Review

Molecular mechanisms and physiological roles of Atg5/Atg7-independent alternative autophagy

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Abstract: ATG5 and ATG7 are considered to be essential molecules for the induction of autophagy. However, we found that cells lacking ATG5 or ATG7 can still form autophagosomes/autolysosomes and perform autophagic protein degradation when subjected to certain types of stress. Although the lipidation of LC3 is accepted as a good indicator of autophagy, this did not occur during ATG5/ATG7-independent alternative autophagy. Unlike conventional autophagy, autophagosomes appeared to be generated in a Rab9-dependent manner by the fusion of the phagophores with vesicles derived from the trans-Golgi and late endosomes. Therefore, mammalian autophagy can occur via at least two different pathways; the ATG5/ATG7-dependent conventional pathway and an ATG5/ATG7-independent alternative pathway.

Keywords: alternative autophagy, reticulocytes, Atg5-independent autophagy

Introduction

Autophagy is a catabolic process in which cellular constituents, including proteins, lipids, and even entire organelles, are digested using lysosomal lytic enzymes. There are at least three types of autophagy in mammals, i.e., macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is believed to be the major pathway for degrading cytoplasmic proteins and organelles.1) Macroautophagy begins with the nucleation of a double-membrane structure (the so-called isolation membrane), which expands to engulf portions of the cytoplasm as well as damaged organelles, and eventually mature into double-membrane structures called autophagosomes. Subsequently, lysosomes fuse with autophagosomes to generate autolysosomes, in which cellular constituents are broken down by acid hydrolases.2) The second type of autophagy is microautophagy, which occurs by direct invagination of the lysosomal membrane to engulf cellular constituents, followed by closure of the membrane pocket and degradation of the constituents within the lysosomal lumen.3) Microautophagy can deliver entire organelles, such as peroxisomes, directly into lysosomes. The third type of autophagy is chaperone-mediated autophagy, in which soluble cytosolic proteins containing a specific targeting motif are delivered by the cytosolic heat shock cognate 70 chaperone to the lysosomal membranes.4) After docking onto the cytosolic tail of the lysosomal receptor, the substrate protein unfolds and crosses the lysosomal membrane through a multimeric pore complex and is degraded in the lysosomal lumen. Despite the presence of these distinct types of autophagy, the term “autophagy” is usually used synonymously with macroautophagy (hereafter referred to simply as “autophagy” unless otherwise indicated).

Autophagy occurs constitutively at low levels but is accelerated by a variety of cellular stressors, such as nutrient starvation, growth factor withdrawal, DNA damage, accumulation of abnormal proteins, and organelle damage. Autophagy is necessary for development and is also crucial for maintaining homeostasis in adult organisms.5) In many physiological and pathological contexts, autophagy is a protective mechanism that facilitates the degradation of superfluous or damaged cellular constitutents.
constituents for the subsequent recycling of amino acids, lipids, nutrients, and metabolites. Therefore, a disturbance in autophagy is associated with various pathologies, such as cancer, neurodegenerative disorders, inflammatory bowel diseases, and etc.5)

The molecular basis of autophagy was first analyzed in autophagy-defective mutant yeast.1) The subsequent identification of vertebrate homologs of the yeast autophagy proteins has greatly expanded our understanding of the molecular mechanisms of autophagy. It is currently accepted that autophagy is driven by more than 30 autophagy related-proteins (Atgs), which are well conserved from yeast to mammals.6) Among these proteins, several core proteins, including Atg5 and Atg7, have long been believed to be molecules essential for autophagy. However, several years ago we identified an Atg5/Atg7-independent type of autophagy and named it “alternative autophagy”.7) Here, we describe the molecular mechanisms and physiological roles of alternative autophagy.

1. Conventional autophagy

Before describing alternative autophagy, we will briefly summarize the well-established process of Atg5-dependent autophagy (conventional autophagy) (Fig. 1). Readers who are interested in the detailed mechanism of conventional autophagy should refer to other reviews. Conventional autophagy begins with Unc-51-like kinase 1 (Ulk1), a homologue of yeast Atg1. Atg1 is a serine/threonine kinase that forms the Ulk1 complex together with Fip200, Atg13, and Atg101.6),8) In healthy conditions, Ulk1 is phosphorylated and inactivated by mammalian target of rapamycin complex 1 and AMP-activated protein kinase at different serine/threonine residues.9–11) After nutrient starvation, Ulk1 is dephosphorylated by Protein phosphatase 2A and subsequently translocates to pre-autophagosomal membranes, which are the initial platforms of the isolation membrane. In the case of DNA damage, Ulk1 is dephosphorylated by protein phosphatase, Mg2+/
Mn$^{2+}$-dependent 1D in a p53-dependent manner. This Ulk1 dephosphorylation also triggers Ulk1 translocation to pre-autophagosomal membranes.

