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Post-translational Modifications of Three Members of the Human MAP1LC3 Family and Detection of a Novel Type of Modification for MAP1LC3B*

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The molecular machinery required for autophagy is highly conserved in all eukaryotes as seen by the high degree of conservation of proteins involved in the formation of the autophagosome membranes. Recently, both yeast Apg8p and its rat homologue Map1lc3 were identified as essential constituents of autophagosome membrane as a processed form. In addition, both the yeast and human proteins exist in two modified forms produced by a series of post-translational modifications including a critical C-terminal cleavage after a conserved Gly residue, and the smaller processed form is associated with the autophagosome membranes. Herein, we report the identification and characterization of three human orthologs of the rat Map1LC3, named MAP1LC3A, MAP1LC3B, and MAP1LC3C. We show that the three isoforms of human MAP1LC3 exhibit distinct expression patterns in different human tissues. Importantly, we found that the three isoforms of MAP1LC3 differ in their post-translation modifications. Although MAP1LC3A and MAP1LC3C are produced by the proteolytic cleavage after the conserved C-terminal Gly residue, like their rat counterpart, MAP1LC3B does not undergo C-terminal cleavage and exists in a single modified form. The essential site for the distinct post-translation modification of MAP1LC3B is Lys-122 rather than the conserved Gly-120. Subcellular localization by cell fractionation and immunofluorescence revealed that three human isoforms are associated with membranes involved in the autophagic pathway. These results revealed different regulation of the three human isoforms of MAP1LC3 and implicate that the three isoforms may have different physiological functions.

Autophagy is a highly regulated bulk degradation process that plays an important role in cellular maintenance and development and has been implicated in a number of genetic diseases (1–3). Upon induction of autophagy, a portion of the cytoplasm is sequestered within double-membrane vesicles known as autophagic vacuoles/autophagosomes (4, 5) and delivered to a degradative organelle. Of the various aspects of autophagy, the formation of autophagosome has been least characterized to date. Yeast Apg8p/Aut7p (6–8) and rat homologue microtubule-associated protein light chain 3 (Map1LC3)[1] (9, 10) have attracted attention recently for their unique localization to the autophagosome membranes.

As one of essential proteins in autophagy, Apg8p exists in two modified forms; the smaller form was the first molecule found to localize to autophagosomes in yeast (11). The membrane-bound Apg8p is formed by a multi-step post-translational modification process. Apg8p is synthesized with a C-terminal arginine (Arg-117) that is immediately removed by Aut2p (6), a cysteine protease, exposing a glycine (Gly-116). Upon activation by Apg7p (12), the processed Apg8p is transferred to Apg3 (13), followed by conjugation of the newly exposed C-terminal Gly-116 to phosphatidylethanolamine on a pre-autophagosomal sac (14). Recently, rat Map1LC3, the rat homologue of yeast Apg8, was identified as a novel constituent of autophagosome membrane (10). Like the yeast Apg8, rat Map1LC3 also exists in two modified forms, an 18-kDa cytoplasmic form that was originally identified as a subunit of the microtubule-associated protein 1 (9, 15), and a 16-kDa form that is associated with the autophagosome membranes (10). The autophagosome membrane-bound form is generated by a multi-step post-translational modification process including a critical proteolytic cleavage after the C-terminal Gly-120 of the newly synthesized Map1LC3 (10). Thus, a common post-translational processing step for both yeast Apg8 and rat Maplc3 is the proteolytic cleavage to expose a conserved Gly residue, indicating a high degree of conservation of post-translational processing in autophagy from yeast to mammals.

Although a number of proteins involved in autophagy in yeast have been reported, the identification and characterization of their mammalian counterparts did not begin until recently. In particular, the molecular machinery involved in autophagy in human cells remains poorly understood. To identify human genes involved in autophagy, the nucleic acid sequences of the rat Map1LC3 was used to search the Human Expressed Sequence Tag (EST) data base in GenBank™ for its human

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[1] The abbreviations used are: Map1LC3, microtubule-associated protein 1 light chain 3; EST, expressed sequence tag; HEK, human embryonic kidney; MTN, multiple tissues Northern; HRP, horseradish peroxidase; TBS, Tris-buffered saline; GFP, green fluorescent protein.

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TABLE I

Nucleotide sequences of oligonucleotides used for the cloning of MAP1LC3A,B,C and the construction of mutant MAP1LC3A,B,C cDNAs

Underlined nucleotides were exchanged to obtain the desired mutation. Restriction sites are indicated by boldface. His tag is showed by italics. Deletion mutants are indicated by Δ. Mutations at a specific residue number are indicated by one letter amino acid abbreviations. The first letter is the wild-type residue, and the last letter is the amino acid to which it is changed by the mutated codon. A, sense primer; B, antisense primer.

Primers	Sequence
cDNA cloning primers	
LC3A-A:	5′-CCCAAACCGCAGACACATCC
LC3A-B:	5′-ACGGTAGAGGCAGCTCAGTTCAG
LC3B-A:	5′-GCACCATGCCGTCGGAGAAGACC
LC3B-B:	5′-CACTCCTAGGTGGGAACACTACTG
LC3C-A:	5′-GACACAGCTACTGAGTGCAAT
LC3C-B:	5′-ACACGTCTGTCAGAGCACTC
RATLC3-A	5′-CGCGCCATGCCGTCCGAGAAGAC
RATLC3-B	5′-GTCTGTCACAAGCATGGCTCTCT
Gene expression primers	
Myc-LC3A-A	5′-TAG**GAATTC**TAATGCCCTCAGACCGGCCTTTC
Myc-LC3A-B	5′-TTG**CTCGAG**TCAGAAGCCGAAGGTTTCCTC
Myc-LC3B-A	5′-ACG**GAATTC**GCATGCCGTCGGAGAAG
Myc-LC3B-B	5′-GGC**GGTACC**TTACACTGACAATTTC
Myc-LC3C-A	5′-TTA**GAATTC**ATATGCCGCCTCCACAG
Myc-LC3C-B	5′-TTT**CTCGAG**CTAGAGAGGATTGCAG
Myc-RATLC3-A	5′-CG**GAATTC**TAATGCCGTCCGAGAAGAC
Myc-RATLC3-B	5′-CTT**CTCGAG**CAAGCATGGCTCTCT
Myc-LC3A-His-B	5′-AAT**CTCGAG**TCA *ATGGTGATGGTAGATGATG* GAAGCCGAAGGTTTC
Myc-LC3B-His-B	5′-ATT**GGTACC**TTA *ATGGTGATGGTGATGATG* CACTGACAATTTC
Myc-LC3B-K122A-His-B	5′-ATT**GGTACC**TTA *ATGGTGATGGTGATGATG* CACTGACAA**GGC**CATC
Myc-LC3C-His-B	5′-ATA**CTCGAG**CTA *ATGGTGATGGTGATGATG* GAGAGGATTGCAG
Mutation analysis primers	
Myc-LC3AΔ-B	5′-GCG**CTCGAG**TCAGCCGAAGGTTTCCTGGG
Myc-LC3BΔ-B	5′-GCG**GGTACC**TTACCCGAACGTC
Myc-LC3CΔ-B	5′-AGA**CTCGAG**CTAGCCAAATGTCTCC
Myc-RATLC3CΔ-B	5′-CTT**CTCGAG**TCACCCGAACGTCTCCTGGG
Myc-LC3AΔG120A-B	5′-GCG**CTCGAG**TCAGGCGAAGGTTTCCTGGG
Myc-LC3BΔG120A-B	5′-GCG**GGTACC**TTACGCGAACGTC
Myc-LC3CΔG120A-B	5′-AGA**CTCGAG**CTAGGCAAATGTCTCC
Myc-RATLC3CΔG120A-B	5′-CTT**CTCGAG**TCACGCGAACGTCTCCTGGG
Myc-LC3B · G120A-B	5′-GGC**GGTACC**TTACACTGACAATTTCAT**GGC**GAACG
Myc-LC3B · M121A-B	5′-ATT**GGTACC**TTACACTGACAATTT**GGC**CCCGAACGTCTCC
Myc-LC3B · K122A-B	5′-TTT**GGTACC**TTACACTGACAA**GGC**CATCCCG
Myc-LC3B · K122E-B	5′-TTT**GGTACC**TTACACTGACAA**TTC**CATCCCG
Myc-LC3B · K122R-B	5′-TTT**GGTACC**TTACACTGACAA**TCT**CATCCCG
Myc-LC3B · L123A-B	5′-ATT**GGTACC**TTACACTGA***GGC***TTTCATCCCGAAC
Myc-LC3B · S124A-B	5′-TTT**GGTACC**TTACAC**GGC**CAATTTCATCCCG
Myc-LC3B · V125A-B	5′-GGC**GGTACC**TTA**GGC**TGACAATTTCATC
Location primers	
GFP-LC3A-A	5′-TAG**CTCGAG**CTATGCCCTCAGACCGGCCTTTC
GFP-LC3A-B	5′-TTG**GGATCC**TCAGAAGCCGAAGGTTTCCTG
GFP-LC3B-A	5′-TAG**CTCGAG**CTATGCCGTCGGAGAAG
GFP-LC3B-B	5′-GGC**GGATCC**TTACACTGACAATTTC
GFP-LC3C-A	5′-TAG**CTCGAG**CTATGCCGCCTCCACAG
GFP-LC3C-B	5′-TTT**GGATCC**CTAGAGAGGATTGCAG
GFP-LC3C-B	5′-TTT**GGATCC**CTAGAGAGGATTGCAG
GFP-RATLC3C-A	5′-TAG**CTCGAG**CTATGCCGTCCGAGAAGAC
GFP-RATLC3C-B	5′-CTT**GGATCC**CAAGCATGGCTCTCT
Prokaryotic expression primers	
GEX4T-1-RATLC3&LC3A/B/C-A	5′-CG**GGATCC**ATGGCATCAATGCAGAAG
GEX4T-1-LC3A-B	5′-TTG**CTCGAG**TCAGAAGCCGAAGGTTTCCTG
GEX4T-1-LC3B-B	5′-GGC**CTCGAG**TTACACTGACAATTTC
GEX4T-1-LC3C-B	5′-TTT**CTCGAG**CTAGAGAGGATTGCAG
GEX4T-1-RATLC3A-B	5′-CTT**CTCGAG**CAAGCATGGCTCTCT

