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Semisynthesis of autophagy protein LC3 conjugates

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Abstract

Autophagy is a conserved catabolic process involved in the elimination of proteins, organelles and pathogens. Autophagosome formation is the key process in autophagy. Lipidated Atg8/LC3 proteins that are conjugated to phosphatidylethanolamine (PE) play a key role in autophagosome biogenesis. To understand the function of Atg8/LC3-PE in autophagosome formation and host-pathogen interaction requires preparation and structural manipulation of lipidated Atg8/LC3 proteins. Herein, we report the semisynthesis of LC3 proteins and mutants with modifications of different PE fragments or lipids using native chemical ligation and aminolysis approaches.

Keywords: lipidated proteins; native chemical ligation; aminolysis; LC3; RavZ; Atg4; autophagy.

1. Introduction

Autophagy is an evolutionarily conserved “self-eating” process in eukaryotes. It plays an essential role in cellular homeostasis in response to various environmental and cellular stresses. During autophagy the cytosolic components are sequestered within double-membrane structure, termed phagophore (or isolation membrane), which elongates and closes to form an autophagosome. Subsequent fusion with a lysosome leads to degradation of internal contents. Autophagy plays an important role in physiology such as development and has been associated with diverse human diseases, including cancer, neurodegeneration and pathogen infection.¹

The ubiquitin-like protein Atg8 is required for the biogenesis of autophagosomes and needs to be C-terminally conjugated to phosphatidylethanolamine (PE) for membrane association and function.² Microtubule-associated protein light chain 3 (LC3) and GABARAP family proteins are the mammalian orthologs of yeast Atg8.^{3,4} Lipidated Atg8/LC3 has been a *bona fide* marker of autophagosomes and progression of autophagy.⁵ Biogenesis of lipidated Atg8/LC3 is mediated by two ubiquitin-like conjugation systems. Newly synthesized Atg8/LC3 is cleaved by a protease, Atg4, to expose a C-terminal glycine. The processed Atg8/LC3 serves as a substrate in a ubiquitin-like conjugation reaction catalyzed by Atg7 and Atg3 and is conjugated to phosphatidylethanolamine (PE), which is mediated by the E3-like Atg12-Atg5:Atg16 complex. The Atg12-5:16 complex is generated by another ubiquitin-like conjugation system. Atg12 is conjugated to the lysine side chain of Atg5 in sequential reactions catalyzed by Atg7 and Atg10. There is no E3-like enzyme implicated in Atg12-Atg5 conjugation. The Atg12-Atg5 conjugate further forms a complex with a multimeric protein, Atg16. Atg4 releases lipidated Atg8/LC3 from the surface of autophagosomes.⁶

Autophagy is also used to combat infection with pathogenic microbes. The intracellular bacterium *Legionella pneumophila* is a common cause of community and hospital-acquired pneumonia. *Legionella pneumophila* inhibits autophagy by injecting an effector protein called RavZ into the cytoplasm. RavZ serves as a cysteine protease and irreversibly deconjugates mammalian Atg8s from PE to inhibit autophagosome formation.⁷

However, the function of Atg8/LC3-PE in autophagosome formation and host-pathogen interaction remains poorly understood. This is largely due to the insurmountable difficulties in preparation and structural manipulation of lipidated Atg8/LC3 proteins. Native chemical ligation (NCL) and expressed protein ligation (EPL) provide almost unlimited possibilities to modulate the structure of a polypeptide chain by synthetic chemistry.^{8,9} Especially, these protein ligation methods have been successfully applied to produce lipidated proteins and allow the elucidation of biological functions of these lipidated protein previously not possible through traditional biochemical approaches.¹⁰ In this study, we report the semisynthesis of LC3 proteins and mutants with modifications of different PE fragments or lipids using EPL and aminolysis approaches. These proteins conjugates pave the way for mechanistic studies of Atg8/LC3 proteins in autophagy and host-pathogen interaction.¹¹

