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Molecules and their functions in autophagy

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Abbreviations: AMPK, AMP-activated protein kinase; Atg, Atg proteins; CMA, chaperone-mediated autophagy; ESCRT, endosomal sorting complex required for transport; IP3, myo-inositol-1,4,5-triphosphate; mTOR, mammalian Target of rapamycin; mTORC1, mTOR complex 1; NSF, orthologue of the N-ethylmaleimide-sensitive fusion; PAS, pre-autophagosome; PE, phosphatidylethanolamine; PI(3)P, phosphatidylinositol 3-phosphate; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species

Abstract

Autophagy is a self-degradation system of cellular components through an autophagosomal-lysosomal pathway. Over the last 15 yr, yeast genetic screens led to the identification of a number of genes involved in the autophagic pathway. Most of these autophagy genes are present in higher eukaryotes and regulate autophagy process for cell survival and homeostasis. Significant progress has recently been made to better understand the molecular mechanisms of the autophagy machinery. Especially, autophagy process, including the regulation of autophagy induction through mTOR and the nucleation and elongation in autophagosome formation through class III phosphatidylinositol 3-kinase complex and ubiquitin-like conjugation systems, became evident. While many unanswered questions remain to be answered, here, we summarize the recent process of autophagy with emphasis on molecules and their protein complexes along with advanced molecular mechanisms that regulate the autophagy machinery.

Keywords: ATG2 protein, human; ATG5 protein, human; ATG9B protein, human; ATG12 protein, human; ATG16L1 protein, human; autophagy; class III phosphatidylinositol 3-kinases; MTOR protein, human; ULK1 protein, human

Introduction

Autophagy is an evolutionarily conserved and highly regulated lysosomal pathway that degrades macromolecules (e.g. proteins, glycogen, lipids and nucleotides) and organelles (Cuervo, 2004; Levine and Klionsky, 2004). Recent progress has demonstrated that autophagy plays an essential role in cellular development and differentiation (Levine and Klionsky, 2004) and its dysregulation is implicated in various diseases, including cancer, infectious disease, obesity, aging and neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases (Huang and Klionsky, 2007; Mizushima *et al*., 2008; Lee *et al*., 2012).

Depending on the delivery route of the cytoplasmic material to the lysosome, there are three major types of autophagy in eukaryotes; 1) chaperone-mediated autophagy (CMA), 2) microautophagy and 3) macroautophagy, hereafter referred to as autophagy (Klionsky, 2005). CMA allows the direct lysosomal import of unfolded, soluble proteins which contain a particular pentapeptide motif. In microautophagy, cytoplasmic material is directly engulfed into the lysosome at the surface of the lysosome by membrane rearrangement. Autophagy involves the sequestration of cytoplasm into a double-membrane cytosolic vesicle, referred to as an autophagosome that subsequently fuses with a lysosome to form an autolysosome for the degradation by lysosomal hydrolases (Klionsky and Emr, 2000). Autophagy is the major regulated- cellular pathway for degrading long-lived proteins and is the only known pathway for degrading cytoplasmic organelles (Yang and Klionsky, 2009). Autophagy consists of several sequential steps, which are induction, autophagosome formation, autophagosome-lysosome fusion and degradation. Although autophagy has been extensively studied at the cellular level for more than four decades, its molecular mechanisms have just started to be elucidated in the past few years, mainly due to the application of yeast genetics. In this review, we summarize the

Table 1. Identification of Atg genes and their functions in mammals.

Autophagy-related gene (Atg) - products	
Mammalian Atg gene	Functions
ULK1, ULK2	Protein Kinase: Atg1-Atg13-Atg17-Atg29 complex
Atg2	Atg9/Atg2-Atg18 complex
Atg3	E2-like enzyme for Atg8s-lipidation
Atg4A,B,C,D	Cysteine protease: Atg8s-activation and delipidation
Atg5	Atg12-Atg5 conjugate: E3-like activity for Atg8s-lipidation
Beclin-1	Subunit of Vps34 PI3K complex
Atg7	E1-like enzyme for Atg12-and LC3-conjugation
LC3, GATE-16, GABARAP	Modifier: Conjugates to PE to localize to Autophagosome
Atg9L1, L2	Atg9 interacts Atg2-Atg18 complex: membrane-bound
Atg10	E2-like enzyme for Atg12-conjugation
Atg12	Modifier: Conjugates to Atg5
Atg13	mTor signaling: Atg1-Atg13-Atg17-Atg29 complex
Atg14	Subunit of Vps34 PI3K complex
Atg16L	Complex between Atg16 and Atg12-Atg5 conjugate
FIP200	Atg1-Atg13-Atg17-Atg29 complex
WIPI-1,2,3,4	Atg9/Atg2-Atg18 complex

