Skeleton binding protein 1 (SBP1) of Plasmodium falciparum accumulates in electron-dense material before passing through the parasitophorous vacuole membrane

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Skeleton binding protein 1 (SBP1) of *Plasmodium falciparum* accumulates in electron-dense material before passing through the parasitophorous vacuole membrane

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Abbreviations: PVM, parasitophorous vacuole membrane. PV, parasitophorous vacuole. PPM, parasite plasma membrane. SBP1, Skeleton binding protein 1. EDM, electron-dense material.

Abstract

Plasmodium falciparum proteins involved in vascular endothelial cell adherence are transported to the surface of infected erythrocytes. These proteins are exported through parasite-derived membrane structures within the erythrocyte cytoplasm called Maurer's clefts. Skeleton binding protein 1 (SBP1) is localized in the Maurer's clefts and plays an important role in transporting molecules to the surface of infected erythrocytes. Details of the translocation pathway are unclear and in this study we focused on the subcellular localization of SBP1 at an early intraerythrocytic stage. We performed immunoelectron microscopy using specific anti-SBP1 antibodies generated by immunization with recombinant SBP1 of *P. falciparum*. At the early trophozoite (ring form) stage, SBP1 was detected within an electron dense material (EDM) found in the parasite cytoplasm and in the parasitophorous vacuolar (PV) space. These findings demonstrate that SBP1 accumulates in EDM in the early trophozoite cytoplasm and is transported to the PV space before translocation to the Maurer's clefts formed in the erythrocyte cytoplasm.

Keywords: malaria, *Plasmodium falciparum,* SBP1, electron dense material,

parasitophorous vacuole membrane, Maurer's clefts.

During intraerythrocytic stage development malaria parasites undergo repeated cycles of invasion, proliferation, and egress from host erythrocytes. The most virulent species of malaria parasites, *P. falciparum,* exports various proteins to the host erythrocyte to facilitate nutrient acquisition and adhesion to the vascular endothelium [1,2]. These parasite proteins are firstly exported from the parasite endoplasmic reticulum across a lipid bilayer membrane referred to as the parasitophorous vacuole membrane (PVM) [3]. Maurer's clefts, parasite-derived membrane structures in the erythrocyte cytoplasm, are thought to function as a later sorting compartment between the parasite PVM and the host erythrocyte membrane [4]. Live fluorescence microscopy of green fluorescent protein (GFP)-tagged Maurer's cleft proteins, membrane associated histidine-rich protein 1 (MAHRP1), and ring-exported protein 1 (REX1) indicated that Maurer's clefts are formed at the ring stage [5, 6]. Another Maurer's cleft resident protein, the transmembrane skeleton binding protein 1 (SBP1) [7], is essential for trafficking of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) to the

54 erythrocyte surface, which is required for adhesion of infected erythrocytes to the
55 vascular endothelium [8, 9]. It is not clear how SBP1 is transported from the parasite
56 cytoplasm and beyond the PVM to Maurer's clefts. In this study, immunoelectron
57 microscopy was performed focusing on SBP1 localization during early intraerythrocytic
58 stage parasite development.

59 To characterize SBP1, we generated recombinant SBP1 protein corresponding
60 to the C-terminal region after the transmembrane domain [7] fused with a GST tag at
61 the N-terminus as described [10]. Briefly, a DNA fragment encoding SBP1 (amino acid
62 positions 259–337) was amplified by PCR from cDNA, obtained from intraerythrocytic
63 stage *P. falciparum* 3D7 strain parasites, using sense primers tailed with an XhoI site
64 and antisense primers tailed with a NotI restriction site (SBP1-F1: 5'-
65 ctcgagCAAAACCCAGAACAAAACCCAGAAC-3', SBP1-R1:
66 5'-gcggccgcCTATTAGGTTTCTCTAGCAACTGTTTTTG-3'). The amplified DNA
67 fragment was cloned into the pEU-E01-GST-TEV-N2 plasmid (CellFree Sciences,
68 Matsuyama, Japan) at XhoI and NotI restriction enzyme recognition sites. Recombinant
69 SBP1 protein (rSBP1) was expressed using the wheat germ cell-free protein synthesis

system (CellFree Sciences). rSBP1 was affinity-purified using a glutathione-Sepharose 4B column (GE Healthcare, Camarillo, CA, USA) [10]. As shown in Fig. 1A, GFP-fused rSBP1 was eluted as a major protein at approximately 45 kDa.