2. Results and Discussion

2.1. The strategies for semisynthesis of LC3 conjugates

Previously, we reported semi-synthesis of LC3-PE using a combination of lipidated peptide synthesis and EPL. An maltose binding protein (MBP)-assisted solubilisation strategy was used

to facilitate ligation under folding conditions and to solubilize the lipidated protein without detergents.¹² Here we use the strategy to produce the LC3-PE proteins with C-terminal mutation or LC3 proteins with other lipid modification, such as 1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine [DHPE (6:0)] and 16-carbon hexanoyl chain (C16). To this end, the lipidated peptides were synthesized by solid phase peptide synthesis (SPPS) and the protein MBP-LC3¹⁻¹¹⁴-MESNA thioester was obtained by intein strategy (Scheme 1).

Aminolysis reaction has been used as a variant of EPL. It has provided an effective means for installation of bioorthogonal handles into proteins.¹³⁻¹⁵ We prepared LC3 proteins with modifications of different fragments derived from PE by aminolysis strategy. To this end, the nucleophiles were synthesized and the protein LC3¹⁻¹²⁰ MESNA thioester was obtained by intein strategy (Scheme 1).¹¹

Scheme 1. The strategies for semisynthesis of LC3 conjugates

2.2. Synthesis of lipidated peptides

Since the lipid is conjugated to the C-terminal carboxyl group, we couple the lipid moiety to the peptide after release of the peptide from resin. A series of C-terminal peptides with different mutations were synthesized using Fmoc SPPS strategy with the acid-sensitive chlorotrityl resin. Treatment of the resin with low concentration of trifluoroacetic acid (TFA, 1%) releases the protected peptides (Figure 1).

Lipidation of the peptides was performed using different methods, which depends on the type of the lipid modification. For PE-modified peptides, the protected peptides were subsequently activated as a pentafluorophenyl ester and coupled in solution to 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine [DPPE (16:0)] or DHPE (6:0) to produce protected lipidated peptides. For the C16-modified peptides, the protected peptide was coupled with 1-hexadecanol in the presence of EDC-HCl and HOBt (Figure 1). The lipidated protected peptides were purified by flash column chromatography. Finally, the peptides were deprotected using high concentration of TFA (30%) with a final yield of 12-56% (Table 1). The lipidated peptides were characterized by high resolution-mass spectroscopy (HR-MS) (Table 1).

Figure 1. Synthesis of C-terminal lipidated peptides of LC3. a) DIPEA, CH₂Cl₂; b) 1) 20% piperidine in DMF; 2) AA, HCTU, DIPEA, DMF; repeat; c) 1% TFA, 3% TES, CH₂Cl₂; d) PFP-TFA, TEA, CH₂Cl₂; e) DPPE (16:0) or DHPE (6:0), TEA, CHCl₃/CH₃OH=3/1; f) 30% TFA in CH₂Cl₂, 4 hours; g) 1-Hexadecanol, EDC-HCl, HOBt, TEA, CH₂Cl₂, 0°C 3 h and then 22°C overnight.

