Trends in Parasitology

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Review

Roles of Phosphoinositides and Their binding Proteins in Parasitic Protozoa

Lenka Cernikova,[1] Carmen Faso,[1,2] and Adrian B. Hehl[1,*]

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.

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.

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], bisphosphorylated [PI(3,4)P_2, PI(3,5)P_2, and PI(4,5)P_2], and tris-phosphorylated [PI(3,4,5)P_3]. 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, moiesin (FERM), Fab1, YOTB, Vac1, and EEA1 (FYVE), Golgi phosphoprotein 3 (GOLPH3), postsynaptic density 95, disk large, zonula occludens (PDZ), Pleckstrin homology (PH), β-propellers that bind PIs (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.

[1]Institute of Parasitology, University of Zurich (ZH), Zurich, Switzerland

[2]Institute of Cell Biology, University of Bern (BE), Bern, Switzerland

*Correspondence: adrian.hehl@uzh.ch

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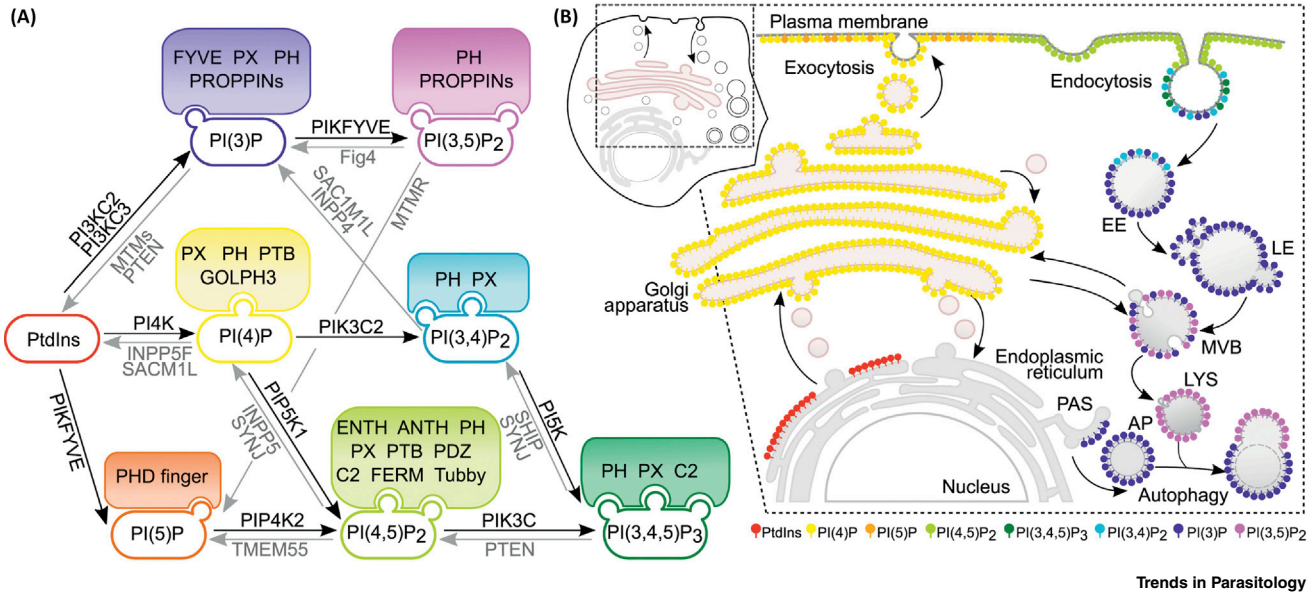


Figure 1. Metabolism and Subcellular Localization of Phosphoinositides (PIPs) in Mammalian Cells.

