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(Degree)

博士 (保健学)

(Date of Degree)

2024-03-25

(Date of Publication)

2025-03-01

(Resource Type)

doctoral thesis

(Report Number)

甲第8920号

(URL)

<https://hdl.handle.net/20.500.14094/0100490145>

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博 士 論 文

Skeleton binding protein 1 localizes to the Maurer's cleft and interacts with PfHSP70-1 and PfHSP70-x in *Plasmodium falciparum* gametocyte-infected erythrocytes

(熱帯熱マラリア原虫の生殖母体期において Skeleton binding protein 1 は Maurer's cleft に局在し、PfHSP70-1 および PfHSP70-x と相互作用する)

令和 6 年 1 月 10 日

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Abstract

Plasmodium falciparum accounts for the majority of malaria deaths, due to pathology provoked by the ability of infected erythrocytes to adhere to vascular endothelium within deep tissues. The parasite recognizes endothelium by trafficking and displaying protein ligands on the surface of asexual stage infected erythrocytes, such as members of the large family of pathogenic proteins, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). Parasite-encoded skeleton binding protein 1 (SBP1) plays an important role in the transport of these binding-related surface proteins, via cleft-like membranous structures termed Maurer's clefts, which are present within the cytoplasm of infected erythrocytes. Erythrocytes infected with gametocyte stages accumulate in the extravascular compartment of bone marrow; and it was suggested that their surface-expressed adhesion molecule profile and protein trafficking mechanisms might differ from those in asexual stage parasites. Protein trafficking mechanisms via Maurer's clefts have been well investigated in asexual stage parasite-infected erythrocytes; but little is known regarding the gametocyte stages. In this study, we characterized SBP1 during gametocyte maturation and demonstrated that SBP1 is expressed and localizes to dot-like Maurer's cleft structures in the cytoplasm of gametocyte-infected erythrocytes. Co-immunoprecipitation and mass spectrometry assays indicated that SBP1 interacts with the molecular chaperones PfHSP70-1 and PfHSP70-x. Localization analysis suggested that some PfHSP70-1 and/or PfHSP70-x localize in a dot-like pattern within the cytoplasm of immature gametocyte-infected erythrocytes. These findings suggest that SBP1 may interact with HSP70 chaperones in the infected erythrocyte cytoplasm during the immature gametocyte stages.

Keywords; *Plasmodium falciparum*, gametocyte, SBP1, PfHSP70-1, PfHSP70-x

Abbreviations: SBP1: skeleton binding protein 1, PfEMP1: *P. falciparum* erythrocyte membrane protein 1, PV: parasitophorous vacuole, PVM: parasitophorous vacuole membrane, IFA: indirect immunofluorescence analysis, IEM: immunoelectron microscopy, co-IP: co-immunoprecipitation, LC/MS/MS: liquid chromatography tandem mass spectrometry.

1. Introduction

Malaria is an infectious disease that is endemic in tropical areas of the world and remains a serious threat to humans, with approximately 608,000 deaths reported in 2022 [1]. During the parasite asexual cycle, merozoites invade host erythrocytes and develop into trophozoites and schizonts; which, after maturation, release invasive merozoites to continue proliferation. *Plasmodium falciparum* accounts for the majority of malaria deaths; in part due to the adhesion of infected erythrocytes to vascular endothelium at various sites in the body, which in turn causes severe symptoms such as cerebral and placental malarias. Host tissue recognition and binding are mediated by parasite-encoded adhesive proteins; such as members of the broad family of erythrocyte surface-displayed receptors, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) [2]. Following the invasion of erythrocytes by merozoites, the parasite develops within a parasitophorous vacuole; and PfEMP1 is exported into the host erythrocyte cytoplasm via a translocon within the parasitophorous vacuole membrane (PVM) [3–5]. PfEMP1 is subsequently transported as a soluble chaperone complex [6,7]; and is inserted into the membrane bilayer [8]. There, PfEMP1 is repackaged for transport via vesicles to the erythrocyte surface and is ultimately displayed as a receptor within knob-like structures, which are a major erythrocyte cell surface modification involved in adhesion of asexual stage parasites to endothelial cells [9]. Skeleton binding protein 1 (SBP1) and membrane-associated histidine-rich protein 1 (MAHRP1) were identified as essential proteins for PfEMP1 trafficking to the erythrocyte surface [10–13]. SBP1 is a trans-membrane protein which is located within the infected-erythrocyte cytoplasm in parasite-induced membranous structures known as Maurer's clefts [14,15]. SBP1 is likely involved in the intracellular distribution of Maurer's clefts, because the location of Maurer's clefts within infected erythrocytes is altered in *sbp1* gene-deficient parasites [10]. It has also been shown that the Hsp70/Hsp40 chaperon/co-chaperon complex is involved, although is not essential, in transporting molecules such as PfEMP1 to infected erythrocytes [16,17].

Our understanding of parasite-encoded adhesion molecules, their display on the erythrocyte surface, and their binding to vascular endothelium has accumulated through studies with asexual stage parasites. Erythrocytes that are infected with gametocytes have morphologies distinct from asexual stage parasites; and, during early gametocyte

development, accumulate in the extravascular compartment of the bone marrow [18,19]. Immature gametocytes lack erythrocyte surface knobs and express little, if any, PfEMP1 [20]. Such differences in gene expression and host cell surface modifications might explain the inability of stage I gametocytes to bind the same endothelial cell receptors recognized by the asexual parasite [21]. Therefore, we hypothesized that differences in the sequestration of asexual versus gametocyte stage infected erythrocytes may be due to changes in adhesion-related molecules transported through the Maurer's cleft and other structures. However, there is limited information about protein transport through Maurer's clefts in gametocytes. In this study, we focused on SBP1, which plays a role in the transport of adhesion molecules in asexual stage parasites, and demonstrated that SBP1 is exported to Maurer's clefts during gametocyte development. We then attempted to identify molecules that interact with SBP1 in immature gametocyte-infected erythrocytes, to elucidate the molecular mechanism of SBP1 transport via Maurer's clefts in these stages.

