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Mechanism of Cargo Recognition During Selective Autophagy

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1. Introduction

Autophagy is an intracellular bulk degradation system conserved among eukaryotes from yeast to mammals. It is responsible for the degradation of cytosolic components and organelles in response to nutrient deprivation. There are three main types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Microautophagy sequesters cytoplasmic components and delivers them for degradation by direct invagination or protrusion/septation of the lysosomal or vacuolar membrane (Mijaljica et al., 2011; Uttenweiler and Mayer, 2008). CMA targets specific cytosolic proteins that are trapped by the heat shock cognate protein of 70 kDa (hsc70) and, through interaction with lysosome-associated membrane protein type 2A (LAMP-2A), they are then translocated into the lysosomal lumen for rapid degradation (Orenstein and Cuervo, 2010). Macroautophagy, hereafter referred to as autophagy, is the most well characterized process of the three. During autophagy, double membrane structures called autophagosomes sequester a portion of the cytoplasm and fuse with the lysosome (or vacuole in the case of yeast and plants) to deliver their inner contents into the organelle lumen (Mizushima, 2007; Mizushima et al., 2010). Analyses of autophagy-related (Atg) proteins have unveiled dynamic and diverse aspects of mechanisms that underlie membrane formation during autophagy (Mizushima et al., 2010; Nakatogawa et al., 2009). As the contents of autophagosomes are indistinguishable from their surrounding cytoplasm (Baba et al., 1994), autophagy has long been considered a nonselective catabolic pathway. Recent studies, however, have provided evidence for the selective degradation of various targets by autophagy. In autophagy-deficient neuronal cells, intracellular protein aggregates accumulate and eventually lead to neurodegeneration, suggesting that autophagy selectively degrades harmful protein aggregates (Hara et al., 2006; Komatsu et al., 2006). Damaged or superfluous organelles, such as mitochondria and peroxisomes, and even intracellular infectious pathogens are also selectively degraded by autophagy (Goldman et al., 2010; Gutierrez et al., 2004; Manjithaya et al., 2010; Nakagawa et al., 2004; Noda and Yoshimori, 2009). In the budding yeast *Saccharomyces cerevisiae*, α-mannosidase and aminopeptidase I are selectively transported to the vacuole through autophagic pathways (Baba et al., 1997; Hutchins and Klionsky, 2001).

Although the precise molecular mechanisms of cargo selection by autophagy are yet to be established, an increasing number of autophagic receptors that are responsible for recognition of specific cargoes have been identified. These include Atg19 and Atg34 in the

selective transport of vacuolar enzymes to the vacuole through autophagy (Leber et al., 2001; Scott et al., 2001; Suzuki et al., 2010), p62 and neighbor of BRCA1 gene 1 (NBR1) in the autophagic degradation of ubiquitinated protein aggregates (Bjorkoy et al., 2005; Kirkin et al., 2009), PpAtg30 in pexophagy (autophagic degradation of peroxisome) (Farre et al., 2008), and Atg32 and Nix1 in mitophagy (autophagic degradation of mitochondria) (Kanki et al., 2009; Novak et al., 2010; Okamoto et al., 2009). Most of these receptors interact directly with Atg8-family proteins, which are crucial factors in autophagosome biogenesis.

We have been studying the mechanisms of specific cargo recognition during autophagy, especially those of the selective delivery of vacuolar enzymes into the vacuole in yeast. We summarize here the current knowledge of such mechanisms as revealed by biochemical and structural studies.

2. Recognition of vacuolar enzymes by Atg19 and Atg34

2.1 Selective transport of vacuolar enzymes by autophagic pathways

In the budding yeast *S. cerevisiae*, α-mannosidase (Ams1) and a precursor form of aminopeptidase I (prApe1) are selectively delivered into the vacuole through the cytoplasm to vacuole targeting (Cvt) pathway under vegetative conditions, and via autophagy under starvation conditions. The Cvt pathway is topologically and mechanistically similar to autophagy (Lynch-Day and Klionsky, 2010); therefore, studies on the molecular mechanisms of cargo recognition in the Cvt pathway will provide insight into the basic mechanism of selective autophagy. prApe1, the primary Cvt cargo, is synthesized in the cytosol as a precursor form with a cleavable propeptide consisting of 45 amino acid residues at the N terminus (Klionsky et al., 1992) and assembles into a dodecamer. The prApe1 dodecamer further self-assembles into a higher order structure called the Ape1 complex. The existence of a specific receptor for prApe1 was proposed when it was observed that prApe1 transport to the vacuole by the Cvt pathway is both specific and saturable.

