Identification of endoplasmic reticulum-shaping proteins in *Plasmodium* parasites

**Letter**

Dear Editor,

In eukaryotic cells, the endoplasmic reticulum (ER) is a continuous membrane system involved in many critical cellular processes, including protein synthesis, lipid synthesis, and calcium storage. Morphologically, the ER is composed of cistern-like sheet structures and a reticular network of tubules. Classes of integral membrane proteins that shape the ER have been identified: the reticulons and DP1/Yop1p proteins generate ER tubules by inducing high curvature in the membrane (Voeltz et al., 2006), the atlasin GTPases and Sey1p/RHD3 proteins mediate fusion of ER membranes, forming a tubular network (Hu et al., 2009; Orso et al., 2009), and Climp63, Kinetin, and p180 play a role in stabilizing ER sheets (Shibata et al., 2010). Mutations in the determinants of ER tubules cause growth defects, short root hairs, and a neurodegenerative disease called hereditary spastic paraplegia (HSP) (Hu et al., 2009), and Climp63, Kinetin, and p180 play a role in stabilizing ER sheets (Shibata et al., 2010).

ER morphology has rarely been studied in protozoan parasites, likely due to difficulties posed by the lack of genetic manipulation and relatively small scale of these cells. In *Entamoeba histolytica*, the intestinal protozoan that causes invasive amebiasis, the ER was first thought to be composed of vesicles of varying size but recently shown to be a continuous network (Teixeira and Huston, 2008). In *Toxoplasma gondii*, the infectious agent resulting in toxoplasmosis, expansion and partitioning of the ER has been followed during the cell cycle (Nishi et al., 2008). The ER in *Plasmodium* parasites, the causative agents of malaria, has been visualized during the erythrocytic cycle (van Dooren et al., 2005); it transforms from a perinuclear structure with no distinctive morphological characters into a reticular network throughout the cytoplasm. *Plasmodium* develops first in the *Anopheles* mosquito, and then invades the liver upon injection into mammalian hosts. Whether the ER adopts its characteristic shapes in these stages is yet to be determined, and the molecular determinants of the ER in *Plasmodium* parasites remain unclear.

To identify *Plasmodium falciparum* orthologs of ER-shaping proteins, we conducted Blast searches of the *P. falciparum* genomic database (PlasmoDB, www.plasmoDB.org, v. 6.3, released December 22, 2009) using *Saccharomyces cerevisiae* Yop1p (ScYop1p) and human DP1 protein sequences as queries. Both searches revealed 35%–42% identity (55%–60% homology) with PFC0730w (Gene ID PF3D7_0316700 in PlasmoDB v2.6, released October 15, 2015). The PFC0730w sequence was further used to query *P. berghei* proteins using the tblastn tool. This search returned PBANKA_0414500 and PBANKA_1135000 as the closest *P. berghei* homologs (Fig. S1). PBANKA_0414500 was recently annotated as a putative HVA2/TB2/DP1 family protein and PBANKA_1135000 as a HVA22-like protein. Because HVA2 is the Yop1p homolog in plants and TB2 is a previously used alias for DP1, our homologous searches confirm the annotation of the genomic database. We renamed PBANKA_0414500 as *PbYOP1* and PBANKA_1135000 as *PbYOP1L* (Fig. 1A and 1B). When searching for the reticulon homolog in the *P. berghei* genome, we found PBANKA_1139900 and renamed it *PbRTN1* (Fig. 1B). According to PlasmoDB database, the three potential *Plasmodium* tubule-forming proteins have distinct expression profiles during the asexual cycle: the expression of *PbYOP1* gradually increases and peaks in trophozoites and schizonts, the levels of *PbYOP1L* peak in rings and trophozoites but decrease in schizonts, and levels of *PbRTN1* are relatively constant. These data suggest non-redundant functions of these proteins.

