Phosphatidylserine in Addition to Phosphatidylethanolamine Is an in Vitro Target of the Mammalian Atg8 Modifiers, LC3, GABARAP, and GATE-16*

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In yeast, phosphatidylethanolamine is a target of the Atg8 modifier in ubiquitylation-like reactions essential for autophagy. Three human Atg8 (hAtg8) homologs, LC3, GABARAP, and GATE-16, have been characterized as modifiers in reactions mediated by hAtg7 (an E1-like enzyme) and hAtg3 (an E2-like enzyme) as in yeast Atg8 lipidation, but their final targets have not been identified. The results of a recent study in which COS7 cells were incubated with [14C]ethanolamine for 48 h suggested that phosphatidylethanolamine is a target of LC3. However, these results were not conclusive because of the long incubation time. To identify the phospholipid targets of Atg8 homologs, we reconstituted conjugation systems for mammalian Atg8 homologs in vitro using purified recombinant Atg proteins and liposomes. Each purified mutant Atg8 homolog with an exposed C-terminal Gly formed an E1-substrate intermediate with hAtg7 via a thioester bond in an ATP-dependent manner and formed an E2-substrate intermediate with hAtg3 via a thioester bond dependent on ATP and hAtg7. A conjugated form of each Atg8 homolog was observed in the presence of hAtg7, hAtg3, ATP, and liposomes. In addition to phosphatidylethanolamine, in vitro conjugation experiments using synthetic phospholipid liposomes showed that phosphatidylserine is also a target of LC3, GABARAP, and GATE-16. In contrast, thin layer chromatography of phospholipids released on hAtg4B digestion from endogenous LC3-phospholipid conjugate revealed that phosphatidylethanolamine, not phosphatidylserine, is the predominant target phospholipid of LC3 in vivo. The discrepancy between in vitro and in vivo reactions suggested that there may be selective factor(s) involved in the endogenous LC3 conjugation system.

Ubiquitylation and ubiquitylation-like posttranslational reactions play indispensable roles in many cellular functions (1–8). Ubiquitin is synthesized as a precursor, which is cleaved to expose a C-terminal Gly (17, 18). After cleavage, each Atg8 homolog is activated by Atg7, an E1-like enzyme, and transferred to Atg3 via a thioester bond dependent on ATP and hAtg7. A conjugated form of each Atg8 homolog was observed in the presence of hAtg7, hAtg3, ATP, and liposomes. In addition to phosphatidylethanolamine, in vivo conjugation experiments using synthetic phospholipid liposomes showed that phosphatidylserine is also a target of LC3, GABARAP, and GATE-16. In contrast, thin layer chromatography of phospholipids released on hAtg4B digestion from endogenous LC3-phospholipid conjugate revealed that phosphatidylethanolamine, not phosphatidylserine, is the predominant target phospholipid of LC3 in vivo. The discrepancy between in vitro and in vivo reactions suggested that there may be selective factor(s) involved in the endogenous LC3 conjugation system.

Ubiquitylation and ubiquitylation-like posttranslational reactions play indispensable roles in many cellular functions (1–8). Ubiquitin is synthesized as a precursor, which is cleaved to expose a C-terminal Gly (9) and subsequently activated by reaction with a ubiquitin-activating enzyme (E1).2 Ubiquitin and E1 form an E1-substrate intermediate via a thioester bond between the E1 active site Cys and the C-terminal Gly of ubiquitin. Thereafter, the activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2), which forms an E2-substrate intermediate with ubiquitin via a thioester bond between the E2 active site Cys and the C-terminal Gly of ubiquitin. Finally, the ubiquitin is conjugated to a target protein via an amide bond between the C-terminal Gly of ubiquitin and a Lys within the target protein by a ubiquitin ligase (E3). These enzyme-reaction systems (E1, E2, and E3) are essentially conserved among the other ubiquitylation-like modifications of ubiquitin-like proteins (10).

