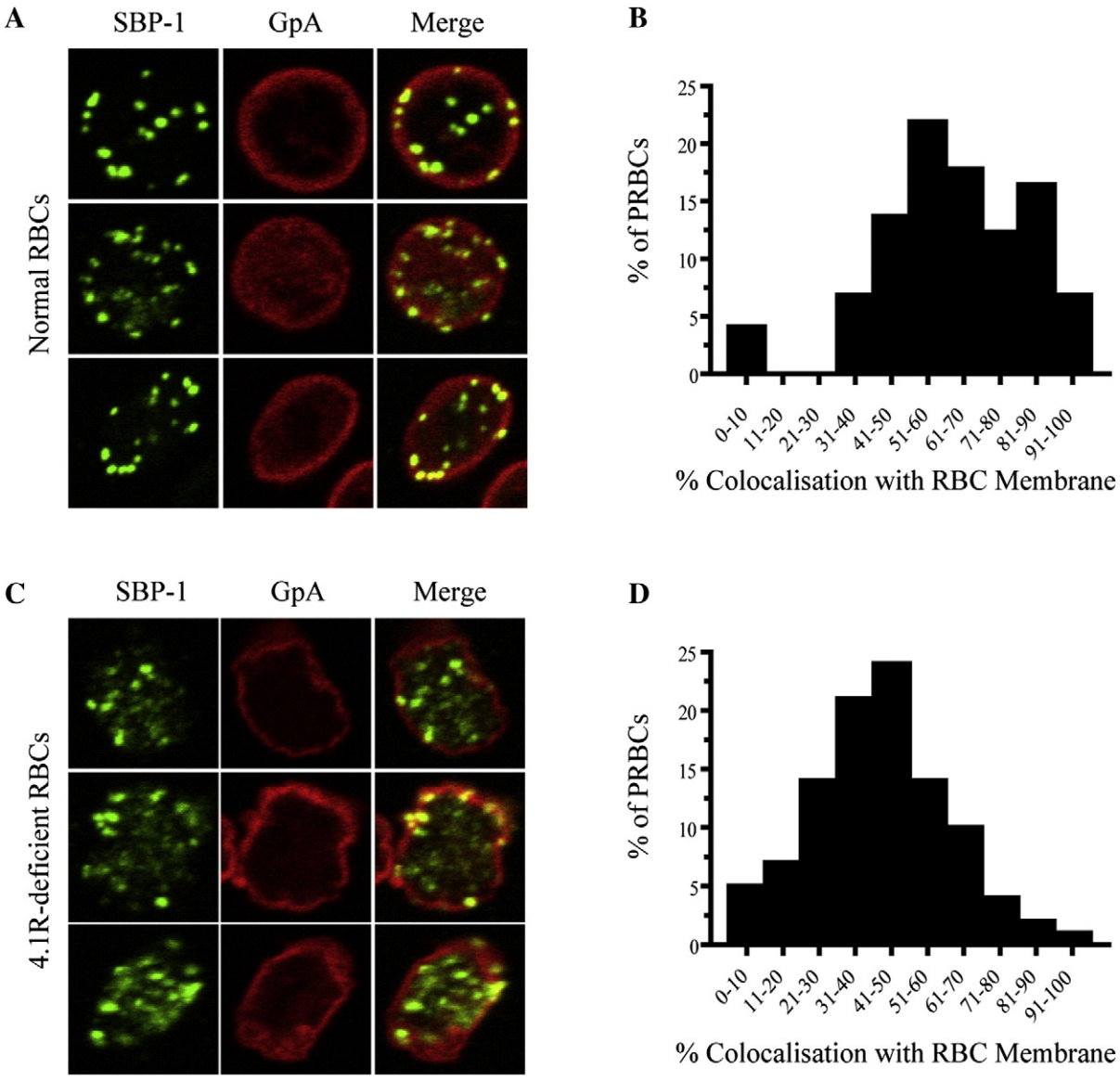

Fig. 4. Localisation of SBP-1 in normal and protein 4.1R-deficient red blood cells. Representative confocal immunofluorescence images of normal (A) and 4.1R-deficient (C) RBCs immunolabelled for Maurer's clefts (SBP1; green) and the RBC membrane skeleton (Glycophorin A, GpA; red). Co-localisation of SBP1 and GpA was analysed using ImageJ and the fraction of MCs (SBP1) co-localised with the RBC membrane skeleton in 73 normal (B) and 102 protein 4.1R-deficient (D) RBCs plotted. A significant shift in the mean co-localisation of SBP1 with GpA is observed between normal and 4.1R-deficient RBCs.

α-spectrin. Importantly, these interactions are highly specific and of sufficient magnitude (K_D ~ 0.02–0.06 μM) to be capable of playing a physiological role *in vivo* [3]. Furthermore, the number of MCs associating with the membrane skeleton in mutant human RBC that lack protein 4.1R, was considerably reduced, suggesting that binding to protein 4.1R by SBP1 likely plays a significant role in MC localisation. To date, a number of exported malaria proteins have been shown to interact directly with the RBC membrane skeleton, including at least 3 (KAHRP, PfEMP3 and RESA) that interact specifically with distinct domains within spectrin, and one (MESA) with protein 4.1R [34]. Considering that both KAHRP and PfEMP3, in addition to SBP1, are required for the correct trafficking and display of functional PfEMP1 on the surface of PRBCs [6,10,11,40], this strengthens the importance of the α4 repeat region of spectrin as playing a highly significant role in PfEMP1 trafficking and PRBC surface display.

