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REVIEW

Autophagosome Formation in Mammalian Cells

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ABSTRACT. **Macroautophagy is an intracellular degradation system for the majority of proteins and some organelles. The molecular mechanism of autophagy has been extensively studied using the yeast, *Saccharomyces cerevisiae*, during these past 10 years. These studies suggested that the molecular machinery of autophagosome formation is well conserved from yeast to higher eukaryotes. Identification and characterization of the mammalian counterparts of the yeast autophagy proteins has facilitated our understanding of mammalian autophagy, particularly of autophagosome formation. These findings are now being applied to studies on the physiological roles of autophagy in mammals.**

Key words: autophagy/autophagosome/Apg12/Apg5/LC3

Most intracellular short-lived proteins are selectively degraded by the ubiquitin-proteasome pathway (Hochstrasser, 1996; Hershko and Ciechanover, 1998), while most long-lived proteins are degraded in lysosomes (Mortimore and Poso, 1987). The mechanism to deliver cytoplasmic components to the lysosomes is called autophagy in general. Three types of autophagy have been proposed: macroautophagy, microautophagy and chaperon-mediated autophagy (Seglen and Bohley, 1992; Dunn, 1994; Blommaart *et al.*, 1997). Among them, macroautophagy is believed to be responsible for the majority of the intracellular protein degradation, particularly of the starvation-induced proteolysis (Mortimore and Poso, 1987). In macroautophagy (simply referred to as autophagy hereafter), cytoplasmic constituents, including organelles such as mitochondria, are first enwrapped by a membrane sac called isolation membrane (Fig. 1). Closure of the isolation membrane results in formation of double membrane structures, called autophagosomes, which are also known as initial autophagic vacuoles (AVi). Autophagosomes, then, fuse with endosomes to become amphisomes or intermediate AV (AVi/d) (Tooze *et al.*, 1990; Liou *et al.*, 1997; Berg *et al.*, 1998; Nara *et al.*, 2002). Eventually, autolysosomes or degrading AV (AVd) are generated by the fusion of the outer membranes of the autophagosomes and lysosomes. Lysosomal hydrolases degrade the cytoplasm-derived contents of the autophagosome, together with its inner membrane.

However, the molecular mechanism of autophagy has been poorly understood for some time. About 10 years ago, genetic approaches using the yeast *Saccharomyces cerevisiae* were introduced in this research field and at least 16 genes (*APG* and *AUT* genes) required for autophagosome formation were identified (Tsukada and Ohsumi, 1993; Thumm *et al.*, 1994, for review, Klionsky and Emr, 2000; Khalfan and Klionsky 2002; Reggiori and Klionsky, 2002) (Table I). Most of them have their counterparts in higher eukaryotes including mammals. Having these homologues, we are now able to better study the mammalian autophagy in detail. This review focuses on the molecular basis of autophagosome formation in mammalian cells.

Tracing of autophagosome formation with mammalian Apg proteins

Although the size of autophagosomes varies among mammalian cells (usually 0.5–1.5 μm), it is principally

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Abbreviations: ES cells, embryonic stem cells; GFP, green fluorescent protein; PtdIns, phosphatidylinositol; PAS, pre-autophagosomal structure; TGN, *trans*-Golgi network.

larger than that of yeast cells (500–900 nm) (Takeshige *et al.*, 1992). In some particular cells such as ES cells, embryonic fibroblasts, hepatocytes and pancreatic acinar cells, autophagosomes are large enough to be visible as ring-shaped structures by light microscopy. Fluorescent labelling of certain mammalian Apg proteins enables us to trace each step of autophagosome formation.

