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Molecular cloning and characterization of rat *LC3A* and *LC3B*—Two novel markers of autophagosome

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Abstract

Rat microtubule-associated protein light chain 3 (LC3) is a homologue of yeast Atg8, an essential component of autophagy. Following synthesis, the C-terminus of rat LC3 is cleaved by a cysteine protease-Atg4, to produce LC3-I, which is located in cytosolic fraction. LC3-I can be converted to LC3-II through the processing by Atg7 (E1-like enzyme) and Atg3 (E2-like enzyme). LC3-II is modified by phosphatidylethanolamine on C-terminus and binds tightly to autophagosomal membrane. Here we reported the cloning of two novel variants of rat *LC3*, named *LC3A* and *LC3B*, respectively, and *LC3B* is an alternative splicing variant of *LC3*. LC3A, LC3B, and LC3 showed different expression patterns in rat tissues, suggesting a functional divergence among these proteins. When LC3A and LC3B were overexpressed, both exhibited two forms (18 and 16 kDa, representing types of I and II, separately), which might be due to post-translational modification including the characteristic C-terminal cleavage at these two proteins as similar to that found in rat LC3 and yeast Atg8. Subcellular localization demonstrated that both LC3A and LC3B are colocalized with LC3 and associated with the autophagic membranes. Mutation analysis further revealed that the conserved Gly120 residues of LC3A and LC3B are essential for their characteristic C-terminal cleavage and localization to autophagic membranes. Present data suggested that LC3A and LC3B could also be used as two novel autophagosomal markers.

Keywords: Rat *LC3*; *LC3A*; *LC3B*; Post-translational modification; Subcellular localization; Autophagy

Autophagy is a highly regulated process for bulk degradation of proteins and organelles, and it has been shown to be essential for differentiation and development as well as for cellular maintenance (reviewed in [1,2]). Light chain 3 (LC3) was identified originally as a protein co-purified with microtubule-associated proteins (MAPs)—MAP1A and MAP1B. And LC3 was regarded as a common subunit of both MAPs [3–5]. Rat LC3 shows 28% amino acid identity with yeast Apg8/Aut7/Cvt5 (Atg8), which is a crucial factor for yeast autophagy [6]. Rat LC3 was proposed to be a homologue of yeast Atg8 and a novel constituent of autophagosomal membrane [7]. Following synthesis, the C-terminal fragment of rat LC3 is cleaved immediately by Atg4, a cysteine protease. The cleavage yields its cytosolic form LC3-I and exposes the carboxyl terminal Gly [8–10]. LC3-I is further activated by Atg7 (an E1-like enzyme), transferred to Atg3 (an E2-like enzyme), and finally modified to a membrane-bound form, LC3-II [11,12]. LC3-II is localized to autophagosomal membranes and made this protein an autophagosomal marker [7]. LC3-II also has been identified to be a phosphatidylethanolamine (PE)-conjugated form (LC3-PE) [10], like its yeast homologue Atg8 (Atg8-PE) [13]. LC3-PE is in turn delipidated by Atg4B [10]. Recently, the solution structure of LC3-I was determined and it was divided into N- and C-terminal subdomains. The N-terminal subdomain of LC3 is essential for its binding of tubulin and microtubules, suggesting that LC3 can act as an adaptor protein between microtubules and autophagosomes [14].

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In addition, Rat LC3 was found to bind to an adenosine-uracil rich element (ARE) in the 39-untranslated region (39-UTR) of fibronectin mRNA and it is identified as a Fibronectin mRNA-binding protein [15]. LC3 can facilitate fibronectin mRNA recruitment to membrane-bound polyribosomes in vascular smooth muscle cells [16]. Rat LC3 was reported to interact with Sos1, a guanine nucleotide exchange factor for Ras, and thereby it negatively regulates the Sos1-dependent Rac1 activation, leading to membrane ruffling [17].

