LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation

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Summary

Rat LC3, a homologue of yeast Atg8 (Aut7/Apg8), localizes to autophagosomal membranes after post-translational modifications. The C-terminal fragment of LC3 is cleaved immediately following synthesis to yield a cytosolic form called LC3-I. A subpopulation of LC3-I is further converted to an autophagosome-associating form, LC3-II.

Because yeast Atg8 is conjugated with phosphatidylethanolamine (PE) by a ubiquitin-like system, it has been hypothesized that LC3 is modified in a similar manner. Here, we show that [^{14}C]-ethanolamine was preferentially incorporated into LC3-II, suggesting that LC3-II is a PE-conjugated form. LC3-II can be a substrate of mammalian Atg4B, a homologue of yeast Atg8-PE deconjugase, supporting the idea that LC3-II is LC3-PE. Moreover, two other mammalian homologues of yeast Atg8, γ-aminobutyric-acid-type-A-receptor-associated protein (GABARAP) and Golgi-associated ATPase enhancer of 16 kDa (GATE16) also generate form II, which are recovered in membrane fractions. Generation of the form II correlates with autophagosome association of GABARAP and GATE16. These results suggest that all mammalian Atg8 homologues receive a common modification to associate with autophagosomal membrane as the form II.

Key words: Autophagy, ATG, Autophagosome, UBL, Deconjugation, Phosphatidylethanolamine

Introduction

Autophagy is a process responsible for the bulk degradation of intracellular material that is evolutionarily conserved between all eukaryotes. In autophagy, cytoplasmic components are engulfed by double-membrane-bound structures (autophagosomes) and delivered to lysosomes/vacuoles for degradation. We have previously reported that Atg8 (Aut7/Apg8) functions in the formation step of autophagosomes in the yeast *Saccharomyces cerevisiae* (Kirisako et al., 1999). Atg8 is post-translationally modified by a ubiquitylation-like system (Ohsumi, 2001). The C-terminal Arg residue of Atg8 is cleaved by Atg4 (Aut2/Apg4), a novel cysteine protease (Kirisako et al., 2000) (see Fig. 2A). The exposed Gly of Atg8 is conjugated to phosphatidylethanolamine (PE), which is catalysed by Atg7 (an E1-like enzyme) and Atg3 (an E2-like enzyme) (Ichimura et al., 2000). Subsequently, Atg8-PE is deconjugated, again by Atg4. The cycle of conjugation and deconjugation is important for the normal progression of autophagy (Kirisako et al., 2000).

There are at least three families of mammalian Atg8-related proteins: microtubule-associated protein 1 light chain 3 (LC3); Golgi-associated ATPase enhancer of 16 kDa (GATE16); and γ-aminobutyric-acid-type-A ($GABA_A$)-receptor-associated protein (GABARAP). Each family occasionally has subfamilies, and three human LC3 subfamilies (LC3A, LC3B and LC3C) have been identified (He et al., 2003). Since we previously found that LC3 localizes to autophagosomal membranes (Kabeya et al., 2000), LC3 is thought to be an orthologue of yeast Atg8. The precise function and localization of the other two homologues are unclear. GABARAP might be involved in the $GABA_A$-receptor clustering (Chen et al., 2000) or transport (Kneussel et al., 2000). GATE16 has been suggested to be an intra-Golgi transport modulator that interacts with *N*-ethylmaleimide-sensitive factor (NSF) and the Golgi v-SNARE GOS-28 (Sagiv et al., 2000). These three mammalian Atg8 homologues all possess a conserved Gly residue near their C-termini, which corresponds to the PE-acceptor site of yeast Atg8 (see Fig. 2A).

We previously suggested that LC3 is cleaved after Gly120 within 6 minutes of synthesis in the cytoplasm (Kabeya et al., 2000). As a result, a cytosolic 18-kDa LC3-I, which lacks the C-terminal 22-amino acid fragment, is produced. Subsequently, a subpopulation of LC3-I is converted to LC3-II, a 16-kDa protein that localizes to autophagosomal membranes. Conversion of LC3-I to LC3-II is greatly enhanced by culturing cells under starvation conditions

(without amino acids and serum) that efficiently induce autophagy. Accordingly, the amount of LC3-II correlates well with the number of autophagosomes (Kabeya et al., 2000). This characteristic conversion of LC3 can be used to monitor autophagic activity. However, the nature of LC3-II still remains unknown. Recently, existence of the form-II was also reported as for GATE16 and GABARAP (Tanida et al., 2003). By analogy to yeast Atg8, LC3-II has been speculated to be a PE-conjugated form, which is also supported by the following observations. (1) LC3-II is a tightly membrane-bound form that can be solubilized only by detergents (Kabeya et al., 2000). (2) Formation of LC3-II requires Gly120, which corresponds to the PE-conjugated residue of yeast Atg8. (3) Formation of LC3-II depends on mammalian Atg7 and Atg3 (Tanida et al., 2003). (4) LC3-II generation depends on Atg5 and its conjugation with Atg12 (Mizushima et al., 2001), which is consistent with our finding that PE-conjugation of yeast Atg8 is severely suppressed in the mutants defective in the Atg12 conjugation system (Suzuki et al., 2001). However, in sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE), LC3-II (16 kDa) migrates much faster than LC3-I (18 kDa), whereas the mobility of yeast Atg8-PE is only slightly higher than unconjugated Atg8; this difference is apparent only in the presence of 6 M urea (Kirisako et al., 2000). This discrepancy raises the question of whether LC3-II is simply a conjugated form or has undergone other unknown modifications. Here, we show that LC3-II is probably the PE-conjugated form and that it could be deconjugated by a mammalian Atg4 homologue. Additionally, we show that conversion of GATE16-I and GABARAP-I to GATE16-II and GABARAP-II confers the capacity for binding to autophagosomes as well as LC3.

