Roles of Phosphoinositides and Their binding Proteins in Parasitic Protozoa

Lenka Cernikova,1 Carmen Faso,1,2 and Adrian B. Hehl1,*

Phosphoinositides (or phosphatidylinositol phosphates, PIPs) are low-abundance membrane phospholipids that act, in conjunction with their binding partners, as important constitutive signals defining biochemical organelle identity as well as membrane trafficking and signal transduction at eukaryotic cellular membranes. In this review, we present roles for PIP residues and PIP-binding proteins in endocytosis and autophagy in protist parasites such as Trypanosoma brucei, Toxoplasma gondii, Plasmodium falciparum, Entamoeba histolytica, and Giardia lamblia. Molecular parasitologists with an interest in comparative cell and molecular biology of membrane trafficking in protist lineages beyond the phylum Apicomplexa, along with cell and molecular biologists generally interested in the diversification of membrane trafficking in eukaryotes, will hopefully find this review to be a useful resource.

Phosphatidylinositol Derivatives and Binding Domains in Eukaryotes

Phosphoinositides (PIPs) are phosphorylated derivatives of phosphatidylinositols (PtdIns) found throughout Eukarya as crucial components of cell membranes. Although their synthesis is tightly regulated, and they account for only 1% of the total lipid pool [1], they are involved in all major signal transduction pathways, cytoskeleton regulation, development, as well as in the regulation of intracellular membrane traffic for endocytosis and autophagy [2,3]. PtdIns are synthesized in the endoplasmic reticulum (ER) and delivered to distal endomembranes either by vesicular transport or by cytosolic PtdIns transfer proteins [4]. The inositol headgroup can be reversibly phosphorylated at positions 3, 4, and 5, giving rise to seven different PtdIn derivatives that are accordingly divided into three PIP groups: monophosphorylated [PI(3)P, PI(4)P, and PI(5)P], bis-phosphorylated [PI(3,4)P2, PI(3,5)P2, and PI(4,5)P2], and tris-phosphorylated [PI(3,4,5)P3]. PIP species interconversion is catalyzed by specific phosphatidylinositol (PI) kinases and phosphatases (Figure 1A) [2,3,5], with each lipid species displaying a distinct subcellular distribution (Figure 1B) (pathways of PIP interconversion in mammalian cells and related PI kinases and phosphatases are summarized in Box 1).

The significance of PIPs as important spatiotemporally controlled membrane markers is underscored by the identification of protein effectors that recognize and bind individual PIPs. Negatively charged phosphates on the inositol ring interact electrostatically with a protein-binding module to generate a low-affinity bond which is further strengthened by the interaction of adjacent hydrophobic amino acids with the membrane bilayer [4]. Furthermore, higher affinity, and thus a more stable interaction, can be produced by engaging additional PIP-binding sites in the membrane [2,5]. Depending on the configuration of the PIP–protein interaction, more than 11 different globular PIP-binding domains, divided into two broad classes, can be identified: (i) high-specificity domains involving stereospecific recognition of membrane components such as phospholipids or phosphatidic acid, and (ii) a group of domains with low specificity that bind membranes based on general physical properties such as charge, amphiphilicity, and curvature [2,5]. A full list of PIP-binding protein domains, often named according to the proteins they were first identified in, consists of the AP180 N terminal homology (ANTH), Bin, amphiphysin and Rvs (BAR), conserved region-2 of protein kinase C (C2), epsin N terminal homology (ENTH), 4.1, Erin, radian, moesin (FERM), Fab1, YOTB, Vac1, and EEA1 (FYVE), Golgi phosphoprotein 3 (GOLPH3), postsynaptic density 95, disk large, zonula occludens (PDZ), Pleckstrin homology (PH), b-propellers that bind Pts (PROPPINs), phosphotyrosine binding (PTB), Phox homology (PX), and Tubby modules [2,5]. The list can be extended further by plant homeodomain zinc finger (PHD) with binding specificity for PI(5)P [6]. PIP-binding specificities for each module are summarized in Figure 1A.

Highlights

PIPs are lipid species with multiple roles in subcellular trafficking, signaling, and cell growth.

Parasitic protists employ a variety of PIPs and PIP-binding proteins in both canonical and noncanonical functions.

