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The amino-terminal region of Atg3 is essential for association with phosphatidylethanolamine in Atg8 lipidation

Takao Hanada [a], Yoshinori Satomi [b], Toshifumi Takao [b], Yoshinori Ohsumi [a,*]

[a] Division of Molecular Cell Biology, National Institute for Basic Biology, Myodaiji, Okazaki 444-8585, Japan
[b] Institute for Protein Research, Osaka University, Suita, Osaka 565-0871, Japan

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ABSTRACT

Autophagy is a bulk degradation process conserved among eukaryotes. In macro-autophagy, autophagosomes sequester cytoplasmic components and deliver their contents to lysosomes/vacuoles. Autophagosome formation requires the conjugation of Atg8, a ubiquitin-like protein, to phosphatidylethanolamine (PE). Here we report that the amino (N)-terminal region of Atg3, an E2-like enzyme for Atg8, plays a crucial role in Atg8–PE conjugation. The conjugating activities of Atg3 mutants lacking the 7 N-terminal amino acid residues or containing a Leu-to-Asp mutation at position 6 were severely impaired both in vivo and in vitro. In addition, the amino-terminal region is critical for interaction with the substrate, PE.

Structured summary:
MINT-7010457: *ATG8* (uniprotkb:P38182) and *ATG3* (uniprotkb:P40344) *bind* (MI:0407) by *biochemical* (MI:0401)

1. Introduction

Autophagy is a bulk degradation process conserved among eukaryotic cells from yeast to mammals. In macro-autophagy in yeast, a double membrane-bound structure, the autophagosome, engulfs a portion of cytoplasmic components and fuses with the vacuole, releasing the autophagic body into the vacuole for degradation of the contents.

Formation of autophagosomes requires two ubiquitin-like conjugation systems [1]. One system consists of Atg12, Atg7, and Atg10. The other system consists of Atg8, Atg7, and Atg3. The target of Atg12 is the protein Atg5, whereas that the target of Atg8 is the phospholipid, phosphatidylethanolamine (PE). Recently, we have developed an in vitro Atg8–PE conjugation system consisting of Atg8, Atg7, Atg3 and PE-containing liposomes [2]. Utilizing this system, we showed that Atg12–Atg5 facilitates Atg8–PE conjugation in a manner similar to E3 ubiquitin ligases [3], and that lipidation of Atg8 induces membrane tethering and hemifusion [4]. Formation of Atg8–PE is tightly associated with the development of autophagosomal membranes [4–6]. Thus, Atg8–PE formation should be spatio-temporally regulated by a conjugation cascade for the generation of spherical autophagosomal membranes.

Atg3, an E2-like enzyme for Atg8, catalyzes protein–lipid conjugation, in contrast to other E2 enzymes. Atg3 has a core domain similar to canonical E2 enzymes, but it lacks conserved residues around the active site cysteine [7]. It therefore seems that Atg3 must adopt a special mode of action in achieving protein–lipid conjugation. Here, we report that the amino (N)-terminal region of Atg3 is essential for the interaction with PE in Atg8–PE conjugation.

2. Materials and methods

2.1. Mass spectrometry

Lysyl endopeptidase (LEP) digests were separated using an Ultimate nano-flow LC system on a C_{18}-PepMap column (Dionex). The column effluent was continuously introduced into the nano-flow electrospray ion source of a Q-TOF mass spectrometer (Micromass). The peptide ions were automatically subjected to MS/MS analysis in the data dependent mode of the Q-TOF.

2.2. Yeast strains and media

We used yeast *Saccharomyces cerevisiae* strains SEY6210 (*MATα ura3 leu2 his3 trp1 lys2 suc2*), YYI001 (SEY6210; *Δatg3::TRP1*),

Abbreviations: PE, phosphatidylethanolamine; LEP, lysyl endopeptidase; ALP, alkaline phosphatase; PS, phosphatidylserine; SCF, Skp1-Cullin-F-box protein

* Corresponding author. Fax: +81 564 55 7516.
E-mail address: yohsumi@nibb.ac.jp (Y. Ohsumi).