Materials and Methods

Plasmids

cDNAs encoding human Atg4A (DDBJ/EMBL/GenBank databases accession number AB066214) and Atg4B (AB066215) were obtained by reverse-transcription polymerase chain reaction (RT-PCR) based on previously reported sequences (Kirisako et al., 2000), and cloned into the yeast constitutive expression vector pKT10 (Tanaka et al., 1990). Replacement of Cys74 with Ser of HsAtg4B (HsAtg4B^{C74S}) was performed with a Quick Change Site-directed Mutagenesis Kit (Stratagene). To generate the FLAG-tagged HsAtg4s, we amplified the cDNA encoding HsAtg4A, HsAtg4B or HsAtg4B^{C74S} were amplified by PCR using the specific primers: hAtg4A-5 (5′-TGA-CCCCGGGAGAATGGAGTCAGTTTTATCCAAG-3′); hATG4A-3FLAG (5′-TGACCCCGGGCTATTTATCGTCGTCGTCTTTATAG-TCCACACTCAGAATCTCAAAATCTTC-3′); hAtg4B-51 (5′-AGC-TCCCGGGAAGATGGACGCAGCTACTCTGACCTACGACACT-3′); hATG4B-3FLAG (5′-CGATCCCGGGTCATTTATCGTCGTCGTC-TTTATAGTCAAGGGACAGGATTTCAAAGTCTTC3′). The PCR products were inserted into the *Sma*I site of pKT10. Human *GATE16* cDNA was a kind gift from K. Ogura (The Tokyo Metropolitan Institute of Medical Science) and *GABARAP* cDNA was obtained by RT-PCR. cDNA encoding GATE16 or GABARAP tagged with three Myc epitopes at the N-terminus and with three haemagglutinin (HA) epitopes at the C-terminus (Myc-GATE16-HA or Myc-GABARAP-HA) was cloned into the yeast expression vector pKT10ADE (Mizushima et al., 2002). Myc-LC3-HA and Myc-LC3^{G120A}-HA (Kabeya et al., 2000) were also subcloned into pKT10ADE. To obtain pECFP-LC3, pEYFP-GATE16 and pEYFP-GABARAP, LC3, GATE16 and GABARAP were first cloned into the pECFP or pEYFP vector (Clontech) to produce cyan- or yellow-fluorescent protein (CFP and YFP) fusion proteins. The CFP or YFP fusion constructs were excised from the pECFP or pEYFP vectors by *Nde*I and *Hin*dIII digestions, blunted by T4 polymerase and subcloned into the *Sma*I site of the pCE-neo vector containing the CMV enhancer and elongation factor promoter derived from pCE-FL (a gift from S. Sugano, The Institute of Medical Science, The University of Tokyo, Japan). Myc-LC3-HA, Myc-GATE16-HA and Myc-GABARAP-HA were also subcloned into the pCE-neo vector. The Myc- or YFP-tagged C-terminal deletion mutants with a point mutation to replace the C-terminal glycine to alanine (LC3$^{\Delta C22,\ G120A}$, GATE16$^{\Delta C1,\ G116A}$, and GABARAP$^{\Delta C1,\ G116A}$) were generated by PCR-based site-directed mutagenesis. His$_6$-Myc-LC3 was also subcloned into the pCI-neo vector.

Cell culture and transfection

Media and reagents for cell culture were purchased from Invitrogen. HeLa cells and F9 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS), 5 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Mouse embryonic stem (ES) cells were grown in DMEM containing 20% FBS, 0.1 mM non-essential amino acid solution, 1 μM 2-mercaptoethanol, 2 mM glutamine, 1000 U ml^{-1} leukemia inhibitory factor (LIF) (Chemicon), 5 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. For amino acid starvation, cells were cultured in Hanks' solution containing 10 mM Hepes, pH 7.5 (without amino acid and FBS) for 1-1.5 hours at 37°C.

Transfection was performed as previously described using FuGENE 6™ reagent (Roche) (Mizushima et al., 1998b). To obtain stable transformants, 5×10^6 F9 cells were electroporated with 10 μg linearized expression vector, and with 2 μg pPGKpurobpA when required. Cells were selected in the presence of 0.5-1 mg ml^{-1} geneticin (Invitrogen) or 5-10 μg ml^{-1} puromycin (Sigma).

In vivo labelling experiments

The HeLa cells transfected with Myc-LC3 were washed with methionine- or serum-free DMEM and labelled with 40 μCi [^{35}S]-methionine/cysteine (NEN Life Science Products) for 1 hour at 37°C or 100 μCi [2-^{14}C]-ethan-1-ol-2-amine hydrochloride (Amersham Life Science) for 38 hours at 37°C, respectively. After a 2-hour starvation, cell lysates were prepared with lysis buffer (1% Triton X-100 in PBS) on ice and immunoprecipitated with antibody against the Myc epitope as previously described (Yoshimori et al., 2000). Immunoprecipitated materials were separated by 12% SDS-PAGE and visualized by a bioimage analyser BAS2000 (Fuji Film, Tokyo, Japan).

Yeast strains

The *S. cerevisiae* strain used in this study was KVY57 (*MAT*a *leu2 ura3 his3 trp1 lys2 ade2 suc2 pho8Δ60 Δatg4::LEU2*). Yeast cells were cultured either in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in SD medium (0.17% yeast nitrogen base without amino acid and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose) supplemented with nutrient requirements.

Immunoblotting

Total cell lysates were prepared from yeast, HeLa cells and ES cells, and immunoblotting was performed using antibodies against Myc epitope (9E10, BAbCo), HA epitope (16B12, BAbCo), FLAG-tag (M2, Sigma) and the recombinant LC3 as previously described (Mizushima et al., 1998a; Mizushima et al., 1998b).

