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Review

LC3 conjugation system in mammalian autophagy

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Abstract

Autophagy is the bulk degradation of proteins and organelles, a process essential for cellular maintenance, cell viability, differentiation and development in mammals. Autophagy has significant associations with neurodegenerative diseases, cardiomyopathies, cancer, programmed cell death, and bacterial and viral infections. During autophagy, a cup-shaped structure, the preautophagosome, engulfs cytosolic components, including organelles, and closes, forming an autophagosome, which subsequently fuses with a lysosome, leading to the proteolytic degradation of internal components of the autophagosome by lysosomal lytic enzymes. During the formation of mammalian autophagosomes, two ubiquitylation-like modifications are required, Atg12-conjugation and LC3-modification. LC3 is an autophagosomal ortholog of yeast Atg8. A lipidated form of LC3, LC3-II, has been shown to be an autophagosomal marker in mammals, and has been used to study autophagy in neurodegenerative and neuromuscular diseases, tumorigenesis, and bacterial and viral infections. The other Atg8 homologues, GABARAP and GATE-16, are also modified by the same mechanism. In non-starved rats, the tissue distribution of LC3-II differs from those of the lipidated forms of GABARAP and GATE-16, GABARAP-II and GATE-16-II, suggesting that there is a functional divergence among these three modified proteins. Delipidation of LC3-II and GABARAP-II is mediated by hAtg4B. We review the molecular mechanism of LC3-modification, the crosstalk between LC3-modification and mammalian Atg12-conjugation, and the cycle of LC3-lipidation and delipidation mediated by hAtg4B, as well as recent findings concerning the other two Atg8 homologues, GABARAP and GATE-16. We also highlight recent findings regarding the pathobiology of LC3-modification, including its role in microbial infection, cancer and neuromuscular diseases.

Keywords: Atg8; Autophagy; Autophagosomes; GABARAP; GATE-16

Contents

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1. Introduction

Autophagy is the bulk degradation of proteins and organelles, a process essential for cellular maintenance and cell viability (for reviews (Kadowaki, Venerando, Miotto, & Mortimore, 1996; Klionsky, 2004; Ohsumi, 2001; Reggiori & Klionsky, 2002; Seglen et al., 1996; Thumm, 2002; Yoshimori, 2004)). In mammals, autophagy has been observed in many tissues, and has been shown to be essential for differentiation and development as well as for cellular maintenance (for a review (Meijer & Dubbelhuis, 2004; Riddle & Gorski, 2003; Thummel, 2001)). In addition, autophagy has been shown to have significant associations with neurodegenerative diseases, cardiomyopathies, cancer, programmed cell death, and bacterial and viral infections (for reviews (Baehrecke, 2003; Bursch, 2001; Cuervo, 2003; Dorn, Dunn, & Progulske-Fox, 2002; Edinger & Thompson, 2003; Eskelinen, Tanaka, & Saftig, 2003; Larsen & Sulzer, 2002; Lemasters et al., 2002; Lockshin & Zakeri, 2002; Meier & Silke, 2003; Nakanishi, 2003; Nishino, 2003; Ogier-Denis & Codogno, 2003; Perlmutter, 2002; Petersen & Brundin, 2002; Rich, Burkett, & Webster, 2003; Saftig, Tanaka, Lullmann-Rauch, & von Figura, 2001; Tolkovsky, Xue, Fletcher, & Borutaite, 2002)). During the autophagic process, a cup-shaped structure, also referred to as an isolation membrane or preautophagosome, forms in the cytosol. It engulfs cytosolic components, including organelles, and later become enclosed to form an autophagosome. The autophagosome subsequently fuses with a lysosome, enabling the intra-autophagosomal components to become degraded by lysosomal hydrolytic enzymes.

The proteins involved in the autophagic process in yeast have been isolated and characterized (Harding, Morano, Scott, & Klionsky, 1995; Thumm et al., 1994; Tsukada & Ohsumi, 1993) (for reviews (Klionsky, 2004; Klionsky & Emr, 2000; Ohsumi, 2001; Stromhaug & Klionsky, 2001; Thumm, 2000)). Recently, nomenclature for yeast autophagy-related genes has been unified to *ATG* from *APG*, *AUT*, and *CVT*; therefore in this review, we have employed the Atg nomenclature for mammalian Atg/Apg/Aut/Cvt genes (Klionsky et al., 2003). Many mammalian homologues of yeast *Atg* genes have been identified and characterized, suggesting that the molecular mechanisms of autophagy have been conserved from yeasts to mammals (Table 1) (Hemelaar, Lelyveld, Kessler, & Ploegh, 2003; Ichimura et al., 2000; Kametaka, Matsuura, Wada, & Ohsumi, 1996; Kametaka, Okano, Ohsumi, & Ohsumi, 1998; Kabeya et al., 2000; Kim, Dalton, Eggerton, Scott, & Klionsky, 1999; Lang et al., 1998; Liang et al., 1999; Mann & Hammarback, 1994; Marino et al., 2003; Matsuura, Tsukada, Wada, & Ohsumi, 1997; Mizushima, Sugita, Yoshimori, & Ohsumi, 1998; Mizushima, Noda, & Ohsumi, 1999; Mizushima, Yoshimori, & Ohsumi, 2002; Mizushima et al., 2003; Okazaki et al., 2000; Sagiv, Legesse-Miller, Porat, & Elazar, 2000; Scherz-Shouval, Sagiv, Shorer, & Elazar, 2003; Shintani et al., 1999; Tanida, Tanida-Miyake, Ueno, & Kominami, 2001; Tanida, Tanida-Miyake, Komatsu, Ueno, & Kominami, 2002a; Tanida, Nishitani, Nemoto, Ueno, & Kominami, 2002b; Tanida et al., 2002c; Tanida et al., 1999; Wang, Bedford, Brandon, Moss, & Olsen, 1999; Yuan, Stromhaug, & Dunn, 1999). Most of the characterized *ATG* gene products, including Atg3, Atg5, Atg7, Atg10, Atg12, and LC3, are involved in two ubiquitylation-like modifications of target proteins, Atg12-conjugation and LC3-modification (Atg8-lipidation in yeast), which are essential for

