ATG Systems from the Protein Structural Point of View

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Contents

1. Introduction 1587
2. Atg Proteins 1587
   2.1. Classification of Atg Proteins 1587
   2.2. Atg Conjugation Systems 1588
3. Structural Aspects of Atg8 Homologues and Their Functions 1589
   3.1. Structure of Atg8 Homologues 1589
   3.2. Structural Aspects of Atg8 Functions in Target Recognition 1591
   3.3. Structural Aspects of Atg8 Functions in Autophagosome Formation 1591
4. Structural Aspects of Enzymes Modifying Atg8 1592
   4.1. Processing and Deconjugating Enzyme Atg4 1592
   4.2. Activating (E1-like) Enzyme Atg7 1593
   4.3. Conjugating (E2-like) Enzyme Atg3 1593
   4.4. The E3-like Atg12—Atg5—Atg16 Complex 1594
5. Conclusions 1596
6. Acknowledgments 1596
7. References 1596

1. Introduction

Cellular homeostasis is maintained by a balance between the synthesis and breakdown of a number of proteins. Most of the selective degradation of these proteins occurs in the cytosol through the ubiquitin/proteasome system.[1] Major bulk degradation takes place in the lytic compartment, the lysosome in mammals, and the vacuole in yeasts and plants. Lysosomal/vacuolar degradation of cellular components, autophagy, is further classified into three types: macroautophagy, microautophagy, and chaperone-mediated autophagy, in which macroautophagy (autophagy hereafter) is the main route for delivery of cytoplasm to the lysosome/vacuole.[2] Further to the above-mentioned differences, autophagy is distinct from the ubiquitin/proteasome system in that it can degrade much larger targets such as protein inclusions and organelles. Autophagy was originally known as a cellular response to enable survival during starvation.[3] However, recent studies have shown a variety of physiological roles of autophagy including intracellular clearance, differentiation, development, programmed cell death, antigen representation, and elimination of invading pathogens, and autophagic dysfunction is associated with a number of diseases, including cancer and neurodegeneration.[4−7]

Autophagy in yeast proceeds as follows in Figure 1:[2,8−10] isolation membranes (phagophores) appear in the cytoplasm and expand to enclose a portion of the cytoplasm, forming a double membrane structure, an autophagosome. The autophagosome fuses with the vacuole, the inner membrane and its contents, termed autophagic bodies, are degraded by vacuolar hydrolases, and the constituents are reused in the synthesis of macromolecules such as proteins. These processes are mediated by a number of unique proteins, Atg proteins (see the next chapter).

Despite the many genetic and biochemical studies on the mechanism of autophagy, many problems remain to be elucidated. For example, it is unknown where the membranes for autophagosome formation come from and how the isolation membranes expand to form autophagosomes. Many Atg proteins have been characterized, but the precise roles in autophagosome formation are largely unknown. The lack of understanding of these issues is partly due to a lack of knowledge of the Atg protein structures. Most Atg proteins have novel sequences and lack characterized domains/motifs, and their functions and structures are difficult to estimate with sequence comparisons. Details of the structures of most Atg proteins still remain unknown, but those of the Atg conjugation systems have been determined experimentally in the most recent five years. The following sections summarize the Atg proteins responsible for autophagosome formation and review the structures of Atg8 homologues and enzymes modifying Atg8 and the functions suggested by the structures.

2. Atg Proteins

2.1. Classification of Atg Proteins

With yeast genetics, mutants deficient in the autophagic pathway have been obtained in *Saccharomyces cerevisiae*,[11,12] and 31 *ATG* (autophagy) genes have been identified, among which 18 *ATG* genes are essential for the autophagosome formation step.[13,14] These 18 *ATG* genes are mostly conserved among higher eukaryotes such as mammals[10] and plants,,[15,16] suggesting that the molecular machinery of autophagosome formation is evolutionarily conserved. In *S. cerevisiae*, most of the 18 Atg proteins essential for autophagosome formation localize to a punctuate structure proximal to the vacuole.[13,14,17−19] This preautophagosomal structure (PAS)[17] is believed to be the site of autophagosome formation although whether the PAS produces the isolation membranes or PAS itself becomes the isolation membranes is not established.[13] The 18 Atg proteins are categorized into five functional groups: (i) Atg1 protein kinase and its regulators (Atg13, Atg17, Atg29, and Atg31),[14,18,20−24] (ii) the autophagy-specific phosphatidylinositol 3-kinase complex (Vps30/Atg6, Atg14, Vps15, and Vps34),[25,26] (iii) integral membrane protein

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Atg9[27] and the Atg2−Atg18 complex,[13,28−31] (iv) the Atg8 conjugation system (Atg5, Atg4, Atg7, and Atg8),[32,33] and (v) the Atg12 conjugation system (Atg5, Atg7, Atg10, Atg12, and Atg16).[34−36]

In group (i), Atg1 is a protein kinase and its kinase activity is essential for autophagy although its substrate is not known,[21,37] Atg13 is hyperphosphorylated under growing conditions and rapidly dephosphorylated after Tor kinase inactivation by nitrogen starvation or rapamycin treatment.[21,38] Dephosphorylated Atg13 binds to Atg1 and activates its kinase activity, which is thought to induce autophagy, although no mechanism has been reported.[21] Atg17 constitutively forms a ternary complex with Atg29 and Atg31[22] and also forms a complex with the Atg1−Atg13 complex in starvation.[21−23] The localization of Atg1−Atg13 and Atg17−Atg29−Atg31 complexes to the PAS does not require other Atg proteins, and the complex is thought to be the core of the starvation-induced PAS.[13,22,37]

The role of the autophagy-specific phosphatidylinositol 3-kinase complex is to produce phosphatidylinositol 3-phosphate (PtdIns(3)P) at the PAS. The localization of this complex at the PAS is determined by Atg14[39] but also requires Atg9 and Atg13.[13] Recently, PtdIns(3)P was shown to be abundant in isolation membranes and autophagosomal membranes, and it is finally transported into the vacuole by autophagy,[40] suggesting that PtdIns(3)P plays a critical role in autophagosome formation. One identified role of PtdIns(3)P in autophagy is to recruit the Atg2−Atg18 complex to the PAS.[31]

Atg9 in group (iii) is the only integral membrane protein among the 18 Atg proteins,[27] and Atg9−GFP occurs in two forms: one in the PAS and the other in the cytoplasm outside of PAS.[27,41] Localization of Atg9 to the PAS requires Atg17,[13] whereas the loss of Atg1 kinase activity results in accumulation of Atg9 to the PAS.[41] The bidirectional movement of Atg9 between the PAS and the cytoplasm is necessary for autophagosome formation.[41−43] Based on these observations, a model, where Atg9 is recycled between the PAS and the cytoplasmic pool, and thus supplying lipids to the PAS, has been proposed.[9,41,44] Further, the Atg2−Atg18 complex as well as the kinase activity of Atg1 have been suggested to control the cycling of Atg9.[41] However, it is still not known whether Atg9 actually supplies lipids to the PAS by shuttling between two sites.