In vitro deconjugation assay

ES cells cultured on two 10 cm gelatinized dishes were amino-acid

starved for 4 hours as previously described (Mizushima et al., 2001) and lysed with 1% Triton X-100 in TBS containing 3 mg ml^{-1} bovine serum albumin (BSA) and 1 mM dithiothreitol. 70 OD_{600} units of yeast cells expressing FLAG-tagged HsAtg4A, HsAtg4B or $HsAtg4B^{C74S}$ were collected and converted to spheroplasts. Cells were lysed in 150 μl of PBS with 1 mM dithiothreitol by vigorous mixing with glass beads. 10 μl ES lysate (~4 mg ml^{-1}) and 10 μl yeast lysate (~15 mg ml^{-1}) were mixed and incubated at 37°C for 1 hour. The reaction was stopped by addition of 4 μl of 6× SDS sample buffer. 6 μl of each sample was subjected to SDS-PAGE and immunoblot analysis using anti-LC3 antibody (Kabeya et al., 2000).

Immunoprecipitation

HeLa cells grown on four 15 cm culture dishes were transfected with His-Myc-LC3. Cells starved for 1 hour were lysed with 1% Triton X-100 in PBS on ice and immunoprecipitated with anti-Myc antibody as previously described (Yoshimori et al., 2000). The immunoprecipitated proteins were separated by SDS-PAGE and analysed by staining with Coomassie Brilliant Blue (CBB) or immunoblotting as described (Mizushima et al., 1998a).

Subcellular fractionation

Subcellular fractionation was performed at 100,000 ***g*** to separate soluble cytosolic material and a pellet containing total membranes as previously described (Mizushima et al., 1998b).

Fluorescence and immunoelectron microscopy

The F9 stable transformants co-expressing CFP-LC3 and YFP-GATE16 or CFP-LC3 and YFP-GABARAP were grown on glass coverslips and cultured under the conditions indicated. The cells were examined immediately using a DeltaVision microscopic system (Applied Precision).

For immunoelectron microscopy, the pre-embedding silver enhancement immunogold method was performed as previously described (Yoshimori et al., 2000).

Results

LC3-II is modified with PE

In animal cells, PE is synthesized from three precursors: fatty acyl CoA, glycerol 3-phosphate and cytidine diphosphoethanolamine (CDP-ethanolamine). To form CDP-ethanolamine, ethanolamine is first phosphorylated by ethanolamine kinase with ATP, and resulting phosphoethanolamine attacks CTP (Kuge and Nishijima, 1997). To determine the possible modification of LC3 with PE, we examined whether LC3 is labelled with exogenously added [^{14}C]-ethanolamine. HeLa cells transiently transfected with Myc-tagged LC3 were labelled with [^{14}C]-ethanolamine and immunoprecipitated with anti-Myc antibody. To assess the relative amounts of LC3-I and LC3-II, transfected HeLa cells were also labelled with [^{35}S]-Met and [^{35}S]-Cys, and immunoprecipitated. It revealed that most Myc-LC3 was present in the LC3-I form and about one-tenth was converted to LC3-II (Fig. 1, lane 1). By contrast, [^{14}C] was preferentially incorporated into LC3-II (Fig. 1, lane 3). LC3-I was also labelled with [^{14}C] but less efficiently. This might indicate that [^{14}C] is incorporated into amino acids and proteins. However, the preferential incorporation of [^{14}C] into LC3-II suggests that LC3-II is the PE-conjugated form.

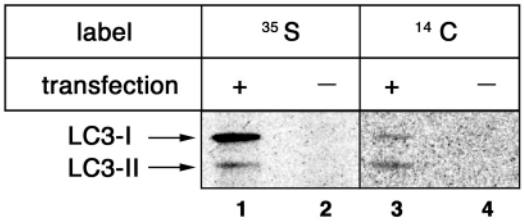

Fig. 1. [^{14}C]-Ethanolamine is incorporated into LC3-II. HeLa cells transfected with Myc-LC3 were labelled with [^{35}S]-methionine/cysteine for 1 hour (lanes 1-2) or [^{14}C]-ethanolamine for 38 hours (lanes 3-4). The cell lysates were immunoprecipitated with anti-Myc antibody and the immunoprecipitates were separated by SDS-PAGE and analysed with a bioimage analyser.

HsAtg4B hydrolyses the C-terminus of LC3

If LC3-II is the LC3-PE conjugate, it is expected to be deconjugated into LC3-I and PE. By analogy to the yeast Atg8 system, mammalian Atg4 homologue(s) would catalyse both C-terminal hydrolysis of newly synthesized LC3 and deconjugation of LC3-PE. So far, four human homologues of Atg4 have been reported: autophagin 1 (HsAtg4B), autophagin 2 (HsAtg4A), autophagin 3 and autophagin 4 (Kirisako et al., 2000; Mariño et al., 2003). Although it was shown that C-terminus of GATE16 is cleaved by HsAtg4A (Scherz-Shouval et al., 2003), it has not been determined whether HsAtg4A can also process the C-terminus of LC3. We cloned HsAtg4A and HsAtg4B, and examined their ability to hydrolyse the C-termini of LC3, GATE16 and GABARAP in yeast cells. We tagged these molecules with a Myc epitope at their N-termini and an HA epitope at their C-termini (Fig. 2A). When these double-tagged proteins were expressed in Atg4-deficient yeast cells, each protein was detected as a single band with both anti-Myc and anti-HA antibodies, indicating that these proteins were not cleaved by unknown yeast proteases (Fig. 2B, lanes 1,5,9,13). The ability of human Atg4 homologues to cleave mammalian Atg8 family proteins was determined by co-expression of HsAtg4A or HsAtg4B with LC3, GATE16 or GABARAP. When HsAtg4B was co-expressed with substrates, LC3, GABARAP and GATE16 were all efficiently cleaved (Fig. 2B, lanes 3,11,15). Immunoblotting with anti-Myc antibody suggested that substrate cleavage occurs at or near the C-termini, probably following the conserved Gly residues. This hypothesis was supported by the demonstration that hydrolysis by HsAtg4B was drastically reduced when Gly120 was replaced with Ala (Fig. 2B, lane 7). HsAtg4A had different substrate specificity. Although HsAtg4A cleaved GATE16 efficiently, it hydrolysed GABARAP less efficiently and LC3 the least efficiently (Fig. 2B, lanes 2,10,14). The substrate specificity of HsAtg4A is quite similar to that of yeast Atg4 (Fig. 2B, lanes 4,12,16). These data suggest that GABARAP and GATE16 could be cleaved by either HsAtg4A or HsAtg4B, but LC3 is preferentially cleaved by HsAtg4B. These results are consistent with a recent report showing that Apg4B has a broad specificity for mammalian Atg8 homologues (Hemelaar et al., 2003).