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YTH115 (SEY6210; *Δatg3::TRP1 pho8::PHO8Δ60*), BJ2168 (*MATα leu2 trp1 ura3 prb1 pep4 prc1 gal2*), and YTH118 (BJ2186; *Δatg3::TRP1*). Autophagy was induced by shift to SD (–N) medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, and 2% glucose) or S (–NC) medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate).

2.3. Plasmid construction

To express Atg3 mutants in yeast cells, the multi-copy plasmid (pRS425) encoding each Atg3 mutant was generated by site-directed mutagenesis (Stratagene) using appropriately designed primers and parental plasmid encoding wild-type Atg3. For recombinant Atg protein preparation, plasmids for Atg7Myc, Atg8, Atg3, and Atg12–Atg5 were used as previously reported [2,3]. Note that a thrombin cleavage site was introduced between GST and Atg3 in the protein used in the experiments depicted in Fig. 1 [2], while an enterokinase recognition sequence (DDDDK) was introduced between GST and Atg3 in the protein used in the experiments depicted in Figs. 3 and 4, which results in no additional sequence at the N-terminus of Atg3.

2.4. Alkaline phosphatase (ALP) assay

Lysates were prepared from cells starved in SD (–N) medium for 4 h. The ALP assay was done as described previously [8] using α-naphthyl phosphate (Sigma) as a substrate.

2.5. In vitro Atg8–PE conjugation reaction

As described previously, Atg7Myc, Atg8^{G116} (C-terminal glycine-exposed form of Atg8), Atg3, Atg12–Atg5, and PE-containing liposomes were prepared and Atg8 lipidation reactions were carried out [2,3].

2.6. Floatation assay

Atg3 was mixed with liposomes in HSM buffer (50 mM HEPES/KOH (pH 8.0), 150 mM NaCl, 1 mM $MgCl_2$) containing 0.2 mM DTT