2.2. Atg Conjugation Systems

Eight Atg proteins constitute two ubiquitin-like conjugation systems: the Atg8 and Atg12 systems[45] (Figure 2). The Atg12 system is comprised of five Atg proteins: Atg5, Atg7, Atg10, Atg12, and Atg16.[34−36] Atg12 is activated by Atg7, an E1-like enzyme,[34] and is then transferred to Atg10, an E2-like enzyme.[36] Finally, the C-terminal glycine of Atg12 is conjugated to the side-chain of Lys-149 of Atg5.[34] Thus far, E3-like enzymes have not been reported for this conjugation system. There are no processing and deconjugating enzymes for Atg12, and the Atg12−Atg5 conjugate behaves like a single protein. The Atg12−Atg5 conjugate further forms a complex with a multimeric protein, Atg16 (Atg16L in the case of mammals and plants), in which Atg5 interacts with Atg16 noncovalently.[35,46]

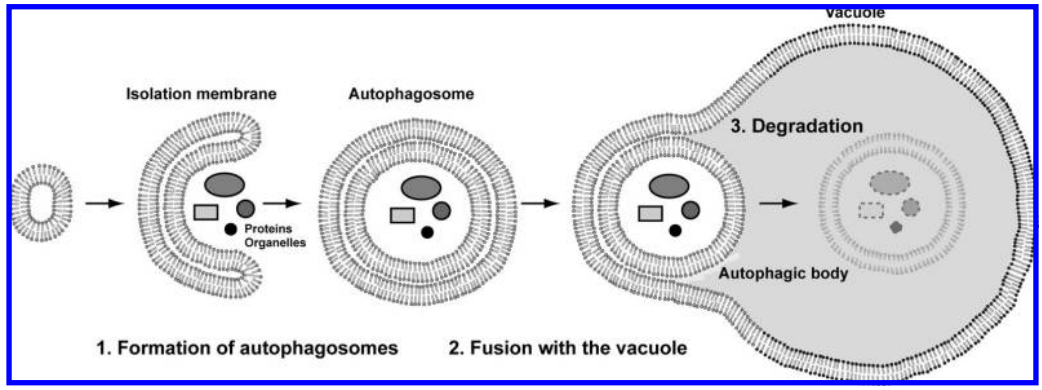

Figure 1. Schematic outline of autophagy in yeast.

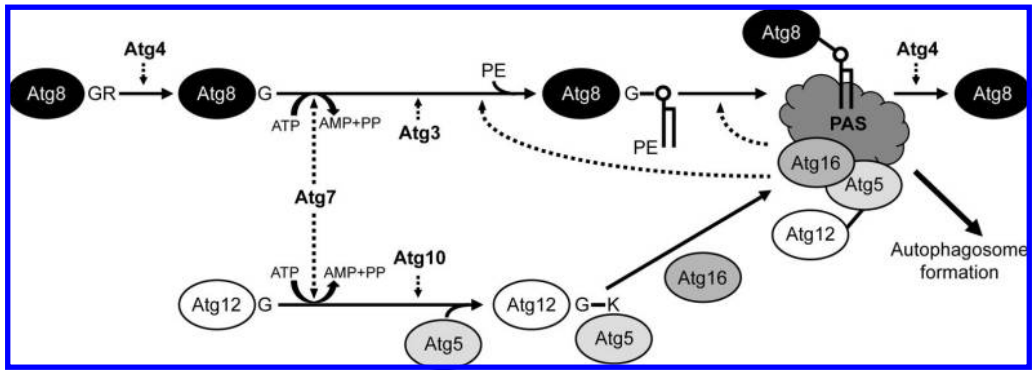

Figure 2. Atg conjugation systems. Atg12 is irreversibly conjugated to Atg5, and the Atg12—Atg5 conjugate further forms a complex with Atg16, whereas Atg8 is reversibly conjugated to PE. Both the Atg12—Atg5—Atg16 complex and Atg8—PE localize to the PAS, a perivacuolar site where most Atg proteins colocalize.

The Atg8 system is comprised of four Atg proteins: Atg3, Atg4, Atg7, and Atg8[32] (LC3 in mammals[47]). Nascent Atg8 is processed by a cysteine protease Atg4 to expose a glycine at its C-terminus.[33] The processed Atg8 is then activated by Atg7, a common E1-like enzyme as in the Atg12 system, and is then transferred to Atg3, an E2-like enzyme.[32] Finally, the C-terminal glycine of Atg8 is conjugated to the amino group of phosphatidylethanolamine (PE).[32] Atg8—PE is deconjugated by Atg4 again. In vitro reconstitution of the Atg8 system has shown that Atg7, Atg3, PE-containing liposomes, and ATP are sufficient for the conjugation of Atg8 with PE;[48] however, the Atg12 system is known to be necessary for Atg8—PE formation in vivo.[17] Recently, the Atg12—Atg5 conjugate was shown to promote Atg8—PE formation in vitro.[49] Thus, the Atg12—Atg5—Atg16 complex may be considered to be a candidate for the E3-like enzyme in the Atg8 system.

The Atg12—Atg5—Atg16 complex localizes to the PAS depending on the autophagy-specific phosphatidylinositol 3-kinase complex.[13] In mammals, the Atg12—Atg5—Atg16L complex localizes to the isolation membranes, and as the isolation membranes expand, the localization becomes restricted to the outer surface.[50] Immediately before or after completion of autophagosome formation, the complex dissociates from the autophagosome.[50] From these features as well as from the ability to oligomerize, the Atg12—Atg5—Atg16 complex was proposed to function as a structural support or a coating for autophagosome formation.[44] However, recent quantitative analysis of Atg proteins by fluorescence microscopy showed that the amount of Atg16 at the PAS is too low to allow the Atg12—Atg5—Atg16 complex to cover the entire surface of an autophagosome.[51]

Atg8—PE conjugates also localize to the PAS, for which the Atg12—Atg5—Atg16 complex is required.[13,17,50] Different from the Atg12—Atg5—Atg16 complex, Atg8—PE conjugates localize to both the inner and outer membranes, and some remain on the autophagosomes after completion and are delivered into the vacuole.[52] Recent studies have shown that Atg8—PE conjugates play a crucial role in autophagosome formation, especially in the membrane expansion step.[53,54] Recent studies also reported that Atg8 plays a role in recognition of specific cargos during selective autophagy (see below).

3. Structural Aspects of Atg8 Homologues and Their Functions

3.1. Structure of Atg8 Homologues

Structural studies of Atg8 have been performed on mammalian homologues. Mammals have at least four Atg8 homologues: GATE-16 (a Golgi-associated ATPase enhancer of 16 kDa),[55] GABARAP (a γ-aminobutyric acid type A receptor-associated protein),[56] LC3 (a microtubule-associated protein light chain 3),[57] and Atg8L.[58] The structural study was performed on GATE-16 by X-ray crystallography[59] (Figure 3A). GATE-16 was isolated in a search for factors that aid in intra-Golgi transport,[55] and it was found to have 55% sequence identity with *S. cerevisiae* Atg8 and is conjugated to a lipid, possibly PE, by a conjugation system similar to the yeast Atg8 system.[60] However, the role of GATE-16 in autophagy remains elusive. The structure of GATE-16 is comprised of the N-terminal helical domain and the C-terminal ubiquitin-like domain.[59] The C-terminal ubiquitin-like domain is comprised of a four-stranded central