homologues. In this paper, we report the identification, isolation, and characterization of three human *MAP1LC3* genes, named *MAP1LC3A*, *MAP1LC3B*, and *MAP1LC3C*. The deduced proteins of *MAP1LC3A*, *MAP1LC3B*, and *MAP1LC3C* show 81, 94, and 55% sequence identity to rat Map1LC3, respectively. Although the amino acid sequence spanning Tyr-113 and Gly-120 of rat MapLC3 is conserved in MAP1LC3A, MAP1LC3B, and MAP1LC3C, we found that MAP1LC3B undergoes a different type of post-translational processing. Although MAP1LC3A and MAP1LC3C are processed in a manner that is similar to the rat Map1LC3, MAP1LC3B exhibits a distinct type of post-translational modification. A C-terminal lysine (Lys-122), rather than the conserved Gly-120, is shown to be required for the modification of MAP1LC3B. Moreover, the three human isoforms display different patterns of subcellular localization and distinct response to amino acid starvation.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli strain DH5α and BL21 cells, the hosts for plasmids and protein expression, were grown in Luria broth in the presence of antibiotics as required. Human embryonic kidney (HEK293) and HeLa cells were from ATCC and grown in 5% CO_2 at 37 °C. DNA primers (see Table I) were custom-synthesized by Sheng-Gong Inc. (Shanghai, China). Eight human tissue cDNA gt10/11 libraries (liver, kidney, skeletal muscle, testis, marrow, placenta, heart, and brain) and multiple tissues Northern (MTN I and MTN II) membranes, pCMV-Myc vector pEGFP-C1 vector, and mouse c-Myc monoclonal antibody were purchased from Clontech. Restriction enzymes, *Taq* DNA polymerase, and T4 DNA ligase were purchased from New England

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Biolabs. Antibodies against human transferrin receptor and human aldolase were purchased from Santa Cruz Biotechnology, Inc. Goat anti-mouse IgG CY5 conjugate was from Southern Biotechnology Associates. Mouse anti-His monoclonal antibody was from Qiagen. Anti-goat IgG HRP conjugate and anti-rabbit IgG HRP conjugate were from Calbiochem. pGEM-T vector and anti-mouse IgG(H+L)HRP conjugate were from Promega. Protease inhibitors aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride, Ponceau S, and protein marker were purchased from Watson Inc. (Shanghai, China). Calcium phosphate transfection regent and lipofectinamino™ reagent were from Invitrogen.

Cloning of cDNA—The amino acid sequence of rat Map1LC3 (GenBank™ accession number U05784) was used to search the human EST data base of GenBank™. Homologous ESTs were obtained from NCBI EST data base and assembled into three EST contigs by the Pileup program in the GCG package. Three pairs of primers (named LC3A/B/C-A and B; see Table I) were designed based on the contig sequences and used to amplify the corresponding cDNA from several human cDNA λgt11 libraries. The primers of rat LC3 were designed based on the sequence of U05784 in NCBI data base and used to amplify rat brain tissue cDNA prepared by reverse transcription-PCR from rat brain tissue RNA. PCR amplification conditions were as follows: 1 μl of template (>10^8 pfu/ml) was amplified in a 50-μl volume containing 5 μl of 10× PCR buffer, 1 μl of 20 mM dNTPs, 1.5 μl of 2.5 mM $MgCl_2$, 2 units of *Taq* polymerase, and 1 μl of 25 mM each specific primer. PCR reactions were carried out at 94 °C for 5 min at 94 °C (60 s), 55 °C (60 s), and 72 °C (60 s) for a total of 34 cycles, with a final extension at 72 °C for 10 min in a PTC-200 DNA Engine (MJ Research, Watertown, MA). The PCR products were subcloned into the pGEM-T vector and sequenced with the primers on either side of the clone site of the vector pGEM-T (primer sequences were as follows: A, 5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′; B, 5′-TCA CAC AGG AAA CAG CTA TGA C-3′) using the BigDye terminator sequencing kit and ABI377 sequencer (PerkinElmer Life Sciences).

Sequence Alignment—The sequence alignment was performed by ClustalW (www.ebi.ac.uk/clustalw/). The cDNAs encoding the proteins used and their accession numbers (in parentheses) are yeast *Apg8* (NC 001134), mouse *Map1lc3–1* (AK003122), mouse *Map1lc3–2* (AK003106), rat *Map1LC3* (*NM_022867*), and human *MAP1LC3A* (AF 276658), *MAP1LC3B* (AF087871), and *MAP1LC3C* (AF276659).

Northern Blotting—Northern hybridizations of *MAP1LC3A*, *MAP1LC3B*, and *MAP1LC3C* were performed on MTN I and MTN II membranes with mRNA samples from 16 adult human tissues as described previously (16). The probes were prepared by labeling the cDNA fragments (amplified from human cDNA libraries as mentioned above) with [α-^{32}P]dATP (Amersham Biosciences) using PCR and purified by a Sepharose G50 column. The MTN membranes were prehybridized in prehybridization/hybridization solution (50% formamide, 5× SSPE (0.9 M NaCl, 50 mM NaH_2PO_4-NaOH [pH 6.5], 5 mM EDTA), 10× Denhardt's, 2% SDS, 100 μg/ml calf thymus (CT-DNA) at 42 °C for 16 h, hybridized with the labeled probe for another 24 h, and shaken continuously in a hybridization oven (Hybaid UK Inc.). The membranes were then washed several times with 0.1× SSC (0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS at 42 and 65 °C, followed by exposure to x-ray film at −80 °C for 5 days. Northern blotting of human β-actin was performed as described above except that the exposure time to x-ray film was 8 h. The intensities of the bands are determined by scanning and analyzing with a complete gel documentation and analysis system (GDS 8000; Gene Company Ltd., Shanghai, China).