Yeast Atg8 is a unique ubiquitin-like protein essential for autophagy; whereas the targets of ubiquitin and the other ubiquitin-like proteins are proteins, the target of Atg8 is a phospholipid, phosphatidylethanolamine (PE). Genetic analyses have indicated that Atg8 is cleaved by Atg4, a predicted cysteine protease, to expose its C-terminal Gly. Thereafter, Atg8 is activated by Atg7, an E1-like enzyme, and transferred to Atg3p, an E2-like enzyme. Finally, Atg8 is covalently conjugated to PE through an amide bond between the C-terminal Gly of Atg8 and an amino group in the hydrophilic head within PE (11, 12), and the target has been identified by mass spectroscopy of the purified Atg8-PE conjugate. Recently, the Atg8 lipidation system has been reconstituted in vitro. These experiments indicated that a mutant Atg8, in which the C-terminal Gly is exposed, as well as Atg7, Atg3, and PE-containing liposomes, are the minimum reaction units in yeast (13).

In mammals, at least three Atg8 homologs, LC3, GABARAP, and GATE-16, have been identified (Fig. 1A), all of which have structural ubiquitin folds (14–16). In vivo and in vitro biochemical analyses have shown that human Atg4B is an authentic cysteine protease essential for cleavage of the C terminus of each Atg8 homolog to expose the C-terminal Gly (17, 18). After cleavage, each Atg8 homolog is activated by human Atg7 (hAtg7), transferred to human Atg3 (hAtg3), and finally forms a conjugate as a modifier (19, 20). We have recently demonstrated that LC3 and GABARAP form protein-phospholipid conjugates, LC3-II and GABARAP-PL, respectively (18). Incubation of COS7 cells with [14C]ethanolamine for 48 h has been reported to result in incorporation of radioactivity into the LC3-phospholipid conjugate (LC3-II), suggesting that the target of LC3, similar to yeast Atg8, is PE (17). The metabolic pathways of the synthesis and degradation of phospholipids, however, are such that 48-h labeling with [14C]ethanolamine cannot lead to conclusive results. Although the target of the third homolog, GATE-16, is not yet known, a conjugated form of GATE-16 (GATE-16-II) shows biochemical characteristics similar to those of LC3-II and GABARAP-PL, suggesting that the target of GATE-16 may also be a phospholipid.

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2 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; PE, phosphatidylethanolamine; DOPE, dioleoyl-phosphatidylethanolamine; PS, phosphatidylserine; DOPS, dioleoyl-phosphatidylserine; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; DTT, dithiothreitol; Atg8-PE, Atg8-phosphatidylethanolamine conjugate; hAtg7, human Atg7 homolog (E1-like enzyme); hAtg3, human Atg3p homolog (E2-like enzyme); hAtg8, human Atg8 homolog; GABARAP, human γ-aminobutyric acid type A (GABA_A) receptor-associated protein; GABARAP-PL, a GABARAP-phosphopholipid conjugate; GATE-16, human Goliagi-associated ATPase enhancer of 16 kDa; GATE-16-II, a GATE-16-phosphopholipid conjugate; LC3, human microtubule-associated protein 1 light chain 3; LC3-II, a LC3-phospholipid conjugate; GST, glutathione S-transferase.
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However, there are differences among the conjugated forms of the three Atg8 homologs. For example, the amount of LC3-II in rat tissues cannot be correlated with the amounts of the other conjugates. In HEK293 cells, conjugation of LC3, but not of GATE-16 or GABARAP, is facilitated by overexpression of hAtg7 and hAtg3. In HeLa cells, LC3-II and GABARAP-PL, but not GATE-16-II, accumulate in the presence of the protease inhibitors, E64d and pepstatin A, under nutrient-rich conditions. In the livers of mice lacking Atg7, the amounts of the unlipidated forms of all three Atg8 homologs increase, suggesting that conjugation of all three molecules is active in the mouse liver.