One of the marker proteins is the Apg12-Apg5 conjugate. Apg12 and Apg5 are covalently attached to each other post-translationally and the resulting conjugate behaves as if it is a single molecule. Apg12-Apg5 localizes on the isolation membrane throughout its elongation process (Mizushima *et al.*, 2001) (Fig. 2). It dissociates from the membrane upon completion of autophagosome formation. Another useful marker protein is LC3 (microtubule-associated protein 1 (MAP1) light chain 3), a mammalian orthologue of yeast Aut7/Apg8 (Mann and Hammarback, 1994). In contrast to Apg12-Apg5, LC3 localizes on the membrane of complete spherical autophagosomes as well as on the isolation membranes (Kabeya *et al.*, 2000). LC3 is also detected on membrane of autolysosomes, which have been fused with lysosomes, although its amount is less than that of autophagosomes. We will describe the roles of mammalian Apg proteins and the related proteins in autophagosome formation, mostly based on the observations using these two marker proteins.

Initial step of autophagosome formation

In vivo visualization of autophagosome formation in living cells using GFP-Apg5 demonstrated directly that autophagosomes are generated by elongation of small membrane structures, known as autophagosome precursors, which are not derived from pre-existing large membrane such as ER cisternae (Mizushima *et al.*, 2001). Although the nature of these autophagome precursors is not known, they are not generated by the treatment of cells with 3-methyladenine or wortmannin, which are inhibitors of phosphatidylinositol (PtdIns) 3-kinase (Mizushima *et al.*, 2001). Requirement of Class III PtdIns 3-kinase for the autophagic pathway has been also demonstrated biochemically (Petiot *et al.*, 2000), and 3-methyladenine is a widely used inhibitor of autophagy (Seglen and Gordon, 1982). Thus, PtdIns 3-kinase activity is required probably at an early step of autophagosome formation. It is consistent with previous observations in yeast cells.

In yeast cells, autophagosomes seem to be generated from a structure near the vacuole, termed the pre-autophagosomal structure (PAS) (Suzuki *et al.*, 2001; Noda *et al.*, 2002). Multiple Apg proteins including Apg1, Apg2, Aut7, Apg9, the Apg12-Apg5·Apg16 complex and Apg14 are targeted to PAS (Suzuki *et al.*, 2001; Kim *et al.*, 2002). PAS is probably distinct from the precursor structure of autophagosomes seen in mammalian cells. Formation of PAS requires a PtdIns 3-kinase complex made up of Vps15, Vps30/Apg6, Apg14, and Vps34 (Kihara *et al.*, 2001b). It should be noted that there is another kind of PtdIns 3-kinase complex in yeast consisting of Vps15, Vps30/Apg6, Vps38 and Vps34 that functions in the vacuolar protein sorting pathway, not for autophagy.

Although the mammalian Apg14 is still missing, mammalian Vps30/Apg6 was identified as Beclin-1 (Liang *et al.*, 1998). Beclin-1 interacts with class III PtdIns 3-kinase also in mammalian cells and localizes in the the *trans*-Golgi Network (TGN) almost exclusively (Kihara *et al.*, 2001a). As the transport of lysosomal hydrolases from TGN to endosomes depends on PtdIns 3-kinase activity in mammalian cells (Brown *et al.*, 1995; Davidson, 1995), TGN-associated Beclin-1 would function for this pathway. Considering that, in yeast, the amount of the Vps15·Vps30·Apg14·Vps34 complex is much smaller than that of the Vps15·Vps30·Vps38·Vps34 complex (Kihara *et al.*, 2001b), and Apg14 is detected on PAS (Kim *et al.*, 2002), it is still possible that a quite small portion of Beclin-1 complex localizes on the autophagosomes or the precursor structures. Alternatively, TGN itself might be directly involved in autophagosome formation by providing some membrane sources. Indeed, the presence of Golgi-derived glycosylated proteins in the autophagosomal membranes was suggested (Yamamoto *et al.*, 1990).