Plasmid Construction and Site-directed Mutagenesis—A total of 35 recombinant constructs were produced in this study. All insert fragments were obtained by PCR amplification, and the oligonucleotide sequences of PCR primers are listed in Table I. First, the wild type rat *MAP1LC3* and human *MAP1LC3A* and *MAP1LC3C* were cloned into the eukaryotic expression vector pCMV-Myc at *Eco*RI and *Xho*I sites using the primers Myc-ratLC3-A and Myc-ratLC3-B, Myc-LC3A-A and Myc-LC3A-B, and Myc-LC3C-A and Myc-LC3C-B, separately. The wild type *MAP1LC3B* was cloned into pCMV-Myc at *Eco*RI and *Kpn*I sites using the primers Myc-LC3B-A and Myc-LC3B-B. Then, wild rat *MAP1LC3* and human *MAP1LC3A*, *MAP1LC3B*, and *MAP1LC3C* were used as a template for mutagenesis. A total of 24 mutants were generated by PCR. All names of the primers and the mutants of amino acids are given in Table I. All 5′ primers of rat *MAP1LC3* and human *MAP1LC3A/MAP1LC3C* variants contain the *Eco*RI restriction site, and 3′ primers of them contain the *Xho*I restriction site, allowing insertion of the PCR-derived fragments into *Eco*RI- and *Xho*I-digested pCMV-Myc plasmid. All 5′ primers of MAP1LC3B variants contain the *Eco*RI restriction site, and 3′ primers of *MAP1LC3*B variants contain the *Kpn*I restriction site, allowing insertion of the PCR-derived fragments into *Eco*RI- and *Kpn*I-digested pCMV-Myc construct. To identify whether MAP1LC3A, MAP1LC3B, and MAP1LC3C proteins undergo a C-terminal cleavage, a His_6 tag (CATCATCACCATCACCAT) was inserted by PCR in the open reading frame of *MAP1LC3A*, *MAP1LC3B*, and *MAP1LC3C* just before the stop codon and followed by insertion of His-tagged amplified products into the pCMV-Myc vector by the same method mentioned above. All mutations were confirmed by sequencing with the primers on either side of the clone site of the vector pCMV-Myc (primer sequences were as follows: A, 5′-GAACTGCTCCTCAGTGGATG-3′; B, 5′-TCACTGCATTCTAGTTGTGG-3′). For the prokaryotic expression and immunofluorescence, all full-length genes amplified with the primers (Table I) were inserted into the pGEX4T-1 vector in the sites of *Bam*HI and *Xho*I and the pEGFP-C1 vector in *Eco*RI and *Bam*HI, respectively.

Cell Culture and Transient Transfection—Media and reagents for cell culture were purchased from Invitrogen. HEK293 cells and HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 5 units/ml penicillin, and 50 μg/ml streptomycin. For transient transfection, HEK293 cells were plated at a density of 1×10^6 cells per 100-mm tissue culture dish 1 day prior to transfection. The next day, the medium was replaced, and cells were transfected with 20 μg of the indicated plasmid DNA per dish using calcium phosphate transfection system or lipofectinamino™ (Invitrogen) according to the manufacturer's instructions. The overexpression of the transfected gene was analyzed in special time post-transfection. For immunofluorescence, HeLa cells were plated at a density of 0.5×10^5 cells per 35-mm dish.

Western Blotting—9×10^6 cells were harvested and washed with 1 ml of ice-cold phosphate-buffered saline and resuspended in 500 μl of cold lysis buffer containing 58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 10 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, and 25 μg/ml aprotinin. The lysate was incubated on a rotating apparatus at 4 °C for 30 min and cleared by centrifugation at 12,000 rpm for 30 min at 4 °C. For Western blot, 10 μl of lysate was mixed with an equal volume of 2× SDS sample buffer, boiled for 3 min, and centrifuged briefly. The proteins in the supernatant were resolved by SDS-PAGE (12–15%) and transferred to a nitrocellulose membrane. After blocking with phosphate-buffered saline containing 0.2% Tween 20 and 5% non-fat dry milk for 1 h, the membrane was probed with a specific mouse anti-Myc antibody (1:500) or anti-His antibody (1:1000) for 2 h and then washed and exposed to horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (1:2500) for 1 h. The bound antibodies were visualized with HRP staining solution or with a Photope-HRP Western detection kit according to the manufacturer's instructions (Cell Signaling Technology).

Recombinant Proteins—Rat MAP1LC3 and human MAP1LC3A/MAP1LC3B/MAP1LC3C tagged with Myc epitope in the N terminus were cloned into pGEX4T-1, an *E. coli* expression vector. Bacterially expressed proteins were produced, cleaved with thrombin, isolated using standard glutathione *S*-transferase fusion protein protocols (Amersham Biosciences), and then analyzed by Western blotting.

Subcellular Fractionation—Subcellular fractionation was carried out as previously described (25). HeLa cells transfected with Myc-MAP1LC3A/MAP1LC3B/MAP1LC3C and Myc-ratMAP1LC3, respectively, were washed, harvested, and homogenized with a sonicator four times, for 5 s each in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride. After low speed centrifugation of the homogenate at 3000 rpm for 5 min, the supernatant was centrifuged at 100,000 × *g* (a S45A rotor; Hitachi) at 4 °C for 30 min. The pellet was sonicated and centrifuged again. The resulting pellet (total membrane) was resuspended in the homogenization buffer. The supernatant (cytosol) in the first centrifugation was supplemented with concentrated lysis buffer for SDS-PAGE. The total membrane was also supplemented with the concentrated lysis buffer, and its volume was normalized to the volume of the supernatant.

Immunofluorescence Microscopy—The transfected HeLa cells were washed with ice-cold phosphate-buffered saline three times and fixed in 4% paraformaldehyde in phosphate-buffered saline for 10 min. After fixation, the cells were permeabilized with 0.2% Triton X-100 in TBS for 5 min, quenched in 0.1% sodium borohydride in TBS for 5 min, and then blocked with 10% goat serum, 1% bovine serum albumin (Amersham Biosciences) in TBS at room temperature for 60 min. The cells were incubated overnight with first antibodies diluted in 1% bovine serum albumin at 4 °C. After washing the cells three times with TBS, they were incubated with the second antibodies diluted in 1% bovine serum albumin for 1 h and then washed three times with TBS. Finally, images were obtained using a PL APO ×63/1.40 oil immersion objective under

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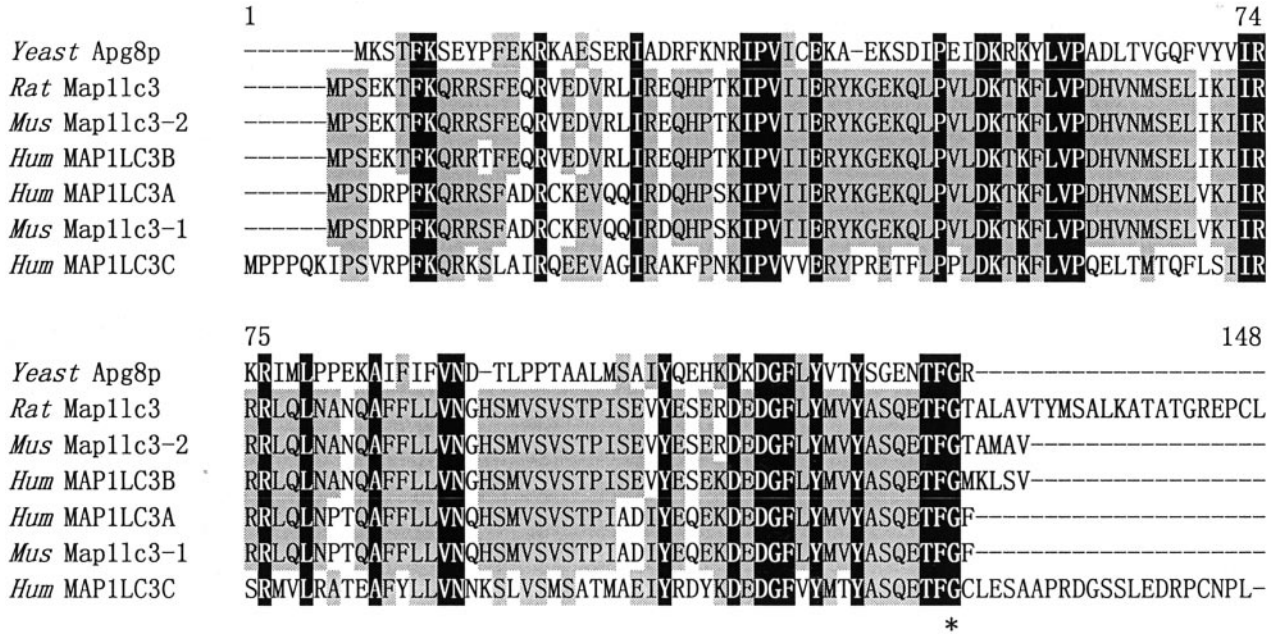

FIG. 1. **Sequence comparison of human MAP1LC3 and their homologues.** Comparison of the deduced amino acid sequences of human MAP1LC3A, MAP1LC3B, and MAP1LC3C and their homologues (including human MAP1LC3A, MAP1LC3B, and MAP1LC3C, rat Map1LC3, mouse Map1lc3–1 and Map1lc3–2, and yeast Apg8p) is shown. Identical residues among the yeast Apg8p and other homologues are *shaded* in *black*. Identical residues among the six members of the mammalian MAP1LC3 family are *shaded* in *gray*. The conserved glycine is marked with an *asterisk*. *MAP1LC3A*, AF276658; *MAP1LC3B*, AF087871; *MAP1LC3C*, AF276659.

a Leica TCS-NT laser scanning microscope system and processed with Adobe Photoshop software.

