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PI(3)P-independent and -dependent pathways function together in a vacuolar translocation sequence to target malarial proteins to the host erythrocyte

Souvik Bhattacharjee^{a,b}, Kaye D. Speicher^c, Robert V. Stahelin^{d,e}, David W. Speicher^c, and Kasturi Haldar^{a,b,*}

Souvik Bhattacharjee: sbhatta1@nd.edu; Kaye D. Speicher: kspeicher@wistar.org; Robert V. Stahelin: rstaheli@nd.edu; David W. Speicher: speicher@wistar.org; Kasturi Haldar: khaldar@nd.edu

^aCenter for Rare and Neglected Diseases, University of Notre Dame, 103 Galvin Life Sciences, Notre Dame, IN 46556, USA

^bDepartment of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA

^cCenter for Systems and Computational Biology and Molecular and Cellular Oncogenesis Program (Room 151), Wistar Proteomics Core Facility (Room 154), The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA

^dDepartment of Biochemistry & Molecular Biology, Indiana University School of Medicine-South Bend, 143 Raclin-Carmichael Hall, 1234 Notre Dame Avenue, South Bend, IN 46617, USA

^eDepartment of Chemistry & Biochemistry University of Notre Dame, Notre Dame, IN 46556, USA

Abstract

Malaria parasites export ‘a secretome’ of hundreds of proteins, including major virulence determinants, from their endoplasmic reticulum (ER), past the parasite plasma and vacuolar membranes to the host erythrocyte. The export mechanism is high affinity (nanomolar) binding of a host (cell) targeting (HT) motif RxLxE/D/Q to the lipid phosphatidylinositol 3-phosphate (PI(3)P) in the ER. Cleavage of the HT motif releases the secretory protein from the ER membrane. The HT motif is thought to be the only export signal resident in an N-terminal vacuolar translocation sequence (VTS) that quantitatively targets green fluorescent protein to the erythrocyte. We have previously shown that the R to A mutation in the HT motif, abrogates VTS binding to PI(3)P ($K_d > 5 \mu\text{M}$). We now show that remarkably, the R to A mutant is exported to the host erythrocyte, for both membrane and soluble reporters, although the efficiency of export is reduced to ~ 30% of that seen with a complete VTS. Mass spectrometry indicates that the R to A mutant is cleaved at sites upstream of the HT motif. Antibodies to upstream sequences confirm that aberrantly cleaved R to A protein mutant is exported to the erythrocyte. These data suggest that export mechanisms, independent of PI(3)P as well as those dependent on PI(3)P, function together in a VTS to target parasite proteins to the host erythrocyte.

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*Corresponding author, Tel.: +1 574 6311474; Fax: +1 574 6311648.

Supplemental information

Supplemental information includes three supplementary figures, supplementary figure legends and one supplementary table.

Conflict of interest

The authors declare no competing financial interests.

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Keywords

Plasmodium falciparum; pathogenic protein secretion; PI(3)P-independent export

Introduction

Malaria is a major parasitic disease responsible for almost a million deaths in children each year [1]. The most virulent of human malarias is caused by the protozoan *Plasmodium falciparum*, which during the blood stages, infects mature erythrocytes [2]. Within the erythrocyte, the parasite resides and proliferates within a parasitophorous vacuole (PV). Proteins synthesized by the parasite are exported across the PV membrane (PVM) into the cytoplasm and membrane of the erythrocyte to induce changes in transport and membrane properties there [3, 4]. *P. falciparum* is predicted to export several hundred proteins into the erythrocyte [5, 6], a subset of which (such as *P. falciparum* membrane protein 1, PfEMP1) are major virulence determinants linked to severe disease [7, 8].

