Metamorphoses of malaria: the role of autophagy in parasite differentiation

Isabelle Coppens

Department of Molecular Microbiology and Immunology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD 21205, U.S.A.

Abstract

Several protozoan parasites undergo a complex life cycle that alternates between an invertebrate vector and a vertebrate host. Adaptations to these different environments by the parasites are achieved by drastic changes in their morphology and metabolism. The malaria parasites must be transmitted to a mammal from a mosquito as part of their life cycle. Upon entering the mammalian host, extracellular malaria sporozoites reach the liver and invade hepatocytes, wherein they meet the challenge of becoming replication-competent schizonts. During the process of conversion, the sporozoite selectively discards organelles that are unnecessary for the parasite growth in liver cells. Among the organelles that are cleared from the sporozoite are the micronemes, abundant secretory vesicles that facilitate the adhesion of the parasite to hepatocytes. Organelles specialized in sporozoite motility and structure, such as the inner membrane complex (a major component of the motile parasite's cytoskeleton), are also eliminated from converting parasites. The high degree of sophistication of the metamorphosis that occurs at the onset of the liver-form development cascade suggests that the observed changes must be multifactorial. Among the mechanisms implicated in the elimination of sporozoite organelles, the
degradative process called autophagy contributes to the remodelling of the parasite interior and the production of replicative liver forms. In a broader context, the importance of the role played by autophagy during the differentiation of protozoan parasites that cycle between insects and vertebrates is nowadays clearly emerging. An exciting prospect derived from these observations is that the parasite proteins involved in the autophagic process may represent new targets for drug development.

Introduction: adaptation of \textit{Plasmodium} to the hepatic environment

Malaria parasites must change their shape and function many times over the course of their life cycle. Although their developmental cycle is very complex, it basically boils down to two basic events: the parasite has to become motile to find a new replicative niche in the vector or the mammalian host, and then it needs to transform into a growth-proficient form, to generate more parasites (Figure 1). In a very basic sense, the cycle is very repetitive as three times over the life cycle the malaria parasite converts from a motile form (sporozoite, merozoite and ookinete) to a trophic form (liver and blood schizonts, and oocyst).

The sporozoite is adeptly equipped for migration through tissues and penetration into mammalian hepatocytes [1,2]. Extremely motile, elongated and finely curved, it possesses a robust membrane cytoskeleton essential for the maintenance of its shape, and therefore its motility. This invasive form contains unique secretory organelles, e.g. micronemes and rhoptries, which discharge their content at the time of host cell contact. The proteins secreted from micronemes contribute to hepatocyte adhesion, whereas the rhoptry content is implicated in the formation of the PV (parasitophorous vacuole). The intrahepatic parasite undergoes a process of conversion, upon completion of which the cytoskeletal structure that was providing mechanical support to the sporozoite plasma membrane is completely dismantled [3,4]. Micronemes and rhoptries are also cleared from converting parasites. At the end of metamorphosis, the parasites only retain organelles involved in biosyntheses e.g. the ER (endoplasmic reticulum), the mitochondrion network and the apicoplast, a relict plastid that contains essential anabolic pathways. Replication then occurs, characterized by multiple fissions of parasite nuclei. The replicative capacity of intrahepatic \textit{Plasmodium} is remarkable as the parasite achieves one of the fastest growth rates among eukaryotic cells. To satisfy its nutritional needs, the parasite actively up-regulates the expression of host genes involved in metabolite transport and anabolism [5,6], and it transforms its PV into a highly permeable compartment accessible to small host molecules [7]. The end of the schizogony marks a phase of biogenesis of organelles necessary for the formation of merozoite forms that are competent to invade red blood cells, which instigates the pathology associated with malaria.
Autophagic removal of organelles