Two groups simultaneously discovered that Atg19 has all of the characteristics needed to be a receptor for prApe1 in Cvt transport (Leber et al., 2001; Scott et al., 2001). Characterization of the protein revealed that Atg19 is needed for the stabilization of prApe1 binding to the Cvt vesicle membrane, and that in *atg19*Δ cells, prApe1 maturation is inhibited while autophagy is not affected (Suzuki et al., 2002). In addition, Atg19 binds to prApe1 in a propeptide-dependent manner, suggesting that the propeptide region is responsible for the recognition of prApe1 by the Cvt pathway machinery (Shintani et al., 2002). A secondary-structure prediction suggested that the prApe1 propeptide forms a helix-turn-helix structure and that the first helix exhibits the characteristics of an amphipathic α helix (Martinez et al., 1997). Our previous study revealed that the region containing the first helix of the prApe1 propeptide (residues 1-20) is sufficient for interaction with Atg19 (Watanabe et al., 2010). This is consistent with a previous report showing that the first helix of the prApe1 propeptide is critical for prApe1processing (Oda et al., 1996). *In vitro* pull-down assays showed that the coiled coil domain of Atg19 (residues 124-253), which contains a predicted coiled coil between amino acids 160 and 187, directly interacts with the prApe1 propeptide. This is consistent with a previous report showing that the prApe1-binding site of Atg19 is located in the region between amino acid residues 153 and 191 (Shintani et al., 2002).

Ams1, another Cvt cargo, oligomerizes after synthesis and associates with the Ape1 complex through the action of Atg19. Atg19 has two stable domains, the N-terminal

domain (residues 1-123) and the Ams1 binding domain (ABD; residues 254-367, see below for further details). Ams1 associates with Atg19 via the ABD that is distinct from the prApe1 binding site and therefore Atg19 can simultaneously interact with both prApe1 and Ams1. prApe1, Ams1, and Atg19 assemble into a large complex called the Cvt complex, which was identified as an electron-dense structure localized close to the vacuole by electron microscopy (Baba et al., 1997). Atg11 interacts with Atg19 to recruit the Cvt complex to the preautophagosomal structure (PAS), which plays a central role in autophagosome formation near the vacuole (Shintani et al., 2002; Suzuki and Ohsumi, 2010). Atg19 further interacts with Atg8, which is localized at the PAS and involved in the elongation of autophagosomes, using the Atg8 family-interacting motif (AIM; 412-WEEL-415) to induce formation of the Cvt vesicle (Noda et al., 2008). Atg8 is conjugated to phosphatidylethanolamine (PE) and associates with autophagosomes or the Cvt vesicle (Ichimura et al., 2000). This explains why the vesicle selectively surrounds only the cargo. After transport to the vacuole, the prApe1 propeptide is removed via a proteinase B-dependent reaction to generate mature Ape1 (mApe1), and the Ape1 complex disassembles back into dodecamers. Atg34, an Atg19 paralog, functions as an additional receptor protein for Ams1 but not prApe1 only under starvation conditions (Suzuki et al., 2010). Although Atg34, similar to Atg19, has the predicted coiled coil (residues 130-157), Atg34 is not capable of interacting with prApe1.

Recently, two cargoes that are selectively delivered to the vacuole have been identified: leucine aminopeptidase III (Lap3) (Kageyama et al., 2009) and aspartyl aminopeptidase (Ape4) (Yuga et al., 2011). Lap3 is transported to the vacuole for degradation only when it is overproduced under nitrogen starvation conditions. Lap3 forms a homohexameric complex of ~220 kDa, which further forms an aggregate independently of prApe1. Although this transport is partially mediated by Atg19, it remains to be determined whether Lap3 can interact with Atg19. Ape4 is the third Cvt cargo, which is similar in primary structure and subunit organization to Ape1. Ape4 lacks the N-terminal propeptide that is used by prApe1 for binding to Atg19. As the Ape4-binding site in Atg19 is located between the prApe1- and Ams1-binding sites (residues 204-247), these enzymes are unlikely to compete with each other for binding to Atg19. As Atg34 did not interact with Ape4, it might not be involved in Ape4 transport. More recently, Suzuki *et al*. elucidated that selective autophagy downregulates Ty1 transposition by eliminating Ty1 virus-like particles (VLPs) from the cytoplasm under nutrient-limited conditions (Suzuki et al., 2011). Although Ty1 VLPs are not vacuolar enzymes, they are targeted to autophagosomes by an interaction with Atg19. The N-terminal domain of Atg19 is specifically required for selective transport of Ty1 VLPs to the vacuole, though Atg19 is able to interact with Ty1 Gag without the N-terminal domain. Selective autophagy might safeguard genome integrity against excessive insertional mutagenesis caused during nutrient starvation by transposable elements in eukaryotic cells.