The active site of HsAtg4B was suggested to be Cys74 by sequence alignment with yeast Aut2/Atg4. Substitution of this Cys with Ser resulted in the complete loss of LC3 cleavage (Fig. 2C).

LC3-II is a substrate of HsAtg4B

Having the above results that HsAtg4B is the C-terminal hydrolase for LC3, we sought to determine whether LC3-II could act as a substrate for HsAtg4B. Lysate from amino-acid-starved ES cells, in which most LC3 is converted to LC3-II, was mixed with yeast cell lysate expressing HsAtg4A, HsAtg4B or HsAtg4B^{C74S}, and incubated for 1 hour at 37°C. When incubated with HsAtg4A-transfected yeast cell lysate (Fig. 3A, Exp. 1, lane 1), most LC3 was detected as the LC3-II form. By contrast, LC3-II was almost absent after incubation with the lysate containing HsAtg4B (Fig. 3A, Exp. 1, lane 2). Such a decrease was not observed when incubated with the lysate from cells expressing HsAtg4B^{C74S} (Fig. 3A, Exp. 1, lane 3). The amount of LC3-I is modestly increased by HsAtg4B. This slight increase was reproducible (Fig. 3A, Exp. 2, lanes 5-8). These results suggest that LC3-II can be a substrate of HsAtg4B, but not of HsAtg4A, and LC3-II might be modified back to LC3-I.

However, the increase in amount of LC3-I was much less than the decrease of LC3-II by immunoblot analysis (Fig. 3A). A possible explanation is that the detection efficiencies of LC3-I and LC3-II could be different by immunoblotting. To address this question, we purified His$_6$-Myc-LC3 from transiently transfected and starved HeLa cells. We compared patterns of CBB staining and immunoblotting of the same sample. Although the band intensity of LC3-I of the sample is similar to that of LC3-II on the immunoblot, the amount of LC3-II is much less than that of LC3-I judged from the CBB staining (Fig. 3B). Silver staining showed a similar pattern to CBB staining (data not shown). Together, these data suggest that LC3-II is much more sensitive (or LC3-I is less sensitive) by immunoblot assay by unknown reasons. Therefore, the small increase in the LC3-I in the Fig. 3A, lane 2 could account for the disappearance of LC3-II. Alternatively, a proportion of LC3-II might be degraded after the action of HsAtg4B, rather than recycled into LC3-I. In either case, it is certain that LC3-II is the substrate of HsAtg4B, supporting the idea that LC3-II is the conjugate formed via an amide bond.

GATE16 and GABARAP are converted to form II

As in the case of LC3, it was suggested that GATE16 and GABARAP are also converted into form II (Tanida et al., 2003). We determined this by transfection of F9 teratocarcinoma cells with double-tagged GATE16 and GABARAP (Fig. 4A). In F9 cells, both GATE16 and GABARAP were detected in two forms (I and II) using anti-Myc antibody but not anti-HA antibody (Fig. 4, lanes 4,7, data not shown). In the case of GABARAP, the pro-form was also detected by antibodies against both Myc and HA epitopes. It should be noted that the amounts of GATE16-II and GABARAP-II increased by the starvation treatment (Fig, 4, lanes 2,5,8).

To confirm further that the lower bands represent GATE16-II and GABARAP-II, we constructed GATE16$^{\Delta C1,\ G116A}$, which lacks Phe117 and has a G116A substitution, and GABARAP$^{\Delta C1,\ G116A}$, which lacks Leu117 and has a G116A substitution (Fig. 4A). Immunoblotting of the F9 cells expressing the mutant proteins revealed that the double mutations prevented the conversion from form I to form II for LC3, GATE16 and GABARAP (Fig. 4, lanes 3,6,9). An additional band was found in the case of GABARAP$^{\Delta C1,\ G116A}$. It was not identical to form II, although we did not further characterize it (Fig. 4, asterisk in lane 9). Taken together, these results suggest that both GATE16 and GABARAP are partly converted to form II, although less efficiently than LC3.

The two forms of GABARAP and GATE16 demonstrated different subcellular distribution in F9 cells (Fig. 5, lanes 4-9). The form II was recovered in the membrane fraction, whereas form I of both proteins was primarily cytosolic. These results suggest that GATE16-II and GABARAP-II are present on some membrane.