Parasite proteins destined for export are recruited into the ER via an N-terminal signal sequence or a transmembrane domain. The presence of a consensus host (cell) targeting (HT) or PEXEL (*Plasmodium* Export Element) signal RxLxE/D/Q downstream of the signal peptide or transmembrane domain, is known to export proteins to the erythrocyte [5, 6]. We originally described the HT signal as a high-value 'core' motif located within a vacuolar translocation sequence (VTS) of 40 amino acids [9, 5] that were functionally equivalent across five secreted proteins and necessary and sufficient for protein export to the erythrocyte. Later studies suggested that VTS residues downstream of the HT signal provided little or no sequence information for protein export [10]. Rather there was need for a 'spacer' arm and the HT motif needed to be placed at least 12 amino acids upstream of a reporter like green fluorescent protein (GFP) in order to function in export to the host erythrocyte [10]. The HT signal is also cleaved immediately after RxL in the parasite's ER [11, 12] via a protease plasmepsin V [13, 14]. Cleavage releases the polypeptide from the ER membrane. Since mutation in R was reported to quantitatively block protein release from the ER/ PV, cleavage of HT motif appeared to be the only event required to target exported proteins to the host erythrocyte.

However our recent work revealed that the export mechanism of the HT motif is not due to cleavage, but rather its high affinity binding of the lipid PI(3)P in the plasmodial ER, which occurs prior to and independent of protease cleavage [15]. We now show that in addition, the VTS can also support PI(3)P/HT-independent export which provides new insights into models of *P. falciparum* secretome trafficking to the host erythrocyte.

2. Materials and methods

2.1. Cloning

The construct pA150-HT-GFP with the wild type HT core of RLLYE and those with single point mutants in HT core like ALLYE, RLAYE and RLLYA were generated as described earlier [15]. The HT signal of *P. falciparum* histidine rich protein II (PfHRPII) in *P. falciparum* 3D7 is indicated as RLLHE in PlasmoDB. This differs in 4th position from the sequence amplified from our laboratory strain [5, 15] and is functionally equivalent to RLLYE.

The construct pA150-HT-GFP^{mem}myc, with the HT core RLLYE and containing sequences encoding for the transmembrane and cytoplasmic region of PfEMP1, fused to myc tag, was derived from pBacII (HT-GFP^{mem}myc) [3]. Briefly, region containing SS-

HT-GFP^{memb}myc was amplified by PCR using HRPIIAvrIIF and mycXhoIR and subsequently cloned into pA150 to generate pA150-HT-GFP^{memb}myc. This construct as well as the emerging transgenic line is also referred to as SS-RLLYE-GFP^{memb}myc during the course of this study to emphasize that the HT region contains wild type RLLYE core. For the generation of single point mutants in HT core RLLYE, like ALLYE, RLAYE and RLLYA, a strategy of overlapping PCR was used using pA150-HT-GFP^{memb}myc as the template. In all cases the products of PCR 1 and PCR 2 were used as templates for PCR 3 that was sub-cloned into pA150. For changing RLLYE to ALLYE, the primer pairs HRPIIAvrIIF and ALLYER yield PCR 1 and ALLYEF and mycXhoIR yield PCR 2. For RLAYE, the primer pairs HRPIIAvrIIF and RLAYER yield PCR 1 and RLAYEF and mycXhoIR yield PCR 2. For RLLYA, the primer pairs HRPIIAvrIIF and RLLYAR generated PCR 1; and RLLYAF and mycXhoIR generated PCR 2. Sequences of primers are shown in table S1.

Constructs for fusion proteins with C-terminus RFP (for testing the reactivity of pre-HT antibody) or GFP (for Surface Plasmon Resonance (SPR) analyses) were generated and proteins affinity purified as described earlier [15].

Fusion constructs expressing red fluorescent protein (RFP) tagged to a Maurer's cleft marker Skeleton binding protein 1 (SBP1) was generated by first amplifying *rfp* from pAdRFP (Addgene) using MRPFbBamHIF and MRFPBgIIR primers and cloning into pBluescript (Stratagene) digested with BamHI. *P. falciparum sbp1* was then amplified from genomic DNA using SBP1XhoIF and SBP1BamHIR and cloned at BamHI and XhoI site to generate pBS(SBP1-RFP), which was further subcloned into pA156 (Blasticidin-resistant version of pA150; [15]) at NheI-XhoI site, using SBP1NheIF and RFPTXhoIR, to yield pA156 (SBP1-RFP).

