Review Article

Forms, Crosstalks, and the Role of Phospholipid Biosynthesis in Autophagy

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1. Introduction

Eukaryotic cells have evolved numerous pathways to improve survival in harsh environments. One such pathway, known as autophagy, specializes in the breakdown of cell components through specific and nonspecific delivering to the lysosome. The products of lysosomal degradation can then be used for the biosynthesis of new proteins and organelles and as an energy source [1]. To date, three forms of autophagy have been identified and characterized. This review will discuss key findings in autophagy research as well as provide new insights on the role of membrane lipids in autophagosomal membrane formation.

Three major forms of autophagy have been identified in cells: chaperone-mediated autophagy (CMA), microautophagy (MiA), and macroautophagy (MaA). CMA is a selective protein delivering system which uses specific heat shock protein complexes (HSPC) to deliver proteins to the lysosome for degradation [2]. CMA is unique in that it specializes in the sequestration and degradation of a single-protein substrate, whereas both MiA and MaA specialize in bulk sequestration and degradation of cytosolic components. MiA is characterized by the engulfment of cytoplasm (including proteins and organelles) by membrane invagination of lysosome and/or endosome in mammals, or vacuole in yeast [3]. Finally, MaA is distinguished by the formation of a specialized double-membrane vesicle termed the autophagosome, which forms around the material to be digested (organelles/proteins). Once the autophagosome is formed, it fuses with a lysosome forming an autolysosome [4]. Though similar in their means of cargo degradation, the three forms differ in the manner that they use to deliver their cargo to the lysosome.

To date, most autophagy research has been directed towards MaA, resulting in large gaps in our understanding of CMA and MiA processes. However this does not mean that these processes are any less important than MaA, as numerous studies have identified an association between various diseases (Parkinson’s disease, Alzheimer’s disease, type-II diabetes, obesity, cardiovascular disease, and cancer) and generally unregulated or defective autophagy processes [5–12]. And, as will be shown in this review, surmounting...
evidence suggests that there is considerable crosstalk between these three pathways.

2. Chaperone-Mediated Autophagy

CMA is a specific form of autophagy targeting only soluble proteins for delivery to the lysosome. In order for a protein to be degraded, its specific motif KFERQ is initially recognized by a large heat shock protein complex (HSPC, Figure 1). The HSPC is made from three heat shock proteins (Hsc70, Hsp40, and Hsp90), Hsc70-interacting protein (Hip), Hsc70-Hsp90-organizing protein (Hop), and Bcl2-associated athanogene 1 protein (BAG-1) (Figures 1(a)–1(c)). Within the complex, Hsc70 specifically recognizes the KFERQ motif of the substrate protein. The interaction between Hsc70 and the substrate protein is controlled by ATP hydrolysis where the ADP-bound form of Hsc70 has the greatest affinity for the substrate [13]. The other components of the HSPC complex act as cochaperones regulating the activity of Hsc70. The ADP bound form of Hsc70 then targets the HSPC to the lysosomal membrane. Transport of the protein substrate into the lumen of the lysosome requires a transmembrane protein called the lysosome-associated membrane protein 2A (LAMP2A), which acts as a receptor for substrate proteins and has been proposed to be a rate limiting step in the CMA pathway [13–15] (Figure 1). LAMP2A is one of three splice variants of the LAMP2 gene. It contains a large heavily glycosylated portion within the lumen, a ~20 amino acid transmembrane component, and ~12 amino acid tail on the cytosolic side of the lysosome [16]. The three splice variants contain a similar luminal and transmembrane portion and differ in their cytosolic tails. The short cytoplasmic tail of LAMP2A contains four positive amino acids (KRHH/KHHH) specifically required for the translocation of the substrate protein into the lysosome [17]. Once the substrate binds to the LAMP2A monomer, a 700 kDa LAMP2A complex forms, and it, as well as a luminal chaperone (lys-Hsc70) aid in protein translocation into the lumen of the lysosome, where it is degraded [14, 18]. Importantly, before the protein can be transported into the lysosomal lumen, it must first be unfolded at the lysosomal surface (Figure 1(d)).

