Commentary

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To a better understanding of the giardial ENTH protein function

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Summary **Epsin *N*-terminal homology (ENTH) domains are present at the N-terminus of either the epsin or epsin-related (epsinR) proteins. These proteins have been involved in clathrin-mediated trafficking and are critical for membrane deformation at the site of vesicle budding. While more than one type of these proteins have been described in many eukaryotic cells, the protozoa parasite *Giardia lamblia* contains only one member of this ENTH-protein family. In the last two years, four works have been published showing that this giardial protein might play diverse functions. This commentary gives a brief overview on the current status of the particular characteristics and functions of this unique protein.**

Keywords: *Giardia lamblia*, epsin

1. Introduction

A feature that defines eukaryotic cells is the compartmentalization of their cytoplasm in different membrane-associated organelles. In order to maintain these compartments, cells have developed mechanisms to ensure that specific proteins are targeted to particular organelles (*1*). Thus, subcellular compartmentalization became an essential feature in these organisms, allowing the correct interrelation of certain intracellular components and enabling reactions to occur efficiently and orderly. To connect all compartments proteins and lipids are trapped into transport vesicles, which are made of "coatomers", specific for a particular compartment. Clathrin-Coated Vesicles (CCVs) are transport vesicles that have been extensively characterized in a wide variety of eukaryotic cells and mediate the delivery of cargo molecules to the endosomal/lysosomal compartments. These vesicles are covered by a layer that is composed of scaffold proteins, clathrin and several oligomeric and monomeric adapter proteins (APs: Adaptor Proteins). The APs binds clathrin and also recognize specific classification signals that are present in the cytosolic domains of transmembrane proteins, producing accumulation of these proteins in the CCVs.

Giardia lamblia is a parasite of great importance not only because it is the cause of a very frequent disease, giardiasis, but also because it shows clear evidence of reductive evolution (*2*). In this scenario, *G. lamblia* presents an atypical endomembranous system (*3,4*), characterized by the presence of ER and lysosome-like peripheral vacuoles (PVs) and by the absence of other organelles characteristic of higher eukaryotes such as endosomes, lysosomes, mitochondria, peroxisomes, and a morphologically recognizable Golgi apparatus. The simplicity of the giardial subcellular organization makes this parasite an excellent model to study the basis of different cellular processes in eukaryotes, such as vesicular protein trafficking. The very existence of CCVs in *Giardia* is controversial since typical clathrin-coated cages or clathrin budding have never been observed in this parasite. However, there is increasing amount of fresh data suggesting that *Giardia* utilizes both conserved and non-conserved mechanisms for protein delivery via clathrin vesicles. Without a morphologically discernible Golgi apparatus, the anterograde vacuolar protein trafficking seems to start at the ER through the action of AP-1 and clathrin while receptor-mediated endocytosis involves the participation of AP-2 and clathrin. Last year, the presence of a monomeric adaptor protein containing an ENTH domain (see below) was discovered. Here, we showed and discussed all the findings about GlENTHp

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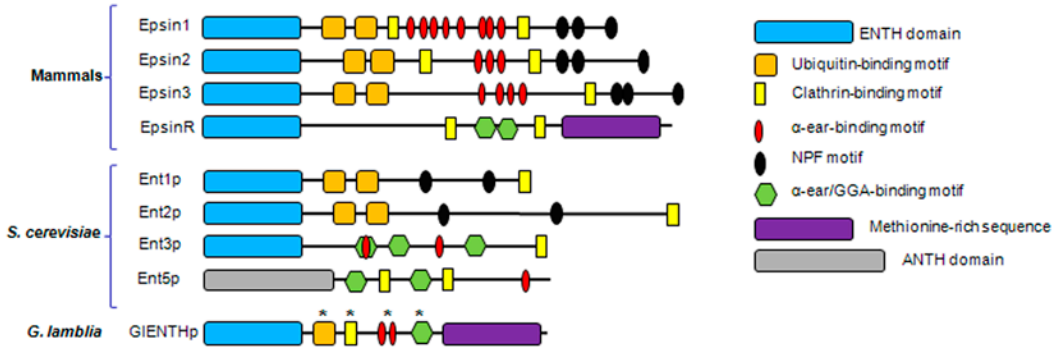

