Maurer’s clefts of *P. falciparum* are secretory organelles that concentrate virulence protein reporters for delivery to the host erythrocyte.

Running title: Protein transport in malaria-infected erythrocytes.

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Abstract

In blood stage infection by the human malaria parasite *Plasmodium falciparum*, export of proteins from the intracellular parasite to the erythrocyte is key to virulence. This export is mediated by a host-targeting (HT) signal present on a ‘secretome’ of hundreds of parasite proteins engaged in remodeling the erythrocyte. But the route of HT-mediated export is poorly understood. Here we show that minimal soluble and membrane protein reporters that contain the HT motif and mimic export of endogenous *P. falciparum* proteins are detected in the lumen of ‘cleft’ structures synthesized by the pathogen. Clefts are efficiently targeted by the HT signal. Further the HT signal does not directly translocate across the parasitophorous vacuolar membrane (PVM) surrounding the parasite to deliver protein to the erythrocyte cytoplasm, as suggested by current models of parasite protein trafficking to the erythrocyte. Rather, it is a lumenal signal that sorts protein into clefts, which then are exported beyond the PVM. These data suggest that Maurer’s clefts, which are unique to the virulent *P. falciparum* species, are pathogen-induced secretory organelles that concentrate HT-containing soluble and membrane parasite proteins in their lumen for delivery to the host erythrocyte.
Introduction

Plasmodium falciparum causes the most virulent form of human malaria, a disease that affects 200-300 million people and kills over a million children every year. Blood stage parasites that infect mature erythrocytes export proteins to dramatically remodel the host cell surface and cause all of the major disease pathologies including death. Further, several hundreds of parasite secretory proteins carry a host targeting (HT) motif (also known as Plasmodium Export Element, PEXEL), which is required for protein export past the parasitophorous vacuolar membrane (PVM) surrounding the parasite, to the erythrocyte. But how the HT motif functions in exporting both soluble and membrane proteins beyond the PVM remains poorly understood.

Prior studies have shown that both soluble and membrane bound antigens and virulence proteins containing the HT motif associate with flattened, lamellar intraerythrocytic structures called the Maurer’s clefts. Over the last decade, clefts have been suggested as possible intermediates of protein transport to the erythrocyte surface. A recent model suggests that a HT-containing protein exported to the erythrocyte membrane is translocated across the PVM as a soluble complex and delivered to the cytoplasmic face of clefts prior to membrane insertion and subsequent vesicular export from clefts to the erythrocyte surface. However, unequivocal biochemical evidence for the presence of soluble or cytoplasmic forms of these proteins in the erythrocyte is lacking. This model also fails to explain the mechanism by which soluble proteins containing the HT signal are released into the cytoplasm and associate with the cytoplasmic face of clefts.
We constructed minimal soluble and membrane reporters and examined their export to the erythrocyte as well as their membrane association. Our studies reveal that a minimal soluble reporter detected in the erythrocyte cytoplasm is also found in the cleft-lumen but shows no association with the cytoplasmic face of cleft membranes. Attachment of a transmembrane domain (derived from the surface adhesin *P. falciparum* erythrocyte membrane protein 1, PfEMP1) to this soluble reporter blocked its release into the erythrocyte cytoplasm and enabled its accumulation anchored in the cleft lumen. Subsequent replacement of the HT signal did not prevent membrane anchoring but resulted in reporter accumulation at the PVM. These data establish that the HT motif does not mediate protein translocation across the PVM bilayer into erythrocyte cytoplasm. Rather, the HT appears to function as a lumenal signal that sorts secretory parasite proteins into clefts, which are then exported beyond the PVM. Thus clefts may be major intermediates of transport across the erythrocyte cytoplasm for both soluble and membrane proteins and thus central to host remodeling.
Materials and Methods

Construct production and transgene expression utilizing piggyBac.

All constructs used for transfections were first assembled in pBluescript (SK+).

Constructs for transfections utilizing piggyBac strategy were further subcloned into a modified piggyBac integration vector pBacII, derived from pXL-BACII-DHFR.

The plasmid pH1solGFP was derived from pDCHRPIIDomainImin.his.GFP.

Construction of pBacII(HT-GFPmembmyc), pBacII(Δ-GFPmembmyc) and pBacII(HT-GFPmembC-term) was carried out as follows. pDC1 was digested with NarI and SapI and the 3746 base pair (bp) fragment containing the dhfr, 5’ cam and 3’ hsp86 was treated with DNA polymerase I Klenow fragment to form blunt ends. In parallel, pXL-BACII-DHFR was digested with EcoRI and XhoI to release the 3961 bp fragment containing oriC, the ampicillin resistance gene and the inverted terminal repeats, which was then treated with DNA polymerase I Klenow fragment to form blunt ends and ligated to the earlier obtained fragment to form pBacII. The construct pBacII(HT-GFPmembmyc) was generated as follows. Firstly, the entire HRPIIDomainImin.his.GFPmyc was amplified by PCR using HRPIIPstIF (CGGCTGAGATGGTTTCCTTCAAATAATAAAGTATTATCC) and MycXhoIR (CCGCTCGAGGTCGACGGTATCGATAAGCTTATAAATCTTCTTCC) primers. The PCR product was cloned at PstI and XhoI site of pBluescript (SK+) to generate pBSHRPIIDomainImin.his.GFPmyc. The gfp-vartm region was amplified by PCR from pBSGFPvarTM using GFPKpnIF (CGGGGTACCATGCATAGATCTAAAGGAGAA) and varTMBglIIR (CCGAGATCTTTATTACTTTAGATAAAAATAAAGTGAATGTAGC), digested with
*BglII* and inserted into similar digested pBSHRPIImyc to generate pBSHRPIIGFPvarTMmyc. Subsequently, pBSHRPIImin.his.GFP was digested with *NcoI* and *XhoI* to replace its *gfp-myc* with *gfp-vartm-myc* derived from a similar digestion of pBSHRPIIGFPvarTMmyc. Finally, the HRPIImin.his.GFPvarTMmyc fragment was cloned into pBacII to generate pBacII(HT-GFP<sup>memb</sup>myc). Plasmid pBacII(Δ-GFP<sup>memb</sup>myc) was generated by a three step PCR. PCR 1 was performed using HRPIIPstIF and HTmutR (CGGGATATCGCATCAACAATCCATGTAGATGATGCCCATCATGC); while PCR 2 used HTmutF (CGGGATATCGCATCAACAATCCATGTAGATGATGCCCATCATGC) and MycXhoIR primers. The products of PCR 1 and 2 were used to constitute the entire Δ-GFP<sup>memb</sup>myc, which was finally cloned into pBacII at the *XhoI* site. For the generation of HT-GFP<sup>memb</sup>C-term, the region containing *gfpvartm exon2* was amplified from Glm42GFPvarTMexon2 using GFPKpnIF and Exon2XhoIR (CCGCTCGAGTTATATATCCCATAAATCTGCTATTG). The PCR product was digested with *NcoI* and *XhoI* and cloned into pBSHRPIImin.his.GFP to replace its *gfp-myc* with *gfpvartm exon2*. Finally, the HRPIImin.his.GFPvarTMexon2 fragment was cloned into pBacII to generate pBacII(HT-GFP<sup>memb</sup>C-term).