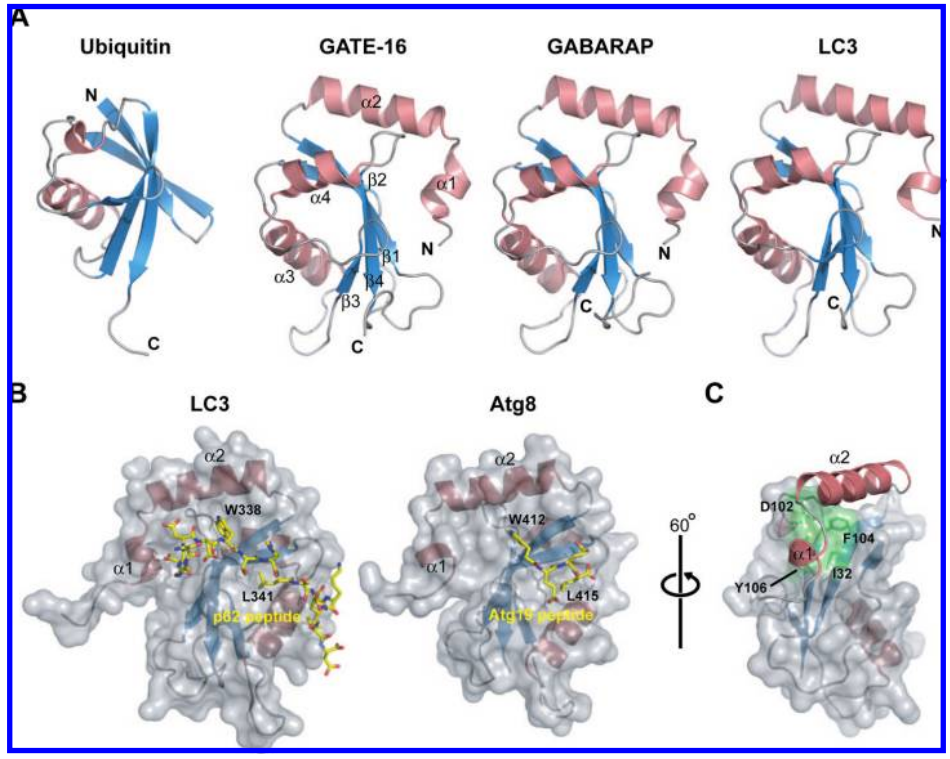

Figure 3. Structure of Atg8 homologues: (A) Ribbon representation of ubiquitin (PDB code 1UBI), GATE-16 (PDB code 1EO6), GABARAP (PDB code 1GNU), and LC3 (PDB code 1UGM) in the same orientation. α-Helices are salmon pink, and β-strands are cyan. (B) Structure of the LC3-p62 peptide (left, PDB code 2K6Q) and the Atg8-Atg19 peptide (right, PDB code 2ZPN) complexes in the same orientation. LC3 and Atg8 are shown with ribbon and surface models, whereas the p62 and Atg19 peptides are shown with stick models. This LC3 model is obtained by rotating the model in part A 180° around the vertical axis. (C) Residues of Atg8 for membrane tethering and hemifusion. Atg8 (PDB code 2ZPN) is shown with a ribbon model on which a surface model is superimposed except for the N-terminal helices. The side-chains of the residues responsible for membrane tethering and hemifusion are shown with a green stick model. This figure is obtained by rotating the Atg8 model in part B 60° around the vertical axis. All structural models in Figures 3−7 were prepared using PyMOL (http://pymol.sourceforge.net/).

β-sheet (β1−β4) and two α-helices, α3 between β2 and β3, and α4 between β3 and β4, which is a very similar topology to that of ubiquitin although the sequence similarity is very low. The N-terminal helical domain is comprised of two α-helices, α1 and α2, and packs against one side of the β-sheet of the C-terminal ubiquitin-like domain. Sequence alignment shows that the N-terminal helical domain is well conserved among Atg8 homologues but not among other ubiquitin-fold superfamily members, suggesting that the N-terminal helical domain is a unique feature of Atg8 homologues.

The structural study was also performed on GABARAP. GABARAP was identified in a yeast two-hybrid search for proteins that bind to the large intracellular loop of the $GABA_A$ receptor, and it was suggested to participate in the intracellular trafficking of the $GABA_A$ receptor.[56] GABARAP is also conjugated to a lipid in a manner similar to Atg8 and GATE-16.[60] Lysosomal turnover of GABARAP−phospholipid conjugates has been shown to be enhanced during differentiation of C2C12 cells to myotubes;[61] however, the role of GABARAP in autophagy remains to be elucidated. Two conformations of GABARAP have been reported: closed and open conformations.[62] The closed conformation of GABARAP, which was reported using both X-ray crystallography[62−64] and NMR,[65] is very similar to that of GATE-16, comprising the N-terminal helical domain packing against the C-terminal ubiquitin-like domain to form a compact fold (Figure 3A). In the open conformation which was reported by X-ray crystallography,[62] the N-terminal ten residues composing α1 in the closed conformation adopt an extended structure and are detached from the ubiquitin-like domain. Residues 2−4 in the open conformation form an intermolecular β-sheet with β2 of an adjacent GABARAP molecule in the crystal lattice. Further, the side-chains of residues 1−5 form complimentary polar and nonpolar interactions with adjacent GABARAP. The open conformation seems to be stabilized by high salt crystallization conditions (2.4 M ammonium sulfate); however, it was suggested that the open conformation of GABARAP would presumably be induced and stabilized via interactions with other proteins such as tubulin or membranes in vivo.[62] The open conformation results in a head-to-tail association of a number of GABARAP molecules; therefore, it is proposed that GABARAP in the open conformation would promote tubulin polymerization and facilitate $GABA_A$ receptor clustering.

A further study on LC3 was performed by both X-ray crystallography[66] and NMR.[67] LC3 was originally identified as a light chain of microtubule-associated proteins 1A and 1B in the rat brain,[57] and LC3 is conjugated to PE in a manner similar to that of the yeast Atg8 system.[47,68,69] Further, LC3−PE conjugates localize to the isolation membranes and autophagosomes, and some of them are finally delivered into the lysosome.[47] In summary, LC3 is considered to be the mammalian Atg8 ortholog and to play a critical role in autophagy. The structure of LC3 is very similar to that of GATE-16 and the closed conformation of GABARAP (Figure 3A). LC3 is unique in the electrostatic surface potential of α1 and α2;[66] α1 of LC3 is highly basic, whereas α1 of GATE-16 and GABARAP are somewhat acidic. Further, α2 of LC3 is acidic, whereas α2 of GATE-16 and

GABARAP are neutral and basic, respectively. These differences in the electrostatic surface potentials of the N-terminal domains may be responsible for the variety of functions of these homologues. In vitro binding assays have shown that the N-terminal domain of LC3 is responsible for binding to tubulins and microtubules.[67]

Recently, the crystal structure of *S. cerevisiae* Atg8 was determined as a complex with a peptide derived from Atg19, the receptor protein for aminopeptidase I[70] (see below). This structure is very similar to that of mammalian homologues comprising the N-terminal helical domain and the C-terminal ubiquitin-like domain, and it was found that the N-terminal domain has the closed conformation (see below).

