Export of virulence proteins by malaria-infected erythrocytes involves remodelling of host actin cytoskeleton

Melanie Rug 1,*, Marek Cyrklaff 2, Antti Mikkonen 3, Leandro Lemgruber 2,¶, Simone Kuelzer 3, Cecilia P. Sanchez 2, Jennifer Thompson 1, Eric Hanssen 4, Matthew O’Neill 1, Christine Langer 1, Michael Lanzer 2, Freddy Frischknecht 2, Alexander G. Maier 3 and Alan F. Cowman 1,5,*

1 The Walter and Eliza Hall Institute of Medical Research, Parkville 3050, Australia
2 Department of Infectious Diseases-Parasitology, University of Heidelberg, 69120 Heidelberg, Germany
3 Research School of Biology, The Australian National University, Canberra, Australia
4 Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Vic., 3010, Australia and 5 Department of Medical Biology, University of Melbourne, Melbourne 3010, Australia

Address correspondence to: cowman@wehi.edu.au and melanie.rug@anu.edu.au

Alan F. Cowman, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3052, Australia.
Phone: +61 3 93452446
Melanie Rug, Centre for Advanced Microscopy, The Australian National University, Canberra 0200, Australia
Phone: +61 2 61257649

Running head: Plasmodium protein and host actin cytoskeleton
Key points:

1. Maurer’s clefts are *Plasmodium falciparum*-derived membranous structures within the host erythrocyte that are essential for parasite survival.
2. PfPTP1 functions in a large complex of proteins and is required for linking of Maurer’s clefts to the host actin cytoskeleton.

Abstract

Following invasion of human red blood cells (RBC) by the malaria parasite, *Plasmodium falciparum*, a remarkable process of remodelling occurs in the host cell mediated by trafficking of several hundred effector proteins to the RBC compartment. The exported virulence protein, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), is responsible for cytoadherence of infected cells to host endothelial receptors. Maurer’s clefts are organelles essential for protein trafficking, sorting and assembly of protein complexes. Here we demonstrate that disruption of PfPTP1 (PfEMP1 trafficking protein 1) function leads to severe alterations in the architecture of Maurer’s clefts. Furthermore, two major surface antigen families, PfEMP1 and STEVOR, are no longer displayed on the host cell surface leading to ablation of cytoadherence to host receptors. PfPTP1 functions in a large complex of proteins and is required for linking of Maurer’s clefts to the host actin cytoskeleton.

Key Words: malaria; virulence proteins; PfEMP1; effector proteins; *Plasmodium falciparum*; protein trafficking
Introduction

*Plasmodium falciparum* causes the most severe form of malaria in humans. Circulating red blood cells (RBCs) are invaded by *P. falciparum* merozoites, which develop into schizonts generating more merozoites that in turn are released to invade new erythrocytes. Profound structural and morphological changes occur in parasite-infected erythrocytes that alter their physical properties and impair circulation *in vivo*\(^1,2\). Parasitised RBCs become rigid and adhere to a variety of cell types. This can lead to perturbation or obstruction of blood flow in the microcirculation of organs and is related to parasite-induced alterations of both their biomechanical and adhesive properties, central to survival and pathogenicity of *P. falciparum*\(^3\).

Cytoadherence is conferred by the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which binds to various ligands displayed on the microvasculature of organs (reviewed in \(^4\)). To reach the erythrocyte surface PfEMP1 traverses the ER of the parasite and is transported across the parasite plasma membrane and parasitophorous vacuolar membrane (PVM), which surrounds the parasite. The further route of PfEMP1 is largely unknown, it is either trafficked through the erythrocyte cytosol via a vesicular pathway or in a complex where it transiently associates with parasite-induced membranous compartments, named Maurer’s clefts (MC)\(^5\). The protein is subsequently translocated onto RBC membranes and anchored in protrusions of the membrane (knobs)\(^6\). Knobs are points of elevation and concentration for PfEMP1 anchoring to host cytoskeleton facilitating binding to receptors on host cells.