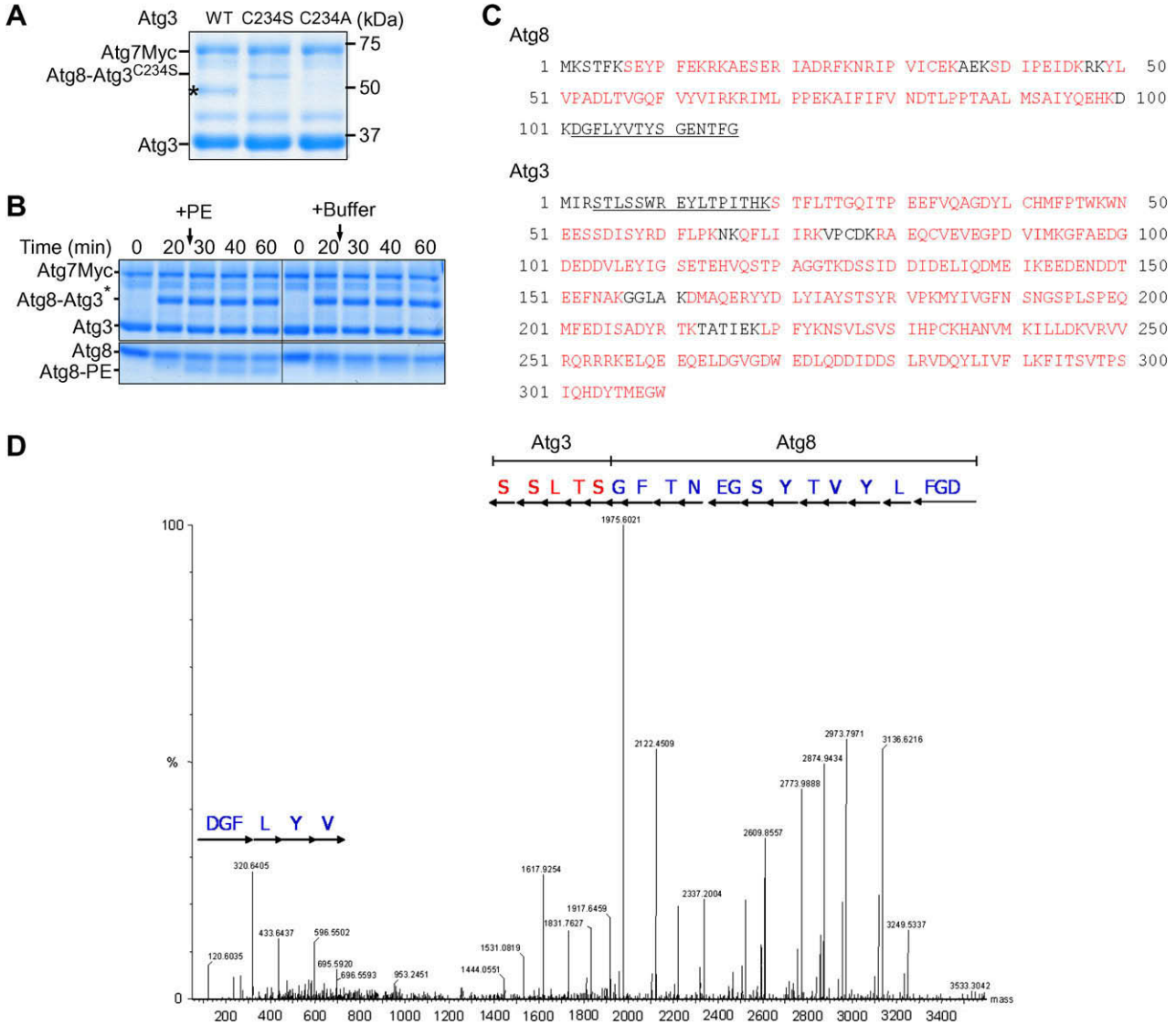

Fig. 1. Atg8 is conjugated to the N-terminus of Atg3. (A) Atg8–Atg3* (asterisk) was formed in in vitro reactions lacking PE-containing liposomes only when wild-type Atg3 was used. (B) After the formation of Atg8–Atg3*, buffer with or without PE-containing liposome was added to the reaction mixture. (C) A band corresponding to Atg8–Atg3* was subjected to in-gel digestion with LEP followed by nano-flow LC/ESI-MS/MS. The red letters indicate amino acid sequences identified for Atg8 and Atg3, and the underlined letters indicate those detected as a conjugated peptide. (D) MS/MS confirmed the conjugated peptide (3568.7 Da) contained the C-terminal Atg8 peptide linked to S4 of Atg3.

and incubated for 30 min at 30 °C in a total volume of 40 μl. The suspension was adjusted to contain 30% (w/v) OptiPrep™ (Axis-Shield) by the addition of 60 μl 50% (w/v) OptiPrep solution. The resulting suspension was overlaid with 350 μl HSM buffer containing 25% (w/v) OptiPrep and 50 μl HSM buffer without OptiPrep. The samples were centrifuged at 206 000×*g* in a Hitachi swing rotor (S55S) for 30 min. Fractions (50 μl) were collected from top to bottom and analyzed by western blotting.

3. Results and discussion

3.1. Atg8 is transferred to the N-terminus of Atg3 in the in vitro reaction

When PE-containing liposomes were omitted from an in vitro Atg8–PE reaction in the presence of Atg12–Atg5, an unexpected band around 50 kDa was observed (Fig. 1A, asterisk). This band migrated faster than Atg8–Atg3^{C234S}, a form in which the C-terminal glycine of Atg8 is linked to Atg3 via serine residue replacing cysteine at position 234 [3]. The new band was detected by both anti-Atg8 and anti-Atg3 antibodies in western blotting, suggesting that it contains Atg8 and Atg3 in a different linkage (hereafter mentioned as Atg8–Atg3*) from that contained in Atg8–Atg3^{C234S}. We examined whether Atg8–Atg3* is a reversible complex by addition of PE-containing liposome following the formation of Atg8–Atg3*, and found that Atg8–Atg3* is a stable conjugate (Fig. 1B).

