ACCELERATED COMMUNICATIONS

Binding to E1 and E3 is mutually exclusive for the human autophagy E2 Atg3

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Abstract: Ubiquitin-like proteins (UBLs) are activated, transferred and conjugated by E1-E2-E3 enzyme cascades. E2 enzymes for canonical UBLs such as ubiquitin, SUMO, and NEDD8 typically use common surfaces to bind to E1 and E3 enzymes. Thus, canonical E2s are required to disengage from E1 prior to E3-mediated UBL ligation. However, E1, E2, and E3 enzymes in the autophagy pathway are structurally and functionally distinct from canonical enzymes, and it has not been possible to predict whether autophagy UBL cascades are organized according to the same principles. Here, we address this question for the pathway mediating lipidation of the human autophagy UBL, LC3. We utilized bioinformatic and experimental approaches to identify a distinctive region in the autophagy E2, Atg3, that binds to the autophagy E3, Atg12~Atg5-Atg16. Short peptides corresponding to this Atg3 sequence inhibit LC3 lipidation *in vitro*. Notably, the E3-binding site on Atg3 overlaps with the binding site for the E1, Atg7. Accordingly, the E3 competes with Atg7 for binding to Atg3, implying that Atg3 likely cycles back and forth between binding to Atg7 for loading with the UBL LC3 and binding to E3 to promote LC3 lipidation. The results show that common organizational principles underlie canonical and noncanonical UBL transfer cascades, but are established through distinct structural features.

Keywords: Atg3; Atg7; Atg12~Atg5; E1-E2 binding; E2-E3 binding; autophagy; ubiquitin-like protein; E1 enzyme; E2 enzyme; E3 enzyme

Abbreviations: BME, β-mercaptoethanol; DTT, dithiothreitol; FR, flexible region; HMM, hidden Markov model; ITC, isothermal titration calorimetry; LC3, microtubule-associated protein 1A/1B-light chain 3; UBL, ubiquitin-like protein

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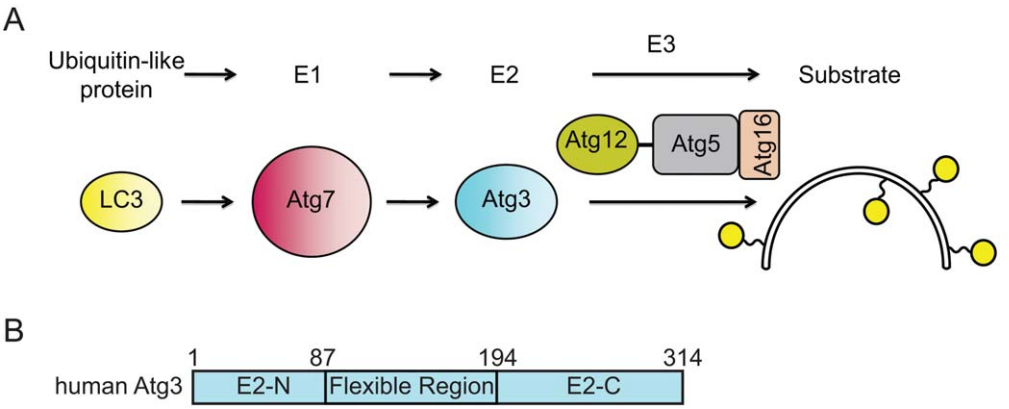

Figure 1. LC3 conjugation pathway. (A) Atg8 family members such as human LC3 are activated by the E1 Atg7 and then transferred to the E2 enzyme, Atg3. Atg12∼Atg5-Atg16 acts as an E3 enzyme that catalyzes lipid conjugation of LC3 and other Atg8 protein family members. (B) Domain diagram representing the primary structure of Atg3 and highlighting the Atg3 Flexible Region. E2-N and E2-C are the N- and C-terminal parts of an E2 fold.