PIP-binding proteins involved in autophagy in model organisms have been co-opted to roles in organelle maintenance and partitioning in several parasitic protist lineages.
Protozoan parasites possess restricted repertoires of PIP-binding proteins associated with reduced or modified pathways often pertaining to atypical endomembrane compartments. Adaptation to narrow biological niches resulted in either innovations in existing organelles – apicoplast (see Glossary), vacuolar compartment, food vacuole (FV), peripheral vacuole (PV), and glycosome – and/or likely repurposing of endosomal organelles in particular (rhoptry, microneme, and dense granule). For these reasons, protist parasites have often served as model organisms to investigate evolutionary origins and minimal essential components of vesicle trafficking/membrane transport in extant eukaryotes as well as in a hypothetical model for the last eukaryotic common ancestor (LECA).

To date, PIP synthesis and related functions in membrane trafficking pathways in parasitic protists have been mostly investigated in representatives of the phylum Apicomplexa – specifically in T. gondii and P. falciparum [7]. In the case of species such as E. histolytica, T. brucei and G. lamblia, we have only limited information. In E. histolytica, PI(4,5)P2 is explicitly compartmentalized to lipid rafts and the actin-rich area of the polarized cell called the uroid [8]. PI(4,5)P2 plays a well-documented role in endocytosis, and was shown to be enriched in the cytostome of members of the Apicomplexa [9], in the flagellar pocket of T. brucei [10], and at endosome-like PVs in G. lamblia [11]. In Giardia, PI(3)P is also enriched at PVs and marks the interface between PVs and the plasma membrane (PM) [11], whereas PI(3)P localization in Plasmodium is restricted to the FV, the apicoplast, and lysosomal/phagosomal vesicles [12–14]. PI(3)P in E. histolytica is concentrated...
on membranes of phagocytic cups and nascent phagosomes [15] and has a role in membrane trafficking of internal vesicles and vacuoles [16]. PI(3,5)P₂ and pathways for its synthesis were identified in T. gondii [17] but not in P. falciparum [9]. Furthermore, PI(4)P in P. falciparum decorates the Golgi complex (GC), the PM, and vesicles localized close to the trans-Golgi network (TGN), whereas in T. gondii the exact distribution of this PIP residue is unknown, although biochemical data point to its presence [13]. In contrast to P. falciparum, PI(4)P in Giardia trophozoites is diffused across the cell [11,18]. PI(5)P, to date experimentally detected only in members of the Apicomplexa, shows unique localization at the transitional ER of Plasmodium merozoites, and otherwise at the nucleus and the PM [9]. Strikingly, PI(3,4,5)P₃ in E. histolytica is involved in an elaborate signaling cascade at the interface of phagocytosis and trogocytosis [19] and localizes to motile pseudopods and phagocytic organelles, and a phagocytic cup [20]. Similar to E. histolytica, PI(3,4,5)P₃ distribution in G. lamblia involves more than one location, including Pvs and a diffuse cytosolic distribution [11,18]. In P. falciparum schizonts and Plasmodium berghei ookinetes, the presence of PI(3,4,5)P₃ was detected by both ³²P labelling followed by chromatography, and by mass spectrometry, although there are no PI(3,4,5)P₃ subcellular localization data yet available [21].

PIP-Binding Proteins and Their Roles in Parasitic Protozoa

Clathrin-mediated endocytosis (CME) and subsequent endosomal trafficking is a fundamental cellular process well characterized in all model and many nonmodel eukaryotes. This process relies heavily on PIPS and their corresponding binding protein partners, as summarized in Box 2 and shown in a simplified model for CME in mammalian cells in Figure 2A. A variety of novel endocytic machineries mediate nutrient uptake as a common requirement for all parasitic protists. Although most investigated species present a certain degree of conservation of core effector proteins, CME in parasitic protists often employs lineage-specific proteins or show evidence of repurposing of otherwise established protein functions. For example, E. histolytica possesses clathrin- and receptor-dependent machinery for holo-transferrin (Tf) fluid-phase uptake [22,23]. A gene coding for a canonical ENTH domain-containing protein (hereafter named EhENTH; Table S1 in the supplemental information online), was found in the Entamoeba genome, and its binding affinities for PI(4,5)P₂ and PI(3,4,5)P₃ were demonstrated in silico using pull-down assays [19].

**Glossary**

**Apicoplast**: a vestigial, non-photosynthetic and pigment-free plastid found in the phylum Apicomplexa.

**Apoptosis**: a predefined program for cell suicide where the cell actively destroys itself.

**Cytostome**: a cellular organelle found in some protozoan lineages involved in uptake processes such as phagocytosis or endocytosis.

**ESCRT**: Endosomal Sorting Complex Required for Transport types I, II, and III are widely-conserved machineries required for multivesicular-body formation and sorting pathways.