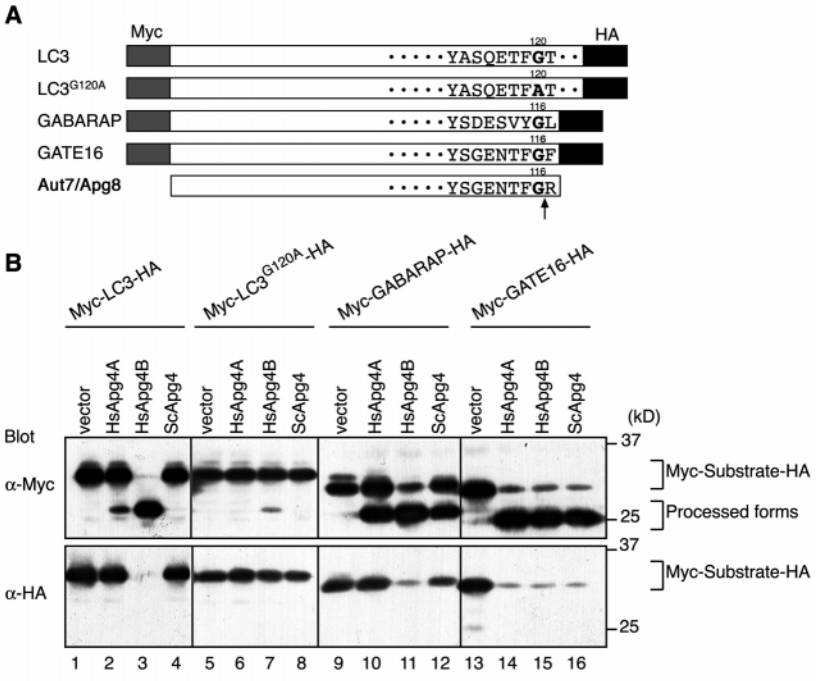

Fig. 2. Human Atg4 homologues process from pro-form to form I of LC3, GABARAP and GATE16 at C-termini. (A) Tagged versions of LC3, GABARAP and GATE16. Positions of Myc and HA epitope tags are indicated. The conserved Gly residues are shown in bold; this residue is replaced by Ala in the LC3^{G120A} mutant. Arrow indicates the cleavage site in yeast Atg8 by Aut2/Atg4 (Kirisako et al., 1999). (B) In vivo analysis of C-terminal hydrolase activities of HsAtg4A and HsAtg4B. The double-tagged mammalian Atg8 family proteins in (A) and the indicated human Atg4 family proteins were co-expressed in *Atg4*-deficient yeast cells. Total lysate of each transformant was analysed by immunoblot analysis using anti-Myc or anti-HA antibodies. Positions of unprocessed substrates and their processed forms are indicated. (C) Cys74 of HsAtg4B is a putative active site. Enzyme activity of wild type HsAtg4B and HsAtg4B^{C74S} mutant was examined as in (B) using Myc-LC3-HA as substrate.

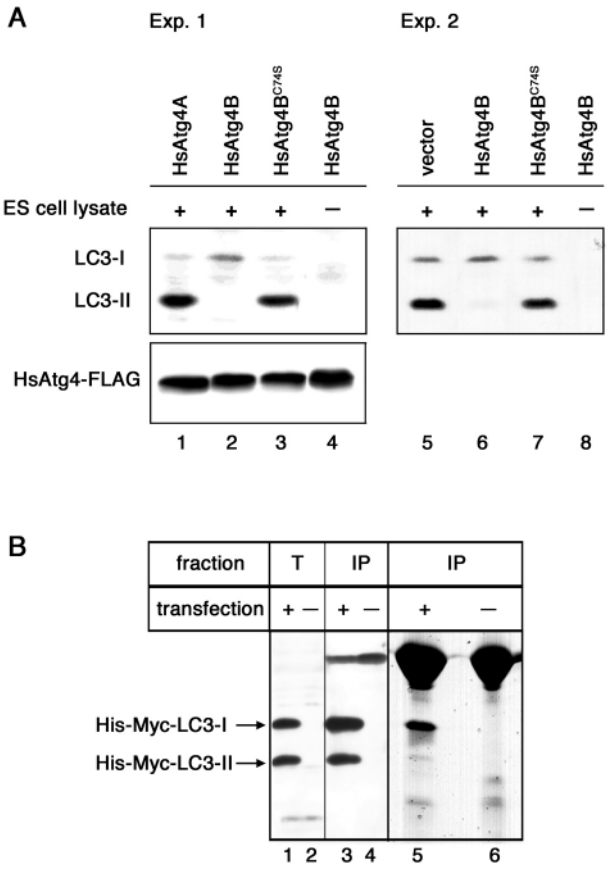

Fig. 3. LC3-II is a substrate of HsAtg4B. (A) LC3-II is cleaved by HsAtg4B in vitro. Lysate from amino-acid-starved ES cells was mixed with lysate from yeast cells expressing FLAG-tagged HsAtg4A, HsAtg4B, HsAtg4B^{C74S}, or vector alone, and incubated for 1 hour at 37°C. After the addition of SDS-PAGE sample buffer to stop the reaction, the products were resolved by SDS-PAGE and subjected to immunoblot using antibody against LC3. The data were shown from the two independent experiments (Exp. 1 and 2). (B) The amount of LC3-II is overestimated by immunoblotting. Total cell lysates (T) were prepared from HeLa cells expressing His-Myc-LC3 after starvation for 1 hour and subjected to immunoprecipitation using anti-Myc antibody (IP). The immunoprecipitates were assayed by immunoblotting (lanes 1-4) or CBB staining (lanes 5 and 6). As controls, mock-transfected cells were used.

GATE16-II and GABARAP-II, like LC3-II, localize to autophagosomes

Both GATE16-II and GABARAP-II were recovered in membrane fractions and their levels were upregulated during starvation, so we hypothesized that GATE16-II and GABARAP-II might, like LC3, be present on autophagosomes. Autophagosomes can be recognized as ring-shaped structures under a fluorescence microscope in living F9 teratocarcinoma cells expressing CFP-LC3 (Fig. 6A,B), a pattern consistent with previous reports in ES cells (Mizushima et al., 2003).

