The Atg12-Atg5 Conjugate Has a Novel E3-like Activity for Protein Lipidation in Autophagy

To whom correspondence should be addressed. Tel.: 81-564-55-7515; Fax: 81-564-55-7516; E-mail: yohsumi@nibb.ac.jp.

ACCELERATED PUBLICATION

Autophagy is a bulk degradation process in eukaryotic cells; autophagosomes enclose cytoplasmic components for degradation in the lysosome/vacuole. Autophagosome formation requires two ubiquitin-like conjugation systems, the Atg12 and Atg8 systems, which are tightly associated with expansion of autophagosomal membrane. Previous studies have suggested that there is a hierarchy between these systems; the Atg12 system is located upstream of the Atg8 system in the context of Atg protein organization. However, the concrete molecular relationship is unclear. Here, we show using an in vitro Atg8 conjugation system that the Atg12-Atg5 conjugate, but not unconjugated Atg12 or Atg5, strongly enhances the formation of the other conjugate, Atg8-PE. The Atg12-Atg5 conjugate promotes the transfer of Atg8 from Atg3 to the substrate, phosphatidylethanolamine (PE), by stimulating the activity of Atg3. We also show that the Atg12-Atg5 conjugate interacts with both Atg3 and PE-containing liposomes. These results indicate that the Atg12-Atg5 conjugate is a ubiquitin-protein ligase (E3)-like enzyme for Atg8-PE conjugation reaction, distinctively promoting protein-lipid conjugation.

Autophagy is a bulk degradation process conserved in eukaryotes from yeast to mammals. In macro-autophagy, a double membrane-bound structure, the autophagosome, engulfs a portion of cytoplasmic components and fuses with the lysosome/vacuole for degradation of the contents (1, 2). Recent studies have shown that autophagy associates with numerous biological processes, including development and differentiation, immunity, infectious disease, neurodegeneration, and cancer (3, 4).

Studies of yeast Saccharomyces cerevisiae have shown that 18 Atg proteins are required for autophagosome formation, and most of them are conserved in higher eukaryotes (5). About half of Atg proteins comprise two ubiquitin-like conjugation systems, the Atg12 and Atg8 systems (6–8). Atg12 is activated by Atg7 (7, 9) and transferred to Atg10 (10). Atg12 is finally conjugated to Atg5, forming the irreversible Atg12-Atg5 conjugate (7). The Atg12-Atg5 conjugate further forms a ~350-kDa multimeric complex with Atg16 (11, 12). The other ubiquitin-like protein, Atg8, is synthesized with additional arginine at its C-terminal end, which is cleaved by Atg4, producing Atg8cyt (7, 11, 12) (hereafter referred to as Atg8 for simplicity). Atg8 is activated by Atg7 and transferred to Atg3 (6). Finally, Atg3 conjugates Atg8 with a phospholipid, phosphatidylethanolamine (PE)3 (6).

Previous studies have indicated that there is an apparent hierarchy between these systems; the absence of the Atg12-Atg5 conjugate severely reduces the level of Atg8 lipidation in vivo (14–16), but the precise relationship has been unclear. Here, we show that the Atg12-Atg5 conjugate directly facilitates the formation of Atg8-PE as an E3-like enzyme.

EXPERIMENTAL PROCEDURES

Materials—Lipids, POPC (850475), DOPE (850725), dioleoyl phosphatidylserine (840035), NBD-PE (810156), NBD-sphin-gosine (810205), and NBD-PS (810195) were purchased from Avanti Polar Lipids. Anti-His antibody (H-5) was purchased from Santa Cruz Biotechnology. Anti-Atg12, Atg3, Atg8, and Atg16 antisera have been described previously (6, 12, 17). The cross-linker, AMAS, was purchased from Pierce Biotechnology. Plasmid Construction—We cloned every ATG gene from S. cerevisiae for the expression of recombinant proteins. For expression of the Atg12-Atg5 conjugate in Escherichia coli, Atg7 and Atg10 were cloned into the pET-11a vector, and Atg12 and Atg5 were cloned into the pACYC184 vector. Briefly, an Atg7 fragment including the Shine-Dalgarno sequence was amplified from pHT1-Atg7 by PCR using primers having an Ndel site (5′-GGAATTCATATGTCGTCAGAA-AGGGTCTT-3′) and a BamHI site (5′-GGGGATCCGCTAGC- TTAAGCAATCTCATCAGATTC-3′). The PCR product digested with Ndel and BamHI was ligated into pET-11a. An Atg10 fragment was prepared from pET-11a-Atg10 by XbaI and BamHI digestion and was ligated into the above pET-11a-Atg7 construct. In a similar fashion, an Atg5 fragment including the

8 This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in-Aid for Specially Promoted Research 15002012 (to Y. O.). A grant from the National Project on Protein Structural and Functional Analyses (to Y. O. and F. I.), and a Grant-in-Aid for Creative Scientific Research 15002012 (to Y. O.). A grant from the National Project on Protein Structure and Function (to T. T.) as well as research fellowships of the Japan Society for the Promotion of Science for Young Scientists (to T. H.).

