Structural Basis for the Specificity and Catalysis of Human Atg4B Responsible for Mammalian Autophagy*

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Reversible modification of Atg8 with phosphatidylethanolamine is crucial for autophagy, the bulk degradation system conserved in eukaryotic cells. Atg4 is a novel cysteine protease that processes and deconjugates Atg8. Herein, we report the crystal structure of human Atg4B (HsAtg4B) at 1.9 Å resolution. Despite no obvious sequence homology with known proteases, the structure of HsAtg4B shows a classical papain-like fold. In addition to the papain fold region, HsAtg4B has a small α/β-fold domain. This domain is thought to be the binding site for Atg8 homologs. The active site cleft of HsAtg4B is masked by a loop (residues 259–262), implying a conformational change upon substrate binding. The structure and in vitro mutational analyses provide the basis for the specificity and catalysis of HsAtg4B. This will enable the design of Atg4-specific inhibitors that block autophagy.

Autophagy is a process that involves the bulk degradation of cytoplasmic components by the lysosomal/vacuolar system (1, 2) and plays a critical role in numerous biological processes. Such processes include neurodegeneration, pathogen infection, muscular disorders, cancer, and programmed cell death (3). In the process of autophagy, a double-membrane structure called an autophagosome sequesters a portion of the cytoplasm and fuses with the lysosome/vacuole to deliver its contents into the organelle lumen.

Genetic approaches in Saccharomyces cerevisiae (4) and subsequent biochemical analyses have identified a novel ubiquitin-like conjugation system called the Atg8 system that is essential for autophagosome formation (5). In the Atg8 system, nascent Atg8 is cleaved at its C-terminal arginine residue by Atg4, a novel cysteine protease (6), and the exposed C-terminal glycine is conjugated to phosphatidylethanolamine (PE) by Atg7, an E1-like enzyme, and Atg3, an E2-like enzyme (5). The Atg8-PE is then further deconjugated by Atg4. The reversible modification of Atg8 is crucial for the normal progression of autophagy (6). Although the conjugation mechanism adopted by the Atg8 system is similar to those of other ubiquitin-like conjugation systems, the Atg8 system has several unique features. The most outstanding feature is that the target for Atg8 is a lipid, not a protein. Therefore, in contrast to other deconjugating enzymes, which function to deconjugate soluble proteins, Atg4 has to deconjugate Atg8-PE, which is localized in the membranes. Another unique feature is that in addition to the ubiquitin-fold region, Atg8 has two α-helices at its N terminus. These helices have been elucidated by structural studies of mammalian homologs (7–10). This feature of Atg8 may also distinguish Atg4 from other deconjugating enzymes.

Recent investigations have suggested that the molecular machinery of autophagosome formation is evolutionarily conserved from yeast to higher eukaryotes (4). In mammals, an Atg8-like conjugation system, called the LC3 (microtubule-associated protein light chain 3) system, has been shown to exist (11–13). LC3, the first identified mammalian homolog of Atg8, was originally found as a light chain of microtubule-associated proteins 1A and 1B in the rat brain (14). Like the Atg8 system in yeast, the C-terminal region of LC3 is cleaved by mammalian Atg4 (mAtg4) homologs (11, 15–17). The processed form, called LC3-I, has a glycine residue at its C terminus (11) and resides in the cytosol. After activation by mammalian Atg7 (mAtg7) and Atg3 (mAtg3) homologs (12, 13), LC3-I is further modified to another form, called LC3-II, which is most likely the PE-conjugated form (15), as in the Atg8 system. Because LC3-II is localized in the autophagosomal membrane, it is now widely used as a probe to monitor autophagosomes and autophagy activity in mammalian systems (11). In addition to LC3, three mammalian Atg8 homologs are identified: Gogli-associated ATPase enhancer of 16 kDa (GATE-16) (18), GABA receptors-associated protein (GABARAP) (19), and most recently, Atg8L (16). GATE-16 and GABARAP have also been shown to be the substrates for mAtg7, mAtg3 (20), and mAtg4 (15–17, 21) in a similar manner to LC3 and can be conjugated to PE. However, among these homologs LC3 is most abundant in autophagosomal membranes, indicating the crucial role of LC3 in mammalian autophagy.