Central to survival of *P. falciparum* is extensive remodelling of the RBC, a host cell lacking an established protein trafficking network. To provide protein trafficking routes in the host cell the parasite exports many proteins. These cross the parasite plasma membrane and the PVM to reach their subcellular destination in the RBC. Most exported proteins contain a signal sequence for entry into the ER\(^7-9\) and a PEXEL/VTS motif\(^10,11\), which confers trafficking to the RBC\(^12,13\). These proteins move through the parasitophorous vacuole and PVM via a translocator machine\(^14-16\). A number of exported proteins do not contain a PEXEL/VTS motif and are thus called PEXEL-negative exported proteins (PNEPs), their transport pathways are unknown\(^5,17\).
Various proteins play a role in trafficking PfEMP1 to the *P. falciparum*-infected RBC membrane and its assembly into knob structures (reviewed in 18). In the absence of PNEPs Skeleton Binding Protein 1 (SBP1) 19,20, Maurer’s Clefts-Associated Histidine-Rich protein 1 (MAHRP1) 21 or Ring Exported Protein 1 (REX1) 22 PfEMP1 cannot be displayed on the RBC membrane. Additionally, a large-scale gene-knockout screen in *P. falciparum* identified other proteins required for trafficking and function of PfEMP1 23. MC are parasite-derived structures that appear in the RBC cytosol in the early ring stage of the parasite blood stage life cycle 5. It has been suggested that PfEMP1 trafficking from the MC to the RBC membrane occurs through vesicular transport along actin filaments 24-26. There are a number of different vesicles described in *P. falciparum* including electron dense vesicles (EDVs), J-dots and vesicle like structures (VLS), which could play a role in PfEMP1 trafficking 27-31.

We have characterised a novel PEXEL-containing protein, that we have named *P. falciparum* PfEMP1 trafficking protein.

**Materials and methods**

See Supplementary material for full methods.

**Parasites and cell culture of *P. falciparum***

Parasites used in this study were *P. falciparum* strain CS2 and CS2/PTP1-HA (Haemagglutinin/Streptavidin-tagged; Fig. S1 C), CS2/PTP1-GFP and CS2ΔPTP1 transfectants 23.

**Fluorescence Microscopy**

Thin blood smears of parasitised cells were fixed in Acetone/Methanol (90/10). Cells were labelled with antibodies α-PTP1 antibody; 1:500, α-SBP1 (1:500), α-REX1 32 (1:2000), α-GFP (1:500), α-HA (1:100), α-MAHRP1 (1:100) 33, α-Pf332 (1:500) 34, α-STEVOR (1:500) 35, α-Rif29 36, α-Hsp70-x (1:200) 37, α-PTP2; (1:250) 38. Cells were viewed with a Zeiss Plan-Apochromat 100 ×/1.4 numeric aperture oil-immersion lens on a Zeiss Axioskop 2 microscope equipped with a PCO SensiCam (12 bit) camera.
Subcellular fractionation analysis, SDS-PAGE and Western Blots

Immunoblots were probed with either mouse α-HA antibodies (Roche; 1:1000) or rabbit α-PfHSP70 (1:1000). Solubility assays were performed as described \textsuperscript{6,39,40} and probed with mouse α-HA antibodies (Roche; 1:1000), stripped and reprobed with control antibody SBP1 (rabbit; 1:500). The trypsin cleavage assays were performed as described \textsuperscript{41}.

Blue Native and two dimensional-PAGE analysis and Electron Microscopy and Tomography

The blue native-PAGE was performed as described \textsuperscript{42}.

For cryo-electron tomography, cells were plunge-frozen and tilt series were recorded in an FEI Polara electron microscope (EMBL Heidelberg, Germany), with an acceleration voltage of 300kV. The tomograms were calculated by back-projection of 50–60 images aligned by fiducial markers using the EM-image processing package and the TOM package on MATLAB. The volumes were denoised with nonlinear anisotropic diffusion methods, and the surface rendering was done using the AMIRA volume processing software (Amira TGS Europe S.A., France).

\textit{In vitro actin polymerisation assay}

The PfPTP1-peptide (JPT, Berlin, Germany) was resuspended in 5 mM Tris-HCl; pH 8.0, 0.2 mM CaCl\textsubscript{2}. Actin polymerization assays were conducted as described in the Actin Polymerization Biochem Kit, from Cytoskeleton Inc.

Results

PfPTP1 is exported and associates with membranous vesicles

PfPTP1 is required for trafficking and display of PfEMP1 on the surface of \textit{P. falciparum}-infected RBCs but its function is unknown \textsuperscript{23}. PfPTP1 is a protein of 290 amino acids with a signal sequence, PEXEL \textsuperscript{10,11} and transmembrane domain consistent with export to parasite-infected RBCs (Fig.1A). To address PfPTP1’s function we showed it was expressed across the asexual cycle and located in punctate structures, surrounding the intracellular parasite, that bud from the PVM (Fig.1B, C). During development these structures increase in size contacting the RBC membrane in the mature parasite (Fig.1C). In rupturing schizonts PfPTP1 associated organelles...
appear as remnants between merozoites (Fig. 1C). Live imaging of green fluorescent protein (GFP)-tagged PfPTP1 showed large and smaller punctate organelles with the larger structures likely being MC ⁸ (Fig. S1B; Movies 1,2). This suggests PfPTP1 is associated with MC in the *P. falciparum*-infected RBC.

