Published in final edited form as:

Mol Biochem Parasitol. 2010 May ; 171(1): 25–31. doi:10.1016/j.molbiopara.2010.01.003.

The Host Targeting motif in exported *Plasmodium* proteins is cleaved in the parasite endoplasmic reticulum

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Abstract

During the blood stage of its lifecycle, the malaria parasite resides and replicates inside a membrane vacuole within its host cell, the human erythrocyte. The parasite exports many proteins across the vacuole membrane and into the host cell cytoplasm. Most exported proteins are characterized by the presence of a Host Targeting (HT) motif, also referred to as a *Plasmodium* Export Element (PEXEL), which corresponds to the consensus sequence RxLxE/D/Q. During export the HT motif is cleaved by an unknown protease. Here, we generate parasite lines expressing HT motif containing proteins that are localized to different compartments within the parasite or host cell. We find that the HT motif in a protein that is retained in the parasite endoplasmic reticulum, is cleaved and N-acetylated as efficiently as a protein that is exported. This shows that cleavage of the HT motif occurs early in the secretory pathway, in the parasite endoplasmic reticulum.

Keywords

Plasmodium; protein export; protease; acetylation

Introduction

There were approximately 247 million malaria cases in 2006, resulting in close to a million deaths, mostly of children under 5 [1]. The malaria parasite, *Plasmodium*, is a unicellular eukaryote, and transmitted to humans via mosquito bites. Symptoms of the disease occur when the parasite enters the bloodstream, where it invades and replicates inside erythrocytes. Within the erythrocyte, *Plasmodium* resides in a membrane-bounded vacuole, the parasitophorous vacuole (PV). By exporting proteins across its own plasma membrane as well as the PV membrane, and into the cytoplasm and to the surface of the infected erythrocyte, the parasite is able to alter the solute permeability, cytoskeleton, and adhesion properties of its host cell [2,3]. Specific soluble proteins are exported across the PV membrane into the erythrocyte cytoplasm. Certain membrane proteins, some of which are linked to disease pathology [4], are also exported and become integrated into the host cell plasma membrane and vesicular membrane structures, called Maurer's clefts, within the infected erythrocyte's cytoplasm.

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The first step in the export pathway involves targeting of a protein to the parasite endoplasmic reticulum (ER), either by an N-terminal signal sequence or a transmembrane domain. Translocation into the ER lumen is likely mediated by the conserved ER co-translational translocation pathway [5]. Several lines of evidence indicate that exported proteins are initially targeted to the parasite endoplasmic reticulum; firstly, soluble exported proteins are characterized by the presence of an N-terminal hydrophobic signal sequence that can be replaced by the signal sequence of an ER targeted protein, without compromising export [6], and secondly, fusion of an ER retention sequence to the C-terminus of an exported protein blocks its export [7].

After targeting to the parasite ER, exported proteins are likely trafficked by a vesicle-mediated pathway to the parasite plasma membrane where they are released into the PV lumen. Most proteins that are transported across the PV membrane also contain a HT motif (also referred to as a PEXEL) [8,9], which is generally located less than 32 residues downstream of the predicted signal sequence cleavage site [10]. The HT motif corresponds approximately to the consensus RxLxE/D/Q (x is any amino acid) and is required for efficient export of most proteins [8,9,11,12]. Mutation of residues within the HT motif prevents protein export across the PV membrane and leads to accumulation of protein within the parasite secretory pathway and in the parasitophorous vacuole lumen [8,9,13]. Additionally, a linker of 11 residues or more must separate the HT motif from any folded C-terminal domain. Placing the HT motif too close to a C-terminal folded domain, such as GFP, blocks export [6].