Thus far, four human homologs of yeast Atg4 have been reported: HsAtg4A/autophagin-2, HsAtg4B/autophagin-1, autophagin-3, and autophagin-4 (6, 22). HsAtg4A cleaves GATE-16 most efficiently (15, 21), whereas HsAtg4B has a broad specificity for mammalian Atg8 homologs (GATE-16, GABARAP, and LC3) (15, 17). Among human Atg4 homologs, HsAtg4B is the sole enzyme reported to efficiently
HsAtg4B was crystallized with a reservoir solution consisting of 0.2 M NaH2PO4, 0.8 M K2HPO4, 0.1 M NaCl, and 0.1 M appropriate buffer at a broad range of pH 5.6 to 10.0. The crystals belong to the orthorhombic space group P2221, with unit cell parameters of a = 51.9, b = 88.3, c = 160.1 Å. The selenomethionine derivative was expressed in E. coli and purified on a glutathione-Sepharose 4B column. The 9-mer peptide QETFGATLA, which corresponds to amino acids 116–124 of unprocessed rat LC3 (homolog of human LC3B) were constructed with different tags fused to the C-terminal Gly-120. One consisted of a 15-amino acid sequence, GSPEFPGRHHHHHH, which originated from the pGEX vector and a hexahistidine tag (this construct is called LC3-His); the other was the GST tag (this construct is called LC3-GST). Single mutant LC3s (Q116A and F119A) were prepared by PCR-mediated site-directed mutagenesis and sequenced to confirm their identities. LC3-His and LC3-(1–115) were inserted into the pGEX6p vector, whereas LC3-GST was inserted into the pET11a vector (Novagen). They were expressed in E. coli and purified on a glutathione-Sepharose 4B column. Each construct was concentrated to 1 mg/ml (LC3-His and LC3-GST) or 3 mg/ml (LC3-(1–115)) in the reaction buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol). The 9-mer peptide QETFGATLA, which corresponds to amino acids 116–124 of unprocessed LC3, was purchased from Sigma Genosys. Constructs of various mutant HsAtg4Bs were also prepared by PCR-mediated site-directed mutagenesis.

**EXPERIMENTAL PROCEDURES**

**Expression, Purification, and Crystallization**—The region coding the full length of HsAtg4B was inserted into the pGEX6p vector (Amersham Biosciences), and HsAtg4B was expressed in Escherichia coli BL21 DE3 cells. The GST-fused protein was first purified using a glutathione-Sepharose 4B column (Amersham Biosciences) followed by excision of GST from the protein with PreScission protease (Amersham Biosciences). Further purification was performed using Resource Q followed by Superdex 75 columns (Amersham Biosciences). The purified protein was concentrated to 7 mg ml⁻¹ in 0.15 M NaCl with 20 mM Tris-HCl at pH 7.4 and 2 mM dithiothreitol. Crystallization trials were performed with the sitting-drop vapor diffusion method at 293 K. HsAtg4B was crystallized with a reservoir solution consisting of 0.2 M NaH2PO4, 0.8 M K2HPO4, 0.1 M NaCl, and 0.1 M appropriate buffer at a broad range of pH 5.6 to 10.0. The crystals belong to the orthorhombic space group P2221, with unit cell parameters of a = 51.9, b = 88.3, c = 160.1 Å. The selenomethionine derivative was expressed in E. coli, B834 (DE3) using an amino acid medium (23) containing selenomethionine instead of methionine and was crystallized under the same conditions as those for native crystals.