Figure 1. Schematic representation of E/ANTH proteins of mammals, *S. cerevisiae* and *G. lamblia*. The ENTH or ANTH domain is depicted in the N-terminus. Specific motifs within their C-terminal region are also represented. NPF motifs bind to Eps15 homology (EH) domain-containing proteins. Asterisks in GlENTHp denote no defined localization or presence of the motifs.

(for *Giardia lamblia ENTH protein*), focussing on its function and origin.

2. The multiple function of GlENTHp

The ENTH/ANTH/VHS superfamily are composed by proteins that possesses either an ENTH (Epsin N-terminal homology), an ANTH (AP180 N-terminal homology) or a VHS (Vps27, Hrs and STAM) domain at their N-terminus and have been identified in proteins that participate in clathrin-mediated budding in mammalian cells (*5*). Among the ENTH family, the epsin subfamily are composed by classical epsin proteins and the epsin-related (epsinR) protein that play a role in clathrin-mediated endocytosis or Golgi-to-endosome protein trafficking, respectively (*6,7*). The ENTH module binds phosphoinositides (PIs), with epsin preferring $PI4,5P_2$ while epsinR favouring PI4P binding. At their C-terminal these proteins contain several short motifs that mediate interactions with endocytic proteins and are characteristic for each protein (Figure 1) (*8*).

In Feliziani *et al*. 2015, we presented our first studies on GlENTHp (GL50803_3256), which possesses the highly conserved ENTH domain at its N-terminal region (*9*). Because, only one member of the family is present in *Giardia*, we wondered whether it might function as a classical epsin or as an epsinR-like protein in this parasite. Analysis of its secondary and tertiary structure showed that the only conserved motif between GlENTHp and other members of this family was the ENTH domain but possesses a methionine-rich sequence with unknown function that is only observed in the epsinR (*10*). Besides the lack of other conserved motifs like the ubiquitin-interacting motif (UIM), clathrin-binding motifs and the α-ear (AP-2)-binding or γ-ear (AP-1)-binding motifs, we found that GlENTHp is able to interact with proteins involved in CCVs formation, besides specific PIs. By immunofluorescence assay (IFA) and confocal microscopy, we showed that GlENTHp localized in the cytosol and somehow in the nuclei. Similarly to classical epsin-type adaptors, it was associated with the giardial αAP-2, clathrin heavy chain, ubiquitin and it was able to interact with $PI3,4,5P_3$, which was observed at the plasma membrane (PM) of the parasite. We also found that GlENTHp played an active role in receptor-mediated endocytosis of low-density lipoproteins. On the other hand, a direct association was found with the γAP-1 and PI4P, present in the *Giardia* ER-sorting sites, and was implicated in the trafficking of the soluble acid phosphatase from the ER to the PVs, suggesting that GlENTHp could also carry out a role of epsinR in the parasite. When the PVs were ultrastructural analyzed in transgenic cells over-expressing GlENTHp, we observed that these oval-shaped vacuoles lost their heterogeneous electron-density when compared with *wild-type* cells. Conversely, enhance electron-density of the PVs was detected in cells in which the GlENTHp expression was either reduced or replaced by a mutant that is unable to bind PIs (*9*). These studies suggest that GlENTHp would be involved in the transport of material to and/or from the PVs, playing an active role in the maturation of these vacuoles. The alteration of the GlENTHp function caused a severe defects in cell growth, which correlated with more electron-dense PVs, reinforcing the idea that GlENTHp could be implicated in the preservation of the PV homeostasis and cell survival. Several evidences support the idea that the proteins involved in endocytosis may also play a role in the nucleus (*11,12*). Surprisingly, we found an increase of the GlENTHp mutant in one of the nuclei, when compared with endogenous GlENTHp. This result was correlated with an increase nuclear localization of GlENTHp, but not of the mutant, when the cells were treated with LY294002 to reduce the synthesis of $PI3,4,5P_3$ and PI4P. These results suggest that GlENTHp might continuously move in and out of the nuclei depending on its ability to bind PIs. Further analyses are necessary to disclose how the mechanism of GlENTHp trafficking through the nuclear membrane takes place and what the meaning of this behavior is.

