Discerning autophagy pathway intermediates – transmission electron microscopy techniques

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Part 1: What will you learn in this chapter

In this chapter, you will learn guidelines on how to identify macroautophagy/autophagy pathway intermediates in transmission electron microscopy samples prepared using conventional techniques including chemical fixation and plastic embedding. We will describe the typical morphological features of phagophores, autophagosomes, amphisomes and autolysosomes induced by amino-acid starvation in mammalian cells. We will point out what you should keep in mind when interpreting transmission electron microscope images taken of very thin sections cut from much thicker cells and organelles. You will also learn how immuno-electron microscopy of endogenous MAP1LC3/LC3 can be used to help identification of autophagy pathway intermediates.

Introduction

Autophagy is a degradative pathway in which cytoplasmic components, including whole organelles like mitochondria and parts of the endoplasmic reticulum, are first enwrapped into membrane-bound vesicles called autophagosomes, which then deliver their cytoplasmic cargo to lysosomes for degradation and recycling (Figure 1). Autophagy was originally discovered using electron microscopy (1). After the autophagy genes and proteins were discovered, it became possible to develop numerous new methods to monitor the activity of the autophagic pathway in cells and tissues (2). These methods include monitoring the lipidation of the autophagy marker protein LC3 (microtubule-associated protein light chain 3) by immunoblotting (3, 4), as well as quantifying the amount of LC3-positive vesicles in cells by immunofluorescence. LC3 can exist in the cytoplasm as a non-lipidated protein called LC3-I. Once autophagy is induced, LC3 is conjugated to a lipid called phosphatidyl ethanol amine. The lipidated form is called LC3-II, and it associates with the autophagic membranes, including phagophores and the inner and outer limiting membranes of autophagosomes. Thus, LC3 is

a marker protein for phagophores and autophagosomes, and to some extent also for amphisomes and even autolysosomes. However, LC3 dissociates from the outer limiting membrane when autophagosomes mature into amphisomes and autolysosomes, and the LC3 that is trapped inside autophagosomes will eventually be degraded together with the cytoplasmic cargo (3, 5).

Tandem fluorescent tagged LC3 can also be used to monitor maturation of autophagosomes to acidic and degradative autolysosomes (6). This is based on the decrease in pH; autophagosomes are neutral while autolysosomes are acidic. The method requires expression of the reporter tandem-tagged LC3 in the cells or tissues. The tandem tag includes both green and red fluorescent protein. In the acidic milieu of autolysosomes, the fluorescence of certain green fluorescent protein (GFP) variants is quenched (7), while the red fluorophores like monomeric red fluorescent protein (mRFP) and mCherry are more acid resistant (6). Thus, the neutral autophagosomes will show both green and red fluorescence, while the acidic autolysosomes show only red fluorescence.

Despite the numerous new techniques for monitoring autophagy, electron microscopy is still used for this purpose. It is a relatively sensitive technique for detecting the accumulation of autophagy pathway intermediates, especially when combined with careful quantification. The advantage of electron microscopy is that there is no need for the expression of exogenous marker proteins. The disadvantage of electron microscopy is that sample preparation is time consuming compared to many of the newer methods. Further, special equipment and know-how are necessary for sample preparation. In addition, interpretation of the images needs special expertise. This chapter introduces the basic principles that help in identification of autophagic pathway intermediates including phagophores, autophagosomes, amphisomes and autolysosomes in electron microscopy samples prepared using chemical fixation and plastic embedding. The examples we show were prepared using cultured mammalian cells induced for autophagy by 1-2-h serum and amino-acid starvation. Detailed protocols that can be used for sample preparation and quantification of the images have been published elsewhere (8, 9). The pre-embedding immuno-electron microscopy protocol for LC3 is described in Biazik et al. (10).