Introduction

Macroautophagy is a lysosomal degradation pathway in which substrates are engulfed within a double-membrane structure called an autophagosome. Fusion of autophagosomes with lysosomes (or with the vacuole in yeast) enables lysosomal hydrolases to degrade the sequestered substrates.[1,2] Macroautophagy is carried out by a large set of proteins that includes ∼35 so-called "Atg" proteins that were originally identified by genetic screens in yeast.[3,4] Among these is the ubiquitin-like protein (UBL) Atg8 in yeast or LC3 in mammals (for simplification, the multiple mammalian orthologs of yeast Atg8 are referred to here as LC3, although similar properties were found for others including the GABARAP family). Atg8/LC3 is ultimately conjugated to lipid to dynamically recruit autophagy machinery and substrates to the membrane to promote autophagosome formation.[5–7]

Like other UBLs, LC3 is conjugated to substrates via the sequential action of a hierarchical cascade of E1, E2, and E3 enzymes [Fig. 1(A)]. The autophagy specific E1 enzyme Atg7 binds LC3 and uses a molecule of ATP to adenylate the LC3 C terminus by a mechanism that is similar to that of other E1s, such as those for the canonical UBLs ubiquitin, SUMO and NEDD8.[8–11] Similar to canonical UBL transfer cascades, Atg7 next forms a covalent thioester-linked intermediate between the UBL C terminus and an E1 enzyme catalytic cysteine residue and transfers the UBL to the catalytic cysteine residue of an E2 enzyme (Atg3). Finally, an E3 enzyme Atg12∼Atg5-Atg16 ("∼" denotes a covalent complex and "-" denotes a non-covalent complex), containing an Atg12∼Atg5 isopeptide-linked covalent conjugate, promotes the lipidation of LC3.[6,12–14]

Despite both using E1-E2-E3 cascades, there are no similarities between structures of E2 binding sites on the E1s for canonical and autophagy-specific UBL transfer enzymes, mechanisms of E1–E2 interactions, or structures of E3 enzymes in canonical and autophagy-specific UBL conjugation cascades.[13–19] Thus, the extent to which common principles underlie both the canonical and autophagy-specific cascades remains incompletely understood. For instance, one important principle of canonical cascades is that E1 and E3 enzymes have overlapping binding sites on the E2 and an E2 must therefore disengage from an E3 before reloading with another activated UBL.[20,21] Thus, we sought to evaluate whether an autophagy UBL cascade likewise displays this organization.

Prior structures of complexes with yeast Atg7 and biochemical studies show that a segment of the "flexible region" (FR) from yeast Atg3 binds to Atg7.[15–18,22] Although mutation of the corresponding Atg3FR-binding region in mouse Atg7 led to impaired LC3 lipidation in mouse embryonic fibroblast cells,[15] alignments of the Atg3FR sequences between yeast, animals, and plants show poor conservation and this region was not ordered in the electron density for a complex of Arabidopsis Atg3 with a domain from Atg7.[19] We therefore generated hidden Markov models (HMMs) and used HMM-HMM comparison methods to detect protein homology among the flexible regions of Atg3 homologs and evaluated the importance of conserved regions by performing affinity capture experiments with human Atg3 deletion constructs. Binding studies and competition experiments demonstrate that overlapping sites in the Atg3FR are important for E3 binding and E1 binding.

Results and Discussion

To investigate whether a peptide-like Atg7-interacting motif within the Atg3FR (termed Atg3FR^{pep}) is conserved in nonfungal proteins, we attempted to construct multiple sequence alignments of fungal,