Synchronous *P. falciparum* 3D7 parasites in culture were transfected with indicated plasmids by standard procedures. Forty-eight hours after transfection, the cultures were selected with 2.5nM WR99210 (Jacobus Pharmaceuticals, USA) and stable cell lines cloned by limiting dilution. Genomic DNA was isolated from respective clonal populations and digested with *EcoRV*. Chromosomal integration in respective *piggyBac*
clones was detected by Southern hybridization using labeled \textit{hdhfr} probe and sequencing. For each transgene, a single site of insertion was identified in three independent clones suggesting clones with specific insertions may dominate in a population. In all the three clones of parasites expressing HT-GFP\textsuperscript{mem} myc (named PfHT-GFP\textsuperscript{mem} myc), the \textit{piggyBac} strategy resulted in insertion at TTAA target site in the 5’ UTR of \textit{pff1505w} (chromosome 6). The common insertion site for each of the three parasite clones expressing Δ-GFP\textsuperscript{mem} myc (called PfΔ-GFP\textsuperscript{mem} myc) was in the 5’UTR of \textit{pf14_0096} (chromosome 14). Both \textit{pff1505w} and \textit{pf14_0096} are hypothetical genes of unknown function. They are not predicted to be secretory proteins and thus not expected to influence parasite protein export to the erythrocyte. Exhaustive clonal analysis has not been undertaken to determine whether these are indeed the dominant sites of insertion. Neither transgenic parasite line showed any growth defects relative to parent 3D7 (data not shown).

\textbf{Generation of parasites with deletion in the putative Hsp40 substrate-binding domain in PFE0055c (\textit{Actermpfe0055c})}

\textit{P. falciparum pfe0055c} codes for a putative protein of 413 amino acids. DNA sequence encoding for amino acid 109-212 of \textit{pfe0055c} was amplified from genomic DNA using PFE0055cXhoIF

\begin{verbatim}
(CGCGCTCGAGGATAAACACAATCATTTGGAAATGAAATATTTAAAAATACAAAAG)
\end{verbatim}

and PFE0055cSpeIR

\begin{verbatim}
(CCGACTAGTTGATCTGCTTGATCTAGGTCTTCTTGAATTCATACTTGCGAAAC
C)
\end{verbatim}

primers and cloned upstream of \textit{neomycin phosphotransferase} gene in the vector
pGTPneo46\textsuperscript{13} to generate pHsp40neo46. Parasites were transfected with DNA by loading\textsuperscript{12}, selected for \textit{bsd} expression with 1\(\mu\)g/ml Blasticidin hydrochloride followed by selection with 400\(\mu\)g/ml G418 for single crossover recombination. Successful replacement of \textit{pfe0055c} with \textit{pfe0055c} fragment-\textit{neo} fusion, which is deleted in region encoding for putative HSP40 substrate-binding domain at the chromosomal locus, was analyzed by PCR using primer 1 (CGGCTCGAGATGTCCATTTTAAATAATACGAAGGAAAAAGAAAAATACAACT)\textsuperscript{1}, 2 (CTTTACATTCATTATGAAGAATGTGTATGATTTTTTCCC), 3 (CGGCTCGAGGATAAACACAACAAATCCATTTGGAAATGAAATATTTAAAAAT ACAAAAAG) and primer 4 (TCAGAAAGAACTCGTCAAGAAGGCAGATGAAAGGC).

**Construction of \textit{Actermsbp} mutant parasites**

\textit{P. falciparum} parasites with chromosomal deletion in \textit{pfsbp1} (\textit{AcTermsbp}) were generated as follows. Briefly, the region in \textit{pfsbp1} between nucleotides 109-744 was amplified using SBP1KOxhoIF (CGGCTCGAGTCGGATGCAGCAACAAATGTTACTGATGCAGTAAG) and SBP1KOxSpeIR (CCGACTAGTTAATGCTTACTCGAAGGCGATGAGGAAGG) and cloned at corresponding sites in pGTPneo46\textsuperscript{13} to generate pSBP1neo46. Parasites were transfected with pSBP1neo46 by DNA loading\textsuperscript{12}, selected for \textit{bsd} expression with 1\(\mu\)g/ml Blasticidin hydrochloride and after establishment of a resistant population, selected with 400\(\mu\)g/ml G418 for single crossover recombination. Successful deletion in \textit{pfsbp1} was confirmed by isolation of genomic DNA from G418-resistant population and
analysis by PCR using primers: primer 1
(CCGGGCTAGCATGTTGAGCCAGCCACGCTTTTACTGATTAGCC), primer 2
(GGCAGGGTCCGCAGCAGCCGTTTCTTAGCAGGTTTTTTGTTGG),
primer 3 (CGGCTCGAGTCGGATGCAGCAACAAATGTTACTGATGCAGTAAG)
and primer 4 (TCAGAAGAACTCGTCAAGAAGGCGATAGAAGGC).