In order to determine the intracellular distribution of GATE16 and GABARAP, we compared them with that of LC3 under starvation conditions. YFP-fused GATE16 localized to punctate structures that were increased in number following starvation and dispersed throughout the cytoplasm (Fig. 6A,b,e). Occasionally, ring structures were also stained, suggesting that GATE16 localized to autophagic vacuoles. Indeed, most GATE16 signal co-localized with CFP-fused LC3 in the F9 cells stably co-transfected with YFP-GATE16 and CFP-LC3 (Fig. 6B,a-c). In a similar manner, GABARAP showed dots and ring-shaped staining (Fig. 6A,c,f) co-localized with LC3 under starvation conditions (Fig. 6B,d-f). We also obtained the similar results in ES cells transfected with the CFP-GATE16 or GABARAP and YFP-LC3 (data not shown). In autophagy defective Atg5$^{-/-}$ ES cells (Mizushima et al., 2001), no punctate structures were visible and only cytoplasmic staining was observed, which indicates that these structures are indeed

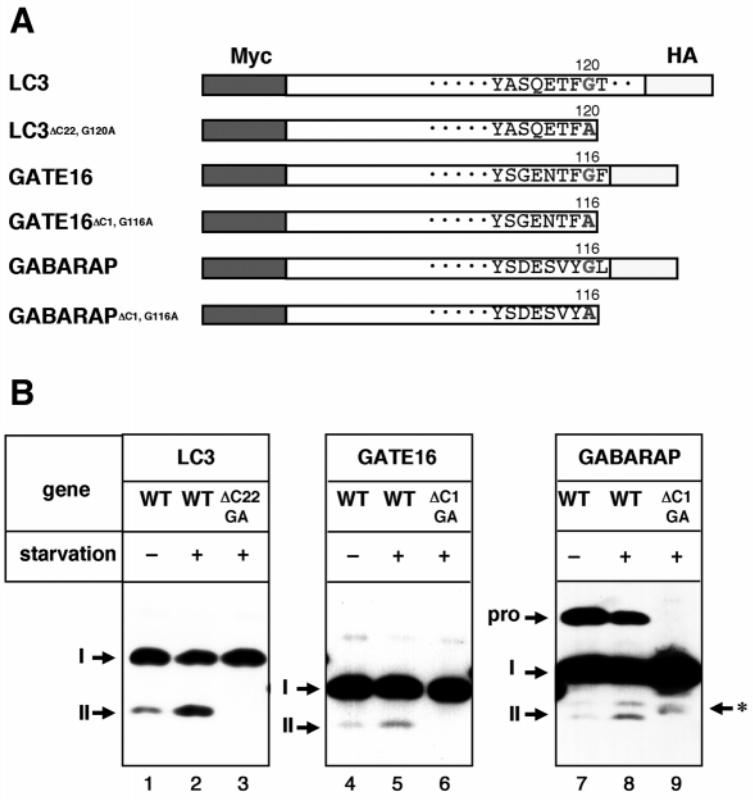

Fig. 4. GATE16 and GABARAP exist in two forms after processing at their C-termini. (A) Tagged versions of LC3, GATE16 and GABARAP. Positions of Myc and HA epitope tags are indicated. The conserved Gly residues are shown in bold. LC3$^{\Delta C22,\ G120A}$ lacks the C-terminal fragment and Gly120 is replaced with Ala. GATE16$^{\Delta C1,\ G116A}$ and GABARAP$^{\Delta C1,\ G116A}$ were also produced. The ΔC, GA mutants were tagged only with the Myc epitope at their N-termini. (B) Two forms of mammalian Atg8 family are produced. F9 cells transfected with the tagged proteins shown in (A) were cultured under nutrient-rich or starvation conditions for 2 hours and subjected to immunoblot analysis using an antibody against the Myc epitope. Position of processed forms (I and II) and unprocessed forms (pro) are indicated. An uncharacterized form (*) appeared in GABARAP-transfected cells.

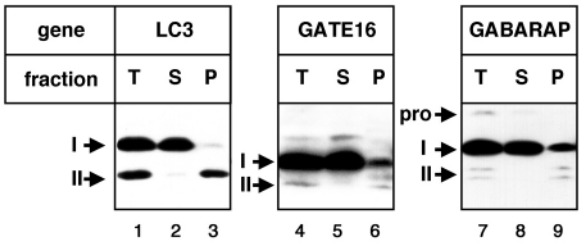

Fig. 5. Two forms of GATE16 and GABARAP differ in subcellular localization. Homogenate of the starved F9 cells expressing Myc-LC3-HA, Myc-GATE16-HA or Myc-GABARAP-HA (T) was centrifuged at 100,000 ***g*** for 30 minutes and then supernatant (S) and pellet (P) fractions were collected. These fractions were analysed by immunoblotting using anti-Myc antibody.

autophagosomes (data not shown). Taken together, these results suggest that GATE16 and GABARAP can be localized on autophagosomal membranes under starvation conditions.

We next examined the localizations of YFP-GATE16$^{\Delta C1,\ G116A}$ and YFP-GABARAP$^{\Delta C1,\ G116A}$, because the formation of form II was suppressed with these mutant proteins (Fig. 4). Both GATE16$^{\Delta C1,\ G116A}$ and GABARAP$^{\Delta C1,\ G116A}$ were detected diffusely in the cytoplasm and did not show autophagosome localization (Fig. 6C), suggesting that form-II formation is required for targeting of GATE16 and GABARAP to autophagosomes.

To examine the localization of GATE16 in more detail, we carried out immunoelectron microscopy of YFP-GATE16-transfected F9 cells using an anti-GFP antibody, In cells subjected to a 2-hour starvation period, silver-enhanced gold particles were found to associate extensively with autophagosome membranes (Fig. 7A,C) and isolation membranes (Fig. 7A,B). By contrast, almost no YFP-GATE16 signal was detected on the autolysosome membranes (Fig. 7A,D). YFP-GATE16 associated with both inner and outer sides of autophagosome membranes like LC3.