2. Materials and methods

2.1. *In vitro* cultivation of *P. falciparum*

P. falciparum NF54 strain parasites were obtained from the National Institute of Allergy and Infectious Diseases (NIAID), NIH. Human erythrocytes (blood type O+) and plasma (blood type A+, B+) were provided by the Japanese Red Cross Society (Approval number: 27J0018). NF54 strain asexual stages were cultivated at 37°C under 5% O₂, 5% CO₂, and 90% N₂ in O+ human erythrocytes at 2% hematocrit in RPMI 1640 medium (Life Technologies Corporation, NY, USA) supplemented with 25 mM HEPES (Sigma-Aldrich, MO, USA), 50 µg/mL hypoxanthine (Sigma-Aldrich), 2 g/L NaHCO₃ (Gibco, NY, USA), 0.5% AlbuMAX II (Thermo Fisher Scientific, Waltham, MA, USA), and 7.5% human serum.

Gametocyte stage development was induced in asexual blood stage parasite cultures having parasitemias of more than 10% and consisting mainly of ring stage parasites; by addition to the culture medium of 50 mM N-acetylglucosamine (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) for the first 3 days [22] or 20 U/mL heparin (Nipro,

Tokyo, Japan) for the entire gametocyte culture period [23]. Gametocytes were cultured in conditions similar to the conditions used for the asexual stages. After the addition of N-acetylglucosamine or heparin on day 0, asexual infected erythrocytes were eliminated by continuing the cultures for one round of the asexual life cycle, and immature gametocyte-infected erythrocytes were collected on day 3. Mature gametocyte-infected erythrocytes were collected on day 11 [24].

2.2. Antisera production

Antiserum against a molecular marker of *P. falciparum* gametocytes, Pfs16 (parasitophorous vacuole membrane protein S16) [25,26], was prepared as follows. A DNA fragment encoding full-length Pfs16 (amino acid positions [aa] 1–157, Fig. 1A) was amplified by PCR from Pf3D7 blood-stage cDNA, using the primer pairs Pfs16-F1 (5'-ccaccaccaccaccaATGAATATTCGAAAGTTCATACCATC-3') and Pfs16-R1 (5'-TTAAGAATCATCTCCTTCGTCTCCTTC-3'). The amplified *pfs16* DNA fragment was inserted into a TOPO TA-cloning vector (Invitrogen, Carlsbad, CA), then reamplified using the primer pairs pEU_E01His_S1_XhoI (5'-gagactcgagCACCCACCACCACCAATG-3') and pEU_E01His_S1_NotI_Rev (5'-gagagagagcggccgcGAAACAGCTATGACCATGATTACG-3'), and cloned into the plasmid pEU-E01-GST-TEV-N2 (CellFree Sciences, Matsuyama, Japan) at the restriction sites, XhoI and NotI. Recombinant Pfs16 protein was tagged by glutathione S-transferase (GST) at the N-terminus (rGST-Pfs16), and was expressed using a wheat germ cell-free protein synthesis system (CellFree Sciences). rGST-Pfs16 was affinity purified by passage over a glutathione-Sepharose 4B column (GE Healthcare, Camarillo, CA, USA) and eluted with elution buffer (40 mM reduced glutathione, 50 mM Tris-HCl, 300 mM NaCl, 200 mM imidazole, 2% glycerol, pH 8.0). After separation of eluted proteins by SDS-PAGE, rGST-Pfs16 protein had a molecular weight of approximately 42 kDa, which is the expected size of full-length Pfs16 (16 kDa) fused with a GST-tag (26 kDa) (Fig. 1B; arrowhead). Two smaller molecular weight proteins between 20 to 29 kDa are C-terminally truncated rGST-Pfs16. To generate antiserum against rPfs16, BALB/c mice were immunized intraperitoneally with 20 µg of affinity-purified rGST-Pfs16 with Freund's complete adjuvant, followed by two immunizations at 3-week intervals using 20

μg of the recombinant protein with Freund's incomplete adjuvant. Antisera was collected 14 days after the last immunization (Kitayama Labes Co. Ltd., Ina, Japan). The experiments reported here were conducted in accordance with current Japanese law. The animal work was conducted by Kitayama Labes in compliance with the guidelines based on “Charter for Laboratory Animal Welfare” (Japanese Society for Laboratory Animal Resources).

The method for producing the SBP1 (Pf3D7_0501300) antibody has been reported [14]. Briefly, recombinant SBP1 (rSBP1) protein was generated corresponding to the C-terminal region after the transmembrane domain [15], and was expressed using the above wheat germ cell-free protein synthesis system. To generate anti-SBP1 antisera, a rabbit was immunized with rSBP1 (Kitayama Labes Co. Ltd.) as described [27]. To increase the specificity of the antibody, the anti-SBP1 antisera was affinity purified using rSBP1.