Previously we identified three human LC3 family members (LC3A, LC3B, and LC3C) and detected a novel type of modification for human LC3B based on Lys121 of its non-cleavable C-terminus [18]. However, Tanida et al. [19] reported that human LC3B undergoes the characteristic modifications triggered by C-terminal cleavage after Gly120. Recently we demonstrated that alternative splicing generates two LC3B isoforms with or without an Arg (designated as LC3B-α and LC3B-β, respectively), and showed that LC3B-α and LC3B-β have different pathway of modifications based on the cleavable or non-cleavable C-teminus (unpublished paper). In fact, Tanida et al. and we studied LC3B-α and LC3B-β in a previous study, respectively.

We noticed that in Mann and Hammarback's [5] work, they obtained two bovine amino acid sequences by automated Edman degradation approach. But interestingly, the major sequence used to clone the rat *LC3* gene was highly conserved to the N-terminal fragment of human LC3A and the minor sequence is of high similarity to human LC3B. Therefore, we proposed that there is another variant of rat LC3.

Hence, we conducted a series of experiments to verify it. Here, we reported the identification and characterization of two novel rat variants of LC3, named LC3A and LC3B, respectively. The deduced protein of rat LC3A is identical to that of human LC3A. Rat LC3B is an alternative transcript of LC3 and is similar to human LC3B. The three variants of rat LC3 showed different expression patterns in rat. Additionally, rat LC3A and LC3B are modified in a similar manner to the LC3 and are associated with the autophagosomal membrane, and the conserved Gly120 residues are essential for the characteristic C-terminal cleavage and localization to autophagic membranes of LC3A and LC3B. Present data suggested that LC3A and LC3B could also be used as two novel autophagosomal markers.

Materials and methods

Cloning of LC3A and LC3B cDNAs. The amino acid sequences of human LC3A (GenBank Accession No.: AF276658), LC3B (GenBank Accession No.: AF087871) and LC3C (GenBank Accession No.: AF276659) were used to search the rat expressed sequence tag (EST) database at GenBank (http://www.ncbi.nlm.nih.gov). Homologous ESTs were obtained and assembled into three EST contigs and checked manually. Two pairs of primers (named LC3A/B/-A and B; see Table 1) were designed based on the contig sequences and used for PCR amplification, cDNAs reverse transcribed from rat brain total RNA used as templates. PCR amplification conditions were: 1 μl of template was amplified in a 50 μl volume containing 5 μl of 10× PCR buffer, 1 μl of 20 mM dNTPs, 2 U *Taq* polymerase, and 1 μl of 25 mM each specific primer. PCRs were carried out at 94 °C for 5 min, 94 °C (30 s), 55 °C (30 s), and 72 °C (30 s) for a total of 30 cycles, with a final extension at 72 °C for 10 min in a PTC-200 DNA Engine (MJ Research, USA). The PCR products were cloned into the pGEM-T vector and sequenced using the BigDye terminator sequencing kit and ABI377 sequencer (PerkinElmer Life Sciences) according to the manufacturer's instructions.

Northern blotting. To prepare the probes for Northern blot, first, PCR products of the *LC3A* and *LC3B* from rat brain cDNA were purified after electrophoresis in 1% agarose, subsequently radioactively labeled with

Table 1
Nucleotide sequences of primers used for the cloning of rat LC3A, B and the construction of wild type and mutant LC3A, B cDNAs