Deciphering the mechanisms involved in sporozoite metamorphosis and organelle elimination is important to control malaria infection at the onset of the disease. In many biological systems, efficient detection and removal of superfluous or damaged cell constituents are crucial to maintain cellular homoeostasis and assure cell survival. In higher eukaryotic cells and yeast, most intracellular degradation is carried out by two mechanisms: the proteasome and autophagy. The proteasome degrades proteins that have
been tagged with ubiquitin and is sterically limited [8], whereas autophagy has the capacity to degrade protein complexes and entire organelles [9,10]. Autophagy encompasses all processes delivering cytosol and organelles to the vacuole/lysosome for macromolecule turnover and recycling of building blocks. This definition includes non-vesicular cargo-delivery modes, such as chaperone-mediated autophagy, and vesicular subtypes, such as macroautophagy and microautophagy. Whereas microautophagy sequesters cytosol directly into vacuolar/lysosomal invaginations, macroautophagy (commonly referred to as autophagy) involves the enclosure of a large portion of the cytoplasm within a nascent cup-shaped membrane structure termed the phagophore that is subsequently moulded into a double-membrane spherical vesicle, the autophagosome. Cup-shaped protrusions from the ER, named omegasomes, have been suggested to serve as platforms for autophagosome biogenesis [11]. The outer membrane of the autophagosome fuses with a lysosome to become an autolysosome. Nonselective autophagy, in which cytoplasmic structures are randomly engulfed into autophagosomes, is governed by extracellular stimuli (e.g. nutrient starvation or hormone limitation) to sustain anabolic needs [12]. By contrast, selective autophagy, in which a precise cargo is incorporated into autophagosomes, is triggered by intracellular components derived from the cargo itself [13]. For example, Uth1 on mitochondria is involved in the selective degradation of these organelles. Peroxisomes are specifically targeted to autophagy through the interaction of the Pex14 (peroxisomal membrane protein 14)–Pex3 protein complex with ATG30 (autophagy-related gene 30). Yeast genetic screens identified about 34 ATG genes [14] and most of them are harboured by multicellular organisms, implying conserved mechanisms [15].

Four major functional groups of ATG gene products have been identified (Figure 2): (i) the class III PI3K (phosphatidylinositol 3-kinase) complex [with ATG14 and several Vps (vacuolar sorting proteins)], necessary for the docking of ATG proteins to the phagophore; (ii) the cycling system of ATG9 (with ATG1, ATG2, ATG13 and ATG18), which transports membrane for phagophore biogenesis; (iii) the ubiquitin-like system of ATG8 (with ATG3, ATG4 and ATG7), which mediates membrane fusion between vesicles and the phagophore, contributing to its expansion; and (iv) the ubiquitin-like system of ATG12 (with ATG5, ATG10 and ATG16), which assembles into a coat-like structure and dictates the curvature of the phagophore and facilitates its closure to form an autophagosome. Besides primary roles in baseline turnover of organelles during nutrient deficiency, autophagy also occurs under normal growth conditions and is important for the dynamic processes that accompany cell differentiation [16]. For example, the cytoplasm of embryonic erythroid cells becomes scant and devoid of most organelles during gestation and the digestion of organelles occurs in autolysosomes [17].

Among the autophagy-related proteins, ATG8 is the most abundantly expressed and it associates with various autophagic structures. Its crystal
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structure reveals a conserved ubiquitin-fold region [18]. ATG8 is attached to its substrate through enzymatic pathways that are similar to the ubiquitin system. The C-terminal arginine residue of this protein is proteolytically removed by a cysteine protease, ATG4, to expose a glycine residue that forms a thioester bond with the C-terminal cysteine of an E1-like enzyme, ATG7. Activated ATG8 is then transferred to an E2-like enzyme, ATG3, also through a thioester bond. The C-terminal glycine residue of ATG8 is finally conjugated to PE (phosphatidylethanolamine) through an amide bond, resulting in ATG8–PE membrane association [19]. Interestingly, it has been shown that the outer membrane of mitochondria, which is rich in PE, may be one of the main lipid sources for autophagosomes [20]. The ubiquitin β-grasp fold of ATG8 provides specific surfaces for interaction with ubiquitin-binding sites, which contributes to the selective degradation of components. In mammals, for example, ATG8 interacts with the ubiquitin-binding protein p62, which facilitates the degradation of ubiquitinated protein aggregates. During ribophagy in yeast, ATG8 binds to the ubiquitin protease Ubp3 to form a complex with Bre5 (Brefeldin-A sensitivity protein 5) [13].

Critical role of autophagy for protozoan parasite development

Macroautophagy is an evolutionarily ancient process in eukaryotes, however, bioinformatics surveys for ATG candidates encoded by the genome of protozoa reveal a limited number of proteins compared with yeast and higher eukaryotes.