Table 1
Mammalian autophagy-related genes

Gene Designation				
Yeast	Human	Mouse	Comment	Reference
ATG1	ULK1		Unc-51-like kinase interacts with GATE-16 and GABARAP	Larsen and Sulzer, 2002; Lemasters et al., 2002
ATG3	hATG3/hAPG3	mATG3/mAPG3	an E2-like enzyme for LC3, GABARAP, and GATE-16	Liang et al., 1999; Lockshin and Zakeri, 2002; Mann and Hammarback, 1994
ATG4	hATG4A/HsATG4A/ HsAPG4A/autophagin-2		Cysteine protease for GATE-16	Liang et al., 1999; Marino et al.,2003; Matsuura et al., 1997; Meier and Silke, 2003
	hATG4B/HsATG4B/ hAPG4B/autophagin-1		Cysteine protease for LC3,	Matsuura et al., 1997; Meijer and Dubbelhuis, 2004
			GABARAP, and GATE-16	unpublished results
			Delipidating enzyme for LC3-II and GABARAP	unpublished results
	hAtg4C/HsAUTL1/ autophagin-3		Cysteine protease	Matsuura et al., 1997
	hATG4D/autophagin-4			Matsuura et al., 1997
ATG5	hATG5/hAPG5		target protein of Atg12	Mizushima et al., 1998; Mizushima et al., 2001; Mizushima et al., 2003
ATG6	beclin 1	beclin 1	related to tumorigenesis	Mizushima et al., 1999; Mizushima et al., 1998
ATG7	hATG7/HsGSA7/hAPG7	mATG7/mAPG7	an E1-like enzyme for Atg12 and Atg 8 homologues	Mizushima et al., 2001; Mizushima et al., 2004; Mizushima et al., 2002; Mizushima et al., 2003; Nakanishi, 2003; Nemoto et al., 2003
ATG8	LC3		modifier for autophagosomes	Marino et al., 2003; Nishino et al., 2000; Nishino, 2003
	GABARAP		modifier	Noguchi et al., 2004
	GATE-16		modifier	Ogier-Denis and Codogno, 2003
	ATG8L/APG8L		unknown	Meijer and Dubbelhuis, 2004
ATG10		mATG10/mAPG10	an E2-like enzyme for Atg12	Ohsumi, 2001; Okazaki et al., 2000
ATG12	hATG12/hAPG12	mATG12/mAPG12	modifier for autophagosomal	Mizushima et al., 2001; Mizushima et al., 2003; Nemoto et al., 2003
ATG16		ATG16L/APG16L	interacts with Atg5	Otto et al., 2004; Otto et al., 2003

the dynamic process of autophagosome formation (Kabeya et al., 2000; Mizushima et al., 1998; Mizushima et al., 2002; Tanida et al., 2001, 2002a) (for reviews (Klionsky, 2004; Mizushima, Yoshimori, & Ohsumi, 2003)). Atg12-conjugation is essential for the formation of preautophagosomes, whereas LC3-modification is essential for the formation of autophagosomes (Fig. 1) (Kabeya et al., 2000; Mizushima et al., 2001). Atg12 is activated by the E1-like enzyme Atg7, transferred to the E2-like enzyme Atg10, and conjugated to Atg5 to form an autophagosomal precursor (Mizushima et al., 1998, 2001, 2002; Nemoto et al., 2003; Tanida et al., 2001). ProLC3 is processed to its cytosolic form, LC3-I, exposing a carboxyl terminal Gly (Kabeya et al., 2000). LC3-I is also activated by Atg7, transferred to Atg3, a second E2-like enzyme, and modified to a membrane-bound form, LC3-II (Tanida et al., 2001, 2002a). LC3-II is localized to preautophagosomes and autophagosomes, making this protein an autophagosomal marker (Kabeya et al., 2000). Following the fusion of autophagosomes with lysosomes, intra-autophagosomal LC3-II is degraded by lysosomal hydrolytic enzymes (Kabeya et al., 2000). LC3 is therefore characterized as an autophagosomal ortholog of yeast Atg8. In contrast, the roles of three

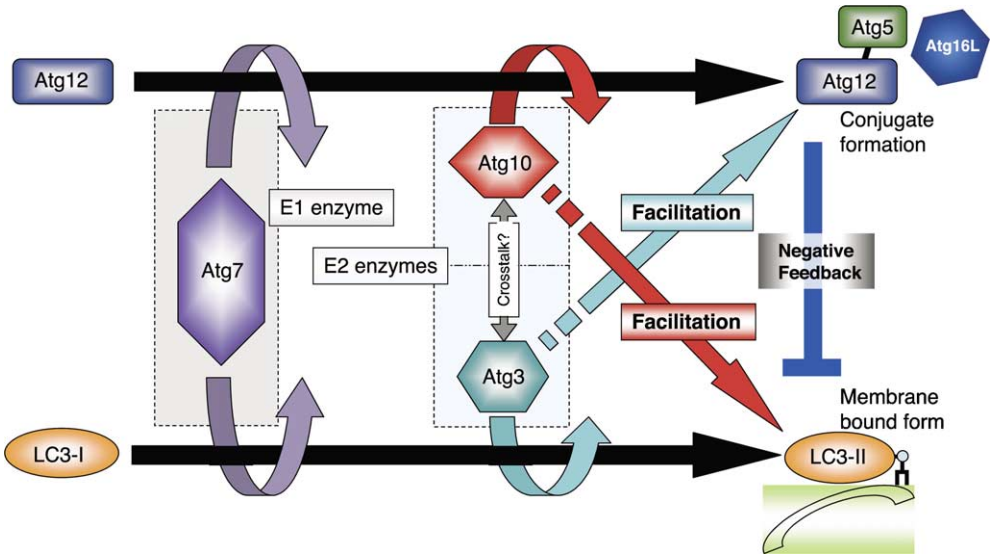

Fig. 1. Schematic representation of Crosstalk between two ubiquitylation-like modifications essential for mammalian autophagy. Atg12 is a first modifier essential for the formation of autophagosomal precursors and isolation membranes. Atg12 is activated by Atg7, transferred to Atg10, and conjugated to Atg5, which subsequently forms a complex with Atg16L. LC3 is a second modifier essential for the later formation of autophagosomes. After translation, most proLC3 is processed to LC3-I, which is localized in the cytosol. LC3-I is activated by Atg7, transferred to Atg3, and conjugated to phospholipid. Overexpression of Atg10 facilitates the modification of LC3-I to LC3-II (Facilitation, Red color). Overexpression of Atg3 facilitates the conjugation of Atg12 to Atg5 (Facilitation, Blue color). Excess amount of the Atg12-Atg5 conjugate inhibits LC3-modification (Negative Feedback).

other homologues of Atg8, GATE-16, GABARAP, and Apg8L/Atg8L, during autophagy are less well known.