In the present study, we focused on whether hAtg7, hAtg3, and phospholipid(s) are the minimum essential requirements needed for conjugation of the three mammalian Atg8 homologs and on the nature of the targets of these three human Atg8 homologs. We hypothesized that PE and phosphatidylserine (PS) may be targets, because the hydrophilic heads of both PE and PS have amino groups, which are required in ubiquitylation-like reactions. We reconstituted the conjugation systems for the mammalian Atg8 homologs in vitro using purified recombinant hAtg7, hAtg3, LC3, GABARAP, and GATE-16, as well as synthetic phospholipid liposomes. Furthermore, we directly analyzed the conjugation products of LC3-II purified from HeLa cells by TLC.

EXPERIMENTAL PROCEDURES

Strain, Cells, Culture, Biochemical Materials, and Molecular Biological Techniques—Molecular biological and biochemical techniques were performed as described previously (18). The Escherichia coli strain JM109 was used for plasmid construction and protein expression. Total lipid was extracted from HeLa cells as described (22). The plasmid pGEX-6P-1 was purchased from Amersham Biosciences. The synthetic phospholipids, 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dioleoyl-phosphatidyethanolamine (DOPE), and dioleoyl-phosphatidylserine (DOPS), were purchased from Avanti Polar Lipids (Alabaster, AL).

Plasmids—For expression of GST-tagged human Atg7, Atg3, LC3, GATE-16, and GABARAP, and the GST-tagged mutant human Atg8 homologs (Fig. 1), cDNAs containing the respective open reading frame were inserted into pGEX-6P-1 (Amersham Biosciences) by PCR mutagenesis and molecular biological techniques (Fig. 1B).

Antibodies—Polyclonal antibodies against human LC3, GABARAP, Atg3, Atg4B, and Atg7 have been described (18, 23, 24). To generate polyclonal serum against GATE-16, a rabbit was immunized with purified GST-GATE-16, and antibody in the serum was affinity-purified by a thioredoxin-GATE-16-immobilized Sepharose column. To remove cross-reactive antibodies against LC3 and GABARAP, the affinity-purified antibody was passed through thioredoxin-GABARAP- and thioredoxin-LC3-immobilized Sepharose columns. Anti-GABARAP antibody recognizes lipidated GABARAP better than unlipidated GABARAP, and anti-GATE-16 antibody recognizes lipidated GATE-16 better than unlipidated GATE-16.

Expression of GST-tagged Recombinant Human Atg Proteins in E. coli and their Purification—GST-tagged proteins were expressed in E. coli and purified using glutathione-Sepharose 4B and PreScission protease according to the manufacturer’s protocol (Amersham Biosciences).

In Vitro Assay for E1- and E2-Modifier Intermediates via Thioredoxin Bond—Purified recombinant proteins (0.1 μM each) were mixed in TN buffer (50 mM Tris–HCl, pH 7.0, 150 mM NaCl), and 5 mM ATP, 10 mM MgCl₂, and 1 mM DTT were added to the mixture where indicated. The mixture was incubated at 25 °C for 1 h, the reaction was stopped by the addition of an equal volume of SDS solution (1% SDS, 50 mM Tris–HCl, pH 6.8, 15% glycerol) in the absence or presence of a reducing reagent, DTT (100 mM), and the mixtures were incubated for 10 min at 37 °C (25, 26).

Preparation of Total Lipids from HeLa Cells and Formation of Liposomes—Total lipids in HeLa cells were extracted as described (13, 22, 27) and stored as a chloroform solution. To prepare dried lipid films, the chloroform was evaporated with nitrogen gas, and the samples were placed in bell jars under vacuum at room temperature for 12 h. The resultant dried lipid films were hydrated to a final concentration of 1 mM phospholipids in a buffer consisting of 25 mM Tris–HCl (pH 7.5), 137 mM NaCl, 2.7 mM KCl, vortexed vigorously at room temperature, and sonicated for 5 min at 4 °C. After centrifugation at 20,000 × g for 20 min, the supernatant was used as small unilamellar liposomes (13, 27, 28).