3.2. Structural Aspects of Atg8 Functions in Target Recognition

Autophagy is in principle a nonselective, bulk degradation process. However, recent studies suggest that autophagy also mediates selective degradation of various specific targets such as aggregated proteins, damaged organelles, and invading microorganisms.[4−6] As all the components sequestered by an autophagosome are delivered into the lysosome/vacuole and degraded, it may be assumed that there is a mechanism of selective degradation of targets in the sequestration step. The Atg8−PE, one of few proteins identified on isolation membranes and autophagosomal inner membranes, has the potential for selective recognition of degradation targets. In yeast, *S. cerevisiae*, two vacuolar hydrolases, aminopeptidase I (ApeI) and α-mannosidase (Ams1), are known to be selectively delivered into the vacuole independent of the secretory pathway.[71,72] This is called the cytoplasm-to-vacuole targeting (Cvt) pathway,[2,73] which is considered as a model of selective autophagy, since it is mechanically similar to autophagy. In the Cvt pathway, ApeI and Ams1 are recognized by their receptor protein, Atg19,[74] and via the interaction of Atg19 with Atg8 and Atg11, the adaptor protein for ApeI, these cargos are selectively sequestered by a double-membrane structure called a Cvt vesicle and are delivered into the vacuole.[75,76] Under starvation conditions, ApeI and Ams1 are selectively sequestered by an autophagosome and are delivered into the vacuole by autophagy in a similar manner to the Cvt pathway. Thus, Atg8 plays a key role in the selective incorporation of target compounds into the vesicles through the receptor protein Atg19. In mammals, polyubiquitinated aberrant proteins have been recently shown to be selectively degraded by autophagy.[77,78] In this process, p62 functions as a receptor protein for aberrant proteins,[78,79] p62 interacts with ubiquitin *via* its C-terminal UBA domain,[80] and it self-assembles *via* its N-terminal PB1 domain[81] and thereby can form large aggregates containing ubiquitinated proteins.[77,78] p62 further interacts with LC3 *via* a 22-residue sequence (residues 321−342),[79] and as a result, protein aggregates are selectively sequestered by an autophagosome and delivered into the lysosome. Thus, LC3 plays a key role in the selective incorporation of targets into vesicles through the receptor-like protein, p62. Recently, the structure of Atg8 complexed with the C-terminal Atg8-binding region of Atg19 was determined by X-ray crystallography (Figure 3B, right), and the structure of LC3 complexed with the LC3-binding region of p62 was determined by both X-ray crystallography[82] and NMR[70] (Figure 3B, left).

It has been found that LC3 interacts with p62 and Atg8 interacts with Atg19 in a quite similar manner. Peptides of p62 and Atg19 assume an extended β-conformation and form an intermolecular β-sheet with the β2 of LC3 and Atg8, respectively. This interaction is similar to that between SUMO and SUMO interacting motifs (SIMs), in which SIMs assume a β-conformation and form an intermolecular β-sheet with the β2 of SUMO.[83,84] However, LC3 and Atg8 also interact with the side-chains of Trp and Leu in a four-amino acid motif, Trp-X-X-Leu, in p62 (residues 338−341) and Atg19 (residues 412−415) using hydrophobic pockets conserved among Atg8 homologues but not among other ubiquitin-like proteins, including SUMO; therefore, the Trp-X-X-Leu motif is a binding sequence specific to Atg8 homologues. Mutational analysis has shown that both Trp and Leu residues in the motif of p62 and Atg19 are required for the interaction with LC3 and Atg8, respectively.[70,82] In addition to the Trp-X-X-Leu motif, three consecutive Asp residues (residues 333−335) of p62 and Glu413 of Atg19 have also been shown to be important for the interaction.[79,82] Further, in vivo studies showed that these interactions are crucial for the delivery of ubiquitin−positive protein aggregates to the lysosome[82,85] and Ape1 to the vacuole.[70] As Atg19 and p62 are entirely unrelated over their whole sequence as well as in their targets, it is quite unexpected that they utilize the same Trp-X-X-Leu motif for the interaction with Atg8/LC3 and it would be of interest to determine whether this motif takes part in other cases of selective autophagy.

3.3. Structural Aspects of Atg8 Functions in Autophagosome Formation

Studies using the in vitro reconstituted Atg8 conjugation system have shown that Atg8−PE conjugates mediate tethering and hemifusion of PE-containing liposomes,[53] and electron microscopic studies found that Atg8−PE conjugates were abundant at the junction of the liposomes.[53] Further, cross-linking experiments showed that Atg8−PE conjugates self-assembled into dimers, trimers, and higher multimers although unlipidated Atg8 remained as a monomer.[53] These data suggest that liposomes were tethered and hemifused by the interaction between Atg8−PE conjugates, and mutational analysis suggested that membrane tethering and hemifusion in vitro represent actually occurring functions of Atg8 in autophagosome formation, especially in the expansion of autophagosomal membranes in vivo.[53] Mutations leading to dysfunction of Atg8 in both tethering and hemifusion of liposomes are clustered at one face of the ubiquitin-like domain that is covered by the N-terminal helical domain[53] (Ile32, Asp102, Phe104, and Tyr106; green in Figure 3C). Therefore, if Atg8−PE conjugates self-assemble into multimers using this face of the ubiquitin-like domain, the N-terminal domain would detach itself from the ubiquitin-like domain. The N-terminal domain itself was also shown to be required for tethering and hemifusion of liposomes.[53] In the case of GABARAP, the N-terminal Met-X-X-Val sequence in the open conformation is bound to the Trp-X-X-Leu motif-binding site of another GABARAP, resulting in multimerization of GABARAP in a head-to-tail manner.[62] With Atg8, the N-terminal region does not have a sequence (hydrophobic-X-X-hydrophobic), and it is unlikely that Atg8 self-assembles in a similar manner to GABARAP. The region responsible for the membrane-expansion activity of Atg8 is spatially close to that for the Trp-X-X-Leu motif recognition. It is not established how Atg8 performs the two distinct functions using a similar surface. Structural studies of

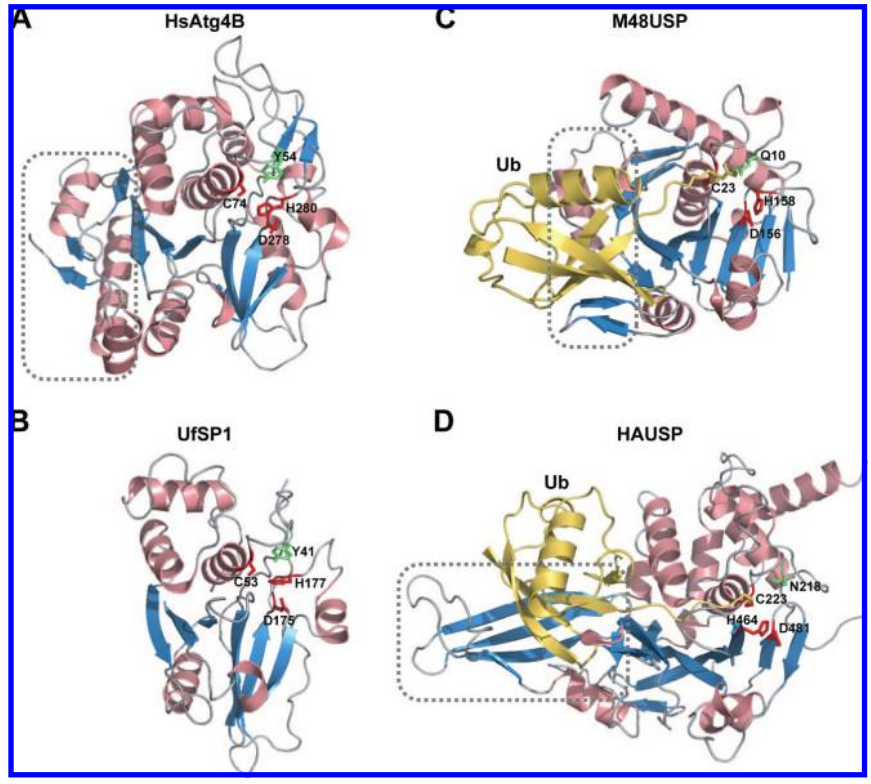

Figure 4. Structures of HsAtg4B and other deconjugating enzymes: (A) HsAtg4B (PDB code 2CY7); (B) UfSP1 (PDB code 2Z84); (C) M48USP (PDB code 2J7Q); (D) HAUSP (PDB code 1NBF). α-Helices are salmon pink, and β-strands are blue. Residues in a catalytic triad are shown with red stick models, and those comprising an oxyanion hole are shown with green stick models. In parts C and D, bound ubiquitin is yellow. The unique insertions observed in HsAtg4B, M48USP, and HAUSP are enclosed within a broken line.