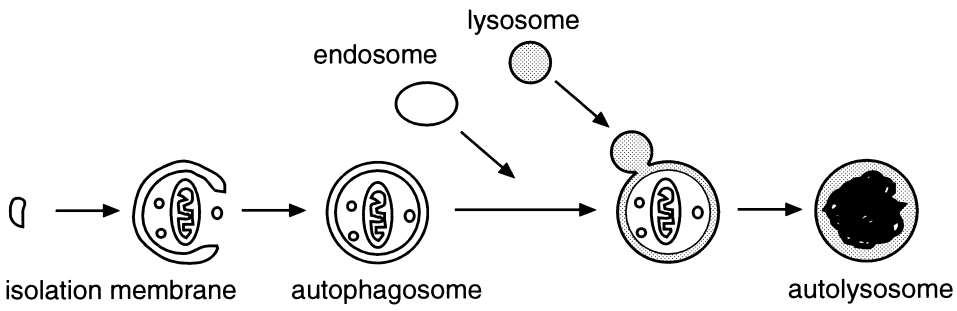

Fig. 1. Scheme of macroautophagy in mammalian cells. A portion of cytoplasm is enclosed by autophagic isolation membrane to form an autophagosome. The outer membrane of the autophagosome then fuses with lysosome to degrade the inside materials. This pathway can also degrade organelles such as mitochondria.

Table I. AUTOPHAGY-RELATED PROTEINS IN YEAST AND MAMMALS

Apg	Aut	Cvt	Gsa	Paz	mammals	function etc.
Apg1	Aut3	Cvt10	Gsa10	Paz1	ULK1	Protein kinase, interacts with Apg13, Apg17 and Cvt9, PAS localization
Apg2	Aut8		Gsa11	Paz7	Apg2	PAS localization
Apg3	Aut1		Gsa20		Apg3*	Aut7-conjugating enzyme (E2-like)
Apg4	Aut2			Paz8	Apg4A, Apg4B*	C-terminal hydrolase and deconjugase of the Aut7 system, cysteine protease
Apg5					Apg5*	Conjugated with Apg12, required for elongation of isolation membrane
Apg6					Beclin-1*	Subunit of PtdIns 3-kinase complex, identical to Vps30
Apg7		Cvt2	Gsa7	Paz12	Apg7*	Apg12- and Aut7-activating enzyme (E1-like)
Apg8	Aut7	Cvt5		Paz2	LC3*, GATE16, GABARAP	Ubl, conjugates to PE, localizes on PAS and autophagosomes
Apg9	Aut9	Cvt7	Gsa14	Paz9	Apg9?	Trans-membrane protein, PAS localization?
Apg10					Apg10*	Apg12-conjugating enzyme (E2-like)
Apg12					Apg12*	Ubl, conjugates to Apg5
Apg13					?	Subunit of the Apg1 protein complex, dephosphorylated upon induction of autophagy
Apg14		Cvt12			?	Subunit of PtdIns 3-kinase complex (autophagy-specific)
Apg16		Cvt11		Paz3	Apg16L*	Interacts with the Apg12-Apg5 conjugate
Apg17					?	Interacts with Apg1, not required for the Cvt pathway
	Aut10	Cvt18	Gsa12			Functions in an early step of macroautophagy and pexophagy
	Aut4					Required for degradation of autophagic bodies in the vacuole
	Aut5	Cvt17				Required for degradation of autophagic bodies in the vacuole, lipase-like
		Cvt9	Gsa9	Paz6		Required for the Cvt pathway and micropexophagy, but not for macroautophagy
		Cvt19				Receptor for aminopeptidase I on the Cvt vesicle

APG and *AUT* genes are required for macroautophagy, *CVT* is for the transport for aminopeptidase I from cytoplasm to the vacuole, *GSA* and *PAZ* are for microautophagy of peroxysome (micropexophagy) in *Pichia pastoris*. *indicates the mammalian homologues that were shown to be related to autophagy in mammals. PAS; the pre-autophagosomal structure.

Beclin-1 was originally isolated as a Bcl-2-interacting protein (Liang *et al.*, 1998). Later it was suggested that Beclin-1 is a negative regulator of tumorigenesis (Liang *et al.*, 1999) (see below).