**Flagellar pocket**: a specialized plasma membrane invagination surrounding the base of the flagellum in some members of the Trypanosomatidae.

**Food vacuole (FV)**: also known as a digestive vacuole, found in parasites which cause malaria.

**Last eukaryotic common ancestor (LECA)**: a progenitor of all extant eukaryotes derived from prokaryotes by developing an endomembrane system about 800–1500 million years ago.

**Lineage-specific proteins**: part of an emerging paradigm where a conserved protein-core is supplemented with a secondary ‘shell’ represented by lineage-specific proteins. The ‘shell’ frequently retains common architecture features or motifs but is specific to a particular lineage.

**Lipid raft**: a cholesterol-, glycosphingolipid- and protein-receptor-rich domain found on the cell’s surface as a part of the plasma membrane.

**Merozoite**: a cell that develops from a schizont during sexual reproduction in members of the Apicomplexa.

**Microneme**: a specialized secretory organelle connected to the extreme apical pole in members of the Apicomplexa.

**ookinete**: motile zygote of Plasmodium species that penetrates the mosquito stomach to form an oocyst under the outer gut lining.

**Peripheral vacuoles (PVs)**: lysosomal/endosomal vacuoles localized underneath the plasma membrane in Giardia lamblia.

**Phagocytosis**: the process by which a cell membrane engulfs a foreign large object as, for
Polarization of endocytosis and exocytosis to the flagellar pocket is a hallmark of the Trypanosoma genus. A number of conserved T. brucei core CME-related proteins have been found and characterized, including clathrin heavy chain (CHC), clathrin light chain (CLC), adaptor protein complex 1 (AP1) or auxilin (Table S1). However, some proteins were also lost, such as adaptor protein complex 2 (AP2) [10,24,25]. Furthermore, another CME hallmark, PIP(4,5)P₂, was shown to be enriched at the cytosolic face of the T. brucei flagellar pocket via activity of TbPIPKA, a PIP(4,5)P₂ kinase [26]. The T. brucei CME-related proteins TbEpsinR and TbCALM (Table S1), carrying ENTH and ANTH domains, respectively, were described as ancient PIP-binding proteins with distinct and vital roles in AP2-independent endocytosis [10]. The high level of sequence and domain architecture conservation compared with opisthokont orthologs is suggestive of conserved functions in PIP binding [10]. Both TbEpsinR and TbCALM are predicted to link the flagellar pocket via PIPs, in particular PIP(4,5)P₂, to clathrin-coated vesicle (CCV) formation. Furthermore, TbCALM appears to also be important for proper duplication and segregation of the T. brucei lysosome [10]. As a counterpart to the core, ‘shell’ proteins TbCAP80 and TbCAP141 are examples of lineage-specific proteins carrying N terminal lipid-interacting domains and disordered C termini with predicted clathrin-binding sites, resembling the architecture of ANTH and ENTH proteins. TbCAP80 and TbCAP141 control not only CME, but also the architecture and organization of the broader endomembrane system in T. brucei (Figure 2B; Table S1) [27]. Furthermore, two genes encoding BAR domains were identified in the genome of T. brucei and named TbFlabarin and TbFlabarin-L [28] (Table S1). However, these nonessential proteins are not involved in regulating endocytic vesicle fission but rather show dual association with the flagellar membrane and components of the paraflagellar rod. Given that T. brucei has been used as a tractable experimental cell biology model for trypanosomes, almost all data regarding membrane trafficking relate to this species. However, given the diversity among the order Trypanosomatida, it is likely that endocytic adaptations may be genus- or species-specific. Indeed, although T. brucei lacks AP2, Trypanosoma cruzi possesses a complete set of AP1-4 complexes; however, all four subunits of the AP5 complex are missing. In contrast, the closely related genus Leishmania lacks genes coding for AP4 subunits [29].