Discussion

We focused on characterization of form II of the mammalian Atg8 homologues. Because isotope-labelled ethanolamine is preferentially incorporated into LC3-II and LC3-II can be the substrate of HsAtg4, we propose that LC3-II is the LC3-PE conjugate. We have not yet succeeded to prove this hypothesis by purification of LC3-II and subsequent mass spectrometry, probably because the absolute amount of LC3-II is low. It is also possible that LC3-II is difficult to elute from the gel and/or the C-terminus of LC3-II might not be ionized.

Form II of GATE16 and GABARAP appears in cultured cells as well as LC3 (Fig. 4) (Tanida et al., 2003). Because all of LC3, GATE16 and GABARAP are modified by mammalian Atg7 and Atg3 (Tanida et al., 2003; Tanida et al., 2002; Tanida et al., 2001), GATE16-II and GABARAP-II are also probably the PE-conjugated forms. Roles of GATE16-II and GABARAP-II are unknown in their previously described functions. Interestingly, we found that GATE16-II and GABARAP-II also localize to LC3-positive autophagosomes that are induced by starvation. Thus, it remains a possibility that they participate in autophagy in addition to or instead of their involvements originally described. This might

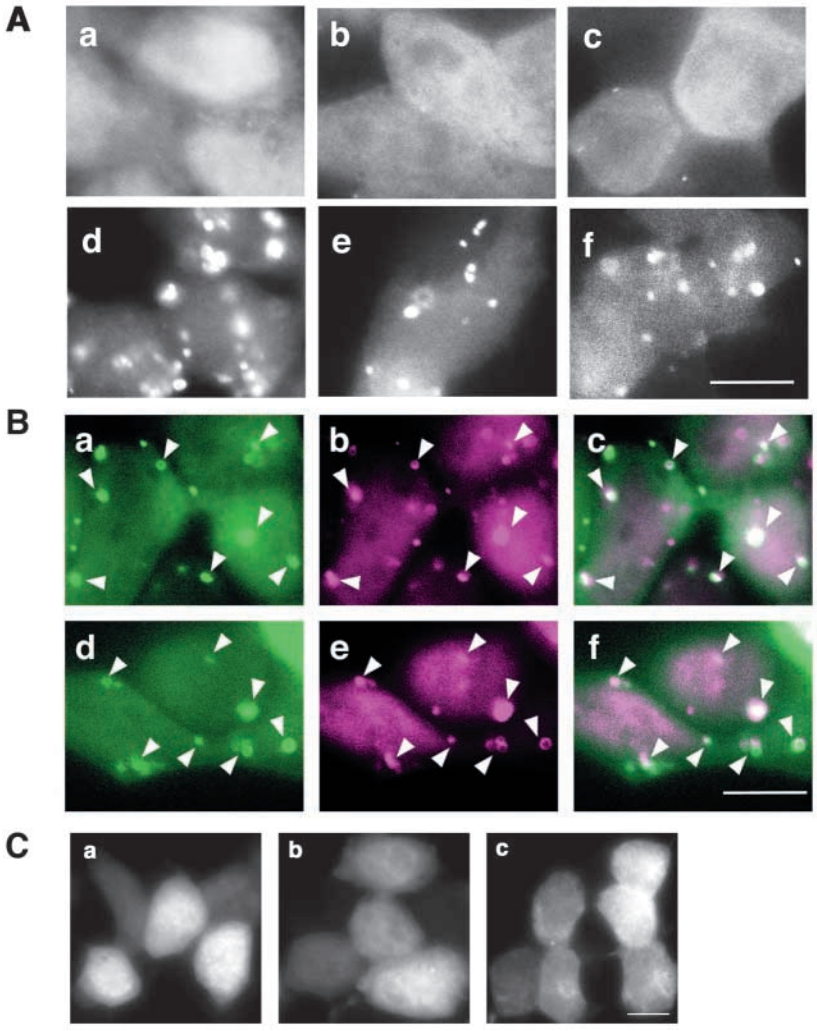

Fig. 6. GATE16 and GABARAP localize to the LC3-positive autophagosome under starvation conditions. (A) F9 cells stably transfected with CFP-LC3 (a,d), YFP-GATE16 (b,e), or YFP-GABARAP (c,f) were cultured under nutrient-rich conditions (a-c) or starvation conditions (d-f) for 2 hours at 37°C. Living cells were observed using a Delta Vision microscopic system. Scale bar, 10 μm. (B) F9 cells co-expressing YFP-GATE16 and CFP-LC3 (a,b), YFP-GABARAP and CFP-LC3 (d,e) were starved for 2 hours and subjected to fluorescence microscopic analysis. Merged images (c,f) are shown. Arrowheads indicate the co-localization of both the punctate and ring-shaped structures. Scale bar, 10 μm. (C) The C-terminal glycine is required for autophagosome-targeting of GATE16 and GABARAP. F9 cells expressing YFP-LC3$^{\Delta C22,\ G120A}$ (a), YFP-GATE16$^{\Delta C1,\ G116A}$ (b), or YFP-GABARAP$^{\Delta C1,\ G116A}$ (c) were starved for 2 hours and subjected to fluorescence microscopic analysis. Scale bar, 10 μm.