Elongation step of isolation membrane

One characteristic phenomenon of autophagy is the elongation of the membrane sac, called isolation membrane. Many Apg proteins are likely involved in this process. It is now clear that a ubiquitin-like conjugation system, the Apg12 system, is required in the elongation of the isolation membrane. The Apg12-Apg5 protein conjugation system, originally discovered in yeast, is well conserved among eukaryotes (Mizushima *et al.*, 1998a; Mizushima *et al.*, 1998b; Ohsumi, 2001; Mizushima *et al.*, 2003). As in yeast cells (Kuma *et al.*, 2002), most endogenous Apg12 and Apg5 are conjugated to each other and unconjugated forms are almost absent in mammalian cells (Mizushima *et al.*, 2001). At the initial step of the conjugation reaction, the carboxy-terminal glycine residue of mammalian Apg12 is activated by Apg7 (E1-like), resulting in formation of an Apg12-Apg7 thioester intermediate (Tanida *et al.*, 2001). Apg12 is then transferred to mammalian Apg10 to form an Apg12-Apg10 thioester intermediate (Mizushima *et al.*, 2002). Finally the carboxy-terminal glycine of Apg12 is covalently attached to lysine 130 of Apg5 via an isopeptide bond (Mizushima *et al.*, 1998b). This conjugation reaction is quite similar to that of the ubiquitin system.

In contrast to yeast cells, in which the Apg12-Apg5 conjugate forms a ~350-kDa multimeric protein complex with a small coiled-coil protein, Apg16 (Mizushima *et al.*, 1999; Kuma *et al.*, 2002), mammalian Apg12-Apg5 is contained in a ~800-kDa protein complex with a novel WD repeat protein, which we named Apg16L (N. Mizushima, A. Kuma, Y. Kobayashi, A. Yamamoto, M. Matsubae, T. Takao, T. Natsume, Y. Ohsumi, T. Yoshimori, manuscript submitted). Apg16L is a 63 to 74-kD protein with several spliced isoforms and is quite larger than yeast Apg16 (17 kD). The N-terminal region of this novel protein contains several features similar to yeast Apg16. Apg16L, however, has a large C-terminal domain containing seven WD repeats, absent from yeast Apg16. Since WD repeat proteins generally provide a platform for protein-protein interac-

tions, mammalian Agp16L is expected to further interact with other proteins.

Although most Apg12-Apg5·Apg16L complex resides in the cytosol, a small fraction localizes on the isolation membrane throughout its elongation process (Fig. 2). Apg12-Apg5·Apg16L initially associates evenly with the membrane of small vesicles. As the membrane elongates, Apg12-Apg5·Apg16L shows asymmetric localization, with most of them associating with the outer side of the isolation membrane. Finally, Apg12-Apg5·Apg16L dissociates from the membrane upon completion of autophagosome formation. Such a characteristic localization of the complex suggested that Apg12-Apg5·Apg16L likely functions at the elongation step of autophagosome formation. Actually, a gene targeting study using ES cells revealed that Apg5 is required for autophagosome formation (Mizushima *et al.*, 2001). Since the presence of Apg5 is the best definition of the precursor structures, it is difficult to tell whether the autophagosome precursors are generated in *APG5*$^{-/-}$ cells. This question was addressed by a different approach. In the mammalian Apg12 system, Apg12 is attached to Lys130 of Apg5 (Mizushima *et al.*, 1998b). When the Apg5^{K130R} mutant, in which Lys130 is replaced with Apg, is expressed in *APG5*$^{-/-}$ ES cells, Apg5 is no longer conjugated with Apg12. Even in such cells, Apg5^{K130R} is able to localize to the small autophagosome precursors with Apg16L, suggesting that the covalent modification of Apg5 with Apg12 is not required for membrane targeting of Apg5 and Apg16L (Mizushima *et al.*, 2001) (manuscript submitted). However, the membrane does not elongate to form a cup-shaped isolation membrane and autophagosomes. Thus, the Apg12-Apg5·Apg16L complex is essential for the elongation of the isolation membranes, but not for the generation of the precursor structures.