**PfPTP1 is an integral Maurer’s clefts membrane protein**

To confirm PfPTP1 localisation to MC, *P. falciparum* lines expressing PfPTP1-GFP or PfPTP1-HA (Fig. S1C) were used in immunofluorescence experiments. Antibodies against PfPTP1 recognised the same structures as α-GFP and α-HA antibodies reflecting the localisation of the endogenous protein (Fig. 2A, 2B). Other antibodies raised against the C-terminus of PfPTP1 showed an identical localisation.

PfPTP1 localises to the same structures as MC protein SBP1; however, there was not complete overlap suggesting differences within these organelles (Fig. 2C). MCs are comprised of sub-compartments and resident proteins can display different labelling patterns ²⁹,⁴³. Similar results were obtained for MAHRP1, a second MC protein (Fig. S1A). To determine if PfPTP1 was localised to transport vesicles within the RBC cytoplasm we performed co-localisation studies with PfPTP2, a marker for electron dense vesicles (EDV) ³⁸ and PfHsp70-x which is associated with J-dots ³⁷. PfPTP1 did not overlap with PfPTP2 indicating it was not localised to EDVs (Fig. 2D). In some instances EDVs were closely associated with PfPTP1-labelled MC confirming a connection between these structures in transport processes. PfHsp70-x co-localised with PfPTP1 in some areas (Fig. 2E). The small mobile vesicles found in PfPTP1-GFP time-lapse studies (Movies 1; 2) combined with overlap of PfHsp70-x signal suggests localisation of some PfPTP1 to J-dots as well as MC.

To determine if the transmembrane region of PfPTP1 was embedded in membranes of MC its solubility characteristics were determined. PfPTP1 was partially extractable with urea and Triton X-100 but insoluble in sodium carbonate (Fig. 2F) ³⁹. PfPTP1 showed similar properties to SBP1, a known MC transmembrane protein ⁴⁰ (Fig. 2F) suggesting PfPTP1 is a transmembrane protein embedded in MC membranes.

To determine orientation of PfPTP1 in MC *P. falciparum*-infected RBCs were treated with detergents and proteases (Fig. 2G). Tetanolysin O forms pores in RBC membranes and exposes proteins present in infected host cells (Fig 2G) ⁴⁴. Saponin
exposes proteins of the RBC cytoplasm, the PVM and MC (Fig. 2G). Triton X-100 solubilises all membranes and releases proteins from each compartment. Triton X-100 treatment solubilized PfPTP1 consistent with results from solubility assays (Fig. 2F) and upon proteinase K treatment it was degraded and therefore accessible. In saponin-treated samples most PfPTP1 was in the pellet but degraded by proteinase K as the MC lumen becomes accessible by this treatment. Tetanolysin O treatment renders the infected-RBC cytoplasm accessible and PfPTP1 was present in the pellet. After proteinase K treatment PfPTP1 was not detectable with αPTP1-C but a 17 kDa band was observed with α-PTP1-N antibodies. Control antibodies for MC, PVM and PV gave the expected results (Fig. 2G). Taken together this data shows the C-terminal region of PfPTP1 is exposed to the RBC cytosol whereas the N-terminus faces the MC lumen.

**PfPTP1 function is required for trafficking of PfEMP1 and STEVOR to Maurer’s clefts**

To determine if PfPTP1 was required for subcellular localisation of proteins trafficking to MC we analysed their subcellular localization in CS2ΔPTP1. PfEMP1 accumulates in MC in trophozoites (Fig. 3A) and then moves to the surface of parasite-infected RBCs. However, in CS2ΔPTP1 PfEMP1 was stalled and accumulates in the parasite (Fig. 3A). STEVOR, an exported protein family that traffics through MC, has been detected on the parasite-infected RBC surface. Similar to the trafficking defect observed for PfEMP1, absence of PfPTP1 function leads to accumulation of STEVOR at the PV (Fig. 3B). These results show PfPTP1 is required for trafficking of PfEMP1 and STEVOR across the PVM to MC and the parasite-infected RBC surface consistent with an overlapping pathway.