Recent reports show that the HT motif is cleaved by a protease and that the newly generated N-terminus is N-acetylated. It is unclear where in the cell this processing occurs or indeed whether the cleaved or uncleaved HT motif targets a protein for export [13,14]. In all exported proteins examined, the HT motif is cleaved after the leucine residue (in the sequence RxLxE/D/Q). Surprisingly, in a protein where the HT motif is placed close to a folded GFP domain, preventing export, the HT motif is still cleaved and acetylated [14]. This suggests that processing occurs within the parasite secretory pathway or in the PV lumen, before a protein is exported. Furthermore, treatment of parasites with Brefeldin A for 24 hours, which leads to an accumulation of exported proteins in the parasite ER, does not prevent their cleavage and acetylation [14]. This suggests that processing of the HT motif occurs in the parasite ER. However, interpretation of this experiment is complicated as even brief treatment of mammalian cells with Brefeldin A causes Golgi resident proteins to be relocalized to ER [15] and results in certain ER resident proteins being abnormally modified with o-linked glycans [16]. It is therefore possible that a parasite protease, normally localized to the Golgi apparatus, is mislocalized to the ER where it mediates cleavage of the HT motif upon treatment with Brefeldin A. The consequences of prolonged 24-hour incubation in Brefeldin A, used to block protein export [14], have not been characterized. They likely include an accumulation of non-ER proteins in the parasite ER, as newly synthesized proteins cannot exit from the organelle normally. After such treatment the parasite ER may contain significant amounts of non-resident ER proteins that would normally be localized to later compartments in the secretory pathway, the PV lumen, or even exported to the red cell cytoplasm. Therefore, based on this experiment it is difficult to conclude where in the parasite secretory pathway the HT motif is normally cleaved.

To establish whether the HT motif is cleaved in the parasite ER we have generated parasites that express HT motif containing proteins that are localized to different cellular compartments. We find that, as previously shown, the HT motif in an exported protein is cleaved and acetylated. This cleavage does not occur during cell lysis, as it is not seen in a control protein. Using quantitative mass spectrometry we show that an ER resident protein, containing a HT motif, is cleaved with similar efficiency to an exported protein, indicating that cleavage of the HT motif occurs in the parasite ER. Significantly, this experiment is done without blocking

general protein trafficking from the parasite ER or disruption of the parasite secretory pathway, avoiding any potential artifacts associated with use of drugs such as Brefeldin A.

Materials and methods

Protein immunoprecipitation

Proteins were isolated from mixed parasite cultures at a parasitemia of 3–7%. 800µl of packed, infected, red blood cells were lysed at 4°C by addition of an equal volume of lysis buffer (0.1M Tris pH 8.5, 250mM NaCl, 5mM EDTA, 1% Triton X100, 2% SDS, 1µM Pepstatin, 0.1mM PMSF, 20mM DTT, complete protease inhibitor cocktail at 2X concentration (Roche)). Lysis buffer lacking SDS and DTT was added to a final volume of 8ml. Insoluble material was removed by centrifugation at 22,000g for 10 minutes and 40µl of anti-GFP beads (MBL) were added to each clarified lysate. Samples were rotated end over end at 4°C for 3 hours. Beads were washed 6 times with lysis buffer containing 0.2% SDS. Bound proteins were eluted by heating beads to 95°C in SDS sample buffer and separated on 12% polyacrylamide gels. For western blotting analysis, GFP tagged proteins were immunoprecipitated from 10ml cultures at 2–5% parasitemia. After SDS PAGE and transfer to PVDF membrane, proteins were detected with a rabbit anti-GFP antibody (Invitrogen), followed by a HRP coupled secondary antibody and enhanced chemiluminescent reagent.

Mass Spectrometry and peptide quantitation

Protein bands of interest were excised from the gel, destained, alkylated with iodoacetamide, and digested with modified trypsin as previously described [17]. Tryptic digests were analyzed by LC-MS/MS using reverse phase capillary HPLC with a 75µm nanocolumn interfaced on-line with a Thermo Electron LTQ Orbitrap XL mass spectrometer. The mass spectrometer was operated in data dependent mode with full scans performed in the Orbitrap and parallel MS/MS analysis of the six most intense precursor ions in the linear ion trap. Resulting precursor masses and spectra were searched against a custom database using TurboSequest (Thermo Scientific) with the Proteomics Browser interface (provided by William Lane, Harvard Microchemistry and Proteomics Analysis Facility). The custom database consisted of a combination of the expected sequences of the proteins used in this experiment, the *E. coli* proteome (to provide a reasonable sized database), and expected common contaminants including keratins, trypsin, etc.. A reversed sequence database was appended to the front of the forward database and used to estimate peptide false positive rates. Data were searched using partial tryptic specificity, a maximum of three missed cleavages, a mass tolerance of 100 ppm, cysteine fixed as the carboxymethyl derivative, and dynamic methionine oxidation and N-terminal acetylation. Resulting data was filtered on 5ppm and dCn of 0.07. The false positive rate for peptide identifications using these database search and data filtering parameters was less than 2%. Extracted Ion Chromatograms (XIC) of full MS scans were obtained by simultaneously using all observed m/z values for peptides of interest with a mass error window of ±5ppm and peak areas were integrated using Xcalibur Qualbrowser software (Thermo Scientific). The peptide identities of integrated peaks were verified based on retention times of matching MS/MS spectra.