Expression-purification of rHT-GFP and loading into erythrocyte ghosts
The region encoding HT-GFP fusion was cloned into pET29b (EMD Biosciences,
Germany) as follows. The HT-GFP fusion was amplified from plasmid
HRPIIDomIminHisGFP with primers HRPII-4
(TGGCATTTAATAATAACTTGTGTAGCAAAAATG) and GFP3’-3
(GGACctcgagTTTGTATAGTTCATCCATGTG) and digested with XhoI. The
expression vector pET29b was digested with NdeI, blunt ended using T4 polymerase, and
ligated with XhoI digested PCR product to form pET29b(rHT-GFP). E. coli BL21 (DE3)
(Invitrogen, USA) cells were transformed with pET29b(rHT-GFP). An overnight grown
culture was used to prime 500 ml culture. At exponential phase (O.D.600nm of 0.5-0.6)
cells were induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 h.
Recombinant HT-GFP was purified from cell pellet under native conditions using
ProBond nickel purification resin (Invitrogen, USA) and following manufacturer’s
instructions. Eluted fractions were pooled, dialyzed extensively against PBS and
concentrated using Centricon concentrators (Millipore, USA). The purity of the eluted
fractions was checked by SDS-PAGE and protein concentration estimated by Bradford’s reagent (Bio-Rad, USA).

Erythrocytes ghosts were loaded with 1 mg/ml of purified rHT-GFP following published protocol 14. As a control, non-loaded erythrocyte ghosts were also prepared. Preparations of rHT-GFP loaded erythrocyte ghosts and non-loaded erythrocyte ghosts were infected with purified \( P. falciparum \) schizonts and the percentage parasitaemia compared to invasion in intact human erythrocytes. Cells were also harvested and processed for live cell fluorescence imaging, indirect immunofluorescence assay or immunoelectron microscopy.

**Immunofluorescence assay and immunoblotting**

Immunofluorescence assay of erythrocytes infected with parasite expressing HT\textsuperscript{sol}-GFP (PfHT\textsuperscript{sol}-GFP) and permeabilized with tetanolysin was performed using antibodies to GFP and PfStomatin as described earlier 15. Indirect immunofluorescence assay of rHT-GFP loaded/non-loaded erythrocyte ghosts infected with non-transfected parasites or erythrocytes infected with parasite expressing HT-GFP\textsuperscript{memb} myc and Δ-GFP\textsuperscript{memb} myc (PfHT-GFP\textsuperscript{memb} myc and PfΔ-GFP\textsuperscript{memb} myc, respectively) was performed according to a published protocol 9. For quantitative colocalization between green fluorescence GFP signal and red fluorescing PfSBP1 signal, optical images from 200 sections were analyzed and result expressed in percentage.
Under specific instances erythrocytes infected with PfHT-GFP^{memb}myc parasites were labeled with 0.5μM Texas-red ceramide (Molecular Probes, USA) to view the body of parasite as well as erythrocyte membrane. Briefly, cells were harvested from culture, washed with serum-free RPMI and labeled with 0.5μM Texas-red ceramide for 30 min at 4°C. Samples were washed with serum-free RPMI, stained with Hoechst and imaged live using fluorescence microscope.

Erythrocyte ghosts were also prepared in the presence anti-GFP antibodies conjugated to Alexa 594 and infected with percoll-purified schizonts of PfHT-GFP^{memb}myc or PfΔ-GFP^{memb}myc parasites. At trophozoite stages, cells were harvested and processed for live cell imaging as described later. A fraction of erythrocyte ghosts, not loaded with Alexa-594 conjugated anti-GFP antibodies and infected with parasite expressing HT-GFP^{memb}myc or Δ-GFP^{memb}myc, was harvested, adhered to poly-L-lysine coated coverslips and permeabilized with 0.01% saponin. Saponin-permeabilized cells were probed with anti-GFP antibodies conjugated to Alexa 594. Parasite nucleus was stained with 5µg/ml Hoechst 33342 and the cells viewed under deconvolution fluorescence microscope.

Western blotting of the pellet and supernatant fractions from tetanolysin-permeabilized erythrocyte infected with parasite expressing HT-GFP^{memb}myc, Δ-GFP^{memb}myc or HT-GFP^{memb}C-term was performed as follows. Briefly, 10% cell suspension of infected erythrocytes were permeabilized with 100U/ml tetanolysin for 30 min at 37°C and separated into pellet and supernatant fractions after a brief centrifugation at 3200 × rpm
for 10 min. Samples were subsequently analyzed by western blotting using antibodies to GFP, PfStomatin and parasite cytosolic marker PfFKBP.

Detection of neomycin phosphotransferase (NPT) fusion products for parasite lines expressing a C-terminal deletion of PfSBP1 (Δctermsbp), or a deletion in putative Hsp40 substrate-binding region in PFE0055c (Δctermpe0055c), was performed using antibodies to NPT.

All other western blots were performed by directly solubilizing the infected red blood cells in Laemmli’s sample buffer, separating by SDS-PAGE immunoblotting with respective antibodies.

Immunoelectron microscopy

Immunoelectron microscopy of erythrocyte ghosts; either non-loaded and infected with parasite expressing HTsol-GFP or loaded with rHT-GFP and infected with untransfected parasites; as well as erythrocytes infected with parasite expressing HTsol-GFP, HT-GFPmembmyc or Δ-GFPmembmyc, was performed as described earlier 9.

A comparison between the numbers of gold particles associated with Maurer’s clefts in rHT-GFP loaded (and infected) erythrocyte ghosts and non-loaded erythrocyte ghosts infected with parasite expressing HTsol-GFP was performed by manual counting in 20 cells and averaging the number of cleft-associated gold particles per cell.