2.3. Western blotting analysis

Asexual, immature gametocyte (on day 3 of gametocyte cultivation), and mature gametocyte (on day 11 of gametocyte cultivation) stage parasites were purified using Percoll-sorbitol gradients [22]. The infected erythrocytes were lysed with 0.1% saponin in PBS-PI (PBS containing Protease Inhibitor Cocktail Set I; Fujifilm Wako Chemicals) for 8 minutes and centrifuged at 15,000×g for 10 minutes at 4°C. The parasite pellets were washed twice with ice-cold PBS-PI, and then lysed in Pierce IP Lysis Buffer (Thermo Fisher Scientific) and centrifuged at 17,360×g for 10 minutes at 4°C. The supernatants were collected and stored at -80°C until further use.

The parasite proteins were subjected to electrophoresis under reducing and non-reducing conditions on a 12.5% polyacrylamide gel (ATTO, Tokyo, Japan); and then transferred to a PVDF membrane (ATTO) and incubated with mouse anti-Pfs16 antibody (1:3000) or rabbit anti-SBP1 antibody (1:2000 [14]). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG (1:3000; Invitrogen, OR, USA) were used as secondary antibodies. Bands were visualized with Immobilon Western Chemiluminescent HRP substrate (EMD Millipore Corp., MA, USA) and images were captured by a Chemiluminescence Imaging System FUSION SOLO.6S.EDGE (M&S Instruments Inc., Osaka, Japan). To determine gametocyte protein-specific loading per lane, the PVDF

membranes which were immunostained with anti-SBP1 were de-probed by incubation in Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific) at 37°C for 1 hour. Subsequently, they were washed 3 times for 5 minutes with PBS containing 0.01% Tween-20, and then the membranes were re-stained with anti-Pfs16 as a gametocyte marker and loading control.

2.4. Indirect immunofluorescence analysis

Thin smears of infected erythrocyte were prepared on glass slides, fixed in cold acetone for 2 min, and were blocked with 5% skim milk for 1 hour at 37°C. The slides were then incubated for 1 hour at 37°C with primary antibodies; specifically, rabbit anti-SBP1 antibody (1:2000 [14]), mouse anti-Pfs16 antibody (1:2000-1:4000), and rabbit anti-PfHSP70 antibody (1:100; StressMarq Biosciences, BC, Canada). The blood smears were then incubated for 1 hour at 37°C with Alexa fluor 488- or 555-conjugated goat anti-mouse or rabbit IgG secondary antibodies (1:500; Thermo Fisher Scientific) and 1 µg/mL Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) or 2 µg/mL 4',6-diamidino-2-phenylindole (DAPI). After mounting with ProLong Gold antifade reagent (Thermo Fisher Scientific), the samples were observed with a fluorescence microscope (Axio Vert.A1; Carl Zeiss, Oberkochen, Germany), and images were acquired with an DP74 Microscope Digital Camera and cellSens Imaging Software (Evident Corporation, Tokyo, Japan).

2.5. Immunoelectron microscopy

In vitro cultured gametocytes were fixed with 1% paraformaldehyde and 0.2% glutaraldehyde in HEPES-buffered saline (pH 7.05) and embedded in LR white resin (Polysciences, Warrington, PA, USA). Ultrathin sections were blocked for 30 min in 0.1 M PBS containing 5% skim milk and 0.01% Tween 20 (PBS milk-Tween), followed by overnight incubation with rabbit anti-SBP1 antibody (1:100 [14]) and mouse anti-Pfs16 antibody (1:100) in PBS milk-Tween. After washing with PBS containing 1% BlockAce (DS Pharma Biomedical, Tokyo, Japan) and 0.01% Tween 20, the samples were incubated for 1 hour in PBS milk-Tween containing goat anti-mouse IgG(H+L) conjugated to 10 nm of gold particles and goat anti-rabbit IgG(H+L) conjugated to 15 nm of gold particles

(BBI International, Minneapolis, MN, USA). The grids were then rinsed with distilled water, dried, and stained with 2% uranyl acetate in 50% methanol and lead citrate. The samples were examined with a transmission electron microscope (HT-7000; Hitachi, Tokyo, Japan).

2.6. Co-immunoprecipitation

Immature gametocyte-infected erythrocytes on day 3 of gametocyte cultivation were treated with 0.1% saponin in PBS-PI for 8 min and centrifuged at $15,000\times g$ for 10 min at 4°C. The parasite pellets were then washed twice with ice-cold PBS-PI and stored at -80°C until further use. Parasite pellets corresponding to approximately 10^7 infected erythrocytes were lysed in 1 mL of Pierce IP Lysis Buffer (Thermo Fisher Scientific) and centrifuged at $17,360\times g$ for 10 min at 4°C. Extracted proteins were used for co-immunoprecipitation (co-IP) using the SBP1 antibody or normal rabbit IgG (Fujifilm Wako Pure Chemical Corporation) and a Dynabeads Co-IP kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Proteins which did not interact with the antibody bound Dynabeads Epoxy were collected as the supernatant, and interacting proteins were collected as the eluted sample. The co-IP samples were stored at -80°C until further use.