Parasite culture and live cell imaging

3D7 parasites, obtained through MR4 (MRA102, deposited by D.J. Carucci) were propagated in A+ human erythrocytes in RPMI supplemented with 0.5% Albumax II, 0.2mM Hypoxanthine, 11mM Glucose, 0.17% NaHCO₃, 10µg/ml gentamycin and maintained in 5% Oxygen, 5% Carbon dioxide, and 90% Nitrogen. Prior to live cell imaging, parasites were washed in RPMI (without phenol red, supplemented with 25mM HEPES 7.4, and 1µg/ml Hoechst 33342) and transferred to an 8 wellLab Tek II chamber slide (Nunc). Parasites were maintained at 37°C during imaging. Images were acquired using an inverted Olympus IX

fluorescence microscope and a CoolSnap HQ2 CCD camera driven by DeltaVision software from Applied Precision Inc. (Seattle, WA). Images were deconvolved using Softworx and single optical sections are presented.

Indirect immunofluorescence

Plasmeprin 5 was localized by indirect immunofluorescence using a mouse monoclonal antibody obtained from MR4 (MRA-815A, deposited by D.E. Goldberg). Briefly, cells were adhered to poly-L-Lysine coated coverslips and fixed in 1% paraformaldehyde in phosphate buffered saline for 20 minutes at room temperature. After permeabilization for 10 minutes in Tris buffered saline (TBS) containing 0.1% TX100, cells were incubated in TBS containing 1% fish skin gelatin, and subsequently labeled with anti-plasmeprin 5 antibody, diluted 1:6 [18,19], followed by rhodamine labeled anti-mouse antibody. Images were acquired and processed as above.

Parasite transfection

Parasites were co-transfected with pHTH, encoding the piggybac transposase, and a second plasmid encoding the selectable marker, human DHFR, and the GFP fusion protein of interest [12].

Construction of plasmids

Fragments of PFI1755c, encoding residues 1-61, were PCR amplified using cDNA derived from 3D7 parasites, and inserted into a SalI site between the Calmodulin promoter and eGFP in vector pA2. For PFI1755c:GFP and PFI1755c:GFP:SDEL constructs, the fragment was amplified with the 5' primer, CATATCGTCGACATGCAAACCCGTAAATATAATAAG and the 3' primer ActagtcgacTGTTTTTTTTTAAATCCTGTTCTTCTAC. To generate PFI1755c:GFP:SDEL the PFI1755c fragment was cloned into a similar vector that contained the final 10 residues of *P.falciparum* BIP (GDEDVDSDEL) fused to the C-terminus of eGFP. To generate the Δ SS:PFI1755:GFP construct, a fragment of PFI1755c was amplified from cDNA and cloned into the SalI site of pA2. The fragment was amplified using two consecutive PCR reactions using the 3' primer described above and the 5' primers GAGAATGTATAAATATACCACCTCATACGAAGG, followed by CATCAAGTCGACTTAGAGAATGTATAAATATACCACCTCA.