RESULTS

Identification, Isolation, and Sequence Comparison of Three Different Isoforms of Human MAP1LC3—To identify human homologues of the rat Map1LC3, we searched the human EST data base with the amino acid sequence of rat Map1LC3 and obtained three human EST contigs with lengths of 1.0 kb, 2.0 kb and 500 bp, respectively. A fragment was amplified by PCR from the human heart cDNA library with primers LC3A-A and LC3A-B. A second specific fragment was amplified from the human testis cDNA library with primers LC3B-A and LC3B-B. A third specific fragment was amplified from the human placenta cDNA library with primers LC3C-A and LC3C-B. The three DNA fragments were sequenced and confirmed to be identical to the corresponding contig sequences. These three cDNAs were named *MAP1LC3A* (AF276658), *MAP1LC3B* (AF087871), and *MAP1LC3C* (AF276659). Conceptual translation of each open reading frame yielded three putative polypeptides containing 121, 125, and 147 amino acids for MAP1LC3A, MAP1LC3B, and MAP1LC3C, respectively. Sequence comparison revealed a high degree of sequence similarity of the three human proteins to the rat Map1LC3 (Fig. 1). The predicted amino acid sequences of MAP1LC3A, MAP1LC3B, and MAP1LC3C show 81, 94, and 55% identity and 91, 96, and 72% similarity to rat Map1LC3, respectively. In particular, the region corresponding to Tyr-113-Gly-120 of rat Map1LC3 is highly conserved among all three human proteins and rat Map1LC3. Although a stretch of amino acids N-terminal to Gly-120 showed a high degree of sequence similarity among the rat Map11C3 and human MAP1LC3A-C, the C-terminal tails diverge in sequences.

Distinct Tissue Distribution of MAP1LC3A, MAP1LC3B, and MAP1LC3C mRNAs—The existence of three isoforms of human MAP1LC3 raised the question of whether they play redundant roles in different tissues and cell types. We used Northern blot analysis to determine the tissue distribution of the three isoforms of human MAP1LC3. *MAP1LC3A* and *MAP1LC3C* were detected as a single transcript of 1.1 and 1.9 kb, respectively. *MAP1LC3B* was detected as two transcripts of 2.5 and 1.0 kb, which correlates with two poly(A) signals found in its cDNA sequence (data not shown). The three isoforms of MAP1LC3 showed distinct patterns of expression in different human tissues (Fig. 2*A*). *MAP1LC3A* is most abundantly expressed in the heart, brain, liver, skeletal muscle, and testis but is absent in thymus and peripheral blood leukocytes. Like *MAP1LC3A*, abundant expression of *MAP1LC3B* is also seen in heart, brain, skeletal muscle, and testis. *MAP1LC3B* is least abundant in the liver, among other tissues, where *MAP1LC3A* is most abundant. Furthermore, *MAP1LC3B* is expressed at a high level in peripheral blood leukocytes in which *MAP1LC3A* mRNA is absent. In comparison with *MAP1LC3A* and *MAP1LC3B*, *MAP1LC3C* expression is much lower in all tissues. Its highest level of expression is seen in placenta, lung, and ovary (Fig. 2*B*).

Expression of the Human MAP1LC3 Isoforms in HEK293 and E. coli cells—To characterize the three human MAP1LC3 isoforms, the cDNA of rat *MAP1LC3* and human *MAP1LC3A*/*MAP1LC3B*/*MAP1LC3C* were cloned into the eukaryotic expression vector pCMV-Myc, and the four fusion proteins containing an N-terminal c-Myc epitope tag were expressed in HEK293 cells. When using the anti-Myc monoclonal antibody, Myc-tagged rat MAP1LC3 was detected as two bands migrating at about 18 kDa (termed RLC3-I) and 15 kDa (termed RLC3-II). Like rat MAP1LC3, we also detected tagged MAP1LC3A as two bands migrating at about 18 kDa (termed LC3A-I) and 15 kDa (termed LC3A-II) (Fig. 3*B*, *lane 1*). Myc-tagged MAP1LC3C was detected as two bands migrating at about 16 kDa (termed LC3C-I) and 15.5 kDa (termed LC3C-II) (Fig. 3*B*, *lane 7*). The amounts of RLC3-I, LC3A-I, and LC3C-I were more abundant than that of RLC3-II, LC3A-II, and LC3C-II. However, Myc-tagged MAP1LC3B was detected as a single band migrating at about 16 kDa (termed LC3B) (Fig. 3*B*, *lane 4*).

It is known that apg8 or rat LC3 undergo post-translational

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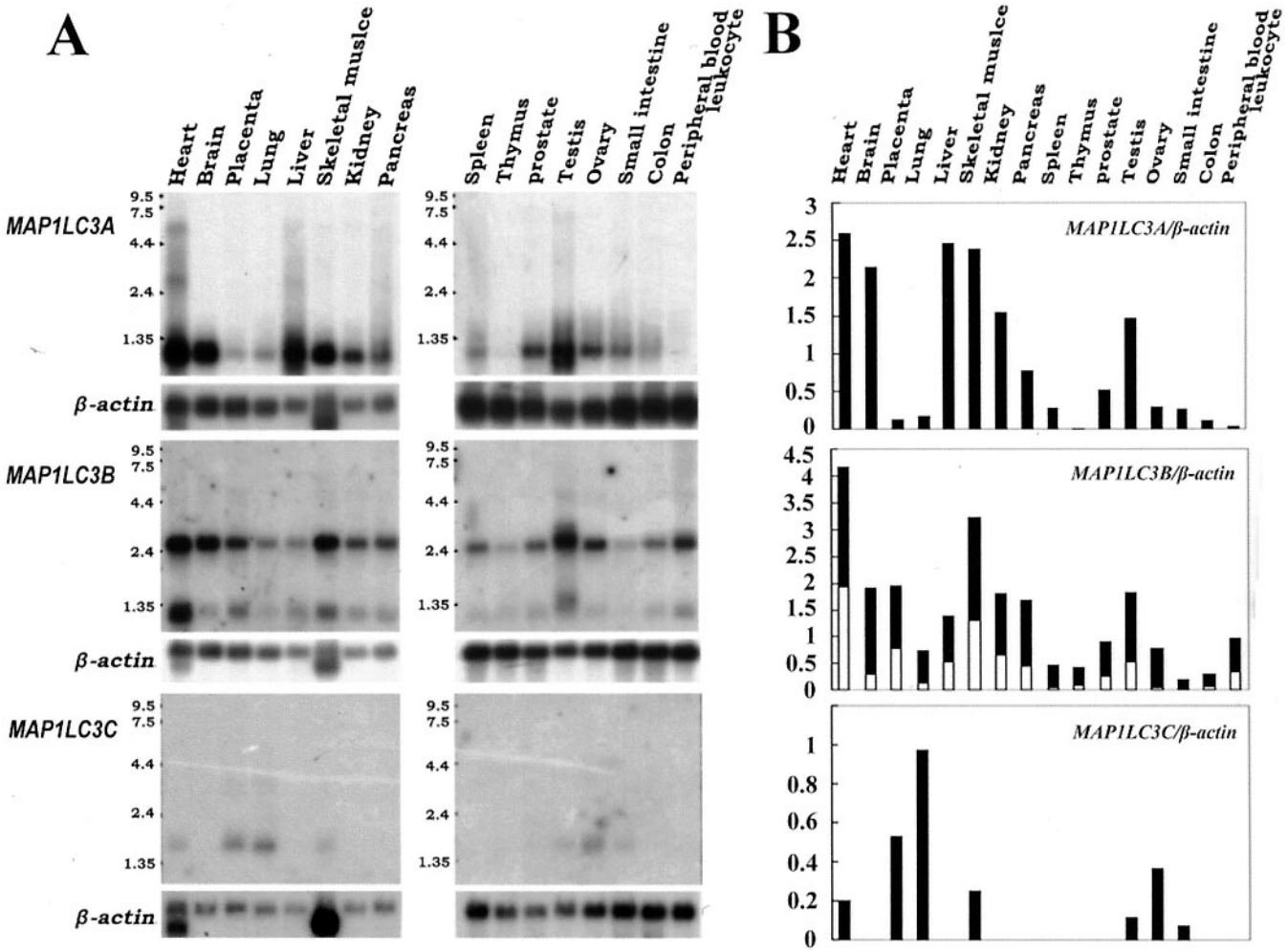