2.2. Generation of anti-peptide antibodies

We used peptides>NNLCSKNAKGLNLNKRL (pre-HT) and YETQAHVDDVHHAAHHADV (post-HT), based on sequence N-terminal or C-terminal, respectively to the deduced plasmepsin V cleavage site of PfHRPII. Rabbit polyclonal antibodies raised to each were designated as anti-(pre-HT) and anti-(post-HT), respectively [15].

2.3. Parasite culture, transfection and live cell imaging

P. falciparum 3D7 parasites were propagated in A+ human erythrocytes in RPMI supplemented with 0.5% Albumax II, 0.2 mM Hypoxanthine, 11 mM Glucose, 0.17% NaHCO₃, 10 µg/ml gentamycin and maintained at 37°C in 5% Carbon dioxide. Synchronous *P. falciparum* 3D7 parasites in culture were transfected with indicated plasmids by standard procedures as described earlier [3]. Forty-eight hours after transfection, the cultures were selected with 2.5 nM WR99210 (Jacobus Pharmaceuticals) until stable cell lines were obtained.

Double transgenic parasite line with HT/RLLYE-GFP^{memb}myc background and additionally expressing SBP1-RFP was generated by transfecting HT-GFP^{memb}myc with pA156 (SBP1-RFP) and selecting with 1 µg/ml Blasticidin.HCl 72 hours after transfection. Drug selection was continued until infected erythrocytes were detected in giemsa smears (typically 4 weeks).

For live cell imaging, parasites were processed as described previously [12]. In both live and fixed cells, the parasite nuclei were stained with Hoechst 33342 and cells were imaged with a 100X, NA 1.4 objective 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, WA).

Quantitative analysis for the percentage of GFP signal exported to the red cell cytoplasm for transgenic parasite lines expressing HT/RLLYE-GFP^{memb}myc and ALLYE-GFP^{memb}myc was undertaken using DeltaVision software. Briefly, 20 optical z-sections per cell were first converted into a 0° projection, the total pixel intensities of GFP signal was measured and assigned as 100%. The parasite boundary was then marked (using brightfield images as a reference) and the pixel intensities for the GFP signal within the parasite and those exported to the red cell were measured and expressed as a percentage of the total GFP signal for the cell. A total of 200 optical sections were thus examined for each transgenic line and the quantitative information obtained was represented graphically.

2.4. Immunoprecipitation and Western blotting of tagged transgenic proteins

Proteins were extracted from mixed parasite cultures at 8-10% parasitemia and processed for immunoprecipitation with anti-GFP beads (MBL) or western blotting as described earlier [15].

To check the reactivity of pre-HT and post-HT antibodies, 2×10^6 *P. falciparum* 3D7 parasites at 15% parasitemia were permeabilized with 0.05% saponin, which permeabilizes the red cell membrane and the PVM but not the parasite plasma membrane. The pellet and the supernatant fractions were solubilized in Laemmli's sample buffer containing 1 mM dithiothreitol (DTT) and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membrane, blocked with PBS containing 5% skim milk and probed with respective primary antibodies. After washes, the membrane was probed with secondary antibody HRP conjugate and developed using chemiluminescence.

2.5. Mass Spectrometry and peptide quantitation

Protein bands of interest were excised from the gel, destained, alkylated with iodoacetamide, and digested with AspN and analyzed by LC-MS/MS using reverse phase capillary HPLC with a Thermo Electron LTQ OrbiTrap XL mass spectrometer as described earlier [15]. The peptide identities of integrated peaks were verified based on retention times of matching MS/MS spectra.

2.6. Immunolocalization by indirect immunofluorescence assays (IFA)

IFAs were performed on non-transfected *P. falciparum* 3D7 or transgenic parasites fixed with glutaraldehyde/paraformaldehyde as described previously [16] using the following antibody concentrations: polyclonal rabbit anti-GFP (Invitrogen) 1:200; monoclonal mouse anti-Exp2 (MR4) 1:200, 2 µg/ml custom-made polyclonal rabbit anti-(pre-HT) (Affinity Bioreagents), and 2 µg/ml custom-made polyclonal rabbit anti-(post-HT) (Affinity Bioreagents). The appropriate FITC- or TRITC labeled secondary IgG antibodies (ICN Biochemicals) were used at 1:200 dilution. Parasite nuclei were stained with 5 µg/ml Hoechst 33342 (Molecular Probes) and slides were mounted with DABCO.