After Ulk1 complex activation, vesicle nucleation occurs via activation of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex, which comprises PtdIns3K, Beclin 1, Vps15, and Atg14L. The subsequent elongation and closure of isolation membranes are mediated by two ubiquitin-like conjugation pathways, namely, the Atg5–Atg12 pathway and the microtubule-associated protein 1 light chain 3 (LC3) pathway. Atg7 is required for the conjugation of Atg12 to Atg5 as an E1-like enzyme. Conjugation of phosphatidylethanolamine (PE) to LC3 is mediated by the actions of Atg3 and the Atg5–Atg12 complex, as E2-like and E3-like enzymes, respectively. This event is coupled with the translocation of LC3 from the cytosol to the isolation membrane, and hence this translocation is considered to be a reliable marker of autophagy. In the final step, UV radiation resistance associated gene and the PtdIns3K complex excluding Atg14L facilitate autophagosome–lysosome fusion. Syntaxin17 is also required for this fusion. Various lines of evidence indicate that among these molecules, members of the Atg5–Atg12 conjugation system are essential for autophagy; (1) yeast Atg5 is crucial for autophagy, (2) autophagy is suppressed in some cell types in Atg5-deficient mice, (3) LC3-PE formation has never been detected in Atg5-deficient cells, and (4) Atg5 is essential for development and tissue homeostasis. However, by the assistance of maternal effect, most Atg5-deficient mouse embryos develop normally until the perinatal period, suggesting that an alternative autophagic pathway may exist that compensates for the lack of conventional autophagy in embryonic mutant mice at mid- and late-embryonic days.

2. Discovery of alternative autophagy

Autophagy is characterized by the formation of specific double-membrane vesicles that engulf intracellular components for their subsequent degradation by lysosomal enzymes. Thus, autophagy is best studied in cells by serial ultrastructural analysis during their response to autophagic inducers. However, detailed ultrastructural analyses of Atg5-deficient cells had not been performed. Therefore, to investigate the possibility that the other autophagic pathways may exist, we examined ultrastructural changes within Atg5-deficient cells after various treatments. When cells were treated with rapamycin, a specific inhibitor of mTor and a well-established autophagy inducer, we observed typical autophagic structures in wild-type mouse embryonic fibroblasts (MEFs) but not in Atg5-deficient MEFs, indicating that Atg5 is crucial for rapamycin-induced autophagy. In contrast, the addition of etoposide, a topoisomerase inhibitor that induces DNA strand breaks in mitotic cells, resulted in the formation of autophagic structures even in Atg5-deficient MEFs. The numbers and sizes of these autophagic vacuoles were equivalent in wild-type and Atg5-deficient MEFs (Fig. 2A), and the morphology of these etoposide-induced autophagic structures was indistinguishable from the autophagic vacuoles observed during rapamycin-induced conventional autophagy. Moreover, all stages of autophagosome biogenesis were observed in Atg5-deficient MEFs, including formation of isolation membranes (membrane cisternae curving around a part of the cytoplasm), autophagosomes (double-membrane vacuoles generated by sealing of the edges of the isolation membrane), and autolysosomes (single-membrane vacuoles generated by fusion of an autophagosome and lysosome). Thus, MEFs appear to perform two distinct types of autophagy, an Atg5-dependent type and an Atg5-independent type, which show similar morphological characteristics.