Thus, three potential ER tubule-forming proteins were identified in *P. berghei*. A common feature of ER tubule-forming proteins is a reticulon-homology domain (RHD) consisting of two tandem transmembrane hairpins (TMH) (Fig. 1B). The sequences of all three candidates for ER tubule formation in *P. berghei* exhibited characteristics of the RHD domain. Notably, the intervening loop between the two TMHs in *PbRTN1* is much longer than that of Yop1 family proteins, including *PbYOP1* and *PbYOP1L* (Fig. 1B). In contrast, *PbYOP1* bears a much longer N-terminus than *PbYOP1L* and *PbRTN1*. The length of the TMHs is also variable, with the first TMH of *PbYOP1L* and the second TMH of *PbRTN1* being shorter than other TMHs (Figs. 1B and S2), suggesting that it partially traverses the lipid bilayer. Importantly, the primary structure of *PbYOP1* is highly conserved among *Plasmodium* species (Fig. S2), implying a fundamental role of the protein.
We performed a similar search for the homolog of Sey1p in the *P. berghei* genome and identified PBANKA_1026600 as *PbSEY1* (Fig. 1C and 1D). Four conserved motifs were identified in the GTPase domains of the dynamin superfamily: the P-loop (G1, β1–α1), the switch 1 region (G2, α1–β2), the switch 2 region (G3, β3–α2), and the G4 motif (after β5). Most residues in these signature motifs are identical between *ScSey1p* and *PbSEY1* (Fig. S3). The predicted stalk domain of *PbSEY1*, which is between the GTPase domain and the transmembrane domains, is ~150 amino acids longer than that of *ScSey1p* and ~400 amino acids longer than those found in ATLs. The length of the stalk domain of *ScSey1p* has been shown to be essential for fusion (Yan et al., 2015) and to possibly contribute to the unique GTP cycle of *ScSey1p* when compared to ATL. If this region forms a helical bundle as predicted, it would be taller than that of *ScSey1p*, implying likely different dynamics during membrane fusion reactions. Taken together, the ER-...
shaping proteins identified in *P. berghei* may function similarly to those of yeast and mammalian orthologs and may possess unique features.

Purified ScYop1p, when reconstituted into proteoliposomes, leads to the generation of membrane tubules in vitro, indicating that Yop1p is sufficient to induce high curvature in membranes (Hu et al., 2008). To test whether *Pb*YOP1 is capable of bending membranes as demonstrated for ScYop1p, we performed in vitro reconstitution assays. Recombinant *Pb*YOP1 (residues 39–186) lacks the predicted flexible region in the N- and C-termini for optimized expression and stability. *Pb*YOP1 was expressed and purified from *Escherichia coli* (Fig. 2A), mixed with preformed liposomes, and detergents were removed by the addition of bio-beads. When the reconstituted products were visualized by transmission electron microscopy (TEM), tubular structures similar to those of ScYop1p and ScRtn1p were observed (Fig. 2B). The diameters of these tubules are relatively uniform (15–20 nm), which is consistent with the ScYop1p tubules. In the absence of *Pb*YOP1, liposomes exhibited a characteristic round shape (Fig. 2B). These results suggest that *Pb*YOP1 alone is adequate to induce high curvature in membranes and cause tubule formation upon reconstitution with lipids.

The two TMHs of the RHD domain are thought to occupy more space in the outer leaflet than the inner leaflet of the lipid bilayer, deforming membranes as a wedge insertion. A conserved amphipathic helix C-terminal to the second TMH of ScYop1p was recently identified to provide additional wedging (Brady et al., 2015). Deletion of the helix in ScYop1p abolished its tubule-forming ability in vitro. Through secondary structure prediction and helical wheel analysis, we found two potential helices that exhibit an amphipathic nature, one on each side of the RHD domain of *Pb*YOP1. To test the role of these helices, we synthesized a peptide corresponding to the sequences of the C-terminal helix (Fig. S4A, residues 171–184) and determined its membrane association as expected for other membrane-deforming amphipathic helices. Circular dichroism spectrum analysis revealed that the peptide only adopted an α-helical configuration when liposomes were present (Fig. S4B), indicating stabilization of the peptide in a membrane environment. To our surprise, mutant *Pb*Yop1 (residues 39–170) missing the entire C-terminus, including the predicted amphipathic helix, could still form tubules in vitro (Fig. 2C). Therefore, we investigated the role of the N-terminal helix. We found that deletion of the N-terminal region of *Pb*YOP1 did not yield any usable recombinant protein (Fig. S4C). Instead, we obtained a major contaminant after attempting to purify truncated *Pb*YOP1 (residues 73–186). When the contaminant underwent the same reconstitution process, no tubular structures were formed (Fig. S4D), further confirming that the tubules we observed could only be specifically generated by functional *Pb*YOP1 proteins. As an alternative, we generated point mutations in the predicted hydrophobic face of the helix. However, mutant *Pb*YOP1 (residues 39–186, L45D/L49D/V52D) formed tubules like the wild-type protein when reconstituted with lipids (Fig. S4D). Taken together, these results suggest that the C-terminal region of the RHD domain is dispensable for generating membrane tubules and the N-terminus is important for the stability of the protein but may not function as an amphipathic helix to augment wedge insertion.

In addition to the “wedge” mechanism, ScYop1p and ScRtn1p can form homo- and hetero-oligomers through which these proteins may act as arc-like scaffolds to shape the membrane tubules (Shibata et al., 2009). We tested the oligomerization tendency of *Pb*YOP1 using a sucrose-density gradient. When reconstituted *Pb*YOP1 was solubilized by digitonin, a relatively mild detergent, it migrated in the gradient at a position that corresponds to a much higher molecular weight than that of a monomer (Fig. 2D). The oligomerization was largely disrupted when Triton X-100 or SDS was used instead (Fig. 2D). Notably, SDS-resistant dimers were often observed when *Pb*YOP1 was separated by SDS-PAGE and analyzed by immunoblotting (Fig. 2D). These results confirm that *Pb*YOP1 forms oligomers.

