Review

Atg8-family interacting motif crucial for selective autophagy

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Abstract

Autophagy is a bulk degradation system conserved among most eukaryotes. Recently, autophagy has been shown to mediate selective degradation of various targets such as aggregated proteins and damaged or superfluous organelles. Structural studies have uncovered the conserved specific interactions between autophagic receptors and Atg8-family proteins through WXXL-like sequences, which we term the Atg8-family interacting motif (AIM). AIM functions in various autophagic receptors such as Atg19 in the cytoplasm-to-vacuole targeting pathway, p62 and neighbor of BRCA1 gene 1 (NBR1) in autophagic degradation of protein aggregates, and Atg32 and Nix in mitophagy, and may link the target-receptor complex to autophagic membranes and/or their forming machineries.

1. Introduction

Autophagy is an intracellular bulk degradation system conserved among eukaryotes from yeast to mammals. In autophagy, isolation membranes enclose a portion of the cytoplasm to form double-membrane vesicles, called autophagosomes, and deliver their contents to the lysosome/vacuole [1]. Although autophagy is in principle a non-selective degradation process, recent studies have shed light on other autophagy modes that selectively degrade aggregated proteins, surplus or damaged organelles, and even invasive bacterial cells [2–5]. 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 in the cytosol-to-vacuole targeting (Cvt) pathway [6], p62 and neighbor of BRCA1 gene 1 (NBR1) in the autophagic degradation of ubiquitinated protein aggregates [4,5,7–10], and Atg32 and Nix1 in mitophagy (autophagic degradation of mitochondria) [11–13]. Recently, we have shown that Atg19 and p62, which share little sequence homology with each other, use a common WXXL motif for direct interaction with Atg8-family proteins [14]. Subsequently, NBR1, Atg32 and Nix were also shown to use a similar motif for interaction with Atg8-family proteins [8,11,13,15]. We refer to these WXXL-like sequences as the Atg8-family interacting motif (AIM). Atg8, initially identified in Saccharomyces cerevisiae, is a ubiquitin-like modifier that is conjugated to phosphatidylethanolamine in a similar manner to ubiquitination [16]. It localizes to isolation membranes and autophagosomes [17] and plays crucial roles in the formation of autophagosomes [18,19]. Atg8 and its conjugation system are evolutionarily conserved, and Atg8-family proteins in various species appear to have important roles in autophagy as in the case of yeast Atg8 [20,21]. The interaction between autophagic receptors and Atg8-family proteins through AIMs contributes, at least in part, to the selection of specific cargoes [8,11,14,22], possibly by linking the specific cargoes to autophagic membranes and/or their forming machineries. In this review, we focus on the structural basis of the interaction between AIMs and Atg8-family proteins, and summarize their interaction mode. Based on current knowledge, we discuss predictions of functional AIMs in uncharacterized proteins from their primary sequence.

2. Structure of the AIM binding site of Atg8-family proteins

Thus far, the three-dimensional structures of S. cerevisiae Aut8 and its mammalian and protozoan homologues have been reported...
All of these structures are composed of a C-terminal ubiquitin-like domain and an N-terminal helical domain (Fig. 1A). The N-terminal helical domain, consisting of tandem $\alpha$-helices, $\alpha_1$ and $\alpha_2$, is a conserved feature unique to Atg8-family proteins and distinguishes the Atg8-family proteins from other ubiquitin-like proteins. All Atg8-family proteins have an exposed $\beta$-strand ($\beta_2$; corresponding to residues 48–52 of Atg8), which is responsible for AIM binding through an intermolecular $\beta$-sheet (see following sections). They also possess two characteristic hydrophobic pockets near $\beta_2$, one located between $\alpha_2$ and $\beta_2$, and the other between $\beta_2$ and $\alpha_3$. In the case of Atg8, the former pocket is composed of the side chains of Glu17, Ile21, Pro30, Ile32, Lys48, Leu50, and Phe104, whereas the latter pocket is composed of the side chains of Tyr49, Val51, Leu55, Phe60, and Val63 [14]. These pockets are named the W-site and the L-site, respectively, since they are typically responsible for the interaction with Trp and Leu in AIM, respectively (see following sections). These residues are conserved among Atg8-family proteins, particularly those constructing the W-site (Fig. 1B). Therefore, the two hydrophobic pockets are a conserved feature of Atg8-family proteins, although the L-site structure is slightly more variable among Atg8 homologues.