Primers	Sequences
cDNA cloning primers	
RLC3A-A	5′-TCCGACCGGCCTTTCAAGCAG-3′
RLC3A-B	5′-GAGAACCTGACCAGAACTCCCAG-3′
RLC3B-A	5′-CCATGCCGTCCGAGAAGACCTTC-3′
RLC3B-B	5′-GACCAGCTTCCGCTGGTAACGTC-3′
Gene expression primers	
Myc-RLC3A-A	5′-CG**GAATTC**GGATGCCCTCCGACCGG-3′
Myc-RLC3A-B	5′-CGG**CTCGAG**TCAGAAGCCGAAGG-3′
Myc-RLC3B-A	5′-CG**GAATTC**GGATGCCGTCCGAGAAG-3′
Myc-RLC3B-B	5′-CGG**CTCGAG**TTACACAGCCAGTGC-3′
Myc-RLC3A-his-B	5′-CCG**CTCGAG**TCA*ATGATGATGATGATGATG* GAAGCCGAAGGTTTC-3′
Myc-RLC3B-his-B	5′-CCG**CTCGAG**TCA*ATGATGATGATGATGATG*CACAGCCAGTGCTGT-3′
Mutation analysis primers	
Myc-RLC3AG120A-B	5′-CGG**CTCGAG**TCAGAAGGCGAAGGTTTCTTGGG-3′
Myc-RLC3BG120A-B	5′-CGG**CTCGAG**TTACACAGCCAGTGCTGTCGCGAAC-3′
Myc-RLC3AG120A-His-A	5′-CCTCCCAAGAAACCTTCGCCTTCCATCATCATCATC-3′
Myc-RLC3AG120A-His-B	5′-GATGATGATGATGGAAGGCGAAGGTTTCTTGGGAGG-3′
Myc-RLC3BG120A-His-A	5′-CCCAGGAGACGTTCGCGACAGCACTGGCTGTG-3′
Myc-RLC3BG120A-His-B	5′-CACAGCCAGTGCTGTCGCGAACGTCTCCTGGG-3′

Underlined nucleotides were changed to obtain the desired mutation. Restriction sites are indicated by boldface. His tag is showed by italics. 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.

[α-^{32}P]dATP (Amersham Biosciences) and purified by a Sepharose G50 column. The probes were hybridized to the MTN membranes with mRNA samples from 12 adult rat tissues (Shenzhen King Grace Bbiotechnologies). β-Actin gene probes were also used for hybridization as control.

Plasmid construction and site-directed mutagenesis. We subcloned LC3A and LC3B to pCMV-Myc vector by adding a 6× His tag (CATCATCACCATCACCAT) to their C terminus using the primers Myc-LC3A-A and Myc-LC3A-his-B, Myc-LC3B-A, and Myc-LC3B-his-B, separately. To construct Myc-LC3A-G120A-His and Myc-LC3B-G120A-His, we used site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit was from Stratagene) according to the manufacturer's protocol using desired mutation primers and templates, respectively. We also constructed the wild type Myc-LC3A, Myc-LC3B, and their mutants Myc-LC3A-G120A, Myc-LC3B-G120A, using the indicated primers. All names of the primers and the mutants of amino acids are given in Table 1. All 5′primers (except for site-directed mutation primers) contain the *Eco*RI restriction site, and 3′primers (except for site-directed mutation primers) contain the *Xho*I restriction site, allowing insertion of the PCR-derived fragments into *Eco*RI- and *Xho*I-digested pCMV-Myc plasmid. GFP-LC3 vector was constructed as previously [18]. All plasmids were confirmed by sequencing.

Cell culture, transient transfection and Western blotting. HEK293 cells and HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 5 U/ml penicillin, and 50 μg/ml streptomycin. For transient transfection, HEK293 cells were plated in 35-mm tissue culture dish a day prior to transfection. The next day, cells were transfected with 2 μg of the indicated plasmid DNA per dish using Lipofectinamine (Invitrogen, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested and washed with 1 ml ice-cold phosphate-buffered saline and resuspended in 500 μl cold lysis buffer (Cell Signaling Technology) containing 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 8 min, and centrifuged briefly. The proteins in the supernatant were resolved by SDS–PAGE (4–15%) and transferred to a nitrocellulose membrane. After blocking with phosphate-buffered saline containing 0.2% Tween 20 and 5% non-fat dried milk for 1 h, the membrane was probed with a specific mouse anti-Myc antibody (1:1000) or anti-His antibody (1:1000) for 2 h, and then washed and exposed to horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (1:10,000) for 1 h. The bound antibodies were visualized with a Photope-HRP Western detection kit according to the manufacturer's instructions (Cell Signaling Technology).

Subcellular localization. HeLa cells were grown on glass coverslips and co-transfected transiently with the indicated plasmids. Forty-eight hours later, cells were induced in Hanks' solution for 2 h, followed by fixed in −20 °C methanol (10 min) and then in −20 °C acetone (1 min). After washing three times for 5 min each in TBS, the fluorescent signal was observed under Leica TCS-NT laser scanning microscopy.