Protease treatment of tetanolysin or saponin permeabilized erythrocytes

Infected erythrocytes, intact or tetanolysin permeabilized (as described earlier), were treated with 1mg/ml of trypsin (Sigma, USA) in ice for 30 min. Trypsin inactivation was
carried out by adding an equal volume of PBS containing 2 mg/ml soybean trypsin inhibitor (Sigma, USA) and protease inhibitor cocktail (Roche, Switzerland) at 4°C. Samples were analyzed in western blots with antibodies to GFP, c-myc and human spectrin.

**Deconvolution Fluorescence Microscopy and image acquisition**

Fluorescence microscopy and digital image collection of both fixed and live infected erythrocytes were performed on an Olympus IX inverted fluorescence microscope with a temperature controlled stage and a Photometrix cooled CCD camera (CH350/LCCD) driven by DeltaVision software from Applied Precision Inc. (Seattle, USA). Briefly, DeltaVision software (softWoRx) was used to deconvolve the bright field and fluorescent optical images taken through cells. For quantitative projections, a minimum of 200 optical images containing fluorescent parasites was subjected to the "additive" method of data collection. Fluorescence quantification was carried out with a 60X, NA 1.4 objective. Use of a constitutive cam promoter results in continuous GFP chimera expression associated with the parasite.
Results

An exported, minimal soluble reporter is detected in the lumen of Maurer’s clefts.

Parasite proteins exported to the erythrocyte cytoplasm via the HT motif vary greatly in size, amino acid composition and sequence. Thus, to understand the role of HT motif in all soluble proteins, we followed a minimal reporter comprising of a cleavable endoplasmic reticulum (ER)-type signal sequence (SS), HT motif and green fluorescent protein (GFP), known to result in the delivery of green fluorescence in the erythrocyte cytoplasm \(^9\) (Figure 1A). In these cells green fluorescence is also detected in association with the parasite likely due to constitutive action of the promoter that leads to overproduction of the protein and its accumulation in the parasite during blood stage growth \(^9\). In addition, structures in the erythrocyte cytoplasm also appear to be labeled by the fluorescent reporter (Figure 1A, arrows). Immunoelectron microscopy confirmed the presence of gold particles in the erythrocyte cytosol as well as in flattened lamellar structures called Maurer’s clefts (Figure 1B, arrow) in the host cell. When the infected erythrocyte membrane was permeabilized with tetanolysin and cells were subsequently, fixed, permeabilized and probed with antibodies; GFP was largely associated with punctuate structures that colocalized with a resident protein of Maurer’s clefts (Figure 1C). This cleft-associated GFP was detected both proximal to the parasite (arrow heads) and in the periphery of the erythrocyte (arrow). Previous studies have shown that full length parasite proteins exported to the erythrocyte cytoplasm have also been located to the Maurer’s clefts \(^9\), suggesting that a minimal secretory reporter mimics this feature of parasite protein export to the erythrocyte.
To investigate whether localization of GFP in clefts was due to protein exported to the erythrocyte cytoplasm adhering to the cytoplasmic face of intraerythrocytic structures, we first permeabilized erythrocyte membrane to release protein freely diffusible in the erythrocyte cytoplasm. Antibodies introduced into these permeabilized cells failed to detect GFP on the cytoplasmic face of intraerythrocytic membranes although Pfstomatin known to be cytoplasmic face of the vacuole was readily detected (Figure 2A). GFP was readily detected when saponin was used to permeabilize infected cells (Figure 2A), suggesting that reporter was in the lumen of punctate intraerythrocytic clefts.

To examine whether GFP in the cleft lumen could be obtained by uptake of protein from the erythrocyte cytoplasm, we expressed recombinant HT-GFP (rHT-GFP) using bacterial expression system. We purified rHT-GFP, loaded into resealed erythrocyte ghosts and subsequently infected those with *P. falciparum* (also see Supplementary Figure S1). As shown by live cell image in Figure 2B (top), rHT-GFP was found to be uniformly distributed in the infected erythrocyte cytoplasm as well as the food vacuole of parasite (presumably via cytostome-mediated uptake of host cytoplasm). Areas devoid of green fluorescence in the erythrocyte cytoplasm were visible in the live cell image (Figure 2B top, arrowhead), which were identified as cleft structures by indirect immunofluorescence assay (Figures 2C, top panel; 2E) as well as immunoelectron microscopy (Figures 2D, top panel; 2F). In contrast, when resealed ghosts were infected with parasites that biosynthetically expressed secretory HTsol-GFP, green fluorescence was quantitatively associated with the clefts (Figures 2B-D, bottom panels; 2E and 2F). Together, the data in Figures 1 and 2 suggest that the exported
minimal soluble GFP reporter associated with Maurer’s clefts is found in the luminal and not cytoplasmic face of these structures.

**The malarial HT motif is a luminal signal that sorts protein into Maurer’s clefts without translocating soluble complexes into the erythrocyte cytoplasm.**

We next investigated the effect of the presence of transmembrane domain in the minimal soluble reporter, and thus synthesized the chimera HT-GFP\textsuperscript{memb}myc by adding the transmembrane domain of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1; also see Supplementary Figure S2). Remarkably, this appeared to deplete green fluorescence from erythrocyte cytoplasm and limit its detection in punctuate structures dispersed in the erythrocyte (compare Figure 1A to Figure 3A, top panel). These punctuate structures colocalized with Maurer’s clefts as confirmed by both indirect immunofluorescence and immunoelectron microscopy (Figures 3B and 3C, top). This suggested that addition of the transmembrane domain to the soluble reporter blocked its efficient release to the erythrocyte cytoplasm. Since the membrane chimera accumulated in clefts rather than PVM, clefts may be the preferred site of soluble protein translocation into the erythrocyte cytoplasm.