2.2 Structural basis for Ams1 recognition by Ag19 and Atg34

Scott *et al*. suggested that Ams1 is delivered to the vacuole in an Atg19-dependent manner (Scott et al., 2001). Ams1 was found to associate with Atg19, and a defect in the Ape1-Atg19 complex formation was shown to severely affect the import of Ams1 into the vacuole, whereas Ams1 was dispensable for transport of the Ape1-Atg19 complex. This suggests that Ams1 might exploit the prApe1 import system to achieve its own effective transport to the vacuole and that it is tethered to the Ape1-Atg19 complex through interaction with Atg19. In

our recent study, we identified the Ams1 binding domain (ABD) in Atg19 and Atg34 by limited proteolysis of full-length Atg19, an *in vitro* pull-down assay as well as sequence alignment (Watanabe et al., 2010). In *atg19*Δ cells expressing Atg19$^{\Delta ABD}$, Ams1 transport to the vacuole was inhibited, suggesting that the Atg19 ABD is required for Ams1 transport to the vacuole through the Cvt pathway. In such cells, prApe1 transport to the vacuole is the normal process. These results indicate that the Atg19 ABD is specifically responsible for the transport of Ams1, but not prApe1, to the vacuole through the Cvt pathway.

The Atg19 and Atg34 ABD structures were determined in solution using NMR spectroscopy (Figure 1A and B) (Watanabe et al., 2010). Both ABDs comprise eight β-strands (A-H), of which A, B, E, and H form an antiparallel β-sheet; the surface of this sheet faces a second antiparallel β-sheet comprising C, D, F, and G, thus forming a typical immunoglobulin-like β-sandwich fold. The Atg19 and Atg34 ABD structures are similar to each other with a root mean square difference of 2.1 Å for 102 residues (Z-score calculated by the Dalilite program (Holm and Park, 2000) is 12.8). There are relatively large structural differences between the Atg19 and Atg34 ABDs in the loops located at the bottom of the immunoglobulin fold (the loop connecting strands A and B (AB loop), CD, EF, and GH loops). In contrast, the loops located at the top of the immunoglobulin fold (the BC, DE, and FG loops) have a similar conformation. Furthermore, the residues comprising the top loops, especially those of the DE loop, are more strongly conserved between the Atg19 and Atg34 ABDs than those comprising the bottom loops (Figure 1). In the DE loop, His-310/296, Glu-311/297, Ile-314/300, and Lys-315/301 of Atg19/Atg34 are exposed. Among these exposed residues, His-310/296 and/or Glu-311/297 of the Atg19/Atg34 ABD are essential for Ams1 recognition. Further analysis showed that in *atg19*Δ*atg34*Δ cells expressing Atg19^{H310A} (substitution of His-310 with alanine) but not Atg19^{E311A}, transport of Ams1-GFP to the vacuole under autophagy-inducing conditions is inhibited. This indicates that the conserved His residue in the DE loop of the Atg19 ABD plays a critical role in Ams1 recognition and that Ams1 binding of the Atg19 ABD is essential for Ams1 transportation to the vacuole. Similar experiments using Atg34 mutants showed that His-296 of Atg34 ABD, which corresponds to His-310 of Atg19 ABD, also plays a critical role in Ams1 recognition.

The ABDs in Atg19 and Atg34 have a β-sandwich fold that is observed in a variety of immunoglobulins and immunoglobulin-like domains responsible for recognizing various proteins. Because antibodies generally recognize antigens using the hypervariable loops from both the VH and VL regions, their manner of antigen binding should differ from that of monomeric ABDs with Ams1. Interestingly, however, the ABD-Ams1 interaction resembles that observed between camelid antibody fragments and their antigens, as camelid antibodies lack a light chain and function as a monomer where hypervariable loops of the VH are responsible for antigen binding (Muyldermans, 2001). It also mimics the interaction of monobodies (artificially designed proteins that use a fibronectin type III domain as a scaffold) and their targets, as monobodies interact with their targets using similar loops in a monomeric immunoglobulin fold. Camelid antibody fragments and monobodies interact with their target proteins using loops clustered at one side of their immunoglobulin fold; these loops are topologically equivalent to the BC, DE, and FG loops of the Atg19 and Atg34 ABDs, one of which was shown to be crucial for Ams1 recognition as mentioned above. Therefore, they might recognize Ams1 using these loops in a similar manner with camelid antibodies and monobodies. In order to further elucidate the recognition mechanism of Ams1 by the ABD, structural determination of the Ams1-ABD complex by X-ray

crystallography is needed. We have already succeeded in overexpressing *S. cerevisiae* Ams1 in *Pichia pastoris* and purifying it on a large scale (Watanabe et al., 2009). Crystallization and structural determination of the Ams1-ABD complex are now in progress.

