PEXEL-independent trafficking of *Plasmodium falciparum* SURFIN$_{4.2}$ to the parasite-infected red blood cell and Maurer's clefts

Jean Semé Fils Alexandre[a,b], Kazuhide Yahata[a], Satoru Kawai[c], Motomi Torii[d], Osamu Kaneko[a,*]

[a]*Department of Protozoology, Institute of Tropical Medicine (NEKKEN) and the Global Center of Excellence Program, Nagasaki University, Sakamoto, Nagasaki 852-8523, Japan*
[b]*Centro Nacional de Control de Enfermedades Tropicales, Santo Domingo, República Dominicana*
[c]*Center for Tropical Medicine and Parasitology, Dokkyo Medical University, Tochigi 321-0293, Japan*
[d]*Department of Molecular Parasitology, Ehime University Graduate School of Medicine, Shitsukawa, Toon, Ehime 791-0925, Japan*

Abbreviations: aa, amino acid(s); CRD, cysteine-rich domain; ER, endoplasmic reticulum; GFP, green fluorescence protein; IFA, indirect immunofluorescence assay; iRBC, infected red blood cell; PBS, phosphate buffered saline; PEXEL, *Plasmodium* export element; PNEP, PEXEL negative exported protein; PVM, parasitophorous vacuole membrane; TM, transmembrane; Tx, Triton-X 100; Var, variable region; WR, tryptophan-rich

***Corresponding author**: Tel.: (+81) 95 819 7838; Fax: (+81) 95 819 7805; E-mail address: okaneko@nagasaki-u.ac.jp (O. Kaneko)

Abstract

SURFIN$_{4.2}$ is a parasite-infected red blood cell (iRBC) surface associated protein of *Plasmodium falciparum*. To analyze the region responsible for the intracellular trafficking of SURFIN$_{4.2}$ to the iRBC and Maurer's clefts, a panel of transgenic parasite lines expressing recombinant SURFIN$_{4.2}$ fused with green fluorescent protein was generated and evaluated for their localization. We found that the cytoplasmic region containing a tryptophan rich (WR) domain is not necessary for trafficking, whereas the transmembrane (TM) region was. Two PEXEL-like sequences were shown not to be responsible for the trafficking of SURFIN$_{4.2}$, demonstrating that the protein is trafficked in a PEXEL-independent manner. N-terminal replacement, deletion of the cysteine-rich domain or the variable region also did not prevent the protein from localizing at the iRBC or Maurer's clefts. A recombinant SURFIN$_{4.2}$ protein possessing 50 amino acids upstream of the TM region, TM region itself and a part of the cytoplasmic region was shown to be trafficked into the iRBC and Maurer's clefts, suggesting that there are no essential trafficking motifs in the SURFIN$_{4.2}$ extracellular region. A mini-SURFIN$_{4.2}$ protein containing WR domain was shown by Western blotting to be more abundantly detected in a Triton X-100-insoluble fraction, compared to the one without WR domain. We suggest that the cytoplasmic region containing the WR may be responsible for their difference in solubility.

Keywords: malaria; Maurer's clefts; *Plasmodium falciparum*; protein trafficking; SURFIN

1. Introduction

During its asexual replication in the human host, *Plasmodium falciparum*, the apicomplexan parasite responsible for malaria, dramatically remodels the infected red blood cell (iRBC) [1]. This process involves the generation of a parasitophorous vacuole (PV) in which parasites reside and replicate, the transportation of parasite proteins into the iRBC across the PV membrane (PVM), the generation of parasite-derived membranous structures in the cytoplasm of the host RBC called Maurer's clefts that play a major role as protein-sorting points, and the formation of knobs on the iRBC surface [2 - 4]. Some of the most severe malaria pathologies caused by *P. falciparum*, such as cerebral and placental malaria, are specifically linked to the adherence of the iRBCs to capillary vessels (cytoadhesion) and to uninfected RBCs (rosseting). *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), a parasite protein transported to the surface of the iRBC has previously been shown to mediate these phenomena [5 – 7]. Understanding the molecular mechanisms and pathways by which parasite-proteins such as *Pf*EMP1, are trafficked to the cytosol and thence to the surface of the iRBC is, therefore, critical for a clear insight into the pathogenesis of *P. falciparum* malaria.

RBCs lack a protein secretory apparatus so the parasite must establish *de novo* secretion machinery within the host cell cytoplasm in order to transport its own proteins into the iRBC across the PVM. The mechanisms that enable such trafficking are incompletely understood; however, many *P. falciparum* proteins destined for export into the iRBC contain both an N-terminal hydrophobic signal sequence and a short conserved pentameric host cell-targeting motif (RxLxE/Q/D) termed the *Plasmodium* export element (PEXEL) or the vacuolar transport signal (VTS) [8, 9]. The N-terminal signal sequence is required for the proteins to enter the constitutive secretory pathway *via* the endoplasmic reticulum (ER) [10, 11], where the PEXEL/VTS motif is cleaved by plasmepsin V, an ER residing aspartic protease [12, 13], and the newly formed N-terminus (xE/Q/D) allows translocation into the iRBC cytosol by a PVM residing translocon called the "*Plasmodium* translocon of exported proteins" (PTEX) complex [14]. Our understanding of the mechanisms behind the transport of proteins within the iRBC remains vague, but some tentative explanations have been raised; transport may be mediated through vesicles, through complex membrane networks, non-lipid enclosed protein aggregates, or lipid enclosed structures such as Maurer's clefts [4, 15].