2.7. SPR Binding Protein-Lipid Interactions

All SPR measurements were performed at 25°C in PBS as described earlier [15]. Sensor chip surfaces were injected with POPC/POPE/Phosphoinositide (77:20:5) and POPC/POPE (80:20) vesicles at 5 µl/min onto the active surface and the control surface, respectively, to give the same resonance unit (6000 RU) values. Equilibrium SPR measurements were done at the flow rate of 5 µl/min. Each data set was repeated three times to calculate the mean ± standard deviation.

3. Results

3.1. The R to A mutation in the HT motif blocks PI(3)P binding but only partially inhibits export of a membrane protein reporter to the erythrocyte

The wild type HT motif of the parasite protein PfHRP2 is RLLYE, where underlined residues indicate conserved motif positions. Mutation of the R to A in the motif abrogates PI(3)P binding ([15]; Fig. 1A). Its presence in a membrane bound secretory reporter reduced, but surprisingly, did not block protein export to the erythrocyte (Fig. 1B). The wild type reporter was comprised of the signal anchor and the next 40 amino acid VTS of PfHRP2 followed by GFP, the transmembrane and cytoplasmic domains of the parasite protein PfEMP1, and a myc tag (Fig. 1B). We have previously demonstrated that this reporter is efficiently exported to structures called the Maurer's clefts which are punctate structures induced by infection at the periphery of the erythrocyte [3]. This is also evident in Fig. 1B, where virtually all of the green fluorescent protein associated with the RLLYE motif is detected in punctate structures (Fig. 1B, top panel). However in the R to A mutant there is significant accumulation of GFP outside of punctate structures and within the body of the parasite and only a fraction is discernible in exported clefts (Fig. 1B, bottom panel).

Quantitative analyses of 200 single optical sections (Fig. 1C) revealed that in cells expressing protein with the RLLYE motif (also called wild type protein), greater than 90% of GFP was in punctate structures exported from parasites. Some GFP signal surrounded the parasite but most was observed in the periphery of the erythrocyte. The punctate GFP fluorescence co-localized with Maurer's cleft resident protein (Fig. 1D). In contrast in cells expressing the R to A mutant transgene, only 30% of GFP was exported to the erythrocyte (Fig. 1C). These results are not entirely in agreement with previous studies that suggested the R to A mutation blocked export by 90% [17], which may be due to several reasons. Prior analysis was limited to soluble reporters which may be difficult to detect when they are present at low levels and dispersed in the erythrocyte cytoplasm. In contrast, protein export achieved by our membrane form chimera is efficient, where the wild type form does not substantially accumulate within the parasite. Finally, recent increases in sensitivity of detection reveal that single point mutations reduce but do not abrogate export. Nonetheless our findings shown in Fig. 1 were unexpected. This is because the R to A mutation is known to completely abrogate PI(3)P binding ($K_d > 5 \mu\text{M}$; [15]; Fig. 1A) and hence the 30% export associated with the R to A mutant in Fig. 1 B-C, must be independent of PI(3)P. In addition it is well established that export requires cleavage of the signal anchor. Indeed an ER protease has been shown to cleave the HT motif [13, 14] and *in vitro* studies suggest that it may be plasmepsin V; and the R to A mutation is expected to quantitatively block cleavage of the HT motif by this protease [13]. This raised question on how exported protein with the R to A mutation in Fig. 1B, was released from the signal anchor.

To address the mechanism of signal anchor cleavage, we immunopurified wild type and mutant proteins and subjected them to mass spectrometry (Fig. S1A-B). As expected, the wild type protein is cleaved within the HT motif just after the RxL (Fig. 1E and Fig. S1C). However for the R to A mutant, the most N terminal peptide starts from sequence NLN upstream of the expected cleavage site (Fig. 1E and Fig. S1D), thus suggesting that R to A mutant is cleaved upstream of the HT motif. In contrast, the L to A and E to A mutants revealed N terminal sequences starting in YETQAMVD and YATQAMVD (Fig. 1E and Fig. S1E-F) strongly indicating that, as expected by the specificity of plasmepsin V, they were cleaved within the HT motif.