To generate anti-SBP1 antisera, a Japanese white rabbit was immunized three times with 250 µg of rSBP1 (Kitayama labes Co. Ltd. Ina, Japan) as described [11]. The specificity of anti-SBP1 polyclonal antibodies (diluted 1:500) was examined by Western blotting analysis using approximately 1 x 10^6 trophozoites/schizonts stage parasites derived from the *P. falciparum* 3D7 strain that were cultured and maintained as described [12]. Anti-SBP1 antibodies detected a double band at approximately 42 kDa, which is consistent with the predicted molecular weight and a report that SBP1 is phosphorylated post-translationally [13] under reducing and non-reducing conditions (Fig. 1B). By IFA using anti-SBP1 antibodies (diluted 1:1000) signal was detected as a patchy pattern in the cytoplasm of erythrocytes parasitized with mature trophozoites (Fig. 1C). To determine the precise localization of SBP1, infected erythrocytes were fixed with 1% formaldehyde and 0.2% glutaraldehyde in HEPES buffered solution and embedded in LR White resin (Polysciences, Warrington, PA). Ultrathin sections were

immuno-stained and examined using a transmission electron microscope (JEM-1230; JEOL, Tokyo, Japan) [14]. Gold particles indicated SBP1 accumulation in Maurer's clefts in the cytoplasm of infected erythrocytes (Fig. 1D) [7]. Taken together, it was confirmed that anti-SBP1 antibodies specifically recognize parasite SBP1.

We focused on changes in the subcellular localization of SBP1 during the early phase of intraerythrocytic stage of malaria parasite infection. Samples from synchronized culture samples were fixed for IEM and their developmental stage determined by microscopy of Giemsa stained blood smears on glass slides. In the early trophozoite (ring form) cultured for 8-11 hours after invasion, the accumulation of a material with high electron density was observed in the parasite cytoplasm, within which gold particles were deposited indicating the localization of SBP1 (Fig. 2A). Similar electron dense material (EDM) containing SBP1 was observed in the PV space between the parasite plasma membrane (PPM) and PVM (Fig. 2B, 2C). PVM in contact with EDM containing SBP1 was observed to invaginate into the cytoplasm of the early trophozoite infected erythrocyte (Fig. 2D). Gold particles were also detected within a membranous cleft formed in the erythrocyte cytoplasm in the vicinity of PVM

and having the appearance of a newly formed Maurer's cleft (Fig. 2E). EDM with similar morphological features were observed just below the erythrocyte membrane at the knob structure, around the Maurer's clefts in the erythrocyte cytoplasm, and in the PV space between the PVM and PPM. It was proposed that these EDM could be responsible for the molecular transport from the parasite to the surface of infected erythrocytes via Maurer's clefts [5, 15]. However, a detailed molecular analysis of the relationship between the formation of Maurer's clefts and EDM has only recently been performed. Studies using the semi-conserved head structure of PfEMP1 fused with GFP (mini-PfEMP1-GFP) have reported that the protein accumulates in electron-dense bulging regions of the PV between the PPM and PVM [16]. Proteins containing *Plasmodium* export elements (PEXEL), such as *P. falciparum* erythrocyte membrane protein 3 (PfEMP3) and knob-associated histidine-rich protein (KAHRP), are cleaved by an aspartyl protease (plasmepsin V) and transported to the PV via the secretory pathway [17]. These proteins are then translocated across the PVM via the *Plasmodium* translocon of exported proteins (PTEX) [18, 19]. Although many PEXEL-negative exported proteins, such as PfEMP1 and SBP1, are known to translocate from the PV

118 across the PVM to the cytoplasm of infected erythrocytes, the molecular mechanism has
119 not been clarified.