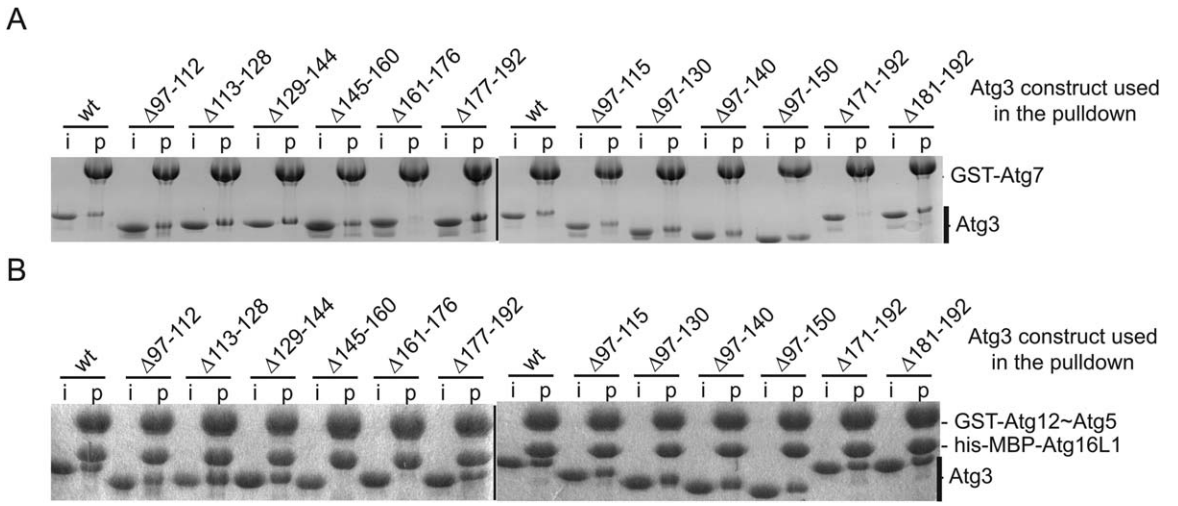

Figure 2. Atg7 and Atg12∼Atg5-Atg16L1 bind to an overlapping region in the Atg3 flexible region (FR). (A) Coomassie-stained SDS-PAGE gels of affinity pulldowns with purified recombinant GST-Atg7 and wild-type (wt) or deletion mutant versions of Atg3. Lanes showing input material are labeled "i" and lanes with affinity pulldowns are labeled "p." (B) Affinity pulldown with purified recombinant GST-Atg12∼Atg5-his-MBP-Atg16L1 complex and wild-type or deletion mutant versions of Atg3.

plant and animal homologs. Because the Atg3FR is highly divergent and difficult to align, we first constructed three separate alignments for representative Atg3 homologs from the fungal, animal and plant kingdoms[23] (Supporting Information Fig. 1). Hidden Markov models derived from the three alignments were then compared to each other by the HHpred method.[24] The result of this analysis suggests that the Atg7-binding motif conserved in the fungal proteins corresponds to a conserved motif in animals (purple bars in Supporting Information Fig. 1). This motif appears to be duplicated in plants. The alignment suggests that features of the $Atg3FR^{pep}$ that binds yeast Atg7 appear to be conserved and to encompass at least residues 156–167 of human Atg3.

Since multiple lines of evidence suggested the importance of the Atg3FR for Atg3 function,[15,16,22] we performed affinity capture experiments with purified human Atg3 deletion mutants and GST-tagged versions of either Atg7 or Atg12∼Atg5-Atg16L1 to identify Atg3FR residues essential for binding to its cognate E1 and E3, respectively. Of the Atg3FR deletion mutants tested, Atg3(Δ161–176) and Atg3(Δ171–192) did not pull down with GST-Atg7 [Figs. 1(B) and 2(A)], consistent with prior work demonstrating the importance of the Atg3FR for binding to Atg7 in yeast.[15,16,22] Notably, deletion mutants Atg3(Δ145–160) and Atg3(Δ161–176) prevented binding to GST-Atg12∼Atg5-Atg16L1 [Fig. 2(B)]. The common binding defect caused by the 16-residue deletion mutant Atg3(Δ161–176) raised the possibility that Atg3 may interact with its E1 and E3 enzymes via overlapping binding sites.