9 A series of experiments were performed in part at Research Institute for Molecular and Cellular Biology, National Institute for Basic Biology, Myodaiji, Okazaki, 444-8585, the Department of Structural Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, N-21, W-11, Kita-ku, Sapporo 001-0021, and the Institute for Protein Research, Osaka University, Suita, Osaka 565-0871, Japan.

10 Correspondence should be addressed. Tel.: 81-564-55-7515; Fax: 81-564-55-7516; E-mail: yohsumi@nibb.ac.jp.
Shine-Dalgarno sequence was amplified from pHT1-Atg5 by PCR using primers having an Ndel site (5’-GGGGCCGCATAGTACCAAAAAATT-3’) and a PstI site (5’-GGCTTGGAGTCTAGTATGACTGAATCGAACGTTTGAAGTGCAGAGGAAAGCTT-3’). PCR product digested with Ndel and PstI was ligated into pACYC184. An Atg12 fragment was prepared from pHT1-Atg12 by Xbal and PstI digest and ligated into the above pACYC184-Atg5 construct. Expression plasmids encoding Atg7Myc (C-terminal Myc$_3$-tagged Atg7), Atg3 (thrombin-cleavage from GST), and Atg8$_{Gly116}$ (C-terminal glycine-exposed form of Atg8) were used as described previously (18). Atg5 was cloned into the pHT1 vector, and Atg16 and Atg10 were cloned into the pGEX6p vectors. Atg12 was cloned into the pYNGHisB vector for expression in the superworm system (Katakura industries). DNA sequences were confirmed using an automated DNA sequencer.

**Protein Preparation**—Atg proteins were expressed in *E. coli* cells except for Atg12 that expressed within silkworm. Plasmids encoding Atg proteins were transformed into the *E. coli* BL21 or BL21 (DE3) strains (Stratagene), and cells were cultured in LB medium. Expression of each protein was induced by the addition of 50 µM (Atg7Myc, Atg3, and Atg8), 100 µM (Atg16), or 500 µM (Atg12-Atg5 and Atg5) isopropyl β-D-thiogalactopyranoside to the medium. After induction, cells were disrupted by sonication in TBS buffer (50 mM Tris/HCl (pH 7.5), 150 mM NaCl) with 2 mM dithiothreitol for Atg7Myc, Atg3, and Atg8 or high salt buffer (50 mM Tris/HCl (pH 8.0), 300 mM NaCl for Atg12-Atg5 or 20 mM Tris/HCl (pH 8.0), 500 mM NaCl for Atg5). Phosphate-buffered saline buffer was used for Atg16, Atg8, Atg7Myc, Atg3, and Atg16 were purified by a single step purification using glutathione-Sepharose 4B (GE Healthcare) and cleaved from GST by the appropriate protease. For purification of Atg12-Atg5, lysates were first subjected to nickel-nitrilotriacetic acid resin (Qiagen). Resins were washed with wash buffer (20 mM Tri/HCl (pH 8.0), 0.5 M NaCl 10 mM imidazole) several times, and proteins were eluted with elution buffer (20 mM Tris/HCl (pH 8.0), 0.1 M NaCl 200 mM imidazole). After concentration, samples were subjected to gel filtration (HiLoad Superdex 75 pg 16/60) followed by a weak cation exchange chromatography (carboxy methyl (CM) Sepharose FF). Finally, samples were subjected to a second gel filtration. For purification of Atg5, lysates were first subjected to nickel-nitrilotriacetic acid resin, washed with wash buffer (20 mM Tri/HCl (pH 8.0), 0.5 M NaCl 10 mM imidazole), and eluted with elution buffer (20 mM Tri/HCl (pH 8.0), 0.5 M NaCl 200 mM imidazole). Eluates were concentrated and subjected to gel filtration (HiLoad Superdex 75 pg 16/60). Atg12 expressed in silkworm was recovered in a pellet fraction. Atg12 denatured by 6 M guanidine chloride was subjected to HiTrap chelating column (GE Healthcare) and refolded on the column. All proteins were stored in buffer containing 50% glycerol at −80 or −20 °C until use.