A few hundred proteins are known to contain a PEXEL/VTS motif, defining a large *Plasmodium* exportome [9, 16]. However, several parasite proteins that are transported to the iRBC lack both an N-terminal signal sequence and a PEXEL/VTS motif, and are termed "PEXEL negative exported proteins" (PNEPs) [17]. The most well characterized PNEPs are skeleton-binding protein 1 (SBP1)

[18], the membrane associated histidine-rich protein 1 (MAHRP1) [19] and the ring-exported proteins 1 (REX1) and 2 (REX2) [20, 21, 17]. Most of the reported PNEPs are believed to be trafficked to the iRBC *via* the classical secretory pathway, involving initial transport to the ER, but no shared signal or transport-related sequence has been identified to date for subsequent transport to the iRBC. SBP1, MAHRP1 and REX2 lack a signal peptide but contain a transmembrane (TM) region which, along with sequences at their N-terminal region, has been implicated in protein transport [22 – 24]; however, the hydrophobic N-terminal region of the REX1 protein has been shown to be the only region required for transport of this particular protein. [25].

The recently identified *P. falciparum* surface-associated interspersed gene (*surf*) family encodes high molecular mass proteins, and one of these, $SURFIN_{4.2}$, has been shown to be co-transported along with *Pf*EMP1 and RIFIN to the iRBC surface [26]. The N-terminal of $SURFIN_{4.2}$, predicted to be extracellular, contains a moderately conserved cysteine-rich putative globular domain (CRD) preceding a variable segment (Var) followed by a putative TM region. The C-terminal region of $SURFIN_{4.2}$ contains three tryptophan-rich (WR) domains which are highly conserved among SURFIN protein members, intersected by stretches of higher variability. The protein does not appear to contain either a hydrophobic signal peptide sequence or a classical PEXEL motif. Although there are two PEXEL-like sequences located at the N-terminal segment amino acid positions (aa) 24 - 30 (R_KI_FE) and aa 118 - 122 (R_TL_ED), they may not be true signals because the first does not completely agree with the consensus PEXEL motif, and the second was located in the putative globular domain CRD. This suggests that $SURFIN_{4.2}$ may be transported *via* a PEXEL-independent pathway. Using a serial deletion approach, we have attempted to identify the regions involved in *P. falciparum* $SURFIN_{4.2}$ transport into the iRBC. We generated a panel of transgenic parasite lines expressing green fluorescent protein (GFP)-tagged recombinant $SURFIN_{4.2}$ and show that the protein is trafficked as a PNEP. We show that the TM region, the only predicted hydrophobic region of the protein, is necessary for entry into the parasite's secretory pathway and for subsequent trafficking into the iRBC. These findings confirm the importance of hydrophobic regions for the trafficking of PNEPs.

2. Materials and Methods

2.1. Plasmid construction

A panel of plasmids that were used to make final *P. falciparum* transfection constructs were prepared based on the Multisite Gateway® system (Invitrogen, Carlsbad, CA) [27]. DNA fragments containing attB1 and attB2 sites were inserted into pUC19, resulting the pB12 plasmid. DNA fragments encoding aa 1 - 419 of $SURFIN_{4.2}$ (CRD and a part of Var), a triple HA tag, and aa 734 - 764 of $SURFIN_{4.2}$ (TM) were ligated into pB12 to make the pB12-$SURF_{4.2}$CRD-Var1-HA-TM plasmid. DNA fragments encoding aa 765 - 1347, 1320 - 1728 or 1712 - 2380 of $SURFIN_{4.2}$ (first, second or third WR domain) were ligated into pB12-$SURF_{4.2}$CRD-Var1-HA-TM, resulting in the pB12-$SURF_{4.2}$CRD-Var1-HA-TM-WR1, pB12-$SURF_{4.2}$CRD-Var1-HA-TM-WR2 or pB12-$SURF_{4.2}$CRD-Var1-HA-TM-WR3, respectively. DNA fragments encoding 50 amino acids at aa 684 - 734 adjacent to TM region, a triple HA tag, TM, and WR1 regions were ligated into pB12, resulting in the pB12-$SURF_{4.2}$VarC-HA-TM-WR1. All DNA fragments were amplified from *P. falciparum* 3D7 line parasites using KOD Plus DNA polymerase (Toyobo). pB12-$SURF_{4.2}$CRD-Var1-TM was further modified by site-directed mutagenesis using oligonucleotides with desired modifications as follows: To abolish the PEXEL-like sequences at aa 25 - 29 ($R_{25K}I_{27F}E_{29}$) or aa 118 - 122 ($R_{118T}L_{120E}D_{122}$), these sequences were replaced to $A_{25K}A_{27F}A_{29}$ or $A_{118T}A_{120E}A_{122}$, respectively, yielding pB12-$SURF_{4.2}$CRD-Var1-HA-TM-Pexel-1mut or pB12-$SURF_{4.2}$CRD-Var1-HA-TM-Pexel-2mut. To assess the N-terminal sequence for the trafficking, the N-terminal region at aa 1 - 42 (M_1LFVVELDSRLEKSADKRISVERFRKIFEIYVEDKL EELKRS$_{42}$) of $SURFIN_{4.2}$ was replaced with the N-terminal region at aa 1 - 15 (M_1DVHVNQLKNISPID$_{15}$) of *P. falciparum* adenylosuccinate lyase (ASL, PFB0295w), a *P. falciparum* enzyme not considered to be transported to the iRBC, to yield pB12-$SURF_{4.2}$CRD-Var1-HA-TM-RepN. pB12-$SURF_{4.2}$Var1-HA-TM and pB12-$SURF_{4.2}$CRD-HA-TM were generated from pB12-$SURF_{4.2}$CRD-Var1-TM by removing a region encoding aa 46 - 196 containing the CRD and a region encoding aa 198 - 419 containing the Var1 region, respectively. These pB12-based plasmids were subjected to a BP recombination reaction with pDONR™221 (Invitrogen) according to the manufacturer's instructions, resulting in the corresponding pENT12 Gateway Entry vectors. These pENT12-based plasmids were then subjected to a Gateway MultiSite LR recombination reaction with other Entry vectors, *Pf*CRT 5'-pENTR4/1 (as a promoter component) and GFPm2-pENTR2/3 (as a tag sequence), and a Destination vector, pCHDR-3/4 (a kind gift from Dr. G. McFadden), according to the manufacturer's instruction [27]. Initially, we used the promoter region of $SURFIN_{4.2}$, however, the signal was very weak, thus we decided to use CRT (chloroquine resistance transporter; MAL7P1.27) promoter, which has been used to study *P. falciparum* protein trafficking to the iRBC cytosol and Maurer's clefts, to overexpress recombinant proteins for the visualization of their clear location. Previous transcriptome data indicated that the promoter activity of CRT was stronger than that of $SURFIN_{4.2}$ and that CRT was mainly transcribed at schizont, ring and early trophozoite stages, slightly longer than $SURFIN_{4.2}$ which was mainly transcribed at schizont and early ring stages. [28, 29]. Obtained plasmids were verified by their restriction enzyme digestion pattern and sequencing. Schematic structures of the recombinant proteins expressed from the episomal form of the plasmids in the transfected *P. falciparum* are shown in figure 1. Detailed information may be found in Supplementary material.