120 SBP1 is inserted into the Maurer's cleft with the N-terminus facing the lumen
121 side of the cleft and the C-terminus facing the erythrocyte cytoplasm [20]. In this study
122 IEM showed that gold particles conjugated with antibodies against the C-terminus of
123 SBP1 accumulate inside the EDM of the PV space (Fig. 2D) and outside the Maurer's
124 clefts near the PVM (Fig. 2E). These findings suggest that SBP1 initially accumulates in
125 EDM and then is arranged with the C-terminus exposed to the erythrocyte cytoplasm
126 during Maurer's cleft formation.

127 In this study, we clearly demonstrated that SBP1 is associated with EDM
128 formed in the cytoplasm of early trophozoites and is transported into the PV space.
129 SBP1 is then localized to the EDM closely contacted with the PVM protruding into the
130 cytoplasm of infected erythrocytes prior to be delivered to Maurer's cleft in the
131 erythrocyte cytoplasm. The molecular mechanisms of how parasite molecules
132 accumulated in EDM translocate from PVM to the newly formed Maurer's cleft need to
133 be elucidated.

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150

151 **Figure legends**

152 Figure 1. (A) SDS-PAGE analysis of proteins expressed by the wheat germ cell-free
153 system. Protein mixtures were separated by 12.5% SDS-polyacrylamide gel
154 electrophoresis (SDS-PAGE) under reducing conditions and stained with Coomassie
155 brilliant blue. The samples were as follows: protein molecular weight marker (lane M),
156 total reaction mixture in the wheat germ cell-free system (lane 1), and affinity-purified
157 proteins (lanes 2). Arrowhead, SBP1-GST. (B) Western blot analysis using antibodies
158 against SBP1. Proteins from approximately 1 x 10^6 parasites per lane were separated by
159 SDS-PAGE under reducing (lanes 1) and non-reducing (lanes 2) conditions. Arrowhead,
160 SBP1. (C) Subcellular localization of SBP1 in *P. falciparum* infected erythrocytes.
161 Acetone-fixed trophozoites were incubated with rabbit anti-SPB1 antibodies (green) and
162 Hoechst 33342 (blue). DIC, differential interference contrast microscopy image.
163 Merged: SBP1, Hoechst 33342, and DIC. Bar, 5 μm. (D) Subcellular localization of
164 SBP1 in *P. falciparum* mature trophozoite infected erythrocytes by immunoelectron
165 microscopy. Arrowhead, Maurer's cleft. Bar, 500 nm.

Figure 2. Subcellular localization of SBP1 by immunoelectron microscopy using sections of early phase *P. falciparum* erythrocytic stage development parasites. (A) Gold particles indicating the localization of SBP1 are observed in the electron dense material (EDM) in the cytoplasm of an early trophozoite. Inset: larger magnification image of EDM area. Bar, 500 nm. (B) Two EDMs where SBP1 is localized are observed in contact with PPM (Arrowhead). Bar, 500 nm. (C) Gold particles showing deposition of SBP1 are observed in EDM located between PPM and PVM (Arrowhead). Bar, 500 nm. (D) PVM in contact with the EDM is invaginating toward the cytoplasmic side of the trophozoite infected erythrocyte. Inset: larger magnification image of EDM. Bar, 500 nm. (E) Gold particles are deposited on a newly formed Maurer's cleft located adjacent to PVM. Bar, 500 nm.