molecular mechanisms of autophagy process, especially focusing on autophagosome formation.

The process of autophagy

While autophagy has been studied in mammals since the 1950's, yeast genetics has allowed us to understand this process at a molecular level. To date, 32 genes that are involved in autophagy have been identified in mammals (Table 1) and these have been termed as autophagy-related genes (Atg) (He and Klionsky, 2009). Among these, 16 genes (Atg 1-10, 12-14, 16 and 18) are required for all types of autophagy mentioned above (Suzuki and Ohsumi, 2007; Xie and Klionsky, 2007; Longatti and Tooze, 2009). These Atg proteins function at several physiologically continuous steps in autophagy and are generally classified into six groups. (1) The ULK1 kinase complex (ULK1-mAtg13-FIP200-Atg101) for the induction of autophagy, (2) Atg9 for recycling membrane, (3) class III phosphatidylinositol 3-kinase (PI3K) complex (Vps34-Beclin1-Vps15-mAtg14) for vesicle nucleation, (4) phosphatidylinositol 3-phosphate[PI(3)P]-binding Atg2-Atg18 complex (WIPI1/2 in mammals), (5) Atg12-Atg5-Atg16L conjugation system and (6) Atg8 conjugation system involving phosphatidylethanolamine (Atg8-PE) for membrane expansion (Mizushima, 2010).

Regulation of autophagy induction through mTOR and ULK1 complexes

Under stress conditions such as amino acid starvation, autophagy is strongly induced in many types of cultured cells. The effects of individual amino acids differ in their abilities to regulate autophagy. Amino acids including Leu, Tyr, Phe, Gln, Pro, His, Trp, Met and Ala suppress autophagy in ex vivo perfused liver (Mortimore and Pösö, 1987). However, such profiles depend on cell types showing their different amino acid metabolisms in tissues. The questions on how cells sense amino acid concentration and physiological significance of autophagy regulation by amino acid starvation are not fully understood yet. Accumulated reports demonstrated that amino acid signaling pathways exist, which involve activation of serine/ threonine kinase mammalian Target of rapamycin (mTOR) and the subsequent regulation of the class III PI3K. The mTOR is involved in the control of multiple cell processes in response to changes in nutrient conditions (Nobukuni *et al*., 2005). Especially, mTOR complex1 (mTORC1) requires Rag GTPase, Rheb and Vps34 for its activation and subsequent inhibition of autophagy in response to amino acids (Wullschleger *et al*., 2006; Sancak *et al*., 2010). Energy levels are primarily sensed by AMP-activated protein kinase (AMPK), a key factor for cellular energy homeostasis. In low energy states, AMPK is activated and the activated AMPK then inactivates mTORC1 through TSC1/TSC2 and Rheb protein (Gwinn *et al*., 2008). Thus, inactivation of mTORC1 is essential for the induction of autophagy and plays a central role in autophagy. In addition to amino acid signaling, hormones, growth factors and many other factors, including bcl-2 (Levine *et al*., 2008), reactive oxygen species (ROS) (Botti *et al*., 2006), calcium (Green and Wang, 2010), BNIP3 (Tracy *et al*., 2007), p19ARF

(Sherr, 2006), DRAM (Crighton *et al*., 2007), calpain (Xia *et al*., 2010), TRAIL (Mills *et al*., 2004), FADD (Pyo *et al*., 2005) and myo-inositol-1,4,5-triphosphate (IP3) (Sarkar and Rubinsztein, 2006), have also been reported to regulate autophagy. But, not all autophagy signals are transduced through mTOR signaling. A recent study showed that small-molecule enhancers of the cytostatic effects of rapamycin (called SMERs) induce autophagy independently of mTOR (Sarkar *et al*., 2007).