To identify the binding site between Atg8 and Atg3 in Atg8–Atg3*, we subjected samples to mass spectrometry. While most part of Atg8 and Atg3 were detected, the peptides corresponding to C-terminus of Atg8 and N-terminus of Atg3 were not detected (Fig. 1C). Instead, we detected a peptide consisting of the C-terminal glycine of Atg8 conjugated to serine in the N-terminus of Atg3

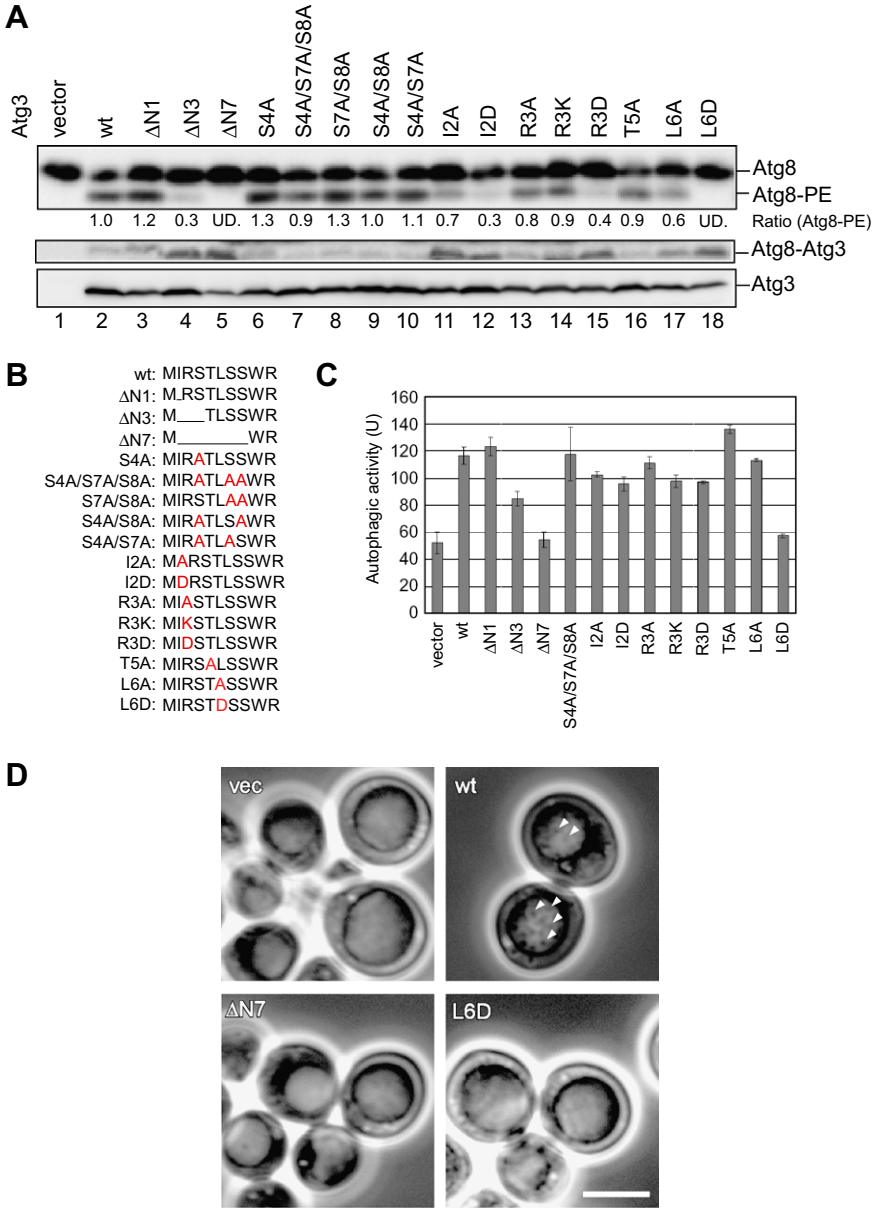

Fig. 2. The amino-terminal region of Atg3 is important for the formation of Atg8–PE in vivo. (A) Levels of Atg8–PE in yeast cells (YYI001) expressing Atg3 mutants, which were constructed as shown in (B). The ratios of Atg8–PE quantified with Scion Image software were shown under top panel, which normalized to wild-type. UD, undetectable. (C) Autophagic activity of yeast cells expressing Atg3 mutants evaluated by ALP assay. (D) Accumulation of autophagic bodies in yeast cells (YTH118) expressing Atg3 mutants after starvation in S (–NC) medium for 5 h. Arrow heads indicate autophagic bodies in the vacuole. Bar, 5 μm.

(Fig. 1D). Atg8–Atg3* was not formed in reactions with the $Atg3^{C234S}$ or $Atg3^{C234A}$ mutants (Fig. 1A), suggesting that the conjugate was formed by transferring Atg8 from C234 of Atg3. The N-terminal residue (T21) defined in the crystal structure of Atg3 [7] locates about 15 Å away from C234 on the same side of the molecule, suggesting that N-terminal region (1–20) could reach the active site. These results led us to speculate that the N-terminal region of Atg3 might be located close to the site of the conjugating reaction or directly contributes to the reaction.