**In Vitro Atg8-PE Conjugation Reaction**—Atg8 lipidation reactions were carried out as described previously (18) with the modification that the ATP regeneration system (phosphocreatine and creatine kinase) is omitted (see the exception in Fig. 1A). Reactions were stopped by the addition of SDS sample buffer.

**Pull-down Assay**—The Atg12-Atg5 conjugate (36 pmol) was incubated with 190 pmol of GST-Atg3, GST-Atg8, and GST at 30 °C for 1 h in 100 mM PIPES/NaOH (pH 6.4) and 150 mM NaCl and further incubated at 4 °C for 1 h after the addition of glutathione-Sepharose 4B (20 µl of slurry, GE Healthcare). Sepharose beads were washed four times in high salt buffer (50 mM PIPES/NaOH (pH 6.4), 1 M NaCl, 2% Triton X-100).

**Cross-linking Experiment**—Liposomes consisting of 50% NBD-PE/50% POPC, 20% NBD-PE/80% POPC, or 50% NBD-sphingosine/50% POPC (50 µM lipids), and cross-linker AMAS (2 mM) dissolved in Me$_2$SO were incubated at 30 °C for 30 min in reaction buffer (100 mM HEPES/NaOH (pH 7.3), 150 mM NaCl) and further incubated for 15 min after the addition of 1 mM glycine (final concentration: 48 mM). Atg12-Atg5 conjugate (36 pmol) were added to the reaction mixtures and incubated at 30 °C for 30 min. Cross-linking was terminated by the addition of 1 M cysteine (final concentration: 36 mM). Samples were resolved by SDS-PAGE and analyzed by LAS image analyzer (Fujifilm) for detection of NBD-fluorescence (excitation 460 nm/emission 515 nm) followed by CBB staining.

**RESULTS AND DISCUSSION**

**The Atg12-Atg5 Conjugate Directly Accelerates the Lipidation of Atg8**—We have reported that Atg8-PE can be formed in an *in vitro* reaction containing yeast Atg8 (a processed form), Atg7, Atg3, ATP, and liposomes containing PE (18). This result suggests that Atg3 is competent for Atg8-PE formation without an E3-like enzyme unlike ubiquitin and other ubiquitin-like protein conjugation reaction. However, efficient formation of Atg8-PE *in vitro* required high PE content within liposomes (optimal PE content is 70%), which is much higher than PE content in yeast organelle membranes (15–30%) (19). Very little Atg8-PE is formed in reactions with low PE-containing liposomes (10–40%). This observation, in addition to the significance of the Atg12 system for the formation of Atg8-PE *in vivo* (15, 20), prompted us to directly examine the molecular function of the Atg12-Atg5 conjugate in Atg8-PE formation using *in vitro* system.

Recombinant Atg12-Atg5 conjugate was formed in *E. coli* co-expressing four components of the yeast Atg12 system (Atg12, Atg7, Atg10, and Atg5) and was purified by successive chromatography to near homogeneity (see “Experimental Procedures”). In the absence of the Atg12-Atg5 conjugate, as reported, little Atg8-PE was formed in the reaction containing 20% DOPE/80% POPC liposomes (Fig. 1A, lane 2), whereas most Atg8 was conjugated to PE in the reaction containing 70% DOPE/30% POPC liposomes (Fig. 1A, lane 4). However, in the presence of the Atg12-Atg5 conjugate, the level of Atg8-PE formed in the reaction containing 20% DOPE/80% POPC liposomes was strikingly enhanced (Fig. 1A, lane 3), whereas most Atg8 was conjugated to PE in the reaction containing 70% DOPE/30% POPC liposomes (Fig. 1A, lane 4). In contrast, individually prepared components of the Atg12 system, Atg12, Atg5, or Atg10 alone, or a mixture of Atg12 and Atg5 were unable to enhance Atg8-PE formation (Fig. 1B, lanes 5–8), indicating that the conjugation between Atg12 and Atg5 is essential for the function. Additionally, we confirmed that the Atg12-Atg5 conjugate had neither E1 nor E2 activities for Atg8 lipida tion (Fig. 1B, lanes 1 and 2).
ACCELERATED PUBLICATION: E3-like Activity of Atg12-Atg5