In this review, we have focused on the crosstalk between the two ubiquitylation—like modifications, the modifications of the four mammalian Atg8 homologues during autophagy, and the relationship of Atg8 to mammalian diseases.

2. Two autophagy-specific ubiquitylation-like protein conjugation systems

2.1. Crosstalk between Atg12-conjugation and LC3-modification in mammals

In both rat tissues and HEK293 cells, endogenous Atg5 and Atg12 homologues are mainly present as conjugates. In contrast, the relative amounts of cytosolic LC3-I and membrane-bound LC3-II vary depending on tissues (Fig. 2) and cell-lines even under nutrient-rich conditions (I. Tanida, T. Ueno, E. Kominami, unpublished results). Enzymatically, these modifications occur independently, but there is crosstalk between them (Fig. 1). In Atg5-deficient mouse embryonic stem cells, which lack the Atg12–Atg5 conjugate, the modification of LC3-I to LC3-II is also impaired (Mizushima et al., 2001). Mammalian Atg3 interacts with mammalian Atg12 and the Atg12–Atg5 conjugate in HEK293 cells, in addition to its substrates, LC3, GATE-16 and GABARAP (Tanida et al., 2002a, 2002b). Excess amounts of the Atg12–Atg5 conjugate inhibit the modification of LC3-I to LC3-II, while overexpression of free Atg12 alone facilitates the LC3-modification in HEK293 cells (Tanida et al., 2002b). Overexpression of mammalian Atg3 facilitates the conjugation of Atg12–Atg5 in the presence of Atg7 in HEK293 cells (Tanida et al., 2002a). These results suggested that interaction of Atg3 with Atg12 and the Atg12–Atg5 conjugate may play a important role in a crosstalk between Atg12-conjugation and LC3-conjugation. Mammalian Atg10 interacts with LC3 in addition to Atg12 (Nemoto et al., 2003). Mammalian Atg10 can form an E2-substrate intermediate with Atg12, but not with LC3 (Nemoto et al., 2003), and overexpression of mammalian Atg10 also facilitates the modification of LC3-I to

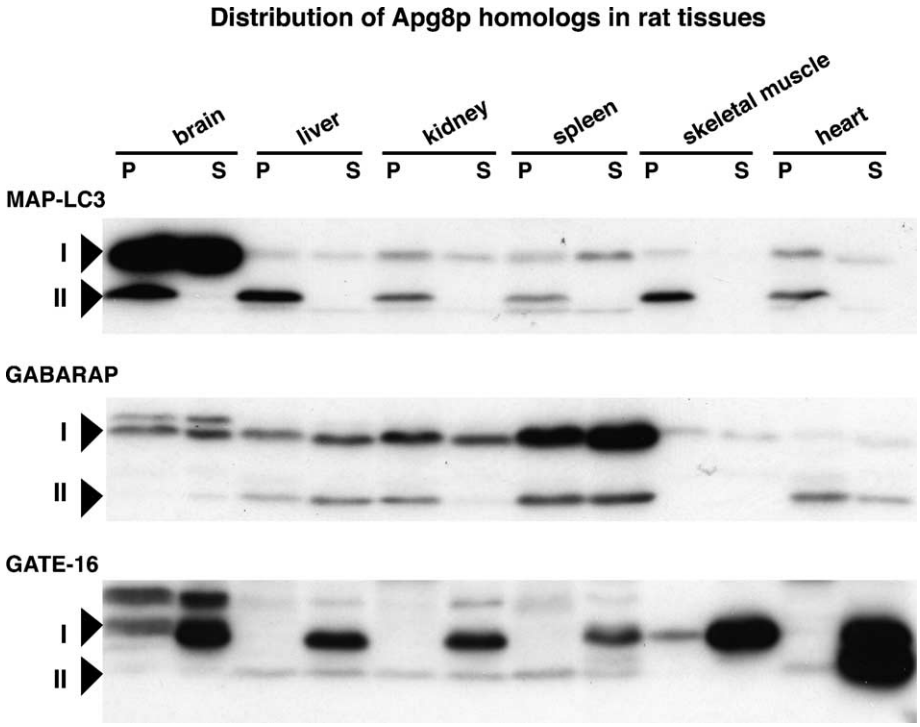

Fig. 2. Tissue distribution of the three Atg8 homologues as analyzed by immunoblotting. Wistar male rats were maintained for at least two weeks in an environmentally controlled room (lights on 06:00 to 20:00) and fed a standard laboratory chow and tap water *ad libitum*. Under these conditions, the feeding-starvation cycle of the animals occurs earnestly dependent on circadian rhythm. Namely, immediately after lights are gone off, they begin to eat chow for ∼3 h and then stop eating. They have been fasting during daytime. Hence, at 11:00 it is assumed that the animals are starved for 12 h. Brain, kidney, liver, spleen, heart, and hind leg muscle were freshly isolated from a Wistar male rat (300 g body weight) at 11:00. Each tissue, suspended in a buffer containing 5 mM Tes-NaOH (pH 7.5), 0.3 M sucrose, 5 μg/ml leupeptin, and 5 μg/ml pepstatin, was homogenized with a glass/Teflon homogenizer. The homogenate was centrifuged at 700 × g for 5 min, and the resulting supernatant was centrifuged at 100,000 × g for one hour. Forty μg protein of the pellet (P) and supernatant (S) was separated on 12.5% SDS-polyacrylamide gels and subjected to immunoblotting analysis for LC3, GABARAP, and GATE-16.

LC3-II in the presence of Atg7 (Nemoto et al., 2003). Atg7 forms a homodimer in yeasts and mammals (Komatsu et al., 2001; Tanida et al., 2001). Taken together, these results show that Atg12-conjugation and LC3-modification are linked to each other, and that the two E2-like enzymes are important in regulating these two ubiquitylation-like modification reactions during autophagy.