In Vitro Assay for LC3, GABARAP, and GATE-16 Conjugation—Each mutant hAtg7 homolog with exposed carboxyl-terminal Gly residues (0.1 μM LC3TFG, GABARAPVYG, and GATE-16TFG), hAtg7 (0.1 μM), hAtg3 (0.1 μM), and liposomes (30 μM) were mixed in TN buffer in the presence of 5 mM ATP, 10 mM MgCl₂, and 1 mM DTT and incubated at 25 °C for 1 h. To stop the reaction, an equal volume of SDS solution containing 5% 2-mercaptoethanol was added, and the mixture was boiled for 5 min.

Identification of Phospholipid Conjugated to LC3—I—Endogenous LC3-II (LC3-phospholipid conjugate) in HeLa cells was accumulated in the presence of the protease inhibitors, E64d and pepstatin A, as described (18). After preparation of an LC3-II-enriched membrane fraction from the inhibitor-treated HeLa cells (about 1 × 10⁶ cells/column), LC3-II was solubilized from LC3-II-rich total membrane fraction in TX solution (2% Triton X-100, 20 mM Tris–HCl, pH 7.5, 150 mM NaCl) (18) and purified by affinity chromatography on an anti-LC3 antibody-immobilized Sepharose column. Purified LC3-II was mixed with recombinant hAtg4B, a delipidating protease (17, 18) from E. coli, the mixture was incubated at 25 °C for 120 min. Released phospholipids were extracted from reaction mixtures using Bligh-Dyer’s method (22) and subsequently separated by TLC on Kieselgel 60 plates (Merck) using chloroform/methanol/deionized water (65:25:4, v/v/v) as a solvent. The TLC plates were dried in a fume hood (5 min) and sprayed with primuline reagent (29) to detect phospholipids under UV light (main wavelength, 365 nm).

RESULTS

In Vitro E1-Substrate Formation between hAtg7 and Each Mutant Atg8 Homolog, LC3TFG, GABARAPVYG, and GATE-16TFG—In HEK293, COS7, and HeLa cells, almost all of the C termini of LC3, GABARAP, and GATE-16 are posttranslationally cleaved to expose a C-terminal Gly residue, which is essential for ubiquitylation-like reactions. Therefore, for in vitro E1-substrate reactions, hAtg7 and a series of mutant Atg8 homologs were expressed as GST fusion proteins in E. coli and purified using glutathione-Sepharose 4B and PreScission protease (Fig. 1). Coomassie Brilliant Blue staining of the resultant proteins on SDS-PAGE showed that all three had been purified almost to homogeneity (Fig. 1C).

We first focused on the formation of the E1-substrate intermediate between hAtg7 and LC3TFG by mixing hAtg7 (0.1 μM) and LC3TFG (0.1 μM) and incubating them at 25 °C for 1 h in the presence or absence of ATP (5 mM). After stopping the reaction, proteins were separated by SDS-PAGE (4–12% linear gradient) under nonreducing conditions, and LC3TFG was recognized by immunoblotting using anti-LC3 antibody. In the presence of ATP, a band of about 100 kDa, corresponding to the hAtg7-LC3TFG (E1-substrate) intermediate was recognized (Fig. 2A). Little intermediate was recognized in the presence of a reducing reagent,
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FIGURE 1. Purified recombinant Atg proteins. A, amino acid sequence alignment of Atg8 (yeast Atg8) and its human homologs (human LC3, GABARAP, and GATE-16). The asterisk indicates the conserved C-terminal Gly essential for conjugation. B, schematic representation of mutant human Atg8 homologs. LC3TGG (LC3 TF), GABARAPVYG (GABARAP VYG), and GATE-16TGG (GATE-16 TF) are a series of mutant human Atg8 proteins with the C-terminal Gly exposed. LC3TT (LC3 TF), GABARAPVY (GABARAP VY), and GATE-16TT (GATE-16 TF) are mutant proteins in which the C-terminal Gly residue is deleted. C, purified recombinant human Atg proteins. Each of the purified proteins (0.4 μM) was subjected to SDS-PAGE (4–12% linear gradient) and stained with Coomassie Brilliant Blue.