Atg8—PE, especially to elucidate its multimeric state, will be necessary to address these issues as well as to understand the mechanism of membrane expansion mediated by Atg8—PE.

4. Structural Aspects of Enzymes Modifying Atg8

Reversible modification of Atg8 with PE is a necessary step in the normal progression of autophagy.[33] Therefore, the modification system of Atg8 can be a good target to control the autophagic process. This system involves ubiquitin-like conjugation reactions, and since the conjugation target is not a protein but a lipid,[32] the mechanism may be expected to be significantly different from that of other ubiquitin-like conjugation reactions. The enzymes involved in the Atg8 system have little sequence homology with those involved in other ubiquitin-like systems.[32,34] This section provides an overview of the current structural knowledge of Atg proteins involved in Atg8 modification and considers the mechanisms which underlie the modification reactions of Atg8.

4.1. Processing and Deconjugating Enzyme Atg4

Nascent Atg8 is rapidly processed by Atg4, a cysteine protease, to expose a glycine residue at its C-terminus.[33] This processing is essential for the further modification reactions of Atg8. In addition to the processing, Atg4 also mediates the deconjugation of Atg8—PE conjugates.[33,68] Thus, Atg4 plays a pivotal role in the regulation of Atg8 modifications. The activity of Atg4 is similar to that of deubiquitinating enzymes (DUBs), which mediate both the processing and deconjugation of ubiquitin. Humans have at least four Atg4 homologues (HsAtg4A-D),[33,86] among which HsAtg4B has been shown to process and deconjugate LC3.[68,87] The structure of HsAtg4B is comprised of an α/β fold with nine α-helices and 13 β-strands, showing high structural similarity to papain family cysteine proteases including DUBs[88,89] (Figure 4A). In addition to the papain-like fold, HsAtg4B has a unique inserted region, which is positionally similar to the unique ubiquitin-binding regions observed in herpesvirus-associated ubiquitin-specific processing protease (HAUSP) (Figure 4D) and the cysteine protease domain of murine cytomegalovirus M48 (M48USP)[90,91] (Figure 4C). Recently, we determined the structure of the HsAtg4B—LC3 complex, and here LC3 is also bound to this unique inserted region of HsAtg4B (unpublished data). The side-chains of Cys74, Asp278, and His280 form a catalytic triad, all of which are strictly conserved among Atg4 homologues. The order (Cys-Asp-His) of the catalytic triad in the primary sequence is unique, since most cysteine proteases of known structure have a catalytic triad of the primary sequence order Cys-His-Asp. Among proteases of known structure, HsAtg4B shows most structural similarity to UfSP1, a processing and deconjugating enzyme for Ufm1,[92] which also has a catalytic triad in the primary sequence order Cys-Asp-His[93] (Figure 4B). These two proteases as well as M48USP appear to form a new subfamily of the cysteine protease superfamily. In addition to the catalytic triad, cysteine proteases have a conserved Asn/Gln residue that participates in the formation of the oxyanion hole crucial for catalysis.[94] For HsAtg4B, Gln80 was suggested to correspond to the conserved Asn/Gln residue;[86] however, the structure of HsAtg4B shows a conserved Tyr residue (Tyr54) but not Gln80 at the Asn/Gln site of cysteine proteases (Figure 4A), and this Tyr residue contributes weakly to the protease activity of HsAtg4B.[88] In UfSP1, there is also a Tyr residue at the same

position[93] (Figure 4B), suggesting an evolutionary link to Atg4. A Cys residue is located four residues downstream of the catalytic Cys residue of both HsAtg4A and 4B, which has been shown to be the target for redox regulation of protease activities.[95] In the structure of HsAtg4B, the side-chain of Cys78 is located near that of the catalytic Cys74; therefore, these two may form a disulfide bond when exposed to reactive oxygen species, and the protease activity of HsAtg4B could be regulated in this manner. The catalytic Cys74 of HsAtg4B is buried under a loop between strands β7 and β8,[88] and substrates could not access the Cys74 in this structure, suggesting that free HsAtg4B is autoinhibited. Autoinhibition has also been observed in other deconjugating enzymes such as ubiquitin C-terminal hydrolase[96] and appears to play a role in preventing nonspecific hydrolysis. Ubiquitin and ubiquitin-like proteins have a conserved Gly-Gly sequence at the N-terminal side of the scissile bond, which is essential for hydrolysis by deconjugating enzymes. The Atg8 homologues also conserve a Gly residue at the scissile site (Gly120 in LC3) and an aromatic residue at the N-terminus of the scissile Gly (Phe119 in LC3), and these two residues appear to be essential for hydrolysis by Atg4 homologues.[88] HsAtg4B processes LC3 at the C-terminal side of Gly120; however, it did not process a peptide corresponding to the C-terminal tail (residues 116−124) of LC3.[88] This indicates that the full-length LC3 but not its C-terminal tail can release the autoinhibited conformation of HsAtg4B and that the full-length LC3 is processed by this protease. Phe80 and Leu82 of LC3 and the equivalent residues in Atg8 were reported to be necessary for the processing by Atg4 proteases;[97,98] therefore, the residues may be necessary to release the autoinhibited conformation of Atg4. The Atg4-specific inhibitors are candidates for autophagy blocking; however, inhibitors to the active site of Atg4 would not be effective, since it is autoinhibited in the free form. Compounds that stabilize the autoinhibited conformation would be candidates for such inhibitors.

