Chapter 11

The Unique Endosomal/Lysosomal System of *Giardia lamblia*

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1. Introduction

Endocytosis, which is important for the internalization of nutrients from the plasma membrane as well as extracellular fluids, has been extensively described in mammalian cells and yeast. The protozoan parasite *Giardia lamblia* (syn. *Giardia intestinalis, Giardia duodenalis*) is an early branching eukaryote that possesses a reduced membrane network with highly polarized vesicles, denominated peripheral vacuoles (PVs), neighboring the plasma membrane (reviewed by Adam, 2001; Faso and Hehl, 2011; Touz, 2011). This is an important zone of interaction between the parasite and its environment (the host intestine *in vivo* or the culture medium *in vitro*), and was shown to be the place involved in fluid-phase and receptor-mediated endocytosis (Gaechter *et al.*, 2008; Rivero *et al.*, 2010). Another characteristic of these PVs is that they contain hydrolytic activity resembling the function performed by lysosomes. These vacuoles have a high lytic capacity and a low luminal pH, both properties of mature lysosomes (Ward *et al.*, 1997; Touz *et al.*, 2002b).

More than twenty years after the first morphological description of the PVs, there is a consensus that these vesicles are rather an unusual combination of endosomal and lysosomal compartments. Conserved markers and mechanisms that govern trafficking to the PVs have been found, but there are particularities that show *Giardia* as a simplified organism compared with higher eukaryotes. This makes *Giardia* a unique biological cell model for investigating the minimal machinery employed by a eukaryote to regulate endocytosis and degradation. Here, we will discuss emerging data that are beginning to shed light on the endosomal-lysosomal system in *Giardia* and the molecules involved in this selective trafficking.

2. The parasite

Giardia lamblia is a flagellated protozoan that inhabits the upper small intestine of its vertebrate hosts and is the most common cause of defined waterborne diarrhea worldwide.

Clinical manifestations of giardiasis vary from asymptomatic infection to acute or chronic disease associated with diarrhea and malabsorption (Adam, 2001). It is the most common cause of diarrheal disease in the United States (Barwick *et al.*, 2000). In developing countries, there is a very high prevalence and incidence of infections, and data suggest that long-term growth retardation can result from chronic giardiasis (Fraser *et al.*, 2000).

Giardia was initially described by van Leeuwenhoek in 1681 from examining his own diarrheal stools under the microscope (Dobell, 1950), but it was not until 1981 that the World Health Organization classified *Giardia* as a human pathogen. Infections initiate with the ingestion of the cyst forms, which excyst in the upper small intestine of the host. The trophozoites replicate and colonize the intestinal surface and some of them encyst in the lower small intestine after sensing the stimulus for encystation (Lujan *et al.*, 1997; Adam, 2001). Interestingly, during differentiation (encystation/excystation), trophozoites undergo important biochemical and morphological modifications involving the secretory machinery of the cell. Recent studies about these changes have provided new insights into the mechanisms of secretion in this organism, but the molecular events leading to intracellular protein trafficking and secretion in *Giardia* remain poorly understood or controversial (Lujan and Touz, 2003; Faso and Hehl, 2011).

The trophozoite is between 10 and 15 microns long and 5 microns wide, pear-shaped and cut along the longitudinal axis (pyriform morphology) (Figure 1A). It presents bilateral symmetry, has two diploid nuclei with nuclear membranes, four pairs of flagella and two media bodies consisting of microtubules (Adam, 2001), suggested to be the storage reservoir of microtubules of the cell (Piva and Benchimol, 2004). The trophozoite also possesses a complex cytoskeleton and endomembrane system including the endoplasmic reticulum (ER), which extends symmetrically throughout the cell body, and the PVs located underneath the plasma membrane. In the front half, on the ventral surface, is the adhesive disc which is used to bind to the intestinal epithelium of the host (Elmendorf *et al.*, 2003). The oval cyst size is between 9 and 12 microns and contains four nuclei, the axostyle (structure at the base of the flagella) and remnants of flagella (Figure 1B). It is characterized by a rigid outer wall glycoprotein, composed of proteins and carbohydrate (Jarroll *et al.*, 1989; Manning *et al.*, 1992; Gerwig *et al.*, 2002). The construction of the extracellular cyst wall (CW; Cyst Wall) is of paramount importance because it allows the parasite to persist in fresh water, survive even the action of disinfectants and resist stomach acid in its new host, and then start infection in the gut.

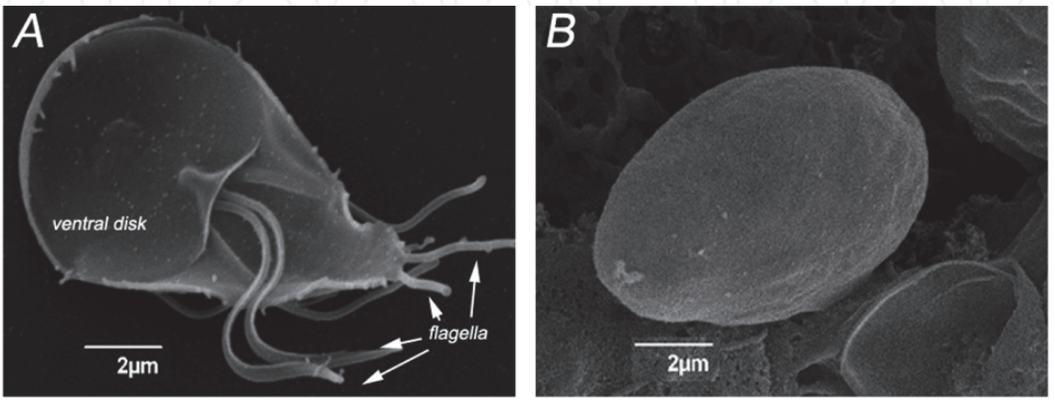

Figure 1. *Giardia lamblia* stages. (A) *Giardia* trophozoite, the ventral disk structure and the flagella are highlighted. (B) *Giardia* cyst.

3. Giardia secretory pathway

Eukaryotic cells have to deal with the fact that, after translation at the ribosomes, most proteins must be specifically targeted from the cytoplasm to the organelle in which they must function. As cellular components became more complex and abundant during evolution, subcellular compartmentalization developed into an essential feature to prevent the inappropriate meeting of certain intracellular components, as well as facilitating efficient ordered reactions (Munro, 2004). To maintain these compartments, cells have evolved mechanisms to ensure that specific proteins are delivered to specific organelles. In most eukaryotes, the Golgi complex serves as a major sorting point in the secretory pathway, selectively targeting proteins and lipids to different organelles (Gu *et al.*, 2001). *Giardia* possesses a distinctive endomembrane system involving the nuclear membrane, the ER, and lysosome-like PVs (Figure 2).