Result and discussion

Identification and isolation of LC3A and LC3B

Mann and Hammarback [5] purified the bovine brain LC3 by molecular sieve chromatography from salt-extracted MAPs, and the sequence of the first 20 amino acids of gel-purified bovine LC3 was determined by Edman degradation. They obtained two amino acid sequences, and they used the major sequence PSDRPFKQRRSFADDVKEVQ to search the GenBank database and cloned the rat LC3 gene, but the sequence similarity is significantly low [5]. We previously identified three human LC3 family members (LC3A, LC3B, and LC3C) [18]. Therefore, we proposed there might exist another variant of rat *LC3*.

In the present work, we confirmed our proposal and isolated two novel transcripts of rat *LC3*, one of which is the homologue to human *LC3A*, the other is an alternative splicing transcript of rat *LC3* and is homologous to human *LC3B*. However, we could not isolate the rat orthologue to human *LC3C*, which might suggest different slicing mechanism in different species. According with the human homologues, the two novel cDNAs were named rat *LC3A* and submitted to GenBank (GenBank Accession No. AY206668) and *LC3B* (GenBank Accession No. AY206669), respectively. Both *LC3A* and *LC3B* contain four exons and three introns (Fig. 1). *LC3B* is an alternative splicing transcript of *LC3*, lacking the fifth exon compared to *LC3* (Fig. 1B). Interestingly, Seidenbecher et al. [20] identified the amino acid sequence of the C-terminal of rat LC3, which is encoded by the last exon, as a minimal caldendrin-binding region to caldendrin, a neuronal Ca^{2+}-sensor protein. These two alternative splicing transcripts might have different functions in vivo based on with or without the last exon. Sequence comparison indicates that LC3 family members are highly conserved in mammals and all known members contain the conserved C-terminal Gly residues (Fig. 2). The amino acid sequence of rat LC3A is identical to mouse and human LC3A, rat LC3 and LC3B are also highly homologous to mouse and human LC3B, in fact they are identical except the C-terminal tails downstream of the conserved Gly-120. However, LC3C shows high similarity to other homologues and is unique to human.

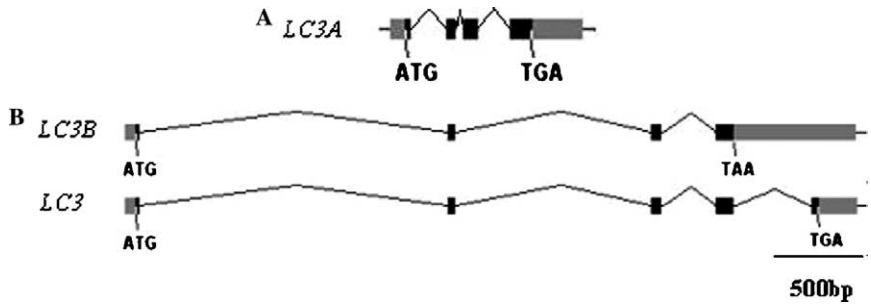

Fig. 1. Genomic organization of *LC3*. (A) The genomic structure of *LC3A*. (B) The genomic structure and alternative splicing forms of *LC3* and *LC3B*. Exons are denoted as black boxes, introns are shown as straight horizontal lines, and untranslational regions shown as gray boxes.