The essential process of autophagy is conserved from yeast to mammals. A distinct difference between yeast and mammalian autophagy is the presence of transient pre-autophagosome (PAS) in yeast. A protein complex composed of Atg1 (serine/threonine kinase), Atg13 (scaffold protein), Atg17, Atg29 and Atg31 is required for the formation of PAS structure and functions in the initial step of autophagosome formation in yeast. Similarly, the ULK1 kinase complex consisting of ULK1 (Atg1), mAtg13 (Atg13), FIP200 (Atg17) and Atg101 exists in mammals. Unlike in yeast, however, the ULK1 kinase complex in mammal is likely to be stably formed for autophagosome formation regardless of nutrient conditions (Ganley *et al*., 2009; Mercer *et al*., 2009; Kuma and Mizushima, 2010).

Activities of the ULK1 kinase complex are regulated by mTOR, depending on nutrient conditions. Under growing and high-nutrient conditions, the active mTORC1 interacts with the ULK1 kinase complex (ULK1-mAtg13-FIP200-Atg101) and phosphorylates ULK1 and mAtg13, and thus inhibits the membrane targeting of the ULK1 kinase complex. During starvation condition, on the other hand, the inactivated mTORC1 dissociates from the ULK1 kinase complex and results in the ULK1 kinase complex free to phosphorylate components, such as mAtg13 and FIP200, in the ULK1 kinase complex, leading to autophagy induction (Mizushima, 2010) (Figure 1).

The Class III PI3K complex in autophagosome nucleation

Autophagosome formation process is composed of isolation membrane nucleation, elongation and completion steps. In mammals, the class III PI3K complex plays an essential role in isolation membrane nucleation during autophagy (Mariño and López-Otín, 2004), while the class I PI3K pathway is also involved in autophagy regulation through insulin signaling cascade to activate mTOR and PKB (Yang and Klionsky, 2009). The class III PI3K (Vps34) is associated with Beclin1 (Atg6) and p150, the homolog of Vps15 (phosphoinositide-3-kinase, regulatory subunit 4), to form the class III PI3K core complex. As the first step of autophagosome formation, autophagosome nucleation

