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***In vivo* Studies Support the Role of Trafficking and Cytoskeletal Binding Motifs in the Interaction of MESA with the Membrane Skeleton of *Plasmodium falciparum*-Infected Red Blood Cells**

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Abstract

In red blood cells (RBCs) infected with the malaria parasite *Plasmodium falciparum*, a 19-residue region of the mature parasite-infected erythrocyte surface antigen (MESA) associates with RBC cytoskeleton protein 4.1R; an interaction essential for parasite survival. This region in MESA is adjacent to a host targeting motif found in other malaria parasite proteins exported to the membrane skeleton. To demonstrate function of these motifs *in vivo*, regions of MESA fused to a reporter were expressed in malaria parasites. Immunochemical analyses confirmed the requirement for both motifs in the trafficking and interaction of MESA with the cytoskeleton and demonstrates their function *in vivo*.

Keywords

Plasmodium falciparum; malaria; MESA; Pexel; trafficking; cytoskeleton; 4.1R

During the development of *Plasmodium falciparum* inside human red blood cells (RBCs), several parasite proteins associate with the RBC membrane skeleton via a multi-step process that involves transport across the parasitophorous vacuolar membrane (PVM) and the RBC cytosol (reviewed in [1]). It has been shown that the knob-associated histidine-rich protein (KAHRP) and *P. falciparum* erythrocyte membrane protein 3 (PfEMP3) are exported across the PVM using a hydrophobic signal sequence and a highly conserved host targeting (HT) motif, also called the *Plasmodium* export element (Pexel), at the N-terminus [2–4]. Once within the RBC cytoplasm, proteins such as KAHRP, PfEMP3 and the mature parasite-infected erythrocyte surface antigen (MESA) attach themselves to the RBC membrane skeleton, as evidenced by a peripheral location by IFA and Triton X-100 insolubility of the proteins in cell lysates. Considerable effort has been devoted to mapping binding interactions of these proteins with the membrane skeleton using a variety of *in vitro* assays with recombinant proteins [5–13]. For example, Bennett and co-workers bound a series of nested fragments or peptides of

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MESA to inside-out RBCs and mapped the binding domain of MESA to a 19-residue region near the N-terminus of the protein [6] (Fig. 1A). Subsequent studies by Waller and colleagues, using recombinant 4.1R and MESA fragments, identified a 51-residue sequence encoded by exon 10 of the *4.1R* gene as the MESA binding partner and showed that the affinity of the interaction was in the micromolar range [10]. There is no published evidence that this MESA sequence is capable of functioning as a binding domain *in vivo* and no information as to what affinity might be indicative of membrane skeleton binding of malaria parasite proteins within intact RBCs.

To demonstrate that the *in vitro* binding studies have physiological relevance and are predictive of actual behaviour within the cell, we fused N-terminal sub-fragments of MESA to a reporter protein (Fig. 1B). The well-characterised *P. falciparum* S-antigen was chosen as the reporter for these studies as it exists in multiple allelic forms which are antigenically distinct, but are haploid within a particular parasite line [14]. Thus, S-antigen from one strain has an epitope unique to that strain, and can be detected by specific antiserum. The various 5' sub-fragments of the *MESA* gene were amplified using PCR from *P. falciparum* genomic DNA (D10 line). Primers p807 (5'-CCGGAATTCATGGAAAAATATGGAGGTAATTTGTAG) and p808 (5'-TCCCCCGGGTAATAAAAAAACACATATTATAGTGC) were used to amplify the entire exon 1 sequence (containing the embedded hydrophobic signal peptide (SP) sequence). Primers p807 and p812 (5'-TCCCCCGGGCATTACATTCACATGTTTTCTAGG) were used to amplify exon 1, the intron and part of exon 2 (including the Pexel and the 4.1R binding site). Primers p807 and p1869 (5'-TCCCCCGGGTTCATCAAATGTTCTCATTATTTC) were used to amplify exon 1, the intron and part of exon 2 (lacking the 4.1R binding site) (Fig. 1B). The PCR products were inserted into the *Sma*I site upstream of the *S-antigen* gene (lacking its signal peptide sequence) (isolate FC27; GenBank accession no. M10129) in a modified pBluescript vector and then cassetted into the *Xho*I site of the transfection vector pHC1, in the correct orientation with respect to the calmodulin promoter. As a control, the *S-antigen* gene alone (with its endogenous signal peptide sequence) was cloned into pHC1 (Fig. 1B).