2.2. Parasite culture and transfection

The *P. falciparum* MS822 line was used in this study. This line was isolated in Mae Sot, Thailand in 1988, maintained in vitro for less than 3 months, and kept at the Institute of Tropical Medicine, Nagasaki University [30]. Parasites were cultured in RPMI-1640 medium containing 5% heat-inactivated pooled type AB human serum and 0.25% Albumax II (Invitrogen), 200 mM hypoxanthine (SIGMA, St. Louis, MO), 10 µg/mL gentamycin (Invitrogen) and human RBC (type O) at 2% hematocrit. Human RBCs and plasma were obtained from the Nagasaki Red Cross Blood Center.

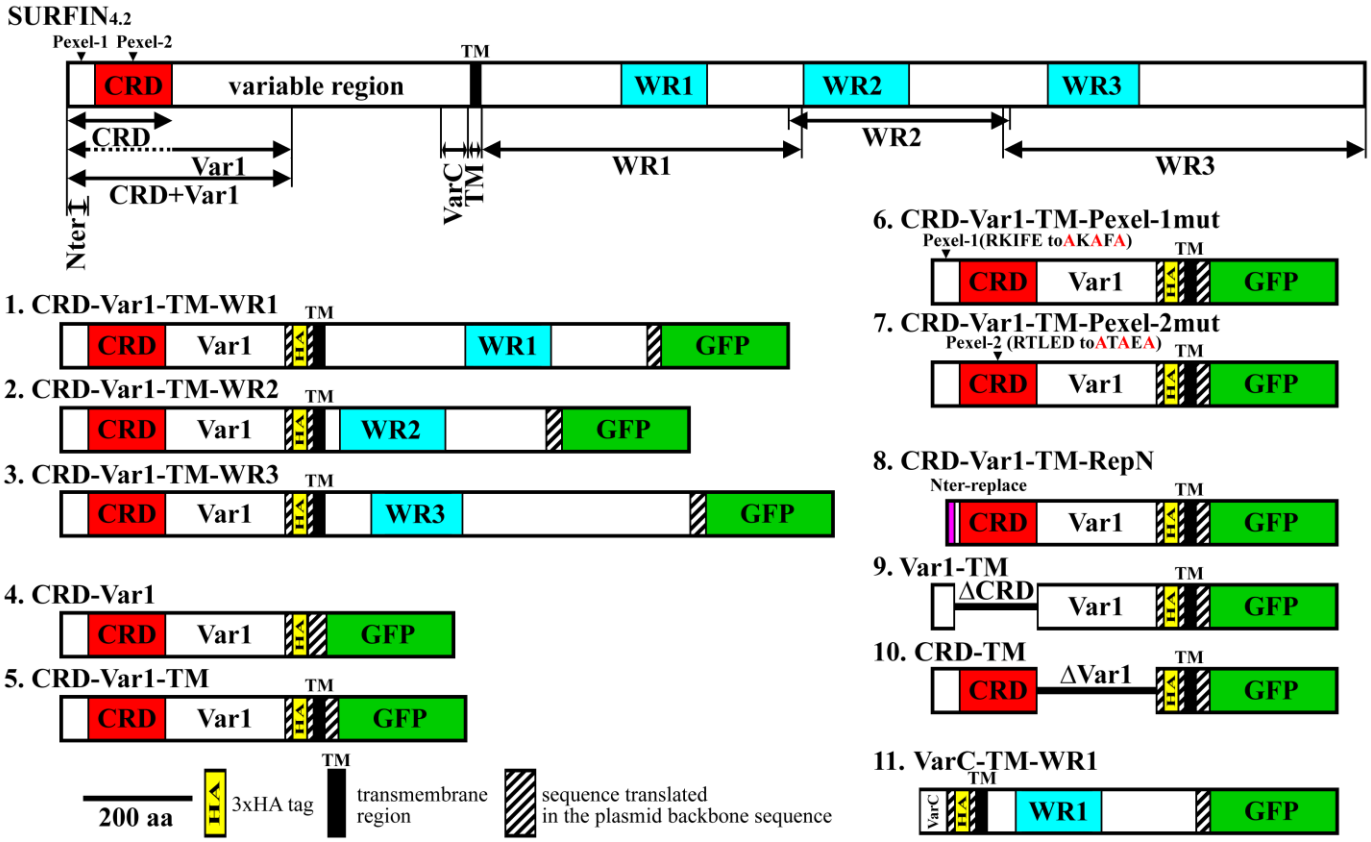

Figure 1. Schematic structure of the recombinant proteins expressed in the transfected *P. falciparum* lines. CRD, cysteine-rich domain; VarC, C-terminal of the variable region; HA, triple hemagglutinin-tag; Nter, N-terminal; TM, transmembrane; Var1, variable region 1; and WR, tryptophan-rich. ΔCRD or ΔVar1 indicate that CRD or Var1 was deleted from the protein.