2.7. In-gel protein digestion before mass spectrometry analysis

The co-IP samples were separated by SDS-PAGE in 12.5% SDS-polyacrylamide gels and visualized using a Sil-Best Stain One kit (Nacalai Tesque Inc., Kyoto, Japan). The bands corresponding to proteins detected only in the elution fraction were excised and then were de-stained by treatment with a mixture of equal volumes of de-staining solutions A and B from a Silver Stain MS Kit (Fujifilm Wako Pure Chemical Corporation). After de-staining, each sliced gel was dehydrated in acetonitrile (500 μ L) at room temperature until the gel pieces became white and shrunken. After removing the acetonitrile, each gel piece was incubated with 10 mM dithiothreitol in 25 mM ammonium bicarbonate for 1 hour at 50°C for reduction, and then with 100 mM iodoacetamide in 50 mM ammonium bicarbonate for 45 min at room temperature for S-alkylation. After processing for reduction and S-alkylation, each gel piece was washed twice with 1 mL of distilled water and was then

dehydrated in 500 μ L of acetonitrile. After removing the acetonitrile, protein digestion was achieved by 200 ng porcine trypsin (Pierce Trypsin Protease; Thermo Fisher Scientific) in 20 μ L of 25 mM ammonium bicarbonate added to each shrunken sliced gel piece in a tube and incubated for 16 hours at 37°C. Endopeptidase digestion was halted by the addition of 25 μ L of 5% formic acid, and then 25 μ L of 5% formic acid/50% acetonitrile was added to each tube for the extraction of peptide fragments from the gels. After incubation for 10 min at room temperature, the supernatant was transferred to a TPX tube (IEDA Trading Co., Tokyo, Japan), and then 50 μ L of acetonitrile was added to each sliced gel and incubated for 15 min at room temperature. Each extract was placed in the same TPX tube collected in the previous procedure, and then dried in a vacuum centrifuge.

2.8. Protein identification by mass spectrometry

Liquid chromatography tandem mass spectrometry (LC/MS/MS) was performed as described [28,29]. Dried peptide fragments were dissolved in 25 μ L of 0.1% trifluoroacetic acid, and the resulting solution was applied to a nanoflow LC/MS/MS system. LC/MS/MS was performed on an LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific) connected to a Dionex UltiMate 3000 pump (Thermo Fisher Scientific) and a HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). Mass spectra were recorded in a range of m/z 300–1500. LC/MS/MS data were interpreted through MASCOT MS/MS ion searches (Matrix Science, London, UK) against amino acid sequence data registered under the *P. falciparum* organism name (OS) in the UniProtKB database, in addition to the SwissProt database. Peptides were identified with a peptide mass tolerance of 4 ppm and a fragment m/z tolerance of 0.8.

3. Results

3.1. Expression profile of SBP1 in the gametocyte stages

To study the expression pattern of SBP1 in gametocytes, we generated an antibody against a PVM protein, Pfs16, as a specific marker of gametocytes [25,26]. Using western blotting, the antibody detected a single band at the expected size of Pfs16 (Fig. 1C). Similarly, indirect immunofluorescence analysis (IFA) revealed the presence of a Pfs16

signal covering the parasite (Fig. 1D). These findings indicate that the Pfs16 antibody is able to specifically recognize parasite-derived Pfs16, consistent with previous reports [25,26,30].

To determine the expression profile of SBP1 in developing gametocytes, we then performed western blotting with approximately 1×10^4 parasite-infected erythrocytes corresponding to each of asexual, immature gametocyte (on day 3 of gametocyte cultivation), and mature gametocyte (on day 11 of gametocyte cultivation) stages. In asexual stage and gametocyte samples, the anti-SBP1 antibody detected an approximately 40 kDa band, consistent with the predicted molecular weight under reducing and non-reducing conditions. SBP1 showed the highest expression in the immature gametocyte sample (Fig. 2A, top). To confirm the quality of the gametocyte sample, the anti-Pfs16 antibody was used to re-probe the same PVDF membrane. The Pfs16 band was detected at approximately 15 kDa in the gametocyte lanes (Fig. 2A, bottom); indicating that the gametocyte sample included gametocyte stage infected erythrocytes.

IFA was used to investigate the localization of SBP1 in gametocyte-infected erythrocytes. As reported, the SBP1 antisera detected a scattered dot-like pattern in the cytoplasm of the infected asexual stage erythrocytes (Fig. 2B, left panels) [15]. Gametocytes were defined by Pfs16 reactivity and were classified into five developmental stages based on their size and shape [24]. SBP1 was observed in the cytoplasm of gametocyte-infected erythrocytes as small, scattered dots in the early stages (I and II), similar to the asexual stages (Fig. 2B, middle panels). In contrast, in the later stages (III to V) SBP1 tended to accumulate at one side of the erythrocyte membrane (Fig. 2B, right panels), as reported by Petter *et al.* [31]. These results demonstrate that SBP1 is exported to the cytosol of gametocyte-infected erythrocytes in all stages of gametocyte development, and it appears to be transported through Maurer's clefts in the cytoplasm of infected erythrocytes.

3.2. Maurer's cleft localization of SBP1 in *P. falciparum* gametocyte-infected erythrocyte confirmed by immunoelectron microscopy

The subcellular localization of SBP1 in gametocyte-infected erythrocytes was further characterized by immunoelectron microscopy (IEM) using rabbit anti-SBP1 and mouse anti-Pfs16 antibodies. Figure 3 shows an IEM image of a gametocyte-infected erythrocyte.

A well-developed inner membrane complex was observed in the vicinity of the parasite cell membrane (Fig. 3, arrowhead). Small oval to elongated electron-dense osmiophilic bodies (Fig. 3, OB) were present in the cytoplasm specifically in gametocytes. The erythrocyte cytoplasm adjacent the PVM shows a low electron density region, called the electron lucent zone [32,33]. The PVM surrounding the parasite was deposited with 10 nm gold colloidal particles corresponding to the localization of the gametocyte marker Pfs16. In a cytoplasm of infected erythrocytes, 15 nm gold colloidal particles bound to anti-SBP1 antibody were observed around the Maurer's cleft (Fig. 3, MC). These findings demonstrated that SBP1 localizes to Maurer's clefts in the erythrocyte cytoplasm of gametocytes.