To explore the potential overlap of Atg3 binding sites for E1 and E3 observed in our deletion analyses, we performed competitive binding experiments. We mixed Atg7 and Atg3 and observed complex formation in a nondenaturing gel mobility shift assay [Fig. 3(A)]. Similarly, Atg3 binding to Atg12∼Atg5-Atg16L1 can also be observed with this technique. When we titrated into the Atg7-Atg3 complex (20 μM) increasing amounts of Atg12∼Atg5-Atg16L1 (0, 4, 10, 20 μM), the Atg7-Atg3 complex was disrupted by Atg12∼Atg5-Atg16L1 and replaced by an Atg3-Atg12∼Atg5-Atg16L1 complex [Fig. 3(A)]. The results suggested that Atg3 binding to its E1 and E3 is mutually exclusive. In support of this conclusion, the Atg7 complex with Atg3(Δ145–160), which does not bind Atg12∼Atg5-Atg16L1 by affinity capture, was not disrupted in the same titration experiments [Fig. 3(B)]. Interestingly, in a reverse titration, increasing amounts of Atg7 had negligible effects on an Atg3-Atg12∼Atg5-Atg16L1 complex [Fig. 3(C)]. In sum, the data suggest that Atg3 has overlapping but nonidentical binding sites for its E1 and E3 enzymes, with E3 capable of disrupting the Atg3–Atg7 interaction but not vice-versa.

To gain further insights into Atg3 binding to Atg7 and Atg12∼Atg5-Atg16L1, we quantified interactions by isothermal titration calorimetry (ITC). We measured an affinity for Atg3 binding to Atg12∼Atg5-Atg16L1 of 80 nM, which is similar to the previously reported value of 51 nM[13] (Table I and Supporting Information Fig. 2). Atg3FR deletion mutants lacking residues ∼150–170 are deficient for Atg12∼Atg5-Atg16L1 binding while GST-Atg3 fragments or peptides containing residues ∼140–170