In contrast, the MC resident protein SBP1 (Fig. 3C), ring-exported protein 1 (REX1) (Fig. 3D), MAHRP 1 and Pf332, which are PNEP exported proteins (Fig. S1D), are exported into the RBC cytoplasm but their localisation was different to CS2. These proteins localise to small puncta within the RBC cytoplasm of CS2ΔPTP1-infected cells instead of MC in the parental line. A similar result was observed for PEXEL-containing A-Rifin (Fig. S1D), which is also a MC protein; however, a high proportion of this protein was trapped within the parasite. The aberrant localisation of MC proteins in CS2ΔPfPTP1-infected RBCs shows the structure of these organelles was disturbed. We could not generate a CS2ΔPfPTP1
parasite line in which the *pfptp1* gene has been complemented despite several attempts. Therefore we generated a second independent CS2ΔPfPTP1 (CS2ΔPfPTP1.2) parasite line (Fig. S2A) and confirmed the phenotype (Fig. S2B and C) 23. This shows PfPTP1 function is required for export of some proteins and causes a significant disturbance of the localisation of other exported proteins.

**PfPTP1 function is required for Maurer’s clefts architecture**

To understand the defect in MC architecture caused by loss of PfPTP1 function the ultrastructure of CS2ΔPTP1-infected RBCs was analysed using transmission electron microscopy. In CS2 typical single lamellae for MC were observed with an electron dense coat and translucent lumen (Fig. 4Aa). In contrast, CS2ΔPTP1 displayed electron dense areas randomly distributed through the RBC cytoplasm (Fig. 4Ab). To identify these structures we used equinatoxin II to selectively form pores in the RBC membrane, which left other membranes intact so haemoglobin, which obscures features in the cytoplasm, was removed 50. Antibodies to PfPTP1 and REX1 were used to identify MC in CS2. The α-PfPTP1 antibodies line the MC lamella similar to REX1 localisation (Fig. 4B). Vesicular structures were observed that appeared to bud off MC and these had a dense lumen and less distinct membranes. These may be vesicles involved in trafficking between PVM and MC and/or MC to the RBC membrane (i.e. J-Dots). These structures were decorated with PfPTP1 and REX1 antibodies (Fig. 4Ba,b) consistent with PTP1-GFP fluorescence on smaller punctate structures in CS2/PTP1-GFP parasites (Movie 1;2). In CS2ΔPTP1 the architecture of MC was disrupted showing large aggregated globular structures (Fig. 4Bc,d). SBP1 and REX1 were located on these structures reflecting the results obtained by IFA (Fig. 3). These structures contained “budding vesicular structures” which were labelled with both REX1 and SBP1 antibodies (Fig. 4Bd). Therefore loss of PfPTP1 function results in disruption of MC architecture.

To understand the globular structures of CS2ΔPTP1 in 3-D the parasite lines were analysed by transmission electron tomography. Analysis of tilt series of tomograms confirmed the globular structures were in close proximity but not connected (data not shown). To exclude artefacts introduced by chemical fixation and dehydration, the cells were studied after vitrification via cryo-electron microscopy preserving the structure in a state close to live 51,52. Single lamellae of MC were observed in CS2 (Fig. 4Ca) whereas globular structures were observed for CS2ΔPTP1
Tilt series were taken for CS2 and CS2ΔPTP1 and representative tomograms and models are shown (Fig. S3A, Fig. S3B). Fig. S3 shows a single MC lamella versus the globular structures in CS2ΔPTP1. In the tomogram of CS2 a tethering structure was observed extending from MC towards the RBC membrane, similar to that described previously (Fig. S3A, small arrowheads) \(^31,41,53,54\). Also large globular structures with the same density were observed (Fig. S3A, large arrowheads). We hypothesise these structures are transport vesicles from MC similar to the previously described electron dense vesicles (EDV) \(^29,43\).

The state of actin filaments was compared between CS2ΔPTP1 and CS2-infected RBCs by iterative denoising of the tomograms. In CS2, MC linked to the RBC via actin filaments were observed consistent with a previous study \(^51\). Foci of actin attachment were more prominent around knob structures (red; Fig. 5A). In contrast, CS2ΔPTP1-infected RBCs displayed short actin filaments that did not link to the globular remnants of MC, the RBC membrane, knobs or the cytoskeleton of the host membrane (Fig. 5B). This suggested that PfPTP1 function is associated with organisation of the host actin cytoskeleton.

To determine whether PfPTP1 interacts with host cell actin we used *in vitro* actin polymerisation assays. This showed no difference in polymerization kinetics if actin was incubated with a peptide from the C-terminus of PfPTP1 (domain in the RBC cytoplasm) or a scrambled peptide (Fig. 5D). However a larger number of longer actin filaments were observed in the presence of the PfPTP1 peptide (Fig. 5C, S4). This reflects the cryo-EM data (Fig. 5A and B) and suggests PfPTP1 plays a role in organisation of host actin filaments, which is required for architecture of MC.