Plasmid DNA was prepared using the Plasmid Mega Kit (Qiagen) and used for transfection of ring-stage *P. falciparum* parasites (3D7 clone) according to standard procedures, except using modified electroporation conditions to enhance DNA delivery [15]. Transfected parasites were cultured in the presence of 0.2 μ M pyrimethamine (Sigma) for 30–35 days until parasites were observed in Giemsa-stained smears. The presence of the correct episome in each of the transfectant lines was confirmed by polymerase chain reaction (PCR) (data not shown). Protein expression was subsequently analysed using SDS-PAGE and immunoblotting with rabbit antiserum raised to recombinant S-antigen (isolate FC27). All of the transfectant parasite lines expressed chimeric proteins of the expected molecular mass (Fig. 1C). Wild-type *P. falciparum* 3D7 was used as a negative control to demonstrate that the FC27-specific S-antigen antiserum does not show cross-strain reactivity.

Immunofluorescence assays (IFA) and confocal microscopy were used to determine the cellular location of the expressed chimeric proteins (Fig. 2A). *In vitro* cultures of *P. falciparum* parasites were grown to ~5% parasitaemia as previously described [16]. Thin blood smears were made when the majority of the parasites were at the trophozoite stage and IFA was performed as previously described [17] using rabbit antiserum raised to recombinant S-antigen (isolate FC27). A mouse antibody to RBC membrane protein Glycophorin A (GpA) was used as a positive control for the IFA experiments. The images show that the proteins expressed by control line (3D7_Sag) which contains S-antigen alone (with its endogenous signal peptide) remained confined within the parasite or the PV, and was not exported into the RBC cytosol. The protein expressed by 3D7_SP (expressing exon 1 of MESA fused to S-antigen) was also retained within the PV, indicating that the embedded signal peptide of MESA is not sufficient to transport the protein across the PVM. The image for 3D7_Pexel+4.1BS,

which contains the putative Pexel and the 4.1R binding site, shows that the protein was trafficked outside the parasite and appeared to co-localise with GpA at the RBC membrane. Transfectant parasite line 3D7_Δ4.1BS(lacking the 4.1R binding site) showed a vastly different staining pattern to that of 3D7_Pexel+4.1BS. It appeared that the Δ4.1BS/S-antigen chimera was being transported outside the parasite, indicating that the Pexel is functional and is the minimum sequence required for passage through the PVM into the RBC cytosol. The Δ4.1BS/S-antigen chimera did not co-localise with GpA at the RBC membrane, supporting the hypothesis that the 4.1R binding site is essential for the association between MESA and protein 4.1R at the cytoskeleton. Studies using 4.1R-deficient RBCs have similarly shown that MESA localises to the RBC cytosol but does not interact with the RBC skeleton [18]. The staining observed for 3D7_Δ4.1BS appeared as a punctate pattern in the cytosol, which is similar to that observed for Maurer's cleft (MC)-associated proteins. However, dual labeling IFA experiments using mouse antiserum to MC-resident skeletal binding protein 1 (SBP1) [19] revealed a distinct difference in the staining pattern (data not shown), indicating that the Δ4.1BS/S-antigen chimera was not co-localising with MCs, but rather appeared to be associating with another vesicular structure in the RBC cytosol. The identity of this compartment remains unknown and needs to be further elucidated by additional trafficking, co-localisation and ultrastructural studies.

To determine whether the chimeric proteins expressed in the transfectant parasite lines were associating with the RBC cytoskeleton, a Triton X-100 (TX-100) extraction experiment was performed (Fig. 2B). Proteins which are associated with the RBC cytoskeleton are insoluble in cold (4°C) TX-100 and can then be solubilised in SDS. Using this method, the only parasite line that was found to be expressing a protein associated with the RBC cytoskeleton was 3D7_Pexel+4.1BS, confirming that the 4.1R binding site is essential for correct localisation of MESA. This result was supported by the finding that the protein lacking the 4.1R binding site (Δ4.1BS/S-antigen) was transported to the RBC cytosol but was unable to associate with the cytoskeleton.