The Atg12-Atg5 conjugate enhances the formation of Atg8-PE.
A, lipidation of Atg8 was carried out in a mixture of purified Atg8 (5.0 μM), Atg3 (1.0 μM), Atg7 (1.0 μM), ATP (1 mM), creatine kinase (CK, 25 μg/ml), phosphocreatine (5 mM), and liposomes (350 μM lipids) consisting of 20% DOPE/80% POPC (lanes 1 and 2) or 70% DOPE/30% POPC (lanes 3 and 4) in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of the Atg12-Atg5 conjugate (0.23 μM). The asterisk indicates a product in which the C terminus of Atg8 binds to the N terminus of Atg3. This complex was observed when PE levels were insufficient for Atg3 activated by the Atg12-Atg5 conjugate. B, Atg8 lipiddation in the reaction with 20% DOPE/80% POPC liposomes, in the absence of Atg7 (lane 1), Atg3 (lane 2), or components of the Atg12 system (lane 3) or in the presence of 1.0 μM Atg12-Atg5 conjugate (lane 4), 2.4 μM Atg12 (lane 5), 1.7 μM Atg5 (lane 6), a mixture of Atg12 and Atg5 (lane 7), or 4.2 μM Atg10 (lane 8). The asterisk indicates the same product mentioned in panel A. The arrowhead indicates protein contaminated in the fraction of Atg5. C, time-dependent Atg8-PE formation was examined in reactions with 20% DOPE/80% POPC liposomes by the different concentration of the Atg12-Atg5 conjugate. The amount of the Atg12-Atg5 conjugate was increased according to the indicated concentrations (0.01, 0.02, 0.1, 0.5, and 1.0 μM), whereas the amount of Atg7 (1 μM), Atg3 (1 μM), and Atg8 (5 μM) was constant. The graph shows the amount of Atg8-PE at each time point (Atg12-Atg5: □, 0 μM; ●, 0.01 μM; △, 0.02 μM; ■, 0.1 μM; ◇, 0.5 μM, X, 1.0 μM).

We also examined the rate of Atg8-PE formation in reactions containing 20% DOPE/80% POPC liposomes in the presence or absence of the Atg12-Atg5 conjugate. The rate of Atg8-PE formation increased in an Atg12-Atg5 dose-dependent manner and reached maximum at 0.5 μM (Fig. 1C). The clear enhancement of Atg8-PE formation was still observed in the presence of ⅓ of the amount of the Atg12-Atg5 conjugate when compared with the E1 and E2 enzymes (0.1 μM). These results show that the Atg12-Atg5 conjugate repeatedly functions in the acceleration of Atg8-PE formation.

The Atg12-Atg5 Conjugate Functions as an E3-like Enzyme—
To further define the function of the Atg12-Atg5 conjugate in Atg8 lipiddation, first we examined the rate of the Atg8-Atg3 complex formation with or without the Atg12-Atg5 conjugate. For this experiment, we used the Atg3C234S mutant, in which the active-site cysteine at position 234 is replaced by serine, which forms a stable Atg8-Atg3C234S complex linked through an ester bond with the C-terminal glycine of Atg8 (supplemental Fig. S1) because the wild-type Atg8-Atg3 intermediate is unstable in SDS-PAGE even under nonreducing conditions. As shown in Fig. 2A, the rates of Atg8-Atg3 complex formation with or without the Atg12-Atg5 conjugate were indistinguishable. Thus, the Atg12-Atg5 conjugate acts on the step after the Atg8-Atg3 intermediate formation.

We previously reported that Atg8-PE is not formed from the Atg8-Atg3C234S complex by itself (18). This would be reasonable considering that Atg8 is stably linked with Atg3C234S mutant through an ester bond in the Atg8-Atg3C234S complex. Unexpectedly, however, a significant amount of Atg8 was transferred from Atg3C234S to PE by the addition of the Atg12-Atg5 conjugate (supplemental Fig. S2). We performed time course experiments of Atg8 lipidation after ATP depletion to observe single round reaction from the Atg8-Atg3C234S complex. It was shown that Atg8-PE was gradually formed in the presence of the Atg12-Atg5 conjugate, accompanied by a decrease of Atg8-Atg3C234S complex as well as an increase of free Atg3C234S (Fig. 2B). These results clearly show that the Atg12-Atg5 conjugate accelerates the step of Atg8 transfer from the E2 enzyme Atg3 to the substrate molecule PE, which corresponds
to that catalyzed by E3 enzyme in ubiquitin and other ubiquitin-like protein conjugation reactions (21).