Table 1. HR-MS characterization of lipidated peptides

Sequence (from N to C)	yield	MS form	M _w cal (Da)	M _w found (Da)
CQETFG-DPPE	30%	C ₆₉ H ₁₂₂ O ₁₈ N ₈ PS ₂ [M+H] ⁺	1445.80506	1445.80896
CSQETFG-DPPE	43%	C ₇₂ H ₁₂₇ O ₂₀ N ₉ PS ₂ [M+H] ⁺	1532.83709	1532.83844
CAQETFG-DPPE	41%	C ₇₂ H ₁₂₇ O ₁₉ N ₉ PS ₂ [M+H] ⁺	1516.84218	1516.84479
CAAQETFG-DPPE	56%	C ₇₅ H ₁₃₂ O ₂₀ N ₁₀ PS ₂ [M+H] ⁺	1587.87929	1587.88119
CQETF-DPPE	35%	C ₆₇ H ₁₁₈ O ₁₇ N ₇ PS ₂ [M+H] ⁺	1389.82478	1389.82961
CAAAG-DPPE	28%	C ₆₄ H ₁₁₅ O ₁₄ N ₇ PS ₂ [M+H] ⁺	1300.76756	1300.77023
CAAAAG-DPPE	12%	C ₅₈ H ₁₁₁ O ₁₄ N ₇ PS ₂ [M+H] ⁺	1224.73626	1224.73873
CQETAG-DPPE	41%	C ₆₃ H ₁₁₈ O ₁₈ N ₈ PS ₂ [M+H] ⁺	1369.77376	1369.77630
CQEAAG-DPPE	35%	C ₆₈ H ₁₂₀ O ₁₇ N ₈ PS ₂ [M+H] ⁺	1415.79450	1415.79738
CQATFG-DPPE	37%	C ₆₇ H ₁₂₀ O ₁₆ N ₈ PS ₂ [M+H] ⁺	1387.79958	1387.80118
CAETFG-DPPE	32%	C ₆₇ H ₁₁₉ O ₁₇ N ₇ PS ₂ [M+H] ⁺	1388.78360	1388.78610
CQETFG-DHPE	31%	C ₄₉ H ₈₈ O ₁₈ N ₈ PS ₂ [M+H] ⁺	1165.49206	1165.49380
CQETFG-C16	38%	C ₄₈ H ₈₂ O ₁₁ N ₇ S ₂ [M+H] ⁺	996.55082	996.55168

2.3. Ligation of lipidated peptides with MBP-LC3¹⁻¹¹⁴ thioester

The expressed protein ligation of MBP-LC3¹⁻¹¹⁴-thioester with lipidated peptides was performed under folding conditions using MBP as a solubilisation tag. We performed the ligation of MBP-LC3¹⁻¹¹⁴-thioester with DPPE (16:0)- or 16-carbon hexanoyl chain (C16)-modified peptides as previously reported.¹² The lipidated peptides were dissolved in dichloromethane/methanol (3:1) in the presence of 1% octyl β -D-glucopyranoside. After removal of the solvent, the mixture was resuspended in ligation buffer containing tris(2-carboxyethyl)phosphine (TCEP) to release StBu group, so that the thiol group of the N-terminal cysteine is free for ligation. The ligation reaction was performed using > 30 eq. lipidated peptides in the presence of 100 mM 4-mercaptophenylacetic acid (MPAA) as a catalyst. After overnight reaction, a quantitative conversion was observed (Figure S1). This result suggests that the strategy is generally applicable to the chemical ligation of lipidated peptides with long alkyl chain.

However, the ligation of MBP-LC3¹⁻¹¹⁴-thioester with DHPE (6:0) under the same conditions was not successful. We sought to carry out the ligation in the absence of the detergent β -D-glucopyranoside. The ligation underwent quantitatively under these conditions (Figure S1). These results suggested that the ligation conditions depend on the type of lipid modification. The LC3-lipid conjugates were characterized by electrospray ionization-mass spectroscopy (ESI-MS) (Figure 2, Figure S1).