The presence of autolysosomes in etoposide-treated Atg5-deficient MEFs was confirmed by the immunostaining of lysosome-associated membrane protein 2 (Lamp2). In general, lysosomes are spread diffusely throughout the cytosol so that Lamp2 immunostaining appears as small well-separated puncta throughout the cytosol. During autophagy, however, lysosomes fuse with autophagic vacuoles, resulting in an increase in the intensity and size of Lamp2 fluorescent puncta (Fig. 2B). Correlative light electron microscopy images, in which Lamp2-GFP expressing cells were examined by fluorescence microscopy followed by electron microscopic (EM) analysis, confirmed that the large Lamp2-positive structures are identical to the autolysosomes observed in etoposide-treated Atg5-deficient MEFs. The induction of alternative autophagy was further confirmed by the addition of bafilomycin A1, which inhibits the fusion between autophagosomes and lysosomes. The formation of autophagosomes and autolysosomes was greatly increased and decreased, respectively, in etoposide/bafilomycin A1-treated Atg5-deficient MEFs. Thus, mammalian cells possess at least two different autophagic pathways; the conventional Atg5-dependent pathway and an alternative Atg5-independent pathway.
3. Origin of alternative autophagic membranes

The source of autophagic membranes is an issue in mammalian autophagy. As for conventional autophagy, the endoplasmic reticulum (ER) has been implicated in the generation of autophagosomal membranes. Firstly, double-FYVE-containing protein 1, which is an ER-localized protein, was found on pre-autophagosomal punctuate structures called “omegasomes” during the early steps of conventional autophagy.17) Moreover, interconnections between the ER and isolation membranes were revealed by 3-D EM.18) Mitochondrial outer membranes, mitochondria-ER contact site membranes, and plasma membranes have also been reported as sources of autophagosomal membranes, indicating that conventional autophagosomal membranes may be derived from multiple sources and at multiple locations.19)

In contrast to the multiple origins of autophagic membranes of conventional autophagy, the membranes observed during activation of the alternative pathway appear to originate exclusively from the Golgi apparatus. This conclusion is based on the following five seminal observations: (1) almost all autophagic vacuoles of alternative autophagy were localized near the Golgi apparatus, (2) Golgiministack formation preceded autophagosome generation, (3) some isolation membranes extended from the Golgi membranes, (4) trans-Golgi proteins were observed on autophagosomes and autolysosomes, and (5) the depletion of Golgi proteins inhibited alternative autophagy but not conventional autophagy.7) Evidence from various sources suggests that there are two types of biological membranes: the 8.5 nm thin type, such as the membranes of the ER and mitochondria, and the 10 nm thick type, such as the membranes of lysosomes, endosomes, and trans-Golgi. These two membrane types do not fuse. In alternative macroautophagy, autophagic membranes are generated from Golgi-derived thick membranes, and thus can easily fuse with the thick membranes of lysosomes.

4. Molecular mechanisms of alternative autophagy

What molecules are involved in alternative autophagy? Similar to conventional autophagy, the Ulk1 protein is required in the initial step of alternative autophagy. The addition of etoposide induced the accumulation and dephosphorylation of
Ulk1 in Atg5-deficient MEFs. Furthermore, few autophagic membranes were observed within Ulk1-silenced, Atg5-deficient MEFs in response to etoposide. Similar results were also observed when Fip200, another component of the Ulk1 complex, was silenced. Thus, these results demonstrated that Ulk1 functions in the initiation of both conventional and alternative autophagy7) (Fig. 1). The PtdIns3K complex is also essential for both types of autophagy. The mechanism determining the specific activation of one pathway or another by different stressors remains a mystery. Perhaps these two forms of autophagy are selectively activated by phosphorylation/dephosphorylation of different serine/threonine residues in Ulk1.

Although Ulk1 and PI3K complexes participate in both conventional and alternative autophagy, neither the Atg5-Atg12 nor the LC3-conjugation pathway is required for alternative autophagy. Furthermore, conversion of LC3 (or LC3-I) to PE-conjugated LC3-II does not occur in alternative autophagy.7) Thus, it is unknown as to how the extension and closure of autophagic membranes are accomplished during alternative autophagy without these two ubiquitin-like systems. Detailed morphological analysis has, however, provided some clues. We demonstrated that the elongation and closure of isolation membranes, which originate from trans-Golgi cisternae, occurs by their fusion with endosomal vesicular membranes. The involvement of trans-Golgi/ endosomal fusion in the extension and closure of isolation membranes was confirmed by the colocalization of mannose-6-phosphate receptors (a trans-Golgi/late endosomal marker) and syntaxin 7 (a late endosomal marker) with autolysosomes in etoposide-treated Atg5-deficient MEFs. The formation of isolation membranes by trans-Golgi/endosomal fusion is also supported by studies showing a requirement for Rab9, which is a GTPase essential for the trafficking of proteins from late endosomes to trans-Golgi membranes. First, GFP-Rab9 was colocalized with autolysosomes in etoposide-treated Atg5-deficient MEFs, and this colocalization was increased by the transfection of GFP-Rab9Q66L, a constitutively active Rab9 mutant,20) and reduced by GFP-Rab9S21N, a GDP-prefering dominant-negative Rab9 mutant.20) Moreover, Rab9 silencing by a targeted siRNA reduced the number of autophagic vacuoles, but induced the accumulation of isolation membranes.7) Numerous isolation membranes are normally generated by etoposide exposure, and hence siRab9 treatment did not merely slow down the progression of autophagy but rather inhibited autophagosome maturation. Presumably, the Rab9-mediated extension and closure of isolation membranes in the alternative autophagy pathway replaces Atg5/Atg7/LC3 in the conventional autophagy pathway (Fig. 1).