Results

Expression of HT motif containing proteins localized to different sub-cellular compartments

To establish where in the secretory pathway the HT motif in exported proteins is cleaved, three parasite lines were generated, expressing chimeric proteins that are localized to different subcellular compartments, but each containing a HT motif. Firstly, we generated parasites expressing a protein, PFI1755c:GFP, comprising the first 61 residues of the exported protein PFI1755c [11], also referred to as Rex3 [20], fused to GFP (Fig. 1A). This protein should be exported as it contains a signal sequence and a HT motif. We also generated parasites expressing a similar protein, PFI1755c:GFP:SDEL that is localized to the parasite ER. This protein contains residues 1-61 of PFI1755c fused to GFP, but at its C-terminus contains the final ten residues encompassing the ER retention sequence from *P.falciparum* BIP (PFI0875w) (Fig. 1B). This sequence has previously been shown to mediate retention of proteins in the parasite ER [7,21,22]. A third protein was expressed, Δ SS:PFI1755:GFP, which only contains residues 33-61 of PFI1755c fused to GFP (Fig. 1C). This protein contains the residues following the predicted signal sequence cleavage site, including the HT motif, but lacks an N-terminal signal sequence and therefore should remain in the parasite cytoplasm. This protein

served as a control in the following experiments. In each case the proteins were expressed from a version of the *P.falciparum* calmodulin promoter.

Expression of each of the protein chimeras in transfected parasites was verified by immunoprecipitation and western blotting using anti-GFP antibodies (Fig. 1D). Each protein migrated at approximately the predicted molecular weight and as a single band.

Next, parasites were imaged to determine the sub-cellular localization of each of the GFP proteins. As expected, PFI1755c:GFP was exported into the host red blood cell cytoplasm (Fig. 2A). The PFI1755c:GFP:SDEL fusion was not exported due to the presence of the C-terminal ER retention sequence; rather it was retained within the parasite (Fig. 2B). This fusion showed a similar localization pattern to that seen previously for ER proteins in *Plasmodium* [21–23]. To confirm this, the localization of PFI1755c:GFP:SDEL fluorescence was compared to the staining pattern for the ER resident membrane protein, plasmepsin 5 [18,19], which was detected by indirect immunofluorescence. The GFP fluorescence pattern showed extensive colocalization with the plasmepsin 5 staining indicating that indeed the PFI1755c:GFP:SDEL is in the parasite ER (Fig. 2D). Δ SS:PFI1755:GFP was localized to the parasite cytoplasm but excluded from the parasite food vacuole in trophozoites and later parasites (Fig. 2C).

Processing of the HT motif in the parasite secretory pathway

To determine where in the secretory pathway the HT motif is cleaved we analyzed the proteolytic processing of the above protein chimeras using mass spectrometry. Each of the proteins was immunoprecipitated from non-synchronized parasite cultures. Parasites were lysed under denaturing conditions to reduce the possibility of any processing occurring during the lysis and immunoprecipitation steps. Immunoprecipitated material was analyzed by SDS-PAGE and the indicated gel bands were excised (Fig. 3A), subjected to tryptic digestion, and the peptides identified using liquid chromatography and tandem mass spectrometry (LC-MS/MS). This analysis confirmed that the bands indeed corresponded to the GFP chimeras. Peptides were identified covering 71%, 70%, and 81% of the amino acid sequences of PFI1755c:GFP, PFI1755c:GFP:SDEL, and Δ SS:PFI1755:GFP, respectively.

Next, we used ion signal intensities corresponding to peptides of interest to quantitatively analyze how efficiently each of the GFP-tagged proteins is processed [24–26]. For each sample, we examined the relative abundance of tryptic peptides derived from the region N-terminal to the HT motif, the intact uncleaved HT motif, and the cleaved and acetylated HT motif. In the LC-MS/MS experiments, tryptic peptides were separated by liquid chromatography (LC) on a reverse phase column that was interfaced directly with the mass spectrometer. Throughout the LC run the mass spectrometer conducted MS scans of precursor ions (tryptic peptides) and MS/MS scans of the most intense ions. The most reliable label-free method of quantitating individual peptides in different samples analyzed using a consistent LC-MS method is to extract the ion intensities from the MS scans for specific peptides using all observed mass/charge (m/z) values and a tight mass tolerance (± 5 ppm). The appropriate peak is then integrated to obtain relative peptide abundance and to compare the amount of this peptide in different samples. This method allows us to compare the relative abundance of identical peptides within different samples; it does not allow us to compare the relative amounts of non-identical peptides either within a sample or between different samples because ion yields vary from peptide to peptide.