C-terminal sequence	lipid	proteins	M _w calculated	M _w found
-C Q E T F G	-DPPE	MBP-LC3-PE	60080	60075
-C S Q E T F G	-DPPE	MBP-LC3-PE_-S-	60167	60168
-C A Q E T F G	-DPPE	MBP-LC3-PE_-A-	60152	60140
-C A A Q E T F G	-DPPE	MBP-LC3-PE_-A-A-	60223	60214
-C Q E T F	-DPPE	MBP-LC3-PE_ Δ G *	58571	58565
-C A A A F G	-DPPE	MBP-LC3-PE_m3	59936	59927
-C A A A A G	-DPPE	MBP-LC3-PE_m4	59859	59849
-C Q E T A G	-DPPE	MBP-LC3-PE_F119A	60004	59995
-C Q E A F G	-DPPE	MBP-LC3-PE_T118A	60050	60045
-C Q A T F G	-DPPE	MBP-LC3-PE_E117A	60023	60012
-C A E T F G	-DPPE	MBP-LC3-PE_Q116A	60024	60015
-C Q E T F G	-EA	LC3-EA	14366	14367
-C Q E T F G	-pEA	LC3-pEA	14446	14447
-C Q E T F G	-GpEA	LC3-GpEA	14520	14523
-C Q E T F G	-DAGpEA	LC3-DAGpEA	14604	14605
-C Q E T F G	-DHPE	MBP-LC3-DHPE(6:0)	59798	59800
-C Q E T F G	-C16	MBP-LC3-C16	59630	59624

Figure 2. ESI-MS characterization of semi-synthetic LC3 conjugates. The letter in red shows the position of mutation at the C terminus of LC3 proteins. The asterisk (*) suggests no 6xHis-tag at the N- terminus of MBP-LC3-PE_ Δ G.

2.4. Aminolysis of LC3¹⁻¹²⁰-thioester by small-molecule nucleophiles

Aminolysis strategy was used to produce LC3 proteins with modification of the soluble fragments derived from PE, including ethanolamine (EA), phosphoethanolamine (pEA), glycerophosphoethanolamine (GpEA) and diacetyl glycerophosphoethanolamine (DAGpEA). First, we treated the fusion protein MBP-LC3¹⁻¹²⁰-Intein-CBD with 1 M EA or pEA on the chitin beads at 4°C or room temperature. Unfortunately, the on-bead cleavage of the intein-derived thioester with nucleophiles was not very successful. Therefore, we prepared LC3¹⁻¹²⁰-MESNA thioester that was subsequently treated with EA or pEA for 48 hours at 4°C or room temperature in presence of MPAA. The aminolysis products LC3¹⁻¹²⁰-EA and LC3¹⁻¹²⁰-pEA were obtained with a yield of ca. 90%. About 10% hydrolyzed product was observed (Figure S1).

Since the molecular weights of LC3¹⁻¹²⁰-pEA and LC3¹⁻¹²⁰-MESNA thioester are very close. To distinguish these two species, they were subjected to hydrolysis under basic condition. As expected, LC3¹⁻¹²⁰-MESNA thioester was hydrolyzed to be LC3¹⁻¹²⁰, whereas the ligated product LC3¹⁻¹²⁰-pEA was resistant to hydrolysis, suggesting formation of LC3¹⁻¹²⁰-pEA conjugate (Figure S2). Similarly, LC3¹⁻¹²⁰-GpEA and LC3¹⁻¹²⁰-DAGpEA were produced using same procedure (Figure 2, Figure S1). However, 60-70% of hydrolyzed products were observed in these reactions (Figure S1).