3. Chaperone-Mediated Autophagy Regulation

Generally speaking, CMA regulation is poorly understood, but it is acknowledged that levels of LAMP2A are controlled mainly through its assembly and disassembly within the lysosome itself, and not through gene expression within mammalian cells. During disassembly, LAMP2A is first truncated before entering the lumen where it is degraded. During nutrient deprivation, degradation rates of LAMP2A are reduced and remain so for a longer duration at the lysosomal membrane, allowing CMA to occur at greater rates [17]. Hsc70 has recently been shown to play a major role in the disassembly of the LAMP2A complex, whereas luminal Hsp90 stabilizes LAMP2A at the lysosome membrane [18] and the lysosomes which contain a higher amount of lys-Hsc70 seemingly have more active CMA [19].

4. Microautophagy

In MiA, a direct invagination of a specialized vesicular membrane takes in cytoplasm from the surrounding environment (Figure 2). This invagination grows and forms a narrow tube which elongates into the interior (Figure 2(a)). This tube is referred to as the autophagic tube and is continuous with the cytoplasm (Figure 2(b)). As the tube reaches the interior of the vesicle, the proximal membrane ends form a bulge, referred to as the autophagic body (Figure 2(c)). This bulging end is thicker than the tube leading into the interior. The walls of the bulging end of the autophagic tube then fuse, pinching off into the inner portions of the lysosome (Figure 2(d)). The cytosolic components trapped within the body are degraded within the vacuole (lysosome) or the vacuole will later fuse with a lysosome and the components degraded [3].

Similar to CMA, the processes involved in the regulation of MiA still need to be elucidated. However within yeast, studies have shown that within the later stages of MiA the pinching of the vesicle at the interior end of the autophagic tube is controlled through a vacuolar transporter cochaperone (VTC) complex, a protein which is localized at the endoplasmic reticulum (ER) and on vacuolar membranes. Although the autophagic tube was produced, the pinching off of the vesicle from the tube was impaired when VTC complex formation was prevented [3]. It also appears that calmodulin plays an important role during invagination of the autophagic tube. Although this interaction is calcium independent, VTC has been identified as a direct target of calmodulin, strengthening the role of VTC during MiA [3, 20].

5. Nuclear or Piecemeal Microautophagy

Extensive research in yeast has identified a form of autophagy in which portions of the nucleus undergo degradation through a MiA-like process. The outer nuclear membrane interacts with the vacuole through a nucleus-vacuole junction mediated by the nucleus vacuole junction 1 protein (Nvj1p) localized on the outer nuclear membrane and vacuolar 8 protein (Vac8p) found on the vacuolar membrane [21] (Figure 3). After the contact, the vesicle invaginates inward pulling on the outer membrane of the nucleus and the components into the autophagic body where they are later released into the vacuole [22] (Figure 3). After upregulation of piecemeal MiA with rapamycin, an increased presence of the chaperone VTC was observed at the nuclear-vacuole junction, suggesting a specific role of VTC in this process [23]. Interestingly, the nuclear protein Nvj1p is also responsible for the recruitment of Tsc13, a protein which plays a role in the synthesis of very-long-chain fatty acids, and Osh1, a protein responsible for nonvesicular lipid transport. The recruitment of Tsc13 and Osh1 in MiA suggests that additional lipids may be required for the formation
of the inner membranes forming within the vacuole [24]. Finally, similar to CMA, MiA and MaA also occur at similar times during certain stimuli (nutrient deprivation) and the proteins specific for MaA are actively involved in piecemeal MiA. Autophagy-related proteins, Atg3, Atg7, Atg12, Atg16, Atg1, and VPS (vacuolar protein sorting) 30, and many other proteins which play key roles in MaA were shown to play active roles in piecemeal MiA [25].

6. Endosomal Microautophagy

There has been a selective and nonselective form of endosomal MiA identified [26]. In the selective form (similar to CMA, Figure 1) proteins containing the KFERQ sequence were targeted for degradation after binding to Hsc70. LAMP2A is not localized on the late endosomes (LE), suggesting that Hsc70 is interacting with the LE
membrane through a mechanism unlike CMA during the delivery of specific proteins (KFERQ sequence) for degradation. Interestingly, Hsc70 has a preference for interacting with regions of membrane containing acidic phospholipids (phosphatidylserine, phosphatidylinositol, and phosphatidylglycerol). When considering the composition of cellular membranes, it is likely that phosphatidylserine (PS) is largely responsible for the interaction between the LE and Hsc70 due to its ability to play a role in protein localization [27]. Additionally, the endosomal MiA relies on ESCRT 1 and 3 systems for the formation of the MiA vesicles. When ESCRT functioning was disrupted, although a thin-like vesicle (autophagic tubule) was formed within the LE, pinching off of the vesicle did not occur [26].