DDT (100 mM), indicating that the intermediate was formed via a thioester bond. Little intermediate was observed when we used a mutant LC3TT in which the C-terminal Gly had been deleted instead of LC3TGG.

Similar results were obtained in the assays for E1-substrate intermediates of GABARAPVYG and GATE-16TGG (Fig. 2, B and C). Bands of about 100 kDa, corresponding to hAtg3-GABARAPVYG and hAtg3-GATE-16TGG (E1-substrate) intermediates were recognized by their respective antibodies only in the presence of ATP, in a manner sensitive to the reducing reagent, DDT (100 mM). There was little intermediate formation when GABARAPVY and GATE-16TT were substituted for GABARAPVYG and GATE-16TGG, respectively. These results indicated that hAtg7, each of the Atg8 homologs, and ATP are the minimum materials essential for the formation of E1-substrate intermediates via thioester bonds.

In Vitro E2-substrate Formation between hAtg3 and Each Mutant Atg8 Homolog—We next investigated whether each of the Atg8 homologs could form an E2-substrate intermediate with hAtg3 in vitro. Similar to the other proteins above, hAtg3 was expressed as a GST fusion protein in E. coli and purified with glutathione-Sepharose 4B and PreScission protease (Fig. 1C). To form an E2-substrate intermediate between hAtg3 and LC3TGG, we incubated hAtg7 (0.1 μM), hAtg3 (0.1 μM), ATP and incubated for 1 h at 25 °C. As a negative control for LC3TT, LC3TF (LC3 TF), which lacks the C-terminal Gly, was added in place of LC3TGG. DTT indicates the addition of the reducing reagent, DTT (100 mM), to the mixture. After stopping the reaction, total proteins were separated by non-reducing SDSPAGE (4–12% linear gradient), and LC3 and hAtg7 were assayed by immunoblotting with anti-LC3 (WB-ω-hAtg7) and anti-hAtg7 (WB-ω-hAtg7) antibodies, respectively.
In Vitro Conjugation Reactions of the Three Atg8 Homologs, Mediated by hAtg7 and hAtg3

Yeast Atg8 is conjugated to PE in vitro and in vivo. It is unclear whether hAtg7 and hAtg3 are the minimum enzymes required for the conjugation reactions and whether GATE-16 is conjugated to PE in vitro and in vivo. It is unclear whether hAtg7 and hAtg3 are the minimum enzymes required for the conjugation reactions and whether GATE-16 is conjugated to PE in vitro and in vivo.
Three Human Atg8 Homologs Conjugate to PE and PS

We found that LC3-II was generated when hAtg7 (0.1 μM), hAtg3 (0.1 μM), liposomes (30 μM), and LC3TTG (0.1 μM) were incubated in the presence of ATP (5 mM) at 25 °C for 1 h, as determined by immunoblotting with anti-LC3 antibody (Fig. 4A). In the absence of hAtg7, hAtg3, ATP, or liposomes, or when LC3TT was substituted for LC3TTG, little conjugate was observed. Similar results were obtained for GABARAP and GATE-16 conjugation reactions (Fig. 4, B and C); i.e. GABARAP-PL was detected by immunoblotting with anti-GABARAP antibody only when all components (hAtg7, hAtg3, ATP, GABARAPVYG, and liposomes) were present in the reaction mixture, whereas little GABARAP-PL was formed when GABARAPVYG was substituted for GABARAPTT (Fig. 4B). GATE-16-II, a conjugated form of GATE-16, was detected by immunoblotting with anti-GATE-16 antibody only when all components (hAtg7, hAtg3, ATP, GATE-16TTG, and liposomes) were present in the reaction mixture, but little GATE-16-II was formed when GATE-16TT was substituted for GATE-16TTG (Fig. 4C). These results indicated that the minimum essential requirements for in vitro conjugation of the hAtg8 homologs include hAtg7, hAtg3, ATP, liposomes, and each hAtg8 homolog.