**Box 2. Endocytosis – in General**

Clathrin-mediated endocytosis (CME) is probably the best described process for the selective uptake of cargo into eukaryotic cells via cell-surface receptors at the PM and into the cytoplasm (Figure 2A). PIPs, their binding partners, and several other proteins, collaborate to spatially organize the complex sequence of events required for CME. The organization and dynamics of each component allow for grouping of endocytic proteins into functional modules. Assembly of the first module, the clathrin lattice, occurs during initiation on the inner leaflet of the PM where endocytic proteins are recruited from the cytosolic pool. The initiation complex comprises the clathrin-AP2 complex and monomeric adaptors, such as CALM/AP180 and epsin, that bind to the PM via interaction with PIP(4,5)P₂. Simultaneously, membrane curvature is induced by FCHO1/2, part of the so-called pioneer module, comprising the scaffold proteins EPS15, EPS15R, and intersectins 1 and 2. While all the components cluster and interact, clathrin triskelia are recruited to form the clathrin lattice. Other PIP-binding endocytic proteins, such as SNX9, contribute to increased membrane curvature by binding to PIP(4,5)P₂ via its FYVE domain. Auxilin exerts a similar effect due to its specificity for PIP(3)P and PIP(3,4)P₂. Progressive membrane deformation forces the relatively flat PM first into a clathrin-coated pit and then into a cargo-loaded vesicle of ~100 nm. Subsequently, actin nucleation contributes to membrane bending during cargo-loaded vesicle formation while other regulatory components, such as myosin motor and dynamin, are recruited. Dynamin cooperates with BAR domain proteins, such as endophilins and amphiphysins, to mediate membrane constriction and scission. Dynamins in general carry either a PM domain or other PIP-binding modules/residues important for the interaction with the PM. Auxilin and ATPase Hsc70 are active during the last phase of uncoating and recycling of components. Uncoated vesicles fuse with early endosomes, where their contents are delivered and sorted for transport to lysosomes or, for some receptor types, recycled back to the PM [82–84].

**Lineage-Specific Innovations among Trypanosomatids**

example, bacteria. It gives rise to compartments called phagosomes where cargo is digested and recycled. Rhopty: a specialized secretory organelle connected to the extreme apical pole in members of the Apicomplexa. Schizont: a cell that develops from a trophozoite during asexual reproduction in members of the Apicomplexa. Trogocytosis: initially described in lymphocytes as a process whereby B, T, and natural killer (NK) cells, conjugated to antigen-presenting cells, extract surface molecules from them to expose on their own surface. Trogocytosis may have first appeared in very primitive organisms as a way for specialized cell types to feed off other cells. It has now been also documented in E. histolytica and Trichomonas vaginalis, both protozoa. Uroid: a well-defined ‘rear’ zone of membrane accumulation that contributes, together with a ‘front’ called a pseudopod, to cell motility.
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PIPs and Endocytosis in the Phylum Apicomplexa

Recent studies show that clathrin in members of the Apicomplexa seems to have a dual function in vesicle formation at the surface of the TGN as well as in rhoptries, and microneme, biogenesis [30]. An ENTH epsin ortholog was identified in some Plasmodium species and in Cryptosporidium parvum [31] but has not yet been functionally characterized. In T. gondii, the role of canonical CME at the PM is still controversial as well as the exact function of TgAP2, since no CCVs have been detected [32]. New insights in TgCHC-mediated trafficking at the TGN were provided by the investigation of the TgAP1-interactome which includes a unique ENTH-carrying protein named TgEpsL [33] (Table S1). Similar to Glespin in G. lamblia [34], no interaction with TgAP2 has been detected. The only conserved epsin-related region in TgEpsL is the ENTH domain with binding affinity for P74P52 predicted by in silico analysis [31]. It has been postulated that T. gondii most likely functionally repurposed evolutionarily conserved regulators of the endosomal system to the secretory pathway to form species-specific secretory organelles such as rhoptries and micronemes [35]. In this scenario, TgAP1 likely functions as a heterotetrameric complex regulating epsin-mediated vesicular transport of parasite proteins (Figure 2C) [33]. Interestingly, inhibition of endocytosis was very recently shown to block retrograde flow and parasite motility [32] in T. gondii.

Endocytosis in Plasmodium trophozoites involves formation of a cup-like invagination called the cytostome, where lipid content is enriched in PI(4,5)P2 [9], to channel hemoglobin transport across the PM, via transient endocytic vesicles, to the FV [36]. Despite its apparent structural divergence, Plasmodium endocytosis is, to some extent, reminiscent of classical CME. PIAP2 localizes to the PM as well as to small vesicles close to the PM and the cytostome [37], suggesting that PIAP2 may participate in the endocytic trafficking of hemoglobin. However, no specific PIP-binding protein has yet been identified [37]. Interestingly, the dynamin-like C terminal EPS15 homology domain containing protein, PIEHD (Table S1), was detected in the PIAP2 interactome [37]. Both PIEHD and PIAP2 were independently shown to associate with PIRaB5 on the endocytic vesicles (Figure 2D) [37,38], and PIEHD also labels vesicles which originate from the PM [38]. Furthermore, PIEHD was also shown to bind Pl(3,4,5)P3, Pl(3,4)P2, and Pl(4)P. Lipid synthesis is most likely controlled by Pl(3)kinase PAP5S45 [7,39] (Table S1), which is essential for host cell cytosol uptake via endosomal-like compartments. The same study also suggested that PAP5S45 is involved in fusion of these Pl(3)P-labelled vesicles with the food vacuole [40]. Surprisingly, and despite the presence of PIAP2, clathrin is not involved in these pathways. Unlike PIAP2, and similar to TgAP1, preliminary data from P. falciparum point to the interaction of PICH and PIAP1 at the interface of TGN and rhoptry organelles (Figure 2D; Table S1) [37,41].