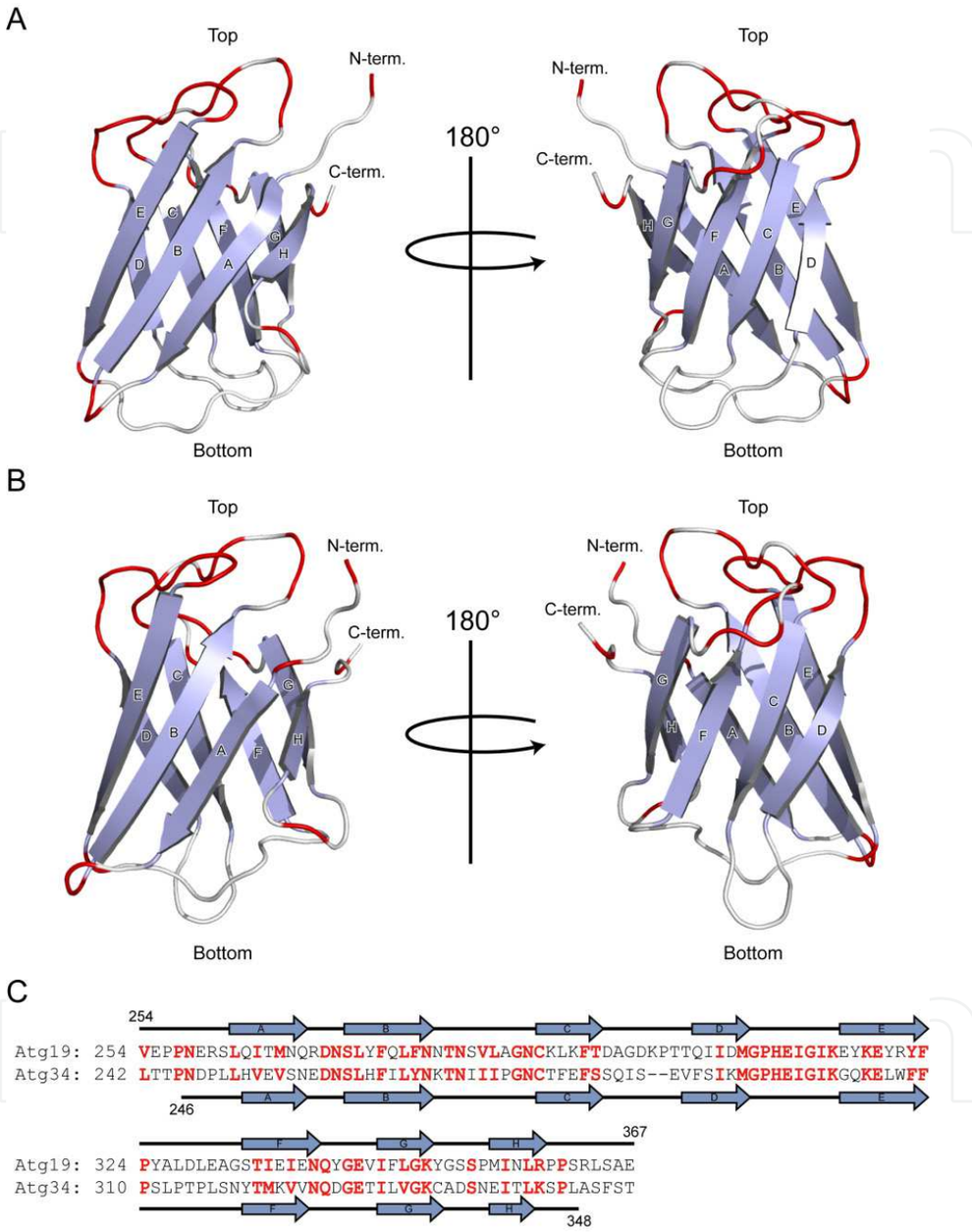

Fig. 1. Solution structures of Atg19 and Atg34 ABDs. (A), (B) Ribbon diagrams of the Atg19 ABD and Atg34 ABD structures, respectively. Strands are colored *light blue* and labeled. Loop residues conserved between Atg19 and Atg34 are colored *red*. *Left* and *right* are related by a 180° rotation along the *vertical axis*. (C) Sequence alignment between Atg19 and Atg34 ABDs. *Gaps* are introduced to maximize the similarity. Conserved or type-conserved residues are colored *red*. Secondary structure elements of the Atg19 and Atg34 ABDs are shown above and below the sequence, respectively.

3. Receptor proteins required for the selective degradation of organelles by autophagy

Damaged or superfluous organelles, such as mitochondria and peroxisomes, are also selectively degraded by autophagy. To date, several receptor proteins which function in these selective types of autophagy have been identified.