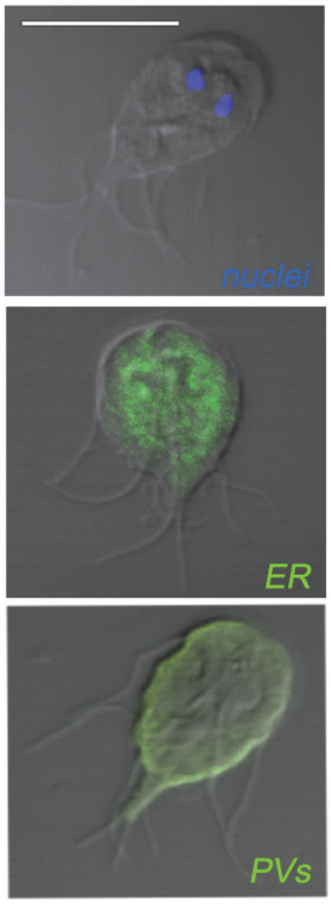

Figure 2. *Giardia* organelles. Confocal microscopy showing the nuclei, endoplasmic reticulum (ER) and PVs labeled with DAPI (4',6-diamidino-2-phenylindole), anti-BiP mAb, and anti-AP2 mAb, respectively. Bar, 10 μm.

On the other hand, it lacks other organelles characteristic of higher eukaryotes such as canonical endosomes, lysosomes, mitochondria, peroxisomes, and Golgi apparatus (reviewed by Lujan and Touz, 2003; Hehl and Marti, 2004). In most eukaryotic cells, the Golgi apparatus consists of a series of flattened cisternal membranes forming a stack

(Ladinsky *et al.*, 2002). The architecture of this organelle is remarkably conserved throughout eukaryotic evolution (Mellman and Simons, 1992); however, a typical Golgi complex with organized and parallel cisternae is not apparent in vegetative *Giardia* trophozoites. Several pieces of evidence suggest that *Giardia* trophozoites may possess organelle(s) in which typical Golgi functions take place, even though they do not have a Golgi-like appearance. The fact that both constitutive and regulated mechanisms for protein transport exist in *Giardia* is an example of Golgi functions, since the sorting and selection process generally occurs in the *trans*-Golgi network in more complex cells (Gu *et al.*, 2001). The constitutive pathway in *Giardia* is represented by continuous expression and trafficking to the plasma membrane of the transmembrane-anchored variant-specific surface proteins (VSPs) (Nash, 2002; Marti *et al.*, 2003a; Touz *et al.*, 2005). The regulated pathway takes place only during encystation and associates with the appearance of encystation-specific vesicles (ESVs), which transport cyst wall components to the plasma membrane of the encysting cell and release their content to the cell exterior during cyst wall formation (Reiner *et al.*, 1989; McCaffery and Gillin, 1994; Lujan *et al.*, 1995; Sun *et al.*, 2003). A selective pathway sorting proteins to the endosomal/lysosome membrane system has been recently demonstrated by our group, although *Giardia* do not possess distinctive endosomes or lysosomes (Touz *et al.*, 2003; Touz *et al.*, 2004; Rivero *et al.*, 2010; Rivero *et al.*, 2011).

Analyses of genes and proteins used for phylogenetic classification indicate that *Giardia* is in fact one of the earliest branching eukaryotes (Sogin *et al.*, 1989; Hashimoto *et al.*, 1998), but some of the particular cellular characteristics of this organism are probably a result of the secondary loss of complex cell structures, as a consequence of its parasitic life style, rather than the primitive simplicity supposed for early diverging protists (Dacks and Doolittle, 2002; Lujan and Touz, 2003).

4. The endosomal/lysosomal system of *Giardia*

Most eukaryotes have a system of endosomes and lysosomes that mediate the internalization, recycling, transport and breakdown of cellular and extracellular components and facilitate dissociation of receptors from their ligands. Early endosomes (EE) internalize endocytosed proteins to allow for their subsequent return to the cell membrane. Later, conversion of the EEs to late endosomes (LE) takes place, undergoing homotypic fusion reactions, growing in size, and acquiring more intraluminal vesicles. What follows is the fusion of an endosome with a lysosome and maturation of the subsequent endolysosome into lysosome, which constitutes a storage organelle for lysosomal hydrolases at acidic pH, and membrane components. Although this is a highly dynamic system, discrete compartments can be distinguished (Huotari and Helenius, 2011).

In contrast to most eukaryotes, *Giardia* has highly polarized vacuoles, located underneath the plasma membrane of the dorsal side, which combine some of the characteristics of endosomes and some of lysosomes (Lindmark, 1988; Lanfredi-Rangel *et al.*, 1998; Touz *et al.*, 2004). These PVs, distinguished by their localization, are about 150 nm in diameter with variable oval shapes and contain a core of low electron density (Figure 3). They are acidic, as

shown by the uptake of acridine orange and the lysosomal markers Lyso-sensor and Lyso-tracker (Lanfredi-Rangel *et al.*, 1998; Touz *et al.*, 2002b; Touz *et al.*, 2003). The first description of hydrolase activity in the PVs came from studies in which acid phosphatase activity was tested, showing a cytochemical localization in these vacuoles as well as in the ER and nuclear envelope cisternae (Feely and Dyer, 1987). The presence of hydrolase activities in the PVs was also proved for cysteine proteases and RNases, demonstrating their lysosomal characteristics (Lindmark, 1988; Ward *et al.*, 1997; Touz *et al.*, 2002b). In addition, their potential role in endocytosis was demonstrated by the uptake of exogenous ferritin and Lucifer yellow (Bockman and Winborn, 1968; Lanfredi-Rangel *et al.*, 1998). Pulse-chase experiments with horseradish peroxidase and fluorescent dextran showed an early and persistent labeling of the PVs, suggesting that there is no distinction between early and late endocytic vesicles in *Giardia*, in contrast to what occurs in higher eukaryotes (Lanfredi-Rangel *et al.*, 1998; Gaechter *et al.*, 2008).