(A) A map of PIP interconversion catalyzed by corresponding kinases/phosphatases in mammalian cells. PIP-binding effectors and their preferentially targeted PIPs are shown. (B) A map of subcellular localization of PIPs in mammalian cells shows their distinct concentrated pools among subcellular compartments, with a focus on intracellular trafficking pathways, that is, exocytosis, endocytosis, and autophagy. Abbreviations: ANTH, AP180 N terminal homology; AP, autophagosome; C2, conserved region-2 of protein kinase C; EE, early endosome; ENTH, epsin N terminal homology; FERM, 4.1, Erin, radian, moiesin; Fig4, Polyphosphoinositide phosphatase; FYVE, Fab1, YOTB, Vac1, and EEA1; INPP4, inositol phosphatases specific for the 5 and 4 positions; LE, late endosome; LYS, lysosome; MVB, multivesicular body; MTMR, myotubularin-related phosphoinositide phosphatase; PAS, phagopore assembly site; PDZ, postsynaptic density 95, disk large, zonula occludens; PI3KC2, phosphatidylinositol 3-kinase C2 domain; PI3KC3, phosphatidylinositol 3-kinase C3 domain; PI4K, phosphatidylinositol 4-kinase; PIK3C2, class I phosphoinositide(3)kinase; PIP, phosphoinositide; PH, Pleckstrin homology; PHD, plant homeodomain zinc finger; PROPPINS, β-propellers that bind PIs; PTB, phosphotyrosine binding; PTEN, phosphatase and tensin homolog; PtdIns, phosphatidylinositols; PIKFYVE, phosphatidylinositol- PI(3)P 5-kinase; PX, Phox homology; SACM1L, SAC1 like phosphatidylinositide phosphatase; SHIP, lipid 5-phosphatase; SYNJ, synaptojanin-1; TMEM55, transmembrane protein 55 or phosphatidylinositol-4,5-bisphosphate 4-phosphatase 1.

A Parasitic Protist's Guide to PIP Distribution

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)P_2 is explicitly compartmentalized to **lipid rafts** and the actin-rich area of the polarized cell called the **uroid** [8]. PI(4,5)P_2 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

Box 1. PIP Distribution in Mammalian Cells

The PM is intimately associated with $PI(4,5)P_2$ which participates in processes that occur at, or involve, the cell surface such as endocytosis or phagocytosis. A minor pool of PI(4)P and PI(5)P in the PM is phosphorylated to $PI(4,5)P_2$ by type I PtdInsP kinases (PIP5K1) or by PtdInsP kinases [77–79]. PI(4)P is a predominant lipid of exocytic membranes , enriched at the Golgi complex (GC) and in the trans-Golgi network (TGN) [80]. PI(4)P is delivered to the PM by membrane carriers derived from the GC and recycling organelles. This phospholipid is also produced directly at the PM [81]. Furthermore, $PI(4,5)P_2$ is phosphorylated to $PI(3,4,5)P_3$ primarily by class I phosphoinositide(3)kinase (PIK3C); although negligible in resting cells, $PI(3,4,5)P_3$ levels increase dramatically following growth factor stimulation. $PI(3,4,5)P_3$ has important roles in cell proliferation, migration, chemotaxis, phago- and macropinocytosis, differentiation, survival, and during metabolic changes [3]. $PI(3,4)P_2$, exhibits specific regulatory roles during late stages of CME [82] and can be produced either by conversion from $PI(3,4,5)P_3$ by lipid 5-phosphatases (SHIP, also called INPP5D), or via phosphorylation of PI(4)P by a phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit alpha protein (PIK3C2) [82]. A minor pool of $PI(3,4)P_2$ serves as a precursor for PI(3)P during the late stage of clathrin-coated vesicle (CCV) formation by inositol phosphatases specific for the 5 and 4 positions (INPP4A). The bulk of PI(3)P is produced from PtdIns by the class II PI(3)kinase VPS34 (PI3K-C2/3). PI(3)P enrichment is a hallmark of early endosome membranes, where it participates in diverse functions, such as membrane tethering and fusion, interaction with the cytoskeleton, signaling, and motility, and associates with autophagosomal structures [80,82]. As early endosomes mature into late endosomes and multivesicular bodies (MVBs), PI(3)P is converted to $PI(3,5)P_2$. Lipid conversion is mediated by a phosphatidylinositol- PI(3)P 5-kinase (PIKFYVE). $PI(3,5)P_2$ is crucial for sorting of cargo destined for degradation in MVBs as well as for interaction with **ESCRT** components [3,80].