Figure 2. The molecular machinery of autophagy in yeast
See the text for details. C, cysteine; G, glycine; PI, phosphatidylinositol; R, arginine. Drawn by B. Jayabalasingham, Department of Molecular Microbiology and Immunology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, U.S.A.
An autophagy-like machinery is activated during the differentiation steps of *Leishmania major*, *Trypanosoma brucei* and *Trypanosoma cruzi*, and during the encystation of *Acanthamoeba* (summarized in [22]).

In humans, autophagy has been implicated in a wide range of diseases, including cancer, neurodegeneration, myopathies, major histocompatibility complex class II antigen processing and the removal of certain bacterial and viral pathogens. Targeting human ATG proteins by small synthetic inhibitors is recognized as an attractive strategy for the treatment of cancers and immune disorders, in which autophagy is either overly active or impaired [23]. Among the small molecules that inhibit autophagy, 3-MA (3-methyladenine) and wortmannin block class III PI3P (phosphatidylinositol 3-phosphate) activity, whereas chloroquine and bafilomycin A1 affect autolysosomal functions [24]. Based on the same idea, it is likely that interference with autophagy-mediated differentiation in protozoa will halt parasite propagation in their hosts. Indeed, impairing the activity of the parasite autophagy-related proteins that are essential for differentiation of *T. cruzi* or *T. brucei* is lethal [22]. Knocking out the *ATG4* gene in *L. major* blocks parasite differentiation [25]. One could then speculate that autophagy inhibitors against trypanosomiasis or leishmaniasis may be efficient in combination with other drugs when the parasites try to overcome the deleterious effects caused by cidal compounds by an autophagic response. In this case, a combined administration with autophagy inhibitors and current antiparasitic drugs may be both effective and offer possibilities to prevent the development of drug resistance.

### Autophagic activities in the malaria parasite

A process of functional autophagy is triggered in sporozoites during metamorphosis (B. Jayabalasingham, M. Labaied, K.A. Metcalf and I. Coppens, unpublished work). This observation is substantiated by the effect of 3-MA that targets Vps34 present in the parasite [26]. Treatment of the parasites with 3-MA results in a significant delay in the sporozoite differentiation process. In addition to Vps34, *Plasmodium* genomes encode clearly identifiable orthologues of ATG: ATG1, ATG17 and ATG18 of the ATG9 cycling system; ATG12 of the ATG12 conjugation system; and ATG3, ATG4, ATG7 and ATG8 of the ATG8 conjugation system. ATG orthologues of the human malaria parasite *Plasmodium falciparum* and the rodent malaria parasite *Plasmodium berghei* are 50–87% identical with each other. The single *P. berghei* orthologues of ATG8 and ATG3 (PbATG8 and PbATG3) share close structural similarity with their yeast and mammalian counterparts.

The PbATG8 sequence predicts two N-terminal α-helices tethered to a ubiquitin-fold core. It is predicted to have two hydrophobic patches as well as a number of conserved basic residues on one face of the protein that form the scaffold for binding to other ATG proteins. Many of the surface-exposed residues located on the opposite face of the protein exhibit low conservation,
which reflects a reduction in evolutionary constraints, perhaps to mediate unique and diverse interactions among ATG8 homologues. To this point, *Plasmodium* ATG8 has a unique inserted loop consisting of nine amino acids between α-helix 3 and β-sheet 3 that is absent in yeast and mammalian ATG8/LC3 homologues, suggesting that such binding partners may exist.

A functionally important residue conserved among all ATG8 homologues is a C-terminal glycine. In most homologues of ATG8, this glycine residue is followed by additional amino acids that are cleaved from the protein by ATG4. In this respect, the *Plasmodium* ATG8 sequences contain a C-terminal glycine that is not followed by additional residues, suggesting that the processing of ATG8 by ATG4 in *Plasmodium* is not necessary. The predicted sequence of PbATG3 contains the catalytic cysteine as found in other ATG3 proteins. Other conserved regions include: the ‘handle region’, which mediates the binding between ATG3 and ATG8, and the ‘flexible region’, which is implicated in the enzyme’s conjugation activity and binding to ATG7 [27]. The N-terminal region of yeast ATG3 interacts with ATG8 to form an intermediate that appears to be essential for the formation of the ATG8–PE moiety. Recombinant *Plasmodium* ATG8 and ATG3 also physically interact with each other. This interaction is different from the yeast ATG8–ATG3 binding activity as it involves several regions on the *Plasmodium* ATG3 protein, including the ‘flexible region’ and the C-terminal region encompassing the catalytic cysteine residue (B. Jayabalasingham, M. Labaied, K.A. Metcalf and I. Coppens, unpublished work). PbATG8 cannot interact with yeast ATG3 and is unable to complement a yeast ATG8Δ mutant, probably due to the structural disparities between *Plasmodium* and yeast ATG3. Additionally, when expressed in mammalian cells, *Plasmodium* ATG8 never associates with autophagosomes, but remains cytosolic. It is possible that the specific conformation of *Plasmodium* ATG8 is responsible for the lack of interaction of this protein with the mammalian autophagy machinery, and that in general the interactions of *Plasmodium* ATG8 and partners have evolved in a species-specific manner.