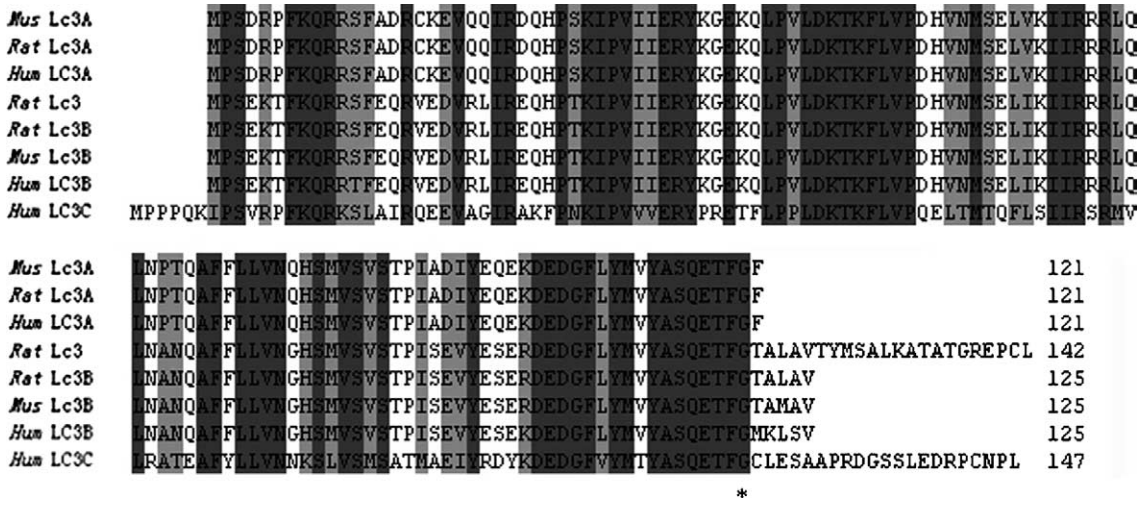

Fig. 2. Sequence comparison of rat LC3 and its mammalian homologues. Listed genes for comparison are: rat LC3A, LC3B, LC3, and their mammalian homologues (including human LC3A/B/C and mouse LC3A/B). Identical residues are shaded in black, similarity residues are shaded in gray. The conserved glycine is marked with an asterisk. Rat *LC3A*, AY206668; rat *LC3B*, AY206669.

Expression analysis

We conducted Northern blot analysis to determine the tissue distribution of the three variants of rat *LC3*. The three variants of LC3 showed distinct expression patterns in multiple rat tissues (Fig. 3). *LC3A* was detected as a single transcript of 1.7 kb, and *LC3A* mRNA was distributed abundant in heart, brain, skeletal muscle, lung, and testis, and undetectable in liver, pancreas, spleen, and small intestine (Fig. 3A). *LC3* and *LC3B* were detected as alternative splicing transcripts of 1.8 and 1.0 kb, respectively. Similar to *LC3A*, *LC3* and *LC3B* were also highly expressed in brain, skeletal muscle, and testis (Fig. 3B). *LC3* and *LC3B* were expressed highly in liver while *LC3A* was not detected. The heart lane of *LC3* and *LC3B* was not shown because it was exposed. The different tissue distribution of these variants suggested a functional divergence of these proteins.

C-terminal Gly-120 is essential for the post-translational modification of rat LC3A and LC3B

Carboxyl terminus of rat LC3 can be cleaved by a cysteine protease—ATG4, and the cleavage leads to the exposition of Gly120 [8–10]. To investigate whether rat LC3A and LC3B also undergo the C-terminal cleavage, we constructed Myc-LC3A-His and Myc-LC3B-His, which produced fusion proteins containing an N-terminal c-Myc epitope tag and a C-terminal 6× His tag. When Myc-LC3A-His protein was expressed in HEK293 cells, two bands corresponding to the cytosolic and lipidated forms of LC3A (termed I or II, respectively) were recognized by immunoblotting with anti-Myc antibody, but not with anti-His antibody (Fig. 4A). Similar results were obtained when Myc-LC3B-His was expressed in HEK293 cells (Fig. 4A). These results demonstrated that the C-terminal His6 tag was removed from both fusion proteins

