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Evidence for Prenylation-Dependent Targeting of a Ykt6 SNARE in *Plasmodium falciparum*

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Abstract

Ykt6 proteins are the most versatile fusogens in eukaryotic cells, and the only SNAREs that can be both prenylated and acylated at a C-terminal CAAX motif. Unlike yeast and mammalian cells where a single Ykt6 gene is expressed, the *Plasmodium falciparum* genome encodes two Ykt6 proteins. We have investigated the expression and prenylation of the Ykt6 orthologue, PfYkt6.1 in intra-erythrocytic stages of *P. falciparum*. PfYkt6.1 localized to the parasite Golgi and other unidentified cytoplasmic compartments, and was partly cytosolic (~50% in early trophozoites). The membrane-association of PfYkt6.1 was dependent on presence of a conserved C-terminal CAAX motif (CCSIM). By expressing full-length and mutant proteins in *Escherichia coli*, we have shown that PfYkt6.1 indeed serves as substrate for prenylation by *P. falciparum* farnesyltransferases. Surprisingly, PfYkt6.1 could also be geranylgeranylated by parasite extracts independent of the C-terminal amino acid residue. Deletion of the CAAX motif inhibited both farnesylation and geranylgeranylation activities. Additionally, the PfYkt6.1 heptapeptide KQCCSIM, corresponding to the C-terminal CAAX sequence, inhibited the parasite farnesyltransferase activity with an IC₅₀ of 1 μM. Our findings underscore the importance of CAAX motif-derived peptidomimetics for antimalarial drug development.

Introduction

The specificity of vesicle fusion with target membranes in the secretory and endocytic pathways in eukaryotic cells is largely determined by the selective transport of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) to distinct intracellular compartments [1–3]. SNAREs belong to the family of tail-anchored proteins that are inserted into cellular membranes following a post-translational process presumably involving specific chaperones [4–7]. Most SNAREs contain a C-terminal transmembrane domain that functions as a membrane attachment signal and might double as a trafficking determinant [8–10]. A few SNARE proteins (Ykt6p, Sec9p, and their eukaryotic

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homologues) lack this C-terminal transmembrane domain, but are membrane-anchored through specific lipid modification processes [1]. Among these lipid modified SNAREs, Ykt6 is unique in that it can be prenylated and palmitoylated at two conserved cysteine residues associated with a C-terminal prenylation motif also known as the CAAX motif (Cysteine-Aliphatic-Aliphatic-X, where X is any amino acid) [11–15]. Protein prenylation involves the covalent attachment of either a 15-carbon (farnesyl) or 20-carbon (geranylgeranyl) isoprenoid moiety to the cysteine residue of CAAX motif-containing proteins [16–20]. These modifications render otherwise cytosolic proteins hydrophobic, triggering their insertion into cellular membranes [15,21]. Previous studies have shown that geranylgeranylation of the CAAX motif is specified by leucine or phenylalanine residues at the X position whereas a methionine, serine or glutamine at this position predicts a potential farnesylation of the protein [22].

Unlike yeast and mammalian cells, which express a single form of Ykt6 proteins, the plant (*Arabidopsis thaliana*) and *Plasmodium falciparum* genomes encode at least two putative Ykt6 isoforms [23,24]. These duplicated Ykt6 proteins in *P. falciparum* are likely to function at distinct transport pathways, mediating vesicle trafficking to unique intracellular compartments. *P. falciparum* is an intracellular parasite of mature red blood cells and the causative agent of the most lethal form of human malaria. The dynamics of vesicle budding and fusion in *P. falciparum* are unusual in that the parasite targets nuclear-encoded proteins to multiple membrane compartments that are absent in all other eukaryotic species [25]. These include a lysosome-like digestive vacuole and various parasite-induced structures in the infected host cell [25–29]. Because Ykt6 proteins represent the most versatile SNAREs in eukaryotes, we speculated that the PfYkt6 proteins would label multiple intracellular compartments in malaria parasites. To continue with our study of Plasmodium SNAREs and protein prenylation, we have investigated the expression, localization, and prenylation of PfYkt6.1 (PlasmoDB ID: PFI0515w) in intra-erythrocytic *P. falciparum* parasites. We show that PfYkt6.1 indeed serves as substrate for prenylation by the parasite farnesyltransferase enzyme. Surprisingly, PfYkt6.1 could also be geranylgeranylated independent of the C-terminal amino acid residue suggesting a PGGT-II activity on the SNARE protein. Our data provides the first experimental evidence for prenylation-dependent transport of Ykt6 in *P. falciparum*, and it is the first report of protein geranylgeranyltransferase activity on a SNARE protein.

Materials and Methods

Antibodies and Immunofluorescence Microscopy

The decapeptide ‘YLTKYKDPLN’, corresponding to residues 125 through 134 of the PfYkt6.1 polypeptide, was synthesized (GenScript) and conjugated to Keyhole Limpet Hemacyanin (KLH) following the manufacturer's instructions (Pierce). This peptide sequence showed no sequence identity with other proteins in PlasmoDB, except for the PfYkt6.1 polypeptide. Polyclonal antibodies were produced in rabbit against the conjugated peptide (Harlan Bioproducts for Science, Inc) and affinity purified using peptide-conjugated agarose beads (Pierce). The reactivity of these antibodies against the endogenous and/or GFP-tagged proteins was analyzed by standard Western blot techniques using Supersignal West Femto detection kit (Pierce).