**Data Collection**—Crystals were immersed in a reservoir solution supplemented with 16% glycerol for several seconds and then flash-cooled and maintained under nitrogen gas at 90 K during data collection. All diffraction data were collected on the ADSC Quantum 315 charge-coupled device detector using the SPring-8 beamline BL41XU. Multi-wavelength anomalous diffraction (MAD) data were collected from a single crystal of selenomethionine-substituted HsAtg4B at three wavelengths. Each data set was independently integrated, scaled, and processed using the HKL2000 program suite (24). Statistics for x-ray diffraction data are summarized in TABLE ONE.

**Structure Determination and Refinement**—The structure of HsAtg4B was determined by the MAD phasing method. All selenium sites with the exception of the N-terminal Met were found and refined, and the initial phases were calculated using the programs SOLVE and RESOLVE (25). About 60% of the residues were automatically modeled as a polyalanine chain by RESOLVE. Further model construction was performed manually using the molecular modeling program O (26), and the refinement was performed using the crystallography and NMR system program (27). The refinement statistics are summarized in TABLE ONE. The coordinates and structure factors of HsAtg4B have been deposited in the Protein Data Bank (code 2CY7).

**In Vitro Cleavage Assay**—For the in vitro cleavage assay, two kinds of rat LC3 (homolog of human LC3B) were constructed with different tags fused to the C-terminal Gly-120. One consisted of a 15-amino acid sequence, GSPEFPGRHHHHHH, which originated from the pGEX vector and a hexahistidine tag (this construct is called LC3-His); the other was the GST tag (this construct is called LC3-GST). Single mutant LC3s (Q116A and F119A) were prepared by PCR-mediated site-directed mutagenesis and sequenced to confirm their identities. LC3-His and LC3-(1–115) were inserted into the pGEX6p vector, whereas LC3-GST was inserted into the pET11a vector (Novagen). They were expressed in E. coli and purified on a glutathione-Sepharose 4B column. Each construct was concentrated to 1 mg/ml (LC3-His and LC3-GST) or 3 mg/ml (LC3-(1–115)) in the reaction buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol). The 9-mer peptide QETFGATLA, which corresponds to amino acids 116–124 of unprocessed LC3, was purchased from Sigma Genosys. Constructs of various mutant HsAtg4Bs were also prepared by PCR-mediated site-directed mutagen-
Crystal Structure of HsAtg4B

**RESULTS AND DISCUSSION**

**Overall Structure of HsAtg4B**—The crystal structure of HsAtg4B was refined against 1.9-Å data to an R-factor of 0.215 and a free R-factor of 0.247. The region corresponding to amino acids 5–377 was modeled with 0.3, 0.03, or 0.003 mg/ml HsAtg4B at a volume ratio of 1:1 and incubated at 293 K for 10 min. The reaction was stopped by the addition of SDS sample buffer and analyzed by SDS-PAGE using Coomassie Brilliant Blue staining. The 9-mer peptide (1 mg/ml) was mixed with 3 mg/ml HsAtg4B or its deletion mutant Δ(259–262) with or without 3 mg/ml LC3 (1–115) and incubated at 293 K for 6 h. The reaction solutions were applied to a Resource RPC column (Amersham Biosciences) equilibrated with 0.1% trifluoroacetic acid solution and eluted using a gradient of 0–80% CH3CN with 0.1% trifluoroacetic acid solution. For mutational analyses using mutant HsAtg4Bs, wild-type and mutant HsAtg4Bs (0.1 mg/ml) in reaction buffer were mixed with LC3-GST (1 mg/ml) at a volume ratio of 1:1. After 10 min at 293 K, the reaction was stopped by the addition of SDS sample buffer and analyzed by SDS-PAGE using Coomassie Brilliant Blue staining.