**PfPTP1 interacts with PfEMP1 and Maurer’s clefts resident proteins**

For PfPTP1 to perform its function in MC it likely interacts with other proteins and to identify potential interacting partners immunoprecipitation experiments were performed. CS2/PTP1-HA- and CS2 infected RBCs were solubilised and PfPTP1-HA immunoprecipitated with α-HA-antibody beads (Fig. S5A-C). Specific protein bands immunoprecipitated from CS2ΔPTP1-HA parasite line and corresponding regions from the CS2 lane were excised from the SDS-PAGE gel and subjected to mass spectrometry (Fig. S5B, Table S1). Table S2 shows specific proteins with a PEXEL motif or known exported proteins identified by mass-
spectrometry with more than two peptides and/or more than 5% coverage. Interestingly, exported proteins SBP1, PFE0060w (PIESP2), PFA0670c (hyp8), PF13_0076 and PFE1600w (PHISTb) were immunoprecipitated (Table S2). 15 peptides corresponded to VAR2CSA, the PfEMP1 variant expressed in CS2. This suggests PfPTP1 may interact directly with PfEMP1, or in a large complex that includes these proteins, to play a role in displaying this virulence protein on the surface of *P. falciparum*-infected RBC.

To confirm the proteins binding to PfPTP1 immunoprecipitations were performed and probed with α-SBP1, α-PfEMP1 and α-PIESA2 antibodies. This suggested binding for these proteins (Fig. 6A) whilst in comparison other MC proteins such as MAHRP1 and REX1 showed no detectable PfPTP1 binding (data not shown). In reciprocal experiments endogenous untagged PfPTP1 was immunoprecipitated from CS2-infected RBCs with α-SBP1 antibodies confirming this interaction (Fig. 6B). However, we could not detect bands in the reciprocal experiments with α-PfEMP1 (against the ATS region of the protein) and α-PIESA2. This might be due to masking of the PfPTP1-interacting region of the two proteins by the antibodies under the experimental conditions.

To determine the PfPTP1 complex size two-dimensional Blue Native Gel analysis was used to separate proteins under native conditions in the first and under reducing conditions in the second dimension. A complex of approximately 420 kDa was detected containing PfPTP1 (Fig. 6C; blue band) and SBP1 (Fig. 6C; green band). Furthermore, a smaller complex of approximately 130 kDa was also detected that included PfPTP1 and SBP1. This indicated PfPTP1 functions as a high molecular weight complex with SBP1 that likely includes additional *P. falciparum* proteins.

**Discussion**

MC are essential trafficking nodes for proteins conferring cytoadherence of parasite-infected RBCs to microvasculature in the host. In particular, they are important for assembly of PfEMP1 into a cytoadherence complex for insertion into *P. falciparum*-infected RBC membranes. A number of proteins resident in MC have been identified required for PfEMP1 trafficking but their functions are unknown. In this study we show PfPTP1 is required for trafficking of PfEMP1 to the *P. falciparum*-infected RBC surface and MC architecture. PfPTP1 functions in a large
complex that includes at least SBP1 and is the first protein shown to have a role in linking MC to the host actin cytoskeleton.

PfPTP1 is exported and inserted into MC membranes with the N-terminus located within the lumen and the short cytoplasmic tail protruding into the cytosol of P. falciparum-infected RBCs adopting the same orientation as other MC-associated proteins, MAHRP1 and SBP1. These PNEPs are required for trafficking of PfEMP1 to the surface of the infected RBC; however, their role is not known. PfPTP1 is required for this same process; however, it is a PEXEL-containing protein. In the absence of PfPTP1 function PfEMP1 remained associated with the parasite indicating its function is required for transfer of PfEMP1 across the PVM to MC. Whilst PfPTP1 showed co-localization with SBP1 and MAHRP1 on MC there were areas of non-overlap consistent with their structural and functional compartmentalization. PfPTP1 was observed on small vesicles in close contact with MC lamellae. These structures were evident in absence of PfPTP1 and therefore its function isn’t required for their formation. They may be related vesicular structures called J-dots observed in the cytosol of parasite-infected RBCs and the partial co-localisation of PfPTP1 with PfHsp70-x supports this notion. J-dots may be chaperone complex transport vehicles and their membrane characteristics are different from MC. It was suggested J-dots could be protein-cholesterol aggregates or vesicular structures of an unusual lipid composition. This may explain absence of a visible membrane in our EM studies around vesicular structures we observed. PfPTP1 and SBP1 occur on these structures and it has been shown that PfEMP1 is found on structures with very similar characteristics.