2.2. Modifications of the three Atg8 homologues, LC3, GABARAP, GATE-16

In mammals, three Atg8 homologues, LC3, GABARAP, GATE-16, have been well characterized as modifiers, whereas Atg8L has not (Fig. 3). LC3 was first isolated as a microtubule-associated protein, and subsequently localized to autophagosomes and isolation membranes during autophagy, similar to yeast Atg8 (Kabeya et al., 2000; Mann & Hammarback, 1994). GABARAP was identified as a $GABA_A$ receptor associated protein (Wang et al., 1999), whereas GATE-16 was identified as a Golgi-associated ATPase enhancer, of molecular weight 16 kDa (Sagiv et al., 2000). Following translation, the carboxyl terminus of the pro-form of each of these three proteins is cleaved to expose a carboxyl terminal Gly (Kabeya et al., 2000; Sagiv et al., 2000; Tanida, Komatsu, Ueno, & Kominami, 2003). When human Atg7 and Atg3 are expressed together with GATE-16 and GABARAP, the cleaved forms of

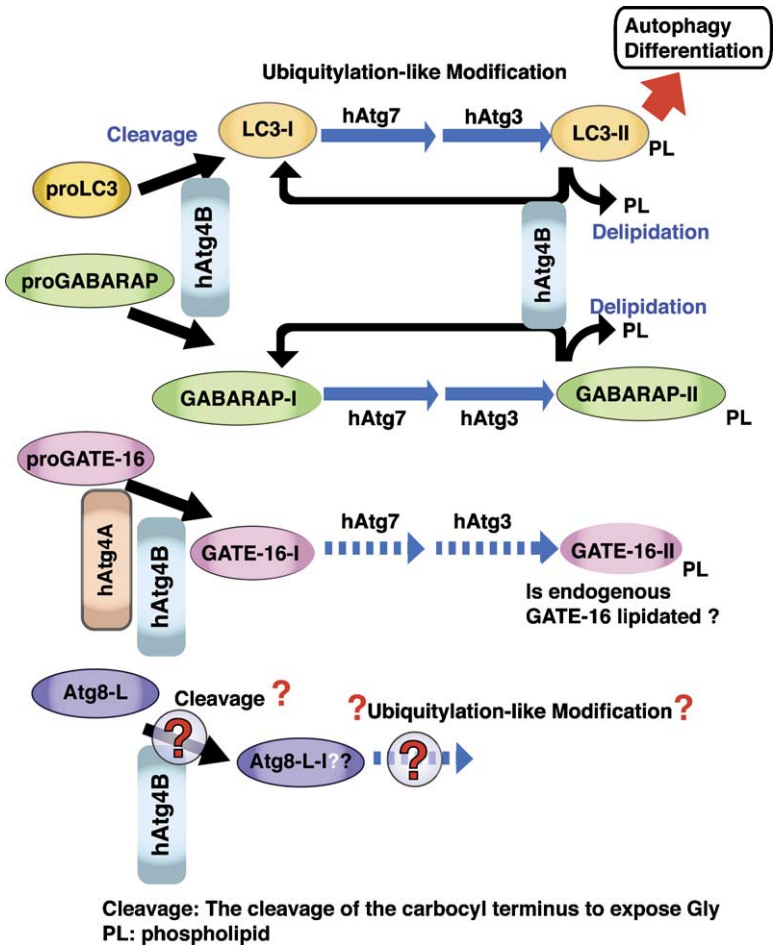

Fig. 3. Lipidation and delipidation cycle of mammalian Atg8 homologues mediated by mammalian Atg4 homologues. The carboxyl termini of the pro-forms of LC3, GABARAP, and GATE-16 are cleaved by hAtg4B (or hAtg4A for proGATE-16) to expose Gly, and the resultant LC3-I, GABARAP-I, and GATE-16-I are modified to LC3-II, GABARAP-II, and GATE-16-II, respectively. While intra-autophagosomal LC3-II is degraded by lysosomal proteolytic enzyme after fusion of autophagosomes to lysosomes, LC3-II on the cytosolic surface of autophagosomes is delipidated by hAtg4B. Membrane-localized GABARAP-II on the cytosolic surface is also delipidated by hAtg4B. Little endogenous GATE-16-II (i.e. lipidated form) is observed in rat and mouse tissues and cell lines. The physiological function of GATE-16-II is not yet known, while GATE-16 itself is an essential component of intra-Golgi transport and post-mitotic Golgi reassembly. Apg8L has been reported to interact with hAtg4B in mammals, but cleavage of the carboxyl termini and ubiquitylation-like modification have not been observed.

the latter, GATE-16-I and GABARAP-I, are modified to secondary ubiquitylation-like modified forms, GATE-16-II and GABARAP-II, in a manner similar to the modification of LC3-I to LC3-II (Tanida et al., 2003). Therefore, all three Atg8 homologues may act as modifiers of reactions mediated by mammalian Atg7 and Atg3. The difference between LC3-I and GABARAP-I or GATE-16-I is that a fraction of the latter two proteins, but not of LC3-I, localizes to membrane compartments prior to modification (Tanida et al., 2003). When endogenous GATE-16 in rat tissues was assayed by immunoblotting using anti-GATE-16 antibody, little GATE-16-II was detected (Fig. 2), whereas GABARAP-II was observed in some rat tissues (Fig. 2). As discussed later in Section 3, these patterns of modifications in rat tissues are

quite different from the pattern of LC3-modifications (Fig. 2). The data suggested that, in mammals, there will be functional divergence between the modification of LC3-I to LC3-II and that of GABARAP-I to GABARAP-II. Recently, Atg8L/Apg8L has been reported to interact with hAtg4B (Hemelaar et al., 2003), but it is still unclear whether Atg8L is an authentic modifier mediated by mammalian Atg7 and Atg3.

While LC3-II is well-established as a marker of autophagosomes during starvation-induced autophagy, LC3-I is also modified to LC3-II during the differentiation of MPC cells into mature podocytes (Asanuma et al., 2003). This reaction is much slower during differentiation than during autophagy, occurring in the order of days rather than hours. In differentiated MPC cells, LC3-II-localized vesicles, which differ from lysosomes and endosomes, are morphologically similar to autophagic vacuoles during starvation-induced autophagy. Inhibition of Tor kinase by rapamycin induces autophagy, indicating that Tor kinase functions as a regulator for autophagy (Klionsky, 2004). However, interestingly, Tor kinase is still active in differentiated MPC cells (Asanuma et al., 2003). During starvation-induced autophagy, autophagosomes fuse with lysosomes, and the LC3-II in autophagosomes is finally degraded by lysosomal proteases (Kabeya et al., 2000). In differentiated MPC cells, however, little LC3-II is degraded by lysosomal proteases, suggesting that there is little fusion between LC3-II-localized vesicles and lysosomes (Asanuma et al., 2003). In addition, the amount of LC3-II level in differentiated MPC cells increases during recovery from damage caused by experimental puromycin aminonucleoside-induced nephrosis (Asanuma et al., 2003). These results indicate that the modification of LC3-I to LC3-II has another function in differentiated cells and tissues.