Replacing the HT motif (LNKRLLYETQA) in HT-GFP\textsuperscript{memb}myc with a non-specific sequence (ISAATDIASTI) blocked export of green fluorescence to clefts (Figures 3A and 3B, bottom panels). Instead we detected GFP accumulation in the periphery of the parasite (Figure 3A, bottom panel). Finally immunoelectron microscopy suggested that Δ-GFP\textsuperscript{memb}myc product was localized in PVM (Figure 3C, bottom). Together these data
suggested that the transmembrane region enabled protein insertion into the membrane, while HT motif directly enables export of GFP to the clefts. To determine whether this export involves soluble intermediates translocated to the erythrocyte cytoplasm, we undertook biochemical analyses of infected erythrocytes permeabilized with tetanolysin. We found that although this treatment quantitatively released hemoglobin (Figure 3D), HT-GFP\textsuperscript{memb}myc, Δ-GFP\textsuperscript{memb}myc as well as HT-GFP\textsuperscript{memb}C-term (containing the cytoplasmic tail of PfEMP1 fused to HT-GFP\textsuperscript{memb}myc and exported to the clefts; Figure 3E) remained cell associated, indicating that none of these HT-containing proteins exist as freely diffusible soluble complexes across the PVM. Further, protease treatments conducted in these permeabilized cells revealed that the c-myc tag on both cleft-associated HT-GFP\textsuperscript{memb}myc and PVM-associated Δ-GFP\textsuperscript{memb}myc faced the erythrocyte cytoplasm (Figure 4A). In contrast, GFP was lumenal. This topology was independently confirmed by demonstrating that anti-GFP antibodies in the erythrocyte cytoplasm could not access GFP of either HT-GFP\textsuperscript{memb}myc or Δ-GFP\textsuperscript{memb}myc (Figures 4 B-C). Together, the data in Figures 3 and 4 strongly support that HT-GFP\textsuperscript{memb}myc and Δ-GFP\textsuperscript{memb}myc respectively spanned the cleft membrane and PVM via their PfEMP1 transmembrane domain and were not translocated across the PVM, via the HT signal. Given these consideration, since the HT clearly drives protein accumulation in Maurer’s clefts it must accomplish this as a lumenal signal that sorts protein into these secretory clefts.

**HT-driven protein accumulation in clefts may occur prior to cleft export to the periphery of the erythrocyte and is apparently not influenced by predicted**
functional changes in cleft cytoplasmic proteins PfSBP1 or a putative heat shock protein PFE0055c.

Our examination of live-infected erythrocytes shown in Figures 1 and 3, suggested that punctuate structures, characteristic of Maurer’s clefts are found in the periphery of the erythrocyte (arrows), in the intraerythrocytic space, at the edge of the parasite (arrow heads) and some may even reside within the parasite. Indeed as shown in Figure 5A, single optical sections of cells labeled with lipid marker Texas-red (TR)-ceramide used to mark the body of the parasite reveal green punctuate spots that are tightly apposed to or even within the periphery of the vacuolar parasite. Hence it is possible that the HT may sort proteins into clefts before these structures are exported from the parasite to the erythrocyte.

Since our data suggest clefts carry export cargo to the erythrocyte, we investigated the effects of truncating their major resident protein PfSBP1 on sorting HT-GFP<sub>memb</sub>myc to clefts or cleft export to the erythrocyte (Supplementary Figure S3). Cooke et al. show that knockout in PfSBP1 does not block export of proteins from the parasite to Maurer’s cleft. However studies of Maier et al. suggest that removal of the C-terminal domain of PfSBP1 which is expected to interact with the erythrocyte membrane, blocks protein export from the parasite to the clefts. We find that HT-mediated sorting to clefts and movement of clefts to the erythrocyte periphery are not blocked by truncation of PfSBP1 (Figure 5B; also see Supplementary Figure S4). This is consistent with studies of Cooke et al. but contradicts studies of Maier et al. Since our work focuses on minimal lumenal export signal, it is more likely that HT-mediated targeting to clefts does not
depend on cytoplasmic interactions of PfSBP1. However, definitive resolution of discrepancies of the data from us and Cooke et al. with those of Maier et al. must await further analyses (note: all three studies have been carried out in the 3D7 strain).

Factors that regulate targeting of the HT to clefts or mediate cleft export beyond the PVM and across the erythrocyte cytoplasm remain poorly understood. Multiple secretome predictions suggest that parasite encoded chaperones including heat shock proteins may be involved in protein export to the erythrocyte and host remodeling. PFE0055c a putative HSP40 (Figure 6A) and our previous data suggested that a GFP chimera of PFE0055c is exported to the host erythrocyte. Since the PFE0055c is also an HT-containing protein, it is expected to package into clefts enroute to the erythrocyte. PFE0055c could have functional consequences for targeting to the clefts as well as additional chaperone activities in the erythrocyte. Indeed, we show that endogenous PFE0055c, a protein of 42-kDa (Figure 6B) localizes primarily to Maurer’s clefts (Figure 6C). This provides the first definitive evidence for the presence of a parasite encoded putative heat shock protein at the clefts. We disrupted the predicted substrate binding domain of PFE0055c by single crossover recombination (Figure 6D) and confirmed the replacement of chromosomal pfe0055c with pfe0055c fragment-npt fusion (Figure 6E). In western blots, using antibodies to NPT, we detected a 45-kDa fusion product in transgenic parasites but not in parental wild type 3D7 (Figure 6F). However, on transfecting parasites expressing PFE0055c (∆ctermpfe0055c, with a deletion in substrate binding region and fused to NPT), we find that disruption of its predicted substrate binding domain thought to be essential for HSP40 function does not block accumulation
of HT-GFP\textsuperscript{memb} myc in Maurer’s clefts (Figure 6G). Nor does this truncation prevent cleft export from the parasite to the erythrocyte periphery (Figure 6G). These data suggest that the substrate-binding domain of PFE0055c heat shock protein does not significantly regulate HT-mediated protein sorting to clefts. It is possible that PFE0055c acts at a later step of transport in the erythrocyte.