PX Domain Proteins and Endocytosis in Giardia

The endocytic machinery of Giardia is a unique example of reductive evolution characterized by the loss of clathrin-coated vesicles and the emergence of PVs as novel endocytic organelles [42].
contrast to other parasitic protists, such as members of the genus Trypanosoma and the phylum Apicomplexa, the Giardia genome codes for several conserved CME components such as GICHC, hypothetical GICLC, GI3DR, and GIAP2 (Table S1), all localized in close proximity to PVs [42]. The status for Glep4 (Table S1), G. Jambila’s single ENTH-domain protein, remains controversial since it was associated with GICHC at PVs [18] and with the ventral disc as a CME-unrelated structural and attachment component [34]. A family of six proteins carrying C terminal PX domains, named GIPX1–6 (Table S1), emerged as lineage-specific proteins involved in Giardia endocytosis [42,43] (Figure 2E). Similar to TbCAP80 and TbCAP141 [27], Giardia GIPX1 and GIPX2 maintain disordered N termini with an abundance of predicted clathrin- and AP2-binding motifs. GIPX1 and GIPX2 exhibit binding preferences mainly for Pi(3)P and Pi(4,5)P2, respectively [44]. A FYVE domain-carrying protein named GIFYVE (Table S1) was localized in close proximity to PVs, and its role in PV-mediated fluid-phase uptake from the extracellular environment was confirmed experimentally (Figure 2E) [11].

**Autophagy**

Autophagy is a highly conserved intracellular degradation pathway (Box 3) where the autophagosome origin is tightly linked to Pi(3)P synthesis at the phagopore assembly site (PAS) where autophagosomal vesicles emerge [45]. Several AuTophaGy (ATG)-related proteins are recruited to autophagosomes in mammalian or yeast cells (Figure 3A). Research on parasitic protists provides evidence for parasite exploitation of host autophagy mechanisms for parasite survival [46]. However, most ATG proteins remain unidentified in unicellular parasites, suggesting either a secondary loss of autophagy or reflecting early divergence prior to the emergence of autophagy [47–50]. Nevertheless, autophagy-like cell death phenomena have been reported following nutrient starvation, ER stress, and drug treatment in T. brucei, Trypanosoma cruzi, Leishmania donovani, T. gondii, and P. falciparum [51,52]. Autophagy-like processes in these parasites appear to be tightly associated with organelle turnover, nutrient utilization, and metabolism [53,54]. Proteins ULK1/ATG1, ATG5, and ATG7A are essential to the initiation of starvation-induced autophagy [55]. However, a recent study of parasitic protist genomes reported on the absence of the activity domain for homologs of ULK1/ATG1, ATG5, and ATG7 [55]. Therefore, it is controversial whether these parasites initiate

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**Box 3. Autophagy – in General**