Models have been suggested for trafficking PfEMP1 to MC and its final destination on the P. falciparum-infected RBC surface including: 1) loading of PfEMP1 onto nascent MC as they bud from the PVM, 2) transport in vesicles to MC, 3) transport as soluble complexes that insert into fully formed MC. Recent studies show MC bud from the PVM and establish in the RBC cytoplasm after invasion before expression of proteins normally resident in or travelling through these organelles, including PfEMP1. Therefore, MC associated proteins translated later have to make their way across the PVM and RBC cytosol to reach these organelles arguing against the first model.

Here, we demonstrate that in the absence of PfPTP1 function PfEMP1 is stalled before the PVM. The presence of PfPTP1 as part of a large complex associated
with SBP1 is consistent with them functioning together in trafficking of PfEMP1 to MC. Complex formation explains why lack of SBP1 function also leads to loss of PfEMP1 on the RBC surface \(^{19,20}\). The presence of mobile vesicular structures containing PfPTP1-GFP in the RBC cytosol supports a hybrid model of the latter two listed above where PfEMP1 traffics to MC in a complex associated with vesicular structures like J-dots \(^{30}\). We hypothesise the complex forms upon translocation of the involved molecules across the PVM or even within certain compartments of the PV and provide a shuttling modality for PfEMP1 to reach MC. A recent report showed PfEMP1 accumulates in membranous structures in the PV in ring stages before translocation across the PVM \(^{31}\). These regions may contain docking signals for proteins to travel as complexes even if they have different expression profiles. The overlapping localisation of SBP1 and PfPTP1 on MC may be docking points for the complex onto these organelles. The additional presence of SBP1 and PfPTP1 in separate compartments on MC points towards further functions for these proteins.

The MC resident proteins studied (SBP1, MAHRP1, Pf332, A-Rifin and REX1) are trafficked to the remnant MC structures in the absence of PfPTP1 function. This suggests PfPTP1 is not required for trafficking of these proteins. Interestingly, STEVOR cannot be trafficked to MC in CS2ΔPTP1-infected RBCs similar to PfEMP1. STEVORs are PEXEL-containing proteins, which are processed by plasmepsin V whereas PfEMP1 is a PNEP and not cleaved by this enzyme \(^{58}\). In contrast to MC proteins, which are trafficked across the PVM in absence of PfPTP1, the final destination for STEVORs and PfEMP1 is the RBC surface. Therefore, although these proteins might be processed differently in their early trafficking pathway they share trafficking modalities once reaching the PVM. We could not identify STEVOR as one of the interacting partners of PfPTP1 so its exact transport across the RBC remains unknown.

A recent study involving cryo-EM analysis showed the parasite mines the human actin filament network to establish an efficient transport system within the cytosol of its host \(^{51}\). It was demonstrated that RBCs from patients with haemoglobinopathies the actin network was rudimentary and MC, which in normal RBCs were hinged on the actin network, are no longer stretched lamellae but large globular structures. It was concluded MC are linked to the RBC via actin filaments from the trophozoite stage onwards and transport vesicles are shuffled along these actin filaments to deliver cargo to RBC membranes. We have demonstrated in
absence of PfPTP1 function, MC are reduced to globular structures and the actin network was not intact with fewer long filaments. Our interpretation is the peptide does not change the total amount of actin filaments, but influences actin filament length or organization, possibly due to actin filament stabilization of the PfPTP1 peptide. An in vitro actin polymerisation assay confirmed that although the presence of the C-terminus of PfPTP1 does not change the total amount of rabbit actin filaments, actin forms longer filaments than in the presence of PTP1-scrambled/no peptide. Interestingly, we readily observed vesicular structures in close proximity to remnant MC in CS2ΔPTP1-infected RBCs; however, if these structures were budding off the MC they would not be able to effectively transport any proteins to the RBC membrane since the extended actin filaments spanning the distance between MC and the RBC membrane were aberrant. Therefore, our data suggest that additionally to transport of PfEMP1 to MC PfPTP1 functions in physically linking MC to the host actin skeleton and that in its absence these organelles lose their normal architecture. Previous data has suggested that SBP1 binds actin and the direct link for interaction of the detected higher order complex may be required for F-actin nucleation at MC.59

This is the first report where both tethers and actin filaments have been found in the same EM preparation (cryo-EM). Both structures play a role in docking MC to the RBC membrane.31,41,53,60 However, only the actin network seems to be disturbed in the absence of PfPTP1 whereas the tethers are still present. Therefore the initiation of the docking process takes place but it may not fulfil trafficking requirements for proteins destined for the RBC surface like STEVORs and PfEMP1.