Discussion

Proteins of the malarial secretome exported to the erythrocyte underlie major virulence and antigenic functions. Thus it is important to understand the transport signals underlying these events of erythrocyte remodeling. Since its discovery, how the HT motif functions in exporting both soluble and membrane proteins beyond the PVM has been debated extensively \cite{6}. Our data that the HT motif is a sorting signal that mediates protein concentration in clefts which are exported to the erythrocyte provides an explanation of how this export signal can be shared by both soluble and membrane proteins destined for the host cell (Figure 7). Our evidence that the HT motif does not translocate across the PVM bilayer conflict with prior suggestions that HT-containing chimeras such as HT-GFP\textsuperscript{memb} C-term diffuse as soluble complexes across the erythrocyte cytoplasm \cite{21}. However, these conclusions were based on measurements of rapid rates of photobleaching of GFP-protein chimeras \cite{21}, lacked unequivocal biochemical evidence of membrane association and protein topology, and do not preclude rapid diffusion of membrane proteins. Studies by Papakrivos et al. \cite{22} suggest that there may be a soluble form of native PfEMP1 protein within the parasite. However, Papakrivos et al. \cite{22} as well as Kriek et al. \cite{23} do not report soluble PfEMP1 in the erythrocyte cytoplasm.
Recent studies on the biogenesis of clefts suggest that they may bud from the PVM. However, it is also possible that they undergo hitherto unknown steps of biogenesis earlier within the parasite and mature into well-developed clefts at the PVM from where they disassociate and move to the erythrocyte periphery (Figure 7). Alternatively, the HT motif may also sort soluble and membrane proteins from the lumen of the PVM to the lumen of clefts that assemble at this vacuole (Figure 7). Regardless of whether sorting occurs inside the parasite or in the PV, as a lumen sorting signal the HT motif may enable soluble proteins to be packaged within clefts, and efficient delivery into the host cytoplasm when clefts dock at the erythrocyte skeleton, a major target of these soluble secretome determinants. This may explain why even proteins such as PfHRPII which have no clear binding partner at the host skeleton, nonetheless appear to be restricted to the periphery of the infected erythrocyte. Parasite membrane proteins sorted by a lumenal HT signal into clefts can be brought proximal to the erythrocyte membrane as is seen for the major membrane bound antigenic families of *P. falciparum*.

Since the HT is present on 300-400 secretory proteins, clefts may thus provide a major conduit for parasite soluble and membrane protein export across the host cytoplasm. However, little is known about the cleft movement from the parasite to the erythrocyte periphery. This is expected to occur in absence of COP-coated vesicles that drive classical secretion in higher eukaryotes (consistent with the most recent evidence that parasite encoded components of eukaryotic secretion are not exported to the erythrocyte). Our data also suggests that cleft interactions with the erythrocyte...
skeleton via PfSBP1 or with putative substrates of PFE0055c in the erythrocyte may not
play a significant role in cleft export across the host cytoplasm. In addition, these
interactions have no effect on HT-mediated protein accumulation in clefts. In the case of
PFE0055c, it is possible that redundant chaperone functions may exist, but for the
present it is the only putative parasite HSP40 shown to be exported to the erythrocyte.

Finally, although the HT signal mediates efficient transport of GFP from PVM to clefts,
we find little or none of minimal reporters are delivered to the erythrocyte surface,
suggesting additional signals and transport structures may be needed for quantitative
protein export from clefts to the host membrane. We would like to indicate that like us,
Knuepfer et al. used minimal reporters. However, they reported surface exposure only
in knobby strains (Figure 4A, Knuepfer et al.). In knobless strains (Figure 4C,
Knuepfer et al.) they fail to detect surface exposure of the reporter. We do not
understand why this should be the case, but all of our studies reported here were carried
out in knobless parasites. Thus with respect to surface exposure, there is no discrepancy
between our findings and those of Knuepfer et al. It should also be noted that in their
export to the erythrocyte and subsequently delivery of protein to the surface, clefts may
associate with a variety of intraerythrocytic structures. This may provide one explanation
for the range of intraerythrocytic protein export structures reported in the literature.
Nonetheless our present data strongly support that sorting into clefts prior to crossing the
PVM, may be the critical secretory decision underlying HT-mediated export of hundreds
of putative effectors across the cytoplasm of the mature erythrocyte infected by this
major human pathogen.
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Authorship

Contribution: S.B. and K.H. designed the experiments, analyzed the data, and drafted the manuscript; S.B., C.vO. and B. B. performed experiments and contributed to data analysis; B.B. and J.H.A. contributed new reagents and carried out insertion site analyses.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
References


Figure legends

Figure 1. HT$^{\text{sol}}$-GFP, a minimal soluble reporter exported to the erythrocyte cytoplasm and detected in clefts. A. 0° projection of an erythrocyte infected with transgenic parasites expressing HT$^{\text{sol}}$-GFP. Arrow indicates GFP labeled intraerythrocytic structure, possibly a cleft. B. Immunoelectron micrographs of trophozoite parasite (p)-infected cells expressing HT$^{\text{sol}}$-GFP. Ultrathin sections were probed with antibodies to GFP and secondary antibody gold (10nm) conjugate. Arrows indicate gold particles at intraerythrocytic Maurer’s clefts (MC). No gold labeling was detected in absence of primary antibody or when a non-specific primary was used (not shown). Bar 200nm. C. Single optical section of an infected erythrocyte expressing HT$^{\text{sol}}$-GFP. Samples were treated to release soluble GFP, fixed and probed with antibodies to GFP (green) and P. falciparum Skeletal Binding Protein1 (PfSBP1, red). Arrow, GFP labeled cleft structures at the periphery of infected erythrocyte; arrowheads, clefts proximal to the parasite. In fluorescence micrographs, p denotes parasite nucleus stained with Hoechst 33342; bar, 2µm. Schematic representation of the construct is indicated above with ER-type signal sequence (red), sequence containing HT signal (blue) fused to GFP (green) and myc (orange).