7. Microautophagy Regulation

The target of rapamycin (TOR), the central regulator in cell growth in yeast, and mammalian cells can exist in two forms, TOR1 and TOR2 (mTOR1 and mTOR2 in mammalian cells). Interestingly, it is TOR1 which, when active, decreases the rate of protein synthesis, causing an arrest in the cell cycle and mimicking the effects seen during nutrient starvation, and preventing macroautophagy from occurring. TOR is a positive regulator of microautophagy due to its relationship with the EGO complex, which is made up of EGO1, EGO2, GTR1, and GTR2 [28, 29]. TOR1 and the EGO complex colocalize with Vam6 at the vacuolar endosomal membrane [30]. In fact it is now believed that the subunits GTR1 and GTR2 of the EGO complex may play a vital role in regulating the activity of TOR1 in yeast [30, 31] (Figure 4).

Interestingly the EGO complex is also located on the vacuolar membrane in yeast and was shown to play a role in monitoring the size of the vacuole during microautophagy. Through continuous fusion of autophagosomal membranes with the vacuole, the size of the vacuole continues to increase, but as microautophagy levels increase, the internalization of the membrane of the vacuole occurs preventing enlargement. The vesicle then buds off into the lumen of the vacuole, and the high lipid content of the vesicle is degraded. In fact mutants of EGO1 and EGO2 developed larger vacuoles, suggesting that the EGO complex is unable to induce internalization of the membrane to prevent vacuolar growth [32].

8. Macroautophagy

In MaA, a double-membrane structure termed an autophagosome is formed (Figure 5). This specialized vesicle delivers its engulfed material to a lysosome via membrane fusion, forming an autolysosome. Once fusion with a lysosome occurs, the components within the autolysosome are degraded (Figure 5).

Three events must occur in order to form a fully functional autophagosome in mammalian cells. The first step is the nucleation event, where recruitment of an early kinase complex is believed to trigger the synthesis of the autophagosomal isolation membrane (IM) [33, 34] (Figure 5). The specific site of autophagosome formation is believed to occur in a region of the endoplasmic reticulum (ER) called the omegasome [35]. The second step of autophagosome formation is characterized by the expansion of the IM. Finally, a fully functional autophagosome is formed through membrane fusion of the IM.

During the nucleation event, recruitment of a large serine/threonine complex consisting of autophagy-related protein 13 (ATG13), focal adhesion kinase family interacting protein (FIP200), Unc-51-like kinase 1 (ULK1), and ATG-101 translocates to the ER region of the omegasome [33, 36] (Figure 5). Translocation of this complex induces recruitment of the large-class-III phosphoinositide 3 kinase (PI3K) complex consisting of vacuolar protein sorting (VPS)-34, VPS-15, BAX interacting protein-1 (ULK1), and ATG-13, focal adhesion kinase family interacting protein 13 (ATG13), translocate to the same region [33, 37]. WIP12 and DFCP1 then promote the recruitment of the phospholipid carrier LC3-II (microtubule-associated light chain 3) to the isolation membrane. Since it is understood that the ATG5-ATG12-ATG16L specifies the site for LC3-I lipidation, it is possible that WIP12 and DFCP1 may be positively effecting LC3-II formation via an interaction with ATG5-ATG12-ATG16L [38, 39]. The fully formed autophagosome then delivers the cytosolic components to a lysosome via vesicle fusion, forming an autolysosome (Figure 5). Lysosomal digestive enzymes contained within the autolysosome degrade the proteins and/or organelles, and degradation products are recycled for use in energy generation or other processes.
Figure 4: Activation of TOR1 in microautophagy. Vam6 activates Grl1 of the EGO complex leading to the activation of TOR1. Through the activation of TOR1, microautophagy levels increase and the cell increases protein synthesis as it returns to a growing state. MaA, typically activated in nutrient deprived conditions, is inhibited.