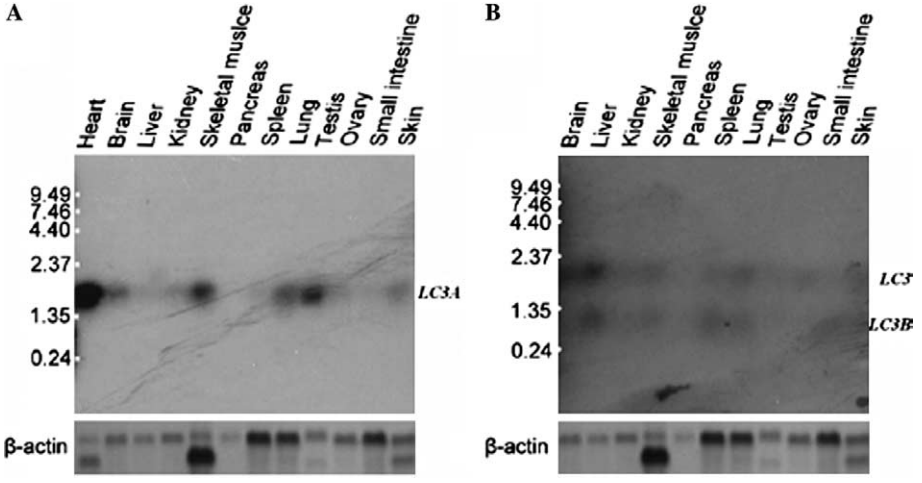

Fig. 3. Northern blotting analysis of *LC3*, *LC3A* and *LC3B* in rat multiple tissues. The tissues are indicated above the panels and β-actin as a loading control. The molecular sizes of markers (kb) are marked. The heart lane of *LC3* and *LC3B* was not shown because it was exposed.

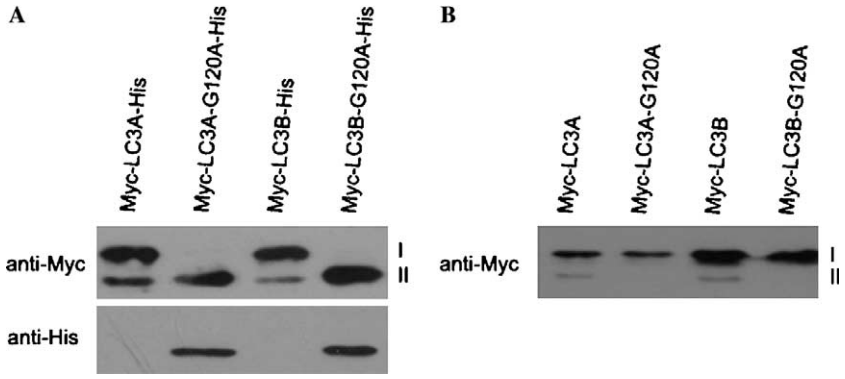

Fig. 4. Expression of rat LC3A, LC3B, and their mutants in HEK293 cells. (A) HEK293 cells were transiently transfected with expression vectors encoding Myc-LC3A-His, Myc-LC3A-G120A-His, Myc-LC3B-His, and Myc-LC3B-G120A-His. Cell lysates were analyzed by Western blotting with anti-Myc antibodies and anti-His antibodies. (B) Myc-LC3A, Myc-LC3A-G120A, Myc-LC3B, and Myc-LC3B-G120A were transiently transfected into HEK293 cells and the cell lysates were analyzed by Western blotting with anti-Myc antibodies.

upon expression and the C-terminal cleavage occurred in LC3A and LC3B, consistent with the observation in rat LC3 [7].

The only known residue for post-translational modifications of yeast Atg8 and rat LC3 is the conserved Gly-120, which is conserved in rat LC3A and LC3B (Fig. 2). It has been shown that mutation of Gly-120 into Ala of LC3 resulted in the abrogation of a series of post-translational modifications [7,21]. To clarify in a straightforward manner whether cleavage of the carboxyl terminus of LC3A and LC3B in HEK293 cells requires Gly120, we constructed Myc-LC3A-G120A-His and Myc-LC3B-G120A-His to determine the effect of this mutation on their processing. Expression of mutant Myc-LC3A-G120A-His and Myc-LC3B-G120A-His, in which Gly120 of LC3A and LC3B was changed to Ala, resulted in a single band that was recognized by both anti-Myc and anti-His antibodies, with an apparent mobility similar to those of LC3A-II, LC3B-II, respectively (Fig. 4A). Mutation of Gly120 to Ala showed that His6 at the carboxyl terminus affects the mobility of non-cleaved mutant Myc-LC3A-G120A-His and Myc-LC3B-G120A-His on SDS–polyacrylamide gel, as observed in human LC3B [19]. To eliminate this effect, we further constructed Myc-LC3A, Myc-LC3B, and their mutants Myc-LC3A-G120A, Myc-LC3B-G120A, and transiently transfected HEK293 cells. Similar to Myc-LC3A-His and Myc-LC3B-His, both Myc-LC3A and Myc-LC3B exhibited two bands (Fig. 4B). However, their mutants contain only single band with an apparent mobility similar to those of LC3A-I, LC3B-I, respectively (Fig. 4B). These results are in agreement with the obligatory role of conserved Gly-120 in the subsequent modification to the mature LC3A-II and LC3B-II.