Laser scanning microscopy was performed using a LSM 510 confocal microscope (Carl Zeiss) as previously described [30,31]. The purified rabbit anti-PfYkt6.1 antibodies were used at a 1 in 1000 dilution. Secondary antibodies consisted of goat anti-rabbit Alexa Fluor-555 or goat anti-rat Alexa Fluor-594 (Molecular Probes), each used at a dilution of 1 in 1,000. Nuclear staining was done with DAPI fluoromount G (Southern Biotech).

Epifluorescence imaging was done on a DeltaVision microscope system (Applied Precision). DeltaVision software (SoftWorx) was used for image deconvolution.

Plasmids, Transgenic parasites and Live Cell Imaging

For live cell imaging, transgenic parasites expressing the N-terminal GFP-tagged proteins were developed using a modified pDC *Plasmodium* expression vector [32,33]. cDNAs corresponding to the full-length ORF of PfYkt6.1 (GFP-PfYkt6.1) or the CAAX motif deletion segment (GFP-PfYkt6.1.CSIM) were amplified by RT-PCR and ligated into the pGEM-T Easy vector (Promega). The constructs were sequence confirmed and subcloned into the AvrII/BglIII site of the pDC vector. *P. falciparum* 3D7 ring stage cultures (at 5% parasitemia) were transfected with Qiagen-purified plasmid DNA (100 µg) by electroporation using a Bio-Rad Gene pulser II (0.31 KV and 950 microfarads). Cells were then maintained in drug media plus 2.5 nM WR99210 until appearance of fluorescent parasites. The GFP signals were captured at a spectra setting of 488/505 nm using a laser confocal microscope (Zeiss).

Triton X-114 Phase Partitioning and Immunoblot Assays

P. falciparum parasites were purified from infected erythrocytes by saponin treatment and solubilized using the Membrane Protein Extraction Reagent kit (Pierce). The solubilized samples were clarified by centrifugation at $10,000 \times g$ for 3 minutes at 4°C, and then clouded in a 37°C water bath for 20 minutes. The samples were again centrifuged at room temperature at $10,000 \times g$ for 2 min to separate the aqueous (hydrophilic) and detergent (hydrophobic) phases. Equal volumes of the phase-separated samples were prepared in Laemmli sample buffer and analyzed by Western blotting using either anti-GFP antibodies (Santa Cruz Biotechnology, Inc) or purified anti-PfYkt6.1 antibodies.

cDNA Synthesis and Recombinant PfYkt6.1 proteins

cDNAs encoding the PfYkt6.1 protein or mutants were amplified by reverse transcriptase PCR and cloned into the pET30EKLIC expression vector (Novagen). The recombinant proteins were over-expressed in *E. coli* BL21 (DE3) CodonPlus-RIPL cells following IPTG induction. Briefly, overnight bacteria cultures were subcultured in 500 ml of Luria Bertani (LB) media containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and 75 µg/ml streptomycin at 37°C to an OD₆₀₀ between 0.4 and 0.5. The cells were further grown for 1 hr at 20°C prior to induction with 1 mM IPTG, and harvested after 4 hrs by centrifugation. The pellets were resuspended in 30 ml of lysis buffer (50 mM sodium phosphate, pH 7.6 containing 300 mM NaCl, 50 U Benzonase, 0.1 mg/ml lysozyme and Complete EDTA-free protease inhibitors) followed by French press (3 times at 1500 psi) lysis and metal-affinity purification using a Talon Super-flow resin (Novagen). Fractions containing the pure proteins were concentrated by Vivaspin centrifugation and then stored at -80°C until needed.

In vitro Prenylation Assays

In vitro farnesylation of full-length PfYkt6.1 proteins was performed at 16°C for 30 minutes with or without the PFTase inhibitor FTI-276 in 50 mM Tris-HCl (pH 7.5), 20 µM ZnCl₂, 5 mM MgCl₂, 20 mM KCl, 1 mM DTT, 5 µg of Mono Q PpPFTase fraction and 2 µCi ³H-farnesyl diphosphate [30,34]. Protein geranylgeranylation was assayed using ³H-GGPP as the geranylgeranyl moiety donor and an ammonium sulfate fraction presumably containing most of the prenyltransferase activity as source of enzyme. The reactions were stopped with Laemmli sample buffer and then subjected to SDS-PAGE and fluorographic analysis using an “Amplify” enhancer (GE Life-Sciences).