2.5. Structure-function relationship study of LC3-deconjugation by RavZ

With the LC3 conjugates available (Figure 2), we were able to study the structure-function relationship of LC3-deconjugation by RavZ. We found that RavZ specifically process LC3-PE but neither LC3 proteins conjugated to soluble fragments derived from PE nor LC3-C16 conjugate, whereas Atg4B is active towards LC3-PE, LC3-DHPE(6:0), LC3-EA, LC3-pEA, LC3-GpEA, LC3-DAGpEA, LC3-C16 (Figure 2). Moreover, RavZ preferentially cleaves LC3-PE with long fatty acid chain. The catalytic efficiency of RavZ for LC3-DPPE (16:0) ($k_{cat}/K_m = 1140 \text{ M}^{-1}\cdot\text{s}^{-1}$) is 12 times higher than that for LC3-DHPE (6:0) ($k_{cat}/K_m = 93.8 \text{ M}^{-1}\cdot\text{s}^{-1}$).¹¹ Therefore, Atg4B only recognizes the LC3's structure upstream of the C-terminal glycine, while RavZ activity is strictly dependent on conjugated PE structures. Based on these findings, an extraction mode was proposed and verified, i.e. RavZ extracts LC3-PE from the membrane before deconjugation. Therefore, we have elucidated a novel mechanism that *Legionella* has evolved to specifically evade host autophagy.¹¹ Interestingly, RavZ-mediated deconjugation of LC3-DHPE (6:0) also resulted in LC3¹⁻¹¹⁹ (Figure 3). This is in keeping with the RavZ-cleavage site of LC3-DPPE (16:0), which leads to irreversible delipidation of LC3.¹¹ The results suggest that the length of PE fatty acid chain determines the catalytic efficiency of RavZ, but does not affect the binding mode of RavZ:LC3-PE interaction.

It has been shown before that C-terminal amino acid residues (116-120) of LC3 play an important role in binding with Atg4B.¹⁶ It remains unclear whether these residues are also involved in RavZ binding. Since Atg4 and RavZ process LC3 at distinct C-terminal sites, it is interesting to investigate the relationship of the C-terminal structure of LC3-PE with RavZ-mediated deconjugation. The LC3 conjugates prepared here will serve as a useful tool to understand the interaction of the C-terminus of LC3-PE with RavZ and to elucidate determinants for the distinct species of Atg4- and RavZ-mediated deconjugation. Further studies will be performed to address these questions.

Figure 3. Cleavage of the semisynthetic MBP-LC3-DHPE (6:0) by Atg4B and RavZ. (A) ESI-MS spectra of MBP-LC3-DHPE (6:0) before and after Atg4B and RavZ treatments. The protein MBP-LC3-DHPE (6:0) was treated with Atg4B or RavZ for 10 h at 37°C. (B) Cleavage site of Atg4B and RavZ on LC3-PE.

3. Conclusions

In summary, using expressed protein ligation and aminolysis approaches, we produced semisynthetic LC3 proteins or mutants conjugated C-terminally to DPPE (16:0), DHPE (6:0), 1-hexadecanol (C16) or soluble fragments of phosphatidylethanolamine. Depending on the modification, different ligation strategies and conditions have been used and proved. This strategy can be generally applied to the production of other lipidated proteins and lipidated LC3 variants for various applications. The obtained LC3 conjugates have made it possible to study the structure-function relationship of LC3-deconjugation by *Legionella* factor RavZ, thereby facilitating elucidation of a novel anti-autophagy mechanism of a pathogenic microbe. The chemical tools are very essential for the study, since progress would be impossible due to the lack of methods for producing LC3 with different structures.^{7,17} In addition, the LC3-PE proteins with C-terminal mutations (Figure 2) will facilitate further study on the mechanism underlying Atg4 and RavZ-mediated deconjugation. It is conceivable that the presented approach will contribute to the synthesis of lipidated proteins and will open a new avenue in the autophagy research field.