Serum was produced from the acid-citrate-dextrose-containing plasma by removing the clot that had formed after adding calcium. *P. falciparum* transfection was performed essentially as previously described [31]. Briefly, RBCs were resuspended in 400 µL of incomplete Cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, and 25 mM Hepes) containing 100 µg of plasmid DNA. Electroporations were performed in 2 mm cuvette using a Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA) with a condition of 320 mΩ, 0.32 kV and 975 µF. Observed time constants were 15-30 msec. Parasites were synchronized to ring stage by 5% sorbitol treatment, then 40 hours later, mature trophozoites/schizonts-iRBCs were resuspended with the plasmids-preloaded RBCs (final 0.2% parasitemia). At day 3 post transfection, 5 nM of the anti-folate drug WR99210 (kind gift from Dr. D. Jacobus) was supplied to the culture medium, and was maintained until drug-resistant parasites reappeared. Resistant parasites were usually detected before the 30th day of culture in the presence of the drug and were subsequently maintained in culture containing 25 nM WR99210.

2.3. Fluorescence live imaging and indirect immunofluorescence assay (IFA)

For fluorescence live imaging, 10 µL of parasite culture were incubated with 1 µg/mL of Hoechst 33342 (Moleculer Probe) for 30 min at 37°C and placed on a glass slide for observation. Parasites expressing GFPm2 were visualized using a fluorescence microscope (Eclipse 80i; Nikon, Japan) and a digital camera (VB-7010; Keyence, Japan) equipped with 100 × oil immersion lens. For IFA, thin smears of *P. falciparum* iRBCs were prepared on glass slides, fixed with 4% paraformaldehyde/0.075% glutaraldehyde in PBS at room temperature for 5 min, rinsed with 50 mM glycine in PBS, and blocked with PBS containing 3% BSA (SIGMA) for 30 min [32]. For single staining, the smears were reacted with rabbit anti-GFP polyclonal antibody (ab6556; Abcam, Cambridge, UK), followed by Alexa-Fluor 488-conjugated secondary anti-rabbit IgG antibody (Invitrogen). For double staining, rabbit anti-GFP antibody and rabbit anti-SBP1 antibody (a kind gift from Dr. T. Tsuboi) were labeled with Alexa-Fluor 488 and -594, respectively, using Zenon® Rabbit IgG labeling kit (Invitrogen). The smears were then incubated with rabbit anti-GFP antibody (1:500) and rabbit anti-SBP1 antiserum (1:1000) in PBS containing 3% BSA for 1 hour at 37°C. Parasite nuclear staining was carried out by adding 4′, 6-diamidino-2-phenylindole (DAPI; Invitrogen; final 1 µg/mL). Stained parasites were mounted with ProLong® Gold antifade reagent (Invitrogen) and visualized as described above. Some images were analyzed by using ImageJ software (1.44p; http://rsbweb.nih.gov/ij/).

2.4. Extraction of parasite proteins and Western blot analysis

Mature trophozoite and schizonts-iRBCs were collected by centrifugation on a 40/70% Percoll-sorbitol gradient. The enriched parasite fractions ($2 - 4 \times 10^8$ parasites) were subjected to protein extraction during which process the water-soluble fraction was collected following a freeze-thaw procedure in PBS containing a mixture of protease inhibitors (PI; cOmplete; Roche, Basel, Switzerland) repeated three times. The pellets were washed twice with PBS-PI, and proteins further extracted in PBS-PI containing 1% Triton X-100 (Tx; Calbiochem) for 30 min on ice. The insoluble materials were washed twice with PBS-PI-Tx, then proteins were further extracted by incubation with PBS-PI

containing 2% SDS (Nacalai, Japan) for 30 min at room temperature.

Parasite extracts were subjected to electrophoresis on 5-20% SDS-polyacrylamide gradient mini gels (ATTO, Japan) under reducing conditions. The protein bands were transferred from gels to PVDF membranes (Millipore, Billerica, MA). The membranes were then probed with rabbit anti-GFP polyclonal antibody (1:500; ab6556; Abcam), for 1 hour at room temperature followed by a secondary incubation with the horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Promega) at a concentration of 1:25,000. Purified mouse anti-glycophorin A antibody (CD235a; BD Pharmingen) was used to detect glycophorin A as a positive control. Bands were visualized with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) and detected using a chemiluminescence detection system (LAS-4000EPUVmini; Fujifilm, Japan). The relative molecular sizes of the proteins were calculated based in reference to the molecular size standards (Precision Plus Dual Color Standards; Bio-Rad).

