Minireview

Biological Roles of Alternative Autophagy

Shigeomi Shimizu*

Department of Pathological Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan
*Correspondence: shimizu.pcb@mri.tmd.ac.jp
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Atg5 and Atg7 have long been considered as essential molecules for autophagy. However, we found that cells lacking these molecules still form autophagic vacuoles and perform autophagic protein degradation when subjected to certain stressors. During this unconventional autophagy pathway, autophagosomes appeared to be generated in a Rab9-dependent manner by the fusion of 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, Atg5, erythrocyte maturation, Golgi membrane, proteolysis

INTRODUCTION

Autophagy is a catabolic process in which cellular contents, including proteins and organelles, are degraded using autophagic vacuoles. Autophagy continuously occurs at low levels and is activated by a variety of cellular stressors, including nutrient starvation, DNA damage, the accumulation of damaged proteins, and in response to organelle damage. In many physiological and pathological contexts, autophagy functions to protect cells from such stressors.

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 (Nakatogawa et al., 2009). In this type of autophagy, subcellular constituents are enclosed by double-membrane structures called autophagosomes, and cargos are degraded by lysosomal enzymes. The second type of autophagy is microautophagy, which occurs by the direct invagination of lysosomal membranes to engulf cellular constituents (Li et al., 2012). Microautophagy can deliver entire organelles 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 (Kaushik and Cuervo, 2012). After docking with the lysosomal membrane receptor, the substrate proteins unfold, penetrate into the lysosomes, and are degraded. Although these three protein degradation systems are mediated by different mechanisms and different molecules, they are categorized as “autophagy” because lysosomes are involved in their proteolysis.

CHARACTERISTICS OF MACROAUTOPHAGY

Macroautophagy (hereafter referred to simply as “autophagy” unless otherwise indicated) begins from the generation of an omegasome, which is the starting point of the isolation membrane. The isolation membrane expands and its leading edges fuse together to form a double-membrane vesicle called the autophagosome, in which intracellular constituents are enclosed (Mizushima et al., 2002). By the fusion between an autophagosome and lysosome, cargos are degraded by lysosomal proteases, lipases, and DNases (Fig. 1).

Autophagy can be classified into different types by its induction mechanism; i.e., constitutive autophagy and stimul-
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Fig. 1. Hypothetical model of autophagy. There are at least two modes of macroautophagy, i.e. conventional and alternative autophagy. Conventional autophagy requires Atg5 and Atg7, is associated with LC3 modification, and is thought to originate from the ER membrane. In contrast, alternative autophagy occurs independently of Atg5 and Atg7, as well as LC3 modification. The generation of autophagic vacuoles in alternative autophagy is mediated by the fusion of isolation membranes with vesicles derived from the trans-Golgi as well as late endosomes, in a Rab9-dependent manner.

**Table 1. Classification of autophagy from the view of inducers and substrates**

| Inducer                   | Substrate                                      | Substrate                                      |
|---------------------------|------------------------------------------------|------------------------------------------------|
|                          | Bulk autophagy (non-specific degradation)      | Selective autophagy (specific degradation)     |
| Constitutively working autophagy (maintain homeostasis) | Cellular metabolism                           | Cell protection                               |
| Inducible autophagy (response to various stimuli) | Replace old proteins/organelles with new ones | Degrade unfavorable, damaged protein and organelles |
|                          | Nutrient supply                               | Cellular adaptation                            |
|                          | Stravation-induced                            | Cellular stress (DNA damage etc.)              |
|                          | Rapamycin-treated                             |                                                |