Results

Expression and Localization of PfYkt6.1 in *P. falciparum* Parasites

Eukaryotic Ykt6 proteins are highly versatile SNAREs that localize to the Golgi, multivesicular endosomes and yeast vacuoles [12,13,35–37]. To characterize the steady-state dynamics of the *P. falciparum* Ykt6 gene product PfYkt6.1, we generated monospecific anti-peptide antibodies for immunolocalization studies. By immunofluorescence analyses, the PfYkt6.1 protein appeared partly cytosolic and partly organellar as seen by its accumulation at multiple intracellular spots that were predominant at the trophozoite stage (supplementary Fig. S1). This distribution pattern is consistent with a lipid modification of PfYkt6.1 and suggests a tight regulation of its function in *P. falciparum*. To characterize the PfYkt6.1-associated structures and elucidate the trafficking mechanisms, we generated transgenic parasites expressing an N-terminal GFP tagged chimera. This GFP-tagged protein also appeared partly cytosolic and accumulated at multiple cytoplasmic spots similarly to the untagged protein (Fig. 1A). This finding suggests a correct targeting of the fusion protein in the transgenic parasites. As shown in Fig. 1B and C, the GFP-tagged protein also localized partially to the Golgi (Fig. 1B) and ER (Fig. 1C), suggesting a functional association of PfYkt6.1 with the parasite secretory system. Further characterization of the unidentified PfYkt6.1-associated structures in this study was precluded by lack of appropriate organellar markers to rule out components of the parasite endosomal system.

Role of the CAAX Motif in PfYkt6.1 Trafficking

Ykt6 transport to multivesicular endosomes and vacuoles in higher eukaryotes is a multi-step process requiring a prenylation-dependent uptake of the cytosolic protein and membrane stabilization following a specific palmitoylation reaction. PfYkt6.1 contains two conserved C-terminal cysteine residues within the CAAX motif region (CCSIM) that potentially can be prenylated. To assess the role of the PfYkt6.1 CAAX motif, first we investigated whether or not PfYkt6.1 is lipid modified in malaria parasites. Studies elsewhere have demonstrated that prenylation of Ykt6 renders the soluble protein hydrophobic, resulting in a detergent phase partitioning of the lipid modified protein following Triton X-114 treatment [11,12,14]. As shown in Fig. 2, a 23 kDa protein corresponding to the predicted size of PfYkt6.1, and a 49 kDa protein corresponding to the GFP chimera, was detected in both fractions following Triton X-114 phase partitioning. These findings confirm the expression of the PfYkt6.1 gene in *P. falciparum* and further indicate a lipid modification of the protein in malaria parasites. Next, we generated transgenic parasites expressing the CAAX deletion mutant GFP-PfYkt6.1ΔCSIM for localization analyses. As shown in Fig. 3A, deletion of the CSIM sequence resulted in a cytosolic protein that failed to associate with the parasite secretory system (Fig. 3B and C), consistent with lack of the membrane targeting signal. To confirm that the CAAX motif is required for the lipid modification of PfYkt6.1, whole cell extracts from trophozoite parasites were subjected to Triton X-114 phase partitioning. Compared to the wild-type untagged protein (Fig. 3D), the mutant protein fractionated entirely into the aqueous phase indicating its high hydrophilicity. These findings strongly suggest that PfYkt6.1 is lipid-modified in *P. falciparum* and that the isoprenylation CAAX signal is required for the membrane-association and function of PfYkt6.1.

In vitro Prenylation of PfYkt6.1

Next, we investigated whether or not PfYkt6.1 is a substrate for prenylation by *P. falciparum* prenyltransferases. Full-length PfYkt6.1 protein and a CAAX motif deletion mutant were produced in *E. coli* and purified by metal affinity chromatography. The *in vitro* prenylation of these recombinant proteins in the presence or absence of the

farnesyltransferase inhibitor FTI-276 was then analyzed by fluorography using ^3H -FPP as previously described [30,38]. Consistent with a requirement of the CAAX motif cysteine for protein isoprenylation, only the full-length PfYkt6.1 protein was farnesylated *in vitro* (Fig. 4A and B). As shown in Fig. 4A, more than 50% of the prenylation activity was inhibited by 5 nM of FTI-276. This inhibitor concentration is consistent with the IC_{50} value for PfPFTase as previously determined using various synthetic peptides, or a recombinant protein tyrosine phosphatase enzyme as substrate [30,38].

To confirm the specificity of the prenyltransferase activity, we investigated the role of the extreme C-terminal residue in the prenylation efficiency of PfYkt6.1. Full-length proteins were produced in which the C-terminus methionine (position +3 relative to the CAAX motif cysteine) was changed to glutamine, leucine or serine, and then subjected to prenylation analyses as described above. As shown in Fig. 4B, replacing the +3 methionine with glutamine or serine significantly reduced the farnesylation activity of PfPFTase in the order $\text{S} > \text{Q}$. Meanwhile, no farnesylation activity was observed when a leucine residue was present at this position. These findings confirm the presence of a specific protein farnesyltransferase activity in our Mono Q extract and suggest that the C-terminal residue dictates the efficiency of protein farnesylation in *P. falciparum*. To further define the role of the C-terminal motif (CCSIM) in lipid modification of PfYkt6.1, we tested whether or not the full-length proteins can be geranylgeranylated *in vitro* as this sequence conforms to both a CaaX farnesylation motif and a CCxxx geranylgeranylation motif [39]. In these reactions, ^3H -GGPP was used as the geranylgeranyl donor and an ammonium sulfate fraction of *P. falciparum* presumably containing the total prenyltransferase activity used as source of enzyme. Surprisingly, both the wild-type PfYkt6.1 protein and the mutant forms containing either Q, S, or L residues at the +3 position were geranylgeranylated *in vitro* (Fig. 4C). Additionally, no difference was observed in the geranylgeranylation efficiency of the L-containing mutant when compared with the M, Q, and S-containing polypeptides. These findings establish the potential of PfYkt6.1 to serve as substrate for PfPGGTs and suggest that the activity of geranylgeranyltransferase I (PGGT-I) was unlikely as this isoform is highly selective for CAAX motifs containing leucine residues at the +3 position. In addition, no geranylgeranylation reaction was detected when the CAAX deletion mutant protein was used as substrate (data not shown). Collectively, these findings suggest that PfYkt6.1 can be farnesylated and geranylgeranylated in malaria parasites.