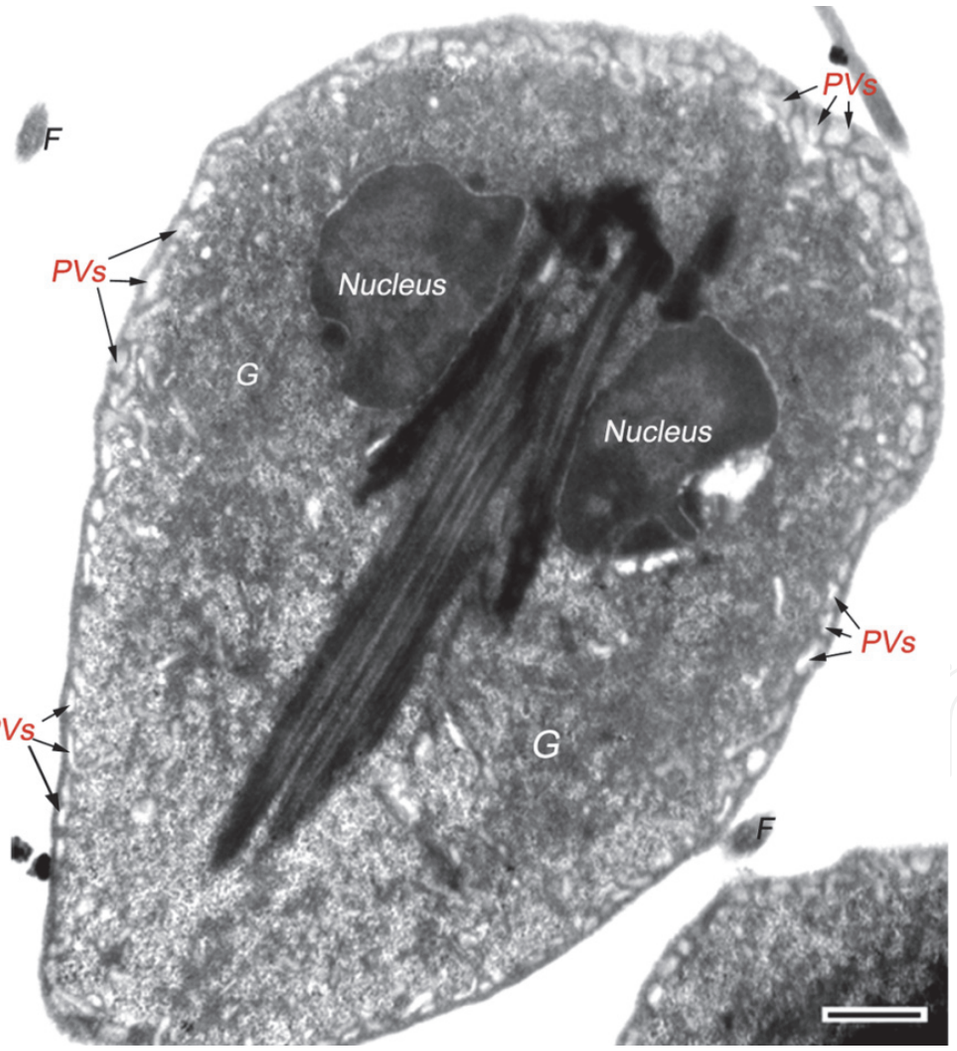

Figure 3. *Giardia* trophozoite ultrastructure. Electromicrograph of a growing *Giardia* trophozoite, showing the peripheral vacuoles (PVs) located underneath the plasma membrane (arrows). Nuclei are also denoted. G: electron-dense glycogen deposits. Bar, 0.5 μm. From Rivero *et al.*, 2010.

Analysis of fluid-phase endocytosis demonstrated that there is no lateral exchange of fluid phase markers between individual PVs (Gaechter et al., 2008). It was observed that, after internalization, some fluid-phase markers translocated rapidly to the ER or to an associated membrane compartment termed the tubulo-vesicular network (TVN) (Abodeely et al., 2009). Moreover, the presence of protease functions within the TVN, plus 3-D reconstruction and electron microscopy tomography of trophozoites stained for acid phosphatase and glucose-6-phosphatase, suggest that there might be a connection between some vesicles and profiles of the ER (Lanfredi-Rangel *et al.*, 1998; Abodeely *et al.*, 2009a). However, a recent work showed, by immunofluorescence and 3-D reconstruction, that the ER membranes are found throughout the cytoplasm, but do not permeate the space occupied by PVs (Faso and Hehl, 2011). Recently, it was shown that a mechanism of receptor-mediated endocytosis occurs in this organism, with specific molecules selectively directed to the PVs through a classical endocytic mechanism (Rivero *et al.*, 2010; Rivero *et al.*, 2011). These data suggest that uptake of soluble material into PVs is not selective at this step but is still capable of redirecting specific molecules to the TVN (Hernandez *et al.*, 2007; Abodeely *et al.*, 2009b). In terms of receptor-mediated endocytosis, movement between vesicles could be observed (Figure 4), which suggests, not only that *Giardia* possesses a refined and conserved mechanism of endocytosis, but also that the PVs population might not be as homogeneous as was thought but rather organized depending on their functions (Rivero & Touz, unpublished).