3.1 Receptor proteins in mitophagy

The mitochondrion is an organelle that produces energy through oxidative phosphorylation and simultaneously generates reactive oxygen species (ROS), causing oxidative damage to mitochondrial DNA, protein and lipids, and often inducing cell death. Therefore, an appropriate quality control of mitochondria is important to maintain proper cellular homeostasis. Selective degradation of mitochondria via autophagy, known as mitophagy, is the primary mechanism for mitochondrial quality control. Two groups independently identified Atg32 as a receptor protein for mitophagy in yeast (Kanki et al., 2009; Okamoto et al., 2009). Atg32 is a single-pass mitochondrial outer membrane protein, and its N- and C-terminal domains are oriented towards the cytoplasm and the intermembrane space, respectively. Mitochondria-anchored Atg32 binds Atg11 during mitophagy to recruit mitochondria to the PAS. When mitophagy is induced, Atg32 is phosphorylated, for which Ser-114 and Ser-119 of Atg32 are required. , The phosphorylation of Atg32 is required for Atg32-Atg11 interaction and mitophagy (Aoki et al., 2011). By controlling the activity and/or localization of the kinase that phosphorylates Atg32, cells may regulate the amount of mitochondria or remove damaged or aged mitochondria through mitophagy. Similarly to other receptor proteins, Atg32 binds to Atg8 using the AIM sequence, 86-WQAI-89, and this binding is required for the efficient sequestration of mitochondria by the autophagosome. The Atg32-Atg8 interaction may restrict autophagosome formation to the neighborhood of the targeted mitochondrion by gathering surrounding Atg proteins. Although no homolog of Atg32 has been identified in mammals, Nix was recently shown to be a mammalian mitophagy receptor protein (Novak et al., 2010). Similarly to Atg32, Nix has a transmembrane domain in its C terminus that can target the protein to the mitochondrial outer membrane and has a functional AIM, 35-WVEL-38, that directly interacts with mammalian Atg8 homologs. Thus Nix may fulfill the function of Atg32 in mammals.

3.2 Receptor protein in pexophagy

Peroxisomes have diverse functions, including the decomposition of hydrogen peroxide and the oxidation of fatty acids. The specific degradation of peroxisomes by autophagy, pexophagy, is conserved from yeast to humans and is triggered physiologically to allow cells to clear an excess of peroxisomes. The study of methylotrophic yeasts, particularly *P. pastoris* and *Hansenula polymorpha*, has led to the current understanding of the molecular mechanism governing pexophagy. Farre *et al.* identified Atg30 as a receptor protein for pexophagy in *P. pastoris* (Farre et al., 2008). PpAtg30 interacts with peroxisomes via two peroxisomal membrane proteins, Pex3 and Pex14, and with autophagy machinery via PpAtg11 and PpAtg17, which organize the PAS. Several residues on PpAtg30 are phosphorylated under pexophagy conditions and, similarly to Atg32, such phosphorylation, especially that of Ser-112, is required for PpAtg11 interaction. The isolation membrane then expands and surrounds the PpAtg30-localizing peroxisomes, in

order to selectively degrade surplus peroxisomes. Unlike other receptor proteins, PpAtg30 has no AIMs, so that PpAtg30 is unable to interact directly with PpAtg8. It is important to understand how the isolation membrane expands around the peroxisome surface while excluding cytosolic contents, and this is speculated to involve the interaction of PpAtg30 with an unidentified protein in the isolation membrane, or the interaction of PpAtg8 with another protein on the peroxisome surface.

4. Receptor protein for selective autophagy in *C. elegans*

Germ granules are restricted to the germ cells of many higher eukaryotes and are believed to carry germ cell determinants (Strome and Lehmann, 2007). Germ granules in *Caenorhabditis elegans,* also known as P granules, are maternally contributed and dispersed throughout the cytoplasm of a newly fertilized embryo. During *C. elegans* embryogenesis, some P granules are left in the cytoplasm destined for the somatic daughter cell and these P granules are quickly disassembled and/or degraded (Hird et al., 1996). Recently, Zhang *et al.* provided evidence that the P granule components PGL-1 and PGL-3 that remain in the cytoplasm destined for somatic daughters are selectively removed by autophagy through the receptor protein SEPA-1 (Zhang et al., 2009). In autophagy-deficient somatic cells, PGL-1 and PGL-3 extensively accumulate in the P granules, and SEPA-1 mediates the accumulation of these P granules into aggregates, termed PGL granules, through its self-oligomerization and direct interaction with PGL-3. SEPA-1 can also directly interact with LGG-1, an Atg8 homolog. Thus, PGL granules associated with SEPA-1 could be incorporated into autophagosomes through the interaction of SEPA-1 with LGG-1. Because the expression of SEPA-1 is restricted to somatic cells, the selective exclusion of P granules is ensured only in these cells. An *in vitro* pull-down assay showed that the SEPA-1 fragment containing amino acids 39 to 160 is required for both self-oligomerization and interaction with PGL-3 and that the SEPA-1 fragment containing amino acids 289 to 575 is required for interaction with LGG-1. The SEPA-1 fragment that interacts with LGG-1 contains a canonical AIM sequence, 469-YQEL-472 (Noda et al., 2010). Thus, the YQEL sequence in SEPA-1 is a potential candidate for a functional AIM.