on membranes of phagocytic cups and nascent phagosomes [15] and has a role in membrane trafficking of internal vesicles and vacuoles [16]. $PI(3,5)P_2$ 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, $PI(3,4,5)P_3$ 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*, $PI(3,4,5)P_3$ 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 $PI(3,4,5)P_3$ was detected by both ^{32}P labelling followed by chromatography, and by mass spectrometry, although there are no $PI(3,4,5)P_3$ subcellular localization data yet available [21].

PIP-Binding Proteins and Their Roles in Parasitic Protozoa

Endocytosis

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 *Eh*ENTH; Table S1 in the supplemental information online), was found in the *Entamoeba* genome, and its binding affinities for $PI(4,5)P_2$ and $PI(3,4,5)P_3$ 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 Trypanosomatida.
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 asexual 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

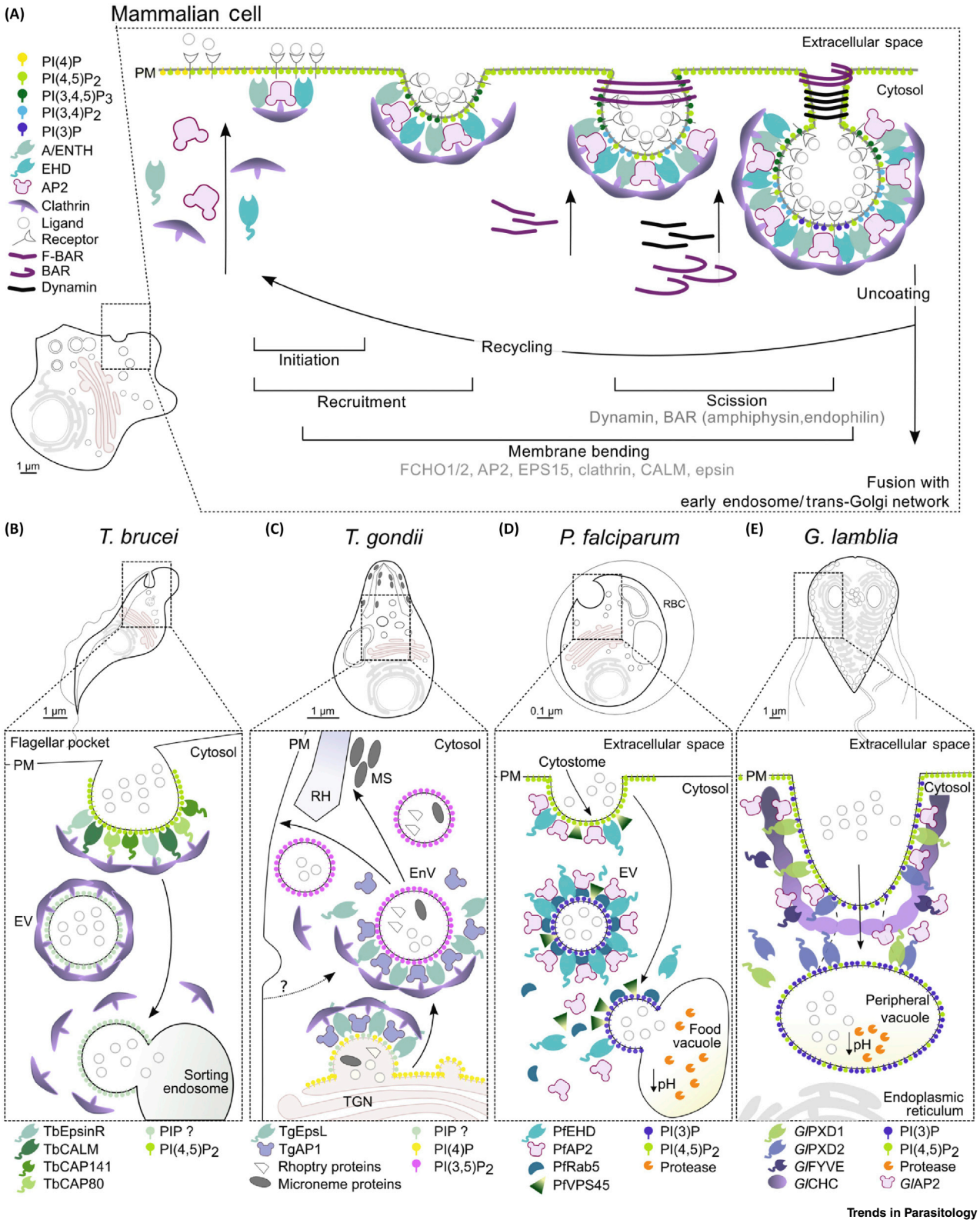
Box 2. Endocytosis – in General

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 spatiotemporally 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 $PI(4,5)P_2$. 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 $PI(3,4)P_2$ via its FYVE domain. Auxilin exerts a similar effect due to its specificity for PI(3)P and $PI(3,4)P_2$. 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 PH domain or other PI-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].

example, bacteria. It gives rise to compartments called phagosomes where cargo is digested and recycled.
Rhoptry: 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 protist parasites.
Uroid: a well-defined 'rear' zone of membrane accumulation that contributes, together with a 'front' called a pseudopod, to cell motility.

Lineage-Specific Innovations among Trypanosomatids

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 , $PI(4,5)P_2$, was shown to be enriched at the cytosolic face of the *T. brucei* flagellar pocket via activity of *Tb*PIPKA, a $PI(4,5)P_2$ kinase [26]. The *T. brucei* CME-related proteins *Tb*EpsinR and *Tb*CALM (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 *Tb*EpsinR and *Tb*CALM are predicted to link the flagellar pocket via PIPs, in particular $PI(4,5)P_2$, to clathrin-coated vesicle (CCV) formation. Furthermore, *Tb*CALM appears to also be important for proper duplication and segregation of the *T. brucei* lysosome [10]. As a counterpart to the core, 'shell' proteins *Tb*CAP80 and *Tb*CAP141 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. *Tb*CAP80 and *Tb*CAP141 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 *Tb*Flabarin and *Tb*FlabarinL [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].



Trends in Parasitology

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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 rhoptry, 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 *Tg*AP2, since no CCVs have been detected [32]. New insights in *Tg*CHC-mediated trafficking at the TGN were provided by the investigation of the *Tg*AP1-interactome which includes a unique ENTH-carrying protein named *Tg*EpsL [33] (Table S1). Similar to *Gl*epsin in *G. lamblia* [34], no interaction with *Tg*AP2 has been detected. The only conserved epsin-related region in *Tg*EpsL is the ENTH domain with binding affinity for $PI(4,5)P_2$ 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, *Tg*AP1 likely functions as a heterotetrametric 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 $PI(4,5)P_2$ [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. *Pf*AP2 localizes to the PM as well as to small vesicles close to the PM and the cytostome [37], suggesting that *Pf*AP2 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, *Pf*EHD (Table S1), was detected in the PfAP2 interactome [37]. Both *Pf*EHD and *Pf*AP2 were independently shown to associate with PfRab5 on the endocytic vesicles (Figure 2D) [37,38], and *Pf*EHD also labels vesicles which originate from the PM [38]. Furthermore, *Pf*EHD was also shown to bind $PI(3,4,5)P_3$, $PI(3,4)P_2$, and PI(4)P. Lipid synthesis is most likely controlled by PI(3)-kinase *Pf*VPS45 [7,39] (Table S1), which is essential for host cell cytosol uptake via endosomal-like compartments. The same study also suggested that PfVPS45 is involved in fusion of these PI(3)P-labelled vesicles with the food vacuole [40]. Surprisingly, and despite the presence of *Pf*AP2, clathrin is not involved in these pathways. Unlike *Pf*AP2, and similar to *Tg*AP1, preliminary data from *P. falciparum* point to the interaction of *Pf*CHC and *Pf*AP1 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]. In