The cleavage of the R to A mutation upstream of the HT motif, as shown in Fig. 1E (and Fig. S1D) was unexpected. Thus, we were interested in investigating whether any of the GFP exported to the erythrocyte (as shown in Figure 2A) was due to the products of aberrant

cleavage. To do this we used antibodies that selectively react with upstream sequences called the pre-HT region [15]. The specificity of these antibodies is indicated schematically in Fig. 2B and characterization of their selectivity [15] is summarized in Fig. S2. Indirect immunofluorescence assay (IFA) studies revealed that pre-HT antibodies did indeed recognize protein exported to the erythrocyte in the R to A mutant (Fig. 2C, second panel) but failed to recognize exported precursor protein forms in the erythrocyte for both wild type protein (Fig. 2C, top panel) as well as for mutants L to A and E to A (Fig. 2C, third and bottom panel, respectively), suggesting that the pre-HT region was cleaved.

These localization data along with mass spectrometry conclusively establish that in the R to A mutant, the HT motif was not cleaved to release protein from the ER membrane. Rather signal anchor cleavage occurred at an upstream site and this aberrantly cleaved form was exported to the erythrocyte and detected by anti-(pre-HT) antibodies. In case of the L to A and E to A mutants, cleavage occurred at the mutated HT motif and hence exported protein could not be detected by anti-(pre-HT) antibodies. Hence the data in Fig. 1 and 2 definitively establish that the R mutation (which abrogates both PI(3)P binding and HT cleavage), inhibits but does not completely block export, suggesting that the VTS appears to contain additional sequence information that is responsible for PI(3)-independent export.

3.2. PI(3)P-independent export mechanisms are located in the VTS for transport of a soluble reporter to the host erythrocyte

Since pathways of export of membrane and soluble proteins can differ, we next examined export of a soluble reporter protein to the host erythrocyte (Fig. 3) by live cell imaging (Figure 3A) as well as by IFA using pre-HT antibodies (Figure 3B). Unlike, membrane reporters, similar comparison for quantitation of the percentage of export in case of soluble reporters is not feasible because there is substantial GFP fluorescence seen in the body of the parasite even for the wild type HT-GFP (data not shown). However, a fraction of GFP is exported to the red cell for all single point mutation in the HT signal (Figure 3A). Here again by IFA using pre-HT antibodies (Figure 3B), the R to A mutation that completely abrogates PI(3)P binding, protein recognized by the pre-HT antibody is seen to be exported to the erythrocyte (top panel). In contrast, in the L to A and E to A mutants, the pre-HT antibodies do not recognize protein in the erythrocyte (middle and bottom panel, respectively). These data also suggested that cleavage of exported protein in the R to A mutant occurred upstream of the (mutated) HT motif, while for proteins exported in L to A and E to A mutants cleavage occurred within the HT motif or downstream.

To address the site of signal anchor and HT cleavage, we immunopurified wild type and mutant proteins and subjected them to mass spectrometry (Fig. S3A and Fig. S3B). Mass spectrometry data confirmed that for the R to A mutant, the most N-terminal peptide starts from sequence FNNL immediately downstream of the putative signal peptidase cleavage site (Fig. S3D), while the wild type motif is cleaved after the RxL (Fig. S3C). The soluble L to A and E to A mutants were cleaved within the HT motif (Fig. S3E-F). These data (summarized in Fig. 3B) complement those in Figure 2, to further strengthen the idea that despite the absence of high affinity interaction with PI(3)P, protein export may occur in an independent manner and this information resides within the VTS.