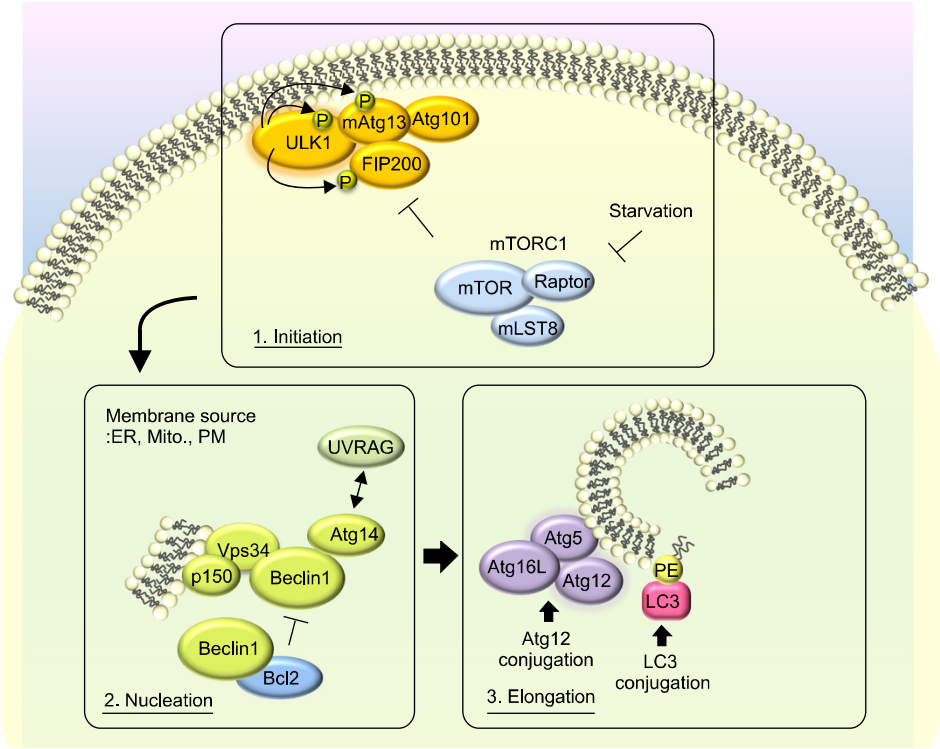

Figure 1. Molecular regulation of autophagosome formation in mammalian macroautophagy. Three major steps consisting of the initiation, nucleation and elongation in autophagosome formation are described. ER, endoplasmic reticulum; Mito, mitochondria; PM, plasma membrane; PE, phosphatidylethanolamine.

requires Beclin1. Mammalian Beclin1, which was identified as an interaction partner of Bcl-2 (Liang *et al*., 1998), associates with the class III PI3K core complex to generate PI(3)P (Funderburk *et al*., 2010). The interaction of Beclin1 with Vps34 is known to promote the catalytic activity of VPS34 and increase levels of PI(3)P, but is dispensable for the normal function of Vps34 in protein trafficking or recruitment of endocytic events (Wurmser *et al*., 1999; Zeng *et al*., 2005).

Beclin1 plays an essential role in the initiation step of autophagy and is also involved in the pathogenesis of diseases such as pathogen infection, cancer and neurodegeneration (Levine and Kroemer, 2008). Despite the proposed roles, molecular function of Beclin1 is poorly understood. Some hints on the molecular function of Beclin1 are likely to be found in its many binding partners and several studies actually provide biological significance of these interactions. Depending on the proteins recruited by Beclin1, class III PI3K complexes differentially regulate the process of autophagosome formation (Proikas-Cezanne and Codogno, 2011). Various additional components of Beclin1 complex were recently identified. (1) Atg14L (the probable mammalian homologue of yeast Atg14) exists primarily in a Beclin1-Atg14L- Vps34-Vps15 complex that is essential for the formation of autophagosome (Itakura *et al*., 2008; Funderburk *et al*., 2010). (2) UV radiation resistance-associated gene (UVRAG) is present in a Beclin1-UVRAG-Vps34-Vps15 complex. Atg14L and UVRAG are located in the Beclin1-Vps34-Vps15 complex in a mutually exclusive manner (Liang *et al*., 2008). (3) Activating molecule in Beclin1-regulated autophagy (AMBRA1) is a positive regulator of the Beclin1-dependent autophagy and regulates developments of the nervous system (Fimia *et al*., 2007). (4) Bax-interacting factor 1 (Bif1) interacts with Beclin1 through UVRAG. Bif1 positively regulates autophagy and suppresses of tumorigenesis (Takahashi *et al*., 2007). Other additional proteins, including PTEN-induced putative kinase 1 (PINK1), neuronal isoform of protein-interaction, specifically with TC10 (nPIST) (Yue *et al*., 2002), IP3 receptor (IP3R) (Vicencio *et al*., 2009), the pancreatitis- associated protein, vacuole membrane protein 1 (VMP1) (Ropolo *et al*., 2007) and high mobility group box 1 (HMGB1) (Kang *et al*., 2010), have also been identified as Beclin1-interacting proteins.

In contrast to these positive regulators, there are negative regulators among Beclin1-interacting partners. (5) RUN domain- and cysteine-rich domain-containing Beclin1-interacting protein (Rubicon) negatively regulates autophagosome maturation by interacting with Beclin1 contrastively to Atg14L (Matsunaga *et al*., 2009). (6) Bcl2 and BclXL also bind to Beclin1 through their BH3 domain and inhibit autophagy by disrupting the interaction between Beclin1 and class III PI3K complex (Pattingre *et al*., 2005; He and Levine, 2010). Despite identification of numerous aforementioned molecules, it is not clear how class III PI3K complex regulates autophagosome nucleation. According to a recent study, one of Beclin1-interacting protein, Barkor/ Atg14(L), was suggested to directly bind to membrane composed of PI(3)P generated by PI3KC3 (Fan *et al*., 2011). By binding preferentially to the curved membranes incorporated with PI(3)P, Barkor may be capable of sensing and maintains membrane curvature to initiate autophagosomal membrane formation. However, the assembly of the class III PI3K complexes and how they act with other components in the class III PI3K complex need to be further characterized.