In the yeast, *Saccharomyces cerevisiae*, Atg8 has been shown to be conjugated to phosphatidylethanolamine (Ichimura et al., 2000), making it of interest to determine whether mammalian LC3-II, GABARAP-II, and GATE-16-II are also lipidated. When endogenous LC3-II and GABARAP-II and recombinant GATE-16-II were treated with phospholipase D, their mobility on SDS-polyacrylamide gels (PAGE) were changed (I. Tanida, Y. Sou, T. Ueno, E. Kominami, manuscript in preparation), suggesting that LC3-II, GABARAP-II, and GATE-16-II are lipidated forms of their respective Atg8 homologues.

2.3. Lipidation and delipidation cycles in mammalian Atg8 homologues

In the yeast *S. cerevisiae*, Atg4, a cysteine protease, is essential for the cleavage of the carboxyl terminus of Atg8 to expose Gly (Ichimura et al., 2000). In mammals, there are at least four Atg4 homologues, hAtg4A/HsAtg4A/HsApg4A/autophagin-2, hAtg4B/HsAtg4B/hApg4B/autophagin-1, hAtg4C/HsAutl1/autophagin-3, and hAtg4D/autophagin-4 (Hemelaar et al., 2003; Ichimura et al., 2000; Kirisako et al., 2000; Marino et al., 2003; Scherz-Shouval et al., 2003). Since the carboxyl terminal regions of LC3, GABARAP and GATE-16 are cleaved soon after translation (Kabeya et al., 2000; Sagiv et al., 2000; Tanida et al., 2003), it is possible that they are cleaved by one or more of these Atg4 homologues. hAtg4A cleaves the carboxyl terminus of GATE-16 in vitro (Fig. 3) (Scherz-Shouval et al., 2003), and hAtg4C has NEM-sensitive proteolytic activities for a synthetic substrate, Mca–Thr–Phe–Gly–Met–Dpa–NH2, in vitro (Marino et al., 2003). hAtg4B has been identified as the only protein that interacts with vinyl sulfone (VS) conjugated LC3, GATE-16, GABARAP, and Atg8L, an as yet uncharacterized fourth Atg8 homologue, from the EL-4 mouse thymoma cell line (Fig. 3) (Hemelaar et al., 2003). hAtg4B cleaves the carboxyl termini of LC3, GABARAP, and GATE-16 in vitro (Klionsky, 2004) (I. Tanida, Y. Sou, T. Ueno, E. Kominami, manuscript in preparation). Overexpression of hAtg4B or hAutl1 has been found to suppress the *Apg*$^-$ and *Cvt*$^-$ phenotypes of the *atg4* mutant (Marino et al., 2003). Moreover, one of the two *Drosophilla*Aut2/Apg4/Atg4 homologues has been shown to play an essential role in the Notch-signaling pathway (Thumm and Kadowaki, 2001), suggesting that the four human Atg4 homologues may have divergent functions.

Interestingly, in *S. cerevisiae*, expression of a mutant form of Atg8 with a carboxyl terminal Gly (Atg8-FG) along with the *atg4* mutant results in the significant accumulation of the lipidated form of Atg8 (Atg8-PE) (Kirisako et al., 2000), suggesting that yeast Atg4 may be important for the delipidation of Atg8-PE as well

as for the cleavage of the carboxyl terminal region of Atg8. It has not been shown, however, if Atg4 directly delipidates Atg8-PE to Atg8 or indirectly activates an as yet unidentified delipidating enzyme.

In mammals, hAtg4B delipidates endogenous LC3-II and GABARAP-II in vitro (Fig. 3) (I. Tanida, Y. Sou, T. Ueno, E. Kominami, manuscript in preparation). When a purified GST-hAtg4B was incubated with a pelletable fraction enriched for endogenous LC3-II and GABARAP-II, the mobility of LC3 and GABARAP was altered, similarly to treatment with phospholipase D. A mutant GST-hAtg4B^{C74A} with a Cys to Ala mutation at the predicted active site, however, had no effect on LC3-II or GABARAP-II. The activity of hAtg4B is sensitive to NEM. Interestingly, when this protein was overexpressed in HeLa cells, the amount of endogenous LC3-II was decreased, and the amount of LC3-I was increased. When CFP-hAtg4B and YFP–LC3 were expressed in HeLa cells, more of the latter was distributed in the cytosol. These results indicate that, in mammals, hAtg4B delipidates LC3-II and GABARAP-II, in addition to being a cysteine protease that cleaves the carboxyl termini of LC3, GABARAP, and GATE-16.

3. Autophagy and endogenous LC3 in health and diseases

3.1. Role of LC3 in mammalian autophagy

As an intrinsic component of autophagosomal membranes, the lipidated form of Atg8 has drawn great attention in recent research on autophagy. In yeasts, nutrient deprivation has been shown to activate the transcription of Atg8 mRNA, leading to elevated expression of Atg8 protein (Kirisako et al., 2000). A significant fraction of these Atg8 molecules is processed by Atg4 protease, activated by Atg7, and transferred to Atg3 to be conjugated with phosphatidylethanolamine (PE) (Ichimura et al., 2000), which is subsequently recruited onto autophagosomal membranes. In starvation-induced autophagy of mammalian cells, the conjugation system is more complicated, in that there are three Atg8 homologues, all of which can be substrates for Atg7 and Atg3 (Tanida et al., 2001, 2002a). When the three homologues were coexpressed in cultured cells, all three were concentrated together following nutrient deprivation (T. Yoshimori, Y. Kabeya, N. Mizushima, Y. Ohsumi, personal communication), suggesting that, similar to yeast Atg8, the three mammalian Atg8 homologues can undergo lipidation to be recruited onto autophagosomes, although the target phospholipid has not yet been identified. Of the three homologues, LC3 has been best characterized as an autophagosomal marker in mammalian autophagy.