3.3. SBP1 interacts with PfHSP70-1 and PfHSP70-x in immature gametocytes

Co-IP studies using the anti-SBP1 antibody were used to screen for proteins interacting with SBP1 in gametocyte-infected erythrocytes. Anti-SBP1 antibody was coupled to magnetic beads and co-IP experiments were performed using cell lysates of immature gametocyte-infected erythrocytes harvested on day 3 of gametocyte culture (Fig. 4A). The supernatant and elution samples were subjected to SDS-PAGE. By western blotting using the anti-SBP1 antibody, SBP1 accumulation in the elution sample was confirmed, as detected by an approximately 40 kDa band (Fig. 4B, bottom).

Twenty bands were detected in the elution sample obtained by co-IP experiments using the anti-SBP1 antibody (Fig. 4B); versus no bands observed in the elution sample using normal rabbit IgG (Supplementary Fig. 1). The positive bands were excised from silver-stained SDS-PAGE gels and analyzed by LC/MS/MS after in-gel digestion (Fig. 4B, E). The results showed that four of the proteins were of human erythrocyte origin; namely, spectrin alpha chain, beta chain, cytoplasmic 1 of actin, and hemoglobin beta subunit. The remaining three proteins were specific to *P. falciparum*: heat shock protein 70-1 (PfHSP70-1), heat shock protein 70-x (PfHSP70-x), and SBP1 (Table 1). PfHSP70-1 and PfHSP70-x share about 86% similarity within the central 547 aa of the two proteins according to the BioEdit program ClustalW (mbio.ncsu.edu/BioEdit/bioedit.html; [34]). To confirm the identity of the detected proteins, we compared the peptide sequences detected by LC/MS/MS and Mascot search with full-length sequences of PfHSP70-1

(PF3D7_0818900) and PfHSP70-x (PF3D7_0831700) present in PlasmoDB. For PfHSP70-1 by the Mascot search, sixteen peptides were detected (TTPSYVAFTDTER, NPENTVFDAK, FTESSVQSDMK, *SGVDEKPMIEVTYQGEK*, NAVITVPAYFNDSQR, *DAGTIAGLNVMR*, *ATAGDTHLGGEDFDNR*, *DTLIPVEK*, *SVHEVVLVGGSTR*, *IQTLIK*, *LSQDEIDR*, MVNDAEK, *YKAEDEENR*, *NSLENYCYGVK*, *SSLEDQK*, *NQLAGKDEYEAK*); of which twelve peptides matched with regions specific to PfHSP70-1 (shown in italic letters and Fig. 5, blue bold) versus those in common with other HSP70 proteins. The remaining four peptides coincide with the regions of both PfHSP70-1 and PfHSP70-x (Fig. 5, black bold). For PfHSP70-x by the Mascot search, six peptides were detected (TTPSYVAFTDTER, NPENTVFDAK, NAVITVPAYFNDSQR, *QNQITITNDK*, MVNDAEK, *IYQGASAQEPQK*); of which two matched with regions specific to PfHSP70-x (shown in italic letters and Fig. 5, red bold). The remaining four peptides coincide with the regions of both PfHSP70-1 and PfHSP70-x (Fig. 5, black bold). Thus, two or more peptides showing specific regions for each PfHSP70-1 and PfHSP70-x peptide sequences were detected by LC/MS/MS and Mascot search, indicating the presence of PfHSP70-1 and PfHSP70-x in the elution sample by co-IP with SBP1 antibodies. These results suggested that PfHSP70-1 and PfHSP70-x interact with SBP1 in immature gametocytes of *P. falciparum*. However, because the co-IP experiments involved protein extracts, it must be borne in mind that interactions might be artificial due to the mixing of distinct biological compartments during the preparation of the extracts.

3.4. PfHSP70 is localized in the infected erythrocyte cytoplasm in immature gametocyte stages

IFA was performed to determine the localization of PfHSP70-1 in immature gametocyte-infected erythrocytes, using rabbit anti-PfHSP70 antibody and mouse anti-Pfs16 antibody. Anti-PfHSP70 antibody strongly recognized the cytoplasm of trophozoite and gametocyte stage I parasites, and was additionally present at lower levels as a dotted pattern in the cytoplasm of infected erythrocytes (Fig. 6, upper and middle panels). In gametocyte stage V infected erythrocytes, the signals corresponding to PfHSP70 were weaker, both within the parasite and in the infected erythrocyte (Fig. 6, lower panels).

These findings suggested that some members of the PfHSP70 multigene family might be expressed and transported into the infected erythrocytes, and localize within structures revealed by a dotted pattern in both the trophozoite and stage I gametocytes, possibly through interaction with SBP1.

4. Discussion

SBP1 is localized to Maurer's clefts, and during asexual stage development is involved in the transport of PfEMP1 to the surface of infected erythrocytes [10,11]. PfEMP1 has not been observed on the surface of gametocyte stage infected erythrocytes [21] and thereby the function of SBP1 in gametocytes is unknown. In this study, we demonstrated SBP1 expression during gametocyte maturation, and by IFA and IEM determined that throughout gametocyte maturation SBP1 also localizes to Maurer's clefts. We additionally discovered that PfHSP70-1 and PfHSP70-x proteins interact with SBP1 in immature gametocytes of *P. falciparum*. These findings suggest that protein transport to the surface of infected erythrocytes involves SBP1 at Maurer's clefts not only in asexual stage parasites but also in gametocytes.