Inhibition of PfPFTase Activity by a PfYkt6.1 CAAX-containing Peptide

To test the possibility of using peptides derived from the prenylation motif of PfYkt6.1 as PfPFTase peptidomimetics, we investigated the inhibitory effect of the C-terminal sequence “KQCCSIM”. To this end, we measured the ability of PfPFTase to farnesylate a biotin-conjugated lamin B substrate in the presence or absence of different competing peptides. The positive and negative control peptides consisted of the C-terminal CAAX sequence of *P. falciparum* protein tyrosine phosphatase (RKCHFM) and the heptapeptide “NRSCAIV”, respectively [30]. Compared to the enzyme activity in the absence of any competing peptides, the PfYkt6.1 peptide exhibited potent inhibitory properties against the malaria enzyme with an IC_{50} value approximately 1 μM (Fig. 5). These findings indicate that PfYkt6.1 is a genuine isoprenoid acceptor in *P. falciparum*, and demonstrate that CAAX motif sequences are potent inhibitors of PfPFTase activities *in vitro*.

Discussion

Ykt6 SNAREs are highly versatile proteins involved in membrane transport to distinct intracellular organelles depending on the cell type [11–14,40–42]. In yeast cells, Ykt6 plays an essential role in ER-to-Golgi vesicle trafficking and associates predominantly with the Golgi, the multivesicular endosomes and vacuoles [12–14]. In contrast, mammalian Ykt6

protein localizes predominantly to the Golgi, and in highly specialized cells such as neurons, the majority of membrane-associated Ykt6 is found in unknown membrane compartments that display little overlap with known Golgi, lysosomal and dense-core granule markers [11,37,40]. Unlike yeast and mammalian cells, which express a single form of Ykt6, genomics studies and transcript analyses have suggested the expression of two forms of Ykt6 in *Arabidopsis thaliana* (AtYkt61 and AtYkt62) [23] and the malaria parasite *P. falciparum* (PfYkt6.1 and PfYkt6.2) [24]. In this study, we investigated the localization and potential prenylation of the *P. falciparum* Ykt6 protein PfYkt6.1. To achieve these objectives, we generated anti-peptide antibodies for immunolocalization studies. Furthermore, we developed transgenic parasites for live cell imaging of GFP-tagged chimeras and for co-localization analyses. Our data indicate a partial localization of PfYkt6.1 to multiple cytoplasmic structures that could be distinguished from the parasite ER and Golgi structures (Fig. 1B and C). We speculated that the peripherally located PfYkt6.1 compartments might represent transport intermediates operating in post-Golgi membrane trafficking in *P. falciparum*. Indeed, brefeldin A-treatment ablated PfYkt6.1 transport to the peripheral compartments, resulting in retention at one or two perinuclear spots within the perinuclear region in trophozoite parasites (data not shown). Collectively, our data suggest an association of PfYkt6.1 with the parasite secretory system, and vesicle-mediated transport to the peripheral compartments.

PfYkt6.1 appeared to be partly cytosolic and partly membrane-associated inside the parasite cytoplasm, suggesting a tight regulation of the protein activity in malaria parasites. In yeast and mammals, the activity of Ykt6 proteins is regulated by both prenylation and palmitoylation of distinct cysteine residues located within a C-terminus isoprenylation motif [11,12,14,37]. It has been suggested that farnesylation of the C-terminal CAAX motif is essential for its membrane-insertion whereas the addition of a palmitoyl moiety at the -1 position enhances its membrane stability and trafficking [11,43]. PfYkt6.1 contains a CCSIM signal at the C-terminal end of the protein that potentially can be prenylated and acierated. Consistent with this prediction, hydrophobicity analyses of both the untagged and GFP-tagged pools of PfYkt6.1 in this study suggested that approximately 50% of each protein pool in trophozoite parasites is lipid-modified (Fig. 2). Furthermore, deletion of the C-terminal CSIM sequence resulted in a highly soluble protein that appeared diffused within the parasite cytoplasm (Fig. 3A–D). These findings strongly suggested that PfYkt6.1 targeting in *P. falciparum* parasites is mediated by lipid modifications requiring the CAAX motif residues. By taking advantage of an *in vitro* prenylation assay previously established by our group [30,38], we confirmed the prenylation of PfYkt6.1 by *P. falciparum* prenyltransferases (Fig. 4A). Furthermore, by targeted mutagenesis of the C-terminal methionine residue of PfYkt6.1, we have shown that this amino acid is essential for farnesylation by *P. falciparum* farnesyltransferase. Replacing the C-terminus methionine with glutamine or serine residues significantly decreased the farnesylation efficiency of the enzyme. Meanwhile, a leonine at the C-terminal position abrogated the protein prenylation (Fig. 4B). These findings are consistent with the sequence selectivity of the malarial and most eukaryotic protein farnesyltransferases [38,44].