Phagophores are curved membrane cisterns with a narrow intermembrane space

Phagophores, also called isolation membranes, are the first autophagic structures that can be identified in electron-microscopy samples by morphology. They are rarely seen in thin sections prepared from cells kept under non-starved, basal conditions, but they appear soon when the cells are kept under amino-acid free conditions. Because phagophores are short lived, their number in normal wildtype cells is lower than that of autophagosomes. However, phagophores accumulate in cells if certain autophagy genes have been knocked out or silenced. For instance, cells deficient in the autophagy genes *Atg3*, *Atg5* or *Atg7* accumulate phagophores (11). Of note, the contrast of the phagophore membranes can be selectively enhanced by using reduced osmium tetroxide during sample preparation. Either potassium ferrocyanide or imidazole can be added as reducing agents during postfixation (8, 12) .

Phagophores are membrane cisterns of varying length and curvature (red arrows in Figure 2A-E). Their membranes do not have bound ribosomes, which differentiates them from rough endoplasmic reticulum (Figure 2B-E). The shape of elongated phagophores is typically ellipsoidal or U-shaped (Figure 2 A-E). After closure of the phagophore, the newly-formed autophagosome may have an elongated shape (Figure 2F). Phagophores are frequently located close to cisterns of endoplasmic

reticulum, which in many cell types have ribosomes attached to them, especially when autophagy is induced by amino-acid starvation (13, 14). Ribosomes are very helpful for identifying cisterns of endoplasmic reticulum (Figure 2B-E). In many cell types, amino-acid starvation induced phagophores can be lined by cisterns of rough endoplasmic reticulum on both sides (10, 14, 15).

The width of the space between the membranes of phagophores varies depending on the sample preparation protocol, but generally it is significantly narrower than that of cisterns of endoplasmic reticulum (Figure 2B-E). Careful analysis of samples immobilized using cryofixation, which is able to preserve the native ultrastructure remarkably better than traditional chemical fixation with glutaraldehyde and osmium tetroxide, showed recently that the intermembrane distance of phagophores decreases when the phagophores elongate (16). However, this phenomenon may not be visible in samples prepared using chemical fixation.

Autophagosomes are spherical, limited by two lipid bilayers, and contain cytoplasmic cargo

When the phagophore closes, it forms and autophagosome that is limited by two lipid bilayers (Figure 1). Similar to phagophores, autophagosome membrane do not have bound ribosomes, and their contrast can be enhanced by postfixation in reduced osmium tetroxide (12). The space between the two limiting membranes may vary depending on sample preparation protocol (Figure 3A-E). In samples incubated in reduced osmium tetroxide, the two limiting membranes are typically very close to each other, which may look like one thick limiting membrane (Figure 3D). In samples immobilized by cryofixation, autophagosomes are almost perfectly spherical, and the distance between the inner and outer limiting membranes is very uniform and approximately the same as the thickness of one lipid bilayer (16). The diameter of autophagosomes varies depending on the cell type. In fibroblasts, the diameter typically varies between 400 nm and one micrometer, while in hepatocytes the size can be considerably larger. In selective autophagy, such as autophagy of intracellular bacteria, the diameter of the autophagosome is determined by the cargo (17). Similar to phagophores, autophagosomes can localize close to endoplasmic reticulum, which typically has ribosomes attached to it, especially in amino-acid-starved cells (Figure 3A,B) (10, 14, 15).

In addition to the two limiting membranes, autophagosomes are characterized by the cytoplasmic cargo (Figure 3). The cytoplasmic cargo is an important feature for identification of autophagosomes in electron microscopy samples. Because autophagosomes have not yet fused with any degradative organelles, the cytoplasmic cargo is still intact. This means that the morphology of the cytoplasmic cargo is similar to that of the cytoplasm that surrounds the autophagosome.

During amino-acid starvation, autophagosomes typically engulf ribosomes and rough endoplasmic reticulum (Figure 3A-F), and occasionally mitochondria and other organelles (Figure 3C,E). Especially ribosomes are practically always visible among the cargo in starvation-induced autophagosomes and serve as an important feature for identification (Figure 3). There are also several types of selective autophagy, in which the phagophores selectively engulf cargo such as mitochondria (mitophagy), endoplasmic reticulum (reticulophagy), peroxisomes (pexophagy), lipid droplets (lipophagy), protein aggregates or condensates (aggrephagy) or intracellular bacteria (xenophagy) (18). Endogenous LC3 can be localized on autophagosome limiting membranes by immuno-electron microscopy (Figure 3F).