Figure 2. Endocytosis in Mammalian Cells and Selected Parasitic Protists.

(A) In mammalian cells, proteins involved in clathrin-mediated endocytosis (CME) are recruited from the cytosolic pool and sequentially assembled at the plasma membrane (PM) during the initiation and recruitment phases. While the cargo is loaded, accessory proteins achieve membrane curvature to form a clathrin-coated vesicle (CCV) that is eventually pinched off from the donor membrane during scission. Vesicle uncoating releases accessory proteins back to the pool, and vesicles fuse with early endosomes [83,84]. (B) Endocytosis in *Trypanosoma brucei* takes place at the flagellar pocket, involving phosphoinositide (PIP)-binding proteins containing either epsin N terminal homology (ENTH) or AP180 N terminal homology (ANTH) modules and giving rise to CCVs that fuse with sorting endosomes [10,27]. (C) Endocytosis in *Toxoplasma gondii* has not been fully investigated yet; however, *Tg*AP1 and the ENTH-carrying protein *Tg*Epsl are repurposed for delivery of microneme and rhoptry protein to the apical side of the trophozoite [31,33]. (D) *Plasmodium falciparum* endocytosis involves an EHD module carrying dynamin, *Pf*EHD, as well as *Pf*AP2. The scaffold-building element, clathrin, is lacking, and a vesicle delivers cargo to the food vacuole (FV) [37,38,94]. (E) In *Giardia lamblia*, no CCVs have been detected. Instead, so-called clathrin assemblies surround PM invaginations that fuse with peripheral vacuoles (PVs), presumably with the assistance of PIP-binding proteins *Gl*PXD2, *Gl*PXD1, and/or *Gl*FYVE [11,42]. Abbreviations: AP2, adaptor protein complex 2; BAR, Bin, amphiphysin and Rvs; CALM, calmodulin; EnV, endosomal vesicle; EPS15, epidermal growth factor receptor substrate 15; EV, endocytic vesicle; F-BAR, extended FCH homology of Bin, amphiphysin and Rvs; FCHO 1/2, F-BAR domain-containing Fer/Cip4 homology domain-only proteins 1 and 2; MS, micronemes; RBC, red blood cell; RH, rhoptry; TGN, trans-Golgi network.