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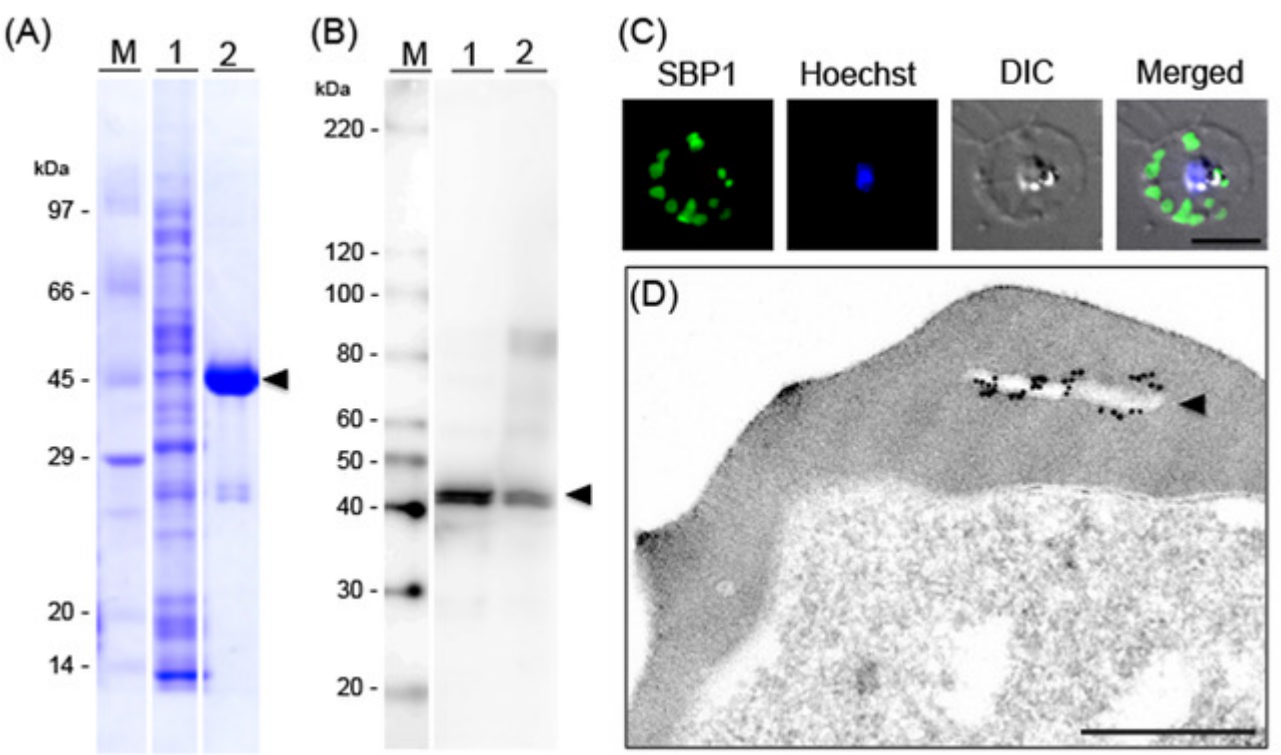
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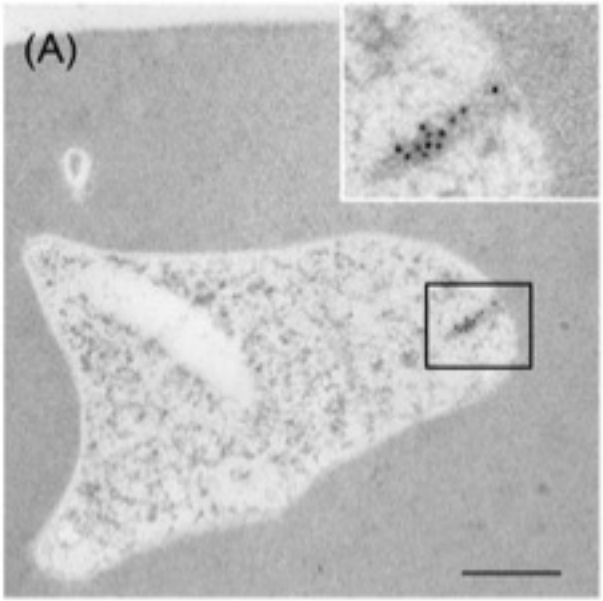
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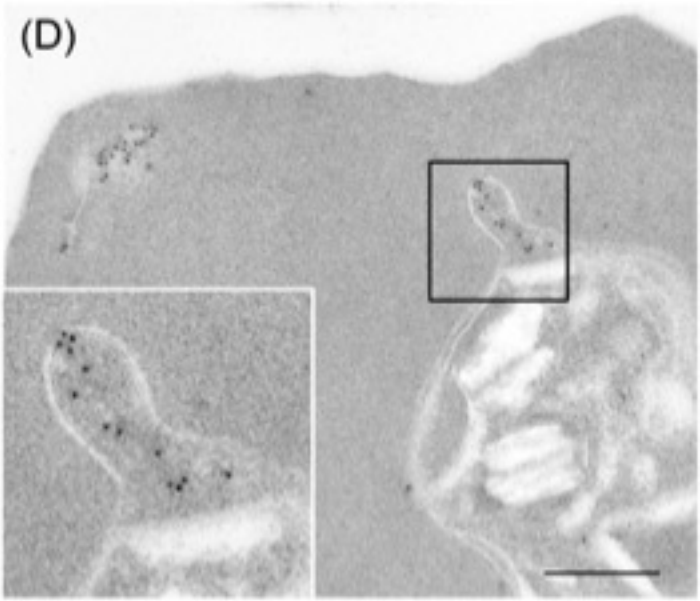