Ubiquitin-like protein conjugation systems in autophagosome expansion

The expansion of the isolation membrane is basically the simultaneous elongation and nucleation of little cistern. It is not known yet how the Atg12-Atg5 complex recruits additional membranes, but two ubiquitin-like protein conjugation systems are involved in the expansion of autophagosome membranes (Figure 2). The first ubiquitin-conjugation

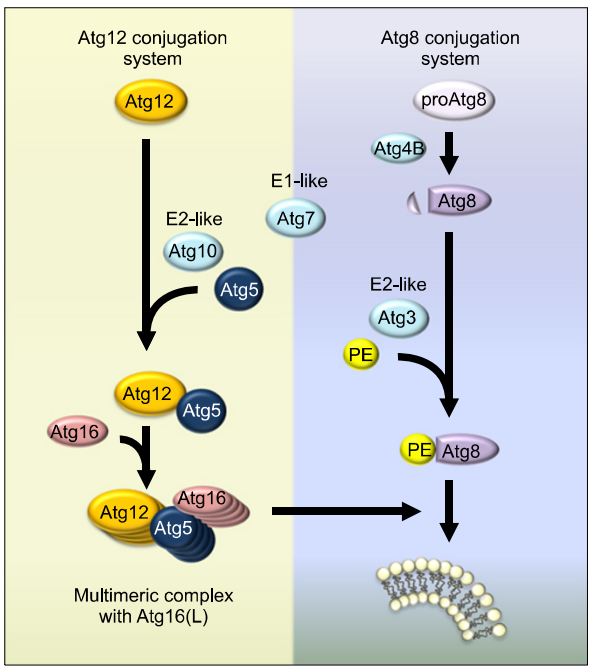

Figure 2. Ubiquitin-like protein conjugation systems in autophagy.

system is Atg12-Atg5-Atg16L which is essential for the formation of pre-autophagosomes. Atg12 is a 186-amino acid protein and is conjugated to Atg5 (Kuma *et al*., 2002). The carboxy-terminal glycine residue of Atg12 is activated by E1-like Atg7 through a high energy thioester bond in an ATP-dependent manner (Mizushima *et al*., 1998; Kim *et al*., 1999; Yuan *et al*., 1999; Tanida *et al*., 2001). Atg12 is then transferred to E2-like Atg10 (Shintani *et al*., 1999) and finally attached to lysine 149 of Atg5 *via* an isopeptide bond (Mizushima *et al*., 1998). The Atg12-Atg5 conjugate further interacts with Atg16L1 to form a ~350 kDa multimeric Atg12-Atg5-Atg16 protein complex through the homo-oligomerization of Atg16 (Mizushima *et al*., 1999) (Figure 2, left). Once autophagosome formation is completed, Atg proteins are released back to the cytoplasm by a yet uncharacterized mechanism.

The second ubiquitin-like protein conjugation system is the modification of LC3 (a mammalian homolog of Atg8) by the phospholipid phosphatidylethanolamine (PE) (Ichimura *et al*., 2000), an essential process for the formation of autophagosomes (Figure 2, right). LC3 is cleaved by cysteine protease Atg4 and then conjugated with PE by Atg7 and Atg3, a second E2-like enzyme. This lipidated LC3-II then associates with newly forming autophagosome membranes. LC3-II remains on mature autophagosomes until its fusion with lysosomes (Burman and Ktistakis, 2010). The conversion of LC3 to LC3-II is thus well-known as a marker of autophagy-induction. However, the increase of LC3-II alone is not enough to show autophagy activation because the inhibition of LC3-II degradation in the lysosome by the impaired autophagy flux can also cause its accumulation.