In summary, we have shown PfPTP1 plays a critical role in MC architecture and trafficking of PfEMP1 and STEVOR. PfPTP1 forms a complex with SBP1 (and possibly PfEMP1) and these interact with other exported proteins to form a large complex involved in trafficking to and/or from the MC membrane. Furthermore, the actin network linking MC and transport vesicles for transport of proteins to the RBC membrane was disrupted in the absence of PfPTP1. Therefore PfPTP1 is a key protein required for the export of the cytoadherence complex of P. falciparum-infected RBCs.
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Author Contributions

M.R. performed parasite culturing, transfection, light and electron microscopy experiments, orientation, solubility and pulldown studies etc., M.C. and L.L. performed cryo electron tomography and in vitro actin assays and analysed the data, A.M. performed Blue Native Gel experiments, S.K. performed orientation assays, C.P.S. performed actin in vitro assays, J.T. generated independent knock-out cell line, E.H. performed electron tomography, M.O’N. and C.L. performed transfections, M.L. and F.F. analysed and interpreted in vitro actin assays,. M.R., A.F.C. and A.G.M designed and interpreted experiments. M.R. and A.F.C. wrote the manuscript and all other authors contributed to the final version.

Conflict of interest disclosure: The authors declare no conflicting financial interests.

References


**FIGURE LEGENDS**

**Figure 1. Domain structure and localization of PfPTP1 in *P. falciparum*-infected RBCs.** (A) Schematic of the PfPTP1 protein with its signal sequence in green, its PEXEL in red and the putative transmembrane domain in purple. Regions against which antibodies were raised are indicated with a black line. (B) PfPTP1 expression during the intracellular cycle (8-48 hrs) in infected RBCs. (C) Localisation of PfPTP1 during the intracellular cycle in immunofluorescence assay. The fixed cells were incubated with α-PTP1 antibody. Parasite nucleus was stained with DAPI. ER = early ring, LR=late ring, ET=early trophozoite, MT=mid trophozoite, LT=late trophozoite and S=rupturing schizont. BF = bright field.

**Figure 2. PfPTP1 is localised on Maurer’s clefts in *P. falciparum*-infected RBCs.** (A) Immunofluorescence assay on CS2/PfPTP1-GFP cell line: the GFP signal (green) colocalises with that of the endogenous protein (PfPTP1; red); inset: enlargement of...
colocalisation pattern. (B) Immunofluorescence assay on CS2/PfPTP1-HA cell line: the HA signal (green) colocalises with the α-PfPTP1 signal (red); inset: enlargement of colocalisation pattern. (C) Immunofluorescence assay on CS2/PfPTP1-HA cell line: the HA-signal (green) colocalises partially with the MC resident protein SBP1 (red); inset: enlargement of colocalisation pattern. (D) Immunofluorescence assay on CS2 parental cell line: the PfPTP2-signal (green) does not overlap with the PfPTP1-signal (red); inset: enlargement of area where MC (PfPTP1) and electron dense vesicles (PfPTP2) are in close proximity. (E) Immunofluorescence assay on CS2 parental cell line: the PfHsp70-x-signal (green) colocalises partially with the PfPTP1-signal (red); inset: enlargement of partial colocalisation on J-Dots (PfHsp70-x). Scale bar: 1 μm; same size of ROI in each image. (F) Western Blot of solubility study on CS2/PfPTP1-HA cell line: upper panel: parasite-infected RBC samples were treated with different detergents and supernatants (S) and pellets (P) detected on a Western Blot with α-HA antibodies; lower panel; blot from upper panel was stripped and probed with an antibody against the MC resident transmembrane protein SBP1. The 15 kDa is non-specific since it is found in the other blots treated with α-PfPTP1 antibodies. (G) Schematic and Western Blot of protease protection assay on CS2/PfPTP1-HA-infected RBCs; parasite-infected RBC samples were lysed and either treated with Tetanolysin O, saponin or TritonX 100 (1%). Each of the samples was then treated with either PBS or Proteinase K. The Western Blot was then either probed with an antibody recognising the N-terminal (upper panel) or C-terminal part (lower panel) of PfPTP1. SBP 1 is a MC marker with its C-terminus exposed to the RBC cytosol, EXP 1 and EXP 2 are integral membrane proteins of the PVM and SERP is a soluble marker of the PV.