Recently, conventional autophagy has been reported to progress slowly in the absence of Atg5.21) However, this autophagy is different from alternative autophagy, because (1) syntaxin17 is crucial for conventional autophagy, but not for alternative autophagy, (2) autophagosome membrane originates from mitochondria-associated ER membrane in conventional autophagy but from Golgi membrane in alternative autophagy.

5. Physiological roles of alternative autophagy

A variety of potential physiological functions of conventional autophagy have been identified by the analysis of systemic and tissue-specific Atg-gene knockout mice, including its crucial roles in preimplantation fetal development, resistance to early neonatal starvation, clearing of neuronal protein aggregates, maintenance of cardiac function, and cell differentiation during erythropoiesis, adipogenesis, and lymphopoesis.23) In contrast, investigation of the biological roles of alternative autophagy has just begun. However, we have already discovered one remarkable physiological role of alternative autophagy in erythrocyte maturation.22) Erythrocytes undergo enucleation and the clearance of mitochondria during terminal differentiation, and autophagy is considered to be involved in the latter process (Fig 3A). However, erythrocyte maturation proceeds normally in Atg5-deficient embryos.23) Ultrastructural analysis also demonstrated that autophagic vacuoles in reticulocytes engulfed and digested mitochondria in both wild-type and Atg5-deficient embryos (Fig 3B), indicating that conventional autophagy is not involved in the elimination of mitochondria from erythrocytes. In contrast, the elimination of mitochondria was not observed in Ulk1-deficient reticulocytes (Fig 3B). Because Ulk1, the initiator of conventional and alternative autophagy, is involved in erythrocyte maturation, but Atg5, an essential molecule for conventional autophagy, is not required, mitochondrial clearance from erythroid cells should be largely dependent on alternative autophagy (Fig 3C). Alternative autophagy also has other biological roles; i.e., it is crucial for mitochondrial clearance during the transformation of differentiated cells to induced pluripotent stem cells.24) Furthermore, it also functions to suppress
inflammatory bowel disease.\textsuperscript{25) A deeper understanding of the physiological and pathological relevance of alternative autophagy will be obtained from the analyses of knockout mice with targeted deletions of genes specific to this alternative pathway.

It is considered that the molecules involved in conventional and alternative autophagy are used in a cell type-dependent and stimulus-dependent manner. For example, starvation-induced autophagy is largely dependent on conventional autophagy, whereas DNA damage simultaneously induces both types of autophagy. More importantly, these two autophagic pathways degrade different molecules and organelles, and thereby play different physiological roles. As described, mitochondria are eliminated from reticulocytes via alternative autophagy. However, conventional autophagy is simultaneously activated in reticulocytes. Conventional autophagy is not involved in mitochondrial clearance, but is involved in the clearance of ribosomes and ERs. Therefore, these two autophagic systems play different roles within the same cells. p62 is a protein that acts as a cargo receptor for the autophagic degradation of substrates, but it is only degraded by conventional autophagy. Therefore, alternative autophagy cannot degrade many of the substrates that interact with p62. To understand the physiological roles of alternative autophagy, it is crucial to identify the molecules that deliver substrates to the alternative autophagy machinery.

**Closing remarks**

In this review, we describe two distinct autophagic pathways; conventional autophagy and alternative autophagy, and compare their molecular mechanisms. The presence of at least two mechanistically distinct forms of autophagy in mammalian cells indicates that autophagy is a highly adaptable cellular stress response. Further elucidation of the biological roles of autophagy will require a more complete understanding of (1) the molecular mechanisms of alternative autophagy, (2) the unique functional roles of these two pathways in vivo, and (3) the contribution of each pathway to disease pathology.
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Profile

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