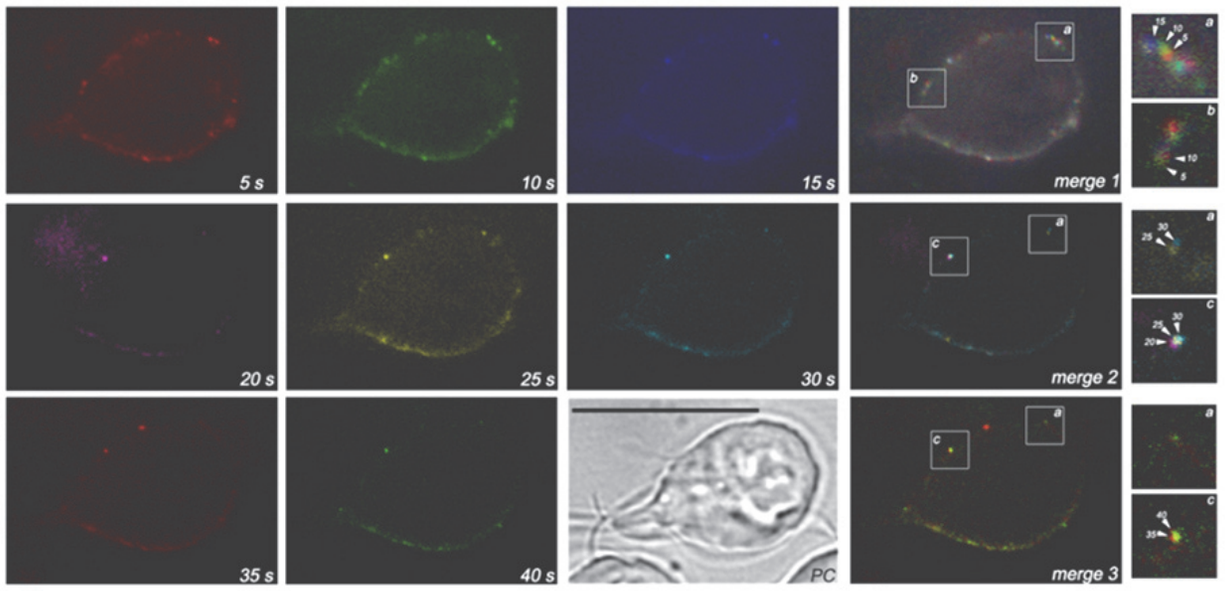

Figure 4. Epifluorescence microscopy shows the internalization and delivery of LDL to the PVs. Eight frames from 5 s to 40 s were artificially colored and combined (merge 1-3) to determine the movement of endocytosed BODIPY-LDL. The lateral movement of the LDL between the PVs in living cells is observed following the sequence red, green, blue (5 s, 10 s, 15 s) for merge 1; magenta, yellow, cyan (20 s, 25 s, 30 s) for merge 2; and red and green (35 s and 40 s) for merge 3. In the insertions a, b and c, movement of BODIPY-LDL is observed in detail. PC: phase contrast. Bars, 10 μm. From Rivero & Touz, unpublished.

During evolution, eukaryotic cells differentiated, adapting to their increasingly complex environment by acquiring new abilities for nutrient uptake, internalization of growth factors, and entry of pathogens, among others. A complex array of endosomal compartments are preserved as modules that are able to perform specific functions in modern eukaryotic cells. In the case of less-evolved eukaryotes, the variety and simplicity of these systems is only starting to become apparent. Thus, *Giardia* possesses the endosomal-lysosomal system concentrated in the PVs, which may represent an ancient organellar system that later subdivided into compartments as early and late endosomes and lysosomes.

5. Endosomal-lysosomal protein trafficking

5.1. Clathrin

Clathrin-mediated endocytosis (CME) regulates many cell physiological processes, such as the internalization of growth factors and receptors, entry of pathogens, and synaptic transmission. Within the endocytic network, clathrin functions as a central organizing platform for coated pit assembly and dissociation via its terminal domain. As isolated from coated vesicles, clathrin is a trimer of 190-kDa heavy chains, each with an associated 25-kDa light chain forming a spiderlike molecule, the 'triskelion' (Kirchhausen, 2000). The unusual geometry of the triskelion allows it to assemble into regular polyhedral structures, the 'clathrin coats', which eventually give rise to clathrin-coated vesicles (CCVs). The dense protein coat of the CCV and its bristle-like morphology were first described by Roth & Porter (1964), who noted the involvement of these vesicles in RME of yolk proteins in mosquito oocytes (Roth and Porter, 1964). The formation of CCVs occurs at the plasma membrane, *trans*-Golgi network and endosomes (Kirchhausen and Toyoda, 1993), and follows a sequence of coordinated steps, in which membrane invagination is coupled to growth of the clathrin lattice, leading to lattice closure and vesicle budding (Kirchhausen, 2000).

The *Giardia* genome encodes an ortholog of the clathrin heavy chain (CHC) (Morrison *et al.*, 2007), and has a molecular weight of about 200 kDa, with three C-terminal clathrin repeats and one N-terminal propeller, according to a protein family database (Finn *et al.*, 2008). Analysis of GlCHC expression showed that clathrin is expressed almost equally in both stages of the parasite and is located in close association with the PVs in trophozoites, and in the ESVs in immature cysts (Marti *et al.*, 2003a; Marti *et al.*, 2003b; Hehl and Marti, 2004; Gaechter *et al.*, 2008). On the basis of these observations, it was suggested that recruitment of clathrin to late ESVs could serve to disperse large ESVs into smaller transport vesicles in response to the secretion signal (see below). The identification of a clathrin light chain (CL) ortholog in the GDB has so far been unsuccessful, probably because the sequences of CLs are not uniformly conserved among species. The differential expression of the CLs (e.g. one from yeast, two mammalian tissues with the presence of isoforms in mammalian neurons) might be associated with the high degree of specialization involved in clathrin vesicle trafficking.

Several groups have presented evidence for a role of clathrin in endocytosis in *Giardia* (Touz *et al.*, 2004; Hernandez *et al.*, 2007; Gaechter *et al.*, 2008; Rivero *et al.*, 2010). However, neither typical membrane-associated clathrin lattices nor emerging clathrin-coated pits have been observed in this parasite. Instead, uncharacteristic coated pits were seen in close association with the PVs (Lanfredi-Rangel *et al.*, 1998), suggesting that a distinct arrangement of clathrin might occur in this parasite. It is then possible that, as was observed *in vitro* (Zhang *et al.*, 2007), clathrin may be organized in a hexagonal array, forming tubes instead of vesicles. Indeed, a different type of clathrin-coated transport carriers (TCs), consisting of larger tubular/vesicular structures having one or more clathrin-coated buds, have been identified (Polishchuk *et al.*, 2006). These TCs travel long distances from the juxtanuclear area of the cell until they fuse with peripheral endosomes. The function of the TCs might be to mediate long-range distribution of mannose 6-phosphate receptors (MPR) and their cargo hydrolases to the peripheral cytoplasm (Puertollano *et al.*, 2003). Similar to TCs in HeLa cells (Polishchuk *et al.*, 2006), the tubules might not break down into CCVs en route to PVs in *Giardia*. In mammalian cells, it has been shown that TCs contain mannose-phosphate receptors, clathrin, Golgi-localizing Gamma-ear containing ARF-binding proteins (GGAs), and/or adaptor protein 1 (AP-1), and it was suggested that these might be uncoated during the TC-endosome fusion or could become integrated into the endosome membrane (Polishchuk *et al.*, 2006). Possibly supporting this hypothesis, giardial clathrin and AP-1 were observed not only on ER-exist sites but also in PVs (Marti *et al.*, 2003a; Marti *et al.*, 2003b; Touz *et al.*, 2004; Gaechter *et al.*, 2008). As we said, the ER tubular-vesicular network apparently extends to and contacts the PVs in the periphery of the cell (Abodeely *et al.*, 2009b) but it was recently reported that no ER membranes invade the space occupied by PVs (Faso and Hehl, 2011). An explanation that reconciles these observations might be that at least some of the clathrin-dependent trafficking in *Giardia* involves tubular carriers that extend from the ER-exit sites to the peripheral cytoplasm until they meet with distally located PVs.