Figure 5: Macroautophagy. Recruitment of the large ULK1 complex to the omegasome (1) induces the migration of the class-III PI3K kinase complex to the same site (2). PI(3)P3 recruitment (3) promotes the localization of ATG12-ATG5-ATG16L (4) and LC3-II (5). The fully formed autophagosome engulfs the MOI and fuses with a lysosome, forming an autolysosome, where lysosomal hydrolases contained within the lysosome degrade the MOI. MOI: material of interest; 12-5-6: ATG12-ATG5-ATG16L; PI3P1P: phosphatidylinositol triphosphate-interacting protein; PSE: phospholipid synthesizing enzyme; LC3: microtubule-associated light chain 3; PI3K: phosphoinositide 3 kinase; ULK1: unc-51-like kinase 1; ER: endoplasmic reticulum; IM: isolation membrane.

9. Macroautophagy Regulation

The most important regulator of MaA is the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase. During nutrient rich states, mTOR is active and phosphorylates ATG13, preventing its association with a large activating complex (ATG13-FIP200-ULK1-ATG101) vital for the initiation of autophagosome formation (Figure 6). When ATG13 is phosphorylated, it does not associate with FIP200, Unc 51 like kinase 1 (ULK1), and ATG101, which prevents autophagy from occurring. During periods of low-energy, amino acids and/or growth factors, mTOR is inhibited through phosphorylation of AMPK [32]. Since mTOR is a negative regulator of autophagy, its inhibition allows the formation of the large ATG13-FIP200-ULK1-ATG101 complex and it subsequently translocates to the ER to initiate the nucleation of the isolation membrane (Figure 6) [25, 35]. Translocation of the serine/threonine complex then induces recruitment of the large-class-III PI3K complex and ATG-14 to the ER [36, 40].

10. Crosstalk between MaA and CMA

In conditions of stress similar to which induce MaA, an upregulation in CMA results, and both processes appear to be vital to cell survival. For instance, studies in fibroblasts have shown that MaA activity rates peak within the first few hours of starvation. However, if the cell remains in this state for longer than 6 hours, CMA activity levels rise above those of MaA and reach maximal activity at about 20 hours [41]. Since MaA is a nonselective process, if its rates were to remain high, it would eventually lead to cell death due to the non-specific degradation of organelles and/or proteins required for survival and proper functioning of the cell. Through activation of CMA, the cell is able to target only nonvital proteins, allowing it to survive and function properly in a nutrient-deprived environment. Therefore it is the consecutive pairing of these two processes that maintains a cell’s viability under adverse conditions. Furthermore, in situations that do not allow for CMA activity, such as the presence of large protein complexes which obstruct
CMA machinery, MaA is upregulated to eliminate protein aggregates and ensure cell survival [41]. It is becoming increasingly clear that there is an important link between MaA and CMA, as both pathways use similar machinery to carry out their respective actions. Moreover both pathways are upregulated by similar conditions including nutrient deprivation, oxidative stress, and/or improper protein folding in the ER. The crosstalk between CMA and MaA was most evident when MaA became upregulated in cells defective in the CMA-specific protein LAMP2A. The exact manner in which the cells compensated for decreased CMA activity is not fully understood but an overlap in proteins involved in both autophagy pathways was offered as a plausible mechanism. In the absence of LAMP2A activity, the assembly of the large CMA complex became prevented and cells had an increased availability of proteins required for CMA. If these same proteins are also required for MaA, that would lead to increase this process [42, 43].

11. Phospholipid Synthesis and the Formation of the Isolation Membrane

Despite the advances in autophagy research over the last few decades, one area of research still remains poorly understood. Due to the lack of lipid specific biomarkers and adequate functional assays, scientific understanding of the origin and source of the autophagosomal membranes remains incomplete. Recent evidence of the contribution of phospholipids to various processes including membrane fusion and cell survival pathways however points to a functional capacity of phospholipids outside of their normal structural role [42–45].

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) are essential bilayer forming lipids in all cells. PC is predominately found in the outer leaflet of the cell membrane and PE and PS make up the inner leaflet phospholipids [46, 47]. PE and PS have been shown to redistribute within the membrane under certain stress conditions [44, 48] where higher concentrations of PE and PS were found in the outer leaflet of the plasma membrane preceding cell-to-cell fusion of myoblasts to form myotubes [45]. Similarly, during cytokinesis, PE was found on the outer leaflet of the plasma membrane and at the cleavage furrow during late telophase [49]. These and other findings demonstrate a necessary capacity of the inner membrane phospholipids PE and PS in membrane fusion. Finally, several studies have identified a distinct function of PS in cell survival whereby localization of PS to the outer membrane leaflet of cells under stress or apoptotic conditions acts as a recognition signal for phagocytic binding [48, 50, 51].