LC3, GABARAP, and GATE-16 Are Conjugated to both PE and PS in Vitro—Conjugates of LC3 and GABARAP have been reported to be sensitive to phospholipase D, suggesting that these hAtg8 homologs target a phospholipid(s). Ubiquitin and other modifiers are conjugated to targets via amide bonds between the carboxyl group in their C-terminal Gly residue and the side chain amino group in a Lys residue or in the hydrophilic head of PE. Since PE and PS have amino groups in their hydrophilic heads, these phospholipids are potential targets of the human Atg8 homologs. To determine whether phospholipids are conjugated to the Atg8 homologs, we prepared liposomes composed of pure synthetic phospholipids (POPC, a molecular species of PC; DOPE, a molecular species of PE; and DOPS, a molecular species of PS), and reconstituted the conjugation reactions in vitro (Fig. 5).

We first focused on the effects of synthetic phospholipid-based liposomes on the conjugation of LC3. When liposomes consisting of 100% DOPE were used in the in vitro reconstitution of LC3 conjugation, LC3-II was observed by immunoblotting with anti-LC3 antibody (Fig. 5A, lane 2). In contrast, when liposomes containing 100% DOPE and 100% POPC were employed, no LC3-II was observed (Fig. 5A, lanes 1 and 3). We further investigated the optimum concentration of PS (DOPS) in the liposomes for in vitro conjugation of the liposomes to the conjugation reactions by varying the concentration of DOPS in the liposomes from 0 to 100%. LC3-II was observed when 10–100% DOPS liposomes were used (Fig. 5A, lanes 8–15), with the optimum DOPS concentration for in vitro LC3 conjugation being 55–85%. These results indicated that PS is a target of LC3.

Since 100% DOPE is not structurally suitable for formation of liposomes, we prepared a series of DOPE-containing liposomes and investigated their effects on LC3 conjugation (Fig. 5A, lanes 4–7). Using a series of DOPE-containing liposomes (33% DOPE, 33% DOPS, 33% POPC, 50% POPC, 50% DOPE; and 50% DOPE, 50% DOPS), we observed LC3-II formation in 100% DOPS liposomes (Fig. 5A, lanes 4, 6, and 7). Using liposomes containing 0–100% DOPE, we observed LC3-II formation in the presence of liposomes containing 10–70% DOPE (Fig. 5A, lanes 8–15), with the optimum DOPE concentration for in vitro LC3 conjugation being 25–55%. These results indicated that PE is also a target of LC3 conjugation in vitro.

We next focused on the effects of synthetic phospholipid-based liposomes on in vitro GABARAP conjugation. Since there are differences in the amounts of the modified forms of LC3, GABARAP, and
GATE-16 in rat tissues, there may be divergence among these hAtg8 homologs regarding their target phospholipids or their optimum concentrations. However, we observed little difference between LC3 and GABARAP conjugation reactions in vitro (Fig. 5B). GABARAP-PL was observed using 100% DOPS liposomes (Fig. 5B, lane 2) but not using liposomes composed of 100% DOPE or 100% POPC. Use of a series of DOPE- and DOPS-containing liposomes in the reaction mixture (Fig. 5B) indicated that their optimum concentrations for in vitro GABARAP conjugation were similar to those observed for LC3 conjugation (Fig. 5B, lanes 8–23). These results indicated that both PS and PE are targets of GABARAP conjugation in vitro.