3. AIMS in autophagic receptors

Most autophagic receptors directly interact with both Atg8-family proteins and their specific targets, thus likely tethering their targets to autophagic membranes and/or their forming machineries. The interaction between autophagic receptors and Atg8-family proteins is mediated by AIM in each receptor. In this section, we re-

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**Fig. 1.** AIM binding sites are evolutionarily conserved among Atg8-family proteins. (A) Structures of *S. cerevisiae* Atg8 (left; PDB ID 2zpn), *Rattus norvegicus* LC3 (middle; PDB ID 1ugm) and *Trypanosoma brucei* Atg8b (right; PDB ID 3h9d). Two hydrophobic pockets responsible for the recognition of Trp and Leu in the AIM (WXXL) motif are labeled W-site and L-site, respectively, and circled. Residues constituting these pockets are colored red and green, respectively, and their side chains are shown with a stick model. (B) Sequence alignment of Atg8-family proteins. The secondary structural elements of *S. cerevisiae* Atg8 are shown above the alignment. Residues constituting W- and L-sites are colored red and green, respectively. Basic residues responsible for ionic interaction with acidic residues in AIM are enclosed with a blue square. Residue numbers of *S. cerevisiae* Atg8 are shown above the alignment. Sc, *S. cerevisiae*; Hs, Homo sapiens; At, Arabidopsis thaliana; Tb, T. brucei.
view the interaction between AIMs in autophagic receptors and Atg8-family proteins.

In the yeast *S. cerevisiae*, aminopeptidase 1 (Ape1) and α-mannosidase (Ams1) are selectively and constitutively transported into the vacuole via an autophagy-like process called the Cvt pathway [27,28]. Although it is a biosynthetic process, the Cvt pathway is regarded as a well-studied model of selective autophagy. Immediately after being translated in the cytoplasm, Ape1 self-assembles into a dodecamer and further aggregates into a much larger complex [29] that interacts with the receptor protein Atg19 [6]. Atg19 interacts with Ams1 and Atg8 [30], thus linking Ape1 and Ams1 to autophagic machineries. In vivo studies have shown that deletion of the C-terminal six residues of Atg19 abolished the Atg8–Atg19 interaction [30], and in vitro studies have shown that the WEE1 sequence in these residues is actually crucial for the interaction [14]. The crystal structure of *S. cerevisiae* Atg8 complexed with a WEE1 peptide (corresponding to residues 412–415 of Atg19) revealed that the peptide adopts an extended β conformation and forms an intermolecular parallel β-sheet with β2 of Atg8 (Fig. 2A) [14]. The side chains of Trp412 and Leu415 of the peptide are docked into the W- and L-sites on Atg8, respectively. Substitution of these residues, particularly Trp412, with alanine, abolished the interaction, suggesting that the WXXL sequence is important for Atg8-binding [14]. In vivo mutational studies have revealed that the WXXL sequence of Atg19 is crucial for the Cvt pathway [14]. Thus the C-terminal WEE1 sequence of Atg19 is a functional AIM. In addition to the hydrophobic interactions, the side chains of Glu413 and Glu414 of the peptide form ionic interactions with those of Arg67 and Arg28 of Atg8, respectively (Fig. 2A), and may contribute to the interaction to some extent.