It is suggested that the Atg12-Atg5 conjugate stimulates the conjugating activity of Atg3. This result provides an explanation for the observation that the C terminus of Atg8 was covalently bound to the N terminus of Atg3 upon the addition of the Atg12-Atg5 conjugate (Fig. 1, A and B, asterisk);

Atg3 stimulated by the Atg12-Atg5 conjugate mis-transfers Atg8 to its own N terminus when insufficient PE is present.

It has not been reported that ubiquitin linked with the E2 enzyme through the ester bond is transferred to the substrate. The core structure of Atg3 resembles the canonical E2 enzymes but lacks the conserved residues around the active cysteine (22). Atg3 may take the characteristic mechanism in conjugation differed from other E2 enzymes.

**The Atg12-Atg5 Conjugate Associates with Both Atg3 and PE-containing Liposomes**—Further, we assessed the interaction among proteins and liposomes. First, we examined the interaction between the Atg12-Atg5 conjugate and Atg3 or Atg8 using a pull-down assay. The Atg12-Atg5 conjugate firmly associated with GST-Atg3 but not with GST-Atg8 (Fig. 3A), suggesting that the Atg12-Atg5 conjugate works on Atg8 lipidation via an interaction with Atg3.

Next, we tested whether the Atg12-Atg5 conjugate binds to PE-containing liposomes. We were not able to demonstrate an interaction using sedimentation analysis. Therefore, we utilized a cross-linker that reacts with amino and sulfhydryl groups (the head group in PE and cysteine residues in proteins, respectively). The Atg12-Atg5 conjugate was incubated with liposomes containing PE modified at the fatty acid moiety with the fluorescent dye NBD. We used NBD-sphingosine having a free amine group at the lipid head as a negative control. The Atg12-Atg5 conjugate showed NBD fluorescence depending on the PE content of the liposomes and no fluorescence signal with NBD-sphingosine (Fig. 3B). Together, these results suggest that the Atg12-Atg5 conjugate promotes Atg8-PE conjugation by interacting with both Atg3 and PE-containing liposomes.

**Effect of Atg16 on the Stimulation of Atg8 Lipidation by the Atg12-Atg5 Conjugate**—In yeast, the Atg12-Atg5 conjugate forms a multimeric complex with Atg16, which is essential for autophagosome formation (12) and important for efficient production of Atg8-PE in vivo (15, 23). Recombinant Atg12-Atg5 conjugate incubated with recombinant Atg16 formed an ∼350-kDa complex, similar in size to the complex observed in yeast lysates (Fig. 4A) (12). This suggests that the ∼350-kDa complex formed in vivo consists of only the Atg12-Atg5 conjugate and Atg16. We tested whether Atg16 is involved in the facilitation of Atg8-PE formation. As shown in Fig. 4B, the coexistence of Atg16 with the Atg12-Atg5 conjugate only slightly increased the rate of Atg8-PE formation. Atg16 alone had no effect on Atg8 lipidation (data not shown). Therefore, Atg16 is not essentially required for the effective formation of Atg8-PE in vitro.

However, Atg16 is required for Atg8-PE formation in vivo.

Microscopic studies have shown that both Atg5 and Atg16

---

4 T. Hanada, Y. Satomi, T. Takao, and Y. Ohsumi, unpublished results.
5 K. Obara and Y. Ohsumi, unpublished results.
The Atg12-Atg5 conjugate, forming the Atg12-Atg5 complex, would contribute to the expansion of autophagosomal membrane by promoting Atg8 lipidation. In mammalian cells, it has been observed that the Atg12-Atg5 complex preferentially localizes on the outer membrane of the forming autophagosome (14, 24). Possibly, the Atg12-Atg5 complex coordinates asymmetric production of Atg8-PE on the autophagosomal membrane.

There are two major families of E3 ligases for ubiquitin, HECT-, and RING-type E3 ligases. Both Atg12 and Atg5 lack authentic features of ubiquitin E3 ligases, conserved cysteine, or RING finger motif. As revealed by structural analysis, Atg12 takes a ubiquitin-like fold (27), whereas Atg5 consists of two ubiquitin-like folds that flank a helix-rich region (23). Accordingly, the Atg12-Atg5 conjugate comprises three ubiquitin-like folds, which structurally differs from known E3 ligases for other ubiquitin-like proteins (28). The Atg12-Atg5 conjugate promotes “protein-to-lipid” conjugation, whereas E3 ligases promote “protein-to-protein” conjugation. The Atg12-Atg5 conjugate would exert a novel action on protein-lipid conjugation reaction. Further characterization of the E3-like catalysis will lead to new mechanistic and structural insights.