To allow a meaningful comparison of peptide abundance in the three samples it was first necessary to determine the relative amounts of GFP chimera in each immunoprecipitated sample. To do this we determined the peak areas attributable to two tryptic peptides derived from GFP, HNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSK and FEGDTLVNR (for the location of peptides in GFP see Fig. 3B). The abundance of each of

these peptides varied less than $\pm 40\%$ between each of the samples indicating that the samples contain similar amounts of each respective GFP chimera (Fig. 3C and Table 1). As there are similar amounts of GFP chimera in each sample this allowed us to directly compare the relative abundance of peptides derived from the HT motif and the surrounding sequence and hence to determine the efficiency with which each protein is processed.

In each of the polypeptides analyzed, the HT motif comprises the sequence RQLSE. The most N-terminal peptide, identified by LC-MS/MS, from the exported protein chimera, PFI1755c:GFP, corresponded to the sequence SEPVVEEQDLK (residues within the HT motif are underlined) in which the N-terminus is N-acetylated. This peptide is derived from the HT motif, cleaved between the leucine and serine residues in the sequence RQLSE. Cleavage is not mediated by trypsin, which cleaves with high specificity on the C-terminal side of lysine and arginine residues, and furthermore, trypsin cleavage would produce an unmodified α -amino group whereas this N-terminus is acetylated. This is consistent with previous reports indicating that the HT motif is cleaved on the C-terminal side of the leucine residue at the third position in the HT motif.

To determine whether the HT motif is cleaved in the parasite ER we analyzed the processing of PFI1755c:GFP:SDEL. Using LC-MS/MS, the C-terminal peptide containing the SDEL sequence (LEGDEDVDSDEL) was recovered from this protein indicating that the full-length protein was expressed. As with the exported PFI1755c:GFP we were able to recover a peptide corresponding to the sequence SEPVVEEQDLK in which the N-terminal serine is N-acetylated. To determine whether the processing of these two chimeras was of similar efficiency we analyzed the abundance of the peptide, Acetyl-SEPVVEEQDLK, by measuring areas of peaks in extracted ion chromatograms (Fig. 3D). In both PFI1755c:GFP and PFI1755c:GFP:SDEL the peptide was present in similar amounts indicating that the HT motif is processed in the parasite ER (Table 1). To ensure that this result was not due to efficient cleavage and acetylation of the HT motif during cells lysis or immunoprecipitation we determined the abundance of the peptide in the Δ SS:PFI1755:GFP sample. The acetyl-SEPVVEEQDLK peptide derived from the cleaved acetylated HT motif was detected in the cytoplasmic protein sample, but it was more than two orders of magnitude less abundant than in the other two samples (Table 1). This indicated that the HT motif is specifically cleaved in the parasite ER and that the processing is not the result of degradation or modification that might occur during sample preparation.

We were also able to detect the N-acetylated peptide (acetyl-SEPVVEEQDLKK), which is identical to the above peptide derived from the cleaved HT motif, but with an additional lysine residue at its C-terminus (Fig. 3B). Because trypsin does not function effectively as an exopeptidase, such heterogeneous incomplete cleavage at clusters of tryptic sites is expected. This peptide was equally abundant in both PFI1755c:GFP and PFI1755c:GFP:SDEL but could either not be detected in the cytosolic Δ SS:PFI1755:GFP sample (Table 1) or was three orders of magnitude less abundant (Supplementary Table 1). We were also able to detect a peptide SEP VVEEQDLK that was not acetylated. This peptide was present in all three samples and slightly more abundant in the Δ SS:PFI1755:GFP sample (Table 1). The physiological significance of the unacetylated peptide is currently unclear, but it is almost certainly a very minor component relative to the acetylated forms of this peptide in the PFI1755c:GFP and PFI1755c:GFP:SDEL proteins. As noted above, ion signal yields for different peptides can be quite variable and unambiguous conclusions concerning relative yields of these different forms of the HT motif region can not be made at this time. However, minor structural variations in a peptide such as addition of a single residue or acetylation of the N-terminus often, but not always, will have a relatively minor effect on ion signal intensity. In support of the argument that signal intensities for the HT motif peptides are probably similar is the fact that the sums of intensities of the four peptides from the HT motif region for all three samples are in the

range of 500 to 900 million. Hence, to a first approximation, on the order of 99% of the HT motif is cleaved and acetylated in the PFI1755c:GFP and PFI1755c:GFP:SDEL proteins, and on the order of 99% of the N-terminal region is unprocessed in the Δ SS:PFI1755:GFP sample.