To selectively and nonselectively target cytoplasmic components and entire organelles to lysosome(s) or vacuolar degradation, eukaryotes have evolved an ancient mechanism called autophagy (for a cartoon visualization, see Figure 3A) [85,86]. Autophagy plays roles in: (i) survival under starvation, (ii) removal of damaged, abnormal, or redundant cellular constituents, and (iii) induction of apoptosis, in case of excessive autophagy [87]. Upon an autophagic stimulus, a serine/threonine kinase (ATG1/ULK1) complex initiates the process by phosphorylating autophagy machinery components which directs it to the ER membrane together with Atg9/ATG9 vesicles and the PI3K complex I. The latter plays a critical role during initiation when it synthesizes PI(3)P from PtdIns enriched in ER membranes [45]. Subsequently, assembly of the ATG complex leads to the formation of a phagophore assembly site (PAS) where membranes of different origins, for example, the Golgi complex, recycling late endosomes and PM [88], contribute to phagophore nucleation. Autophagosomal biogenesis and maturation is dependent on subsequent recruitment of two ubiquitin-like conjugation systems and the presence of PiP4s, in particular PI(3)P and PI(3,5)P2, which contribute to recruiting distinct proteins [89]. Once maturation is complete, PI(3)P turnover accompanies autophagosomal detachment from ER membranes in conjunction with Atg4/ATG4 delipidation and termination of Atg1/ULK1 activity. Mature autophagosomes then fuse with endosomes and lysosomes to become autolysosomes. Cargo is degraded by resident lysosomal hydrolases, and metabolites are eventually recycled as a source of energy or for building blocks. Since the entire process is dependent on correct PiP4 synthesis, several PiP-binding protein families are linked to autophagy. The most eminent is a family of PROPPINs that plays a role during the recruitment of ubiquitin-conjugating systems, in particular the Atg2 system to the PAS. In humans, four component proteins were identified (WIP1–4) and three in yeasts (Atg18, Atg21, and Hsv2) [90–92]. Atg18 binds PI(3)P at the PAS, where it mediates retrieval of Atg9 from autophagosomes together with Atg2. Atg21 was shown to be essential for the selective autophagy route known as the Cvt pathway or microautophagy. Finally, Hsv2 affects partially piecemeal microautophagy of the nucleus. For further reading on PiP-binding proteins not included in the autophagy process, the authors recommend the review by Lystad and Simonsen [93].
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(A) Mammalian/Yeast cell

Stress

P(3)P
P(4)P
P(5)P
P(3,5)P2
P1P
Rab7
WIP1/Apg18
LC3 complex/Apg8
Cargo
Proteases

1 μm

PM
Receptor recycling
endosome

Membrane sources

Endoplasmic reticulum

Nucleus

Atg9 vesicles

Atg1/ULK1 complex

PI3KC3 complex

Degradation or recycling

Lysosome

Cytoplasm

Extracellular space

Cytosol

Lewis acid

Phagophore

Autophagosome

Omegasome

Initiation
Expansion/sealing
Maturation
Fusion

(B) P. falciparum

Canonical catabolic autophagy

0.1 μm

RBC

(C) T. gondii

Non-catabolic atypical function

1 μm

PAS

AP

VAC

EnV

FV

(D) P. falciparum

T. gondii

Growth division

Centrosomes

Cytokinesis

PI3KC3

TgATG8

TgProp1

TgProp2

TgPI3K

TRENDS IN PARASITOLOGY

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(See figure legend at the bottom of the next page.)
autophagy via this route at all. Alternative regulatory mechanisms for this phenomenon await full characterization.

**Autophagy Routes at the Apicoplast**

*Plasmodium falciparum* presents a reduced, albeit putatively functional, repertoire of autophagy-related proteins (Table S1). Investigations of the blood stage uncovered the presence of *P*Vps34–*P*PI3K [7,39], which is essential for the generation of PI(3)P-enriched membranes at PAS, in addition to two conjugation systems, including ATG8 (ATG3, ATG4, ATG7, and ATG8) and ATG12 (ATG12) [56]. Many of the proteins were identified by *in silico* data mining and have not yet been fully characterized. Recent studies centered on *Pf*ATG8 supported the notion that, aside from its association with autophagy, *Pf*ATG8 also has a lineage-specific function in apicoplast formation [57] and parasite replication [62]. *Pf*ATG8 is conjugated to the outermost membrane of the apicoplast [59,60] which is enriched in PI(3)P [61], but not to the FV that is also decorated with PI(3)P [12,13]. Amino acid starvation induces the formation of *Pf*ATG8-positive structures (presumably autophagosomes) that fuse to *Pf*Rab7 vesicles (suggested to be late endosomes) prior to fusing to the FV [57] (Figure 3B). In blood-stage parasites, *P*Vps34 was shown to localize to the food vacuole and vesicular structures near the PM [39], while for liver parasite stages, *P*Nvps34 deposition remains undefined. If it were found to localize to the apicoplast membrane it could be tempting to speculate on a possible role for *P*Nvps34 in biogenesis of PAS-associated structures. [61].