To further determine the role of SBP1-C *in vivo*, we attempted to truncate the endogenous SBP1 protein in parasites, but despite multiple attempts, we were unable to generate transgenic parasites lacking only the C-terminal domain of SBP1. This, however, is not unprecedented as the inability to delete short, cytoplasmic C-terminal domains (C-terminal tails) that follow the transmembrane domain from a number of other eukaryotic proteins without altering the normal trafficking of the whole protein [41,42], including those from *P. falciparum* [43] has been described. Since we could not specifically delete SBP1-C, we decided to replace it with a C-terminal domain of similar length from an unrelated malaria protein. We selected the cytoplasmic tail of apical membrane antigen 1 (AMA1-C) since it has no sequence similarity to SBP1-C, nor a previously described function in protein–protein interactions, and its timing of expression and localisation in PRBCs are different from that of SBP1-C [33,44–46]. This SBP1-N–AMA1-C chimeric protein localised to MCs as expected, but interestingly, MCs showed no significant difference in their association with the PRBC membrane skeleton when compared to RBCs infected with wild type parental parasites. This indicated to us that although SBP1-C is capable of interacting with the RBC membrane skeleton, it is not essential for the interaction of MCs with the membrane skeleton in PRBCs. It is therefore possible that this interaction with the RBC cytoskeleton is a redundant function which could be performed by one or other proteins that are resident in MCs and exposed on the