3. Results

3.1. Successful generation of mini-SURFIN$_{4.2}$ proteins

The production of *P. falciparum* transfectants expressing very large proteins such as SURFIN$_{4.2}$ (predicted molecular weight of 286 kDa in 3D7 parasite line) is technically challenging. Therefore, in order to investigate which region is responsible for the trafficking of SURFIN$_{4.2}$ into the iRBCs, and thence Maurer's clefts, we first attempted to generate and evaluate mini-SURFIN$_{4.2}$ proteins containing regions that are conserved among SURFIN members; N-terminal 419 amino acids containing N-terminal segment (aa 1 - 50) and CRD (aa 51 - 195), which is conserved among *P. falciparum* SURFIN members, and the N-terminal side of the variable region (Var1; aa 196 - 419), which is relatively conserved between SURFIN$_{4.2}$ and SURFIN$_{4.1}$ (identity 19.5% and similarity 28.7%), TM, and either one of the WR domains (WR1, WR2, or WR3). All mini-SURFIN$_{4.2}$ proteins, SURFIN$_{4.2}$CRD-Var1-TM-WR1, -WR2, or -WR3, were tagged with GFP for visualization (Fig. 1). Although the extracellular region was tagged with triple HA, anti-HA mouse monoclonal antibody (4B2; Wako, Japan) could not detect any signals by IFA and Western blot analysis with unknown reason, thus HA tag was not used in this study. Live imaging using a fluorescence microscope produced only very weak fluorescent signals, and so the precise location of the GFPm2-tagged protein was impossible to assess. By double staining IFA, all mini-SURFIN$_{4.2}$ proteins were observed in the iRBC cytoplasm as punctuate dots that co-localized with the Maurer's cleft protein SBP1 [18], indicating that all 3 mini-SURFIN$_{4.2}$ proteins were transported to the Maurer's clefts (Fig. 2). In addition to the Maurer's cleft localized signal, diffuse fluorescence was also observed in the parasite, as well as in the iRBC. As all of the mini-SURFIN$_{4.2}$ proteins were able to traverse the PVM and reach the iRBC cytosol and the Maurer's clefts, we selected SURFIN$_{4.2}$CRD-Var1-TM-WR1 for further evaluation.

3.2. The TM region, but not the WR domain is essential for the SURFIN$_{4.2}$ trafficking to the iRBC and Maurer's clefts

In order to evaluate the importance of the TM region and WR domain in SURFIN$_{4.2}$ trafficking, we generated parasites expressing only SURFIN$_{4.2}$CRD-Var1

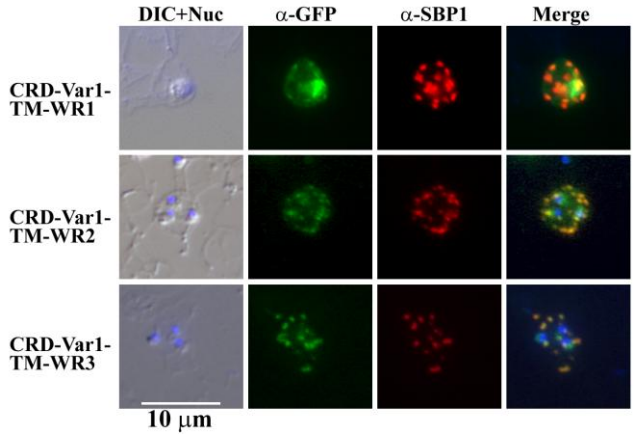

Figure 2. Indirect immunofluorescence assay of three mini-SURFIN$_{4.2}$ proteins. Double staining IFA for 3 mini-SURFIN$_{4.2}$-expressing transfectants are shown. α-GFP and α-SBP1 indicate anti-GFP (mini-SURFIN$_{4.2}$) and anti-SBP1 (Maurer's cleft protein). Negative controls using normal rabbit antibody did not produce detectable signals (not shown). CRD, cysteine-rich domain; TM, transmembrane; Var1, variable region 1; and WR, tryptophan-rich.

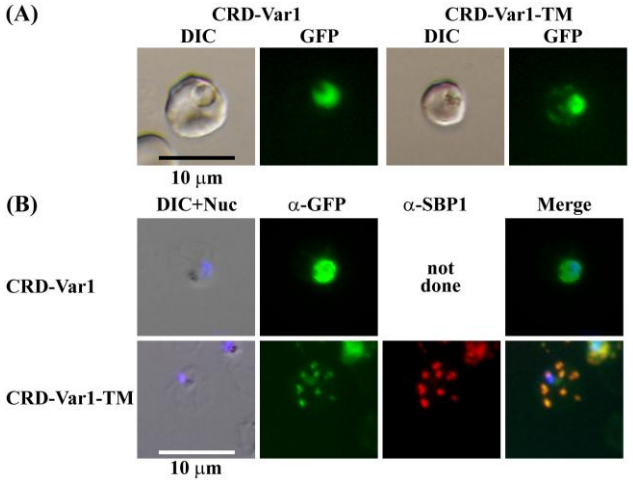

Figure 3. Indirect immunofluorescence assay of SURFIN$_{4.2}$CRD-Var1 and SURFIN$_{4.2}$CRD-Var1-TM proteins. (A) Live imaging of GFP-expressing parasites. (B) Single staining for SURFIN$_{4.2}$CRD-Var1 and double staining for SURFIN$_{4.2}$CRD-Var1-TM with anti-GFP (α-GFP, green) and anti-SBP1 (α-SBP1, red). Nuclei were stained with DAPI. Negative control using normal rabbit antibody did not produce detectable signals (not shown). CRD, cysteine-rich domain; TM, transmembrane; and Var1, variable region 1.

(TM and WR were removed) or SURFIN$_{4.2}$CRD-Var1-TM (WR was removed). By live imaging, weak, but detectable GFP signals were observed for these transfectants, and we found that SURFIN$_{4.2}$CRD-Var1 was exclusively located in the parasite cytoplasm, as would be expected for a protein lacking a signal sequence for transport to the ER. We speculate that the protein was located within the parasite cytosol (Fig. 3A). In contrast, SURFIN$_{4.2}$CRD-Var1-TM was observed in a punctate dot pattern in the iRBC, in addition to diffuse fluorescence in the parasite cytoplasm. By double staining IFA, SURFIN$_{4.2}$CRD-Var1 was observed only in the parasite, confirming the live imaging results, whereas SURFIN$_{4.2}$CRD-Var1-TM colocalized with SBP1, indicating a Maurer's cleft localization (Fig. 3B). Thus, the IFA data indicate that the SURFIN$_{4.2}$ cytoplasmic region containing WR domain is not required for trafficking to the iRBC or Maurer's clefts, but the TM region is essential.