4. Discussion

As indicated earlier, the HT motif (and thus its binding to PI(3)P) functions in the larger context of a ~40 amino acid VTS needed to mediate parasite protein export to the erythrocyte [9, 5]. All three conserved residues in the RxLxE contribute to PI(3)P binding, but loss of the R alone, shifts export away from a high affinity PI(3)P-binding, dependent pathway [15]. Prior studies have shown that the HT motif can function in export as long as

8-12 amino acid 'spacer' separates it from GFP [10]. This led to the suggestion that remaining sequences in the VTS failed to contain information for protein export to the erythrocyte. However we recently reported that sequences downstream of the HT motif of two proteins, PfHRP2 and *P. falciparum* erythrocyte membrane protein 3 (PfEMP3), could indeed export green fluorescence to the erythrocyte [15], but whether they function in the context of the HT motif and in a complete VTS was not known. Quantitative analyses of export and the development of specialized antibodies allowed us to demonstrate that when a polypeptide is cleaved by signal peptidase or aberrantly cleaved in a VTS that cannot bind PI(3)P, the VTS can nonetheless function in protein export to the erythrocyte, definitively establishing that sequences outside of the HT motif contribute to targeting to the erythrocyte and act in conjunction with PI(3)P binding.

That we detect export of the R to A mutant provides strong evidence that export is neither mediated by PI(3)P or plasmepsin V, but rather by independent mechanisms. Our prior data [15] suggest that sequences downstream of the HT when engineered to be exposed as the free N-terminus were necessary and sufficient to export GFP to the host cell, on the basis of which we suggest that these sequences in the VTS at least partly contribute to PI(3)P-independent binding (shown schematically in the model in Fig. 4). However, in prior models [15], PI(3)P-dependent and -independent export signals were proposed to act at two independent sites in the ER. Our data in this study suggest that PI(3)P-dependent and -independent mechanisms may act together in protein export and thus we correct prior models where we have proposed they act in separate routes of protein export (Fig. 4). To the best of our knowledge PI(3)P-independent mechanisms in a VTS do not display any sequence specific 'logos' although they may depend on charge as has been proposed for parasite proteins exported to the host erythrocyte [18].

Early models of secretion [19, 20], proposed separation in pathways of export, targeted to the erythrocyte from those delivered to the PV. Wiser et al (1997) proposed a 'secondary ER' that directly exported protein to the erythrocyte. This was supported by rapid kinetics of secretory protein exit [21]. Two export mechanisms acting in conjunction in the VTS reported here may reflect the need for efficiency in export, because the load of antigenic cargo is high at potentially 400 proteins and most are rapidly secreted to for red cell remodeling early in intraerythrocytic infection. Further, once the HT motif is cleaved by plasmepsin V, the PI(3)P independent pathway must dominate especially in terminal steps of transport to the erythrocyte (Fig. 4).

The role of the Golgi in PI(3)P-dependent and independent pathways is not known. The extent to which these pathways interact with a translocon proposed to mediate protein export into the erythrocyte [22] remains unknown. The translocon was isolated on the basis of specificity of interacting with the HT signal, which is lost in the ER. This then suggests a need for an alternative mechanism for PI(3)P independent export across the parasite plasma and vacuolar membranes. Finally, the number of PI(3)P-independent export pathways to the host cell are unknown. It is possible that a subset of export pathways act in complete absence of PI(3)P binding mechanisms, such as the export of *P. falciparum* skeleton binding protein 1 (PfSBP1 of the Maurer's clefts) that are devoid of an HT signal. Perhaps what is most intriguing is that PI(3)P-targeted protein cargo are delivered to PfSBP1-labeled Maurer's cleft structures [3], suggesting mechanisms of export to the host erythrocyte may be more complex than we have yet recognized.

Plasmodium has been shown to harbor one phosphatidylinositol-3-kinase (PI3K) responsible for PI(3)P synthesis, which has been shown to localize to the food vacuole and the parasite plasma membrane [23]. However, how PI(3)P is synthesized or transported into the lumen of the ER is unknown. One possible mechanism of PI(3)P import is flip-flop mediated by