The function of the Apg12 system is closely linked to another ubiquitin-like system, the LC3 system. LC3, a mammalian orthologue of Aut7/Apg8, is the first protein identified on the autophagosome membrane (Kabeya *et al.*, 2000). Before targeting to autophagosomes, LC3 undergoes quite complicated post-translational modifications, which are essentially similar to those of the yeast Aut7 modification system (Ichimura *et al.*, 2000; Kirisako *et al.*, 2000). Immediately after synthesis, the C-terminal region of LC3 (22 and 5 amino acids in rat and human LC3, respectively) is cleaved (Kabeya *et al.*, 2000). This processing is catalyzed by mammalian Aut2/Apg4 homologues (our unpublished observation). The processed form, having a glycine residue at the C-terminal end called LC3-I, resides in the cytosol. After activation by mammalian Apg7 (Tanida *et al.*, 2001), which functions also as an Apg12-activating enzyme, LC3 is transferred to a specific E2 equivalent enzyme, the mammalian Aut1/Apg3 homologue (Tanida *et al.*, 2002). In yeast, Aut7 is finally conjugated to phosphatidylethanolamine. This discovery was a great surprise because nobody expected that a ubiquitin-like conjugation system is used for protein lipidation (Ichimura *et al.*, 2000). Although the target molecule of LC3 has not been identified, LC3 surely undergoes an unusual modification. The expected final product of LC3, called LC3-II, tightly associates with autophagosomal membrane and migrates faster than LC3-I on SDS-PAGE. Consequently, immunoblotting of LC3 usually gives two bands: LC3-I (apparent mobility, 18 kD) and LC3-II (16 kD) (Kabeya *et al.*, 2000). Since the amount of LC3-II or the LC3-II/LC3-I ratio correlates with the number of autophagosomes, the immunoblot analysis of LC3 is a good and simple method to predict autophagic activity of mammalian cells (Kabeya *et al.*, 2000).

LC3 targets to the isolation membrane throughout the course of membrane elongation and, in contrast to Apg12-Apg5·Apg16, remains on the autophagosomal membrane after the completion of autophagosome formation (Kabeya *et al.*, 2000; Mizushima *et al.*, 2001). Membrane targeting of LC3 depends on Apg5; LC3 is present exclusively in the cytosol in *APG5*$^{-/-}$ cells (Mizushima *et al.*, 2001). Even in

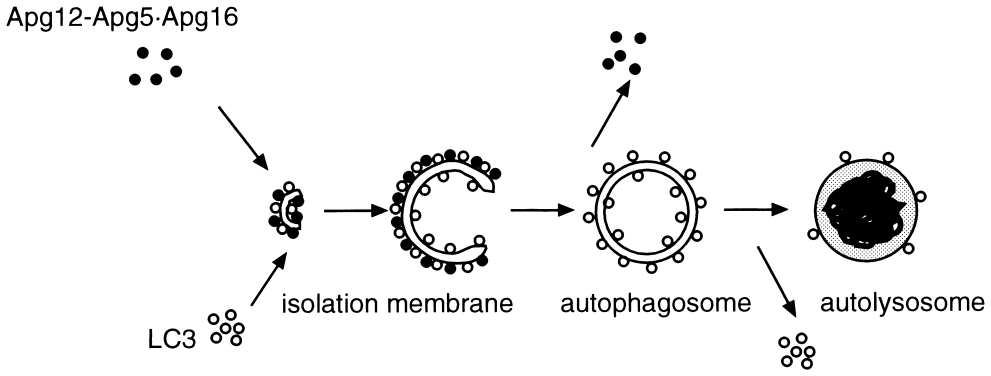

Fig. 2. Model of autophagosome formation in mammalian cells. The Apg12-Apg5 conjugate and Apg16L localize to the isolation membrane throughout its elongation process. LC3 is recruited to the membrane in the Apg5-dependent manner. Apg12-Apg5 and Apg16L dissociate from the membrane upon completion of autophagosome formation, while LC3(-II) remains on the autophagosome membrane. Apg5 and its modification by Apg12 are required for elongation of the isolation membrane.