4.2. Activating (E1-like) Enzyme Atg7

The C-terminal glycine residue of Atg8, which is exposed by Atg4-processing, is adenylated by Atg7 consuming an ATP molecule and is then transferred to the catalytic cysteine of Atg7 to form the Atg8∼Atg7 thioester intermediate.[32,99] The three-dimensional structure of Atg7 has not been determined experimentally, but Atg7 is distally related to the ubiquitin activating enzyme (E1) and has a region with high sequence similarity to the adenylation domain of E1 encompassing the ATP-binding sequence (Gly-X-Gly-X-X-Gly).[34] Further, Atg7 has conserved two zinc motifs (Cys-X-X-Cys; residues 485−488 and 569−572). Structural studies showed that E1 enzymes for SUMO[100] and NEDD8[101] as well as *E. coli* E1 ancestors, MoeB[102] and ThiF,[103] which catalyze the C-terminal adenylation of ubiquitin-like proteins MoaD and ThiS, respectively, all possess two Cys-X-X-Cys motifs that coordinate a single zinc ion to stabilize the protein fold. Therefore, Atg7 may also coordinate a zinc ion using these four cysteine residues to stabilize the structure. In addition to the adenylation domain, E1 enzymes have two further domains: the catalytic cysteine domain and the C-terminal ubiquitin-fold domain that is responsible for E2 binding.[100,101,104−106] The E1s for SUMO and NEDD8 have the ubiquitin-fold domain after the second Cys-X-X-Cys motif,[100,101] while Atg7 has only 58 residues after the second Cys-X-X-Cys motif, too short to assume a ubiquitin fold. The C-terminal 50 residues of Atg7 have been reported to show weak sequence similarity to the carboxy-terminal region of Uba1,[107] which corresponds to the C-terminal half of the ubiquitin-fold domain,[106] but it is not clear whether Atg7 also has an equivalent, ubiquitin-fold domain. Atg7 functions as a homodimer, and it has two ATP-binding sites and two catalytic cysteines. The canonical E1 enzymes function as either a heterodimer (E1s for SUMO and NEDD8) or a monomer with two homologous repeats (Uba1), and in both cases the enzyme assumes a structure with a pseudo-2-fold symmetry but with only one ATP-binding site and one catalytic cysteine.[100,101,106] Atg7 activates both Atg8 and Atg12,[45] different from the case of canonical E1 enzymes, which activate only one Ubl.[108] But it is not known whether this feature relies on the homodimeric structure of Atg7. Recently, Uba6/E1-L2 was also reported to activate two different modifiers, ubiquitin and FAT10.[109] FAT10 comprises two Ubl domains in a tandem array, each of which shows high sequence similarity to ubiquitin.[110] This is in contrast to the low sequence similarity between Atg8 and Atg12. Further, Uba6 shows high sequence similarity to Uba1 but not to Atg7. Therefore, the activation mechanism of two different modifiers by Uba6 may be different from that by Atg7. The interaction mechanism of Atg7 with Atg3 and Atg10 remains to be explained, since the ubiquitin-fold domain, which is conserved in other E1s as a binding site for E2s, is not identified in Atg7. Structural and mutational studies of yeast Atg3 showed that Atg3 interacts with Atg7 via a domain that is unique to Atg3[111] (see below), suggesting that the mode of interaction between Atg7 and Atg3 is different from that between Atg7 and Atg10. The in vitro analysis of *Arabidopsis thaliana* (At) ATG systems showed that AtATG3 inhibits the AtATG12 system by binding to AtATG7 competitively with AtATG10,[112] suggesting that AtATG3 and AtATG10 bind to a similar site on AtATG7. Structural studies of Atg7 and its complexes with Atg3 and Atg10 will be necessary to sort out these issues.

4.3. Conjugating (E2-like) Enzyme Atg3

Atg8 bound to Atg7 via a thioester bond is transferred to Atg3, an E2-like enzyme, to form the Atg8∼Atg3 thioester intermediate.[32] This Atg3 has little sequence homology with canonical E2 enzymes, and is relatively large (∼36 kDa). The structure of Atg3 has been determined by X-ray crystallography,[111] and Figure 5 shows the structures of Atg3 (A) and Ubc9, a canonical E2 enzyme, bound to the SUMO−RanGAP1 conjugate and an E3[113] (B). The overall appearance of Atg3 has a unique hammer-like shape consisting of a head and a handle, and it is significantly different from that of canonical E2 enzymes. Nevertheless, the head moiety is topologically similar to that of canonical E2 enzymes. All canonical E2 enzymes have a core structure composed of a central four-stranded β-sheet and four surrounding α-helices[114] (Figure 5B), and except for two C-terminal α-helices in canonical E2 enzymes, the head moiety of Atg3 has these structures. Ufc1 (an E2-like enzyme for Ufm1) and ubiquitin E2 variant domains also lack two C-terminal α-helices.[115,116] The catalytic cysteine of Atg3 (Cys234) is located similarly to the position on canonical E2 (Figure 5); however, Atg3 does not have a conserved Asn residue in the proximity of Cys234, which is conserved in canonical E2 and is essential for the conjugation reaction by functioning as an oxyanion hole.[84,108,117] Further, residues such as Asn85, Tyr87, and Asp127 of Ubc9 that are

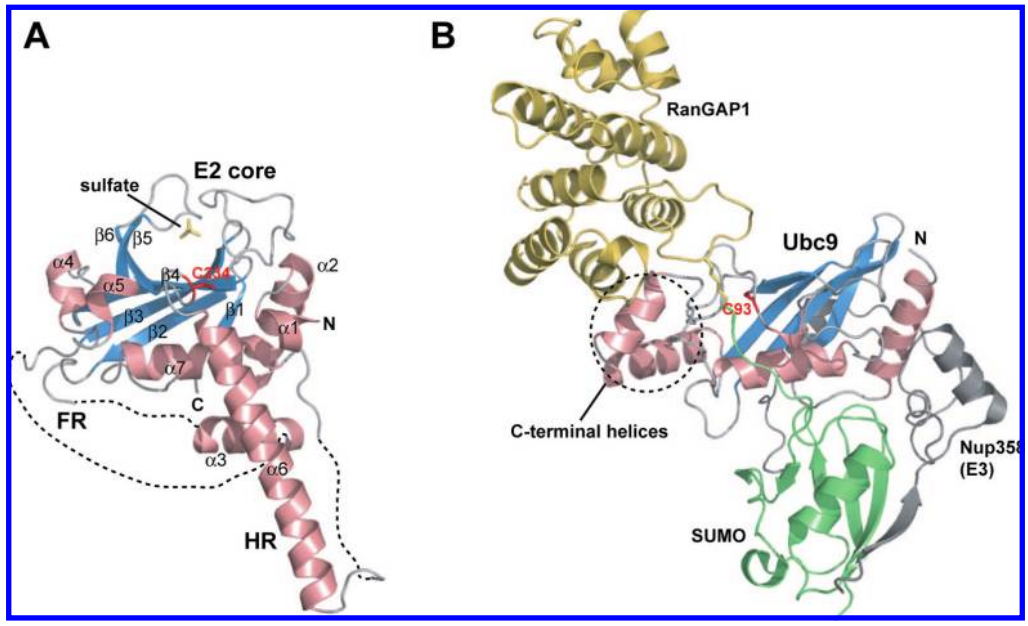

Figure 5. Structures of Atg3 and Ubc9: (A) Structure of Atg3 (PDB code 2DYT); (B) Structure of Ubc9 bound to a SUMO−RanGAP1 conjugate and a fragment of Nup358 (E3) (PDB code 1Z5S). α-helices of Atg3 and Ubc9 are salmon pink, and β-strands are blue. Catalytic cysteine residues are shown with red stick models: RanGAP1, yellow; SUMO, green; Nup358, gray.

responsible for lysine activation of substrates[84,113] are not conserved in Atg3.

In addition to the head moiety which has an E2-core fold, Atg3 has two unique inserted regions. One is the handle moiety, consisting of a long α-helix and a following flexible loop (the handle region: HR). The other unique region consists of about 80 residues with high acidity between β-strands 2 and 3. Most of the residues constituting this region are disordered in the crystal, NMR analysis showed that in solution this region has a mobile, random-coil structure,[111] and this region was termed the flexible region, FR. Mutational analysis showed that both HR and FR of Atg3 are necessary for the conjugation activity of Atg3.[111] Further, in vitro binding assays showed that HR is responsible for Atg8-binding, while FR is responsible for Atg7-binding.[111] HR of Atg3 occupies a location similar to SUMO/ubiquitin in Ubc9/Ubc1[113,118] (Figure 5), and the orientation between Atg8 and Atg3 in the complex appears similar to that between SUMO/ubiquitin and their E2. The Ubl domain of E1 binds to the amino-terminal α-helix of E2[104,105] which is equivalent to α2 of Atg3. The sites where FR is inserted and the second α-helix are located on opposite sides of Atg3. These differences suggest that the interaction between Atg7 and Atg3 is significantly different from that between canonical E1 and E2.