Insight on *Plasmodium* autophagy was provided by the detailed characterization of a PIP-binding, yeast/human Atg18/ WIPI1 homolog named *Pf*ATG18 (Table S1). *Pf*ATG18 does not bind PI(3,5)P2 in vitro which, along with the lack of PI(3,5)P2 detection in this species, may indicate that this PIP residue is absent in *Plasmodium* membranes [12,13]. However, interaction with PI(3)P was shown to be localization-dependent. Detection of *Pf*ATG18 was associated with vesicles near the branching apicoplast and in proximity of *Pf*ATG8 in dividing cells, although colocalization was excluded [62]. *Pf*ATG18 was also found to localize on and in the FV (Figure 3B). Co-occurrence of *Pf*ATG18 at lysosomal compartments, that eventually fuse with *Pf*ATG8-labelled autophagosomes, supports the hypothesis that autolysosome formation occurs in *Plasmodium* [62]. Although it was suggested that the purpose of *Pf*ATG18 vesicles could be trafficking-related, this has not been experimentally tested in colocalization assays with *Pf*Rab7-labelled endosome-like vesicles. It is tempting to hypothesize that both proteins cover endosomal vesicles and are involved in autophagosome maturation (Figure 3B) [62]. Despite their clear involvement in autophagy-related trafficking pathways, *Pf*ATG8 and *Pf*ATG18 represent additional functions lacking in their yeast/mammalian counterparts and related to organellar biogenesis, cell growth, and proliferation. *Pf*ATG8 knock-down resulted in defective apicoplast inheritance (Figure 3D); progeny after the first reinvasion by *Pf*ATG8 knock-down parasites lacked a functional apicoplast and failed to replicate [58]. Similar to *Pf*ATG8, a conditional knock-down line for *Pf*ATG18 presents a ‘delayed death phenotype’ and impaired apicoplast formation [62].

![Figure 3. Autophagy in Mammalian/ Yeast Cells and Selected Parasitic Protozoa.](https://example.com/figure3)

(A) During mammalian/yeast Autophagy (ATG) initiation, the ULK1/Atg1 complex is recruited together with ATG9/Atg9 vesicles and the PI3KC3 complex I to endoplasmic reticulum (ER) membranes producing PI(3)P (monophosphorylated phosphoinositide) and forming a phagophore assembly site (PAS). The subsequent recruitment of additional autophagy components, such as WIPI2/Atg18 and LC3 complex/Atg8, promotes maturation of the double-membrane autophagosome that sequesters and delivers intracellular components to the lysosome for degradation or recycling [87,88,93]. (B) In *Plasmodium falciparum*, production of PI(3)P on apicoplast membranes is linked to the presence of the PI3K34 kinase. Autophagosome vesicles originating from the PAS on the outer membrane of the apicoplast were shown to fuse with Rab7-decorated vesicles prior to fusion with the food vacuole (FV). *Pf*ATG18-decorated vesicles were found in close proximity to branching apicoplasts as well as on and in the FV. Most likely, *Pf*ATG18 endocytic vesicles are involved in the fusion and maturation of autophagosomes [39,57,59,61,63]. (C) In Toxoplasma gondii tachyzoites, the formation of the autophagosome occurs in close proximity to the TgATG8-decorated apicoplast. These membranes fuse with endocytic vesicles decorated with TgATG9 and TgPROPI/2 prior to fusion with vacuolar compartments [12,13,66]. (D) In members of the Apicomplexa, PI/TgATG8 is enriched on the outermost membrane of the apicoplast during growth and division and play an important role in correct inheritance to daughter cells [13,56,58]. Abbreviations: AP, autophagosome; EnV, endosomal vesicle; PM, plasma membrane; RBC, red blood cell; TGN, trans-Golgi network; VAC, vacuolar compartment.
Despite a reduction in the ATG protein repertoire, T. gondii tachyzoites are able to generate autophagosomes in response to nutrient deprivation [63,64] and ER stress [65]. In contrast to Plasmodium, T. gondii encodes two PROPPIN homologs named TgPROP1 and TgPROP2 (Table S1). Both proteins were shown to bind PI(3)P [66], with TgPROP2 also binding PI(3,5)P2 [62]. The lipid-binding properties of TgPROPs, as well as TgPI3K, are important for their correct membrane targeting [66], and TgPI3K is essential for parasite replication and apicoplast biogenesis [13,17]. During cell starvation, both proteins relocated from a cytoplasmic pool to vesicular structures, presumably of autophagosomal nature, and endocytic vesicles, and partially colocalized with TgATG8 and extensively with TgATG9, respectively (Figure 3C) [13,17]. Knock-down of TgPROP1 had little impact on parasite viability, whereas studies on TgPROP2 point to its essentiality for parasite survival [66,67]. These data raised the question of whether both TgPROPs are equally involved in an autophagy-related pathway or if one adapted to a diverse cellular mechanism such as membrane fission [68] or scaffolding for signaling pathways [69]. Phylogenetic analyses suggest that TgPROP1 is more closely related to yeast Atg18 and its mammalian counterpart WIPI2 than TgPROP2. Hence, its function might be exclusively autophagic, while that of TgPROP2 might be more specifically geared towards apicoplast biogenesis (Figure 3E) [66].