5.2. Adaptor proteins

The classic model for clathrin-dependent sorting comprises the participation of cargo receptors, adaptor heterotetramers and clathrin triskelia. Because clathrin has no affinity for biological membranes, its recruitment to membranes and capture of transmembrane cargo requires the action of clathrin-associated adaptor proteins (AP), which bind to clathrin through the amino-terminal domain of the CLH (Bonifacino and Traub, 2003). Among these adaptors are AP-1, AP-2 and AP-3, which comprise two large chains (one each of γ/α/δ and β1-3, respectively), one medium-sized chain (μ1-3), and one small chain (σ1-3) (Boehm and Bonifacino, 2002). These complexes are localized to different subcellular compartments, where they function in cargo selection (Boehm and Bonifacino, 2002). At least one of the large subunits in each AP complex (γ/α/δ) mediates binding to the target membrane. The other large subunit, β1-3, recruits clathrin through a 'clathrin-box' motif (Boehm and Bonifacino, 2001; Brodsky *et al.*, 2001). The μ1-3 subunits are involved in the recognition of tyrosine-based, YXXØ signals (where X represents any amino acid and Ø indicates a residue

with a bulky hydrophobic side chain), and combinations of ασ2, γσ1 and δσ3 recognize dileucine-based, [DE]XXXl[LI] signals (Collins *et al.*, 2002; Bonifacino and Traub, 2003). A fourth AP complex, AP-4, is thought to be a component of a non-clathrin coat and to recognize a different type of signal (Burgos *et al.*). Besides the putative GlCHC, orthologs of two large, one medium, and one small subunit of each AP-1 and AP-2 are present in the *Giardia* genome. The colocalization of AP-1 with lysosomal proteins, its interaction with the GlCHC, together with the observation that lysosomal protein trafficking is altered in μ1-depleted trophozoites, support the participation of this complex in the forward transport of proteins towards the PVs in *Giardia* (Touz *et al.*, 2004). AP-1 also plays a central role during parasite differentiation, since μ1 depletion impairs encystation (Touz *et al.*, 2004). On the other hand, AP-2 is localized to the PVs and plasma membrane in trophozoites and also neighboring the ESV in encysting cells (Rivero *et al.*, 2010). AP-2 participates in RME and is crucial in the internalization of lipoproteins (Rivero *et al.*, 2010). Although the β1–2 and μ2 mRNA transcripts change little during the completion of the cell cycle (Marti *et al.*, 2003b; Rivero *et al.*, 2010), the role of the corresponding AP complexes appears essential for the adaptation of the parasite. AP-1 is not critical for *Giardia* trophozoite survival and multiplication, but it is necessary for cyst formation, acting indirectly in this process by transporting a transmembrane protein to the PVs (Touz *et al.*, 2002b; Touz *et al.*, 2003; Touz *et al.*, 2004). In contrast, AP-2 is essential for *Giardia* growth and survival, being involved in the endocytosis of essential molecules (*e.g.*, exogenous lipids) (Rivero *et al.*, 2010) and in the fragmentation of ESVs into small transport vesicles containing cyst wall proteins during encystation (Rivero & Touz, unpublished). The fast secretion and deposition of cyst wall material has been reported to involve clathrin- and dynamin-dependent breakup of ESVs into small vesicles targeted for the plasma membrane (Hehl and Marti, 2004; Gaechter *et al.*, 2008). It is possible that this parasite requires the concerted action of clathrin and adaptors as well as accessory proteins at the time of cyst wall formation.

Taken together, these results support the hypothesis that *Giardia* possesses molecular mechanisms for lysosomal protein trafficking involving adaptor proteins similar to those of other eukaryotes. AP-1 and AP-2 appear to be the only two adaptors involved in lysosomal protein trafficking in *Giardia*, since there is no evidence of the participation of other adaptor proteins such as AP-3, AP-4, and monomeric adaptors (i.e., the GGAs). It has been suggested that the two prototypic *Giardia* AP complexes predict the point of separation of *Giardia* after the first coordinated round of gene duplications, resulting in an AP-3 and an AP-1/2/4 ancestor (Marti *et al.*, 2003b). Phylogenetic reconstruction from comparative genomics has shown that all four AP complexes were present in the Last Common Eukaryotic Ancestor (LCEA), as was the F-COP subcomplex (Boehm and Bonifacino, 2001). However, the GGAs, which also exhibit homology to the ear region of the AP-1 γ protein, are restricted to animal and fungal lineages (Field *et al.*, 2007). Therefore, individuality of the species lineage and secondary loss are common characteristics in the evolutionary history of the adaptins. Secondary losses of adaptors can be observed in *Drosophila melanogaster*, *Caeanorhabditis elegans*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe,* which lack the AP-4 complex (Field *et al.*, 2007). In addition, comparative genomic and phylogenetic

analyses of protozoan parasites have shown loss of the AP-3 complex in the species *Theileria*, *Cryptosporidium parvum*, and *Babesia bovis*, while *Trypanosome brucei* and *Leishmania major* lack AP-2 and AP-4, respectively (Nevin and Dacks, 2009). Thus, examination of the role of the reduced set of AP complexes in protozoa provides insight into the depths of our cellular history and highlights the importance of essential cell biology adaptations of the ancestral cellular organization.

5.3. Lysosomal proteins

Lysosomal integral membrane proteins (e.g., LAMP/LIMP family proteins) are transported to lysosomes by binding of their cytosolic motifs to AP complexes (Bonifacino and Lippincott-Schwartz, 2003). The carboxy-terminal β-sandwich domain of the μ subunits of AP-1, AP-2, and AP-3 binds directly to YXXØ-type sequences, while the γσ1, ασ2 and δσ3 hemicomplexes bind to the [DE]XXXL[LI] sequences (Janvier *et al.*, 2003; Doray *et al.*, 2007). Although lysosomal integral membrane glycoproteins have not been identified in *Giardia*, it was reported that a cysteine protease termed ESCP (encystation-specific cysteine protease) is transported to the PVs through a tyrosine-based motif. This enzyme is homologous to cathepsin C enzymes of higher eukaryotes and possesses a transmembrane domain and a YRPI motif within the cytoplasmic tail. ESCP localizes to the PVs in growing trophozoites and also to the plasma membrane in encysting cells (Touz *et al.*, 2003). Deletion of the YRPI motif or suppression of μ1 mislocalizes this protein to the plasma membrane or to the ER-exit sites, respectively (Touz *et al.*, 2003; Touz *et al.*, 2004).