FIG. 2. **Northern blot analysis of MAP1LC3A, MAP1LC3B, and MAP1LC3C in human tissues and comparison of their tissue expression patterns.** The molecular sizes of markers (*kb*) are indicated at the *top left* of *panel A*. The tissue names are indicated *above* the *panels*. *A*, MTN I and MTN II were hybridized with *MAP1LC3A*, *MAP1LC3B*, and *MAP1LC3C* cDNA probes, respectively. The same membranes, after stripping, were also hybridized with β-actin as a loading control. *B*, quantitation of the ratios of intensities of three *MAP1LC3A*, *MAP1LC3B*, and *MAP1LC3C* to β-actin. The intensities of β-actin expression in heart and skeletal muscle are represented with the average of those in brain, placenta, lung, liver, kidney, and pancreas. Both long (*black column*) and short forms (*white column*) of *MAP1LC3B* of are included (see *middle panel*).

modification *in vivo* (9, 10, 11). To determine whether the three human MAP1LC3A/MAP1LC3B/MAP1LC3C expressed in HEK293 cells undergo similar post-translational modifications, the three Myc-tagged orthologs were cloned into the pGEX4T-1 vector and expressed in the *E. coli* (products termed reLC3A, reLC3B, and reLC3C), and the mobility of recombinant proteins and their counterparts expressed in HEK293 cells was compared by immunoblot analysis. As shown in Fig. 3*B*, the mobility of reLC3A is almost identical to that of LC3A-I but slower than LC3A-II. In comparison with reLC3C both wild type and mutant LC3C expressed in HEK293 cells migrate faster than reLC3C. As for as LC3B, the mobility of reLC3B is nearly the same as that of LC3B. There is thus clear difference in gel mobility between recombinant proteins and their counterparts expressed in mammalian cells for LC3A and LC3C. The similar gel mobility between recombinant LC3B expressed in bacteria and HEK293 cells suggest that either post-translational modification of LC3B in HEK293 cells does not change its gel mobility, or it does not undergo post-translational modifications. As a control, we also expressed Myc-tagged ratLC3 in *E. coli* (termed reRLC3) and compared its gel mobility with that for the same protein expressed in HEK293 cells. Similar to LC3A, ratLC3 exists in two forms, a predominant slow migrating form and a minor faster migrating form. The gel mobility of recombinant LC3 purified from bacteria is similar to the slow migrating form.

The C-terminal Regions beyond Gly-120 Is Essential for Processing of MAP1LC3B, but Not MAP1LC3A and MAP1LC3C—According to Kabeya *et al.* (10, 11), both mature yeast Apg8p and rat Map1LC3 proteins exist as two forms lacking the C-terminal region beyond the conserved Gly after the conserved post-translational cleavage modification. The appearance of two protein bands of overexpressed MAP1LC3A and MAP1LC3C prompted us to determine whether MAP1LC3A and MAP1LC3C might undergo similar post-translational modifications as yeast Apg8p and rat Map1LC3, given the presence of a highly conserved region upstream the C-terminal Gly-120 in both MAP1LC3A and MAP1LC3C. Although the overexpressed MAP1LC3B is detected as a single band, the conserved region upstream the Gly-120 is also conserved in the C terminus of MAP1LC3B, raising the possibility that MAP1LC3B protein may undergo similar post-translational processing.

To investigate whether the C-terminal regions beyond Gly-120 present in the three isoforms of human MAP1LC3 are involved in the post-translational processing, deletion mutants

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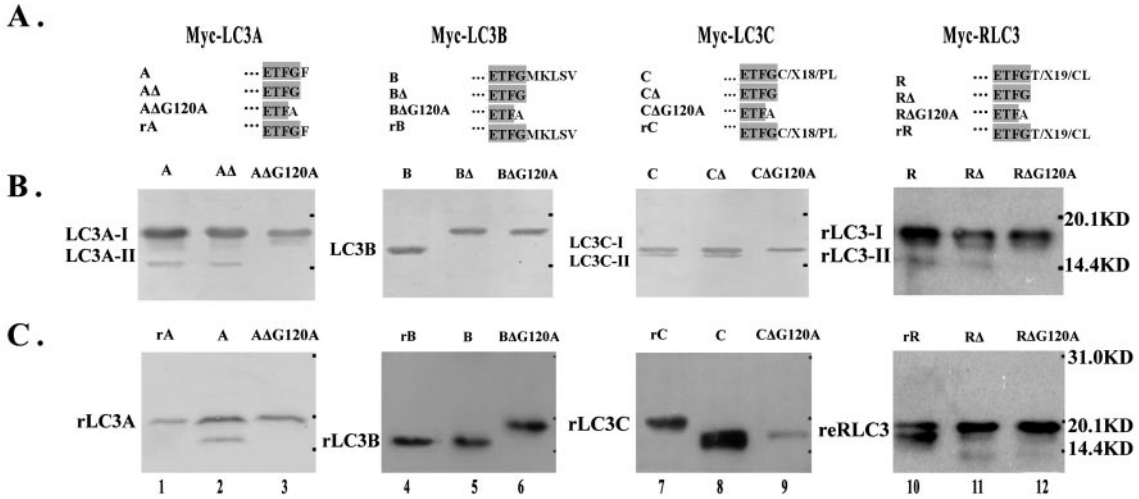

FIG. 3. **Expression of myc-tagged *MAP1LC3A*, *MAP1LC3B*, and *MAP1LC3C* and their mutants in HEK293 cells and *E. coli* cells.** *A*, amino acid sequences of the C terminus of rat MAP1LC3, human MAP1LC3A, MAP1LC3B, and MAP1LC3C, and their mutants. Identical residues are *shaded* in *gray*. *B*, HEK293 cells were transiently transfected with expression vectors encoding Myc-LC3A (*lane 1*), Myc-LC3AΔ (*lane 2*), Myc-LC3AΔG120A (lane3), Myc-LC3B (*lane 4*), Myc-LC3BΔ (*lane 5*), Myc-LC3BΔG120A (*lane 6*), Myc-LC3C (*lane 7*), Myc-LC3CΔ (*lane 8*), Myc-LC3CΔG120A (*lane 9*), Myc-rat LC3 (*lane 10*), Myc-rat LC3Δ (*lane 11*), and Myc-rat LC3ΔG120A (*lane 12*). *C*, the proteins were expressed and purified in *E. coli* cells encoding Myc-LC3A (*lane 1*), Myc-LC3B (*lane 4*), Myc-LC3C (*lane 7*), and Myc-rat LC3 (*lane 10*), and HEK293 cells were transiently transfected with expression vectors encoding Myc-LC3A (*lane 2*), Myc-LC3AΔG120A (*lane 3*), Myc-LC3B (*lane 5*), Myc-LC3BΔG120A (*lane 6*), Myc-LC3C (*lane 8*), Myc-LC3CΔG120A (*lane 9*), Myc-rat LC3 (*lane 11*), and Myc-rat LC3ΔG120A (*lane 12*). The cell lysates were analyzed by Western blotting with anti-Myc antibodies.