Amphisomes are born when autophagosomes fuse with multivesicular endosomes

Autophagosomes often fuse with multivesicular endosomes (also called multivesicular bodies) before fusion with lysosomes (Figure 1). Multivesicular endosomes are characterized by their intraluminal vesicles, which emerge by invagination and pinching off from the limiting membrane. In electron microscopy samples, amphisomes are characterized as membrane-bound vesicles that contain small intraluminal vesicles in addition to the cytoplasmic cargo (Figure 4). The cargo can appear intact, similar to the cytoplasm around the amphisome (Figure 4A), or partially disintegrated, because amphisomes are acidic (Figure 4B-E). Ribosomal cargo can still be identified as electron-dense particulate material (yellow asterisks in Figure 4B-F). Amphisomes typically have one limiting membrane visible in the electron microscopy samples (Figure 4B-E), but if the fusion with the multivesicular endosome is recent, the two limiting membranes may still be visible (Figure 4A). Occasionally, several autophagosomes may be seen to have fused with each other and with a multivesicular endosome, forming an amphisomes containing several cytoplasmic 'packages' lined by one lipid bilayer (the inner limiting membrane of the autophagosome) as well as the small internal vesicles delivered by fusion with the multivesicular endosome (Figure 4A). Endogenous LC3 can still be localized in amphisomes by immuno-electron microscopy (Figure 4F).

Autolysosomes are born when autophagosomes or amphisomes fuse with lysosomes

Autophagosomes can fuse directly with lysosomes, or they can undergo sequential fusion with multivesicular endosomes followed by lysosomes. Because lysosomes are full of degradative enzymes, their fusion with autophagosomes and amphisomes initiates the final degradation of the cytoplasmic cargo. In addition to the cytoplasmic cargo, also the inner limiting membrane of the autophagosome is degraded. Compared to the earlier intermediates of the autophagic pathway, autolysosomes are more difficult to identify by morphology in electron microscopy samples, because the characteristic morphological features have been partially degraded (Figure 5A-F). Autolysosomes are limited by a single (Figure 5A-D), or sometimes still a double (Figure 5E,F), limiting membrane. The contents are disintegrated, but for identification of a structure as an autolysosome, the cytoplasmic cargo should still be identifiable. In particular, the ribosomal cargo can still be identified as electron-dense particulate material (yellow asterisks in Figure 5A-F). In many cases, it is difficult to tell whether a structure is an autolysosome, late endosome or lysosome. Immuno-electron microscopy of a known, degradation-resistant cargo protein such as superoxide dismutase (19) may be used to help identification of the cytoplasmic cargo.

Autophagic vacuole or autophagic compartment are general terms

Sometimes it is not necessary or possible to differentiate all the autophagy pathway intermediates. The terms 'autophagic vacuole' and 'autophagic compartment' can be utilized as general terms to include all intermediates, i.e., phagophores, autophagosomes, amphisomes, and autolysosomes. The term 'initial autophagic vacuole' (initial autophagic compartment) includes phagophores and autophagosomes, while 'degradative autophagic vacuole' (degradative autophagic compartment) includes amphisomes and autolysosomes.

LC3 associates with both phagophore and autophagosome membranes (3, 10, 20). In autophagosomes, it associates with both of the two limiting membranes. Amphisomes and to some extent also autolysosomes are also LC3-positive, although not as strongly as autophagosomes.

Take note of four important points

There are a few issues that you should remember when interpreting images taken using a transmission electron microscope.