In a pioneering work by Kabeya et al. (Kabeya et al., 2000), newly synthesized LC3 precursor was shown to be processed cotranslationally to form a soluble LC3 (LC3-I). Upon starvation, LC3-I was modified to LC3-II, the membrane-bound form, which has greater mobility in SDS-PAGE than LC3-I (Kabeya et al., 2000). This LC3-II was shown to localize on autolysosomal membranes isolated from rat liver. In addition, immunoelectron microscopy showed that overexpressed GFP–LC3 was localized on both autophagosomes and autolysosomes (Kabeya et al., 2000). Recently, important information on the role of LC3 in autophagy has been reported (Mizushima, Yamamoto, Matsui, Yoshimori, & Ohsumi, 2004) using transgenic mice expressing GFP–LC3 systemically. Under fed conditions, GFP–LC3 was distributed diffusively throughout the entire cytoplasm. Under fasting conditions, however, GFP–LC3 fluorescence was present in punctate structures corresponding to autophagosomes and autolysosomes in many tissues, indicating that the fluorescent LC3-construct overexpressed in living animals is a good indicator of autophagy. Strikingly, starvation-induced redistribution of GFP–LC3 onto autophagosomes is more clearly observed in skeletal and heart muscles that were previously thought to be less active in autophagic protein degradation than in ubiquitin-proteasome-dependent degradation. In contrast, GFP–LC3 fluorescence does not respond to nutrient starvation in the brain, indicating that brain autophagy is not controlled by serum amino-acid level, a key determinant for hepatocyte autophagy (Schworer, Shiffer, & Mortimore, 1981). It was also observed that some tissues, including lens and thymus, demonstrated widespread punctate fluorescence irrespective of feeding–fasting conditions, indicating that autophagy is constitutively active in these tissues (Mizushima et al., 2004). Thus, the data clearly indicate that at least LC3 functions as a universal and promising autophagosomal mem-

brane marker, i.e. a functional Atg8 homologue in mammalian autophagy.

In an attempt to evaluate the role of the other two homologues in autophagy, we analyzed endogenous level of these homologues in rat tissues. Rats were starved for 12 h and various tissues were isolated. They were homogenized, centrifuged at 100,000 × *g*, separated into supernatant and pellet, and subjected to immunoblotting analysis with specific antibodies to endogenous Atg8 homologues (Fig. 2). The distribution of these three homologues was quite divergent, in that LC3 is abundantly expressed in brain, liver, and skeletal muscles, GABARAP is relatively abundant in spleen and kidney, and GATE-16 is enriched in brain, skeletal muscles, and heart. LC3-II was recovered in the pellet fraction, whereas LC3-I was mainly present in the supernatant. The abundance of LC3-I in both pellet and supernatant from brain is likely due to the abundance of LC3-I existing as a small subunit of MAP-1A and MAP-1B. In addition, two bands each were observed for GABARAP and GATE-16, presumably corresponding to their soluble and the lipidated forms, although, in contrast to LC3, both forms of GABARAP and GATE-16 were recovered in the supernatant and pellet fractions. The reason for this difference is not known. The divergence in tissue distribution may indicate that the use of GABARAP or GATE-16 in autophagy is more tissue dependent than is LC3. It is also possible that these homologues may have tissue-specific functions other than those in autophagy. For example, GATE-16 is more concentrated than LC3 in skeletal and heart muscles, although overexpressed GFP–LC3 in these tissues of transgenic mice respond to form autophagosomes during starvation (Mizushima et al., 2004). In contrast, although endogenous LC3-II was abundantly present in the brain (Fig. 2), GFP–LC3 signal in the brain is insensitive to nutrient deprivation in GFP–LC3 transgenic animals (Mizushima et al., 2004). More investigations are necessary to better understand the correlations in autophagy among, and possible some other cellular function(s) of, the three homologues.

It should be also noted that cellular levels of LC3, GABARAP, and GATE-16 are indicative of the quantity of autophagic vacuoles (autophagosomes plus autolysosomes) not the autophagic proteolytic activity per se (for detailed discussion, see (Ueno, Tanida, & Kominami, 2004)). Starvation-induced formation of autophagosomes enhances the cellular levels of autophagosomal Atg8 homologues, whereas maturation of autophagosomes into autolysosomes and subsequent degradation of autophagosomal components diminish their levels. Indeed, we have observed with cultured hepatocytes that during long-term starvation (>4 h), LC3-II level decreases, but that in the presence of lysosomal proteinase inhibitors, more LC3-II accumulates (Asanuma et al., 2003). This is consistent with the previous observation that the number of autophagic vacuoles is profoundly increased when lysosomal proteolysis is inhibited (Ishikawa, Furuno, & Kato, 1983).

3.2. LC3 in cell differentiation

In addition to its pivotal role in protein turnover during feeding–fasting cycles, autophagy is essential in development and cell differentiation. Bulky degradation of cell constituents are necessary during dramatic cellular remodeling that accompanies development, metamorphosis and cell differentiation. In fact, *ATG* genes have been shown to be required for proper development of *Drosophila* (Juhasz, Csikos, Sinka, Erdelyi, & Sass, 2003) and the slime mold, *Dictyostelium discoideum* (Otto, Wu, Kazgan, Anderson, & Kessin, 2003). As described previously (Section 2.2), transition of LC3-I–LC3-II was found to occur gradually but concomitantly with the differentiation of cultured podocytes (Asanuma et al., 2003). After differentiation, LC3-II was found to localize on vacuoles morphologically resembling autophagosomes and autolysosomes. It is not known, however, whether these vacuoles are responsible for cell remodeling required for differentiation. More recently, we confirmed that essentially the same transition of LC3-I–LC3-II occurs during differentiation of C2C12 murine myoblasts (I. Tanida, M. Wakabayashi, T. Ueno, & E. Kominami, unpublished observation).

3.3. LC3 in microbial infection

During the last decade, evidence has accumulated regarding the involvement of autophagy in microbial invasion of mammalian cells (for review, see (Dorn et al., 2002)). In the initial step of invasion, bacteria are usually incorporated into phagosomes or endo-

somes. These bacteria then either break out of the phagosome to enter the cytoplasm, or remain and proliferate in the phagosome by preventing its fusion with a lysosome to form a mature phagolysosome, which has an acidic environment with abundant hydrolytic enzymes unfavorable for bacterial survival. Some infectious bacteria, including *Porphyromonas gingivalis* and *Coxiella burnetii*, are internalized into a double-membrane bound, autophagosome-like compartment. In coronary artery endothelial cells infected with *P. gingivalis*, the bacteria were found to be internalized in autophagosome-like vacuoles enriched with Atg7 and rab5 (Dorn, Dunn, & Progulske-Fox, 2001). At later stage of the infection, these compartments acquired BiP, an endoplasmic marker, and lamp1, an endosomal/lysosomal membrane protein, but no cathepsin L. The infection was inhibited by 3-methyladenine and wortmannin, which inhibit autophagy. In *C. burnetii*-infected HeLa cells, the bacteria were sequestered into vacuoles labeled with LysoTracker, a marker of acidic compartments, and monodansylcadaverine, a marker of autophagic vacuoles (Beron, Gutierrez, Rabinovitch, & Colombo, 2002). The bacteria-containing vacuolar membranes also contain LC3, and their formation was sensitive to 3-methyladenine and wortmannin. At later stages of infection, the bacteria-containing vacuoles acquired rab7, a marker for late endosomes (Beron, Gutierrez, Rabinovitch, & Colombo, 2002), further indicating that these vacuoles resemble autolysosomes. These findings suggest it likely that these invasive bacteria can somehow stimulate the signaling pathway, forming autophagosomes and thus enabling the bacteria to enter the cytoplasm of host cells. In case of *Legionella* infection into *Dictyostelium* amoeba, however, the formation of replication vacuoles has been found to occur independently of host *ATG* genes, precluding the possibility that autophagy is required for bacterial replication (Otto et al., 2004).