3.2. The amino-terminal region of Atg3 is important for Atg8–PE formation in yeast

To examine the role of the N-terminal region of Atg3 in the conjugation reaction, we prepared Atg3 mutants lacking one, three, and seven residues at the N-terminus (ΔN1, ΔN3, and ΔN7; see Fig. 2B) and examined Atg8–PE formation in yeast cells expressing these mutants. The amount of Atg8–PE decreased as the N-terminus was deleted (Fig. 2A, lanes 3–5): Atg8–PE was completely absent in cells expressing the ΔN7 mutant. These results show that the N-terminal region of Atg3 is critical for the production of Atg8–PE.

Next, to identify the residue important for Atg8 lipidation, we prepared site-directed mutants, in which residues within the 2-IRSTLSS-8 sequence were substituted to alanine, aspartic acid, or lysine (Fig. 2B). Because Atg8 was mis-transferred to S4 (Fig. 1D), we also introduced several serine to alanine substitutions (S4, S7 and S8). Most of the site-directed Atg3 mutants produced detectable amounts of Atg8–PE; however, the I2D and R3D mutants produced only very small amounts of Atg8–PE and we did not detect Atg8–PE in the L6D mutant (Fig. 2A, lanes 6–18). Altogether, among the Atg3 mutants, Atg8–PE was absent in cells expressing