Atg3 conjugates Atg8 with PE without E3-like enzymes in vitro,[48] and Atg3 itself may have a binding site for PE. Although PE is the sole target for Atg8 in vivo,[32] phosphatidylserine (PS) may be another target for Atg8 in vitro.[119,120] However, a recent in vitro study showed that the formation of Atg8−PS but not Atg8−PE is markedly attenuated at neutral pH, indicating that Atg3 prefers PE to PS at physiological pH.[120] In the crystal structure of Atg3, a sulfate ion contained in the crystallization solution is bound in the proximity of Cys234[111] (Figure 5A). This sulfate ion and HR are located on opposite sides of Cys234, just as RanGAP1 and SUMO are located on opposite sides of Ubc9 Cys93 (Figure 5). Since anions often mimic phosphate groups, this sulfate ion may also mimic the phosphate group of PE and bind to the PE-binding site. However, the PE-preference of Atg3 at neutral pH cannot be explained from the structure alone. The addition of acidic phospholipids into PE-containing liposomes promoted the formation of Atg8−PE in vitro, and this was explained by the enhanced affinity of the Atg8∼Atg3 thioester intermediate to liposomes.[120] Basic residues are abundant on the surface of Atg8[66] as well as on Atg3 surrounding the above-mentioned bound sulfate and Cys234,[111] which may have some affinity to acidic phospholipids. In summation, the mechanism of membrane recognition during Atg8−PE formation must be considered not only for Atg3 and Atg8, but also for the Atg12−Atg5−Atg16 complex, the E3-like enzyme for the Atg8 system.

4.4. The E3-like Atg12−Atg5−Atg16 Complex

In the last step, Atg8 is transferred from Atg3 to PE and an amide bond is formed between the amino group of the head moiety of PE and the carboxyl group of the carboxy-terminal glycine residue of Atg8.[32] In vivo the Atg8−PE formation requires the components of the Atg12 conjugation system, Atg5, Atg10, and Atg12, and partially requires Atg16,[17] although these are not required in vitro.[48] This difference between the in vivo and in vitro situation was recently explained by an in vitro study, which showed that Atg8−PE formation in vitro also required Atg12−Atg5 conjugates when liposomes of a physiological PE content were used.[49] This study also showed that the Atg12−Atg5 conjugates directly facilitate the conjugation reaction between Atg8 and PE through interaction with Atg3.[49] A similar facilitation has also been observed in the in vitro reconstitution system using plant Atg homologues.[112] While Atg16 did not enhance Atg8−PE conjugation in vitro,[49] mammalian Atg12−Atg5−Atg16L complex has been shown to facilitate LC3−PE formation in vivo by recruiting Atg3 to PE-containing membranes, and for this Atg16L was essential.[121] Therefore, it appears likely that the Atg12−Atg5−Atg16 complex has two distinct functions in the Atg8−PE conjugation: one the stimulation of the Atg3 activity, and the other the recruitment of the Atg8∼Atg3 thioester intermediates to the target membranes.

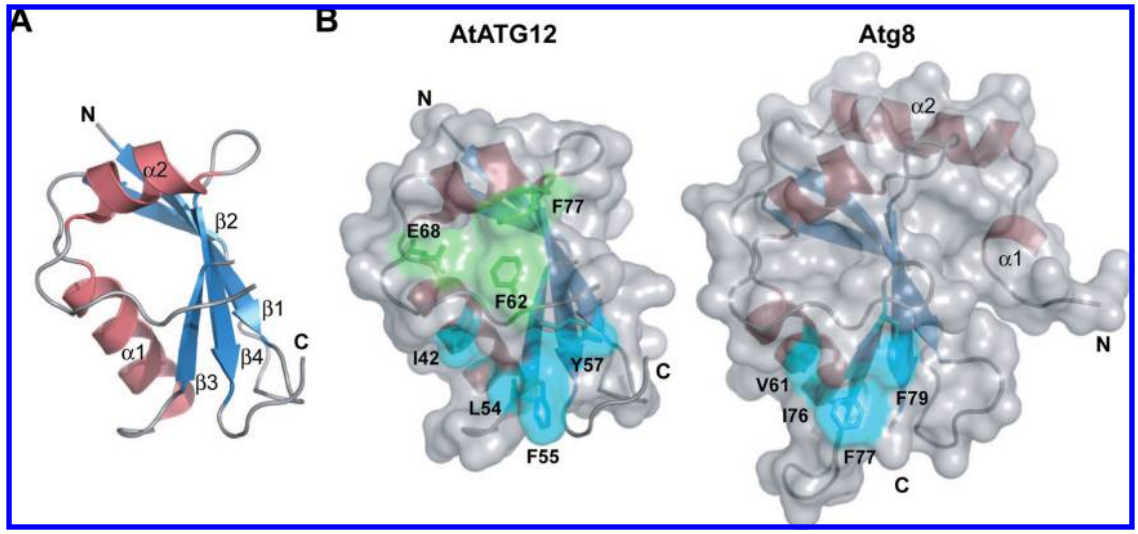

Figure 6. Structure of plant Atg12: (A) Ribbon representation of plant Atg12 (PDB code 1WZ3). This structure is in the same orientation as that in Figure 3A. (B) Surface representation of plant Atg12 and yeast Atg8 superimposed on their ribbon models. Residues conserved in Atg12 but not in Atg8 are green; residues conserved in both Atg12 and Atg8 are cyan.

The Atg12—Atg5—Atg16 complex mediates these E3-like functions, but it lacks a RING finger motif or a catalytic cysteine conserved in HECT. The components of this complex, Atg12, Atg5, and Atg16, have little sequence homology with other known proteins,[34,35] making it impossible to estimate the structures and functions from sequence comparisons. To obtain the structural information, the structure of *Arabidopsis thaliana* ATG12 (AtATG12) was determined by X-ray crystallography.[122]

AtATG12 is a 10 kDa protein consisting of 94 amino acids, much smaller than other Atg12 homologues such as yeast (186 amino acids) and human (140 amino acids).[123] Mutational analysis showed that the carboxy-terminal 86 residues of yeast Atg12, which correspond to residues 11—94 of AtATG12, are necessary and sufficient for both conjugation and autophagy,[123] showing that the structure of AtATG12 provides information for an understanding of the conserved, basic functions of Atg12 homologues. In the crystal, AtATG12 was an intertwined dimer[122] of unknown biological significance. Each monomeric form has a canonical ubiquitin fold (Figure 6A) and shows strong structural similarity to the ubiquitin-like domain of Atg8 homologues (Figure 3A). AtATG12 has no insertions or deletions and contains just a ubiquitin fold. Structure-based sequence alignment of Atg12 and Atg8 homologues showed that three residues, Phe62, Glu68, and Phe77, which gather to form a patch on the surface (Figure 6B, left, green), are conserved in Atg12 homologues but not in Atg8 homologues. Mutational analysis of yeast Atg12 showed Phe154 (which corresponds to Phe62 of AtATG12) to be essential for autophagy and Atg8—PE conjugation but not for Atg12—Atg5 conjugation.[123] Further, mutational analysis of human Atg12 showed Phe108 (which corresponds to Phe62 of AtATG12) to be responsible for the interaction with human Atg3, which is crucial for LC3 lipidation in vivo.[121] Therefore, the conserved three residue patch, which is unique to Atg12 homologues, is important for the E3-like activity of the Atg12—Atg5 conjugate. The sequence alignment also showed that Atg12 and Atg8 homologues have a conserved hydrophobic patch (Figure 6B; Ile42, Leu54, Phe55, and Tyr57 in AtATG12). This hydrophobic patch partially overlaps with the Ile44 surface of ubiquitin and its relatives, where it is the interaction site for various proteins, including E1[124] and E2;[118] therefore, this hydrophobic region of Atg12 and Atg8 may also play a role in the interaction with Atg7 and E2-like enzymes. Here, Tyr149 of yeast Atg12, which corresponds to Tyr57 of AtATG12, has been shown to be crucial for Atg12—Atg5 conjugation.[123]