Soluble acid hydrolases, on the other hand, are synthesized in the ER and transported to the Golgi complex, where their carbohydrate chains are modified by resident enzymes before delivery to lysosomes. In mammalian cells, the hydrolases are modified with mannose 6-phosphate residues that function as recognition signals for MPRs in late Golgi compartments. In yeast, the vacuolar hydrolases lack mannose 6-phosphate, and the sorting receptor is the product of the VPS10 (Vacuolar Protein Sorting 10) gene. In both cases, however, sorting signals present in the cytosolic tails of the receptors interact with clathrin adaptors and direct packaging of the hydrolase-receptor complexes within CCVs or clathrin-coated TCs (Ohno *et al.*, 1995; Ohno *et al.*, 1996). Recently, multi-ligand type-1 receptors Sortilin, SorCS1, SorCS2, SorCS3, and SorLA were discovered, containing an N-terminal Vps10p domain (Rezgaoui *et al.*, 2001; Hermey, 2009). These are transmembrane proteins that convey Golgi-endosome transport and bind a number of unrelated ligands.

In *Giardia*, high hydrolase activity in the PVs has been implicated in protein degradation during growth (Lujan and Touz, 2003), encystation (Touz *et al.*, 2002a; Touz *et al.*, 2003) and excystation (Slavin *et al.*, 2002). A family of three cysteine protease genes (CP1, CP2, and CP3) has been shown to encode members of the cathepsin B subgroup of the peptidase family C1 (Ward *et al.*, 1997), and soluble CP2 has been found in PVs and ER of trophozoites (Ward *et al.*, 1997; Abodeely *et al.*, 2009a). Also, AcPh activity has been examined cytochemically, revealing communication of the PVs with the ER (Feely and Dyer, 1987; Lanfredi-Rangel *et al.*, 1998; de Souza *et al.*, 2004). Unlike AcPh in other eukaryotes,

including protozoa, *Giardia* AcPh is a soluble protein that is transported from the ER-exit site to the PVs via AP-1 (Touz *et al.*, 2004). It is thus possible that a specific receptor, possessing a function similar to the MPR or Vps10p, is involved in the trafficking of soluble hydrolases toward the PVs. Recent studies have identified a type-I membrane protein that interacts with AcPh and contains an YQII (YXXØ-type) motif in its cytosolic tail (Rivero & Touz, unpublished). *In silico* analysis revealed that this protein (GDB: GL50803_28954) might be orthologous to the Vps10p receptor. Further biochemical studies on this putative receptor in both vegetative and encysting trophozoites and its participation in hydrolase delivery are necessary to elucidate the exact function of this protein.

Comparative analysis of lysosomal proteins present in *Giardia* and other cells reveals some intriguing differences. For instance, AcPh is soluble in *Giardia* but exists as a type-I membrane protein containing a YXXØ-type internalization sequence in cells as different as *Leishmania* and humans (Gottlieb and Dwyer, 1981; Waheed *et al.*, 1988; Shakarian *et al.*, 2002), with transport to the lysosome occurring through several cycles of plasma membrane internalization and recycling. In the lysosome of mammalian cells, the luminal domain of AcPh is processed and released in soluble form (Peters *et al.*, 1990). Moreover, while the AcPh tail interacts with AP-2 in these cells, the lysosomal traffic of *Giardia* AcPh depends on AP-1 (Touz *et al.*, 2004). Because much of the machinery involved in lysosomal trafficking is derived from a few protein families (where the various family members perform the same basic mechanistic function), the analysis of the similarities and differences between organisms might provide further insight into eukaryotic cell evolution.

As mentioned above, recent studies have shown that AP-2 participates in endocytosis of the *Giardia* Low-density lipoprotein Receptor-related Protein or LRP (Rivero *et al.*, 2010). *Giardia* LRP is a type-I membrane protein, which shares the substrate-N-terminal binding domain and a FXNPXY-type endocytic motif with human LRP1. This receptor localizes predominantly to the ER but is also found in the PVs and plasma membrane in *Giardia,* and internalizes both low density lipoproteins (LDL) and chylomicrons as shown by *in vitro* studies. The FXNPXY motif of LRP was shown to bind directly to the μ2 subunit of AP-2, with this interaction being necessary for its proper localization, processing, and function.

One common characteristic of LDLR family members such as the LRPs is that they have at least one copy of the FXNPXY-like sequence in their cytosolic tail, which serves as the signal for endocytosis or as a binding element for adaptor proteins involved in signal transduction (Harris-White and Frautschy, 2005). In other eukaryotes, FXNPXY signals are recognized by the adaptor proteins Disabled homolog 2 (Dab2) and Autosomal Recessive Hypercholesterolemia (ARH), which contain a phosphotyrosine-binding (PTB) domain (Traub, 2009). However, no PTB-containing proteins such as Dab2 or ARH are encoded in the *Giardia* genome, supporting the idea that AP-2 might be the key endocytic adaptor in this parasite. Indeed, it has been shown by surface plasmon resonance and photoaffinity labeling that the FXNPXY-like motif binds to μ2 purified from bovine-brain-coated vesicles (Boll et al., 2002). Interestingly, in spite of the strong interaction found between ARH and

LRP1 in an in vitro binding assay, the subcellular localization of LRP1 was not affected in the liver of ARH-deficient mice, whereas LDLR was found to be redistributed from intracellular localizations to the cell membrane (Jones et al., 2003). Thus, the importance of the availability of intracellular adaptor proteins might determine the specific cellular function of lipoprotein receptors. Since *Giardia* trophozoites do not have the capacity of *de novo* synthesis of cholesterol, its acquisition may depend on the internalization of chylomicrons from the host intestine by LRP. Moreover, because the trophozoites normally thrive in an environment where they never come in contact with LDL, it is possible that the binding of LDL to LRP represents a result of exaptation, thereby shifting the function of this protein allowing growth of parasites in culture medium.