To further confirm the ER-shaping function of *Pb*YOP1, we performed ER morphology rescue assays in yeast cells. When Sey1p, the ER fusogen in yeast, and Yop1p or Rtn1p are deleted, the peripheral ER (also termed cortical ER in yeast because it localizes underneath the plasma membrane) displays abnormal morphology. Large areas of the cortex are void of ER structures and most of the tubular ER network becomes sheet-like; the re-introduction of either Yop1p or Rtn1p in these cells restores the ER morphology (Hu et al., 2009). To ensure the expression of *Pb*YOP1 in yeast, we utilized a 2 μv vector with high copy numbers (pESC-URA) and drove protein expression using an inducible GAL promoter. The coding region of *Pb*YOP1 was optimized for yeast codon usage to achieve detectable expression. As expected, most *sey1Δ yop1Δ* cells expressing *Pb*YOP1 exhibited normal ER morphology (Fig. 2E). The *Pb*YOP1 levels were similar to ScYop1p expressed under control of the endogenous promoter (Fig. 2F). These results suggest that *Pb*YOP1 can replace ScYop1p in maintaining proper ER morphology.

Next, we performed ER morphology rescue assays in mammalian cells and tested the function of *Pb*SEY1. ER tubules become long and unbranched when ATL2 and ATL3 are deleted using CAS9/CRISSPR system in COS-7 cells (ATL1 is not detectable in COS-7 cells) (Hu et al., 2015; Wu et al., 2015), indicative of the lack of fusion between ER tubules (Fig. 2G and 2H). As previously reported (Yan et al., 2015), when Sc*sey1p* were expressed in these cells, the defects in ER morphology were largely restored (Fig. 2H). *Pb*SEY1 do not express in yeast or mammalian cells, likely due to different codon usage. When codon-optimized *Pb*SEY1 was expressed in ATL-deleted COS-7 cells, a majority of the mutant cells exhibited normal tubular ER network (Fig. 2H). These results confirm that *Pb*SEY1 is functionally analogous to its yeast or mammalian orthologs.
A  

B  PbYOP1 39–186  

Lipid only  

C  PbYOP1 39–170  

D  5%–25% sucrose gradient  

Fractions 1 2 3 4 5 6 7 8 9 10 11 12 13 14  

% of cells with indicated ER morphology  

E  wt  seyΔyop1Δ + vector  seyΔyop1Δ + ScYop1p  seyΔyop1Δ + PbYOP1  

Periphery  

Center  

% of cells with indicated ER morphology  

F  Vector  ScYop1p  PbYOP1  

IB HA  

IB PGK  

G  wt  DKO  DKO + ScSey1p  DKO + PbSEY1  

Caretinulin  

HA  

Merge  

H  wt  DKO  DKO + ScSey1p  DKO + PbSEY1  

IB HA  

IB ATL2  

IB ATL3  

IB Actin
Our findings reveal four ER-shaping proteins in the *P. berghei* genome. *PbYOP1, PbYOP1L, and PbSEY1* have not been characterized previously. We showed that, similar to *ScYop1p, PbYOP1* generated membrane tubules when purified and reconstituted in vitro, even though a conserved amphipathic helix next to the transmembrane domain is less important than in *ScYop1p. We also confirmed the role of *PbYOP1* and *PbSEY1* in maintaining the tubular ER network in cells. Although the ER shaping activities of these proteins need to be further tested in *Plasmodium*, our results imply that the ER of *Plasmodium* parasites likely forms a tubular network via common mechanisms as described for yeast and mammalian cells. In addition, the ER-shaping proteins of *Plasmodium* parasites possess unique features that may meet specialized demands on the parasitic ER. The homolog of *PbRNT1* in *Plasmodium yoelii nigeriensis* has been termed *Pyn_chl091* due to its transcriptional up-regulation in mosquito vector when treated with chloroquine, an anti-malarial drug (Silveira et al., 2007). These ER-shaping proteins, including *PbRNT1*, likely adopt specialized roles during stressed conditions.

Very little is known about the morphology and function of the *Plasmodium* ER, especially during the pathogenic path of the parasites. Identification and preliminary characterization of *Plasmodium* ER-shaping proteins lays the foundation for further investigation of the *Plasmodium* ER. A lack of individual ER-shaping proteins in unicellular organisms, such as yeast, causes no drastic defects. However, in *Candida albicans*, an infectious fungus, deletion of *Sey1p* decreases its virulence (Yamada-Okabe and Yamada-Okabe, 2002), leading to the possibility that ER morphology determinants play a role in the infection of protozoan parasites, including malaria.

**FOOTNOTES**

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