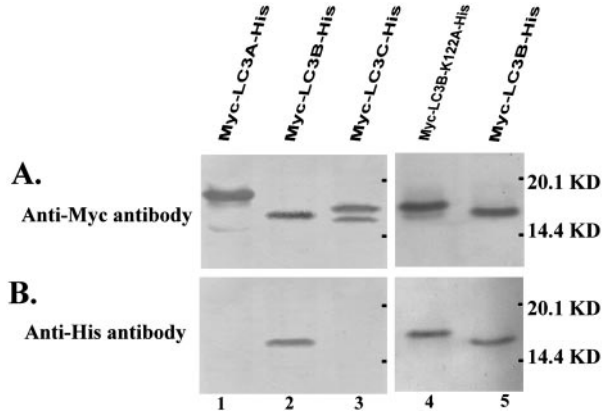

FIG. 4. **C-terminal cleavage occurred in human MAP1LC3A and MAP1LC3C but not in MAP1LC3B.** HEK293 cells were transiently transfected with Myc-LC3A-His (*lane 1*), Myc-LC3B-His (*lanes 2* and *4*), Myc-LC3C-His (*lane 3*), and Myc-LC3BK122A-His (*lane 5*). The cell lysates were analyzed by Western blot analysis with an anti-Myc antibody (*A*) or anti-His antibody (*B*).

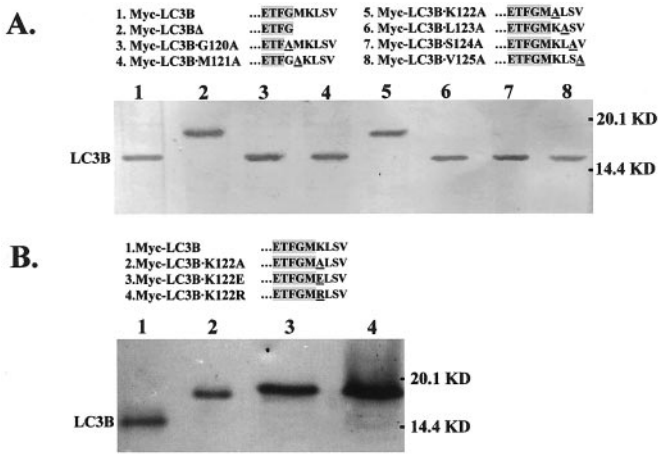

FIG. 5. **The amino acid Lys-122 downstream of Gly-120 is required for the processing of MAP1LC3B.** Identical residues of amino acid sequence of the C-terminal segments of MAP1LC3B are *shaded* in *gray*. The mutated residue is *underlined* in *A* and *B*. *A*, HEK293 cells were transiently transfected with Myc-LC3B (*lane 1*), Myc-LC3BΔ (*lane 2*), Myc-LC3B·G120A (*lane 3*), Myc-LC3B·M120A (*lane 4*), Myc-LC3B·K120A (*lane 5*), Myc-LC3B·L120A (*lane 6*), Myc-LC3B·S120A (*lane 7*), and Myc-LC3B·V120A (*lane 8*). *B*, HEK293 cells were transiently transfected with Myc-LC3B (*lane 1*), Myc-LC3BK122A (*lane 2*), Myc-LC3BK122E (*lane 3*), and Myc-LC3BK122R (*lane 4*). The cell lysates were analyzed by Western blot with anti-Myc antibodies.

of *MAP1LC3A*, *MAP1LC3B*, and *MAP1LC3C* lacking sequences C-terminal to the conserved Gly were constructed in pCMV-Myc vector (named Myc-LC3AΔ, Myc-LC3BΔ, and Myc-LC3CΔ). Using Western blot analysis, we found the protein expression pattern of Myc-LC3AΔ (Fig. 3*B*, *lane 2*) and Myc-LC3CΔ (Fig. 3*B*, *lane 8*) is the same as that of wild-type MAP1LC3A (Fig. 3*B*, *lane 1*) and MAP1LC3C (Fig. 3*B*, *lane 7*), which agrees with the observation by Kabeya *et al.* (10, 11) that loss of carboxyl region of rat Map1LC3 beyond Gly-120 does not affect its mobility on SDS-PAGE gels. In contrast, when a similar deletion mutant of Myc-LC3B was expressed, a new band emerged with a much slower mobility of about 18 kDa (Fig. 3*B*, *lane 5*). This was surprising, as we had expected no change in mobility of the mutant protein. There are two possible explanations for the increase in the apparent molecular mass of the MAP1LC3B deletion mutant. One possibility is that the 16-kDa LC3B (Fig. 3*B*, *lane 4*) represents a post-translationally processed form of MAP1LC3B, and the processing of full-length MAP1LC3B requires C-terminal region beyond Gly-120. The other possibility is that deletion of the five amino acids C-terminal Gly-120 of MAP1LC3B led to a new modification of the newly exposed Gly-120 in the mutant that is absent in the wild type protein. To distinguish between these possibilities, we constructed another mutant of MAP1LC3B by replacing the exposed Gly-120 with Ala in the deletion mutant and analyzed the mutant upon its expression in mammalian cells. The Myc-LC3BΔG120A mutant has the same mobility as Myc-LC3BΔ protein (Fig. 3*B*, *lane 6*), ruling out the possibility that the newly exposed Gly-120 in Myc-LC3BΔ is responsible for the change of its mobility.

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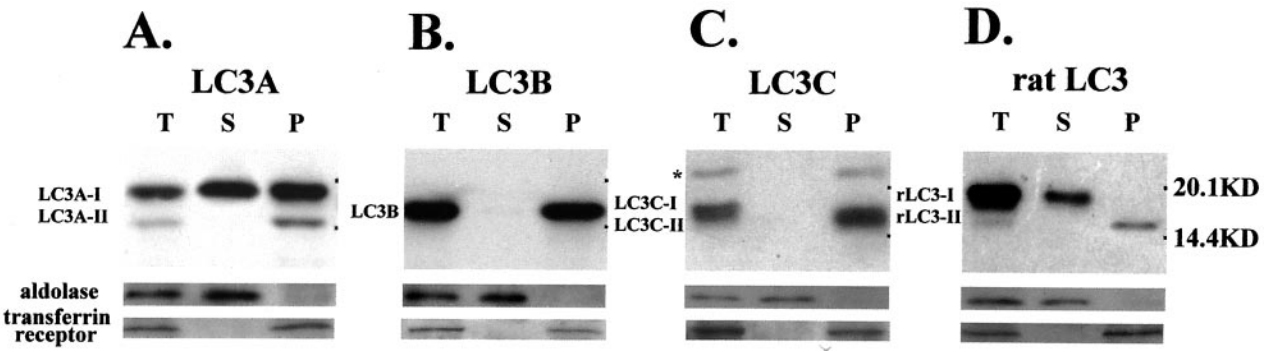

FIG. 6. **Subcellular distribution of rat MAP1LC3 and three human homologues.** HeLa cells (*T*) transfected with *myc-LC3A* (*A*), *myc-LC3B* (*B*), *myc-LC3* (*C*), and *myc-rat LC3* (*D*) were homogenized and fractionated into supernatant (*S*) and pellet (*P*) by ultracentrifugation. These fractions were analyzed by Western blotting using antibodies against Myc tag, transferrin receptor (as a membrane protein marker), and aldolase (as a cytosolic marker). The *asterisk* indicates that the band only existed in the HeLa cells transfected with Myc-LC3C.

C-terminal Cleavage Occurs in MAP1LC3A and MAP1LC3C but Not MAP1LC3B—To further investigate whether the characteristic C-terminal cleavage happens to all three isoforms of human MAP1LC3, we reconstructed MAP1LC3A/MAP1LC3B/MAP1LC3C in pCMV-Myc vector by adding a His_6 tag to their C terminus, with the corresponding proteins dubbed Myc-LC3A/Myc-LC3B/Myc-LC3C-His. In HEK293 cells overexpressing Myc-LC3A/Myc-LC3C-His, two corresponding bands were detectable with anti-Myc antibodies (Fig. 4*A*, *lanes 1* and *3*). As expected, the C-terminal His_6 tag was removed from both fusion proteins upon expression, as judged by the lack of detection using the anti-His antibody (Fig. 4*B*, *lanes 1* and *3*). The results clearly demonstrated that the C-terminal cleavage occurred in MAP1LC3A and MAP1LC3C, as has been observed for the rat Map1LC3. In contrast, in HEK293 cells overexpressing Myc-LC3B-His, a 16-kDa band could be detected with both anti-Myc antibody (Fig. 4*A*, *lane 2*) and anti-His antibodies (Fig. 4*B*, *lane 2*), which indicated that the characteristic C-terminal cleavage does not occur in human MAP1LC3B.