First, keep in mind that when looking at thin sections in the transmission electron microscope, you are looking at very thin slices cut from three-dimensional cells and organelles. The typical thin sections are 50-100 nm thick, while a typical mammalian cell has a diameter of tens or micrometers, and the organelles, including autophagy pathway intermediates, have diameters of hundreds of nanometers. This means that in the thin sections, you do not see the whole organelles, only thin slices of them. The appearance of the organelle in the thin section can vary depending on the orientation of the section. For example, a phagophore can look like a closed autophagosome in a thin section, if the section does not hit the open rim. An amphisome can look like an autophagosome or autolysosome, if the section does not hit the small internal vesicles that would identify it as an amphisome (Figure 5E,F). This may sound confusing. However, in most cases it is acceptable to combine phagophores and autophagosomes in one class and amphisomes and autolysosomes into another class when quantifying the structures. If more specific differentiation between phagophores and autophagosomes or amphisomes and autolysosomes is needed, immunolabelling of phagophore or amphisome markers can be used in either fluorescence microscopy or immuno-electron microscopy.

Second, keep in mind that a double limiting membrane means that autophagosomes are limited by two adjacent lipid bilayers. Occasionally, one lipid bilayer has been mixed with a double membrane in literature (21). While the double limiting membrane is a good criterion for the identification of autophagosomes, it has some drawbacks. Mitochondria are also limited by two lipid bilayers. However, unlike autophagosomes, the mitochondrial inner limiting membrane is characterized by invaginations called cristae, which are relatively easy to recognize in electron microscopy images. Depending on the orientation of the thin section, cisterns of endoplasmic reticulum can look like double-membrane limited vesicles containing cytoplasm, which can be mixed with autophagosomes. However, endoplasmic reticulum cisterns are usually studded with ribosomes, which are not present on autophagosome limiting membranes. In addition, the distance between the two lipid bilayers is wider in endoplasmic reticulum than in autophagosomes (16). Also, the size and shape of autophagosomes can be used as criteria for identification. Autophagosomes are typically spherical, except the situation right after fusion of the phagophore, and in each cell type they have a typical diameter range.

Third, also the appearance of the lipid bilayer can vary in conventional electron microscopy thin sections, depending on the orientation of the section in relation to the bilayer. Occasionally, the lipid bilayer may seem to be abnormally thick, as if it had split into two. An example of this is visible in Figure 3B insert (lower red arrowhead). The reason for this phenomenon is likely alterations occurring during conventional sample preparation that includes dehydration, plastic embedding and thin sectioning.

Last but not least, pay special attention to the cargo: because autophagosomes form in the cytoplasm, they should always contain cytoplasmic cargo. In some publications, membrane-bound, electron-lucent vacuoles with no visible cargo inside them have been mistakenly called autophagic

vacuoles. However, without identifiable cytoplasmic cargo, there is no evidence on the autophagic origin of such empty vacuoles.

Questions

1. What are the main morphological criteria of autophagosomes in conventional thin sections?
2. How to differentiate amphisomes and autolysosomes from late endosomes and lysosomes in conventional thin sections?

Pay attention to these articles

Autophagy was originally described using electron microscopy (1), and later on, electron microscopy has been crucial in bringing autophagy field forward. Three-dimensional electron microscopy was used to confirm the close relationship of nascent phagophores with the endoplasmic reticulum (14, 15). More recently, three-dimensional cryo electron microscopy was used to reveal important details of the morphology of phagophores and autophagosomes (16, 22).

Take-home message

- When using transmission electron microscopy to identify and quantify autophagy pathway intermediates, pay attention to both the limiting membranes and the cytoplasmic cargo.
- Keep in mind that the electron-microscopy sections are considerably thinner than the diameter of the autophagy pathway intermediates – the sections only show a thin slice that is likely to miss part of the features of the organelles.

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Figure legends

Figure 1. Schematic drawing of the macroautophagy pathway when induced by amino acid starvation in a mammalian cell. In many cell types, phagophores form close to rough endoplasmic

reticulum and often also segregate pieces of it. Closed autophagosomes can fuse with multivesicular endosomes, forming amphisomes. Fusion of an amphisome or an autophagosome with a lysosome forms an autolysosome. After degradation of the cytoplasmic cargo by lysosomal hydrolases, the degradation products are transported back to cytosol by special pump proteins in the autolysosome limiting membrane. Lysosomes can reform from autolysosomes, and fuse with new amphisomes or autophagosomes. Autolysosomes can also fuse with the plasma membrane and empty their contents to the extracellular space. This is called exocytosis.