5. Recognition of ubiquitinated cargoes by p62 and NBR1

Autophagic degradation of ubiquitinated protein aggregates is important for cell survival. Defects in autophagy cause the accumulation of ubiquitin-positive protein inclusions, leading to severe liver injury (Komatsu et al., 2005) and neurodegeneration (Hara et al., 2006; Komatsu et al., 2006). The polyubiquitin-binding protein p62, also called sequestosome 1 (SQSTM1), is a common component of protein aggregates found in both the brain and the liver of patients suffering from protein aggregation diseases. These include Lewy bodies in Parkinson's disease, neurofibrillary tangles in Alzheimer's disease, and huntingtin aggregates (Kuusisto et al., 2001, 2002; Nagaoka et al., 2004; Zatloukal et al., 2002). In the liver, Mallory bodies, hyaline bodies in hepatocellular carcinoma, and α1 antitrypsin aggregate contain p62 (Zatloukal et al., 2002); all of these aggregates contain polyubiquitinated proteins. p62 interacts with ubiquitin via its C-terminal UBA domain (Vadlamudi et al., 1996) and self-assembles via its N-terminal PB1 domain (Ponting et al., 2002), thereby forming large aggregates containing ubiquitinated proteins. p62 further interacts with LC3, a mammalian homolog of Atg8, via the LC3 interacting region (LIR;

residues 321-342), so that ubiquitinated protein aggregates containing p62 are selectively degraded by autophagy (Bjorkoy et al., 2005; Komatsu et al., 2007; Pankiv et al., 2007). Therefore, it is implied that p62 functions as a receptor protein for ubiquitinated proteins to be degraded in lysosomes. It is also hypothesized that p62 functions as a receptor for organelles such as peroxisomes and mitochondria (Kim et al., 2008; Kirkin et al., 2009), and for intracellular bacteria (Dupont et al., 2009; Yoshikawa et al., 2009; Zheng et al., 2009). Recently, neighbor of BRCA1 gene 1 (NBR1) has been identified as another autophagy receptor (Kirkin et al., 2009). The structure of NBR1 is similar to that of p62, and NBR1 can bind both LC3 via the LIR and ubiquitinated proteins via the UBA domain. Like p62, NBR1 is sequestered into the autophagosome via LC3-interaction and/or p62-interaction and markedly accumulates in autophagy-deficient tissues.

To clarify the molecular mechanism of ubiquitinated cargo recognition by p62 and NBR1, it is necessary to elucidate which proteins conjugated with either K48-linked or K63-linked polyubiquitin chains are targeted to the autophagy/lysosomal degradation pathway. Classically, proteins conjugated with K48-linked polyubiquitin chains are recognized as the proteolytic substrate by the UBD-containing proteasomal receptors. Recently, K63-linked chains have been implicated in proteolytic degradation of misfolded and aggregated proteins (Olzmann et al., 2007; Tan et al., 2008; Wooten et al., 2008). Given the reported preference of the known ubiquitin-binding autophagy receptors for K63-linked ubiquitin chains, cargoes conjugated with K63-linked ubiquitin chains may be preferentially targeted to the autophagy/lysosomal degradation pathway. However, p62 has been shown to compete for ubiquitinated cargo with the classical proteasomal receptors. Accumulation of p62 resulting from inhibition of autophagy compromised degradation of proteasomal substrates, most likely due to the excessive interaction between p62 and substrates conjugated with K48-linked polyubiquitin chains (Korolchuk et al., 2009). It remains to be clarified how p62 distinguishes between K48-linked and K63-linked polyubiquitin chains.

To date, several structural studies have been performed on p62. Isogai *et al.* determined the crystal structure of the UBA domain of mouse p62 and the solution structure of its ubiquitin-bound form (Isogai et al., 2011). In crystals, the p62 UBA domain adopts a dimeric structure, which is distinct from that of other UBA domains. In solution, the domain exists in equilibrium between the dimer and monomer forms, and ubiquitin-binding shifts the equilibrium toward the monomer to form a 1:1 complex between the UBA domain and ubiquitin. The extreme C-terminal end of the p62 UBA domain is responsible for dimerization of the domain. Mutations that inhibit dimerization of the p62 UBA domain increase the affinity of p62 for ubiquitin. These results suggest an autoinhibitory mechanism in the p62 UBA domain to avoid self-degradation by the ubiquitin-proteasomal system. The interaction between the p62 LIR and LC3 is structurally and functionally well characterized (Ichimura et al., 2008; Noda et al., 2008). In the structure of the p62 LIR in complex with LC3, the tryptophan and leucine residues in the p62 LIR, DDDWTHL, interact with two hydrophobic pockets, the W-site and the L-site, where tryptophan and leucine residues respectively interact (Noda et al., 2010) on the surface of LC3. In agreement with the structure of the p62 LIR in complex with LC3, the tryptophan and leucine residues are involved in the turnover of p62 via autophagy. Recently, the structure of the NBR1 LIR in complex with GABARAP, a LC3 paralog, has also been determined (Rozenknop et al., 2011). Similar to the interaction between p62 LIR and LC3, the tyrosine and the third isoleucine

residues in the NBR1 LIR, EDYIII, interact with GABARAP in a manner typical of the interaction of AIM with Atg8 homologs.