A diffused fluorescence pattern in the iRBC, as observed for the mini-SURFIN$_{4.2}$ proteins, appeared to be reduced for SURFIN$_{4.2}$CRD-Var1-TM with the double staining IFA images. Because the single staining with

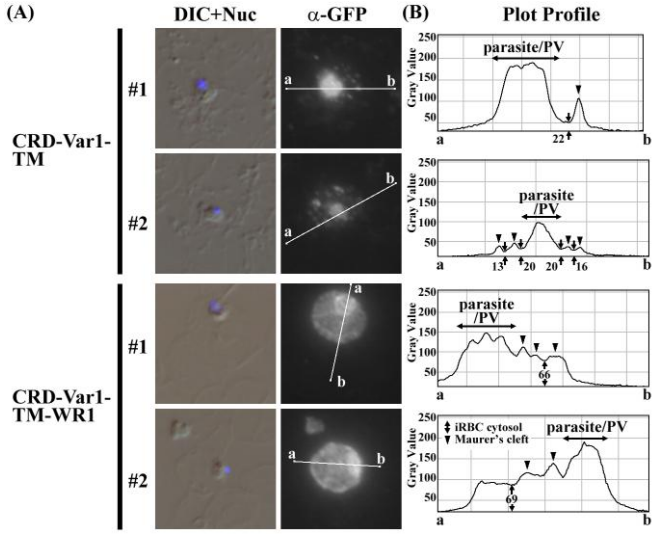

Figure 4. Comparison of the signal intensity between SURFIN$_{4.2}$CRD-Var1-TM and SURFIN$_{4.2}$CRD-Var1-TM-WR1 proteins in the parasite-infected red blood cell. (A) Indirect immunofluorescence assay with anti-GFP antibody was performed for both parasites expressing SURFIN$_{4.2}$CRD-Var1-TM and SURFIN$_{4.2}$CRD-Var1-TM-WR1 at the same time. The fluorescence signal intensities were measured from point (a) to (b). (B) Plot profiles were made by using ImageJ software for the regions shown in panel A. parasite/PV indicates parasite cytosol or parasitophorous vacuole. Signal intensities are shown by gray level pixel intensity values.

Alexa-Fluor 488-conjugated secondary antibody gave clearer images than the double staining using the Zenon antibody-labeling kit, we used two representative single staining images to measure and compare the signal intensity of the recombinant proteins in the iRBC cytosol for SURFIN$_{4.2}$CRD-Var1-TM and SURFIN$_{4.2}$CRD-Var1-TM-WR1. After subtracting background signals, signal intensities in the iRBC cytosol for SURFIN$_{4.2}$CRD-Var1-TM were 13 to 22 units (Fig. 4B; CRD-Var1-TM #1 and #2), whereas those for SURFIN$_{4.2}$CRD-Var1-TM-WR were 66 and 69 units (Fig. 4B; CRD-Var1-TM-WR1 #1 and #2, respectively). This indicates that the fluorescence signal in the iRBC cytosol is weaker for SURFIN$_{4.2}$CRD-Var1-TM than SURFIN$_{4.2}$CRD-Var1-TM-WR1 and suggests that the SURFIN$_{4.2}$CRD-Var1-TM is less abundant in iRBC cytosol than SURFIN$_{4.2}$CRD-Var1-TM-WR1.

In order to evaluate their solubility, parasite proteins were sequentially extracted by a repeated-freeze thaw procedure (FT; water-soluble fraction protein), followed by Tx extraction (Tx; membrane bound protein), and SDS extraction (SDS; Tx-insoluble fraction) and were detected with rabbit anti-GFP antibody. About 105-kDa bands were detected for SURFIN$_{4.2}$CRD-Var1 and SURFIN$_{4.2}$CRD-Var1-TM and a 230-kDa band for SURFIN$_{4.2}$CRD-Var1-TM-WR1 by Western blot. Expected band sizes were 83, 86, and 158 kDa, respectively (Fig. 5A). Although the band sizes detected by Western blot are much larger than the expected size, this is not an uncommon observation for *P. falciparum*-derived proteins which have a deviated amino acid composition due to a highly A/T-rich genome (76.3% in the exon) [33]. In addition to the target protein bands, a ~60-kDa band was observed for all fractions (Fig. 5A), but this band was also observed in the extract from the wild-type non-transfected MS822 parasite, and so was not derived from the recombinant proteins expressed in the transfected parasite lines. Although the identity of this band is unclear, it is likely derived from parasites, because this band was not observed in the extract from the

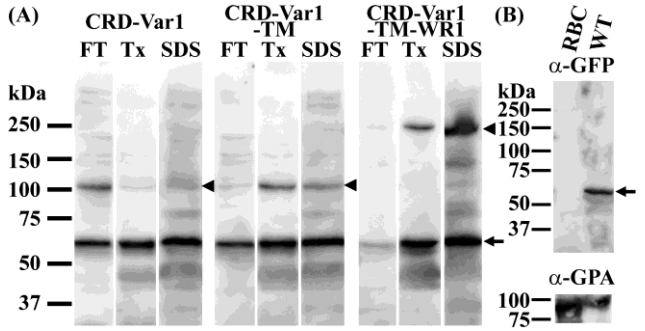

Figure 5. Western blot of SURFIN$_{4.2}$CRD, SURFIN$_{4.2}$CRD-TM, and SURFIN$_{4.2}$CRD-TM-WR1 proteins subjected to different extraction procedures. (A) Parasite proteins were sequentially extracted by repeated-freeze thaw procedure (FT; soluble fraction protein), followed by Triton X-100 extraction (Tx; membrane bound protein), and SDS extraction (SDS, Tx-insoluble fraction) and detected with rabbit anti-GFP antibody. (B) Triton X-100 extracts of parasite-uninfected red blood cell (RBC) and the wild-type MS822 parasite line (WT) were subjected for Western blot analysis with rabbit anti-GFP (α-GFP) or mouse anti-glycophorin A (α-GPA). The arrow marks a ~60-kDa band also observed in wild-type parasites, but not in uninfected RBC. Arrowheads indicate expressed recombinant proteins, SURFIN$_{4.2}$CRD, SURFIN$_{4.2}$CRD-TM, and SURFIN$_{4.2}$CRD-TM-WR1.