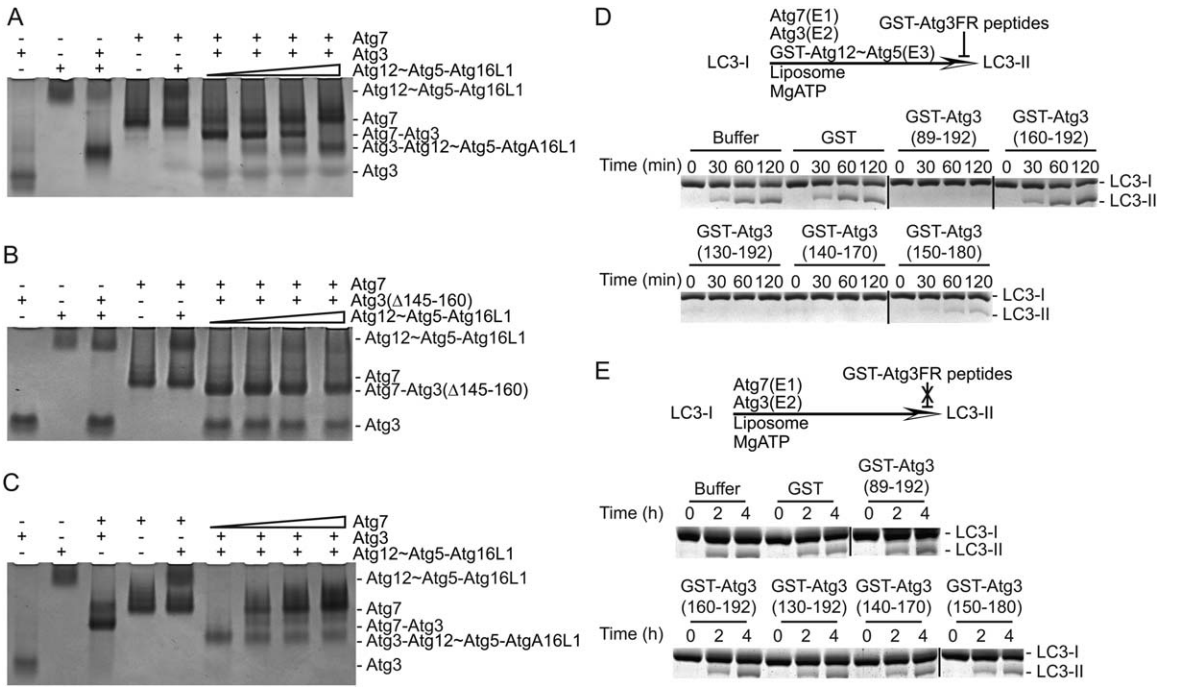

Figure 3. Competition experiments. (A,B) 20 μ*M* Atg7-Atg3 or Atg7-Atg3(Δ145–160) complex was incubated with increasing amounts of Atg12∼Atg5-Atg16L1 complex (0, 4, 10, 20 μ*M*) as described under "Materials and Methods." Atg3, Atg12∼Atg5-Atg16L1 complex, the Atg7-Atg3 complex, and the Atg3-Atg12∼Atg5-Atg16L1 complex were identified by their different migrations on a Coomassie-stained nondenaturing polyacrylamide gel and are indicated at the right. (C) Binding competition assay performed as for A and B showing titration of Atg3-Atg12∼Atg5-Atg16L1 with Atg7. (D) LC3 lipidation competition assays. Coomassie-stained urea SDS-PAGE showing *in vitro* LC3 lipidation in multiple turnover assays containing Atg7, Atg3, Atg12∼Atg5, and LC3. GST or GST-Atg3 peptides were added at a 10-fold molar excess over Atg3. (E) LC3 lipidation competition assays performed in the absence of E3 enzyme. Sypro-stained urea SDS-PAGE showing *in vitro* LC3 lipidation in multiple turnover assays containing Atg7, Atg3, and LC3. GST or GST-Atg3 peptides were added at a 10-fold molar excess over Atg3.

display high affinity for Atg12∼Atg5-Atg16L1 (Table I and Supporting Information Fig. 2). Indeed, functional significance of this region was confirmed by inhibition of LC3 lipidation *in vitro*: addition of GST-Atg3(140–170), but not GST or the non-binding GST-Atg3(160–192), blocked the reaction [Fig. 3(D)]. In similar reactions lacking E3 enzyme, lipidation is not inhibited by GST or GST-Atg3 peptides [Fig. 3(E)], indicating that the inhibition in Figure 3(D) is due to disruption of E2–E3 interaction rather than E1–E2 interaction.

Importantly, the ITC data explain the competitive binding results in Figure 3(C): Atg3 binds Atg12∼Atg5-Atg16L1 with substantially higher affinity than Atg7. Interestingly, Atg7 is a dimer and titrations with full-length Atg3 give rise to an *N*-value that is potentially consistent with one Atg3 binding per Atg7 homodimer, although 1:1 binding is observed for the Atg3FR fragments. Although this differs from the stoichiometric binding measured for yeast Atg7 and Atg3[15–18] (Supporting Information Fig. 3), Atg7 is a multidomain homodimer, and it is conceivable that additional factors, such as LC3 in the Atg7 active site, may be required for both protomers within human Atg7 to achieve a conformation that is competent for Atg3 binding. Future studies will be required to understand factors modulating this interaction in detail.