6. Receptor proteins required for the restriction of infectious bacterial growth by autophagy

Autophagy also serves as a cell-autonomous effector mechanism of innate immunity in the cytosol. It does this through restricting bacterial proliferation by separating bacteria from the nutrient-rich cytosol and delivering them into bactericidal autolysosomes. Several examples showing that these types of autophagy are mediated by cytosolic bacteria-recognizing receptor proteins have been recently reported. In *Drosophila melanogaster*, PGRP-LE, a receptor protein for bacterial peptidoglycans, induces autophagy of wild-type but not listeriolysin-deficient *Listeria monocytogenes*, suggesting that this pathway specifically selects cytosolic bacteria for autophagy (Yano et al., 2008). However, it is unknown how PGRP-LE induces the autophagic degradation of *L. monocytogenes* and whether PGRP-LE binds the *D. melanogaster* Atg8 orthologs.

Salmonella enterica Typhimurium (*S.* Typhimurium) typically occupies a membrane bound compartment, the Salmonella-containing vacuole (SCV), in host cells. In mammalian cells, *S.* Typhimurium and other bacteria enter the cytosol and are released from SCVs, then become coated with a dense layer of ubiquitin (Perrin et al., 2004) and are delivered to lysosomes via autophagy (Birmingham et al., 2006). It was reported that p62 functions as a receptor protein for delivering such ubiquitin-coated bacteria into autophagosomes (Dupont et al., 2009; Zheng et al., 2009). In addition to p62, two other receptor proteins were identified, NDP52 (Thurston et al., 2009) and Optineurin (Wild et al., 2011), both of which recognize ubiquitin-coated bacteria. NDP52 is recruited by ubiquitin-coated *S.* Typhimurium and binds both ubiquitin via a zinc-finger domain (residues 420-446) and LC3. Although NDP52 interacts with LC3, it has not been clarified whether NDP52 has an AIM. NDP52 also coordinates a signaling complex including Tank-binding kinase (TBK1), Sintbad and Nap1. *In vitro* binding studies revealed that a SKICH domain (residues 1-127) in NDP52 is required for direct Nap1 binding. Thereby, NDP52 recruits TBK1 to ubiquitin-coated *S.* Typhimurium. The ability of NDP52 to serve as an adaptor for TBK1 also seems to be critical in the cell-autonomous response, but it remains to be determined what role TBK1 plays in association with NDP52. OPTN is another autophagy receptor protein that binds and co-localizes with LC3 via an AIM (178-FVEI-181) and ubiquitin via its ubiquitin binding in ABIN and NEMO (UBAN) domains. The N-terminal region of OPTN (residues 1-127) also interacts with TBK1 (Morton et al., 2008). When TBK1 is recruited into *S.* Typhimurium via OPTN, it becomes activated and phosphorylates OPTN at Ser-177, one residue N-terminal to the OPTN AIM. The phosphorylation of Ser-177 increases the affinity between OPTN and LC3, which is consistent with the previous review showing that acidic residues are preferred at the N-terminal side of the AIM for higher affinity with Atg8 homologs (Noda et al., 2010). Although p62, NDP52 and OPTN target the same bacteria, NDP52 and OPTN localize to microdomains on the surface of ubiquitinated bacteria where p62 does not co-localize (Cemma et al., 2011; Wild et al., 2011). Because these autophagy receptors have their respective ubiquitin-binding domains, the distinct specificities for different ubiquitin chains may result in partitioning of the receptors to different subdomains on the bacterium. However, it is still unknown which type of ubiquitin chains are conjugated to the bacterial surface components.

7. Concluding remarks

Autophagy receptor proteins have two main functions: recognizing autophagic cargoes, and interacting with Atg8 homologs. Because of these functions, autophagy receptor proteins can tether autophagic cargoes to the isolation membrane (e.g., Atg19; Figure 2) so that the cargoes are selectively and efficiently engulfed by an autophagosome and transported into the vacuole/lysosome. Although the mechanism of direct interaction with Atg8 homologs