Asexual and gametocyte infected erythrocytes have been reported to differ in their manner of sequestration in host tissues; specifically, asexual stages adhere to the vascular endothelium of various organs throughout the body, whereas gametocytes accumulate mainly in the extravascular compartment of the bone marrow [18,19]. While PfEMP1 is expressed in knob structures on the surface of asexual stage parasite-infected erythrocytes, very low expression of PfEMP1 has been reported on the surface of stage I gametocyte-infected erythrocytes, and erythrocyte membrane knobs are absent [21]. These findings raised the possibility that differences in adhesion-related molecules transported via Maurer's clefts may be responsible for the disparity in the sequestration sites of asexual stage and gametocyte infected erythrocytes.

We hypothesized that in asexual stages versus gametocytes SBP1 might interact with different sets of adhesion-related proteins transported to the surface of infected erythrocytes; and therefore, we screened for proteins interacting with SBP1 in gametocyte-infected erythrocytes. Immature gametocytes were used as material for co-IP experiments, since their infected erythrocytes are known to accumulate in the bone

marrow. SBP1 is a type 2 transmembrane protein, with its C-terminal domain exposed to the cytoplasm of infected erythrocytes [15,35]. The protein interacts with human erythrocyte spectrin, band 4.1, and LANCL1; to possibly anchor Maurer's clefts to the host erythrocyte cytoskeleton [36–38]. Our co-IP experiments identified human spectrin as an SBP1 interacting protein, which is consistent with these findings. Regarding parasite-encoded proteins in immature gametocytes, SBP1 interaction with PfHSP70-1 and PfHSP70-x was identified for the first time by LC/MS/MS analysis. However, it is unlikely that they are directly involved as ligands mediating the accumulation of gametocyte-infected erythrocytes in the bone marrow, as PfHSP70s are also expressed in asexual stage parasites. As indicated by the LC/MS/MS results detecting several common peptides, PfHSP70-1 and PfHSP70-x share about 86% similarity within the central 547 aa of the two proteins according to the BioEdit program ClustalW. Therefore, the rabbit anti-PfHSP70 polyclonal antibody used for IFA is likely to react with both PfHSP70-1 and PfHSP70-x.

PfHSP70-1 is an essential molecular chaperone that is detected only in association with the parasite cytoplasm [39–41]. It lacks a signal peptide sequence, in accordance with this predicted cytoplasmic localization. PfHSP70-x doesn't possess a PEXEL/HT signal, but transport to the erythrocyte appears to be directed by a seven amino acid motif found directly following a signal peptide at the N-terminus [6,42]. PfHSP70-x chaperone and cholesterol bound co-chaperone PfHSP40 (PF3D7_0113700) assist PfEMP1 transport across the host erythrocyte [6,43]. This was supported by the finding that deletion of PfHSP70-x led to delayed export of PfEMP1 to the erythrocyte surface and that PfHSP70-x deleted parasite infected erythrocytes had reduced adhesion [17]. In the asexual stage parasite infected erythrocyte, PfHSP70-x localizes to the parasitophorous vacuolar (PV) space and is subsequently exported to 'dotted' structures within the host erythrocyte cytoplasm [6]. Our IFA results indicated that PfHSP70 is expressed in immature stages during gametocyte maturation, as well as in asexual stages, and localizes to the cytosols of both parasites and their infected erythrocytes (Fig. 6). Considering the above-described findings [6,40], it is possible that PfHSP70-1 localizes to the parasite cytoplasm and PfHSP70-x is exported to the erythrocyte cytoplasm. The generation of genetically modified parasites expressing tag-labeled PfHSP70-1 or PfHSP70-x will be required to

determine the exact localization of PfHSP70-1 and PfHSP70-x in gametocyte-infected erythrocytes; because the anti-PfHSP70 antibodies used in this study possibly cross-react with both PfHSP70-1 and PfHSP70-x. Specifically, the anti-PfHSP70 antibody was generated using PfHSP70-1 at the C-terminus (amino acids 365 to 677) as the antigen. This region of PfHSP70-1 shares approximately 71% identity with the sequence from positions 384 to 679 at the C-terminus of PfHSP70-x; and the first half (199 amino acids) of this sequence shares approximately 87% identity. IEM analysis using these parasites will reveal the precise localization of PfHSP70 in gametocyte-infected erythrocytes.

PfHSP70-1 has been shown to localize to the parasite nucleus and cytoplasm of trophozoites. PfHSP70-1 seemed to accumulate towards the periphery of the cytoplasm, where it could be involved in protein transport across the plasma membrane [40]. At the early trophozoite stage, SBP1 was detected within an electron-dense material found in the parasite cytoplasm and in the PV space [14]. SBP1 lacks a classical signal peptide sequence, as well as a PEXEL export element typical of *Plasmodium* erythrocyte-exported proteins [13,44,45], and thus might not be exported via the endoplasmic reticulum (ER) [14,35]. During asexual stage development, PfHSP70-1 accumulates in the periphery of the cytoplasm, where it might be involved in protein transport across the parasite plasma membrane. Together with our findings here, SBP1 transport to the PV space might be mediated by interaction with PfHSP70-1. Alternatively, SBP1 might not interact with PfHSP70-1 *in vivo*, and its observed binding in the co-IP experiments was artificially derived from the generation of a protein extract which allowed the mixing of distinct biological compartments.

In conclusion, this study demonstrates that SBP1 localizes to Maurer's clefts in the cytoplasm of erythrocytes infected with *P. falciparum* gametocytes. Furthermore, it was suggested that SBP1 interacts with PfHSP70-1 and PfHSP70-x, which might be involved in trafficking of parasite proteins to the erythrocyte cytosol.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

We also thank the Japanese Red Cross Society for providing human erythrocytes and plasma. We created a part of the figure using BioRender.com software.