The C-terminal Conserved Gly-120 Is Crucial for Post-translational Modifications of MAP1LC3A and MAP1LC3C but Not MAP1LC3B—The only known residue for post-translational modifications of Apg8p and rat Map1LC3 is the conserved Gly-120, which is also present in MAP1LC3B. It has been shown that mutation of Gly-120 into an alanine in Map1LC3 resulted in the abrogation of its processing (10, 11). To further delineate the difference in C-terminal processing between MAP1LC3B and the other two MAP1LC3 isoforms, we constructed ΔGly120A mutant of each protein and determined the effect of this mutation on their processing. Similar to rat Map1LC3, both Myc-LC3AΔG120A and Myc-LC3CΔG120A were found to be expressed as a single band with an apparent mobility similar to that of LC3A-I and LC3C-I, respectively (Fig. 3*B*, *lanes 3* and *9*), whereas LC3A-II and LC3C-II were not detectable. These results are in agreement with the obligatory role of Gly-120 in the subsequent modification by phosphatidylethanolamine to give rise to the mature autophagosome-associated LC3A-II and LC3C-II. When the Myc-LC3BΔG120A mutant was overexpressed, however, there was no further change in mobility in comparison with the deletion mutant Myc-LC3BΔ (Fig. 3*B*, *lane 6*). To further determine the importance of Gly-120 in the post-translational processing of MAP1LC3B, we mutated it to an alanine in the full-length protein and expressed the mutant in HEK293 cells. The Myc-LC3B·G120A mutant has the same gel mobility as the wild type protein LC3B (Fig. 5*A*, *lane 3*). These results indicated that the conserved Gly was not essential for the post-translational processing of MAP1LC3B.

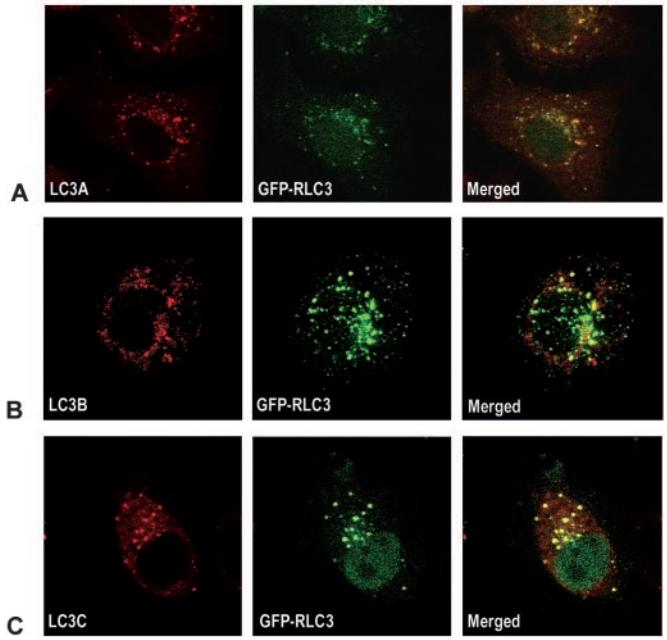

FIG. 7. **The three human isoforms are localized in the autophagosomes.** HeLa cells cotransfected with *GFP-rat LC3* and *MAP1LC3A* (*A*), *MAP1LC3B* (*B*), or *MAP1LC3C* (*C*) were induced with Hanks' solution at 37 °C for 1.5 h. Then the cells were fixed, permeabilized, and observed in the immunofluorescence confocal microscopy using a Cy5-conjugated second antibody. The three fields of *A*, *B*, *C*, and *D* are the same.

Lys-122 Is Essential for Post-translational Modification of Human MAP1LC3B—Having ruled out Gly-120 as an essential residue for the post-translational processing of MAP1LC3B, we wondered which other residues of the protein might take on its role. Based on the observation that Myc-LC3BΔ mutant migrates with a markedly reduced mobility in SDS-PAGE compared with wild type LC3B protein, we considered the possibility that one or more of the five amino acids C-terminal to Gly-120 may be involved in the post-translational processing of MAP1LC3B. To identify the residue(s) involved, we systematically mutated each of the C-terminal five amino acids into an alanine and determined the gel mobility upon overexpression of each mutant in HEK293 cells. Of the five mutants, only one, K122A, exhibited slower mobility with an apparent molecular mass of 18 kDa in comparison with the 16-kDa wild type protein LC3B (Fig. 5*A*). When Lys-122 residual was mutated to glutamate or arginine, the mobility of two mutants is similar to

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the K122A mutant (Fig. 5*B*). These results clearly demonstrate that MAP1LC3B is post-translationally processed in a manner distinct from other members of this family of proteins, and Lys-122, rather than Gly-120, is required for its processing.

Three Human Isoforms of LC3 Homologues Are Associated with the Autophagic Membranes—It is known that Apg8/Aut7p and rat LC3 are located in the autophagic membrane and exhibit a punctate pattern in cells that increase in both number and fluorescence intensity when cells are under the condition of amino acid starvation (10, 26). To further discern the functional similarity and differences between the three MAP1LC3 isoforms and the rat LC3 or yeast Apg8, we determined the subcellular localization of these isoforms by both cell fractionation and fluorescence under normal and stress conditions.

We transiently expressed the three human LC3 homologues in HeLa cells and fractionated the cell homogenates into supernatant and pellet by ultracentrifugation. The presence or absence of the protein in each fraction were determined by immunoblot analysis. As shown in Fig. 6, for MAP1LC3A, the LC3A-II appeared only in the pellet, whereas the LC3A-I was found in both the membrane and the cytosolic fractions. The two bands of MAP1LC3C and MAP1LC3B were only detected in the membrane fraction. Interestingly, we also found one band at about 23 kDa that only existed in the total cell lysate and the pellet when MAP1LC3C was expressed in HeLa cells, but it was not detected in HEK293 cells. When the pellets were sonicated with higher intensity or washed with 0.5 M EDTA, 2 M NaCl (data not shown), the proteins in pellet were not solubilized except LC3A-I and rat LC3-I, suggesting that MAP1LC3A/MAP1LC3B/MAP1LC3C were tightly associated with membranes.

Rat LC3 is the only known mammalian protein present in the autophagosomal membranes in mammalian cells, and it has been used as the marker of autophagosome membranes (10, 26). We further examined the subcellular localization of three human isoforms by indirect immunofluorescence with GFP-rat LC3 fusion protein as a reference. As shown in Fig. 7, all three human isoforms of LC3 are colocalized with GFP-ratLC3, either completely or partially. Both LC3A and LC3C exhibit a nearly identical subcellular localization pattern as GFP-ratLC3. In contrast, the subcellular distribution of LC3B only partially overlaps with GFP-ratLC3. Unlike LC3A, LC3C, or rat LC3, LC3B does not appear in a dotted pattern. Instead, it seems to be localized to some organelle membrane in the cytosol. These observations suggest that LC3B may play a role that is distinct from rat LC3 or LC3A/LC3C in autophagy.

Because LC3A and LC3C are associated with the autophagosome membrane, and autophagy is stimulated by amino acid deprivation, we determined whether their post-translational modification patterns and subcellular location changed under the condition of amino acid starvation. We thus expressed GFP fusion proteins of MAP1LC3A-C, expressed them in HeLa cells, and determined their cellular localization under normal and stress (by exposure to Hank's solution) conditions. Although we found no significant change in the subcellular distribution for MAP1LC3A and MAP1LC3C, MAP1LC3B distribution changed from what appeared to be a Golgi/perinuclear pattern to a dotted autophagosome pattern (Fig. 8). These results suggest that MAP1LC3B is regulated differently from MAP1LC3A or MAP1LC3C and that MAP1LC3B may have distinct functions from the other two human isoforms of MAP1LC3.