Taken together with previous studies,[15–19] our data demonstrate that the autophagy E2 Atg3 acts as a shuttle between E1, from which it receives the UBL to form a transient covalent E2∼UBL intermediate, and E3, which promotes delivery of the UBL to a lipid acceptor in autophagosomal membranes. In the case of canonical UBL cascades, such mutually exclusive binding of canonical E2s to their E1s and E3s is thought to control the nature of the reaction. For example, an E3-target complex with a relatively fast dissociation rate may be modified by a single canonical UBL molecule, whereas relatively long-lived E3-substrate complexes may enable processive modification such as polyubiquitination.[20,21,25–28] In autophagy, it is thought that an individual lipid molecule such as phosphatidylethanolamine is modified with only a single molecule of LC3, although it is possible that processive modification of a segment of membrane could occur depending on the relative timing of Atg12∼Atg5-Atg16

Table I. *E1 and E3 Recognition of the Atg3FR^{pep} Motif*

Molecule: titrant	Kd (μM)	$\Delta H \times 10^3$ (cal mol^{-1})	ΔS (cal mol^{-1} deg^{-1})	N
His-Atg3 : Atg12∼Atg5-Atg16L1	0.08 ± 0.02	−13.20 ± 0.15	−12.0	0.82 ± 0.01
His-Atg3(Δ97–150) : Atg12∼Atg5-Atg16L1	0.27 ± 0.05	−14.80 ± 0.25	−19.5	0.80 ± 0.01
His-Atg3(Δ97–192) : Atg12∼Atg5-Atg16L1	NB	NB	NB	NB
Atg3(89–192) : Atg12∼Atg5-Atg16L1	0.09 ± 0.04	−11.30 ± 0.25	−5.5	0.99 ± 0.01
Atg3(140–170) : Atg12∼Atg5-Atg16L1[a]	0.07 ± 0.004	−10.34 ± 0.03	−1.9	1.00 ± 0.002
Atg3(145–175) : Atg12∼Atg5-Atg16L1[a]	0.05 ± 0.005	−9.08 ± 0.05	3.0	1.04 ± 0.003
Atg3(152–181) : Atg12∼Atg5-Atg16L1[a,b]	≥52.36 ± 17.05	−13.40 ± 5.63	−25.3	0.99 ± 0.35
GST-Atg3(89–192) : Atg12∼Atg5-Atg16L1	0.02 ± 0.002	−11.00 ± 0.04	−1.6	0.83 ± 0.002
GST-Atg3(130–192): Atg12∼Atg5-Atg16L1	0.02 ± 0.005	−10.50 ± 0.07	−0.3	0.88 ± 0.003
GST-Atg3(140–170) : Atg12∼Atg5-Atg16L1	0.02 ± 0.004	−11.10 ± 0.09	−1.9	0.84 ± 0.004
GST-Atg3(150–180) : Atg12∼Atg5-Atg16L1	0.16 ± 0.02	−12.30 ± 0.10	−10.2	0.86 ± 0.01
GST-Atg3(160–192) : Atg12∼Atg5-Atg16L1	NB	NB	NB	NB
His-Atg3 : Atg7	2.22 ± 0.17	2.69 ± 0.03	34.9	0.62 ± 0.004
Atg3(89–192) : Atg7[a,b]	≥72.46 ± 17.01	−2.65 ± 0.82	10.0	0.99 ± 0.25
Atg3(152–181) : Atg7[a,b]	≥101.32 ± 15.30	−2.26 ± 0.42	10.7	0.99 ± 0.15

Thermodynamic parameters for Atg3, Atg3 mutants or Atg3 peptides binding to Atg7 or Atg12∼Atg5-Atg16L1 by ITC. NB = no quantifiable binding.

[a] Concentration was measured by weighing.

[b] The concentrations of the proteins used for ITC were higher than the others to observe the weak binding.

anchoring to autophagosomes compared with Atg3 shuttling to and from Atg7. Interestingly, free Atg3 binds with a submicromolar affinity to its E3, which is the hallmark of one canonical E2 mediating processive polyubiquitination.[27] Atg3's high affinity for its E3 enzyme also raises the question of how flux through the LC3 enzyme cascade is established. Notably, canonical UBL pathways may be driven forward by E1∼UBL intermediates displaying a relatively high affinity for free E2s, and E3s preferentially binding E2∼UBL intermediates and typically displaying weak affinities for free E2s.[9–11,29–31] Thus, it seems likely that future studies will reveal that the substrates—LC3 and its target lipids—influence properties, interactions, dynamics, and organization of their conjugation enzymes to promote UBL modification in autophagy. Nonetheless, our data demonstrate that a segment of the Atg3FR provides a means of simultaneously regulating both E1–E2 and E2–E3 interactions, and raise the possibility that the inhibition of LC3 observed with short peptides could be recapitulated with small molecules.