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Figure legends

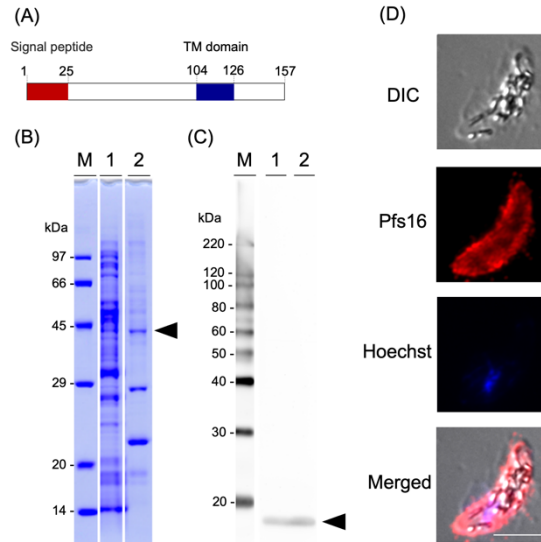


Fig. 1. Preparation of anti-Pfs16 antibody and characterization as a gametocyte marker. (A) A schematic representation of the primary structure of Pfs16. Full-length Pfs16 was tagged with N-terminal GST, and was expressed by a wheat germ cell-free protein production system. (B) rGST-Pfs16 protein production. Proteins expressed by the wheat germ cell-free system were separated by SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue. Lane M: protein molecular weight marker. Lane 1: total reaction mixture in the wheat germ cell-free system; Lane 2: affinity-purified proteins. Arrowhead: rGST-Pfs16. (C) Western blotting analysis using anti-Pfs16 antibody. Cell lysates of approximately 7×10^4 parasite-infected erythrocytes per lane were separated by SDS-PAGE under reducing (lanes 1) and non-reducing (lanes 2) conditions. Arrowhead: Pfs16. (D) Subcellular localization of Pfs16 in *P. falciparum* gametocyte-infected erythrocytes. Acetone-fixed gametocyte stage V parasites were incubated with mouse anti-Pfs16 antibody (red) and Hoechst 33342 (blue). DIC: differential interference contrast microscopy image. Scale bar: 5 μm .

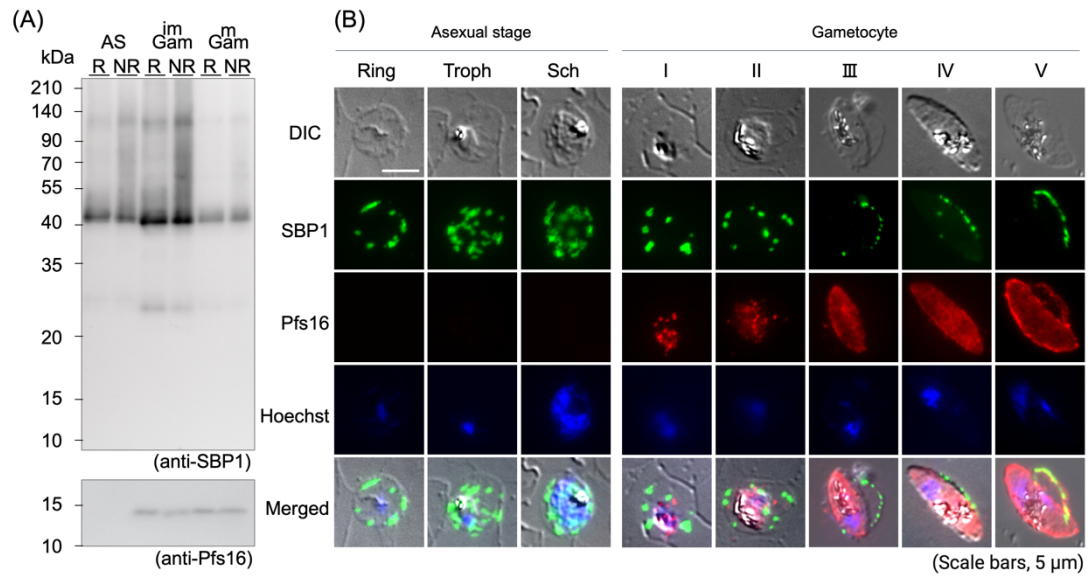


Fig. 2. Expression of SBP1 in *P. falciparum*-infected erythrocytes. (A) Western blotting analysis using anti-SBP1 antibody (upper panel) and anti-Pfs16 antibody (lower panel). Infected erythrocyte extracts were separated by SDS-PAGE under reducing (R) and non-reducing (NR) conditions. Each sample contained 1×10^4 parasite-infected erythrocytes per lane. Arrowhead: SBP1, AS: asexual stage, imGam: immature gametocyte, mGam: mature gametocyte. (B) IFA using anti-SBP1 antibody and anti-Pfs16 antibody against asexual and gametocyte stage parasites. Ring: ring stage, Troph: trophozoite stage, Sch: schizont stage. I-V indicates developmental stages of gametocytes. Red: Pfs16, Green: SBP1, Blue: Hoechst 33342, DIC: differential interference contrast microscopy image. Scale bar: 5 μ m.