In the Δ SS:PFI1755:GFP sample we were able to detect a tryptic peptide (QLSEPVVEEQDLK) derived from the uncleaved HT motif (Fig. 3B). As the HT motif begins with an arginine residue this peptide was interpreted as resulting from the trypsin cleavage of the intact HT motif. This peptide was about 3 orders of magnitude less abundant in the PFI1755c:GFP and PFI1755c:GFP:SDEL compared to Δ SS:PFI1755:GFP (Table 1). In the Δ SS:PFI1755:GFP immunoprecipitate we also detected a peptide (YTTSYEGSSFR) derived from the region N-terminal to the HT motif (Fig. 3B). This peptide was three orders of magnitude less abundant in the PFI1755c:GFP:SDEL sample and was not detected in the PFI1755c:GFP sample (Table 1). The relative abundances of peptides derived from the cleaved HT motif, and the region N-terminal to the HT motif, are consistent with the cleavage of the HT motif in PFI1755c:GFP and PFI1755c:GFP:SDEL but not in Δ SS:PFI1755:GFP.

In a duplicate experiment we compared the peptide abundances of PFI1755c:GFP:SDEL and Δ SS:PFI1755:GFP, which led to similar overall conclusions (Supplementary information and Supplementary Table 1). Taken together this data shows that the HT motif is specifically cleaved early in the secretory pathway, in the parasite ER.

Discussion

We show that the HT motif in a protein, localized to the parasite endoplasmic reticulum, is cleaved and that the newly generated N-terminus is N-acetylated. The cleavage efficiency is similar to that of an exported protein, indicating that cleavage of the HT motif occurs in the parasite ER. This is consistent with a previous study showing that trapping exported proteins in the parasite ER by treatment with Brefeldin A, does not prevent their processing [14]. As proteins containing KDEL-like ER retention sequences cycle into the early Golgi apparatus [27] and are retrieved by the receptor Erd2 [28], it is formally possible that cleavage occurs in this compartment. However, in higher eukaryotes, retrieval of KDEL-containing ER proteins from the Golgi apparatus is efficient. Consequently, in comparison to proteins that traffic through the Golgi, ER-retained proteins acquire Golgi modifications, such as O-glycans, only inefficiently [29]. Hence, if the HT motif is cleaved in the Golgi apparatus then one might expect PFI1755c:GFP:SDEL to be cleaved less efficiently than PFI1755c:GFP. However, the cleavage efficiencies of the HT motif in PFI1755c:GFP:SDEL and PFI1755c:GFP are indistinguishable, suggesting that the cleavage event occurs in the parasite ER. Specific proteolysis of proteins in the ER occurs in other eukaryotes [30–33] but the identity of the protease, likely an ER resident, that cleaves the HT motif is unclear. The residues R and L, in the consensus sequence RxLxE/D/Q, appear to be important for its cleavage; the last position of the HT motif appears to be less important [13].

As the HT motif in a cytoplasmic protein was not efficiently processed it is clear that processing occurs *in vivo* and not after cell lysis or during sample preparation. Significantly, analysis of this protein demonstrated that the peptides derived from the uncleaved HT motif and from the region N-terminal of this HT motif, can be detected by mass spectrometry and quantities can be estimated using a label-free MS approach. This indicates that their absence from the exported and ER retained HT motif containing proteins is because the N-terminus is removed by proteolytic processing of the HT motif and is not due to an inability to detect these peptides in the mass spectrometer.