Previous biochemical studies of GATE-16-II and the results shown in Fig. 4C suggested that PS and PE are potential targets of GATE-16 conjugation. Therefore, we used synthetic phospholipid liposomes to investigate GATE-16 conjugation. When 100% DOPS and a series of DOPE liposomes were employed in the in vitro reaction, GATE-16-II was observed by immunoblotting with anti-GATE-16 antibody (Fig. 5C). The optimum concentrations of DOPS and DOPE liposomes for in vitro GATE-16 conjugation were similar to those observed for LC3 and GABARAP (Fig. 5C, lanes 8–23). These findings indicated that GATE-16-II is a protein-phospholipid conjugate and that the targets of GATE-16 are also PS and PE. In conclusion, both PS and PE are targets of all three Atg8 homologs (LC3, GABARAP, and GATE-16) in these protein-phospholipid conjugation reactions.

The in Vivo Target of Endogenous LC3 Is Phosphatidylethanolamine but Not Phosphatidylserine—In the in vitro conjugation reaction, LC3 could conjugate with both PS and PE. However, it is not known whether this is also the case in vivo. The results of a recent study involving the incubation of [14C]ethanolamine in COS7 cells for 48 h suggested that...
FIGURE 6. LC3 is conjugated with phosphatidylethanolamine but little phosphatidylserine in vivo. A flow chart of analysis of target phospholipid of endogenous LC3 is shown in Fig. 6A. Purified endogenous LC3-II (0.390 pmol/10-ml reaction) was incubated with wild-type hAtg4B (wild) or mutant hAtg4B<sup>C74A</sup> (C74A) (0.039 pmol/10-ml reaction) at 25 °C for 120 min. To confirm the delipidation of LC3-II by hAtg4B in the reaction, total proteins (1 ng/lane for silver staining and 0.1 ng/lane for immunoblotting) in the reaction mixture were separated on SDS-PAGE and analyzed by silver staining (B) and immunoblotting with anti-LC3 and anti-hAtg4B antibodies, respectively (WB: <sup>H9251</sup>-LC3 and WB: <sup>H9251</sup>-hAtg4B) (C). After treatment of LC3-II with wild-type hAtg4B (wild) or mutant hAtg4B<sup>C74A</sup> (C74A), phospholipids were extracted from the reaction containing about 0.31 pmol of LC3, analyzed by TLC, and detected usingprimuline reagent (D). Synthetic phospholipids, POPC, DOPS, and DOPE, were employed as standards. The R<sub>f</sub> values are indicated on the right.

FIGURE 7. Working hypothesis for in vivo selectivity of phosphatidylethanolamine in the LC3 conjugation systems. LC3 is activated by hAtg7, forming high energy thioester bonds between the C-terminal Gly residue in LC3 and an active site Cys in hAtg7 (transient E1-substrate intermediate), and activated LC3 is subsequently transferred to hAtg3 (transient E2-substrate intermediate). In the in vitro reaction (upper panel), hAtg3 directly recognizes PS and PE, and LC3 is conjugated to PE and PS via amide bonds between the C-terminal Gly in LC3 and an amino group of each phospholipid. Since endogenous LC3 is preferentially conjugated to PE, there will be a mechanism(s) to select a target phospholipid after transferring to hAtg3 in vivo (lower panel). One hypothetical factor, X, is an E3-like enzyme to select PE as a target for LC3. Another hypothetical factor, Y, is a regulatory factor that inhibits the association of LC3 (or hAtg3) with PS.
phosphatidylethanolamine is a target of LC3 (17). However, the experiment could not exclude the possibility that phosphatidyleserine may be another endogenous target of LC3 in the ATG conjugation reaction, as in the case of in vitro conjugation.