4. Experimental

4.1. General

Analytical HPLC-MS data were recorded on an Agilent HPLC (1100 series) with a C4 or C18 column coupled to a Finnigan LCQ ESI spectrometer. High resolution mass spectra (HR-MS) of peptides were measured on a Thermo Orbitrap coupled to a Thermo Accela HPLC system using electrospray ionization (ESI). The preparative HPLC purifications were carried on an Agilent HPLC (1100 series) using a reversed-phase C4 (RP C4, flow 20.0 ml/min, from 10 % B to 100 % B over 20 min. (A = water + 0.1% TFA, B = acetonitrile + 0.1% TFA). Resin loading was measured using the UV-Fmoc method. Briefly, 2 to 5 mg of dried resin was treated with freshly prepared piperidine/DMF (1:4 v/v) solution for 10 min and the UV absorption of the solution at 301 nm ($\epsilon = 7800 \text{ M}^{-1}\text{cm}^{-1}$) was determined. Purification of the protected lipidated peptides was performed by flash column chromatography using as eluent a mixture of $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (97:3). LC-MS analysis of modified protein was performed on an Agilent 1100 series chromatography system equipped with an LCQ electrospray mass spectrometer (Finnigan, San Jose, USA) using Jupiter C4 columns (5 μm , 15 x 0.46 cm, 300 Å pore-size) from Phenomenex (Aschaffenburg, Germany). Data evaluation was carried out using the Xcalibur software package and MagTran software programs was used for deconvolution of ESI mass spectra of the modified proteins. The expression and purification of MBP-LC3¹⁻¹¹⁴-thioester and LC3¹⁻¹²⁰-thioester were performed according to our previous protocol.^{11,12}

4.2. Synthesis of C-terminal lipidated peptides of LC3

4.2.1. Synthesis of the side-chain protected peptides

Peptides were synthesized employing an Fmoc-based solid phase peptide synthesis strategy using 2-chlorotrityl chloride resin. Briefly, 2-chlorotrityl 2-chloride resin (initial loading 1.4 mmol/g) was swelled in anhydrous DCM for 10 min. Fmoc-Gly-OH (4.0 eq) or Fmoc-Phe-OH (4.0 eq) in anhydrous DCM was attached to the resin by shaking for 2 h in the presence of DIPEA (4.8 eq), followed by treatment with DCM/MeOH/DIPEA (17:2:1 v/v) (2x3 min) and subsequent washing steps with DCM (3x3min), DMF (3x3 min) and DCM (3x3 min). The resulting loading was determined by quantification of Fmoc deprotection using UV spectrometry (ca. 0.4-0.6 mmol/g). Next, the subsequent N-terminal peptide chain elongation was achieved by standard Fmoc strategy employing the commercially available amino acid building blocks Fmoc-Ala-OH, Fmoc-Phe-OH, Fmoc-Thr(*t*-Bu)-OH, Fmoc-Glu(*O**t*-Bu)-OH, Fmoc-Gln(*Trt*)-OH and Boc-Cys(*S**t*-Bu)-OH according the peptide sequence. Fmoc group cleavage was achieved by shaking the resin twice for 10 min with a solution of piperidine/DMF (1:4 v/v). The coupling was performed for 2 h (4.0 eq. amino acid, 4.0 eq. HCTU and 8.0 eq. DIPEA in DMF). After drying in vacuo overnight, the resin was treated with a solution of DCM (10 mL) containing 1% TFA, 3% TES to release the peptide from the resin. The crude peptides were purified by preparative HPLC.

4.2.2. Synthesis of DPPE (16:0) or DHPE (6:0) modified peptides

Protected peptide (0.016 mmol) was dissolved in dry DCM (10 mL), cooled to 0°C in an ice bath and triethylamine (TEA, 6.8 μL , 0.048 mmol) was subsequently added. After 5 min stirring, pentafluorophenyltrifluoroacetate (PFP-TFA, 3.3 μL , 1.2eq) was added dropwise. The reaction mixture was stirred for 3 h at 0°C and then at room temperature overnight. The solvent was then evaporated and the crude was dissolved in 10 mL of $\text{CHCl}_3/\text{MeOH}$ (3:1) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE, 13 mg, 0.019 mmol) or 1,2-

dihexanoyl-*sn*-glycero-3-phosphoethanolamine (DHPE, 8 mg, 0.019 mmol) was added. The reaction mixture was vigorously stirred for 12 h at room temperature until no starting material was detected. After evaporation, purification of the resulting crude by flash column chromatography using as eluent a mixture of CH₂Cl₂/MeOH (97:3) afforded protected peptide.