Defects in autophagy cause the accumulation of ubiquitin-positive protein inclusions, leading to severe liver injury [31] and neurodegeneration [32,33]. p62/Sequestosome 1 functions as a receptor that links such aggregated proteins to LC3, a mammalian Atg8 homologue [7,10,34]. p62 interacts with ubiquitin via its C-terminal ubiquitin-associated domain [35], as well as self-assembles via its N-terminal PB1 domain [36], and is therefore able to form large aggregates containing ubiquitinated proteins [7,10]. p62 further interacts with LC3 via the LC3 interacting region (LIR; residues 321–342), thus tethering protein aggregates to autophagic machineries [10,37]. p62 LIR is abundant in acidic residues, and possesses a conserved Trp residue (Trp338). X-ray and NMR structures of LC3 complexed with p62 LIR revealed that p62 LIR adopts an extended β-conformation and forms an intermolecular parallel β-sheet with β2 of LC3 (Fig. 2B) [14,22]. The side chains of Trp338 and Leu341 of the peptide are bound deeply into the W- and L-sites on LC3, respectively. Substitution of these residues with alanine abolished the interaction, suggesting that the WXXL sequence is crucial for LC3-binding [14,22,37]. In addition to these hydrophobic interactions, ionic interactions between three Asp residues (Asp335–337) N-terminal to the LIR and basic residues of LC3 (Arg10, Arg11, Lys49, Lys51), appear to also be important for the LC3–p62 LIR interaction. In vivo mutational studies revealed that both the WXXL sequence and the three consecutive Asp residues are crucial for the autophagic degradation of p62 [22,37]. Thus, the WTHL sequence of p62 is a functional AIM, and acidic residues have an auxiliary role.

Recently, neighbor of BRCA1 gene 1 (NBR1) has been identified as another autophagic receptor for ubiquitin-positive protein aggregates [8,9]. NBR1 has little sequence similarity with p62;
however, it has a domain architecture similar to p62, consisting of the N-terminal PB1 domain and the C-terminal ubiquitin-associated domain. NBR1 also has a LIR, EDYIII, and mutational studies in vitro and in vivo have shown that NBR1 directly interacts with LC3 via the LIR [8]. Therefore, this may be another example of a functional AIM and the Tyr and Ile residues in the LIR of NBR1 presumably bind to the W- and L-sites on LC3, respectively.

Mitochondria can also be a target for autophagy, and this process is termed mitophagy. Recently, Atg32 has been identified as a mitophagy-specific receptor in *S. cerevisiae*. Atg32 is a single-spanning mitochondrial outer membrane protein, and exposes its N-terminal region to the cytoplasm. Atg32 directly binds to Atg8 via a WQAI sequence in the N-terminal region, thus tethering mitochondria to autophagic machineries. The crystal structure of Atg8 complexed with a hexapeptide (SWQAIQ), which corresponds to the residues 85–90 of Atg32, showed that the peptide binds to Atg8 in a similar manner to the WEE peptide (Noda et al., unpublished results), suggesting that the WQAI sequence in Atg32 is also a functional AIM.

In mammals, the BCL2 related protein Nix has been reported as an essential factor for selective mitophagy during reticulocyte maturation [38,39]. Very recently, Nix has been shown to function as a mitophagy-specific receptor through the direct interaction with mammalian Atg8 homologues [13]. A NMR study showed that Nix binds to the WXXL binding site on the γ-aminobutyric acid type A receptor-associated protein (GABARAP), a mammalian Atg8 homologue, through a WVEL sequence [15]. Therefore, the WVEL sequence in Nix may also be a functional AIM. Interestingly, this interaction region with LC3B very weakly [13,15], indicating that an AIM can have a preference for binding partners.

4. AIMs in enzymes modifying Atg8-family proteins

Conjugation and deconjugation of Atg8 with a phosphatidylyethanolamine are mediated by several autophagy-related (Atg) proteins. Nascent Atg8 is first processed by the protease Atg4 to expose a Gly at the C-terminus [40]. The exposed Gly is activated by Atg7, an E1-like enzyme, and is then transferred to Atg3, an E2-like enzyme. Finally, Atg8 is conjugated to a phosphatidylyethanolamine [16], in a process in which the E3-like Atg12–Atg5–Atg16 complex plays a crucial role [41–43]. During these reactions, at least Atg3, Atg4, and Atg7 directly interact with Atg8. Therefore, there is a question of whether these proteins also use AIMs for their interaction with Atg8 or not.

Recently, we have reported the crystal structure of the human Atg4B–LC3 complex [44]. In the structure, LC3 was bound to Atg4B using the C-terminal tail and β-strands 3–5, which were located distally from the AIM binding site. However, the YDTL sequence at the N-terminal tail of Atg4B (residues 7–10) was bound to the AIM binding site on the adjacent LC3 (Fig. 2C). The YDTL sequence forms an intermolecular parallel β-sheet with j2 of LC3, and the hydrophobic side chains of Tyr7 and Leu10 are bound to the W- and L-sites on LC3, respectively. In addition to these canonical interactions, Asp8 forms an ionic interaction with Arg70 of LC3.