To identify the endogenous target phospholipid(s) of LC3 in vivo, we first purified endogenous LC3-II from HeLa cells in the presence of lysosomal protease inhibitors, E64d (10 µg/ml) and pepstatin A (10 µg/ml), as described (18), delipidated LC3-II with the delipidating enzyme, hAtg4B, and analyzed the phospholipids released on reaction with purified endogenous LC3-II. Endogenous LC3-II was solubilized from the total membrane fraction with TX solution and affinity-purified on an anti-LC3 antibody-immobilized Sepharose column. Purified LC3-II was incubated with either wild-type hAtg4B or inactive mutant hAtg4B<sup>C74A</sup>. Silver staining and immunoblotting analysis clearly showed that wild-type hAtg4B completely delipidated LC3-II to form LC3-I (Fig. 6, B and C). In contrast, incubation of the mutant hAtg4B<sup>C74A</sup> with LC3-II did not cause delipidation (Fig. 6, B and C). The phospholipids were extracted from the reaction products using the Bligh-Dyer method (22) and further analyzed by TLC. As clearly shown in Fig. 6D, treatment of LC3-II with wild-type hAtg4B released PE but not PS. No phospholipid spots were detected with the sample treated with the inactive mutant, hAtg4B<sup>C74A</sup>. These results indicated that PE, but not PS, is the target phospholipid conjugated with endogenous LC3 in vivo.

**DISCUSSION**

The results of the present study indicated that in vitro conjugation systems for the three Atg8 homologs, LC3, GABARAP, and GATE-16, can be reconstituted using purified hAtg7 (E1-like enzyme), hAtg3 (E2-like enzyme), and synthetic phospholipid liposomes. All three hAtg7-Atg8 homolog intermediates (E1-substrate intermediates) formed via ATP-dependent thioester bonds, and all three hAtg3-Atg8 homolog intermediates (E2-substrate intermediates) formed via thioester bonds dependent on ATP and hAtg7. Finally, all three Atg8 homologs were conjugated to phospholipids, PS and PE. The findings presented here lead to three conclusions. First, the minimum components necessary for human Atg8-phospholipid conjugations were shown to be hAtg7, hAtg3, the respective Atg8 homolog (LC3, GABARAP, or GATE-16), phospholipid (DOPE or DOPS)-containing liposomes, and ATP. Second, the modified form of GATE-16, GATE-16<sup>II</sup>, is a protein-phospholipid conjugate and therefore should be designated GATE-16-PL instead of GATE-16-II. Finally, PS and PE are targets of all human Atg8 modifiers in vitro.

We have further demonstrated that the in vivo target phospholipid of endogenous LC3-II is predominantly PE, as revealed by TLC analysis. This is the first direct evidence for the identity of the target phospholipid of the LC3 conjugation system in vivo.

These observations raise questions regarding the apparent discrepancy between in vitro and in vivo data. Our in vitro conjugation system requires only E1-like enzyme (Atg7) and E2-like enzyme (Atg3) for conjugation of the three Atg8 homologs with PE or PS as in yeast in vitro conjugation of Atg8 with PE (13). However, this does not exclude the possible involvement of some other as yet unidentified factors (Fig. 7) in promoting selective conjugation of LC3-I with PE in vivo. Both positive regulatory factor (X) and negative regulatory factor (Y) should be considered. One potential candidate for X is an E3-like enzyme, which specifically recognizes PE as the substrate of conjugation reaction of activated LC3-I on Atg3 (E2-like enzyme), making efficient and preferential transfer of LC3-I to PE possible.

We hypothesize a presumptive negative regulatory factor (Y), which inhibits LC3 conjugation to PE (Fig. 7). In our in vitro reconstitution experiments, all three Atg8 homologs (LC3, GABARAP, and GATE-16) could be conjugated with both PE and PS. However, cellular expression levels of the three homologs are quite different in native tissues and cultured cells (30). Therefore, the levels of the lipidated forms of the homologs must also be diverse. Hence, the preferential homologs to be lipidated may be different, depending on tissues and cells, and target phospholipid(s) may be also different depending on which of the three homologs is involved in the reaction. Thus, participation of negative regulatory factor (Y) is also a likely mechanism of in vivo lipidation of Atg8 homologs. Experiments are currently under way in our laboratory to screen for possible candidates of X and Y, since it is important to identify and characterize such regulatory factor(s) to improve our understanding of the mechanism of mammalian autophagy.

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