ATG8 is expressed by *Plasmodium* blood and liver forms, but is absent from insect stages. Genetic deletion of *Plasmodium* ATG8 results in a lethal phenotype, indicating that ATG8 is essential for the mammalian stage development. Upon urea-SDS/PAGE separation of *Plasmodium* lysates to detect the lipidated form of ATG8, only a single band corresponding to PbATG8 is detected. One possible explanation is that *Plasmodium* homologues of ATG8 are predominantly present in a lipid-conjugated state, in agreement with the presence of a C-terminal glycine residue of ATG8, making this protein ‘ready’ to bind to membranes following its synthesis. In support of this proposal, *Plasmodium* ATG8 is not present in the parasite cytoplasm but is located in the peripheral membrane of large vesicles. PbATG8-containing structures are prominently present in converting parasites, and some of them co-localize with micronemes. This suggests physical interactions between PbATG8-labelled compartments
and micronemes, and potentially implicates PbATG8 in facilitating microneme disposal. Interestingly, after sporozoite metamorphosis, PbATG8-labelled structures are still visible within the parasite and persist during the replication phase of development. Thus, in addition to a role in organelle degradation via autophagy, it is plausible that \textit{Plasmodium} ATG8 has additional functions e.g. for promoting the vesicular traffickling between organelles during the anabolic phase of intrahepatic development.

\textbf{Conclusions}

Autophagy is involved in various developmental processes \cite{24,28}. Similarly, autophagy plays an important role in parasitic protists, including \textit{Plasmodium} spp., for adaptation to different environmental conditions, which implies morphological remodelling and metabolic reprogramming during progression through the parasite life cycle. Interfering with the function of autophagy-related proteins using either reverse genetics or specific inhibitors results in impairment of parasite differentiation. From a therapeutic point of view, targeting the autophagy machinery could then be envisioned to control malaria infections. Important in this respect is that autophagy-related proteins in \textit{Plasmodium} display unique features compared with other eukaryotic organisms, creating the possibility of developing anti-malaria compounds that selectively interfere with the function of parasite protein, and not with that of the mammalian host counterpart. \textit{Plasmodium} seems to contain only a few \textit{ATG} genes, although this latter statement deserves deeper investigation by using, for example, integrated computational genomics to corroborate the presence of a limited number of autophagy-like proteins in malarial parasites. But if true, this raises the question of the minimal system that is necessary for normal functioning of autophagy in mammalian cells or yeast. In recent years, autophagy has received considerable interest as a target for therapeutic intervention in a variety of human ailments. From a cell biology point of view, the reduced complexity of the plasmodial autophagic machinery provides a simplified model to investigate the core mechanisms of autophagosome formation necessary for selective proteolysis, and will open a completely new area in autophagy research.

\textbf{Summary}

- \textit{In order to efficiently disseminate, the malaria parasites undergo a complex and reiterative life cycle within mosquitoes and mammals.}
- \textit{To adapt to every new environment, the malaria parasites are capable of drastic metamorphoses.}
- \textit{Malaria sporozoites injected by a mosquito into a mammal reach the liver wherein they change their shape and organellar content.}
- \textit{A process of autophagy is triggered during the metamorphosis of sporozoites, leading to the elimination of organelles superfluous for parasite development in the liver.}
• The autophagy machinery of Plasmodium has unique biochemical properties, very distinct from those present in their mammalian hosts.
• Analysis of the multiple steps of the autophagy-like process operational during parasite conversion may inspire new approaches to prevent malaria infections, based on chemotherapeutic interference with the Plasmodium differentiation machineries.

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References