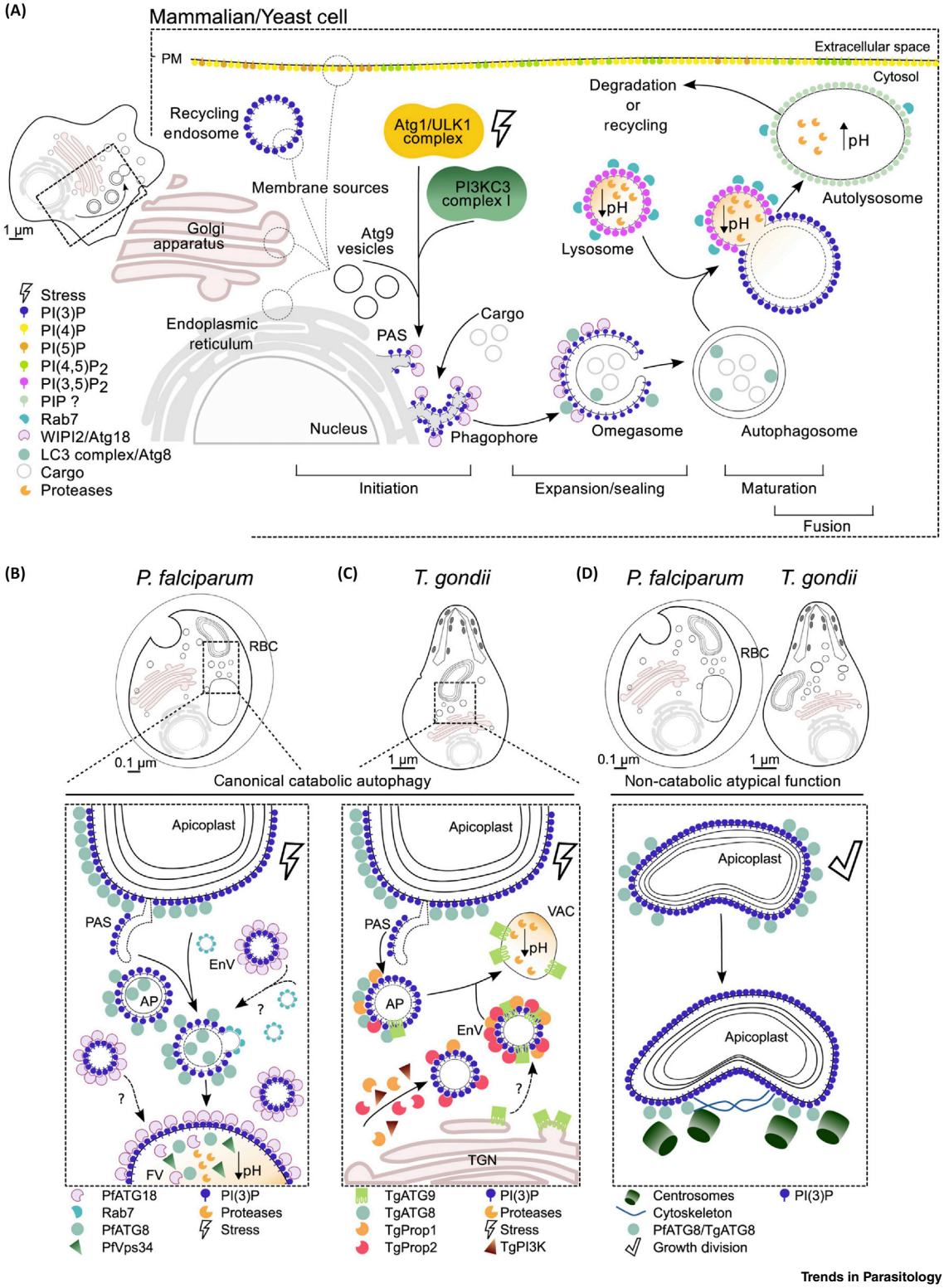
contrast to other parasitic protists, such as members of the genus *Trypanosoma* and the phylum Apicomplexa, the *Giardia* genome codes for several conserved CME components such as *Gl*CHC, hypothetical *Gl*CLC, *Gl*DRP, and *Gl*AP2 (Table S1), all localized in close proximity to PVs [42]. The status for *Gl*epsin (Table S1), *G. lamblia*'s single ENTH-domain protein, remains controversial since it was associated with *Gl*CHC 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 *Gl*PXD1–6 (Table S1), emerged as lineage-specific proteins involved in *Giardia* endocytosis [42,43] (Figure 2E). Similar to *Tb*CAP80 and *Tb*CAP141 [27], *Giardia Gl*PXD1 and *Gl*PXD2 maintain disordered N termini with an abundance of predicted clathrin- and AP2-binding motifs. *Gl*PXD1 and *Gl*PXD2 exhibit binding preferences mainly for PI(3)P and PI(4,5)P_2, respectively [44]. A FYVE domain-carrying protein named *Gl*FYVE (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 autophagopore 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