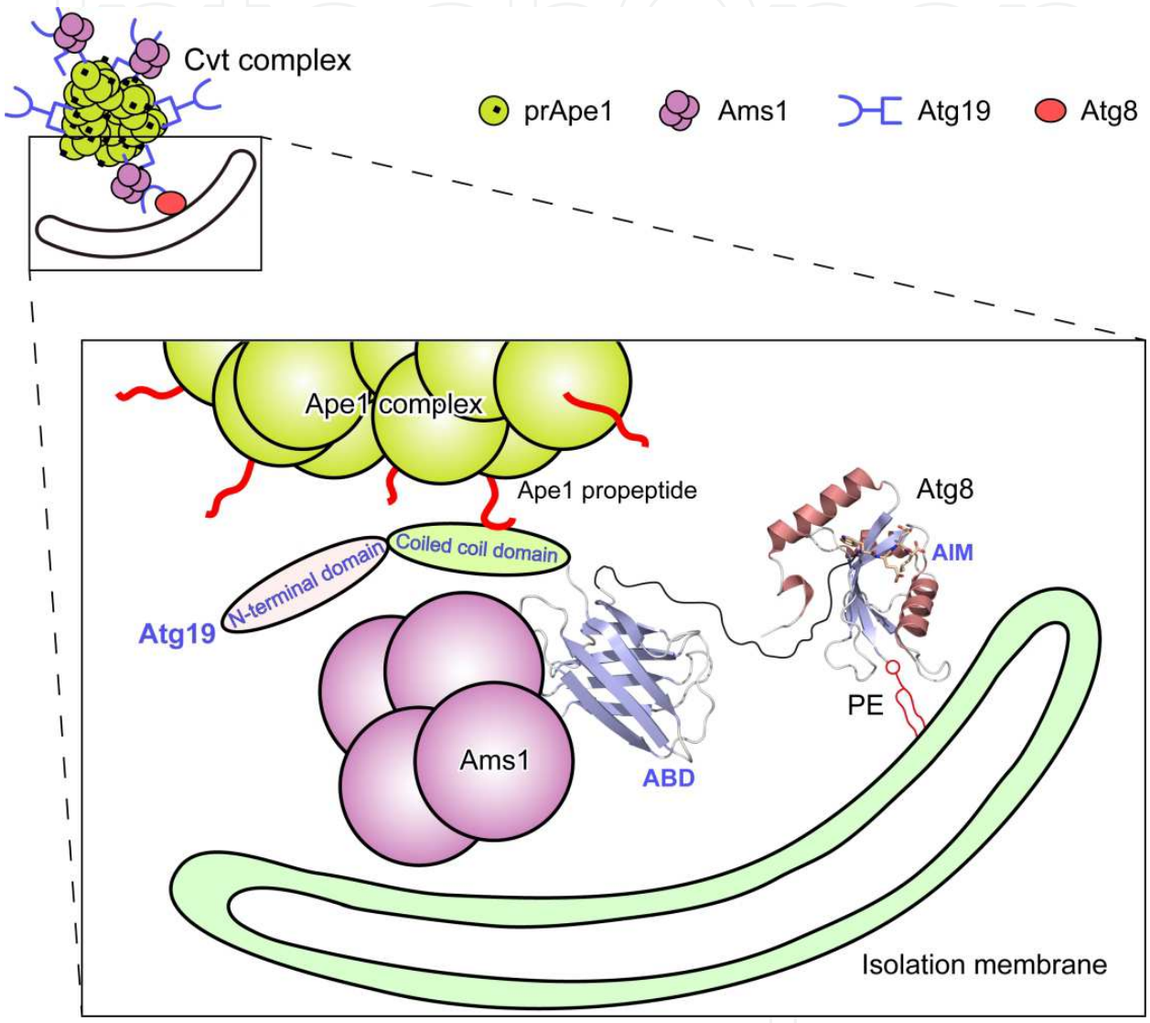

Fig. 2. Atg19 tethers autophagic cargoes to the isolation membrane. Atg19 interacts with prApe1 and Ams1 via the coiled coil domain and ABD, respectively. Atg19 also interacts with Atg8, which is conjugated to phosphatidylethanolamine (PE) and associates with the isolation membrane, via the AIM. Therefore, autophagic cargoes are tethered to the isolation membrane.

via the AIM is common among most autophagy receptor proteins, the recognition mechanisms of autophagic cargoes by autophagy receptor proteins are too divergent to be elucidated. We reported that Atg19 and Atg34 ABDs, similarly to the camelid antibody and monobody, recognize Ams1, an autophagic cargo, using the loops clustered at one side of

their immunoglobulin fold. Recent proteomics analysis has identified proteins that are selectively degraded by autophagy (Onodera and Ohsumi, 2004). Although the recognition mechanism of these target proteins by autophagy has not been established, autophagy-specific receptor proteins possessing an ABD-like fold might be responsible. Identification and structural analysis of other autophagy receptor proteins are required for further clarification of the molecular mechanism of specific cargo recognition during autophagy.

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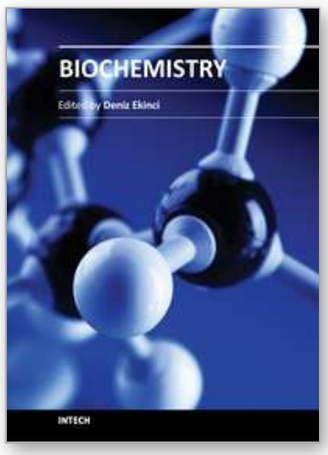

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Over the recent years, biochemistry has become responsible for explaining living processes such that many scientists in the life sciences from agronomy to medicine are engaged in biochemical research. This book contains an overview focusing on the research area of proteins, enzymes, cellular mechanisms and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in biochemistry. Particular emphasis is devoted to both theoretical and experimental aspect of modern biochemistry. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in biochemistry, molecular biology and associated areas. The book is written by international scientists with expertise in protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the book will enhance the knowledge of scientists in the complexities of some biochemical approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of biochemistry.

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