Figure 2. Transmission electron microscopy images of phagophores (A-E) and a putative nascent autophagosome (F) in cultured mammalian cells. Autophagy was induced by amino-acid starvation. The samples were fixed in glutaraldehyde, postfixed in reduced osmium tetroxide, dehydrated in ethanol, and embedded in epoxy resin. The red arrows indicate the phagophore membrane (A-E) or the limiting membrane of the putative nascent autophagosomes (F). Note the close spatial relationship of the rough endoplasmic reticulum (ER) and the phagophore in panels B-E. In panels C and E, the rim of the phagophore seems to be continuous with ER (inserts). Ly, lysosome; m, mitochondrion.

Figure 3. Transmission electron microscopy images of autophagosomes (AP). The samples in panels A-E were prepared as in Figure 2. Panel F shows pre-embedding immunostaining for endogenous LC3 (10). The red arrows indicate the limiting membranes of the autophagosomes. Note that the distance between the two limiting membranes varies. The membranes may be tightly attached to each other (panel D), or a narrow electron-lucent space may be visible between them (panel C). Rough endoplasmic reticulum (ER) surrounds the autophagosome in panel A. The red arrowheads in panel B insert indicate the inner and outer limiting membranes of the autophagosome. The outer limiting membrane appears abnormally thick likely due to alterations during sample preparation. A cistern of rough endoplasmic reticulum is located inside the autophagosome in panel B, very close to the inner limiting membrane. The cytoplasmic cargo includes ribosomes in all panels. The black arrows in the insert in panel D indicate some of the ribosomes in cytosol and inside the autophagosome. Autophagosomes in panels C and E also contain mitochondria (m). Panel C shows cytoplasm of three different cells, and panel E shows cytoplasm of two different cells; PM indicates the adjacent plasma membranes. Immunostaining for endogenous LC3 in panel F shows that the limiting membrane of the autophagosome contains LC3; the black dots are silver-enhanced 1.2-nm gold particles.

Figure 4. Transmission electron microscopy images of amphisomes. The samples in panels A-E were prepared as in Figure 2. Panel F shows pre-embedding immunostaining for endogenous LC3 (10). Amphisomes contain both cytoplasmic cargo and small intraluminal vesicles delivered by fusion with multivesicular endosomes. Cytoplasmic cargo is visible as intact ribosomes in panel A, and as electron-dense particulate material (partially degraded ribosomes), indicated by yellow asterisks, in panels B-F. The intraluminal vesicles are highlighted by magenta arrows in all panels, and enlarged

in the inserts in panels A, D, E and F. Immunostaining for endogenous LC3 in panel F shows that the amphisome contains LC3; the black dots are silver-enhanced 1.2-nm gold particles.

Figure 5. Transmission electron microscopy images of autolysosomes. The samples were prepared as in Figure 2. Autolysosomes contain partially degraded cytoplasmic cargo, which in starvation-induced autolysosomes is typically visible as electron-dense particulate material that represents partially degraded ribosomes (yellow asterisks in all panels). The lower structure in panel B also contains small intraluminal vesicles (black arrow), which identify it as an amphisome. The structures in panels E and F still have parts of the autophagosome inner limiting membrane visible. It is possible that these structures are amphisomes. However, the thin sections shown in the panels did not hit the small internal vesicles that would clearly identify the structures as amphisomes.

Answers

1. The double limiting membrane and the morphologically intact cytoplasmic cargo, which frequently includes ribosomes.

2. In many cases, it is difficult to tell whether a structure is an amphisome, autolysosome, late endosome or lysosome. If partially degraded cytoplasmic cargo (such as ribosome clusters) can still be identified among the contents, the structure can be classified as an amphisome or autolysosome. Immuno-electron microscopy of a known, degradation-resistant cargo protein such as superoxide dismutase may be used to help identification of the cytoplasmic cargo.