4.2.3. Synthesis of 16-carbon hexanoyl-modified peptide

The protected peptide (0.016 mmol) was dissolved in dry DCM (2 mL), cooled to 0°C in an ice bath and 1-hexadecanol (6 mg, 0.024 mmol), EDC-HCl (5 mg, 0.024 mmol), HOBt (7 mg, 0.048 mmol) and triethylamine (TEA, 6.8 µL, 0.048 mmol) were subsequently added into the reaction. The reaction mixture was stirred for 3 h at 0°C and then 22°C overnight. The reaction mixture was evaporated and purified by flash column chromatography using CH₂Cl₂/MeOH (97:3) as eluent to obtain protected peptide.

4.2.4. Deprotection of lipidated peptides

The protected lipidated peptides were stirred in a solution of 30% TFA in DCM for 4 h at room temperature. The reaction mixture was co-evaporated with toluene, and the resulting crude was isolated by trituration with *t*-Butyl methyl ether.

4.3. Ligation of the lipidated peptides with MBP-LC3¹⁻¹¹⁴ thioester

Ligation of the DPPE (16:0) or 16-carbon hexanoyl chain modified peptide with MBP-LC3¹⁻¹¹⁴ thioester was performed as previously reported.¹² Briefly, lipidated peptide and octyl β-D-glucopyranoside (1%) were dissolved in dichloromethane/ methanol (3:1) and dried in a 2 mL tube to a film under a stream of N₂ and then in vacuo. The mixture was resuspended in 100 µL ligation buffer (20 mM NaH₂PO₄, pH 7.2, 30 mM NaCl, 50 mM TCEP) for 5 min in a ultrasonic bath to demask the thiol group. The ligation reaction was performed by mixing 0.35 mM (final concentration) deprotected peptide with 10 µM (final concentration) MBP-LC3¹⁻¹¹⁴-thioester protein in the presence of 100 mM MPAA as the catalyst. After incubation overnight at room temperature, the ligated product was purified by size exclusion chromatography (Superdex 200 10/300 GL).

The ligation of DHPE (6:0) modified peptide with MBP-LC3¹⁻¹¹⁴ thioester was performed in absence of the detergent. Briefly, DHPE (6:0) modified peptide was dissolved in 100 µL ligation buffer (20 mM NaH₂PO₄, pH 7.2, 30 mM NaCl, 50 mM TCEP) for 5 min in a ultrasonic bath to deprotect the thiol group. The ligation reaction is performed by mixing 0.30 mM (final concentration) deprotected peptide with 10 µM (final concentration) MBP-LC3¹⁻¹¹⁴-thioester protein in the presence of 100 mM MPAA as the catalyst.

4.4. Aminolysis of LC3¹⁻¹²⁰-thioester by small-molecule nucleophile

50 µM LC3¹⁻¹²⁰-MESNA thioester was incubated with 0.5 M nucleophiles (ethanolamine, phosphoethanolamine, glycerophosphoethanolamine or diacetyl glycerophosphoethanolamine) in the buffer (100 mM NaH₂PO₄, pH 7.2, 50 mM NaCl, 100 mM MPAA) for 48 h at 4°C. The reaction was subjected to LC-MS analysis. Finally, the solution was dialyzed against dialysis buffer to remove unreacted compound.

4.5. Biochemical assay

To distinguish LC3¹⁻¹²⁰-MESNA thioester and ligated product LC3¹⁻¹²⁰-pEA by hydrolysis, the protein (15 µL) was incubated with 5 µL NaOH (0.5 M) for 30 min at 20°C. The protein samples were desalted using small GF spin columns (DyeEx 2.0 Spin Kit, Qiagen) and subjected to LC-MS analysis.

For Atg4B and RavZ cleavage assay, the reactions were tested in a 20 µL reaction system with a final enzyme concentration of 0.7 µM and MBP-LC3-DHPE (6:0) of 7 µM in the presence of 1 mM DTT. After 10 h at 37°C, the reactions were subjected to LC-MS.

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