5.4. Accessory proteins

5.4.1. Dynamin

Dynamins are large GTPases that belong to a protein superfamily, which are involved in many processes including clathrin-mediated endocytosis, clathrin-independent endocytosis, budding of transport vesicles, organelle division, cytokinesis and pathogen resistance (McNiven *et al.*, 2000; Ochoa *et al.*, 2000; Cao *et al.*, 2003; Krueger *et al.*, 2003). Substantial evidence indicates that dynamin oligomerization around the necks of endocytosing vesicles and subsequent dynamin-catalyzed GTP hydrolysis are responsible for membrane fission (Sweitzer and Hinshaw, 1998). Mammalian dynamins 1, 2 and 3 are the founder members of the dynamin family that, along with other large GTPases, possesses five identifiable domains: GTPase domain, middle domain, a lipid binding Pleckstrin-homology (PH) domain, GTPase Effector domain (GED) and C-terminal proline-arginine rich domain (PRD) (Hinshaw, 2000).

Recent studies indicate that *Giardia* possesses a single dynamin homolog (GlDRP, dynamin-related protein), with the predicted protein containing the N-terminal GTPase domain (33–219), the middle domain (230–523) and a C-terminal GED (628–719) (Marti *et al.*, 2003b). The giardial dynamin (GlDRP, dynamin-related protein), has a PRD of 70 amino acids (538–608) (Gaechter *et al.*, 2008). While the PRDs of dynamins are normally localized at the C-terminus, after the GED, in other eukaryotes, the PDR of the giardial dynamin is inserted between the middle domain and the GED. Interestingly, a typical PH domain that could mediate direct interaction with membrane lipids is missing.

GlDRP partially colocalizes with clathrin at the PVs and is necessary for endocytosis of plasma membrane proteins but not for fluid-phase endocytosis in *Giardia* trophozoites. Moreover, the expression of a mutant GlDRP, with reduced affinity for GTP and GDP, impaired endocytosis and resulted in enlarged PVs, indicative of blocked vesicular fission in these organelles. Also in these cells, GlDRP is detected at the ER, with only a minor proportion being present as a cytoplasmic pool. During encystations, however, both clathrin and GlDRP localize in part in the ESVs containing cyst wall material (Marti *et al.*, 2003b; Hehl and Marti, 2004; Gaechter *et al.*, 2008). Interestingly, matching the

function observed in depleted-μ2 encysting trophozoites (Rivero *et al.*, 2010), expression of a dominant-negative GlDRP affects the formation of small vesicles containing cyst wall proteins (CWPs) and blocks its exocytosis to form the cyst wall. Because close contact of the ESVs and PVs has been frequently reported (Marti *et al.*, 2003b; Touz *et al.*, 2003; Hehl and Marti, 2004; Gaechter *et al.*, 2008), exchange of material between these two structures may occur, with the PVs finally acting as sorting organelles, probably by delivering the CWPs to the plasma membrane and/or returning other proteins to the ER-exit site.

With the characterization of dynamic-like proteins from *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, yeast species, *Arabidopsis thaliana*, and *Giardia,* it may now be possible to reveal the many varied functions of the members of the dynamin superfamily in different organisms.

5.4.2. Snares and rabs

The basic steps underlying vesicle-mediated transport between the secretory and endocytic pathways are vesicle formation from a donor compartment, translocation of transport intermediates to a target organelle, tethering of transport intermediates with the target compartment, and, finally, the docking and fusion of vesicles with the membrane target. SNAREs (soluble N-ethylmaleimide-sensitive factor-attachment protein receptors) function in the final event of docking of vesicles with the target membrane, catalyzing the fusion of the opposing membranes. Compared to other organisms, *Giardia* has a relatively small number of SNARE proteins (Morrison *et al.*, 2007; Elias *et al.*, 2008). Seventeen putative SNAREs have been identified and partially characterized, with five representing Qa-SNAREs, five Qb-SNAREs, four Qc-SNAREs and three R-SNAREs. Although some of these SNAREs localize to the PV area, their function has not been investigated and has rather been inferred from the participation of their orthologs in other cells (Elias *et al.*, 2008). For example, different gSNAREs are present in the PVs/plasma membrane area, Qa1, gQa3, gQa5, gQb2, gQb4, gQb5, and gR3, with the presence of three different gQa SNAREs suggested to be involved in distinct pathways such as exocytosis, endocytosis, and PV-PV fusion (Elias *et al.*, 2008).

The Rab family GTPases regulates many steps of membrane traffic, including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion. So far, over 70 members of the Rab family have been identified in mammalian cells, and each seems to have a characteristic intracellular localization and function. For instance, Rab5 plays roles in endocytosis, early endosome fusion, and caveolar vesicle targeting to early endosomes (Barbieri *et al.*, 2000; Pelkmans *et al.*, 2004), while Rab11 mediates slow endocytic recycling through recycling endosomes, and Rab4 mediates fast endocytic recycling directly from early endosomes (van der Sluijs *et al.*, 1992; Ullrich *et al.*, 1996). There is also a coordinated action of Rab5 and Rab7 in the sorting of cargo receptors by the retromer complex (Rojas *et al.*, 2008).

Similar to the SNAREs, there is a remarkable reduction in the members of this family, with only seven Rab proteins being present in the *Giardia* genome (Morrison *et al.*, 2007). The giardial Rab11 has been localized to the PVs and cytoplasm but relocalizes to the ESVs during encystation (Morrison *et al.*, 2007; Abodeely *et al.*, 2009b). A *Giardia* Rab1 that localizes to ER-exit sites and PVs is also associated with the ESVs during encystation (Langford *et al.*, 2002).

The question of whether Rabs and SNAREs participate in delivery of lysosomal proteins to the PVs remains unanswered and could be addressed in greater detail by functional analysis. Since each member of the Rab and SNARE family retains analogous biological functions in almost all the species analyzed, it will be interesting to determine whether, in *Giardia*, selective pressures might have been operating on distinguishing aspects of the lysosomal trafficking pathway, adapting the specificities of these proteins to accomplish their function.