parasite-uninfected RBC (Fig. 5B). Positive glycophorin A bands for the extracts from both the wild-type parasite and the uninfected RBC indicated the protein extraction from the uninfected RBC was successful. It should be noted that the rabbit anti-GFP antibodies did not show any signal when wild-type parasites were subjected to IFA. We found that SURFIN$_{4.2}$CRD-Var1 was exclusively detected in the soluble FT fraction, indicating that this protein was in soluble form, which is consistent with the observation of its localization in the parasite's cytoplasm (Fig. 3). SURFIN$_{4.2}$CRD-Var1-TM was detected in the Tx-soluble fraction more abundantly than in the Tx-insoluble SDS fraction. Conversely, SURFIN$_{4.2}$CRD-Var1-TM-WR1 was detected in the SDS fraction more abundantly than in the Tx fraction. Thus both proteins appeared to be associated with membrane structures, and the cytoplasmic region containing the WR1 may be responsible for their difference in solubility.

3.3. SURFIN$_{4.2}$ is trafficked to the iRBC cytosol in a PEXEL-independent manner

SURFIN$_{4.2}$ contains two PEXEL-like sequences, one was termed Pexel 1 in this study and was located at the N-terminal region, spanning aa 25 - 29 (R_KI_FE), for which the 3rd position was isoleucine instead of leucine in the authentic PEXEL motif. The other was termed Pexel 2, and was located in the CRD at aa 118 - 122 (R_TL_ED). In order to evaluate their involvement in the transport of the protein into the iRBC, we generated two parasite lines expressing SURF$_{4.2}$CRD-Var1-TM-Pexel-1mut or SURF$_{4.2}$CRD-Var1-TM-Pexel-2mut, for which the conserved residues of the PEXEL-like sequence were replaced by alanine (A_KA_FA or A_TA_EA, respectively). Double staining IFA revealed that SURFIN$_{4.2}$CRD-Var1-TM-Pexel-1mut and -2mut both showed a punctate dot pattern in the iRBC that colocalized with the Maurer's cleft protein SBP1 along with parasite localized fluorescence (Fig. 6A). Thus, there was no appreciable difference between the trafficking of these proteins and that of the original SURFIN$_{4.2}$CRD-Var1-TM recombinant protein. These observations suggest that the PEXEL-like sequences of SURFIN$_{4.2}$ play no evident function in the transport of SURFIN$_{4.2}$ to the iRBC cytosol

and Maurer's clefts. Thus, SURFIN$_{4.2}$ is being trafficked as a PNEP.

3.4. Removal of N-terminal 42 amino acid segment, CRD, or Var1 did not prevent the SURFIN$_{4.2}$ trafficking to the iRBC cytosol and Maurer's clefts.

To further evaluate the importance of the different regions of the SURFIN$_{4.2}$ extracellular region in the trafficking of the protein to the iRBC, we generated three following parasite lines: Two lines expressing SURFIN$_{4.2}$Var1-TM or SURFIN$_{4.2}$CRD-TM, in which the CRD or Var1 region were deleted from SURFIN$_{4.2}$CRD-Var1-TM and the one line expressing SURFIN$_{4.2}$CRD-Var1-TM-RepN, in which the N-terminal first 42 amino acids of SURFIN$_{4.2}$CRD-Var1-TM was replaced by the N-terminal first 15 amino acids of *P. falciparum* adenylosuccinate lyase (*Pf*ASL), an enzyme involved in the purine metabolism in the cell cytosol and is not considered to be transported to the iRBC [34]. Double staining IFA revealed that SURFIN$_{4.2}$Var1-TM and SURFIN$_{4.2}$CRD-TM colocalized with SBP1 in a punctate dot pattern in the iRBC and was also present in the parasite cytoplasm. No difference was observed between these two lines and the line expressing SURFIN$_{4.2}$CRD-Var1-TM (Fig. 6B). A more diffused iRBC localization with less obvious dot pattern formation was observed with the line expressing SURF$_{4.2}$CRD-Var1-TM-RepN compared to that expressing SURFIN$_{4.2}$CRD-Var1-TM. Nonetheless, the transport of the SURF$_{4.2}$CRD-Var1-TM-RepN protein was not completely abrogated and signals, although faint, still colocalized with the Maurer's cleft SBP1. Thus, any of the N-terminal segment (aa 1 - 42), the CRD (aa 46 - 196), or the variable region (aa 198 - 733) at the extracellular region of SURFIN$_{4.2}$ do not carry a specific motif necessary for protein transport to the iRBC.

To confirm these findings, we truncated the entire external domain from the mini-SURFIN$_{4.2}$ protein, and added 50 amino acids (SSGQVRRSGGQGSETYIVGTSQSGFHKNEVIPSIKDK SGKTQIVSNEKGG) preceding the TM region in order to support the integrality of the TM region for membrane insertion, to generate a parasite expressing SURFIN$_{4.2}$VarC-TM-WR1, thus this protein contains 50 amino acids derived from SURFIN$_{4.2}$ followed by a triple HA tag as an extracellular region. The recombinant protein was transported to the iRBC and observed in a punctuate dot pattern in the iRBC cytosol, colocalizing with SBP1 (Fig. 7). This indicates that the extracellular region of SURFIN$_{4.2}$ is not required for the trafficking of the protein to the iRBC.