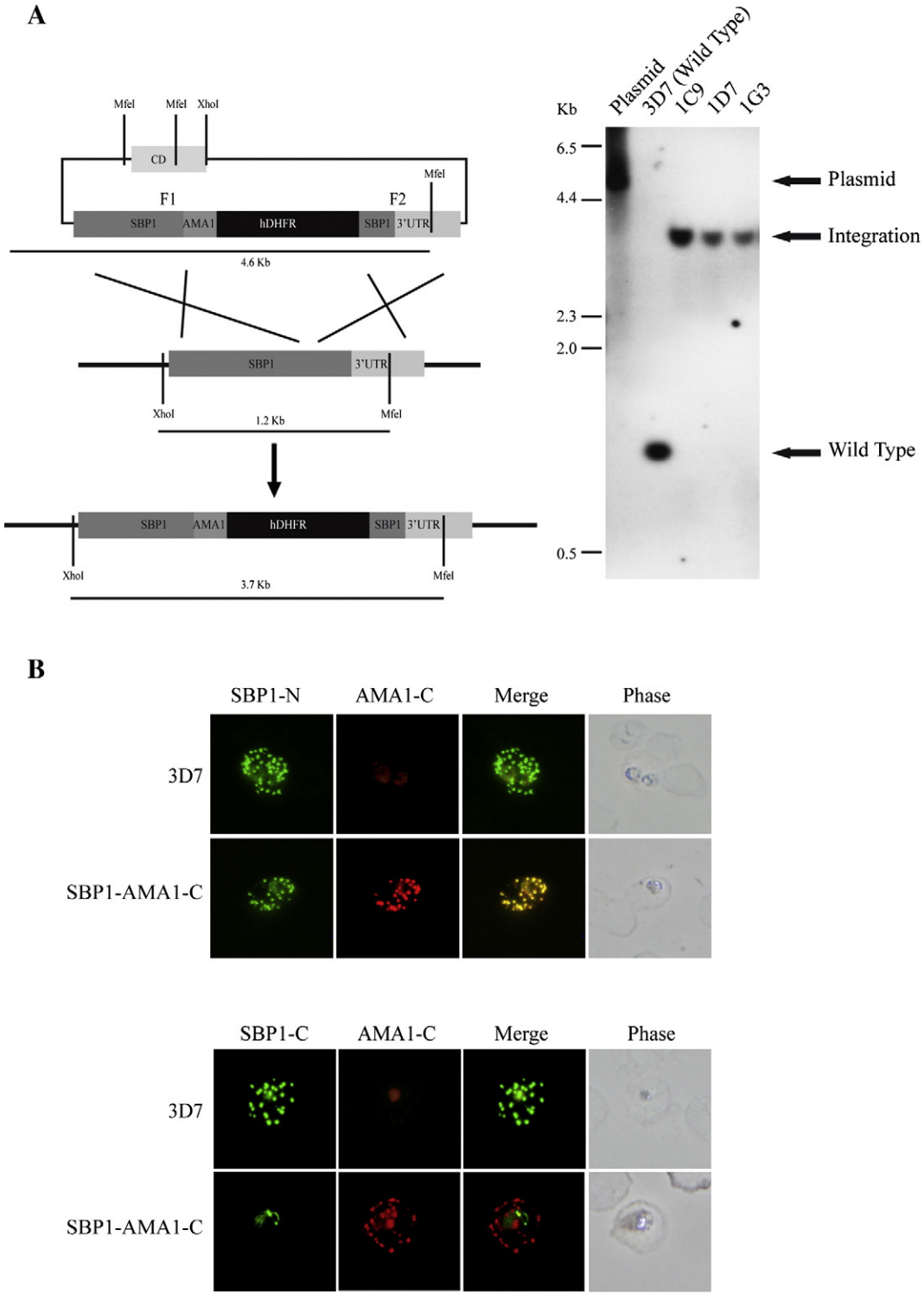

Fig. 5. Localisation of SBP1 C-terminal mutants. A. Southern blot analysis of the SBP1–AMA1-C parasite clones. The left panel is a schematic representation of the expected integration event. The restriction enzymes and the size of the expected bands are indicated. The right panel is the southern blot probed with an SBP1 gene-specific probe. The corresponding bands from the wild type (3D7), transfected plasmid, and integration events in the SBP1–AMA1-C clones (1C9, 1D7 and 1G3) are shown. B. Immunofluorescence analysis of RBCs infected with either normal (3D7) or transgenic parasites expressing a chimeric SBP1 protein in which the C-terminal domain of SBP1 was replaced with the C-terminal domain of *P. falciparum* AMA1 (SBP1–AMA1-C).

cytoplasmic face of the cleft. One other such candidate protein that has been described is REX1 [47]. This is also supported by observations that MCs are attached to the RBC cytoskeleton by membranous tethers that appear to emanate from MCs, but which do not contain SBP1 [35,48]. Additionally, more recent findings suggest that a novel form of actin filaments may also be involved in immobilising MCs, however since MCs can become stationary in the presence of cytochalasin D, which disrupts actin filaments [37] this implies that there are likely to be multiple mechanisms mediating this process in PRBCs. This premise of multiple mechanisms is supported by our findings that in 4.1R-deficient RBCs, MCs were no longer as close to the RBC membrane skeleton as they were in parasitised normal RBCs.