Figure 2. Lumenal association of HT$^{\text{sol}}$-GFP at Maurer’s clefts. A. Schematic representation of the infected erythrocyte (left) and its permeabilization (dotted lines) after treatment with tetanolysin (top) or saponin (bottom). Panels of fluorescent image show infected erythrocyte expressing HT$^{\text{sol}}$-GFP, permeabilized with tetanolysin (top) or
saponin (bottom), and probed with antibodies to GFP (green) and PfStomatin (red). Respective merged images are also shown. Dotted lines indicate erythrocyte periphery. Arrows show intraerythrocytic clefts. B. 0° projections of a rHT-GFP-loaded erythrocyte ghost infected with 3D7 *P. falciparum* (top), or a mock-loaded erythrocyte ghost infected with transgenic parasite expressing HTsol-GFP (bottom). Empty arrowhead, cleft structure not labeled with intraerythrocytic rHT-GFP; solid arrowhead, GFP labeled cleft. C. Cells in B fixed, permeabilized and probed with antibodies to GFP (green) and resident cleft protein PfSBP1 (red). Arrows show clefts. D. Immunoelectron microscopy of cells in B showing distribution of GFP associated with Maurer’s clefts (MC). Bar indicates 500nm. E. Bar graph showing the percentage colocalization between GFP and Maurer’s cleft in indicated samples by fluorescence microscopy. F. Quantitation for the number of gold particles (measuring GFP) associated with clefts by immunoelectron microscopy over 20 infected erythrocytes. In all fluorescence micrographs: p, parasite (nucleus stained with Hoechst; blue); ec, erythrocyte cytosol; bar, represent 2µm.

**Figure 3. The HT motif targets proteins to Maurer’s clefts without translocation into the erythrocyte cytoplasm.** A. Live infected erythrocytes expressing HT-GFP<sup>memb</sup>myc (top) and Δ-GFP<sup>memb</sup>myc (bottom) viewed under bright field image, GFP fluorescence and merged optics. Western blot using antibodies to GFP indicating the detection of 39-kDa fusion product for HT-GFP<sup>memb</sup>myc expressing cells (lane 1), 39/41-kDa doublet fusion product for Δ-GFP<sup>memb</sup>myc expressing cells (lane 2) or no signal for untransfected cells (lane 3) is shown at the right. In lane 2 the 41-kDa band is a precursor which in pulse chase experiments can be chased into the 39-kDa band (not shown).
Vertical lines have been inserted to indicate repositioned gel lanes. **B.** Indirect immunofluorescence assay showing distribution of GFP (green), associated with HT-GFP<sub>memb</sub>myc (top panel) or Δ-GFP<sub>memb</sub>myc (bottom panel) relative to Maurer’s cleft protein PfSBP1 (red). Parasite nucleus (p) is stained with Hoechst 33342. **C.** Localization of HT-GFP<sub>memb</sub>myc in Maurer’s clefts (MC, top) and Δ-GFP<sub>memb</sub>myc in parasitophorous vacuolar membrane (PVM, bottom) by immunoelectron microscopy. Empty arrowheads indicate gold particles showing distribution of GFP chimeras. Bar, 500nm; p, parasite. Micrograph at the bottom has been magnified twice with respect to that at the top, to distinguish the parasite plasma membrane (PPM) from the PVM. **D.** Western blots of tetanolysin-released infected erythrocyte cytoplasm supernatant (S) and pellet (P) fractions of cells expressing HT-GFP<sub>memb</sub>myc (left panel), Δ-GFP<sub>memb</sub>myc (middle panel) and HT-GFP<sub>memb</sub>C-term (right panel) probed for GFP, PVM marker PfStomatin and parasite cytoplasmic protein PfFKBP. Hemoglobin (Hb) released (in S) by tetanolysin is expressed as a percentage of the total Hb detected by hypotonic lysis. **E.** Live infected erythrocytes expressing HT-GFP<sub>memb</sub>C-term viewed under bright field image, GFP fluorescence and merged optics. Schematic representation for all constructs are indicated above with ER-type signal sequence (red), sequence containing HT signal (blue) or its replacement (solid black triangle) fused to GFP (green), transmembrane region (black) and myc (orange). C-terminal region (derived from PfEMP1) is depicted in yellow.

**Figure 4. The HT motif sorts protein into Maurer’s clefts without translocation across the PVM.** **A.** For both HT-GFP<sub>memb</sub>myc and Δ-GFP<sub>memb</sub>myc, western blots show protection of GFP but quantitative degradation of myc and erythrocyte spectrin after
addition of trypsin to cells where the infected erythrocyte membrane was permeabilized with tetanolysin (lanes 2 and 6). Saponin (which additionally permeabilizes PVM and clefts, lanes 4 and 8) renders GFP susceptible to protease. Asterisk, trypsin digested GFP product of 25-kDa. Molecular weight markers are in kiloDalton (kDa). B. Single optical sections of ghosts resealed with Alexa 594 anti-GFP antibodies infected with parasites expressing HT-GFP\textsuperscript{memb}myc (top panel) or Δ-GFP\textsuperscript{memb}myc (bottom panel). Cells were viewed live using optics for GFP (green), Alexa 594/Rhodamine (red) and the merged image is shown in the right panel. Arrows, GFP labeled clefts not labeled with anti-GFP Alexa 594 conjugate. C. Immunofluorescence assay of resealed ghosts infected with parasites expressing HT-GFP\textsuperscript{memb}myc (top panel) or Δ-GFP\textsuperscript{memb}myc (bottom panel) permeabilized with saponin and treated with anti-GFP Alexa 594 conjugated antibodies. Images under GFP (green) and Alexa 594 (red) optics and their respective merge are shown. Arrowhead, region of colocalization (in yellow) between GFP and Alexa 594. In all cells, the parasite (p) nuclei were stained with Hoechst (blue); bar, 2μm. Schematic representation of the construct is indicated above with ER-type signal sequence (red), sequence containing HT signal (blue) or its replacement (filled triangle in black) fused to GFP (green), transmembrane region (black) and myc (orange).