Previous studies using yeast Ykt6 proteins that were chemically coupled with geranylgeranyl moieties had speculated the ability of digeranylgeranylated Ykt6 proteins to enhance intra-Golgi transport in HeLa cells [11]. Until now, no studies have established the potential of Ykt6 proteins to serve as substrates for GGTase enzymes. The prenylation motif of PfYkt6.1 (CCSIM) conforms to both the consensus Coax sequence for protein farnesylation and the CCxxx motif for geranylgeranylation by PGGTII. Here, we have shown for the first time that PfYkt6.1 can serve as a substrate for farnesylation and geranylgeranylation in *P. falciparum* (Fig. 4B and C). These findings are consistent with observations with K-ras, the most frequently mutated form of ras protein in human cancers

whose geranylgeranylation is responsible for the observed resistance to farnesyltransferase inhibitors [45,46]. Deletion of the CAAX motif abrogated both farnesylation and geranylgeranylation reactions, thus indicating the involvement of the C-terminal cysteine residue in both reactions. Unlike the farnesylation activity, geranylgeranylation of PfYkt6.1 was significantly less efficient (Fig. 4C). As expected the geranylgeranylation activity displayed no preference for the C-terminal amino acid residue (Fig. 4C). Two types of protein geranylgeranyltransferase enzymes are expressed in most eukaryotic cells: an enzyme activity with high specificity for CAAX motifs containing a leucine at the C-terminal position (PGGT-I), and a second less specific PGGT-II that is capable of prenylating both CC and CXC signals at the C-terminus of proteins [22,47,48]. Our results suggest that the geranylgeranylation of PfYkt6.1 is catalyzed by a PGGT-II and not by a PGGT-I activity. It is also worth noting that whereas all subunits of the PFT and PGGT-II complexes are encoded by the *P. falciparum* genome, no coding sequence has been detected for the β subunit of PGGT-I [34,49].

Membrane targeting and mediation of protein-protein interactions are well-documented functions associated with lipid anchors [50–52]. Whether or not farnesylation and geranylgeranylation reactions occur simultaneously in malaria parasites is not known. Interestingly, the inhibition of K-ras oncogene requires both an FTase inhibitor as well as a GGTase inhibitor suggesting that both prenylation processes occur independent of one another [53]. Additionally, the inhibition of K-ras functioning is more sensitive to GGTase inhibitors than it is to FTase inhibitors. It is thus possible that the prenylation and geranylgeranylation of PfYkt6.1 might occur interchangeably resulting in different active states of the SNARE protein.

This report on PfYkt6.1, one of two CAAX motif-containing SNARE proteins in *P. falciparum*, is a step towards a better understanding of the role of protein prenylation in membrane traffic and biogenesis in malaria parasites. Additionally, given the importance prenyltransferase inhibitors as potential antimalarial agents [38,54], knowledge of the nature and physiological functions of the prenylated proteins in malaria parasites is critical to understand the drug mechanisms. Such information will provide valuable insights for the synthesis and development of potent pharmacological agents. Interestingly, a heptapeptide derived from the C-terminus of PfYkt6.1 inhibited the parasite farnesyltransferase with an IC₅₀ value approximately 1 μ M (Fig. 5). These findings suggest that PfYkt6.1-derived peptidomimetics can serve as important leads in antimalarial drug discovery and development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Hong W. SNAREs and traffic. *Biochim Biophys Acta* 2005;1744:120–144. [PubMed: 15893389]
2. Jahn R, Scheller RH. SNAREs--engines for membrane fusion. *Nat Rev Mol Cell Biol* 2006;7:631–643. [PubMed: 16912714]
3. Pelham HR. SNAREs and the specificity of membrane fusion. *Trends Cell Biol* 2001;11:99–101. [PubMed: 11306253]
4. Borgese N, Brambillasca S, Soffientini P, Yabal M, Makarow M. Biogenesis of tail-anchored proteins. *Biochem Soc Trans* 2003;31:1238–1242. [PubMed: 14641033]
5. Steel GJ, Brownsword J, Stirling CJ. Tail-anchored protein insertion into yeast ER requires a novel posttranslational mechanism which is independent of the SEC machinery. *Biochemistry* 2002;41:11914–11920. [PubMed: 12269836]