These interactions are not due to a crystallization artifact since an NMR study showed that the N-terminal tail of Atg4B interacts with LC3 in a similar manner in solution [44]. Yeast Atg4 also possesses a similar sequence (WXXL) at the N-terminal region. Although the biological significance of this interaction has not yet been established, it might play some roles in the regulation of Atg4B activity.

A structural study of *S. cerevisiae* Atg3 showed that Atg3 has two unique insertions in addition to the E2 core, the handle region (HR) and the flexible region, and Atg3 HR was shown to directly bind to Atg8 [45]. Since Atg3 HR contains a WEDL sequence, we investigated the interaction between Atg8 and Atg3 HR by NMR and in vitro pull-down assays, which showed that Atg3 HR binds to the AIM binding site on Atg8 using the WEDL sequence (Yamaguti et al., in preparation). Thus AIM is utilized not only in autophagic receptors, but also in enzymes modifying Atg8. It will be interesting to see whether other modifying enzymes, Atg7 and the Atg12–Atg5–Atg16 complex, also possess AIMs and utilize them for their interaction with Atg8.

5. GABARAP interaction with non-autophagic proteins

GABARAP was first identified as a γ-aminobutyric acid type A receptor binding protein and has been implicated in the intracellular trafficking of these receptors. Willbold and co-workers performed phase display screening of a randomized peptide library to identify ligands for GABARAP [46], and determined a sequence position-specific scoring matrix (PSSM) consensus motif, HHHDDDDWVFVPM. Using PSSM information, several potential GABARAP ligands have been identified although their biological significance remains to be elucidated [47].

Calreticulin is a multifunctional, lectin-like protein best known as a luminal Ca2+-dependent chaperone of the endoplasmic reticulum [48]. It directly binds GABARAP [46], and has a PSSM-like sequence, SLEDDWDFFLP, at positions 178–188. The crystal structure of the GABARAP–calreticulin peptide (residues 178–188) complex revealed that the interaction is quite similar to those between Atg8-family proteins and AIMs (Fig. 2D) [49]: the side chains of Trp183 and Leu186 of the peptide bind to the W- and L-sites on GABARAP, respectively. In addition, the side chain of Asp184 forms an ionic interaction with the side chain of Arg69 of GABARAP, and the side chain of Phe185 forms a hydrophobic interaction with the side chain of Tyr27, Arg30, and Leu32 of GABARAP.

GABARAP pull-down experiments with brain lysates identified the clathrin heavy chain as a binding protein for GABARAP [50]. The clathrin heavy chain has a PSSM-like sequence that contains a WVFV sequence (residues 513–516), and an NMR study showed that a peptide corresponding to residues 505–521 of the clathrin heavy chain interacts with the AIM binding sites on GABARAP. From these data, it was suggested that the clathrin heavy chain directly interacts with GABARAP through the WVFV sequence [50].