At the time we were performing this analysis, another group published new results about GlENTHp (*13*). Surprisingly, they found that GlENTHp localized in the giardial ventral disk, a microtubule structure and the primary organelle of attachment to the cell host (*13*). One explanation of this differential behavior of the protein might be just a technical problem since the cytosolic and the membrane distribution of GlENTHp was demonstrated by biochemical and cellular analysis in our work. Moreover, many other differences were reported in this Short Communication, like the partial PI binding specificity of GlENTHp and the lack of binding to any endosomal components, including clathrin. Because we demonstrated each finding utilizing more than one approach, we are confident that we were able to disclose the native function of GlENTHp.

In conclusion, significant steps have been made in the functional characterization of GlENTHp, disclosing that not only AP-1 and AP-2 (as we previously envisioned) are involved in the clathrin-mediated traffic in *Giardia*, but the action of the monomeric GlENTHp is also required. Conversely to AP-1, which was implied in the ER-to-PV protein trafficking or to AP-2, which participates in receptor-mediated endocytosis, GlENTHp seems to play a critical role in both pathways, depending on the specific requirement of the parasite. This is another evidence that *Giardia* might use the same protein to perform multiple functions, making the studies in this parasite increasingly fascinating.

3. Evolutionary origin

The members of the ENTH family are highly conserved, even among organisms as diverse as humans, *Xenopus*, *Drosophila*, *Arabidopsis*, yeasts, and *T. brucei*. However, there are differences in the number of proteins that are members of this family in each species. Thus, while yeast *S. cerevisiae* and most vertebrates (including primates, rodents and zebrafish) contain at least two paralogs of the ENTH family, only one member of this family is present in organisms such as *Toxoplasma gondii, Plasmodium Falciparum and Giardia lamblia* (*14*). In *Saccharomyces cerevisiae* it has been characterized two classical epsin homologues, Ent1p and Ent2p, which are essential proteins with redundant functions, and two epsinR orthologues, Ent3p and Ent5p (*15,16*). In an elegant report, De Craene and co-workers performed *in silico* analysis of this ENTH/ANTH/VHS superfamily, consisting of proteins from 84 genomes representative of the different eukaryotic taxa. They found the ENTH domain of *Giardia* (member of the Excavata taxa), is the unique representative of the ENTH/ANTH/VHS function, thus suggesting that multiple tasks might be performed by the unique ENTH-containing protein (*14*). This statement encouraged us to analyze whether GlENTHp could be a key player in the evolution of the ENTH family by testing its role in *S. cerevisiae*.