6. Stefanovic S, Hegde RS. Identification of a targeting factor for posttranslational membrane protein insertion into the ER. *Cell* 2007;128:1147–1159. [PubMed: 17382883]
7. Abell BM, Rabu C, Leznicki P, Young JC, High S. Post-translational integration of tail-anchored proteins is facilitated by defined molecular chaperones. *J Cell Sci* 2007;120:1743–1751. [PubMed: 17456552]
8. Borgese N, Brambillasca S, Colombo S. How tails guide tail-anchored proteins to their destinations. *Curr Opin Cell Biol* 2007;19:368–375. [PubMed: 17629691]
9. Wattenberg B, Lithgow T. Targeting of C-terminal (tail)-anchored proteins: understanding how cytoplasmic activities are anchored to intracellular membranes. *Traffic* 2001;2:66–71. [PubMed: 11208169]
10. Banfield DK, Lewis MJ, Rabouille C, Warren G, Pelham HR. Localization of Sed5, a putative vesicle targeting molecule, to the cis-Golgi network involves both its transmembrane and cytoplasmic domains. *J Cell Biol* 1994;127:357–371. [PubMed: 7929581]
11. Fukasawa M, Varlamov O, Eng WS, Sollner TH, Rothman JE. Localization and activity of the SNARE Ykt6 determined by its regulatory domain and palmitoylation. *Proc Natl Acad Sci U S A* 2004;101:4815–4820. [PubMed: 15044687]
12. McNew JA, Sogaard M, Lampen NM, Machida S, Ye RR, Lacomis L, et al. Ykt6p, a prenylated SNARE essential for endoplasmic reticulum-Golgi transport. *J Biol Chem* 1997;272:17776–17783. [PubMed: 9211930]
13. Meiringer CT, Auffarth K, Hou H, Ungermann C. Depalmitoylation of Ykt6 prevents its entry into the multivesicular body pathway. *Traffic* 2008;9:1510–1521. [PubMed: 18541004]
14. Dietrich LE, Peplowska K, LaGrassa TJ, Hou H, Rohde J, Ungermann C. The SNARE Ykt6 is released from yeast vacuoles during an early stage of fusion. *EMBO Rep* 2005;6:245–250. [PubMed: 15723044]
15. Wright LP, Phillips MR. Thematic review series: lipid posttranslational modifications. CAAX modification and membrane targeting of Ras. *J Lipid Res* 2006;47:883–891. [PubMed: 16543601]
16. Magee T, Seabra MC. Fatty acylation and prenylation of proteins: what's hot in fat. *Curr Opin Cell Biol* 2005;17:190–196. [PubMed: 15780596]
17. Resh MD. Trafficking and signaling by fatty-acylated and prenylated proteins. *Nat Chem Biol* 2006;2:584–590. [PubMed: 17051234]
18. Michaelson D, Ali W, Chiu VK, Bergo M, Silletti J, Wright L, et al. Postprenylation CAAX processing is required for proper localization of Ras but not Rho GTPases. *Mol Biol Cell* 2005;16:1606–1616. [PubMed: 15659645]
19. Yalovsky S, Rodr Guez-Concepcion M, Gruissem W. Lipid modifications of proteins - slipping in and out of membranes. *Trends Plant Sci* 1999;4:439–445. [PubMed: 10529825]
20. Perez-Sala D. Protein isoprenylation in biology and disease: general overview and perspectives from studies with genetically engineered animals. *Front Biosci* 2007;12:4456–4472. [PubMed: 17485388]
21. Choy E, Chiu VK, Silletti J, Feoktistov M, Morimoto T, Michaelson D, et al. Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. *Cell* 1999;98:69–80. [PubMed: 10412982]
22. Moores SL, Schaber MD, Mosser SD, Rands E, O'Hara MB, Garsky VM, et al. Sequence dependence of protein isoprenylation. *J Biol Chem* 1991;266:14603–14610. [PubMed: 1860864]
23. Sanderfoot AA, Assaad FF, Raikhel NV. The Arabidopsis genome. An abundance of soluble N-ethylmaleimide-sensitive factor adaptor protein receptors. *Plant Physiol* 2000;124:1558–1569. [PubMed: 11115874]
24. Ayong L, Pagnotti G, Tobon AB, Chakrabarti D. Identification of *Plasmodium falciparum* family of SNAREs. *Mol Biochem Parasitol* 2007;152:113–122. [PubMed: 17240462]
25. Cooke BM, Lingelbach K, Bannister LH, Tilley L. Protein trafficking in *Plasmodium falciparum*-infected red blood cells. *Trends Parasitol* 2004;20:581–589. [PubMed: 15522668]
26. Przyborski JM, Lanzer M. Protein transport and trafficking in *Plasmodium falciparum*-infected erythrocytes. *Parasitology* 2005;130:373–388. [PubMed: 15830811]