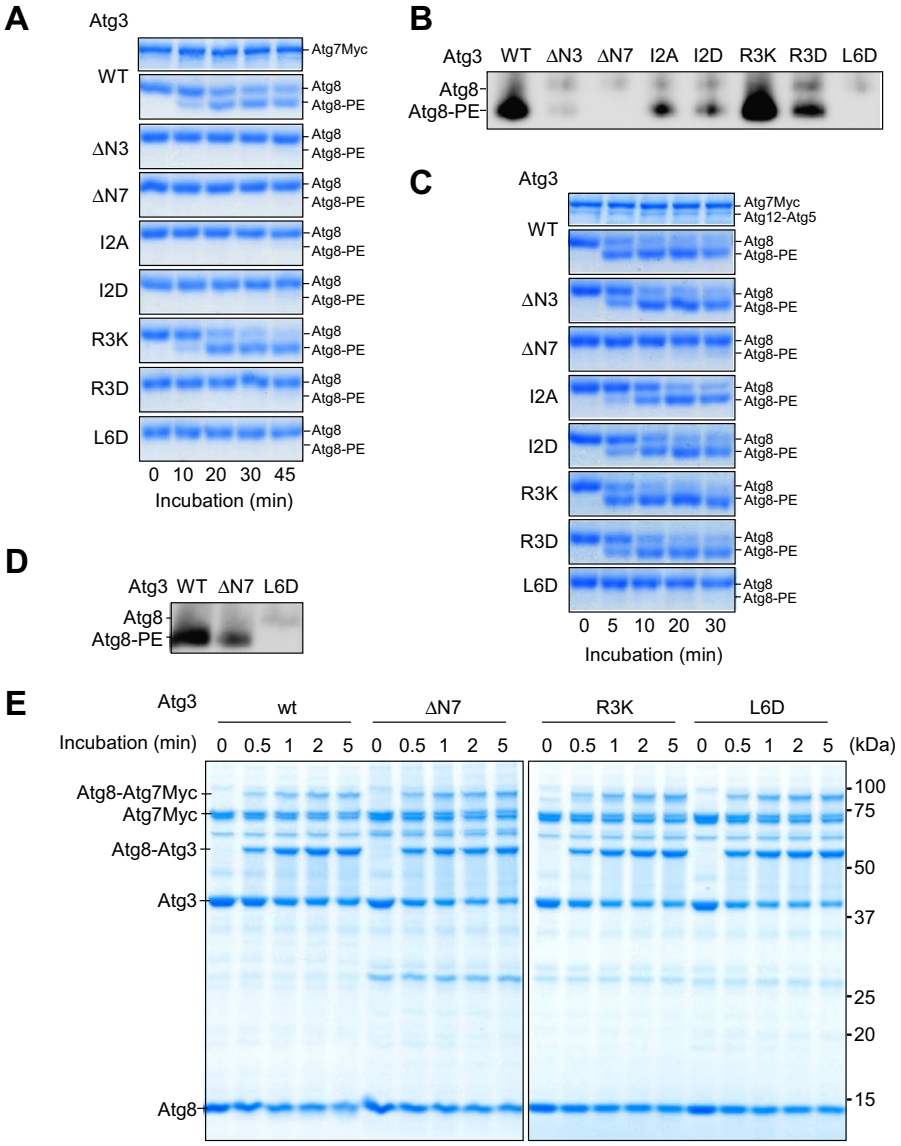

Fig. 3. Conjugating activity of Atg3 mutants in vitro. (A) Atg8 lipidation was carried out in a reaction mixture containing 1.0 μM Atg7Myc, 2.0 μM Atg3, 5.0 μM Atg8, 70% DOPE/20% POPC/10% PI liposomes (350 μM lipids) and 1 mM ATP. Samples were separated by Urea SDS–PAGE and visualized by CBB staining. (B) Atg8–PE in the reactions without Atg12–Atg5 was detected by western blotting with anti-Atg8 antibody. Note that anti-Atg8 antibody used in this analysis preferentially binds the lipidated form of Atg8, about 30 times higher than against un-lipidated form of Atg8 (Ref. [2]). The signals of Atg8–PE were highly enhanced in contrast to those actual amounts. (C) Reactions carried out under the same condition as in (A) in the presence of 0.5 μM Atg12–Atg5. (D) Atg8–PE in the reactions with Atg12–Atg5 was detected by western blotting with anti-Atg8 antibody. (E) Formation of Atg8–Atg3 intermediate was analyzed by NuPAGE. Reaction mixtures without liposomes were incubated and aliquots were mixed with SDS sample buffer without reducing agent at the indicated time points.

the ΔN7 and L6D mutants. In addition, the Atg8–Atg3 intermediate, in which the C-terminal glycine of Atg8 is linked to Atg3 via active cysteine at position 234, accumulated in these cells (Fig. 2A, middle panel), suggesting that these Atg3 mutants have a defect in the conjugating reaction in which Atg8 is transferred from Atg3 to PE.

We next carried out ALP assays to measure the autophagic activity of cells carrying the Atg3 mutants under starvation conditions, and determined the accumulation of autophagic bodies in the vacuoles. Strains expressing the ΔN7 or L6D mutants showed no autophagic activity (Fig. 2C), and autophagic bodies were completely absent in those cells (Fig. 2D). The autophagic activity diminished as Atg8–PE levels decreased (Fig. 2A and C), consistent with previous observations that the amount of Atg8–PE determines the level of autophagy [6].