Rat LC3A and LC3B are associated with the autophagic membranes

Rat LC3 is the only known mammalian protein present in the autophagosomal membranes, and it has been used as the marker of autophagosome membrane [7,22]. We further examined the subcellular localization of rat LC3A, LC3B, and their mutants by indirect immunofluorescence with GFP-rat LC3 fusion protein as a reference under stress conditions (by exposure to Hanks' solution). Subcellular localization result indicates that both LC3A and LC3B exhibit a punctate pattern in the cytoplasm and are colocalized with GFP-LC3 (Figs. 5A and B),

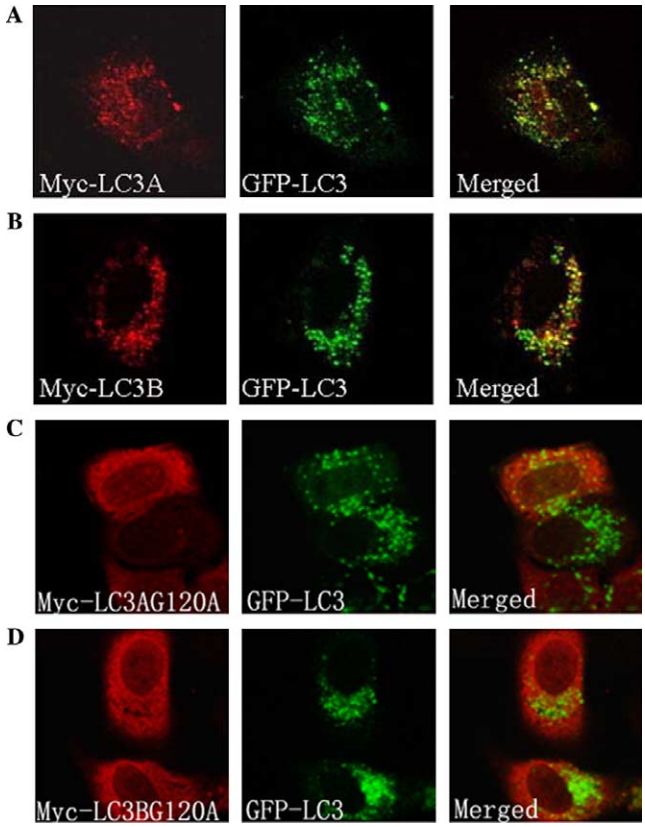

Fig. 5. Subcellular localization of rat LC3A and LC3B. HeLa cells cotransfected with *GFP-rat LC3* and *Myc-LC3A* (A), *Myc-LC3B* (B), *Myc-LC3A-G120A* (C), and *Myc-LC3B-G120A* (D) were induced with Hanks' solution at 37 °C for 2 h. Then the cells were fixed, permeabilized, and observed in the immunofluorescence confocal microscopy using a rhodamine-conjugated second antibody.

suggesting that they are associated with the autophagic membranes. However, their mutants LC3A-G120A and LC3B-G120A disperse in the cytoplasm and are not colocalized with GFP-LC3 (Figs. 5C and D), suggesting that Gly120 is critical for their autophagic localization. These results also indicated that LC3A and LC3B could be used as two novel autophagosomal markers.

Conclusion

In present work, we cloned *LC3A* and *LC3B*, two novel variants of rat *LC3*. The expression patterns of LC3, LC3A, and LC3B were different in 12 normal rat adult tissues. LC3A and LC3B undergo a characteristic C-terminal cleavage and are associated with autophagic membranes. The conserved Gly120 residues of LC3A and LC3B are essential for their post-translational modification and localization to autophagic membranes. Present data also suggested that LC3A and LC3B could be used as two novel autophagosomal markers.

Acknowledgments

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