The fascinating connection between autophagy in the Apicomplexa and apicoplast biogenesis is likely rooted in the mechanisms that led to evolution of the apicoplast. A widely accepted hypothesis concerning apicoplast emergence calls for a secondary endosymbiotic event explaining why the extant apicoplast is surrounded by four membranes, the outermost originating from the engulfing host phagosome [70]. PIATG8/TgATG8 were likely phagosome-associated components that were then co-opted into regulating apicoplast biogenesis (Figure 3D) and inheritance due to their association with the phagosomal membrane.

**ATG8 and 18 Homologs in the Genera Trypanosoma, Entamoeba, and Giardia**

Trypanosomatids retain autophagy-associated proteins, suggesting conservation of the corresponding cell degradation pathways [71,72] (Table S1). Involvement of autophagy in differentiation during the life cycle was suggested in the trypanosomatid genera *Trypanosoma* and *Leishmania* [73,74]. In *Leishmania*, lineage-specific duplication events have been identified for genes coding for some autophagy proteins [71,75]. In *E. histolytica*, almost all major Atg proteins required for autophagy are conserved, although they do not appear to be regulated by starvation [76]. However, Entamoeba Atg8, termed *EhAtg8* (Table S1), plays a role in phagosome/lysosome acidification involving PI(3)P which is enriched during maturation of phagosomal membranes prior to Atg8 recruitment [76]. Interestingly, in *Entamoeba invadens*, autophagy is induced during the differentiation from trophozoite to cyst [76].

The *G. lamblia* genome seems to lack the Atg8 conjugation system; however, two proteins were identified as Atg18 homologs and renamed *GIPROP1* and *GIPROP2* (Table S1). Based on *in silico* analysis of predicted tertiary structures, both GIPROPs present conserved lipid-binding sites [11].

**Concluding Remarks**

Despite their low abundance, PIPs and their binding partners are recognized as important regulators of pathways, including endocytosis, autophagy, or phagocytosis, as demonstrated extensively in well characterized model organisms. This field of research recently expanded its scope by including protozoa. This group of organisms presents a variety of lifestyles ranging from free living and photosynthetic, phagophoric, and parasitic forms, such as genera *Trypanosoma*, *Giardia*, and *Entamoeba*, and the phylum Apicomplexa. Recent reviews on the biological role of PIPs in parasitic protozoa [7,56] have focused exclusively on Apicomplexan species with the exception of one article [43] discussing *G. lamblia* PIP-binding proteins such as members of the PX-domain protein family, albeit without placing them within a specific biological context and/or process. In this review, we have attempted to present and compare data on the role of PIPs in specific biological processes across an evolutionarily wide spectrum of parasitic protists.
A number of questions concerning biological roles for PIPs and their protein-binding partners in parasitic protists remain open (see Outstanding Questions). A certain degree of redundancy in PIP-binding modules is often observed in parasitic protists, despite an overall reduction in endomembrane complexity. The significance of this phenomenon is still not fully understood. Furthermore, it is equally unclear whether the lack of specific PIP-binding modules in certain lineages reflects an evolutionary correlation to absence or reduction of specific subcellular compartments. This question stems from the notion that, in well characterized models, specific PIP residues are almost invariably associated with specific compartments. Last but not least, the role for PIPs and PIP-binding proteins in regulating parasite differentiation and/or stage conversion is an exciting, albeit largely untapped, field of research.

A consequence of adaptive evolutionary forces in protozoa is reflected in the new inventions and re-arrangements within protein domains, trafficking systems, and the repurposing of what are described as canonical pathways in model organisms. Autophagy is a good example of this, with ATG protein homologs in parasitic protists often playing novel roles that may be far removed from their classical function in more complex eukaryotic systems. In terms of protein evolution, it is not yet clear whether some (or all) of these roles are ancestral or acquired during lineage divergence and evolution.

Protozoa include evolutionary early-diverging eukaryotic species; therefore, there is a growing interest in investigating the diversity of trafficking mechanisms and machineries in these organisms. These investigations will illuminate processes that shaped complexity and/or simplifications post-LECA, including elucidation of the origins of vesicle-based membrane trafficking. All of these phenomena directly or indirectly rely on PIPs which are steadily gaining in our appreciation of the diversity and evolution of membrane trafficking mechanisms.

Supplemental Information

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