6. Canonical and additional interactions of AIMs with Atg8-family proteins

Fig. 3 shows the sequence alignment of reported AIMs and the summary of their observed interactions. The residues located at three, two, and one residues N-terminal to the conserved Trp (designated as X3, X2, X1, respectively), and the residues located at one and two residues C-terminal to Trp (designated as X1 and X2, respectively) are also shown. The canonical WXXL sequence is well conserved among AIMs, although Trp can be replaced with Tyr and Leu can be replaced with Ile (and possibly with Val). In the case of the Atg8–Atg19 WEEL interaction, substitution of Trp with Tyr significantly reduced the interaction, indicating that Trp is the preferred amino acid, at least in the case of Atg8 (our unpublished results). The conserved Trp and Leu residues bind to the W- and L-sites, respectively, and the WXXL sequence forms an intermolecular parallel β-sheet with j2 of Atg8-family proteins. This interaction mode is reminiscent of the interaction between SUMO and the SUMO-interacting motif (SIM), in which SIM forms an intermolecular β-sheet with SUMO j2 [51]. However, the W-site interaction is unique to AIM–Atg8-family protein interactions since SUMO lacks the N-terminal domain that constitutes the W-site on Atg8-family proteins. In the case of the SIM–SUMO interaction, the intermolecular β-
sheet can be both parallel and anti-parallel; therefore, it is interesting whether AIMs can also bind to Atg8-family proteins in an anti-parallel direction. In addition to these canonical interactions, some additional interactions are also observed. All five sites (X-3, X-2, X-1, X1, and X2) are preferred by acidic residues. Acidic residues at X-3, X-2, and X-1 may form ionic interaction with Lys46 and Lys48 of Atg8, whereas acidic residues at X1 and X2 may form ionic interaction with Arg67 and Arg28 of Atg8, respectively. In the case of LC3, Arg10 and Arg11 in \( \alpha1 \), which are not conserved among other Atg8 homologues, also appear to be involved in the ionic interaction with the acidic residues at X-3, X-2, and X-1. The highly basic nature of LC3 \( \alpha 1 \) might give LC3 a preference for more acidic AIMs. In addition to acidic residues, hydrophobic residues are also favored at X-2, and appear to form hydrophobic interactions with Phe25 and Leu50 of Atg8. These features may be important for AIMs to have an extended \( \beta \)-conformation upon binding to Atg8-family proteins. As for the residues C-terminal to the conserved Leu, no obvious preference for specific amino acids can be observed.

7. Predicting functional AIMs from primary sequences

In order to bind to Atg8-family proteins, AIM should not only have a WXXL-like sequence, but also an extended \( \beta \)-conformation and exposed hydrophobic side chains for the interaction with the W- and L-sites on Atg8-family proteins. However, essential residues in an AIM are rather hydrophobic and the AIM-like sequence in proteins may tend to have a rigid conformation with buried side chains, which cannot interact with Atg8-family proteins. Therefore, structural information is important to distinguish functional AIMs from non-functional AIM-like sequences. If an AIM-like sequence has a flexible conformation, it is a potential candidate for a functional AIM. The above-mentioned WVFV sequence in the clathrin heavy chain has a helical conformation (PDB ID 1X15). Therefore, mutational studies on the clathrin heavy chain will be required to show that the WVFV sequence actually mediates the GABARAP–clathrin heavy chain interaction.

If structural information is not available, predicting the structurally disordered region from the primary sequence may be effective in some cases. The following is an example to illustrate AIM prediction. SEPA-1 is a specific receptor for autophagic degradation of P granules in Caenorhabditis elegans, which directly binds to LGG-1, a C. elegans homologue of Atg8 [52]. A SEPA-1 fragment (residues 289–575) was shown to be responsible for LGG-1-binding; however, the residues involved in the interaction have not yet been assigned. The SEPA-1 fragment (residues 289–575) contains a canonical AIM sequence, YQEL (residues 469–472), and the region containing YQEL was predicted to be highly disordered using the DRIPPERD server (http://www.sbc.su.se/~maccallr/disorder/). Thus,
the YQEL sequence in SEPA-1 is a potential candidate for a functional AIM. However, it is obviously important to experimentally confirm the direct interaction between predicted AIMS and Atg8-family proteins.

8. Concluding remarks

Intriguingly, autophagic receptors involved in quite different processes, the Cvt pathway in S. cerevisiae, and autophagic degradation of aggregates in mammals, were shown to use the common AIM for interacting with Atg8-family proteins. AIMS have also been shown to be utilized in mitophagic receptors, indicating the motif’s significance in selective autophagy. However, selectivity of autophagy cannot be explained by the receptor–Atg8 interaction alone. In yeast, a dual sorting mechanism has been proposed, in which Ade1 was transported to the vacuole through two independent interactions: one via the receptor–Atg8 interaction, and the other through the receptor–Atg11 interaction [53]. Atg11 plays a crucial role in the Cvt pathway, mitophagy, as well as pexophagy, through direct interactions with the autophagic receptors involved in these processes [11, 54, 55]. Identification of an Atg11-interacting motif and a functional counterpart of Atg11 in mammals is necessary for further understanding the molecular mechanisms of cargo recognition during selective autophagy. In addition to the autophagic receptors, AIMS have also been revealed in Atg8-modifying enzymes and even in non-autophagic proteins. Further studies will be required to understand the various biological roles of AIMS.

References