APG5$^{-/-}$ cells expressing Apg5^{K130R}, in which Apg5^{K130R} and Apg16L localize to the autophagosome precursors, LC3 could not target to the membrane. The functional relationship between the Apg12 and LC3 system was also evidenced by biochemical analysis. In *APG5*$^{-/-}$ cells and *APG5*$^{-/-}$ cells expressing Apg5^{K130R}, LC3-II is not generated at all, although LC3-I is normally detected (Mizushima *et al.*, 2001). The mechanism governing the relationship between these two systems is not known. The Apg12-Apg5 conjugate may act as if it is an E3-like enzyme in the LC3 conjugation system, or may stabilize LC3-II, for example by providing the proper membrane structures.

There are two additional mammalian Aut7 homologues: the Golgi-associated ATPase enhancer of 16 kDa (GATE-16) (Sagiv *et al.*, 2000) and γ-aminobutyric acid $(GABA)_A$-receptor-associated protein (GABARAP) (Wang *et al.*, 1999). The precise function and localization of these two homologues are controversial. GABARAP was suggested to be involved in the $GABA_A$ receptor clustering (Chen *et al.*, 2000) or transport (Kneussel *et al.*, 2000). GATE-16 has been suggested to be an intra-Golgi transport modulator that interacts with N-ethylmaleimide-sensitive factor (NSF) and Golgi v-SNARE GOS-28 (Sagiv *et al.*, 2000). However, transfection experiments showed that both GABARAP and GATE-16 are able to reside on autophagosomal membranes (our unpublished observation). Therefore, these three Aut7 homologues may have redundant functions. The fact that GABARAP self-associates in a head-to-tail fashion, probably forming a dimmer, could be a clue to understand the function of the Aut7 family proteins (Coyle *et al.*, 2002; Stangler *et al.*, 2002; Nymann-Andersen *et al.*, 2002). Both GATE16 and GABARAP are also processed by mammalian Aut2/Apg4 homologues to expose the conserved C-terminal glycine (our unpublished observation), and are catalyzed by mammalian Apg7 (Tanida *et al.*, 2001) and Aut1/Apg3 homologues (Tanida *et al.*, 2002).

Other factors in autophagosome formation

Another distinct functional unit in the yeast autophagic pathway is a protein complex containing Apg1, Apg13 and Apg17 (Kamada *et al.*, 2000). Apg1 is a protein kinase, which is regulated by Apg13. The kinase activity of Apg1 is upregulated upon induction of autophagy. Although the Apg1·Apg13 complex receives a signal from Tor, a master regulator in nutrient sensing, genetic analyses suggested that the Apg1 complex functions at a rather late step of autophagosome formation, not at the PAS formation step (Suzuki *et al.*, 2001). It is also required for the late step of peroxysome microautophagy (micropexophagy) in methylotrophic yeast *Pichis pastoris*, which is induced by glucose adaptation, not by nutrient starvation (Sakai *et al.*, 1998; Stromhaug *et al.*, 2001; Mukaiyama *et al.*, 2002). Therefore, the Apg1 complex would be a common machinery of membrane dynamism shared by macroautophagy and microautophagy, rather than a simple signal transducer.

So far no Apg13- or Apg17-related protein has been identified in higher eukaryotes. Putative Apg1 homologue was identified in *C. elegans* (UNC51) (Matsuura *et al.*, 1997), and mammals (ULK1) (Yan *et al.*, 1998). The striking finding is that ULK1 interacts with GATE-16 and GABARAP (Okazaki *et al.*, 2000). It suggests that Aut7 homologues are functionally related to Apg1 protein complex, although the interaction between ULK1 and LC3 was not detected and involvement of ULK1 in autophagy has not been determined.

Mammalian homologues of other Apg proteins such as Apg2 and Apg9 are found in the database, but have not been studied in detail.