ER flippases. *Trans*-bilayer flip-flop of glycerophospholipids such as PI(3)P is an important step in membrane biogenesis. While in general glycerophospholipids do not cross bilayers in synthetic vesicles, the ER harbors specific flippases that mediate bidirectional movement of glycerophospholipids in an ATP-independent manner. Although specific flip-flop of phosphoinositides has not been demonstrated it has recently been shown that phosphatidylinositol (PI) can be flipped by an ER flippase [24] without specificity for the glycerol-lipid tail or *myo*-inositol head group stereochemistry. Additionally, in the *trans*-Golgi network a Golgi flippase has been identified that is regulated by PI(4)P [25]. Thus, this raises the intriguing possibility that PI(3)P present on the cytoplasmic face of the ER may undergo flip-flop into the lumen where HT signals would bind and possibly trap PI(3)P due to nanomolar affinity. In yeast, PI(3)P has been found in the lumen of multi-endovesicles during autophagy [26] but similar studies of autophagy in malaria parasites have not been done. The mechanism of PI(3)P synthesis or transport into the lumen of the ER remains a burning question in the *Plasmodium* field elucidation of which will lead to a better understanding of mechanisms of PI(3)P-dependent and -independent export.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

► R to A mutation in the HT signal abrogates PI(3)P binding and HT cleavage. ► R to A mutant is exported via HT/PI(3)P-independent pathway. ► Efficiency of HT/PI(3)P-independent export pathway is 30% of that seen with the VTS. ► VTS contains PI(3)P-dependent and -independent signals to maximize export efficiency.

Fig. 1. Mutation in the HT signal partially inhibits protein export. (A) K_d values ($\pm$ standard deviation) determined for recombinant HT/RLLYE-GFP and ALLYE-GFP by equilibrium SPR analysis. To determine K_d values, R_{eq} values were plotted versus [P], and the K_d value was determined by a nonlinear least-squares analysis of the binding isotherm using the following equation: $R_{eq} = R_{max}/(1 + K_d/P_0)$. Experiments were performed in triplicate. (B) Live *P. falciparum*-infected erythrocytes expressing VTS reporter with wild type HT signal (SS-RLLYE-GFP^{memb}myc, top panel) or its mutant (SS-ALLYE-GFP^{memb}myc, bottom panel). Whereas all the GFP associated with the wild type RLLYE motif is detected in punctate cleft structures, the R to A mutant shows accumulation of GFP within the body of the parasite and only a fraction is in exported clefts. Left panels, fluorescence images; right panels, merged brightfield images with Hoechst 33342 nuclear staining (blue). Scale bars, 5 μ m. (C) Quantitative analyses of 200 single optical sections for cells expressing the wild type SS-RLLYE-GFP^{memb}myc motif shows >90% of GFP in exported punctate structures. In contrast in cells expressing SS-ALLYE-GFP^{memb}myc with the R to A mutant, only 30% of GFP was exported to the erythrocyte. (D) Live double transgenic *P. falciparum*

erythrocytes expressing SBP1-RFP and SS-HT/RLLYE-GFP^{memb}myc. The punctate GFP fluorescence in the erythrocyte cytoplasm co-localizes with Maurer's clefts structures (red), as seen yellow/ merge panel. Parasite nuclei are stained with Hoechst 33342; brightfield, fluorescent and merge images are shown as indicated. Scale bars, 5 μ m. (E) Schematic representation for the site of processing in membrane reporters with VTS containing wild type HT motif RLLYE and its respective point mutants (ALLYE, RLAYE and RLLYA), as determined from the most N-terminus peptide obtained from LC-MS/MS after AspN digestion. Arrows indicate the site of cleavage.

Fig. 2.