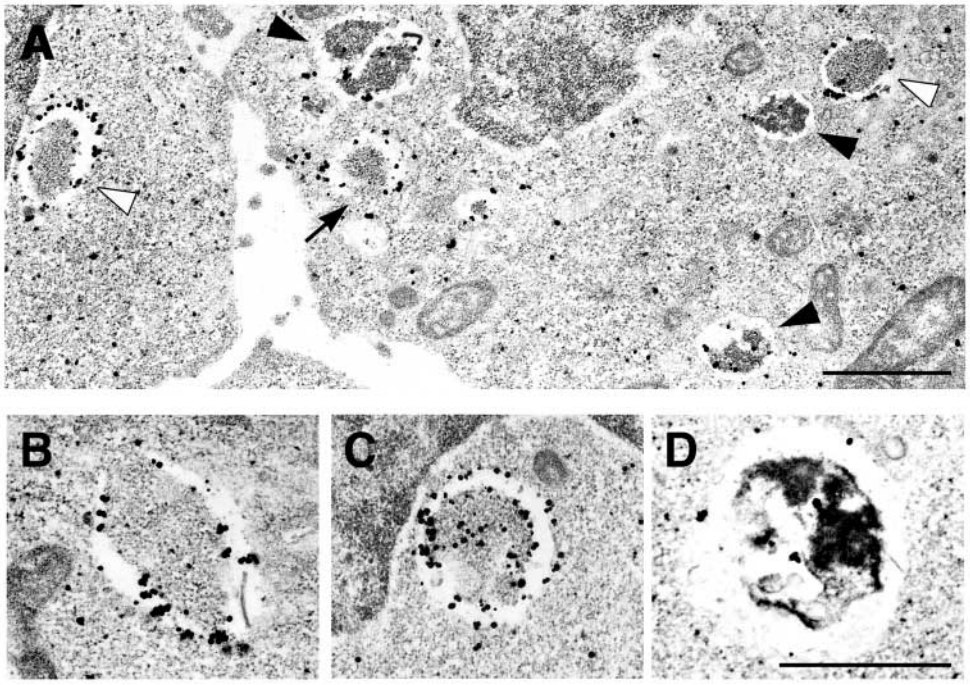

Fig. 7. YFP-GATE16 is present on autophagosome membranes. F9 cells stably transfected with YFP-GATE16 were cultured in Hanks' solution for 2 hours and fixed. The localization of YFP-GATE16 was examined by silver-enhanced immunogold electron microscopy using anti-GFP antibody. The isolation membrane (arrow), autophagosomes (open arrowheads) and autolysosomes (closed arrowheads) are indicated (A). The typical images of isolation membrane (B), autophagosome (C) and autolysosome (D) are shown at higher magnification. Scale bar, 1 μm.

explain why expression of GABARAP is not restricted to GABA-receptor-positive tissues (Wang et al., 1999; Xin et al., 2001). It is also noteworthy that localization of GFP-GATE16 does not show a Golgi pattern, and the distribution of GFP-GABARAP signal is different from previous reports (Chen et al., 2000; Kittler et al., 2001; Kneussel et al., 2000; Sagiv et al., 2000; Wang et al., 1999; Wang and Olsen, 2000) (Fig. 6). Our use of F9 cells might not be appropriate, but RT-PCR analysis showed that F9 cells express mRNA of endogenous LC3, GATE-16 and GABARAP (data not shown). To examine these discrepancies, the precise localization of endogenous GABARAP and GATE16 in various tissues should be examined, rather than relying on cultured cells overexpressing these constructs. We also hope to evaluate the possible redundancy of mammalian Atg8 family members in autophagy.

Here, we also show that the C-terminus of LC3 is processed by HsAtg4B much more efficiently than by HsAtg4A. Although we have not examined the LC3-processing activity of autophagin 3 and autophagin 4, there seems to be some substrate specificity among the mammalian Atg4 homologues. In contrast to the previous report (Mariño et al., 2003), a 3.0-kb *HsAtg4B* mRNA was most abundant in the brain (data not shown). This expression pattern of HsAtg4B is reminiscent of those of LC3 and GATE16, which were reported to be highly expressed in the brain (Mann and Hammarback, 1994; Sagiv et al., 2000).

Through the identification of these different aspects of LC3 biology, we are now able to propose a model for mammalian Atg8 homologues. Step 1 – mammalian Atg4(s), particularly HsAtg4B, remove the C-terminal extension of proLC3 immediately after its synthesis to form LC3-I with a C-terminal Gly. Step 2 – mammalian Atg7 activates LC3-I and forms a Atg7-LC3 thioester (Tanida et al., 2001). Step 3 – LC3 is transferred to mammalian Atg3 to form Atg3-LC3 thioester (Tanida et al., 2002). Step 4 – Atg3 conjugates LC3-I at C-terminus with a putative target molecule, most likely PE, to form LC3-II. Step 5 – mammalian Atg4(s) again remove the target molecule from the C-terminus of LC3-II to again yield LC3-I. This process might be required for the normal progression of autophagy in mammalian cells.

When examined by immunoblot analysis, the amount of LC3-II is overestimated; we cannot simply compare the apparent densities of LC3-I and LC3-II on immunoblots. We have proposed that the amount of LC3-II correlates to the number of autophagosomes (Kabeya et al., 2000). Although the amount of LC3-II is always a reliable indicator for autophagy, the amount of LC3-II seems sometimes larger than that of LC3-I, even in nutrient-rich conditions. This raised the question of whether such a large amount of LC3-II is present under autophagy-suppressed conditions. However, the present study demonstrated that the actual amount of LC3-II is quite small even if it seems to be comparable to that of LC3-I on immunoblots (Fig. 3). Therefore, we must conclude that the amounts of LC3-I and LC3-II cannot be compared directly by immunoblot analysis and summation of LC3-I and LC3-II band densities is meaningless.

In contrast to yeast, which possess only one Atg4 and one Atg8, it appears that multiple homologues might exist in mammalian cells. HsAtg4A and HsAtg4B have different substrate specificities and different biases in tissue distribution, suggesting a specialization of their roles that contributes additional regulation of mammalian Atg8 family proteins. The situation is complex surrounding the mammalian Atg8 family. Family members might be multifunctional and mobilized for autophagy under special conditions such as starvation. Further extensive study will unravel raison d'être of the family in multicellular organisms. A series of investigations of mammalian homologues of yeast autophagic proteins has revealed not only conservation of the underlying machinery

through evolution but also the divergence of this pathway in higher eukaryotes.

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