Some of the leading hypotheses on the source of the autophagosomal membrane include the ER, the Golgi apparatus, the mitochondria, and the plasma membrane [52–55]. Increasing evidence however suggests that the ER plays a significant function in autophagosome formation by providing phospholipids for autophagosome membrane initiation and expansion. Two models propose a role for the ER in autophagosome formation. The first model suggests that the autophagosome originates from a ribosome-free region of the rough ER [56, 57]. However, a lack of certain ER markers (P450 and PDI) on the IM suggests that autophagosomes are not derived from the ER via direct maturation [35, 58–60]. In fact, IMs and mature autophagosomes were reported to appear as nascent membranes, whereby any proteins present on the membrane would have to be stripped for the maturation model to hold true [61–63]. However, protein removal from membranes is an energetically expensive process and the synthesis of new membranes from localized lipid sources is a more plausible explanation [64], which altogether fits into the second model proposing that the ER plays a fundamental role in autophagosome formation through providing newly synthesized lipids, notably the phospholipids PE, PC, and PS as described here in after.

The biochemical pathways for phospholipid synthesis are described in Figure 7. The Kennedy pathway accounts
for bulk synthesis of PE and PC through two independent branches of the pathway: CDP-ethanolamine and CDP-choline, respectively. In the CDP-ethanolamine pathway, ethanolamine (Etn) is phosphorylated by ethanolamine kinase (EK) to form phosphoethanolamine (Etn-P), which is then converted by CTP: phosphoethanolamine cytidylyltransferase (Pcyt2/ET) into CDP-ethanolamine (CDP-Etn). Finally, PE is produced from CDP-ethanolamine and diacylglycerol (DAG) by CDP-ethanolamine 1,2-diacylglycerol ethanolaminephosphotransferase (CEPT) [65]. Alternatively, PE can also be synthesized by the decarboxylation of PS through the action of phosphatidylerine decarboxylase (PSD) within the mitochondria [66, 67] (Figure 7 left).

Analogous to PE synthesis via the CDP-ethanolamine pathway, PC production via the CDP-choline pathway begins with the phosphorylation of choline via choline kinase (CK) to form phosphocholine (P-choline). CDP-choline is produced from phosphocholine via phosphocholine cytidylyltransferase (Pcyt1/CT) and finally condensed with DAG to form PC (Figure 7 right). PC can be synthesized by two additional pathways: PE synthesized through the CDP-ethanolamine pathway can be converted to PC via the enzyme phosphatidylethanolamine N-methyltransferase (PEMT), or through the decarboxylation of PS to form PE, which can then be methylated to form PC [68–70] (Figure 7). Unlike PE and PC, which are primarily synthesized de novo, PS is synthesized largely via an exchange reaction with preexisting PE by phosphatidylerine synthase 1 (PSS1) or with preexisting PC by phosphatidylerine synthase 2 (PSS2).

**Figure 7**: Phospholipid biosynthetic pathways. PE is made by the CDP-ethanolamine pathway in the ER (left) or from PS via the action of PSD in mitochondria. PC is made by the CDP-choline pathway (right) or from PE through the PEMT pathway in the ER and mitochondria-associated membranes of the ER. PS is produced from PE and PC via PSS2 and PSS1 pathways in the mitochondria-associated membranes of the ER. Ethanolamine kinase: EthK; CTP: ethanolamine phosphotransferase: CT; CDP: diacylglycerol ethanolamine and choline phosphotransferases: EPT and CPT; choline kinase: CK; CTP: cholinephosphate cytidylyltransferase: ET; CDP: diacylglycerol ethanolamine and choline phosphotransferases: EPT and CPT; phosphatidylerine synthase 1: PSS1; phosphatidylerine synthase 2: PSS2.
de novo synthesis of membrane is required for these forms of autophagy. Additionally, what is becoming increasingly clear is how Maf, Mia, and CMA are linked and share the same and/or similar proteins. Increased investigation of the crosstalk between these forms of autophagy will help to understand the specific roles these systems play (together and separately) in the removal of cellular material.

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