A HT-independent pathway in the export of membrane reporter to the red cell cytoplasm. (A) Live *P. falciparum*-infected erythrocytes expressing a VTS reporter with the wild type HT signal (SS-RLLYE-GFP^{memb}myc (top panel) or single point mutation in the HT signal, SS-ALLYE-GFP^{memb}myc (second panel); SS-RLAYE-GFP^{memb}myc (third panel) and SS-RLLYA-GFP^{memb}myc (bottom panel). Essentially, all the GFP associated with the wild type RLLYE motif is detected in exported, punctate cleft structures. In contrast R/L/E to A mutant shows different levels of accumulation of GFP within the body of the parasite. This is most prominent with SS-ALLYE-GFP^{memb}myc, where ~70% GFP signal is associated with the parasite. Left panels, fluorescence images; right panels, merged brightfield images with Hoechst 33342 nuclear staining (blue). Scale bars, 5 μ m. (B) Schematic representation of the VTS after signal peptidase cleavage site. Blue bar indicates the pre-HT region used to generate pre-HT antibodies. (B) Localization of non-cleaved HT in membrane reporters as a marker for HT-independent export. Transgenic parasites were fixed, permeabilized and probed with anti-(pre-HT) (green) as well as antibody to endogenous *P. falciparum* marker of the PVM like Exp-2 (red). While precursor, non-cleaved HT signal for wild type secretory HT (SS-RLLYE-GFP^{memb}myc, top panel) and HT point mutants SS-RLAYE-GFP^{memb}myc (third panel) and SS-RLLYA-GFP^{memb}myc (bottom panel) is seen only within the parasite; secretory ALLYE-GFP^{memb}myc (second panel) is seen in punctuate 'spots' and diffused region in infected erythrocyte cytoplasm, thus indicating HT (processing)-independent export to the red cell. Dotted circle indicates red cell membrane. The parasite nucleus was stained with Hoechst 33342 (blue). Brightfield images and fluorescent images are shown as indicated. The scale bars represent 5 μ m.

Fig.3.

A HT-independent pathway in the export of soluble reporter to the red cell cytoplasm. (A) Live *P. falciparum*-infected erythrocytes expressing a soluble VTS reporter with single point mutation in the HT signal SS-ALLYE-GFP (top panel); SS-RLAYE-GFP (middle panel); SS-RLLYA-GFP (bottom panel). A single point mutation in the HT signal does not abrogate GFP export to the red cell. Scale bars, 5 μ m. (B) Localization of non-cleaved HT in soluble reporters as a marker for HT-independent export to the red cell. Transgenic parasites were fixed, permeabilized and probed with anti-(pre-HT) (green) as well as antibody to endogenous *P. falciparum* marker of the PVM like Exp-2 (red). While precursor, non-cleaved HT signal for point mutants RLAYE-GFP (middle panel) and RLLYA-GFP (bottom panel) is seen only within the parasite; secretory ALLYE-GFP (top panel) is seen in punctuate 'spots' and diffused region in infected erythrocyte cytoplasm, thus indicating HT (processing)-independent export to the red cell. Dotted circle indicates red cell membrane. The parasite nucleus was stained with Hoechst 33342 (blue). Brightfield images and fluorescent images are shown as indicated. The scale bars represent 5 μ m. (C) Schematic representation showing the site of processing for soluble reporters with VTS containing wild type HT motif RLLYE and its respective point mutants (ALLYE, RLAYE and RLLYA), as determined from the most N-terminus peptide obtained from LC-MS/MS after AspN digestion. Arrows indicate the site of cleavage.

Fig. 4.

PI(3)P-dependent and independent signals function together in a vacuolar translocation sequence (VTS) to target parasite proteins to the host erythrocyte. Summary model at the LHS, demonstrating that proteins containing PI(3)P-dependent and PI(3)P-independent export signals can be co-translationally inserted via their signal anchor sequence into the ER membrane as shown in steps 1 and 1' respectively. The lipid PI(3)P is shown to be enriched in regions of the ER and recognized the HT motif in step 2 (which may occur co-translationally). Secretory proteins that have a plasmepsin V-refractory HT signal may be cleaved by signal peptidase but remain associated to PI(3)P. Proteins with the malarial HT signal are cleaved by plasmepsin V, which destroys the PI(3)P binding signal and is likely to occur in a newly pinched off vesicle or one whose contents do not freely diffuse with the ER (step 3). Plasmepsin V and PI(3)P may be recycled back to the ER (step 4), while cargo targeted to the erythrocyte moves forward across the parasite plasma membrane and vacuolar membranes (step 5). PI(3)P-independent export signals persist in subsequent export. In default secretion (shown at the RHS) secretory proteins may be co-translationally translocated into the ER (step 1''), undergo cleavage of the signal sequence by signal peptidase (step 2'). Protein maybe delivered through vesicular intermediates to the parasite plasma membrane and released into the parasitophorous vacuole (steps 3' to 5'). Steps 1-5 have ~400 predicted cargo proteins exported to the erythrocyte and thus are likely to be a dominant pathway of protein export to the erythrocyte.