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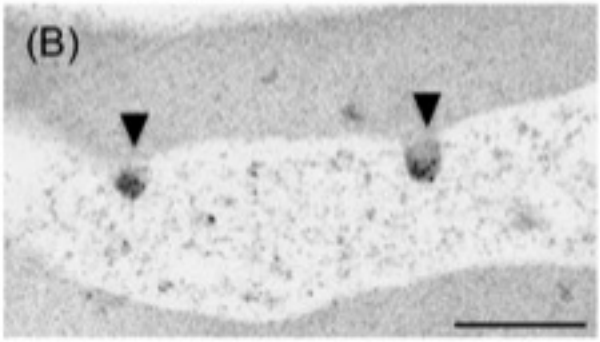
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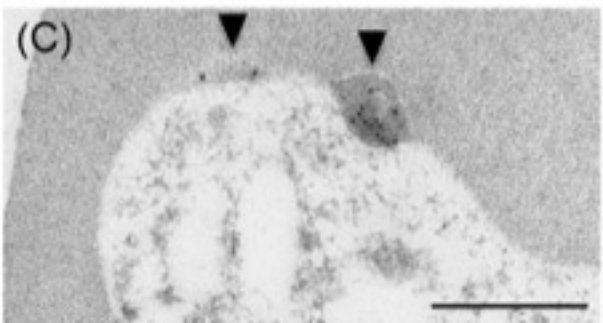
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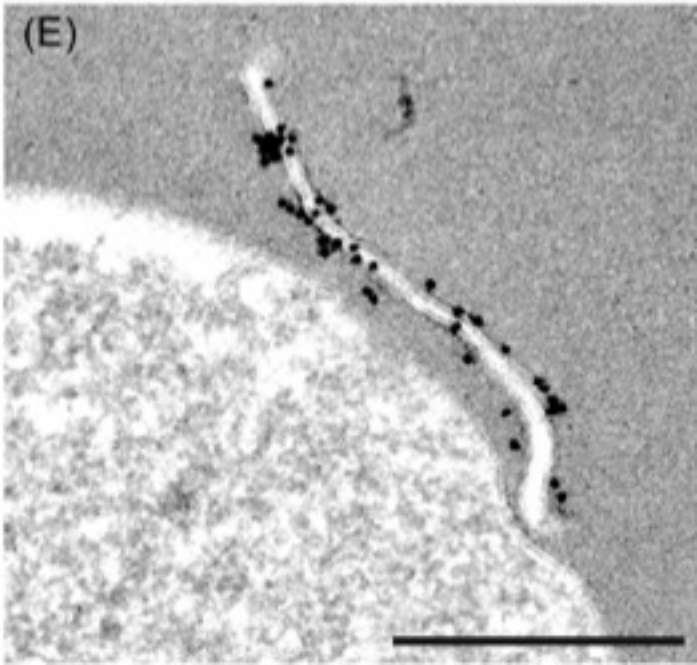
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