While the origin of autophagic vacuoles remains disputable, several hypotheses have been proposed for the source of autophagosomal membrane during autophagosome formation. The first hypothesis is “de novo” formation of autophagosome by Atg9 reservoirs (Mari *et al*., 2010). In the second hypothesis, various organelles such as ER (Hayashi-Nishino *et al*., 2009), mitochondria (Hailey *et al*., 2010) and plasma membrane (Ravikumar *et al*., 2010) are used as an origin for the formation of the phagophore (Figure 1). Recently, cup-shaped structures called omegasome, a discrete region of the ER, was identified as a platform for autophagosome formation (Tooze and Yoshimori, 2010). The Atg5 complex, LC3 and ULK1 were shown to be recruited into the omegasome after starvation, and Atg5- and LC3-positive membranes seem to emerge from the omegasome. It was also observed that omegasomes form in close proximity to the Vps34-containing vesicles which may synthesize the PI(3)P. This hypothesis is also supported by a notion of a physical association between the ER and early autophagic membranes (Hayashi-Nishino *et al*., 2009).

Vesicle completion and lysosomal degradation

Autophagosome then fuses with lysosomes/vacuoles, which is an essential process for completion of the autophagy pathway. Sequestration of cytoplasm into a double-membrane cytosolic vesicle is followed by the fusion of the vesicle with a late endosome or lysosome to form an autophagolysosome (or autolysosome). Then, inner membrane of the autophagosome and autophagosome-containing cytoplasm-derived materials are degraded by lysosomal/vacuolar hydrolases inside the autophagosome. The molecular mechanisms underlying the transport and fusion of autophagosomes are just beginning to be understood, and through active investigations, several major events involved in the process have recently been clarified.

In yeast, the fusion of autophagosomes with the vacuole requires SNARE machinery and proteins such as the vacuolar syntaxin homologue Vam3 (Darsow *et al*., 1997), the SNAP-25 homologue Vam7 (Sato *et al*., 1998), the Rab family GTP-binding protein Ypt7 (Mayer *et al*., 1997) or the orthologue of the N-ethylmaleimide-sensitive fusion (NSF) protein, Sec18 (Ishihara *et al*., 2001). In mammalian cells, autophagosome maturation is a prior step for the fusion between autophagosomes and lysosomes. Like in yeast, the activity of monomeric GTPases such as Rab22 and Rab24 is required for autophagosome maturation (Petiot *et al*., 2000), and mammalian orthologues of SNARE protein family members and the NSF protein may also be involved in the maturation of autophagic vesicles.

Recent studies have identified new regulators of autophagosome maturation and degradation, including UVRAG (Liang *et al*., 2008), Rubicon (Matsunaga *et al*., 2009), presenillin-1 (Lee *et al*., 2010), valosin-containing protein (VCP) (Tresse *et al*., 2010) and syntaxin-5 SNARE complex proteins (Renna *et al*., 2011). In addition, the endosomal sorting complex required for transport (ESCRT), which had originally been identified in the recognition and sorting of ubiquitin-modified cargo proteins into multivesicular bodies (MVBs) (Rothman and Wieland, 1996), was recently found to play a role in autophagosome-lysosome fusion (Rusten *et al*., 2007). Furthermore, ESCRT machinery was shown to be required for phagophore closure (Raiborg and Stenmark, 2009), autophagosome fu-

sion (Lee *et al*., 2007) and lysosome biogenesis (Raiborg *et al*., 2008). The degradation products, including macromolecules, are then exported to the cytosol for re-utilization by the cell. This process is poorly understood.

Conclusion

In the past decade there has been an extraordinary advance in our understanding of the molecular signaling involved in mammalian autophagy. Actually, genetic screens in yeast have identified numerous Atg genes that regulate autophagy process. In spite of those outcomes, many outstanding questions remain to be elucidated, including the origin of the membrane source for autophagosome formation, mechanism of phagophore expansion and autophagosome formation and regulation of ubiquitin-like conjugation system in autophagy process (Chen and Klionsky, 2011). Recently, two interesting approaches have been employed to identify new autophagy regulators: small molecules screening (Zhang *et al*., 2007; Farkas *et al*., 2009) and studies on structural information of Atg proteins. From our knowledge, autophagy is a major contributor to maintain cellular homeostasis and metabolism. It is also involved in the pathogenesis of human diseases. Thus, continued studies to identify key molecules regulating autophagy and a better understanding for the process at molecular level are required to be further proceeded.

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