DEPENDENT INDUCTIBLE AUTOPHAGY (Table 1). The former type of autophagy occurs at low levels but continuously degrades unfolded and aged proteins to maintain cellular homeostasis. The latter type of autophagy occurs in a large scale to prevent cellular damage against various stressors. Autophagy is also classified by the type of cargo it degrades; namely, bulk autophagy and selective autophagy. Autophagy was originally considered to degrade various constituents nonselectively, in contrast to the ubiquitin-proteasome degradation system. However, increasing lines of evidence indicate the presence of a cargo-specific type of autophagy (selective autophagy) that eliminates specific organelles (Komatsu and Ichimura, 2010), including peroxisomes (pexophagy), pathogens (xenophagy), and mitochondria (mitophagy). In selective autophagy, cargos are recognized by cargo receptors and are subsequently enclosed by autophagic structures. When these classification criteria are applied, the well-studied starvation-induced type of autophagy belongs to inducible and bulk autophagy. This type of autophagy is considered to be useful for the compensation of nutrients during times of shortage. On the other hand, genotoxic stress-induced autophagy is classified into inducible and selective autophagy, which degrades damaged proteins and organelles to eliminate unfavorable constituents. Neuron-specific autophagy-deficient mice were found to develop neurodegenerative disorders, with the accumulation of ubiquitin-positive proteins in their neurons (Komatsu et al., 2005), indicating that constitutive autophagy occurs in neurons to selectively degrade ubiquitin-positive proteins. Thus, autophagy plays various essential roles in stress responses and for maintaining cellular homeostasis.

**CLASSIFICATION OF AUTOPHAGY**

Autophagy is also classified into two types based on their molecular mechanisms, i.e., the Atg5-dependent conventional type and the Atg5-independent alternative type (Fig. 1). Atg5 has long been believed to be an essential molecule for autophagy, but recently we discovered an Atg5-independent type of alternative autophagy (Nishida et al., 2009).

As described above, autophagy is defined by the formation of specific double-membrane vesicles that engulf intracellular components, and their subsequent degradation by lysosomal enzymes. This characteristic morphology is easily identified using electron microscopy (EM), and hence ultrastructural analysis has long been used to study autophagy. In particular, starvation-induced and rapamycin (a specific
inhibitor of mTor)-induced autophagy has been extensively examined using EM. In contrast, ultrastructural information of cells receiving other stressors has not been fully elucidated. We thus analyzed ultrastructural features within cells after treatment with etoposide, a DNA damaging reagent, and surprisingly found the representative autophagic structures (isolation membranes, autophagosomes, and autolysosomes) not only in wild-type mouse embryonic fibroblasts (MEFs) but also in Atg5-deficient (Atg5KO) MEFs (Nishida et al., 2009). These autophagic structures were indistinguishable from those observed during starvation-induced conventional autophagy. Thus, MEFs appear to perform two distinct types of autophagy, an Atg5-dependent conventional type and an Atg5-independent alternative type.

Several lines of evidence confirmed the existence of alternative autophagy in etoposide-treated Atg5KO MEFs (Nishida et al., 2009). First, the correlative light-electron microscopy assay using Lamp2-GFP-expressing cells indicated that autolysosomes clearly contain lysosomal proteins. Second, the addition of bafilomycin A1, which inhibits the fusion between autophagosomes and lysosomes, greatly increased and decreased the formation of autophagosomes and autolysosomes, respectively. Third, bafilomycin A1 suppressed not only autolysosome generation, but also autolysosomal proteolysis.

**MEMBRANE SOURCE OF ALTERNATIVE AUTOPHAGY**

Morphological features are basically common to both types of autophagy, but their membrane sources are different (Table 2). Regarding conventional autophagy, the endoplasmic reticulum (ER) membrane, mitochondrial outer membrane, and mitochondria-ER contact site membrane have been reported as sources of autophagosomes (Tooze and Yoshimori, 2010). In contrast, the membranes used in alternative autophagy are thought to originate from the Golgi apparatus and late endosomes. This conclusion is based on the following five seminal observations (Nishida et al., 2009): (1) almost all autophagic vacuoles observed in alternative autophagy were localized near the Golgi apparatus, (2) Golgi ministack 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 (Yamaguchi et al., 2016). Evidence from various sources suggests that there are two types of biological membranes: the thin type (8.5 nm), such as the membranes of the ER and mitochondria, and the thick type (10 nm), such as the membranes of lysosomes, endosomes, and the trans-Golgi. These two membrane types do not fuse. In alternative autophagy, trans-Golgi-derived thick membranes and lysosomal thick membranes fuse to generate autophagosomes.