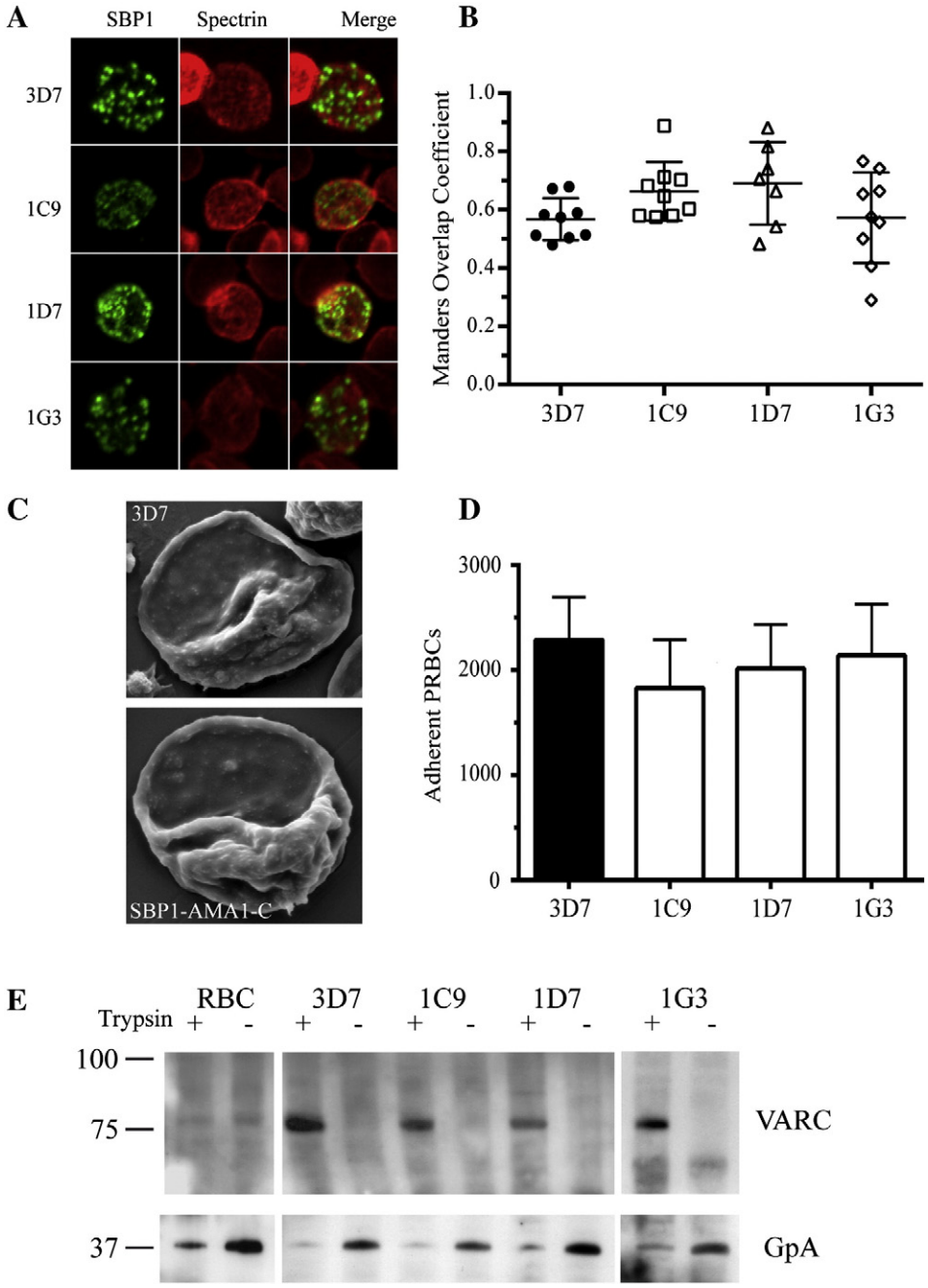

Fig. 6. Functional analysis of the SBP1 C-terminal domain. A. Representative images from immunofluorescence analysis RBCs infected with wild type 3D7 parasites or the three clonal parasite lines for the SBP1–AMA1-C chimeras (1C9, 1D7 and 1G3). Green represents SBP1 and red is spectrin. B. Scatter plot of the co-localisation analysis. Each data point represents the Manders' coefficient calculated from multiple individual images. The horizontal line represents the mean and the error bars are the SEM. C. Representative scanning electron micrographs of RBCs infected with wild type 3D7 (top panel) or SBP1–AMA1-C (bottom panel) parasites showing the similarity in knob number and morphology between the parasite lines. D. The level of adhesion of PRBCs to platelet-expressed CD36 under flow conditions (0.1 Pa). Adherent PRBCs represent the number of 3D7- or SBP1–AMA1-C- (1C9, 1D7 and 1G3) PRBCs that adhered per 10^7 PRBCs perfused through platelet-coated flow chambers. Data represent the mean + SEM for 3 independent experiments. E. Trypsin cleavage assay to determine the surface exposure of PfEMP1 in RBCs infected with SBP1–AMA1-C clonal parasite lines. The cleavage product at 75 kDa detected by anti-VARC (PfEMP1) antibody in wild type 3D7 and all SBP1–AMA1-C clones indicate that trafficking and surface exposure of PfEMP1 is similar for all parasite lines. Trypsin-mediated cleavage of glycophorin A (GpA) was used as a positive control for trypsin cleavage activity.

While the potential importance of protein 4.1R in the anchoring of MCs to the PRBC membrane is intriguing, the difficulty in obtaining large enough quantities of these relatively rare RBCs to perform detailed mechanistic experiments is not currently feasible, so for the time being at least, the precise functional consequences of the interaction between SBP1-C and protein 4.1R remain