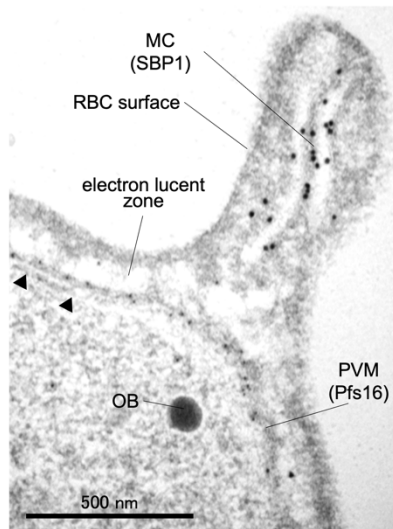


Fig. 3. Subcellular localization of SBP1 in a *P. falciparum* gametocyte-infected erythrocyte after gametocyte cultivation for 12 days. IEM images of a gametocyte-infected erythrocyte using anti-SBP1 and anti-Pfs16 antibodies. The 10 nm and 15 nm gold particles represent the localization of Pfs16 and SBP1, respectively. PVM: parasitophorous vacuole membrane, MC: Maurer's cleft, OB: osmiophilic body. Arrowhead: inner membrane complex. Scale bar: 500 nm.

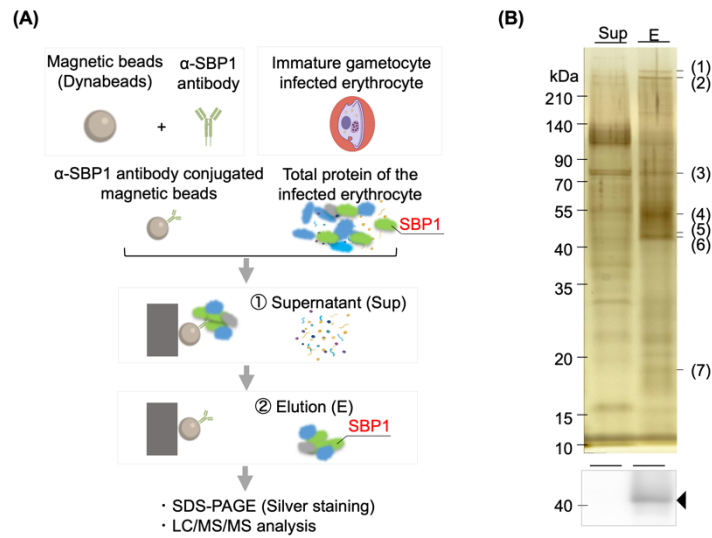


Fig. 4. Protein-protein interaction analysis of SBP1 in immature gametocytes. (A) Schematic diagram of a method for identifying SBP1 binding proteins in a co-IP elution sample using SBP1 antibody conjugated to magnetic beads. (B) SDS-PAGE and silver staining of co-IP samples using anti-SBP1 antibody (upper panel) with western blotting image using anti-SBP1 antibody (lower panel). Arrowhead: SBP1. Sup: supernatant, E: elution.

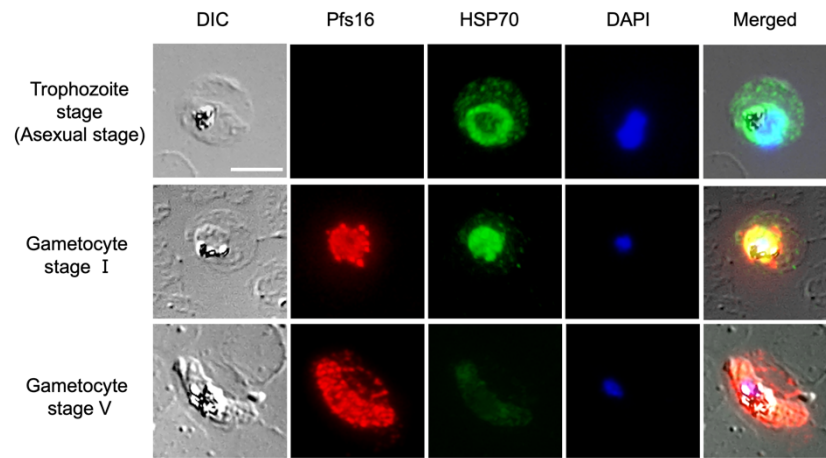
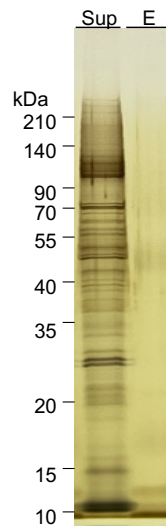


Fig. 6. IFA using anti-PfHSP70 antibody. Red: Pfs16, Green: PfHSP70, Blue: DAPI, DIC: differential interference contrast microscopy image. Scale bar: 5 μ m.

No.	Protein name	Accession	Mass/Da	peptides	Coverage (%)	score
1	Spectrin alpha chain, erythrocytic	SPTA1_HUMAN (UniProt)	281039	76	31	2956
2	Spectrin beta chain, erythrocytic	SPTB1_HUMAN (UniProt)	247171	51	24	2106
3	Pf Heat shock protein 70-1	PF3D7_0818900	74382	13	25	869
	Pf Heat shock protein 70-x	PF3D7_0831700	75576	6	9	311
4	SBP1	PF3D7_0501300	36277	3	13	176
5	SBP1	PF3D7_0501300	36277	3	8	70
6	SBP1	PF3D7_0501300	36277	7	31	265
7	Actin, Cytoplasmic 1, <i>Homo sapiens</i>	ACTB_HUMAN (UniProt)	42052	7	22	290
	Hemoglobin beta subunit, <i>Homo sapiens</i>	HBB_HUMAN (UniProt)	15857	2	15	71

Table 1. List of proteins identified in LC/MS/MS of co-IP experiment samples using SBP1 antibody conjugated to magnetic beads. The numbers indicate the excised position of the gel from which the sample was eluted in Fig.4B.



Supplementary Fig. 1.

SDS-PAGE and Silver Staining of co-IP samples using normal rabbit IgG and immature gametocyte infected erythrocytes. Sup; supernatant, E; elution.