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 where it synthesizes PI(3)P from PtdIns enriched in ER membranes [45]. Subsequently, assembly of the ATG complex leads to the formation of a phagopore assembly site (PAS) where membranes of different origins, for example, the Golgi complex, recycling late endosomes and PM [88], contribute to phagopore nucleation. Autophagosome biogenesis and maturation is dependent on subsequent recruitment of two ubiquitin-like conjugation systems and the presence of PIPs, in particular PI(3)P and PI(3,5)P_2, which contribute to recruiting distinct proteins [89]. Once maturation is complete, PI(3)P turnover accompanies autophagosome 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 PIP 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 (WIPI 1–4) and three in yeasts (Atg 18, 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 microphagy. 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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autophagy via this route at all. Alternative regulatory mechanisms for this phenomenon await full characterization.

Autophagy Routes at the Apicoplast

P. falciparum presents a reduced, albeit putatively functional, repertoire of autophagy-related proteins (Table S1). Investigations of the blood stage uncovered the presence of *Pf*Vps34-*Pf*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 [58]. *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 PfATG8-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, *Pf*Vps34 was shown to localize to the food vacuole and vesicular structures near the PM [39], while for liver parasite stages, *Pf*Vps34 deposition remains undefined. If it were found to localize to the apicoplast membrane it could be tempting to speculate on a possible role for *Pf*Vps34 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 PfRab7-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 organelle 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 PfATG8, 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 Protists.
(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 phagopore 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 *Pf*Vps34 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 *Tg*ATG8-decorated apicoplast. These membranes fuse with endocytic vesicles decorated with *Tg*ATG9 and *Tg*PROP1/2 prior to fusion with vacuolar compartments [12,13,66]. (D) In members of the Apicomplexa, *Pf/Tg*ATG8 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 *Tg*PROP1 and *Tg*PROP2 (Table S1). Both proteins were shown to bind PI(3)P [66], with *Tg*PROP2 also binding PI(3,5)P_2 [62]. The lipid-binding properties of *Tg*PROPs, as well as *Tg*PI3K, are important for their correct membrane targeting [66], and *Tg*PI3K is essential for parasite replication and apicoplast biogenesis [13,17]. During cell starvation, both proteins relocalized from a cytoplasmic pool to vesicular structures, presumably of autophagosomal nature, and endocytic vesicles, and partially colocalized with *Tg*ATG8 and extensively with *Tg*ATG9, respectively (Figure 3C) [13,17]. Knock-down of *Tg*PROP1 had little impact on parasite viability, whereas studies on *Tg*PROP2 point to its essentiality for parasite survival [66,67]. These data raised the question of whether both *Tg*PROPs 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 *Tg*PROP1 is more closely related to yeast Atg18 and its mammalian counterpart WIPI2 than *Tg*PROP2. Hence, its function might be exclusively autophagic, while that of *Tg*PROP2 might be more specifically geared towards apicoplast biogenesis (Figure 3C) [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]. *Pf*ATG8/*Tg*ATG8 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 *Eh*Atg8 (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 *Gl*PROP1 and *Gl*PROP2 (Table S1). Based on *in silico* analysis of predicted tertiary structures, both *Gl*PROPs 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.

Outstanding Questions

Are current roles for ATG proteins in parasitic protists acquired or ancestral?

What is the significance of functional redundancy in PIP-binding modules, especially given the level of reduction in endomembrane complexity often observed in parasitic protists?

Is the observed lack of specific PIP-binding modules in some cells/organisms linked to a reduced complexity of subcellular compartments?

What are the roles of PIPs and PIP-binding proteins in parasite differentiation and stage conversion?

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 rearrangements 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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