3.3. The amino-terminal region of Atg3 directly contributes to Atg8–PE formation

To characterize the conjugating activity of N-terminal Atg3 mutants, we prepared recombinant Atg3 mutants (ΔN3, ΔN7, I2A, I2D, R3K, R3D, and L6D) and carried out in vitro Atg8 lipidation. Atg8–PE was detected by CBB staining only in the reactions with wild-type Atg3 or the R3K mutant (Fig. 3A). Using western blotting, we detected small amounts of Atg8–PE produced in reactions with the ΔN3, I2A, I2D, and R3D mutants and Atg8–PE was undetectable in reactions with the ΔN7 and L6D mutants (Fig. 3B).

Since Atg12–Atg5 enhances the conjugating activity of Atg3 [3], most of the Atg8 was converted to Atg8–PE by the ΔN3, I2A, I2D, and R3D mutants as well as wild-type and R3K mutant in the presence of Atg12–Atg5 (Fig. 3C). No detectable Atg8–PE was formed by the L6D mutant, even in the presence of Atg12–Atg5 (Fig. 3D). Small amounts of Atg8–PE were formed in the reaction with Atg12–Atg5 and the ΔN7 mutant (Fig. 3D), which produced no detectable Atg8–PE in vivo (Fig. 2A). This suggests that the ΔN7 mutant does not completely lose conjugating activity. It should be noted that the R3K mutant showed higher activity than wild-type Atg3 in both the presence and absence of Atg12–Atg5 (Fig. 3A and C).

The rates of Atg8–Atg3 formation in the in vitro reaction [9] were indistinguishable among wild-type Atg3, and the ΔN7, R3K, and L6D mutants (Fig. 3E), indicating that the Atg3 mutants have a specific defect in the conjugating reaction. Taken together, these data indicate that the low production of Atg8–PE in cells expressing the N-terminal Atg3 mutants can be attributed to a defect in the conjugating activity of Atg3.

3.4. The amino-terminal region of Atg3 is important for the interaction with PE-containing membrane

Recognition of the substrate PE by Atg3 must be critical for Atg8–PE conjugation. Thus, we examined the interaction between Atg3 and PE-containing liposomes by floatation assays, in which the protein associated with liposomes is recovered by centrifugation at the top of a high-density solution. Wild-type Atg3 and liposomes containing 0%, 20%, 50%, and 70% PE were incubated and subjected to floatation assay. Atg3 moved to the top fraction in experiments with liposomes containing greater than 50% PE (Fig. 4A), indicating that Atg3 was targeted to the membrane in a PE-dependent manner.

Next, we subjected the Atg3 mutants (ΔN3, ΔN7, R3K, and L6D) to floatation assays using liposomes containing 70% PE (Fig. 4B). As the N-terminal region was deleted (ΔN3 and ΔN7), the affinity toward PE-containing liposomes decreased. Notably, the ΔN7 mutant scarcely bound to PE-containing liposomes. The R3K mutant had higher affinity to PE-containing liposomes than wild-type Atg3, suggesting that the enhanced Atg8 lipidation is due to high affinity toward PE-containing membrane. These results show that the N-terminal region of Atg3 contributes to its interaction with PE-containing membranes. On the other hand, the L6D mutant showed similar affinity to PE-containing liposomes as wild-type Atg3. The L6D mutant associates with PE but is unable to accomplish the conjugation of Atg8 and PE.

It is possible that the N-terminal region of Atg3 alone could associate with PE-containing liposomes. We tested whether a fusion protein of N-terminal regions (1–10 or 1–20) of Atg3 to the C-terminus of GST would have affinity to PE-containing liposomes, but these proteins did not move to the top fraction in the floatation assay (data not shown), suggesting that other regions of Atg3 are required for the interaction with PE-containing liposomes.

