Molecular & Biochemical Parasitology 175 (2011) 162–168

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Evidence for prenylation-dependent targeting of a Ykt6 SNARE in *Plasmodium falciparum*

Lawrence Ayong[a,1], Thiago DaSilva[a,2], Jennifer Mauser[a], Charles M. Allen[b], Debopam Chakrabarti[a,*]

[a] Department of Molecular Biology & Microbiology, Burnett School of Biomedical Sciences, University of Central Florida, 12722 Research Parkway, Orlando, FL 32826, United States
[b] Department of Biochemistry & Molecular Biology, University of Florida, Gainesville, FL 32610, United States

ARTICLE INFO

Article history:
Received 15 March 2010
Received in revised form 1 November 2010
Accepted 5 November 2010
Available online 12 November 2010

Keywords:
Plasmodium falciparum
SNARE
Protein trafficking
Ykt6
Prenylation

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 the 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 IC_{50} of 1 μM. Our findings underscore the importance of CAAX motif-derived peptidomimetics for antimalarial drug development.

1. 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 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

* Corresponding author. Tel.: +1 407 882 2256; fax: +1 407 384 2062.
E-mail address: dchak@mail.ucf.edu (D. Chakrabarti).
[1] Current address: Center for Tropical & Emerging Global Diseases, University of Georgia, Athens, GA 30602, United States.
[2] Current address: University of Miami Miller School of Medicine, Miami, FL 33136, United States.

0166-6851/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2010.11.007

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 showed 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 provide 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.

2. Materials and methods

2.1. 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 1000. 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.

2.2. 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-PfYkt6.1ΔCSIM) was amplified by RT-PCR and ligated into the pGEM-T Easy vector (Promega). The constructs were sequence confirmed and subcloned into the AvrII/BglII 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 μF). 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).

2.3. 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 × *g* for 3 min at 4 °C, and then clouded in a 37 °C water bath for 20 min. The samples were again centrifuged at room temperature at 10,000 × *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.

2.4. 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 overexpressed in *Escherichia 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 OD_{600} between 0.4 and 0.5. The cells were further grown for 1 h at 20 °C prior to induction with 1 mM IPTG, and harvested after 4 h 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.

2.5. In vitro prenylation assays

In vitro farnesylation of full-length PfYkt6.1 proteins was performed at 16 °C for 30 min with or without the PFTase inhibitor FTI-276 in 50 mM Tris–HCl (pH 7.5), 20 μM $ZnCl_2$, 5 mM $MgCl_2$, 20 mM KCl, 1 mM DTT, 5 μg of Mono Q PfPFTase fraction and 2 μCi ^{3}H-farnesyl diphosphate [30,34]. Protein geranylgeranylation was assayed using ^{3}H-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).

3. Results

3.1. 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