27. Taraschi TF, Trelka D, Martinez S, Schneider T, O'Donnell ME. Vesicle-mediated trafficking of parasite proteins to the host cell cytosol and erythrocyte surface membrane in *Plasmodium falciparum* infected erythrocytes. *Int J Parasitol* 2001;31:1381–1391. [PubMed: 11566305]
28. Charpian S, Przyborski JM. Protein transport across the parasitophorous vacuole of *Plasmodium falciparum*: into the great wide open. *Traffic* 2008;9:157–165. [PubMed: 17944805]
29. Halder K, Mohandas N, Samuel BU, Harrison T, Hiller NL, Akompong T, et al. Protein and lipid trafficking induced in erythrocytes infected by malaria parasites. *Cell Microbiol* 2002;4:383–395. [PubMed: 12102685]
30. Pendyala PR, Ayong L, Eatrides J, Schreiber M, Pham C, Chakrabarti R, et al. Characterization of a PRL protein tyrosine phosphatase from *Plasmodium falciparum*. *Mol Biochem Parasitol* 2008;158:1–10. [PubMed: 18096253]
31. Ayong L, Raghavan A, Schneider TG, Taraschi TF, Fidock DA, Chakrabarti D. The longin domain regulates the steady-state dynamics of Sec22 in *Plasmodium falciparum*. *Eukaryot Cell* 2009;8:1330–1340. [PubMed: 19617396]
32. Fidock DA, Nomura T, Wellems TE. Cycloguanil and its parent compound proguanil demonstrate distinct activities against *Plasmodium falciparum* malaria parasites transformed with human dihydrofolate reductase. *Mol Pharmacol* 1998;54:1140–1147. [PubMed: 9855645]
33. Lee MC, Moura PA, Miller EA, Fidock DA. *Plasmodium falciparum* Sec24 Marks Transitional ER that Exports a Model Cargo via a Diacidic Motif. *Mol Microbiol*. 2008
34. Chakrabarti D, Azam T, DelVecchio C, Qiu L, Park YI, Allen CM. Protein prenyl transferase activities of *Plasmodium falciparum*. *Mol Biochem Parasitol* 1998;94:175–184. [PubMed: 9747968]
35. Ungermann C, von Mollard GF, Jensen ON, Margolis N, Stevens TH, Wickner W. Three v-SNAREs and two t-SNAREs, present in a pentameric cis-SNARE complex on isolated vacuoles, are essential for homotypic fusion. *J Cell Biol* 1999;145:1435–1442. [PubMed: 10385523]
36. Kweon Y, Rothe A, Conibear E, Stevens TH. Ykt6p is a multifunctional yeast R-SNARE that is required for multiple membrane transport pathways to the vacuole. *Mol Biol Cell* 2003;14:1868–1881. [PubMed: 12802061]
37. Hasegawa H, Zinsser S, Rhee Y, Vik-Mo EO, Davanger S, Hay JC. Mammalian ykt6 is a neuronal SNARE targeted to a specialized compartment by its profilin-like amino terminal domain. *Mol Biol Cell* 2003;14:698–720. [PubMed: 12589064]
38. Chakrabarti D, Da Silva T, Barger J, Paquette S, Patel H, Patterson S, et al. Protein farnesyltransferase and protein prenylation in *Plasmodium falciparum*. *J Biol Chem* 2002;277:42066–42073. [PubMed: 12194969]
39. Maurer-Stroh S, Koranda M, Benetka W, Schneider G, Sirota FL, Eisenhaber F. Towards complete sets of farnesylated and geranylgeranylated proteins. *PLoS Comput Biol* 2007;3:e66. [PubMed: 17411337]
40. Zhang T, Hong W. Ykt6 forms a SNARE complex with syntaxin 5, GS28, and Bet1 and participates in a late stage in endoplasmic reticulum-Golgi transport. *J Biol Chem* 2001;276:27480–27487. [PubMed: 11323436]
41. Volchuk A, Ravazzola M, Perrelet A, Eng WS, Di Liberto M, Varlamov O, et al. Countercurrent distribution of two distinct SNARE complexes mediating transport within the Golgi stack. *Mol Biol Cell* 2004;15:1506–1518. [PubMed: 14742712]
42. Chen Y, Shin YK, Bassham DC. YKT6 is a core constituent of membrane on machineries at the Arabidopsis trans-Golgi network. *J Mol Biol* 2005;350:92–101. [PubMed: 15919093]
43. Pylypenko O, Schonichen A, Ludwig D, Ungermann C, Goody RS, Rak A, et al. Farnesylation of the SNARE protein Ykt6 increases its stability and helical folding. *J Mol Biol* 2008;377:1334–1345. [PubMed: 18329045]
44. Reiss Y, Stradley SJ, Gierasch LM, Brown MS, Goldstein JL. Sequence requirement for peptide recognition by rat brain p21ras protein farnesyltransferase. *Proc Natl Acad Sci U S A* 1991;88:732–736. [PubMed: 1992464]
45. Sousa SF, Fernandes PA, Ramos MJ. Farnesyltransferase inhibitors: a detailed chemical view on an elusive biological problem. *Curr Med Chem* 2008;15:1478–1492. [PubMed: 18537624]