4. Discussion

In this study, we generated GFPm2-fused mini-SURFIN$_{4.2}$ proteins that, following their transfection into a *P. falciparum* parasite line, was observed to be trafficked into the iRBC and Maurer's clefts. Using this system, we then attempted to identify the specific region of the protein responsible for the iRBC and/or Maurer's cleft localization. We found that the TM region, but not the cytoplasmic region containing WR domain was essential for protein transport. We consider it likely that the TM region is responsible for initiating the trafficking of the protein into the ER. Two PEXEL-like sequences were found not to be essential for the movement of the protein into the iRBC and Maurer's clefts, indicating that SURFIN$_{4.2}$ trafficking is

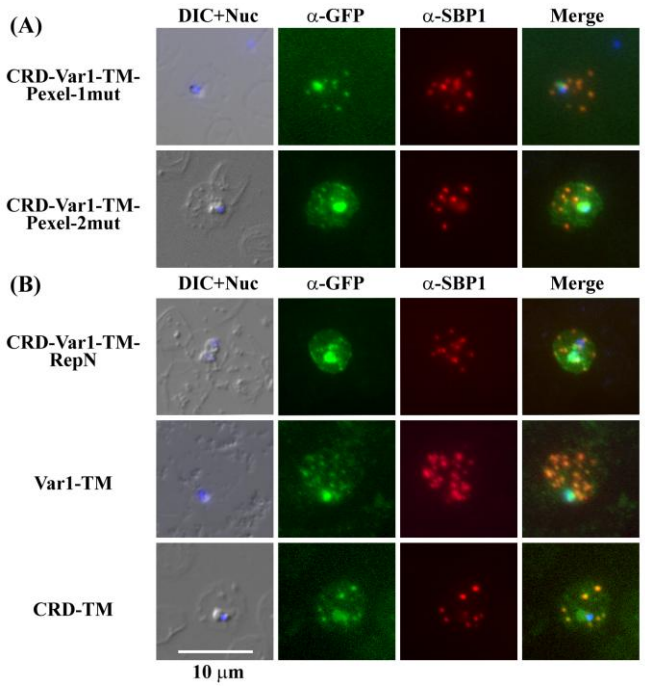

Figure 6. Indirect immunofluorescence assay of modified SURFIN$_{4.2}$CRD-Var1-TM proteins. (A) Double staining for SURFIN$_{4.2}$CRD-Var1-TM-Pexel-1mut and CRD-Var1-TM-Pexel-2mut, and for (B) CRD-Var1-TM-RepN, Var1-TM, and CRD-TM proteins with anti-GFP (α-GFP, green) and anti-SBP1 (α-SBP1, red). Nuclei were stained with DAPI. Negative control using normal rabbit antibody did not produce detectable signals (not shown). CRD, cysteine-rich domain; RepN, N-terminus replacement; TM, transmembrane; and Var1, variable region 1.

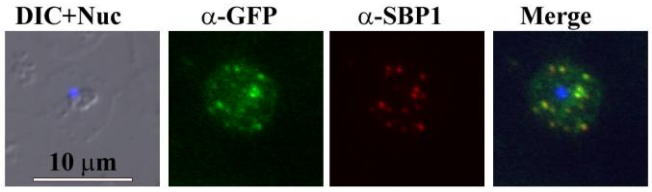

Figure 7. Indirect immunofluorescence assay of SURFIN$_{4.2}$VarC-TM-WR1 protein. Double staining with anti-GFP (α-GFP, green) and anti-SBP1 (α-SBP1, red). Nuclei were stained with DAPI. Negative control using normal rabbit antibody did not produce detectable signals (not shown).

PEXEL-independent. N-terminal replacement, deletion of the CRD or Var region did not prevent iRBC and Maurer's cleft localization, suggesting that no trafficking motif exists in these regions.

By sequential extraction of recombinant SURFIN$_{4.2}$ proteins, we found that mini-SURFIN$_{4.2}$ with an intact WR domain showed more resistance to Triton-X 100 extraction than a similar protein in which WR domain had been removed. As endogenous SURFIN$_{4.2}$ was insoluble in Triton-X 100 but soluble in SDS [26], we suggest that the cytoplasmic region, probably the WR domain, contributes to this difference. Insolubility with a neutral detergent such as Triton-X 100, was also reported for *Pf*EMP1 [35]. A large degree of sequence similarity was shown between the cytoplasmic WR domain of SURFIN$_{4.2}$, *Pf*EMP1, and another iRBC protein *Pf*332 [26]. The cytoplasmic regions of both *Pf*EMP1 and *Pf*332 are known to bind to RBC actin, the former also binding to spectrin [36, 37]. Therefore, we suggest that the WR domain of SURFIN$_{4.2}$ also associates with the RBC cytoskeleton, although further evaluation is required to assess this hypothesis.

Similar to most of the PNEPs reported so far, the SURFIN$_{4.2}$ TM region was found to be essential for protein trafficking. *Pf*SBP1 [11], MAHRP1 [19] and REX2 [24] share this feature, with their TM regions known to play

important roles in protein transport. However, the N-terminal sequence of these PNEPs was also found to be essential for correct protein trafficking. In addition to the TM region, *Pf*SBP1 was shown to require the N-terminal segment at aa 16 – 26, which contain highly negative net charge residues (DEPTQLQDAVP) for transport into the iRBC [11]. This may also be the case for MAHRP1, as the N-terminal 50 amino acids of this protein, which is acidic, along with *Pf*SBP1 TM region is able to transport protein into the iRBC [11]. Conversely, REX2 appears to contain region resembling a PEXEL motif after cleavage in the ER at aa 5 – 10 ($L_5xE_7hhS_{10}$; h indicates hydrophobic residues), for which only the glutamate residue at aa 7 was found to be critical for trafficking [24]. In the mini-SURFIN$_{4.2}$ proteins we expressed in this study, none of the regions from the SURFIN$_{4.2}$ extracellular regions shown to be indispensable for trafficking to the iRBC, thus the trafficking of SURFIN$_{4.2}$ appears not depend on specific sorting signals, nor potential escorter proteins, but other factors in addition to the TM region.

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