To cross the PV membrane, soluble exported proteins must be unfolded [34]. It has been proposed that in the PV lumen, exported proteins bearing a HT motif interact with the pTEX

complex, comprising an Hsp100/ClpB family ATPase and several other proteins [35]. It is likely that the pTEX complex plays a role in unfolding proteins prior to export. In the simplest model the pTEX ATPase would recognize an HT motif containing-protein, unfold it and feed it into a proteinconducting channel in the PV membrane. Exp2, a membrane associated component of the pTEX complex, is a good candidate to form such a channel but this and other aspects of the model, remain to be tested. In the context of this model, cleavage and processing of the HT motif early in the secretory pathway is perhaps surprising as the membrane translocation event is thought to occur at the PV membrane. If the HT motif is recognized for translocation in the PV lumen this suggests that the new N-terminal sequence Acetyl-xE/D/Q might ultimately target a protein for export. Consistent with this idea, mutation of the final residue in the HT motif reduces protein export significantly [9,13]. Alternatively, the HT motif may mediate a sorting event in the parasite ER that targets exported proteins to protein complexes or a vesicle transport pathway that delivers proteins to the translocation apparatus in the PV membrane. Currently, there is little evidence to suggest which of these models is correct.

Recognition of a protein with a cleaved HT motif would likely involve recognition of only a few residues including the acetylated N-terminal residue and the E/D/Q residue in the fifth position of the motif. Although protein recognition often involves extensive protein-protein interactions, recognition of such short sequences is not without precedent. In both prokaryotes and eukaryotes the identity of the N-terminal residue determines whether a protein is recognized by protein degradation machinery according to the N-end rule. In eukaryotes an N-terminal residue mediates recognition of specific proteins by the ubiquitin ligase N-recogin [36]. In prokaryotes, the adaptor protein ClpS recognizes the most N-terminal residue of proteins and targets them to ClpX and ClpP that mediate their unfolding and degradation, respectively [37].

If the cleaved HT motif is recognized by a putative receptor then the loss of a positive charge due to N-acetylation will likely be important for this binding reaction. Chang et al. proposed the N-acetylation might be mediated by the acetyl transferase NatD. However, NatD contains neither a signal sequence nor a transmembrane domain to target it to the ER lumen. Perhaps better candidates encoded in the *Plasmodium Falciparum* genome include PF14_0350 and PFL1345c. Both genes encode proteins with one or more transmembrane domains and an acetyl transferase domain but the membrane topology and substrate specificity of these proteins remains to be established.

Many exported proteins are essential for parasite growth [12,38]. Processing of proteins targeted to the host cell is probably also necessary for parasite survival. This suggests that small molecules that interfere with processing may block parasite growth and might ultimately be used as drugs for the treatment of malaria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

HT	host targeting
PEXEL	<i>plasmodium</i> export element
ER	endoplasmic reticulum
PV	parasitophorous vacuole

LC	liquid chromatography
MS	mass spectrometry
m/z	mass/charge
XIC	extracted ion chromatogram

Acknowledgments

The work was supported by National Institutes of Health grants to K.H. (R01HL69630) and D.W.S (R01HL38794). We thank MR4 for providing us with malaria parasites (MRA-102) and antibodies (MRA-815A) contributed by D.J. Carucci and D.E. Goldberg, respectively.

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Figure 1. Targeting of HT motif containing proteins to different cellular compartments

A. The N-terminal 61 residues of PFI1755c, containing a signal sequence and a HT motif were fused to eGFP to generate the fusion, PFI1755c:GFP. Signal sequence cleavage is predicted to occur between Asn 33 and Tyr 34 (both highlighted in blue text).

B. PFI1755c:GFP:SDEL is identical to PFI1755c:GFP except that an ER retention sequence was fused to the C-terminal end of GFP.

C. Δ SS:PFI1755:GFP was generated by fusing residues 34-61 of PFI1755c to GFP. This construct lacks a signal sequence. The residue following the start methionine (M) is Tyr 34 of PFI1755c (highlighted in blue).

D. Proteins were immunoprecipitated from parasite lysates with rat anti-GFP beads and GFP tagged proteins were detected by western blotting with rabbit anti-GFP. Untransfected 3D7 parasites were used as a control.

Figure 2. Localization of GFP chimeras in transfected parasites

A. Localization of PFI1755c:GFP. GFP fluorescence, in live cells, is seen in the erythrocyte cytoplasm indicating that the protein is efficiently exported.

B. Localization of PFI1755c:GFP:SDEL. GFP fluorescence, in live cells, remains within the parasite and shows a characteristic ER distribution.