Maturation of autophagosome and its fusion with lysosome

Prior to the final fusion with lysosomes, autophagosomes maturate by fusing with endosomes or endosome-derived vesicles (Tooze *et al.*, 1990; Fengsrud *et al.*, 1995; Liou *et al.*, 1997). These structures are called intermediate autophagic vacuoles (AVi/d) or amphisomes, containing endocytic markers but little lysosomal proteins such as cathepsin D and Lgp120. The significance of endosome fusion, however, remains unclear, probably because it is difficult to inhibit the endosome-autophagosome fusion specifically. An interesting approach was using a dominant negative mutant of SKD1 (Nara *et al.*, 2002). SKD1, a member of the AAA-ATPase family, is a mammalian homologue of yeast Vps4 and a key regulator of endosome sorting. Overexpression of its dominant-negative mutant, SKD1^{E235Q}, which is expected not to hydrolyse ATP, induces abnormal endosome morphology accompanied with defects in recycling of plasma membrane receptors and transport to the lysosomes (Yoshimori *et al.*, 2000; Bishop and Woodman, 2000). Perturbation of endosome function by SKD1^{E235Q} also causes massive accumulation of nascent autophagosomes that could not fuse with lysosomes (Nara *et al.*, 2002). One possible explanation is that fusion with endosomes provides nascent autophagosomes with some machinery required for the fusion with lysosomes.

Microtubules are also required for the autophagosome-lysosome fusion (Kovacs *et al.*, 1982; Hoyvik *et al.*, 1986; Aplin *et al.*, 1992). Although LC3 was originally isolated as a protein co-purified with microtubule-associated protein 1A and 1B (Mann and Hammarback, 1994), it is unlikely that LC3 functions at the autophagosome-lysosome fusion step; rather it plays an important role in the autophagosome formation. Acidification of autophagosomes (amphisomes) is also essential for the fusion with lysosomes (Kovacs *et al.*, 1982; Yamamoto *et al.*, 1998).

Role of autophagy in mammals

(i) Starvation response

One of the best known defects of autophagy-defective yeast mutants is the loss of viability during starvation (Tsukada and Ohsumi, 1993). The exact mechanism underlying this phenomenon has not been determined; the most simple explanation is that yeast cells tide over starvation by eating themselves. Similarly, autophagy has been found to be upregulated in various tissues also in mammals, suggesting that it is a fundamental physiological response to starvation (Blommaart *et al.*, 1997).

(ii) Cell death and development

Mammalian homologue of Apg5 was originally identified as "apoptosis-specific protein (ASP)" (Hammond *et al.*, 1998). Based on this finding, the possible relationship between apoptosis and autophagy has been discussed in many literatures. ASP, however, was recently shown to be a distinct protein from mammalian Apg5 (Yung *et al.*, 2002). Since only the detectable phenotype of *APG5*$^{-/-}$ cells is the defect in autophagy (Mizushima *et al.*, 2001), Apg5 would be solely responsible for autophagy. At least, starvation-induced apoptosis normally occurs in *APG5*$^{-/-}$ cells (our unpublished observation).

Nonetheless, the involvement of autophagy in cell death has been suggested in a number of physiological and pathological processes. One of them is a type of cell death during development, so-called autophagic degeneration or type 2 cell death, which is distinct from typical apoptosis (Clarke, 1990; Schweichel and Merker, 1973). Numerous autophagic vacuoles are accumulated in the cytoplasm with relatively intact nuclei. This type of cell death has been observed where large regions of tissues are degenerated, for example in the palatal epithelium during closure. The morphology of autophagy, however, is sometimes quite complicated. To confirm that these vacuoles seen in such kind of cell death are indeed autophagy-related structures, studies using specific marker proteins will be required.

The second obvious phenotype of yeast autophagy mutants is the defect in sporulation (Tsukada and Ohsumi, 1993). It suggests that bulk degradation by autophagy might be important for cellular remodeling during differentiation. Autophagy is also involved in remodeling of mammalian cells; phenobarbital-induced massive endoplasmic reticulum is removed by autophagy after cessation of the treatment (Bolender and Weibel, 1973; Masaki *et al.*, 1987).