46. Sebti SM, Hamilton AD. Farnesyltransferase and geranylgeranyltransferase I inhibitors and cancer therapy: lessons from mechanism and bench-to-bedside translational studies. *Oncogene* 2000;19:6584–6593. [PubMed: 11426643]
47. Finegold AA, Johnson DI, Farnsworth CC, Gelb MH, Judd SR, Glomset JA, et al. Protein geranylgeranyltransferase of *Saccharomyces cerevisiae* is specific for Cys-Xaa-Xaa-Leu motif proteins and requires the CDC43 gene product but not the DPR1 gene product. *Proc Natl Acad Sci U S A* 1991;88:4448–4452. [PubMed: 2034682]
48. Yokoyama K, Goodwin GW, Ghomashchi F, Glomset JA, Gelb MH. A protein geranylgeranyltransferase from bovine brain: implications for protein prenylation specificity. *Proc Natl Acad Sci U S A* 1991;88:5302–5306. [PubMed: 2052607]
49. Nallan L, Bauer KD, Bendale P, Rivas K, Yokoyama K, Horney CP, et al. Protein farnesyltransferase inhibitors exhibit potent antimalarial activity. *J Med Chem* 2005;48:3704–3713. [PubMed: 15916422]
50. Glomset JA, Gelb MH, Farnsworth CC. Prenyl proteins in eukaryotic cells: a new type of membrane anchor. *Trends Biochem Sci* 1990;15:139–142. [PubMed: 2187294]
51. Glomset JA, Farnsworth CC. Role of protein modification reactions in programming interactions between ras-related GTPases and cell membranes. *Annu Rev Cell Biol* 1994;10:181–205. [PubMed: 7888176]
52. Glomset JA, Gelb MH, Farnsworth CC. Geranylgeranylated proteins. *Biochem Soc Trans* 1992;20:479–484. [PubMed: 1397644]
53. Lerner EC, Zhang TT, Knowles DB, Qian Y, Hamilton AD, Sebti SM. Inhibition of the prenylation of K-Ras, but not H- or N-Ras, is highly resistant to CAAX peptidomimetics and requires both a farnesyltransferase and a geranylgeranyltransferase I inhibitor in human tumor cell lines. *Oncogene* 1997;15:1283–1288. [PubMed: 9315095]
54. Maurer-Stroh S, Washietl S, Eisenhaber F. Protein prenyltransferases: anchor size, pseudogenes and parasites. *Biol Chem* 2003;384:977–989. [PubMed: 12956414]

Figure 1. Compartmentalization of PfYkt6.1 in *P. falciparum* parasites

Trophozoite stage parasites exhibiting increased targeting of the PfYkt6.1 protein to organellar compartments were by confocal immunofluorescence microscopy using various organellar markers. (A) Immunofluorescence micrograph showing complete overlap between the GFP fluorescence and anti-PfYkt6.1 antibody signal in GFP-PfYkt6.1 expressing parasites. The GFP-PfYkt6.1 compartments were only partially labeled with antibodies against the Golgi marker PfErd2 (B), or the ER marker PfBip (C). Scale bars, 2 μ M.

Figure 2. Detergent solubility of untagged and GFP-tagged PfYkt6.1 proteins

GFP-PfYkt6.1 expressing parasites were solubilized with Triton X-114 and phase separated prior to immunoblot analyses using specific antibodies to the PfYkt6.1 protein (upper left blot) or GFP tag (upper right blot). The luminal chaperone PfBip and the multi-pass membrane protein PfErd2 were probed as markers for hydrophilic and hydrophobic proteins, respectively. As shown, the untagged and GFP-tagged PfYkt6.1 proteins both partially partitioned into the detergent phase following Triton X-114 phase partitioning. U: untransfected cell lysate, A: Triton X-114 aqueous phase, D: Triton X-114 detergent phase.

Figure 3. Role of the CAAX motif in PfYkt6.1 targeting

(A) Deletion of the C-terminal CAAX sequence CSIM resulted in a diffuse cytosolic localization of the mutant construct, GFP-PfYkt6.1.CSIM. In contrast with the wild-type protein (see Fig. 1), this mutant construct failed to associate with the organellar structures or the ER (B) and Golgi (C) compartments. (D) Triton X-114 phase partitioning of the endogenous PfYkt6.1 protein and GFP-PfYkt6.1.CSIM mutant indicating a complete fractionation of the mutant protein with the Triton X-114 aqueous phase. U: untransfected cell lysate, A: Triton X-114 aqueous phase, D: Triton X-114 detergent phase. Scale bars, 2 μM.

Figure 4. *In vitro* farnesylation and geranylgeranylation of PfYkt6.1 and CAAX mutants
(A) Dose-dependent inhibition of PfYkt6.1 and mutant construct farnesylation *in vitro*. **(B)** Role of the C-terminal residue (position +3 relative to the CAAX cysteine) on the farnesylation efficiency of PfYkt6.1. The +3 residue in each construct is indicated at the top of the respective lanes. **(C)** Geranylgeranylation efficiency of PfYkt6.1 and mutant constructs. Protein farnesylation was achieved using a Mono Q purified fraction of PfPFTase, whereas geranylgeranylation was obtained using an ammonium sulfate fraction presumably containing the total prenyltransferase activities.

Figure 5. *In vitro* inhibition of PfPFTase activity with PfYkt6.1 CAAX-containing peptides
Increasing concentrations of the PfYkt6.1 CAAX sequence KQCCSIM, the PfPTP CAAX sequence RKCHF (potent PfPFTase inhibitor), or the heptapeptide NRSCAIV (negative control) were used to test the farnesylation activity of PfPFTase on the biotinylated lamin B peptide (YRASNRSCAIM). The data represent mean activities $\pm$ standard deviations, and are expressed as a percentage of the total enzyme activity in the absence of any competing peptides.