Figure 3. A-D: Immunofluorescence assay on CS2 (upper panels) versus CS2ΔPTP1 parasites (lower panels). Immunofluorescence assay probed with α-PfEMP1 antibody (A), α-STEVOR antibody (B), α-SBP 1 antibody (C) and α-REX 1 antibody (D). Scale bar: 1 μm; same size of ROI in each image.
Figure 4. Comparison of transmission electron microscopical studies on CS2 versus CS2ΔPTP1 cell line.

(A) Conventional chemical Fixation: a) CS2 parental line. MC (MC) display electron dense membranes and an electron lucent lumen; b) CS2ΔPTP1 cell line shows globular structures and no long lamellar MC structures (arrows). (B) Equinatoxin II-treated cells with pre-embedding labelling: a) α-PfPTP1 antibodies or b) α-REX1 antibodies label the slender MC membranes (arrows) and vesicles (arrowheads) in the CS2 parasite line; c) α-REX1 or d) α-SBP1 antibodies label globular structures (arrows) and vesicular structures (arrowheads) in the CS2ΔPTP1 cell line. RBCM – red blood cell membrane. (C) The frozen hydrated samples show MC (arrow) in which the lumen looks similar to the RBC cytoplasm as described earlier 61. The CS2ΔPTP1 cell line shows aggregated globular structures (arrows).

Figure 5. Cryo electron tomography depicting actin network.

(A) CS2 parental line: Section through cryo-electron tomogram showing a part of an RBC infected with P. falciparum (left) in a trophozoite stage, and corresponding surface rendered view (right). The inserts in the middle show views extracted at different Z-planes and orientations of the tomogram, and are indicated by boxes and marked accordingly (1-4). Actin filaments are indicated with arrows. Scale bar: 100 nm. B) CS2ΔPTP1 cell line: Section through cryo-electron tomogram showing a part of an RBC infected with P. falciparum CS2ΔPTP1 (left) in a trophozoite stage, and corresponding surface rendered view (right). The inserts in the middle show views extracted at different Z-planes and orientations of the tomogram, and are indicated by boxes and marked accordingly (1-5). Actin filaments are indicated with arrows. Color code in both figures: RBC membrane (dark blue); knobs (red); vesicles (cyan); MC (cyan); filaments (yellow); indicated by arrows. Asterisk – edge of a hole in EM-grid carbon support. Scale bar: 100 nm. C) Transmission electron micrograph image of negatively stained human actin filaments in the absence (upper image) or presence (lower image) of PTP1 C-terminal peptide. Scale bar represents 100 nm. D) A significant difference of F-actin length polymerised in vitro is observed in the absence (green line) or presence of scrambled PfPTP1 peptide (red) versus presence of the peptide PfPTP1 (100 μM) (blue line).
**Figure 6. A-C: Interaction partners of PfPTP1.** A) Affinity purification of interacting partners on CS2/PTP1-HA cell line with α-HA coupled beads. Eluted fractions were subjected to Western Blot analysis and Blot was probed with α-HA antibody (upper panel); α-SBP1 antibody (second panel); α-PfEMP1 antibody (third panel) and α-PIESA2 (lower panel). B) Reciprocal immunoprecipitation of interacting partners of PfPTP1 in CS2 parental line with α-SBP1 antibody or rabbit normal serum (NRS; negative control). The Western Blot was probed with α-PfPTP1 antibodies. The arrow points to the specific band for PfPTP1. The 25 kDa band represents the light chain of the SBP1 antibody. C) Blue Native PAGE on CS2/PTP1-HA cell line. The first dimension was run under native conditions, the strip then excised and run in the second dimension under denaturing conditions to resolve potential complexes. The Blot was first probed with α-SBP1 antibody and HRP-conjugated secondary antibody, then stripped and probed with α-HA antibody and specific complexes detected via electrochemiluminescent reagents and x-ray film.
Figure 3

A  PfEMP1

B  STEVOR

C  SBP1

D  REX1
Figure 4

A

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B

0.2 μm

C

100 nm
Export of virulence proteins by malaria-infected erythrocytes involves remodelling of host actin cytoskeleton

Melanie Rug, Marek Cyrklaff, Antti Mikkonen, Leandro Lemgruber, Simone Kuelzer, Cecilia P. Sanchez, Jennifer Thompson, Eric Hanssen, Matthew O’Neill, Christine Langer, Michael Lanzer, Freddy Frischknecht, Alexander G. Maier and Alan F. Cowman