(iii) Autophagy in pathogenesis

Recently, increased numbers of autophagic vacuoles have been found in several human diseases. Intriguingly, most of them are degenerative diseases of the nervous system and muscle (Table II). Turnover of intracellular proteins may be particularly important for these non-dividing cells because, in rapidly-proliferating cells, accumulation of unwelcome proteins can be avoided by dilution with cell proliferation, rather than degradation. In this respect, it should be noted that most of the diseases listed in Table 2 are characterized by accumulation of proteins with abnormal conformations. Kopito (Kopito, 2000) proposed that autophagy could be induced in these circumstances to eliminate these proteins, which might be overflowed from the ubiquitin-proteasome system. Although the size of the typical aggresome is much larger than that of the usual autophagosome, it is still possible that small aggregations of abnormal proteins can be degraded by autophagy. Whether autophagosomes can specifically recognize abnormal proteins and/or aggregates is an important question which remains to be addressed.

Beclin-1 (mammalian Apg6), a subunit of a PtdIns 3-

Table II. HUMAN DISEASES WITH EXCESSIVE AUTOPHAGOSOME FORMATION

Nervous system	
Polyglutamine diseases	(Kegel *et al.*, 2000; Petersen *et al.*, 2001; Ravikumar *et al.*, 2002)
Alzheimer's disease	(Okamoto *et al.*, 1991; Cataldo *et al.*, 1996)
Parkinson's disease	(Anglade *et al.*, 1997)
Familial neurohypophysial diabetes insipidus	(Davies and Murphy, 2002)
Myopathy	
X-linked myopathy with excessive autophagy	(Kalimo *et al.*, 1988)
Distal myopathy with rimmed vacuole (DMRV)	(Nonaka, 1999)
Inclusion body myositis	(Nonaka, 1999)
Marinesco-Sjögren syndrome	(Goto *et al.*, 1990)
Danon disease (Lamp-2 deficiency)	(Nishino *et al.*, 2000)
Liver diseases	
α1-antitrypsin deficiency	(Teckman and Perlmutter, 2000; Teckman *et al.*, 2002)

kinase complex (see above), was proposed to have an anti-tumor effect. The genomic localization of *beclin1* is within a tumor susceptibility locus in 17q21, which is deleted in most of breast carcinomas and a cell line, MCF7. Low autophagic activity of MCF7 was restored by forced expression of Beclin-1, which also inhibited tumorigenesis when MCF7 cells were implanted into nude mice (Liang *et al.*, 1999). These findings suggest that Beclin-1 is required for both autophagy and protection from cancer.

Future prospects

We have described the molecular mechanism of mammalian autophagy formation. Although it was suggested that several groups of mammalian Apg proteins function in distinct steps of autophagosome formation, the precise function of each Apg proteins still remains to be solved. Although the genetic approach is apparently more difficult in mammals than in yeast, studies of mammalian autophagy have several advantages; for example, light microscopy observation of autophagosomes is relatively easy and an established method exists for the generation of semi-intact cells. The understanding of the functions and structures of these proteins and further understanding of their relationship with other known cellular proteins will unravel the mechanism of formation of this mysterious organelle.

Much attention is now paid to the physiological roles of autophagy in mammals, especially in embryogenesis and pathogenesis. There are, however, some limitations to these studies. One is that we do not have good specific inhibitors for autophagy. At the moment, the only reliable method to inhibit autophagy is gene-targeting. Another problem is the limited methods to monitor autophagy, particularly *in vivo*. Mammalian autophagy has been examined mainly by electron microscopy. However, sometimes it is difficult to distinguish autophagic vacuoles from other organelles. We have just generated a transgenic mouse systemically expressing GFP-LC3, which serves as an autophagy-indicator mouse. That promises to provide many new insights into autophagy in mammals.

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