The Pexel motif has been shown to be required for the trafficking of several *Plasmodium* proteins across the PVM [2,3]. In this study we have demonstrated that the putative Pexel motif found in MESA is also functional and, along with the embedded signal peptide, is sufficient to direct translocation of a reporter protein across the PVM. However, once in the RBC cytosol, the 19-residue 4.1R binding site was found to be essential for the correct localisation of MESA at the cytoskeleton. It remains a formal possibility that additional sequences present in the constructs may act in concert with the signal peptide, Pexel motif and 4.1R binding site to contribute to the observed localisation, but the close agreement between *in vitro* and *in vivo* binding studies suggest that 4.1R binding, leading to peripheral localisation, is mainly contributed by the previously described 19-residue sequence. Thus, we have demonstrated for the first time that *in vitro* binding experiments are capable of predicting results that are indicative of real interactions occurring within infected RBCs. The precise function of MESA remains unknown, however, studies have shown that interruption of the MESA/4.1R interaction results in accumulation of unbound MESA within the RBC cytosol and causes parasite death [18]. Thus, definition of a functional minimal binding domain involved in a critical protein-protein interaction in infected RBCs may aid the development of novel therapeutics for malaria.

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Fig. 1.

(A) Schematic representation of the two-exon structure of MESA showing the locations of the embedded signal peptide, intron splice site, putative Pexel, 4.1R binding site and the GESKET repeats. Below the schematic is the amino acid sequence of the N-terminal portion of MESA as indicated by the dashed lines. The exon 1 sequence is shaded in grey, the embedded signal peptide is shown in lowercase, the putative Pexel is underlined and the 19-residue 4.1R binding site is boxed. (B) Reporter constructs used for transfection of *P. falciparum* 3D7. The structural features located within the N-terminal sub-fragments of MESA are highlighted as described in part (A) above. The S-antigen sequence (allele from *P. falciparum* isolate FC27) is shaded in grey. Control plasmid Sag contains S-antigen alone (with its endogenous signal peptide).

Plasmid SP contains the first exon of MESA (amino acids 1 to 50), which encodes an embedded hydrophobic signal peptide, fused to the S-antigen reporter. Plasmid Pexel+4.1BS contains the signal peptide, putative Pexel and 4.1R binding site of MESA (amino acids 1 to 204) fused to the S-antigen reporter. Plasmid Δ 4.1BS contains the signal peptide and putative Pexel of MESA (amino acids 1 to 95) fused to the S-antigen reporter. (C) Percoll purified transfectant parasite lines were resolved on SDS-PAGE (under denaturing conditions) and transferred to polyvinylidene fluoride (PVDF) membranes (NEN) for immunoblotting as previously described [17]. Polyclonal antiserum raised in rabbits against S-antigen (isolate FC27) was used as the primary antibody (1:1000 dilution). The primary antibody was detected using anti-rabbit immunoglobulin (Ig) conjugated to horseradish peroxidase (Silenus) (1:2000 dilution) and developed as previously described [17]. Lane 1, wild-type 3D7 parasites; lane 2, 3D7_Sag parasites; lane 3, 3D7_SP parasites; lane 4, 3D7_Pexel+4.1BS parasites; lane 5, 3D7_ Δ 4.1BS parasites.

Fig. 2.

(A) Immunofluorescence (IFA) analysis of the transfectant parasite lines using rabbit anti-S-antigen (isolate FC27) (1:500 dilution) and mouse anti-Glycophorin A (GpA) (Caltag Laboratories) (1:100 dilution) as primary antibodies. Reactivity was detected using Alexa Fluor 488-conjugated anti-rabbit Ig and Alexa Fluor 568-conjugated anti-mouse Ig as secondary antibodies (Invitrogen) (1:400 dilution). Smears from asynchronous parasite cultures were examined by confocal microscopy (Olympus FluoView FV1000). **(B)** Triton X-100 (TX-100) solubilisation to determine association with the RBC cytoskeleton. Asynchronous *P. falciparum* parasites were cultured to ~6% parasitemia and harvested using 0.15% (w/v) saponin and washed with PBS. The purified parasites were solubilised in cold (4°C) TX-100

and the resulting insoluble material was then solubilised in SDS. The samples include the total parasite material (T), the TX-100 soluble material (S) and the TX-100 insoluble, SDS soluble material (P). The samples were then resolved (under denaturing conditions) on SDS-PAGE and transferred to PVDF membranes (NEN) for immunoblotting as previously described [17]. Polyclonal antiserum raised in rabbits against S-antigen (strain FC27) was used as the primary antibody (1:1000 dilution). The primary antibody was detected using anti-rabbit Ig conjugated to horseradish peroxidase (Silenus) (1:2000 dilution) and developed as previously described [17].