**A Small Domain Unique to HsAtg4B**—Compared with papain, HsAtg4B has an inserted region composed of the α-helices H and I and the β-strands 5 and 13 (enclosed by broken lines in Fig. 2A). HAUSP also has an inserted region, positionally similar to that of HsAtg4B, although its shape and size are different (Fig. 2). The inserted region of HsAtg4B is appreciably shorter; there-
HsAtg4B and HAUSP have an insertion of strand β4 in common, near the inserted domain that is parallel to β6 of the central sheet (Fig. 2B).

**Catalytic Residues**—Fig. 3A shows the active site structure of HsAtg4B. The Ne2 atom of His-280 is 3.4 Å from the S atom of Cys-74, and the Nδ1 atom of His-280 is 2.6 Å from the Oδ1 atom of Asp-278. This is the geometry observed in the canonical catalytic triad of cysteine proteases. These three residues are strictly conserved among Atg4 homologs (22), suggesting that these are the catalytic triad of HsAtg4B. Fig. 3B shows the superimposition of the catalytic residues of HAUSP (PDB code 1NBF), papain (PDB code 1PE6), UCH-L3 (PDB code 1UCH), and Ulp1 (PDB code 1EUV) on those of HsAtg4B. Here, the structure of the HAUSP-ubiquitin aldehyde complex was used for superimposition because the catalytic triad of HAUSP is properly superimposed only when it forms a complex with its substrate (33). Cys-74 and Asp-278 of HsAtg4B are superimposed on the nucleophilic cysteine and the Asp/Asn residue comprising the catalytic triad of cysteine proteases, respectively (Fig. 3B). Cys-74 is located at the N terminus of helix B, and Asp-278 is located at the C terminus of β9. Both of these positions are conserved among papain and DUBs (Fig. 2B). In contrast, although the side chain of His-280 of HsAtg4B can be superimposed on that of the general base histidine of papain and DUBs, its main-chain location is different. His-280 of HsAtg4B is located at a loop between β9 and β11, whereas the general base histidine of papain and DUBs is located at the N terminus of the strand equivalent to β8 of HsAtg4B (Figs. 2B and 3B). Although ULP family proteases have a different topology, their catalytic triad residues are located in the same geometry as those of both papain and DUBs (Figs. 2B and 3B). Thus the different location of the histidine residue is a unique feature of Atg4 family proteases. In addition to the catalytic triad, cysteine proteases have a conserved Asn/Gln residue that participates in the formation of the oxianion hole crucial for catalysis: Gln-19 for papain, Asn-218 for HAUSP, Gln-89 for UCH-L3, and Gln-574 for Ulp1. Atg4 family proteases also have a conserved Gln residue near the nucleophilic cysteine sequence (Gln-80 for HsAtg4B) that was suggested to be a component of the oxianion hole (22). However, a conserved Tyr residue (Tyr-54), not Gln-80, is superimposed on these Asn/Gln residues (Figs. 2B and 3B), and Gln-80 is located distally. This is another unique feature of Atg4 family proteases.