**Figure 5.** HT-dependent protein sorting into clefts may occur at parasite periphery and is not influenced by deletion of the C-terminal domain of PfSBP1. A. 3-dimensional projections of a live infected erythrocyte expressing HT-GFP\textsuperscript{memb}myc and stained with TR-ceramide. Clefts at the periphery of the infected erythrocyte (arrows) as well as at or within the perimeter of the vacuolar parasite (empty arrowheads) are visible.
**B.** 0° projection of live infected erythrocyte expressing HT-GFP\textsuperscript{memb}myc in 3D7 strains with parental (top) or chromosomal deletion of \textit{pfsbp1} (bottom), viewed under GFP optics and merged with brightfield. Arrows indicate that the export of HT-GFP\textsuperscript{memb}myc to cleft structures in parental 3D7 strain is not altered in parasite line with a C-terminal deletion in PfSBP1. Parasite (p) nucleus is stained with Hoechst. Bar, 2\textmu m.

**Figure 6.** HT-dependent protein sorting into clefts is not influenced by deletion of putative substrate binding domain in PFE0055c. **A.** Deduced amino acid sequence of PFE0055c with a N-terminal ER-type signal sequence (brown), HT motif (bold), followed by sequences containing DnaJ region (orange) with the characteristic HPD (green) motif. Further downstream region include a glycine/phenylalanine-rich stretch and C-terminal substrate-binding domain (underlined). Sequences in blue indicate region deleted in parasite line generated by single crossover recombination as shown in panels D-F. **B.** Western blot, using anti-PFE0055c antibodies, detecting the presence of a 42-kDa protein in infected erythrocyte (arrowhead, lane 2) but not uninfected erythrocyte (lane 1). **C.** Single optical section of a trophozoite-infected erythrocyte fixed and probed with peptide antibodies to PFE0055c (green) and the cleft protein SBP1 (red). Arrow in merge image shows proximal location of PFE0055c to clefts. **D.** Strategy for deletion in the C-terminal substrate-binding region of PFE0055c by single crossover recombination with the chromosomal copy of \textit{pfe0055c}. \textit{P. falciparum} parasites were transfected with plasmids containing an in frame fusion of the neomycin resistance gene (\textit{npt}, green) to an internal fragment of \textit{pfe0055c} (orange) without sequences encoding for C-terminal substrate-binding domain. Only chromosomal integration of the vector by single
crossover with the native pfe0055c (pink) drives npt expression under the control of pfe0055c promoter (Ppfe0055c), thus conferring resistance of antibiotic G418. E. PCR-based detection for the loss of chromosomal copy of pfe0055c. Positions for primer pairs used for amplification analyses of single crossover recombination are highlighted in D. F. Western blot analysis showing the detection of PFE0055c-NPT fusion protein of 45-kDa in transfected line (arrowhead, lane 1) but in not parental line (lane 2) using antibodies to NPT (top). Parasite protein PfFKBP serves as a loading control (bottom). G. 0° projection of live infected erythrocyte expressing HT-GFP<sub>memb</sub><sub>myc</sub> in 3D7 strain with chromosomal deletion of pfe0055c viewed under GFP optics and merged with brightfield. Arrow indicates that the export of HT-GFP<sub>memb</sub><sub>myc</sub> to cleft is not altered by truncation in PFE0055c. Parasite nucleus (p) in all cases is stained with Hoechst (blue). Bar represents 2µm.

**Figure 7. Schematic for HT-mediated cleft targeting events in erythrocyte infected with the malaria parasite P. falciparum.** In secretory proteins, a cleavable N-terminal ER-type signal sequence (SS) delivers proteins to the PV (step 1). The HT motif enables protein accumulation in Maurer’s clefts (MC, step 2). Clefts can either bud from the PVM (also step 2) packed with proteins exported to the red cell and function as protein reservoirs underneath the erythrocyte membrane (step 3). The HT motif may also move protein from the lumen of the PVM to lumen of clefts either within the parasite (step 2′) or at the proximity to the parasite plasma membrane (PPM, step 3′). Both steps 2 and 2′ implicate recognition of the HT motif by a putative receptor located at the Maurer’s clefts (not shown).
Figure 1

A Brightfield  GFP  Merge

B

C GFP  PfsBP1  Merge

Brightfield GFP Merge

GFP PfSBP1 Merge

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Figure 2

A

Live cells (GFP + Hoechst)

B

C

D

E

% Colocalization of fluorescence

F

No. of gold particles associated with clefts

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Figure 3

A

Brightfield GFP Merge

B

GFP PfSBP1 Merge

C

GFP PfStomatin FKBP

D

% Hb release 97% 99% 97%

E

Brightfield GFP Merge
Figure 4

A

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B

C

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Figure 5
Figure 6

A

MSILNKYEKGKKNKIFLIINIIIFLYTLEYVLIGSNYDKHNQSFGNEIFKNTKVFDFTSLRSLA
EFNSGSSRESKDETDYLYAVLGLTCDQDDIKAYRKLMWHPDZHLDNEDEKVEAEKRF
LIGEAYEVLSEDREEXKKNYDLFGQSGLLGGTTNDEAYTYSNIDPNLFSRFSSHDAASSFSQG
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YKCYLPLKDALTGFQFSIKSLDNRDINVVDIINPNSSKIIITNEGMPYKSPSVKGDLFIEF
DIVFPKLSPEQKRTLKELENTEKSYTFIMNVT

B

1 2

42-kDa

C

PFE0055c  PfSBP1  Merge

P  P  P

D

3' hrp2

npt

pfe0055c-fragment

5' cam

3' RbDT

5' cam

3' PbDT

3' hrp2

npt

1-2  1-4  3-4

1 kb

E

F

Anti-NPT

Anti-FKBP

1 2

G

GFP  GFP + Brightfield

134

12

45-kDa

35-kDa
Figure 7
Maurer's clefts of *P. falciparum* are secretory organelles that concentrate virulence protein reporters for delivery to the host erythrocyte

Souvik Bhattacharjee, Christiaan van Ooij, Bharath Balu, John H Adams and Kasturi Haldar