In Feliziani *et al*. 2016, we showed that neither the overexpression of GlENTHp nor of the gENTH alone rescued the lethal phenotype in the *ent1Δent2Δ* cells (*17*). It was shown that the essential function of the ENTH motif resided in the conserved Y100 and T104 residues (*18*). Because gENTH domain lacks the conserved T within its sequence, we were wonder whether it might be the reason for the incapacity of GlENTHp to rescue the lethal *ent1Δent2Δ* cells. To test this, we performed complementary assays now using the $GlENTH_{N107Yp}$ and $gENTH_{N107Y}$ mutants. The results showed that none of the mutants were able to complement the *ent1Δent2Δ* cells, suggesting that the presence of the Y/T residues in conserved sites was not sufficient for GlENTHp to supplement the function of Ent1/2p in *S. cerevisiae*. Through functional assays, we evaluated the location of the pheromone a factor receptor Ste3 and the polymerization of the actin-binding protein ABP1 in *wild-type* cells by overexpressing GlENTHp. We found that GlENTHp was not able to act as a dominant negative protein of Ent1/2p by interfering with its normal function. At this point, we concluded that GlENTHp was unable to replace the function of Ent1/2p in fungi, most likely due to the limited degree of homology between sequences of these proteins beyond the ENTH domain. Also, it is possible that the preference of GlENTHp, for $PI3,4,5P_3$ and PI4P over $PI4,5P_2$, altered its interaction with the appropriate target at the PM, resulting in rescue failure in *ent1Δent2Δ* cells. But can GlENTHp be able to perform Ent3/5p functions in *S. cerevisiae* instead? To evaluate this possibility, we assessed deficiencies in the transport of fluorescence carboxypeptidase S, GFP-Cps1 (*19*), and alteration of α-factor maturation (*20*) in *ent3Δent5Δ* cells (*21*). The results showed that the expression of GlENTHp neither re-established the localization of GFP-Cps1 nor restored the maturation of the α-factor, being unable to perform the function of Ent3/5p. To assess whether GlENTHp could act as a dominant negative protein of the Ent3/5p, the localization of Cps1 and maturation of α-factor was again analyzed but now using *wild-type* cells overexpressing GlENTHp. By performing fluorescence microscopy assays in living cells and halo bioassays, we found that overexpression of GlENTHp interfered with the function of epsinR-type proteins in yeasts, evidenced by changes in the route of GFP-Cps1 and by alterations in the maturation of the α-factor. The interaction of Ent3/5p with PI4P has been reported to be crucial for their localization and function (*22*). Thus, we decided to test whether the dominant negative effect of GlENTHp overexpression was related to a change in the localization of Ent3/5p by overexpressing GlENTHp, gENTH and the mutant that is unable to bind PIs. Only when GlENTHp was

over-expressed in cells expressing Ent3p or Ent5p, a change in the localization of Ent3/5p was observed, suggesting that GlENTHp physically interfered with the Ent3/5p recruitment to the *trans*-Golgi network (TGN). In spite of lacking the canonical domains of interaction, we decided to test whether GlENTHp interacted with clathrin, the adapter subunit γAP-1 (APL4) and/or the ADP-ribosylation factor-binding proteins GGA1 and GGA2 (*15*), by performing FRET experiments in live yeast cells. The results showed that GlENTHp interacts with APL4, GGA1 and GGA2. Based on the results obtained, we proposed that GLENTHp transiently interacts with PI4P at the TGN membrane, blocking the binding of GGAs to the membrane and thus preventing the GGA2p/Ent3p binding. GlENTHp could also compete directly with Ent3/5p for the binding to AP-1 and GGAs but not clathrin, modifying the packaging of these proteins in the CCVs. These finding probably explains the clear misslocalization and missfunction of Ent3/5p when GlENTHp is overexpressed. Taken together, our results reinforce the idea that although there is a conserved sorting mechanism between *S. cerevisiae* and *G. lamblia*, a higher degree of similarity in the protein sequence is necessary to replicate the protein function in a different organism.

4. Conclusions

Although clathrin-mediated vesicular trafficking is one of the most conserved through the eukaryotic evolution and involves common regulators, it is more and more evident that it shows distinct particularities depending on the requirements of each cell type. In this sense, the finding that *Giardia* is able to utilize GlENTHp as a monomeric adaptor to fulfill two different clathrin-mediated mechanisms, point emphasis on the behavior of organisms that undergo reductive evolution. More studies are required to disclose other putative functions of GlENTHp performed beyond the cytoplasm. This, at the end, will contribute to understand the plasticity of this parasite to adapt to different environment thorough its evolution.

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