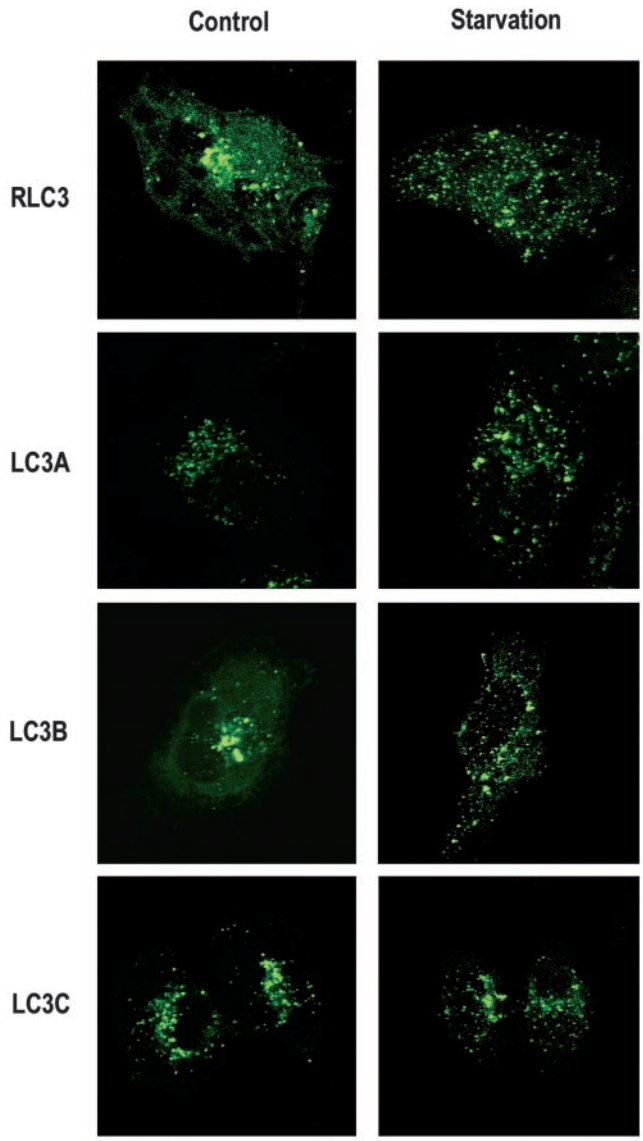

FIG. 8. **The punctuated patterns of the three human isoforms corresponding to amino acid starvation in HeLa cells.** HeLa cells were transiently incubated in full media or in Hanks' solution at 37 °C for 90 min 48 h after transfection with GFP-LC3A, GFP-LC3B, and GFP-LC3C, and then the cells were fixed and observed by immunofluorescence confocal microscopy.

DISCUSSION

The rat Map1LC3 was the first mammalian homologue of the yeast autophagosome-associated protein Apg8 to be identified besides GATE-16 (Golgi-associated ATPase enhancer of 16 kDa) and GABARAP ($GABA_A$ receptor-associated protein) (10, 17–20). The significant sequence similarity between the rat and yeast proteins suggested that the molecular machinery involved in autophagy is highly conserved in eukaryotes. In this study we identified and isolated three human homologues of the rat Map1LC3, named *MAP1LC3A*, *MAP1LC3B*, and *MAP1LC3C*. In comparison with the four members of human GABARAP family (17, 21), the three isoforms of human MAP1LC3 are highly homologous to the rat Map1LC3, implying a similar function for these conserved proteins in both microtubule binding and autophagosome membrane association.

As a first step to unravel the functional similarities and differences among the three isoforms of human MAP1LC3, we assessed the patterns of expression of the three MAP1LC3 isoforms in different human tissues by Northern blot. Whereas some tissues including the heart, brain, skeletal muscle, and testis express both *MAP1LC3A* and *MAP1LC3B*, other tissues such as peripheral blood leukocytes only express one but not the other isoforms. Unlike *MAP1LC3A* and *MAP1LC3B*,

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MAP1LC3C is expressed at a much lower level in all the tissues examined. These observations suggest that these isoforms may play redundant or distinct roles depending on the tissue or cell type.

The existence of two forms of the rat Map1LC3 and its unique post-translational modification including a proteolytic cleavage of its C terminus prompted us to determine whether the same post-translational modifications occur to the three isoforms of human MAP1LC3. We thus expressed the MAP1LC3 isoforms in HEK293 cells and *E. coli* cells with each protein tagged at its N terminus with a Myc epitope to facilitate the detection of the expressed proteins. Like the rat Map1LC3, both human MAP1LC3A and MAP1LC3C were found to exist in two forms, corresponding to the higher molecular mass cytosolic form and a lower molecular mass membrane-associated form. However, MAP1LC3B behaved differently from the other two isoforms of MAP1LC3 in a number of ways. First, both MAP1LC3A and MAP1LC3C are present in doublet whereas MAP1LC3B is present as a single protein band as detected by Western blotting (Fig. 2). Second, MAP1LC3B appeared to undergo a different type of post-translational processing. For MAP1LC3A and MAP1LC3C, a characteristic C-terminal cleavage occurs consistent with the established mechanism for the yeast Apg8p and rat Map1LC3. In contrast, MAP1LC3B does not undergo proteolytic cleavage of it C terminus (Fig. 4). Third, a conserved glycine is required for the post-translational processing of both MAP1LC3A and MAP1LC3C, similar to the rat Map1LC3. But for MAP1LC3B, the mutation of the corresponding glycine (Gly-120) has no effect on its mobility on SDS-PAGE gels. These differences are unexpected, as MAP1LC3B possesses the highest sequence similarity to the rat Map1LC3 among the three isoforms of human MAP1LC3.

Using alanine-scanning mutagenesis of the six amino acids from Gly-120 to the C terminus of MAP1LC3B, we found that only mutation of Lys-122 altered the gel mobility of the protein in comparison with the wild type protein LC3B, and the difference exists even if Lys-122 is mutated to other amino acids. A similar change in gel mobility was observed with the truncation mutant in which the C-terminal five amino acids including Lys-122 were deleted (Fig. 5*B*). It is formally possible that deletion or mutation of a lysine residue can cause a gel mobility shift because of elimination of a positive charge on the protein. This possibility is ruled out by the similar gel mobility shift observed with the K122R mutant, in which the positive charge on the side chain of lysine is retained in arginine (Fig. 5*B*). Moreover, the characteristic C-terminal cleavage did not occur when we expressed the Myc-LC3B-K122A-His in the HEK293 cells. Together with the observation that MAP1LC3B is associated with membrane, these results strongly suggest that Lys-122 undergoes post-translational modification to render it to bind to membranes. Although the precise role of Lys-122 in the post-translational processing of MAP1LC3B remains to be elucidated, a few models are conceivable. The lysine side chain amino group is known to be able to undergo several types of post-translational modifications including acetylation, methylation and ubiquitination, sumoylation, heparin modification, retinylidene modification, and glycosaminoglycan modification (22, 27–31). It is also possible that Lys-122 plays a requisite structural role in the post-translational modification of MAP1LC3B but does not serve as the site of modification itself. It is noteworthy that of all the proteins of the MAP1LC3 superfamily from different species, Lys-122 is unique to MAP1LC3B and is absent in either MAP1LC3A or MAP1LC3C, which may underlie its distinct post-translational processing.

As rat LC3 has been shown to be localized in the autophagosome membrane, we determined the membrane association and subcellular localization of the three human homologues by cell fractionation and immunofluorescence using rat LC3 as a positive control. MAP1LC3A, MAP1LC3B, and MAP1LC3C are all found in the membrane fraction (Fig. 6), suggesting that they are capable of associating with membranes. When the subcellular localization of the three human LC3 homologues were determined by indirect immunofluorescence, however, differences were found between MAP1LC3B and the other two homologues. Whereas Map1LC3A and Map1LC3C are colocalized with GFP-ratLC3, Map1LC3B is localized to some other subcellular compartment yet to be identified (Fig. 7). This difference in subcellular localization is consistent with the difference in post-translational modification between the Map1LC3B and the other two homologues.

Considering the difference of MAP1LC3A/MAP1LC3B/MAP1LC3C in their post-translational modifications, we speculate that the human MAP1LC3 family members may differ in their physiological functions. In addition to its association with the autophagosome membrane and the microtubules (9, 15), the rat Map1LC3 has also been identified as a 16-kDa fibronectin adenosine-uracil rich element-dependent RNA-binding protein that mediates fibronectin mRNA translation via its interaction with microtubules (23, 24). Thus, the MAP1LC3 family of proteins has multiple functions. It remains to be seen whether the human MAP1LC3A/MAP1LC3B/MAP1LC3C retained or shared the same functions as the rat Map1LC3 or have evolved new functions. The distinct post-translational processing and response to starvation of MAP1LC3B suggests that it may have different functions from MAP1LC3A and MAP1LC3C. The identification and isolation of the three human isoforms of the MAP1LC3 family of cDNAs and the discovery of the unique post-translational modification of MAP1LC3B will facilitate the elucidation of their biological functions in autophagy, protein translation, and other cellular processes.

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