6. Conclusion

Over the past few years, our understanding of the cell biological processes underlying the function of the PVs during *Giardia* growth and differentiation has advanced considerably. This is largely because of the complete sequencing and annotation of the *Giardia* genome, the development of transfection systems, highly sophisticated morphological analyses, and the identification of new parasite proteins that participate in endosomal/lysosomal trafficking. Endosomal/lysosomal trafficking pathways exhibit significant complexity and diversity in terms of morphology, function, and mechanisms among different organisms and cell types. As shown by several studies, part of the *Giardia* transport machinery is fairly well conserved. The existence in this organism of the GlCHC and dynamin, the presence of tetrameric adaptor proteins, and endosomal-lysosomal sorting motifs within cargo proteins, support an early acquisition of genes necessary for endosomal/lysosomal trafficking during eukaryotic evolution. Nevertheless, this parasite has experienced considerable diversification (Figure 5). The constraints of living under parasitic conditions have probably been the major driver for the reductive evolution of lysosome/endosome and Golgi compartments to maintain only those components that are essential for specific compartmentalization needs. For example, this parasite possesses only two of the four AP complexes, AP-1 and AP-2, which are involved in sorting signal recognition. No monomeric adaptor proteins have been identified so far. Also, reduced members of the Rab family are present and, although Rab11 has been associated with the PVs, further analysis will be necessary to assess Rab participation in membrane tethering and fusion to preserve the PVs identity. Similarly, investigation on the SNARE proteins closely associated with PVs will shed light on the mechanism of vesicle-vacuole fusion. Despite the progress in the field, it is clear that our molecular understanding of this complex situation remains far from complete. In particular, the current view that this parasite needs a reduced set of organelles and machinery to control nutrient uptake as well as

degradation of intracellular proteins and endocytosed macromolecules is without precedent and raises several conceptual questions, some of which have been addressed in this chapter. In the last decade, PVs are emerging as sorting stations were molecules can be sorted and selected for plasma membrane or ER delivery. The future challenge will be to complete the pieces of this important puzzle to understand and unravel functions of the PVs and to throw light on the fundamental organizational principles of endosome/lysosome biogenesis in all eukaryotes.

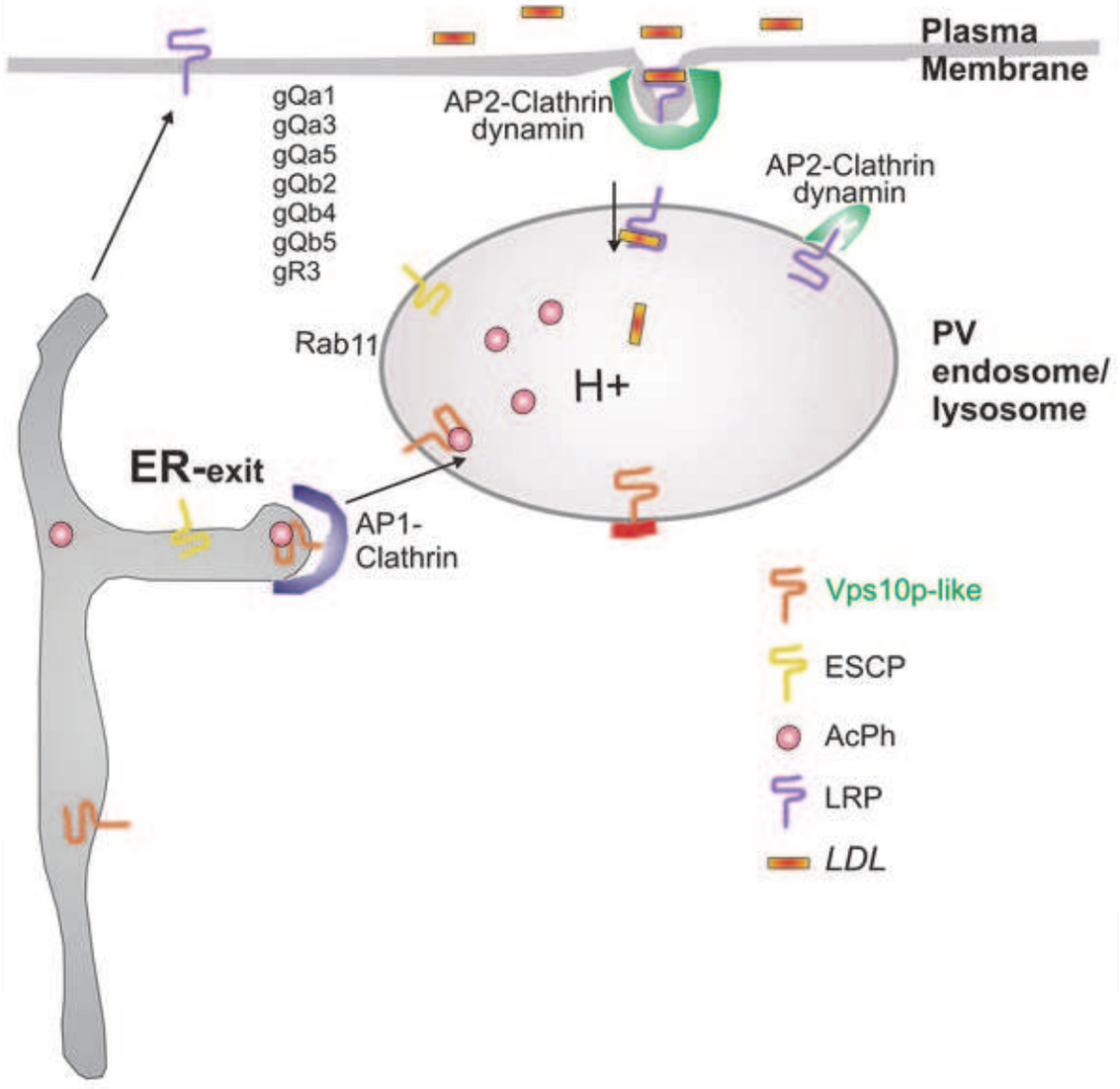

Figure 5. Schematic representation of lysosomal protein trafficking in growing *Giardia* trophozoites. From the ER-exit sorting site, the membrane protease ESCP is directed to the lysosome-like PVs in AP-1 and clathrin-coated vesicles. By the same pathway, the hydrolase AcPh is probably associated with the membrane receptor Vps10p and AP-1. AP-2 is involved in LDL/LRP endocytosis and PV delivery. The cytosolic proteins, clathrin and dynamin, are localized in the PVs. Rab11, and the SNAREs Qa1, gQa3, gQa5, gQb2, gQb4, gQb5, and gR3 may participate in vesicle trafficking to and/or from the PVs. H+ represents the acidic pH of the PV lumen. Unconfirmed protein participation is depicted in green. Modified from (Touz, 2011).

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