**Conformational Changes Prerequisite for Catalysis**—Fig. 3C shows the surface representation of HsAtg4B. Residues conserved among four human Atg4 homologs and yeast Atg4 are colored green, whereas catalytic triad residues are colored red. The green region is localized near the catalytic triad (Fig. 3C), suggesting the importance of the region for substrate recognition. There is a concave surface at the left side of the green region. This surface consists of the short fingers domain, strand β4, and helix E and its N-terminal loop (Fig. 3D). HAUSP also has a similar concave surface consisting of the fingers domain, strand β4, helix E, and other regions (Fig. 2), where the core region of ubiquitin aldehyde binds (33). Therefore, the concave surface of HsAtg4B is the putative binding site for the LC3 core. In contrast, the region between the green region and the catalytic triad is occupied by residues Lys-259, Pro-260, Asn-261, and Ser-262, and no cleft in which the LC3 tail can bind is present (Fig. 3C). The nucleophilic cysteine is buried under these four residues and is not exposed (Fig. 3, C and D). Substrates cannot access Cys-74 in this structure, indicating that free HsAtg4B is autoinhibited. Because the loop between β7 and β8 (corresponding to residues 258–263) masks the active site of HsAtg4B, this is called the inhibitory.
FIGURE 3. The catalytic site structure of HsAtg4B. A, stereoview of the catalytic site structure of HsAtg4B. Final annealed $F_o - F_c$ omit electron density map for Tyr-54, Cys-74, Asp-278, and His-280 at 3.5 $\sigma$ is also indicated. B, stereoview of the superimposition of the catalytic triad residues of four cysteine proteases on Cys-74, His-280, and Asp-278 of HsAtg4B (colored red). Superimposed proteases include papain (PDB code 1PE6; colored green), HAUsp (PDB code 1NBF; colored blue), UCH-L3 (PDB code 1UCH; colored yellow), and Ulp1 (PDB code 1EUV; colored brown). Conserved Gln/Asn residue comprising the oxianion hole of four cysteine proteases and Tyr-54 of HsAtg4B are also shown. A and B are shown in the same orientation. C, surface representation of HsAtg4B. Residues conserved among Atg4 homologs are colored green except for the catalytic triad residues, which are colored red. Catalytic triad residues and residues expected to be involved in the recognition of the LC3 tail are labeled with black letters, whereas residues comprising the inhibitory loop are labeled in blue letters. D, ribbon diagram of HsAtg4B. The side chain of catalytic triad residues, Trp-142, Pro-260, and Asn-261 is shown with stick models. Catalytic triad residues are colored red, the inhibitory loop is colored blue, the loop containing Trp-142 is colored cyan, and the short fingers domain is colored orange. C and D are shown in the same orientation. Panels A, C, and D were prepared using PyMOL [41].
The inhibitory loop has few neighboring interactions; there is one hydrogen bond between the side chain of Asn-261 and the main chain of Gly-71, and there are van der Waals interactions, the majority of which are between Pro-260 and the side chain of Trp-142 (Fig. 3D). Furthermore, the N terminus of the inhibitory loop is composed of two conserved Gly residues (Gly-257 and Gly-258), suggesting the flexibility of the inhibitory loop. Trp-142, the major interacting partner of the inhibitory loop, is located on a loop between helices D and E, and the loop is also expected to be flexible because of its limited interactions with neighboring groups (Fig. 3D). Thus these two loops are expected to be susceptible to conformational changes, which are thought to be a crucial prerequisite for the hydrolysis of substrates by HsAtg4B. As mentioned...
above, the Atg4 family proteases utilize a histidine residue as the general base located at a different position than in other cysteine proteases. In the HsAtg4B structure, Ser-262 in the inhibitory loop is equivalent to the conserved general base histidine of papain family proteases in the sequence. To function as a component of the catalytic triad, the imidazole ring of the histidine residue is required to be fixed in the proper geometry. Because the inhibitory loop may undergo large conformational changes upon complex formation, a histidine residue on the inhibitory loop is not suitable for serving as the general base. Thus, Atg4 family proteases may utilize a histidine residue at a non-canonical position for the general base.