Second, X-ray crystallography showed the structure of Atg5 complexed with the amino-terminal region (residues 1—57) of Atg16 (Atg16N)[125] (Figure 7A). This region of Atg16 does not contain the coiled-coil motif that is responsible for self-assembly,[35] and Atg5 and Atg16N form a heterodimer both in solution and in the crystal.[125] Atg5 is comprised of three domains: N- and C-terminal ubiquitin-like domains and a helix-rich domain in between. These three domains interact with each other and pack into a globular fold. Atg16N assumes a helical structure followed by a loop and is bound to the groove formed by these domains of Atg5. They form a large number of hydrophilic and hydrophobic interactions, in which Arg35 and Phe46 of Atg16N play critical roles (Figure 7B), and Atg16 with a mutation of these residues failed to localize to the PAS and could not restore autophagy in Atg16-deficient yeast strains.[125] Further, the Atg16 mutants (R35A and F46A) could not restore a severe reduction of Atg8—PE formation in Atg16-deficient strains under starvation conditions.[125] Therefore, the direct interaction between Atg5 and Atg16 is crucial for the functions of the Atg12—Atg5—Atg16 complex in autophagy. Lys149 of Atg5, the conjugation site for Atg12, is located opposite to the Atg16N binding site and exposes its side chain (Figure 7A). This is consistent with the observations that (i) Atg16 does not affect the conjugation between Atg5 and Atg12[35] and (ii) Atg12 does not affect the localization of the Atg5—Atg16 complex to the PAS.[13,17] The Atg12—Atg5—Atg16 complex may use the two portions for distinct purposes; the Atg12 moiety is used for the interaction with Atg3,[121] whereas the Atg5—Atg16 moiety is used for the interaction with the membrane.[121,125] Thus, the complex may facilitate the conjugation reaction between Atg8 and PE by juxtaposing the Atg8∼Atg3 intermediate and PE. The mechanism of the interaction of Atg5—Atg16 with membranes remains to be further elucidated, since Atg5—Atg16 has no structural features facilitating membrane binding. Atg5—Atg16 may interact with membranes through unidentified molecule(s), one candidate for which is a small GTPase Rab33, which

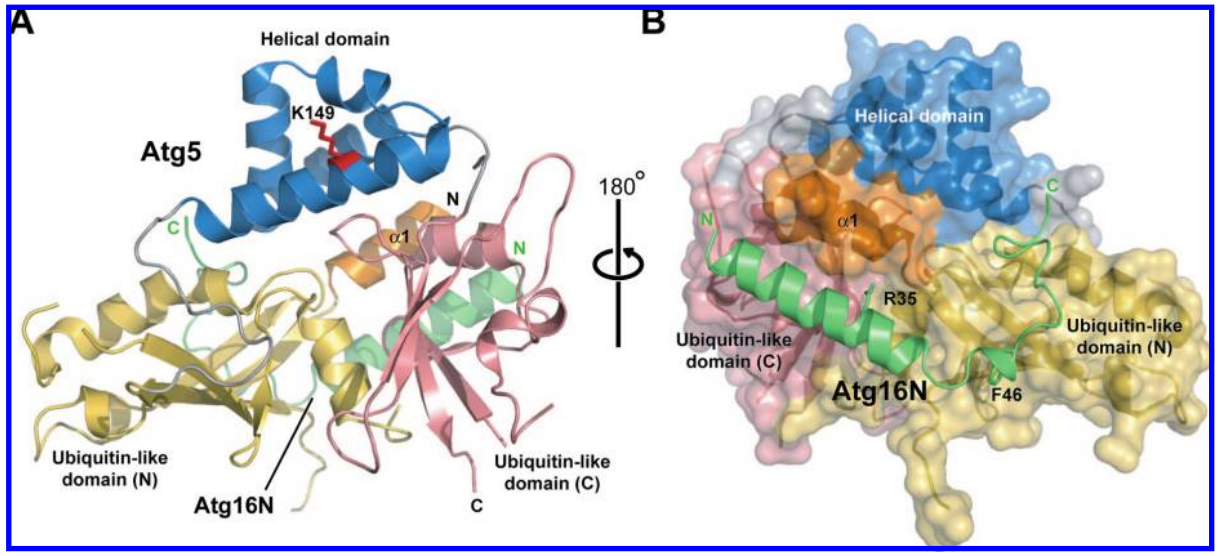

Figure 7. Structure of the Atg5−Atg16N complex: (A) Ribbon representation of the Atg5−Atg16N complex (PDB code 2DYO). Lys149 is shown with a red stick model. The N-terminal ubiquitin-like domain is yellow, the C-terminal ubiquitin-like domain is salmon pink, the helical domain is blue, the N-terminal α1 is orange, and Atg16N is green. (B) Surface representation of Atg5 superimposed on its ribbon model. Atg16N is shown with a green ribbon model with R35 and F46 shown as stick models. This figure is obtained by rotating the figure in part A 180° around the vertical axis.

was recently reported to directly interact with Atg16L in a guanosine triphosphate-dependent manner.[126] The role of the Atg12 modification in the E3-like activity of the Atg12−Atg5−Atg16 complex is reminiscent of that of the NEDD8 modification in the SCF ubiquitin E3 ligase, in which NEDD8 directly recruits the ubiquitin E2 to the ligase.[127,128]

The structures of AtATG12 and the Atg5−Atg16N complex suggest an approximate model of the Atg12−Atg5−Atg16 complex. Atg12 has one and Atg5 has two ubiquitin folds, so the Atg12−Atg5 conjugate contains three ubiquitin folds. Further, the binding of the Atg12−Atg5 conjugates to Atg16, a multimeric protein, results in a ubiquitin-fold-rich structure. This ubiquitin-fold-rich structure lacks similarity to HECT and RING E3s. The structures of two E3 enzymes lacking similarity to both HECT and RING have been reported: a 77-residue fragment of Nup358/RanBP2[113] (Figure 5B) and the A20 zinc-finger domain of Rabex-5.[129,130] Both E3s take a structure distinct from the Atg12−Atg5−Atg16 complex; the Atg12−Atg5−Atg16 complex may be considered to be a novel-type E3. In addition to the E3-like activity, the Atg12−Atg5−Atg16 complex play other essential roles in autophagosome formation, since Atg12-deficient strains show more severe defects in autophagy than Atg8-deficient strains.[131,132] However, the current structural information is not sufficient to verify the roles of the Atg12−Atg5−Atg16 complex. Structural studies of the multimeric Atg12−Atg5−Atg16 complex, in combination with in vitro and in vivo functional analysis, are required to elucidate the molecular functions of this unique protein complex.

5. Conclusions

Some structural information on Atg proteins is becoming available; however, it is restricted to parts constituting the Atg conjugation system. The Atg proteins constitute several functional groups: Atg1 kinase and its regulators, an autophagy-specific phosphatidylinositol 3-kinase complex, the integral membrane protein Atg9, and the Atg2−Atg18 complex. However, there is little structural information on these Atg proteins. Autophagosomes are formed by the concerted function of these Atg proteins; therefore, a comprehensive structural study of Atg proteins would be important to elucidate the mechanism of autophagosome formation.

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