unknown. Earlier published studies from our laboratory however have demonstrated that the adhesion of parasite-infected protein 4.1R-deficient RBCs to C32 melanoma cells is not different to parasite-infected normal RBCs suggesting that trafficking and transport of PfEMP1 and knob-formation in protein 4.1R-deficient RBCs is the same as in normal RBCs [16]. To determine whether the C-terminal tail of SBP1 was required for the trafficking or surface display of PfEMP1, we looked for changes in both the adhesive properties of the PRBC and the surface localisation of PfEMP1 on the SBP1–AMA1-C chimeric parasite lines. In all 3 clones, there was no difference in the trafficking of PfEMP1 or the ability of the PRBC to adhere. This indicated that while SBP1 was able to bind to the membrane skeleton, this binding was not critical for either the localisation of MCs

or for the overall function of SBP1 in the trafficking and translocation of PfEMP1. This seems to be completely discrepant with the known function of SBP1. In previous studies, by us and others, it was clear that SBP1 is critical for the trafficking of PfEMP1 to the surface of the PRBC [10,11]. It was therefore reasonable to expect that SBP1-C, which is exposed on the cytoplasmic face of MCs and binds the membrane skeleton, would be involved in the trafficking of PfEMP1. However, our finding that MCs are able to localise to the RBC membrane in the absence of SBP1-C and the lack of any obvious PfEMP1 trafficking defects, suggests that the important function of SBP1 is not in the C-terminus but lies within the N-terminal domain of the protein. Further characterisation of this region of SBP1 and determination of its role in PfEMP1 trafficking and transport is undoubtedly an important area for future studies of malaria pathogenesis.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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