Materials and Methods

Affinity capture assay

For affinity pulldowns, Atg3 deletion mutants were incubated with GST-Atg12∼Atg5-hisMBP-Atg16L1 or GST-Atg7 bound to 50 μL of glutathione-Sepharose beads (GE life sciences) in binding buffer (25 m*M* HEPES (pH 7.5), 150 m*M* NaCl, 5 m*M* DTT) for 15 min at room temperature. The beads were subsequently washed two times with 500 μL binding buffer. Bound proteins were eluted with 2× SDS sample buffer, resolved by SDS-PAGE and visualized by Commassie staining.

Nondenaturing gel mobility shift assay

Nondenaturing gel mobility shift assays were performed with 20 μ*M* Atg7, 20 μ*M* Atg3, and 4–20 μ*M* Atg12∼Atg5-Atg16L1 in 50 μL volumes in 20 m*M* HEPES (pH 7.5), 150 m*M* NaCl, 10% glycerol, 5 m*M* DTT, for 15 min at room temperature. Free and Atg3-bound Atg7 and Atg12∼Atg5-Atg16L1 were separated on a 4.5% polyacrylamide gel (acrylamide:bis, 37.5:1) in a buffer of 90 m*M* Tris borate, 2% glycerol, pH 8.0, and were visualized with Coomassie staining.

Isothermal titration calorimetry (ITC)

ITC was performed with proteins or peptides in 50 m*M* HEPES (pH 7.5), 150 m*M* NaCl, and 1 m*M* β-mercaptoethanol (BME) at 25°C using a MicroCal ITC200 (GE life sciences). Atg7 and Atg12∼Atg5-Atg16 were placed in the sample cell at concentrations of ∼51–78 μ*M* and 20–51 μ*M*, respectively, and Atg3, Atg3ΔFR, GST-Atg3FR^{pep}, or Atg3FR^{pep} were titrated from the syringe. Data were evaluated using Origin (V 7.0) (OriginLab, Northampton, MA) to determine the values of the thermodynamic parameters. For Atg3(152–181):Atg12∼Atg5-Atg16L1, Atg3(89–192):Atg7, and Atg3(152–181):Atg7 experiments, 1.8, 1.18, and 1.8 m*M* titrant were used, respectively, in order to measure the weak binding.

In vitro *LC3 lipidation assay*

Lipidation assays were performed essentially as outlined[32,33]; *E. coli* polar extract lipids (Avanti Polar Lipids, Alabaster, AL) dissolved in chloroform was used to prepare the liposomes. After drying under nitrogen to remove the chloroform, lipids were hydrated in 20 m*M* HEPES 7.5 for 60 min with periodic vortexing, and then extruded (11 times) with a

microextruder through a 400 nm polycarbonate filter (Avanti Polar Lipids, Alabaster, AL). Liposomes composed of 67% PE, 23.2% PG, 9.8% CA were added to a final concentration of 1 m*M* in reactions with 900 n*M* Atg7, 5 μ*M* Atg3, 20 μ*M* LC3B, 0 or 2.5 μ*M* GST-Atg12(52–140)∼Atg5, 50 μ*M* GST or GST-Atg3FR peptides, 50 m*M* HEPES 7.5, 200 m*M* NaCl, 10 m*M* ATP, 10 m*M* $MgCl_2$. At the indicated times, reactions were quenched by adding an equal volume of SDS loading buffer containing 100 m*M* DTT, and analyzed on 15% PAGE gels containing 6*M* urea.

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