**Initial step of autophagy**

Several of the core molecules required for autophagosome maturation are different between the two types of mammalian autophagy (Table 2). However, both types of autophagy utilize several of the same molecules at the initial step. One such molecule is Unc-51-like kinase 1 (ULK1), a homologue of yeast Atg1. ULK1 is a serine/threonine kinase that forms the ULK1 complex together with Fip200, Atg13, and Atg101 (Wong et al., 2013). 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 (Egan et al., 2011; Kim et al., 2011; Shang et al., 2011). In the case of starvation-induced conventional autophagy, ULK1 is dephosphorylated by protein phosphatase 2A and subsequently translocates to pre-autophagosomal membranes (Wong et al., 2015), which are the initial platforms of the isolation membrane. We demonstrated that during DNA damage-induced conventional autophagy, ULK1 dephosphorylation is facilitated by ULK1 puncta formation, ULK1 kinase function (assessed by Atg13 phosphorylation), DFCP1 puncta formation, and the subsequent progression of autophagy. Because point mutations at Ser637 almost completely inhibited the downstream events, this serine residue was concluded to be crucial for genotoxic stress-induced conventional autophagy (Torii et al., 2016).

The ULK1 protein is also known to be crucial for alternative autophagy. The addition of etoposide induced the accumulation and phosphorylation of ULK1 in Atg5-deficient MEFs. Furthermore, no 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, ULK1 is required for the initial step of both conventional and alternative autophagy (Nishida et al., 2009), and the mechanism for ULK1 activation during alternative autophagy remains to be elucidated.

**CORE MACHINERY OF AUTOPHAGY**

After ULK1 complex activation, vesicle nucleation occurs via activation of the class III phosphatidylinositol 3-kinase
BIOLOGICAL ROLES OF ALTERNATIVE AUTOPHAGY

A variety of potential physiological functions of conventional autophagy have been demonstrated by the analysis of systemic and tissue-specific Atg-gene knockout mice, including resistance to early neonatal starvation, clearing of neuronal protein aggregates, and maintenance of cardiac function (Mizushima and Levine, 2010). On the other hand, research on the biological roles of alternative autophagy has just been started. The first identified biological role is mitochondrial elimination during erythrocyte maturation. Erythroblasts lose their nuclei to become reticulocytes, and reticulocytes transform into erythrocytes by the elimination of their mitochondria. Autophagy is involved in the latter process. Therefore, conventional and alternative autophagy, but mainly functions in mitochondrial elimination during erythrocyte maturation. Mitochondrial elimination mainly occurs via Ulk1-dependent alternative autophagy and only partially via Atg5-dependent conventional autophagy.

Fig. 2. Involvement of alternative autophagy in mitochondrial clearance during erythrocyte maturation. (A) The final stage of red blood cell maturation. During erythrocyte maturation, erythroblasts lose their nuclei to become reticulocytes, and reticulocytes transform into erythrocytes by the elimination of their mitochondria. (B) Mechanism of mitochondrial elimination during erythrocyte maturation. Mitochondrial elimination mainly occurs via Ulk1-dependent alternative autophagy and only partially via Atg5-dependent conventional autophagy.
(pro)insulin granules in glucose-deprived β-cells. When β-cells are subjected to glucose deprivation, crinophagy (Orci et al., 1984) and starvation-induced nascent granule degradation (SINGD) (Goginashvili et al., 2015) are the main pathways for the degradation of unused (pro)insulin. These pathways degrade old and fresh insulin granules, respectively, via their direct fusion with lysosomes. Alternative autophagy also plays a subsidiary function. Interestingly, despite crinophagy/SINGD not being macroautophagy, they are largely suppressed in the β-cells lacking conventional autophagy. In such cells, alternative autophagy mainly contributed to degrade insulin granules. The alternative autophagy also plays a role in mitochondrial elimination from dedifferentiating iPSC cells (Ma et al., 2015) and in the protection of intestinal epithelial cells from inflammatory bowel disease (Ra et al., 2016). A deeper understanding of the physiological and pathological relevance of alternative autophagy should emerge from analyses of knockout mice with targeted deletions of genes specific to the alternative autophagic pathway.

CONCLUSION

In this review, we described two distinct autophagic pathways, namely, conventional and alternative autophagy, and compared their nature. The presence of at least two mechanistically distinct forms of autophagy in mammalian cells underscores autophagy as 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 pathology, particularly of diseases associated with the accumulation of misfolded proteins or damaged organelles.

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