C. Localization of Δ SS:PFI1755c:GFP. GFP fluorescence, in live cells, is localized diffusely throughout the cytoplasm of the parasite.

Panels show a DIC image, GFP fluorescence, GFP and Hoechst fluorescence overlaid, and a merge of DIC, GFP and Hoechst images, as indicated. The scale bar is 4 μm.

D. Colocalization of PFI1755c:GFP:SDEL with the ER marker, plasmepsin 5. The GFP fluorescence in PFI1755c:GFP:SDEL expressing parasites was directly visualized and the localization of plasmepsin 5 was determined by indirect immunofluorescence, using an

antibody raised against plasmepsin 5 [18,19]. DNA was stained with Hoechst. The scale bar is 4 μ M.

Figure 3. Immunoprecipitation of GFP tagged proteins

A. GFP tagged proteins were immunoprecipitated with anti-GFP beads and the bound material was analyzed by SDS-PAGE followed by coomassie staining of the gel. Bands corresponding to GFP tagged proteins, labeled with arrowheads, were excised, subjected to tryptic digestion, and analyzed by mass spectrometry.

B. Peptides used to quantitatively assess efficiency of HT motif cleavage. The sequence of PFI1755c:GFP is shown. The amino acid sequence of PFI1755c, residues 1-61, and GFP are highlighted in yellow and green, respectively. Residues flanking the predicted signal sequence cleavage site are in blue, and those comprising the HT motif are in red. Peptides whose relative abundances were determined in each of the three proteins are shown below the corresponding protein sequences. Peptides, boxed in grey, derived from GFP, varied by less than 40% between the different samples. Peptides, boxed in red, derived from the cleaved and acetylated HT motif,

were more than 2 orders of magnitude more abundant in PFI1755c:GFP or PFI1755c:GFP:SDEL relative to Δ SS:PFI1755:GFP. Peptides, boxed in blue, derived from the uncleaved HT motif or from the region N-terminal to the HT motif, were approximately 3 orders of magnitude more abundant in Δ SS:PFI1755:GFP than in the proteins targeted to the secretory pathway.

C. Extracted ion chromatograms for the GFP derived peptide, HNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSK. The ion current from full scans was extracted by simultaneously using the m/z values for the +3, +4, and +5 ions, 1491.39662, 1118.79928, and 895.240880, respectively, with a ± 5 ppm mass tolerance. Chromatograms for samples PFI1755c:GFP, PFI1755c:GFP:SDEL, and Δ SS:PFI1755:GFP, are shown as indicated.

D. Extracted ion chromatograms for the acetyl-SEPVVEEQDLK peptide derived from the cleaved and acetylated HT motif. The ion current from full scans was extracted using the m/z value for the observed +2 charge state, 657.82482, with a ± 5 ppm mass tolerance. Chromatograms for samples PFI1755c:GFP, PFI1755c:GFP:SDEL, and Δ SS:PFI1755:GFP, are shown as indicated.

Table I

Relative abundance of peptides derived from HT motif containing proteins

Peptide sequence	z	PFI1755c:GFP	PFI1755c:GFP :SDEL	ΔSS:PFI1755c :GFP
FEGDTLVNR	+2	9.18E+08	6.57E+08	8.89E+08
HNIEDGSVQLADHYQ QNTPIGDGPVLLPDN HYLSTQSALSK	+3,+4, +5	3.45E+08	2.73E+08	2.13E+08
[Ac]SEPVVEEQDLK	+2	6.28E+08	4.90E+08	9.55E+05
[Ac]SEPVVEEQDLKK	+2	1.61E+07	1.60E+07	ND
SEPVVEEQDLK	+2	4.53E+05	3.46E+05	1.71E+06
QLSEPVVEEQDLK	+2,+3	3.52E+05	1.42E+06	9.04E+08
YTTSYEGSSFR	+2	ND	2.52E+05	3.25E+08

The areas of peptide peaks, from extracted ion chromatograms, were determined for each of the indicated peptides. Z corresponds to the charge of the peptides. Where the same peptide was detected with multiple different charged states, indicated in the second column, the reported areas are the sum of all charge states. [Ac] indicates the peptide N-terminus was N-acetylated. ND indicates that a specific signal above background was not detected.