Atg8 can also be conjugated to phosphatidylserine (PS) in the in vitro reaction [3,9]. We tested Atg8–PS formation using the Atg3 mutants and obtained similar results as for Atg8–PE formation (data not shown). These results suggest that the N-terminal region is not responsible for the discrimination between PE and PS, but that it is involved in the recognition of a structure that exists in lipid heads of both PE and PS.

Although Atg8–Atg3* was not an intermediate for Atg8–PE (Fig. 1B) and S4, the site where Atg8 conjugates with Atg3 in Atg8–Atg3*, was not important for Atg8–PE formation (Fig. 2A), the determination of the linkage site between Atg8 and Atg3 in Atg8–Atg3* led us to find that the N-terminal region of Atg3 contributes to interaction with the substrate PE in Atg8–PE forma-

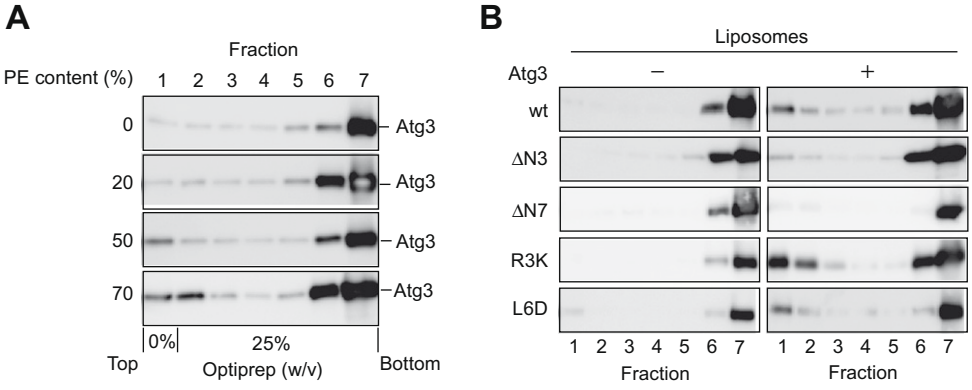

Fig. 4. Interaction between Atg3 and PE-containing liposomes in floatation assays. (A) Liposomes consisting of 90% POPC/10% PI, 20% DOPE/70% POPC/10% PI, 50% DOPE/40% POPC/10% PI, or 70% DOPE/20% POPC/10% PI were incubated with wild-type Atg3 and subjected to floatation assay. Fractions were analyzed by western blotting with anti-Atg3 antibody. (B) Floatation assays with Atg3 mutants. Atg3 mutants were incubated with liposomes consisting of 70% DOPE/20% POPC/10% PI and subjected to floatation assays.

tion (Figs. 3 and 4). It has been known that Cdc34, the E2 enzyme for ubiquitin, undergoes autoubiquitination in a Skp1-Cullin-F-box protein (SCF) E3 ubiquitin ligase-dependent manner but the exact role of autoubiquitination is unclear [10]. Our finding would provide a clue to find the functional region of other E2 enzymes for ubiquitin-like protein; the region containing sites of autoubiquitination might be involved in the ubiquitin conjugating reaction.

By site-directed mutagenesis, we show that I2, R3, and L6 are important for the conjugating activity of Atg3 (Fig. 3A). The 20 amino-terminal residues are not visible in the crystal structure of Atg3 [7], but the region from R3 to L13 is computationally predicted to adopt an amphiphilic α-helix (data not shown). In this prediction R3 resides at the border between the polar and non-polar faces and L6 resides on the non-polar face. The R3K mutation enhanced the affinity toward liposomes (Fig. 4B); this can be explained by the hypothesis that the interfacial lysine in an amphiphilic α-helix increases its affinity to the lipid bilayer [11]. The L6D mutation abolished the conjugating activity although it did not affect the interaction with PE-containing membranes. It is possible that the N-terminal region contributes to the proper coordination of PE for generation of the amide bond between the C-terminal carboxyl group in Atg8 and the amino group in PE.

Acknowledgments

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