**Substrates Susceptible to HsAtg4B in Vitro**—Thus far, the substrate preference of Atg4 family proteases has not been investigated in detail. Therefore, *in vitro* cleavage assays were performed using three forms of LC3: a fusion protein, LC3-GST, in which GST is directly linked to the C-terminal Gly-120 of LC3-I (the processed form of LC3); LC3-I fused to 15 residues containing His15 at its C terminus (LC3-His); and the tail region of the unprocessed LC3 (residues 116–124) (QETFGTALA; 9-mer peptide). These substrates were mixed with purified HsAtg4B and incubated at 293 K for either 10 min (LC3-His and LC3-GST) or 6 h (9-mer peptide), and the reaction solutions were analyzed by SDS-PAGE (LC3-GST and LC3-His) or reverse-phase chromatography (9-mer peptide). As shown in Fig. 4A, both LC3-His and LC3-GST were cleaved with a similar efficiency by HsAtg4B. These results indicate that HsAtg4B has little specificity for the sequence and the size of the C-terminal adducts to the scissile bond of LC3. In contrast, the 9-mer peptide was not cleaved by HsAtg4B, despite its long incubation time (Fig. 4B), indicating that the tail region of LC3 is not sufficient for hydrolysis by HsAtg4B. Thus, the 9-mer peptide could not induce the conformational changes of HsAtg4B and could not access the active site. A deletion mutant of HsAtg4B, lacking the inhibitory loop (residues 259–262), was then constructed and used for reaction with the 9-mer peptide. This deletion mutant was found to retain significant activity against LC3(1–115) (LC3 without C-terminal tail) was also performed, but then constructed and used for reaction with the 9-mer peptide. This deletion mutant was found to retain significant activity against LC3-GST, although the activity is lower than that of wild-type HsAtg4B (Fig. 4D, left panel). A point mutation was also introduced at Phe-119 and another conserved residue, Gln-116 of LC3, with Ala. As shown at the right panel of Fig. 4D, both mutant LC3s, especially the F119A mutant, became resistant to HsAtg4B. Thus, the aromatic residue-Gly sequence at the N terminus of the scissile bond is a unique feature for hydrolysis by Atg4 homologs and is crucial for hydrolysis by HsAtg4B family proteases.

**Structural Basis for the Specificity and Catalysis of HsAtg4B**—The structure of HsAtg4B showed that the active site of HsAtg4B is autoinhibited by the inhibitory loop. Furthermore, an *in vitro* cleavage assay showed that LC3 has to possess both the core and the tail regions connected to each other to be hydrolyzed by HsAtg4B. These observations suggest that during catalysis, the core region of LC3 first binds to HsAtg4B, which then induces conformational changes of the inhibitory loop and opens the active site cleft of HsAtg4B. Thus, the tail region of LC3 can bind to the active site cleft, and the cleavage reaction proceeds.

HsAtg4B has a concave surface at the left side of the conserved region (Fig. 3C), which is the putative binding site for the core region of LC3. The tail region of ubiquitin and ubiquitin-like proteins takes an elongated β conformation when bound to their cognate enzymes (31, 33–38). Hence after the core region of LC3 binds to the concave surface of HsAtg4B, the tail region of LC3 is thought to bind to the exposed active site cleft in an elongated β conformation. Mutational analyses showed that residues comprising the conserved region of HsAtg4B (especially Trp-142) and the tail region of LC3 N-terminal to the scissile bond (especially Phe-119) are crucial for hydrolysis. Therefore, the tail region of LC3 can be recognized by specific interactions with the active site cleft composed of the conserved residues. Especially, the interaction between the aromatic side chains of Phe-119 (LC3) and Trp-142 (HsAtg4B) may be critical for binding and catalysis. The two-step mechanism involving the recognition of the LC3 core and the subsequent recognition of the LC3 tail by HsAtg4B seems to be crucial for enhancing the specificity of Atg4 family proteases. Masking of the active site of free HsAtg4B by the inhibitory loop may function to avoid nonspecific proteolysis.

**Perspectives**—Thus far, there has been no specific inhibitor for autophagy. Because inhibition of Atg4 activity severely blocks autophagy (6), Atg4-specific inhibitors are expected to abrogate the process of autophagy. The design of inhibitors against the active site cleft would not be effective because the active site cleft of HsAtg4B is masked in the free form. As the target for the specific inhibitors, two sites on HsAtg4B can be considered: the site where the ubiquitin core of the substrate binds (possibly the short fingers domain) and the inhibitory loop or its surrounding region. By blocking these sites, the structure of HsAtg4B can be fixed in the masked form. The Atg4-specific inhibitors can be useful tools for further investigations of autophagy.

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