Formation of double-membrane autophagosomes is also very likely to correlate with viral infection and replication. When animal cells were infected with RNA viruses, such as poliovirus and corona virus, the RNA replicative complexes were bounded by cytoplasmic membranes resembling autophagosomes (Prentice, Jerome, Yoshimori, Mizushima, & Denison, 2004; Schlegel, Giddings, Ladinsky, & Kirkegaard, 1996; Suhy, Giddings, & Kirkegaard, 2000). Frequently, these vacuoles contained cytoplasmic materials in the lumen (Schlegel et al., 1996), a characteristic shared by nascent autophagic vacuoles. Recent extensive analyses has revealed more clearly that the molecular machinery for autophagy operates in a cradle for viral replication (Prentice et al., 2004). RNA replication of corona virus takes place on autophagosome-like double-membrane vesicles (DMV), on which LC3 and Atg12 are co-localized. This viral replication is impaired in autophagy-deficient *ATG5*−/− embryonic stem cells, but recovered by overexpression of Atg5 in the cells. During infection, degradation of long-lived proteins is significantly enhanced. In contrast to the replication of viral particles, this degradation is not sensitive to 3-methyladenine, suggesting that *ATG*-dependent formation of DMV is necessary for efficient replication of the virus.

3.4. LC3 in cancer

It has been generally accepted that autophagy is suppressed in many cancer cells and that cellular autophagic activity is inversely correlated with malignancy (for review, see (Ogier-Denis & Codogno, 2003)). For example, the autophagic activity in drug-induced primary hepatocellular carcinomas was observed to be one-fourth that of normal hepatocytes (Kisen et al., 1993). At present, there are two lines of evidence suggesting a relationship between inactivation of autophagy and malignancy at the molecular level.

Beclin 1 is the mammalian homologue of yeast Atg6/Vps30 and forms a multimeric complex with Atg14, Vps34/class 3 PI3kinase, and Vps15. This complex is pivotal in autophagosome formation (Kihara, Kabeya, Ohsumi, & Yoshimori, 2001; Kihara, Noda, Ishihara, & Ohsumi, 2001; Liang et al., 1999). The level of beclin 1 expression was shown to be lower in malignant than in normal breast epithelial cells (Liang et al., 1999). In addition, gene transfer of beclin 1 was observed to promote autophagy in these malignant cells, leading to the loss of malignant morphologic features. Target deletion of beclin 1 gene in mice has been attempted, but homozygous beclin−/− mice die during early embryonic development, indicating that the beclin 1 gene is essential for embryogenesis. Interestingly, heterozygous disrup-

tion of the beclin 1 gene results in a high incidence of spontaneous tumors in mice (Qu et al., 2003; Yue, Jin, Yang, Levine, & Heintz, 2003), although the remaining wild-type allele is neither mutated nor silenced. Hence, beclin 1 is a haplo-insufficient tumor suppressor gene. This is the first example in which a genetic defect of an *ATG* gene was directly linked to carcinogenesis. It is therefore of particular importance to examine the occurrence of tumors in other ATG gene knock-out animals.

It has been shown that inactivation of the PTEN gene, a tumor suppressor gene, frequently occurs in human tumors. The phosphoinositide phosphatase activity of PTEN negatively controls the class 1 PI3kinase/Akt signaling pathway. Inactivation of PTEN results in activation of class 1 PI3kinase/Akt kinase. In contrast to the class 3 PI3kinase, whose activity is essential to autophagy (Ogier-Denis & Codogno, 2003; Kihara et al., 2001), the class 1 PI3 kinase strongly inhibits autophagy (Arico et al., 2001). Not all malignant cells, however, show decreased levels of autophagy. PTEN is active in HT-29 colon cancer cells, which exhibit constitutively active autophagy (Houri et al., 1995).

Temozolomide (TMZ) is an alkylating reagent that has been used clinically to treat primary or recurrent gliomas. Treatment of glioma cell cultures with TMZ (5-1000 μM) was found to inhibit cell viability and increase the number of LC3-positive autophagic vacuoles, showing that TMZ stimulates autophagy in glioma cells (Kanzawa et al., 2004). Interestingly, incubation of glioma cells with TMZ plus bafilomycin, which inhibits autophagic protein degradation but not accumulation of autophagosomes, caused apoptosis of these cells. In order to obtain more insight into the relationship between tumorigenesis and autophagy, more thorough investigations are necessary to clarify signaling pathways controlling cell proliferation, autophagic protein degradation, and cell death.

3.5. LC3 in neuromuscular diseases

In several hereditary neuromuscular diseases, accumulation of cytoplasmic protein aggregates, lipofuscin deposits, and membrane structures resembling autophagic vacuoles (autophagosomes plus autolysosomes) has been reported. The accumulation of protein aggregates and lipofuscin that are not present in normal tissues appears to indicate dysfunction of cellular protein turnover. One common characteristic of many hereditary neuromuscular disorders is that mutations cause conformational changes in particular proteins. Many of these mutant proteins have a strong tendency to form insoluble aggregates, which can be sequestered into autophagic vacuoles but are resistant to lysosomal proteinases. It should be also noted that insoluble protein aggregates in the cytoplasm strongly inhibit the ubiquitin–proteasome system. Thus, the two major protein degradative machineries that greatly contribute to quality control of cytoplasmic components are suppressed, vitiating various cellular activities and eventually causing cell death. In several neuromuscular diseases, dysfunctional autophagy has been well characterized.

3.5.1. Danon disease

Danon disease is an X-chromosome-linked myopathy and cardiomyopathy caused by mutations of the *LAMP-2* gene (Nishino et al., 2000). LAMP-2b is a major lysosomal membrane protein, which is highly glycosylated and expressed mainly in skeletal muscles and heart. In patients with Danon disease, abundant autophagic vacuoles accumulate in skeletal muscles and heart. These characteristics overlap with those of LAMP-2-deficient mice (Tanaka et al., 2000), which have a stronger tendency to die earlier than normal littermates, and in which even survivors display lower body weight. As a principal membrane protein, LAMP-2 may play important, but as yet unknown, roles during autophagosome maturation.

3.5.2. Distal myopathy with rimmed vacuoles (DMRV)

Distal myopathy with rimmed vacuoles (DMRV) is an autosomal recessive disorder characterized by rimmed vacuoles in distal skeletal muscles, such as tibialis anterior and quadriceps. Morphological analyses have revealed that vesicles with single- or double-limiting membranes cluster in the area of rimmed vacuoles that are also positive for lysosomal proteins, ubiquitin, and β-amyloid (Kumamoto, Abe, Nagao, Ueyama, & Tsuda, 1998). LC3 was recently localized in the region of rimmed vacuoles formed in human DMRV patient specimens (Suzuki et al., 2002), suggesting that focal accumulation of autophagic vacuoles in the region of

rimmed vacuoles is a hallmark of this disease. The gene responsible for DMRV has been identified as that encoding the enzyme UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine (Eisenberg et al., 2001). This gene is necessary for sialic acid biosynthesis, making the involvement of a mutant gene in the accumulation of rimmed vacuoles unclear. Mutations of this gene in DMRV patients cause a reduction, but not a total loss, of the proteins enzymatic activity, suggesting that reduced levels of sialic acid in muscles cause hypo-sialylation and abnormal glycosylation of muscle proteins, which precedes the formation of rimmed vacuoles (Noguchi et al., 2004).

3.5.3. Huntington disease

Huntingtons disease (HD) is an autosomal dominant disorder caused by mutations of huntingtin, a cytosolic protein enriched in striatal and cortical neurons. Huntingtin has a polyglutamine (polyQ) tract in its N-terminus. In Huntingtons disease, abnormal expansion of polyQ, caused by reiterations of the codon CAG in exon 1 of the Huntingtin gene, produces mutated huntingtin with an expanded polyQ repeat, consisting of more than 37 units. The mutant huntingtin or N-terminal polyQ fragments cleaved from it by caspases accumulate in the cell to form insoluble aggregates, which cause cell toxicity and cell death. The longer the polyQ repeat, the stronger the tendency to form aggregates, accelerating the onset and strengthening the severity of the disease. Thus, the formation of insoluble polyQ aggregates is an early event leading to manifestation of Huntingtons disease.

The mutant huntingtin is frequently associated with autophagic vacuoles, with or without sequestered components, dense lysosomes, and multilamellar and tubulovesicular structures (Kegel et al., 2000). These autophagic vacuoles and lysosome-like dense vacuoles could be also stained with anti-huntington antibody. However, a significant fraction of huntingtin molecules appear to reside on the outer surface of these vacuoles (Kegel et al., 2000). These morphological features suggest that aggregates of mutant huntingtin stimulate autophagy, but that the entire process of autophagy is halted due to huntingtin sequestered in the lumen or associated with the outer surfaces of autophagic vacuoles.

In an investigation of huntingtin degradation via autophagy (Ravikumar, Duden, & Rubinsztein, 2002), 74 polyQ repeats fused to the N terminus of green fluorescent protein (polyQ74-GFP) were transfected into COS7 cells. Degradation of polyQ74-GFP was inhibited by 3-methyladenine and bafilomycin, but enhanced by rapamycin. This was also confirmed in stable transfectants of PC12 cells expressing polyQ74-GFP. Interestingly, inhibitors of autophagy enhanced cell death, whereas rapamycin reversed these effects. It should be also noted that overexpressed polyQ74-GFP forms insoluble aggregates in a time-dependent manner, and that, once formed, these insoluble aggregates become resistant to rapamycin-induced autophagy.

Glucose metabolism was recently shown to be important in the clearance of insoluble huntingtin aggregates (Ravikumar et al., 2003). In stably inducible PC12 cells expressing either mutant or wild-type huntingtin exon 1, cotransfection of GLUT1, a glucose transporter, was shown to reduce cell death caused by mutant huntingtin exon 1. Moreover, increasing the glucose concentration in the culture medium caused a dose-dependent reduction of cells with huntingtin aggregates as well as cell death, suggesting that increased intracellular glucose concentration is required for efficient clearance of mutant huntingtin aggregates via autophagy. 2-Deoxyglucose, which is phosphorylated to a glucose-6-phosphate analogue but not metabolized further, was found to clear mutant huntingtin more effectively than glucose, as well as to stimulate dephosphorylation of mTor, ribosome S6, and Akt. These findings indicate that glucose-6-phosphate is a key intermediate connecting the signaling pathways of glucose-induced autophagy and amino-acid deprivation-induced autophagy.

4. Conclusions and future aspects

1. As a universal Atg8 homologue in autophagosomal membranes, LC3-II is a useful reporter for starvation-induced autophagy in healthy cells, as well as for dysfunctional autophagy in diseased cells. As the major protein degradative mechanism, autophagy primarily functions as a house cleaning or self-protective process. Au-

tophagy may also contribute to cellular death mechanisms. Type II autophagic cell death is frequently observed when type I apoptosis is inhibited or when a cell-death-related kinase, such as DAP kinase, is overexpressed (for review, see (Bursch, 2001)). It has been recently found that glucose deprivation in the rat cardiomyocyte-derived cell line H9c2 induced cell death insensitive to caspase inhibitors, with accumulation of autophagic vacuoles and a parallel increase in LC3-II observed under these conditions (Aki, Yamaguchi, Fujimiya, & Mizukami, 2003). These processes were completely inhibited by 3-methyladenine. Transfection of these cells with the wild-type, active form of class 1 PI3 kinase accelerated cell death as well as the accumulation of autophagic vacuoles, whereas transfection with a dominate-negative form of PI3 kinase reduced these phenomena. It has therefore become a matter of great interest to distinguish between the self-defending and cell-death-promoting roles of autophagy.

2. The roles of the Atg8 homologues, GABARAP and GATE-16, in autophagy are still poorly understood. These two proteins were originally identified in the brain, as was LC3, and soluble GABARAP and GATE-16 were shown to play distinct roles in membrane trafficking and plasma membrane receptor function, respectively. Since these two proteins are widely distributed among different tissues, with a fraction of each modified to its membrane-bound form, they may be important in starvation-induced autophagy in various tissues. Among the questions still to be answered are whether these two homologues can replace LC3 in autophagosome formation and why the distribution of the three homologues is so divergent.

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