Human Light Chain 3/MAP1LC3B Is Cleaved at Its Carboxyl-terminal Met\textsuperscript{121} to Expose Gly\textsuperscript{120} for Lipidation and Targeting to Autophagosomal Membranes*  

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Human light chain 3/MAP1LC3B, an autophagosomal ortholog of yeast Atg8, is conjugated to phospholipid (PL) via ubiquitylation-like reactions mediated by human Atg7 and Atg3. Since human Atg4B was found to cleave the carboxyl terminus of MAP1LC3B in vitro, we hypothesized that this exposes its carboxyl-terminal Gly\textsuperscript{120}. It was recently reported, however, that when Myc-MAP1LC3B-His is expressed in HEK293 cells, its carboxyl terminus is not cleaved. (Tanida, I., Sou, Y.-s., Ezaki, J., Minematsu-Ikeguchi, N., Ueno, T., and Kominami, E. (2004) J. Biol. Chem. 279, 36268–36276). To clarify this contradiction, we sought to determine whether the carboxyl terminus of MAP1LC3B is cleaved to expose Gly\textsuperscript{120} for further ubiquitylation-like reactions. When MAP1LC3B-3xFLAG and Myc-MAP1LC3B-His were expressed in HEK293 cells, their carboxyl termini were cleaved, whereas there was little cleavage of mutant proteins MAP1LC3B\textsuperscript{G120A}-3xFLAG and Myc-MAP1LC3B\textsuperscript{G120A}-His, containing Ala in place of Gly\textsuperscript{120}. An in vitro assay showed that Gly\textsuperscript{120} is essential for carboxyl-terminal cleavage by human Atg4B as well as for formation of the intermediates Atg7-MAP1LC3B (ubiquitin-activating enzyme-substrate) and Atg3-MAP1LC3B (ubiquitin carrier protein-substrate). Recombinant MAP1LC3B-PL was fractionated into the 100,000 \times g pellet in a manner similar to that shown for endogenous MAP1LC3B-PL. RNA interference of MAP1LC3B mRNA resulted in a decrease in both endogenous MAP1LC3B-PL and MAP1LC3B. These results indicate that the carboxyl terminus of MAP1LC3B is cleaved to expose Gly\textsuperscript{120} for further ubiquitylation-like reactions.

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1 The abbreviations used are: LC3, light chain 3; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; h, human; PL, phospholipid; GABARAP, γ-aminobutyric acid type A receptor-associated protein; GFP, green fluorescent protein; TRX, thioredoxin.

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MAP1LC3B is a modifier protein (9–13). To clarify whether the exposure of Gly120 by carboxyl-terminal cleavage of MAP1LC3B is essential for further ubiquitylation-like reactions, we investigated the effects of Gly120 and Lys122 on carboxyl-terminal cleavage. We also determined whether Gly120 is essential for the formation of E1- and E2-substrate intermediates as well as whether overexpression of MAP1LC3B increases the amount of MAP1LC3B-PL in addition to free MAP1LC3B. We also tested the effect of RNA interference of MAP1LC3B mRNA on endogenous MAP1LC3B-PL and MAP1LC3B. Our results indicate that MAP1LC3B is cleaved in vivo to expose Gly120 and is modified to MAP1LC3B-PL at this residue by hAtg7 and hAtg3.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Materials—**Escherichia coli strain JM109, the host for plasmid and protein expression, was grown in LB medium in the presence of antibiotics (14). pXFLAG-CMV-14 was purchased from Sigma; pCMV-Tag3B was obtained from Stratagene (La Jolla, CA); pEGFP-C1 from Clontech (Palo Alto, CA); pEF4/Myc-His was from Invitrogen; and pSilencer-3.0-H1 was from Ambion Inc. (Austin, TX). To introduce site-directed mutations with MAP1LC3B, the Gene Tailor site-directed mutagenesis system (Invitrogen) was used according to the manufacturer’s instructions.

**Plasmid Construction and Site-directed Mutagenesis—**The mammalian expression vectors pCMV-hAtg3 (expressing wild-type hAtg3), pCMV-hAtg7 (expressing mutant hAtg7 (3,7,22), and pGFP-hAtg3CS (expressing green fluorescent protein (GFP)-tagged hAtg3Thr22) mutant protein) have been described (9, 10). For mammalian cell expression of MAP1LC3B-3xFLAG protein, in which three repeats of the FLAG epitope were fused to the carboxyl terminus of wild-type MAP1LC3B, the open reading frame of MAP1LC3B was excised from pEG-HLc3 (10) and introduced into pXFLAG-CMV-14, and the resulting plasmid was designated pCMV-hLC3-3xFLAG. For expression of MAP1LC3B-3xFLAG, in which Gly120 was changed to Ala, and MAP1LC3B-3xFLAG, in which Gly120 was changed to Ser, we introduced a pair of oligonucleotides, MAP1LC3B-RNAV (5’-GGTACCGGGGAGAACAGAGAGACGAAGAGGAG-3’) and MAP1LC3B-RNAirv (5’-AGGTCCCCCAAAAACCGACACGAGGATGCAGTCGGAATTCGCCCACGTT-3’), into the pSilencer-3.0-H1 vector according to the manufacturer’s protocol. A BLAST search of the sequence for MAP1LC3B RNA interference on the National Center for Biotechnology Information in vitro assay for cleavage of the carboxyl terminus of TRX-MAP1LC3B-Myc has been described (12).

**Results**

**The Carboxyl Terminal of MAP1LC3B Is Cleaved in Vivo, and Gly120, but Not Lys122, Is Essential for Cleavage—**We previously showed that hLC3/MAP1LC3B forms E1- and E2-substrate intermediates with mammalian Atg7 and hAtg3, respectively, and that overexpression of hAtg7 together with MAP1LC3B facilitates the formation of the MAP1LC3B-PL conjugate (9, 10, 12, 15). We also showed that the carboxyl terminus of MAP1LC3B is cleaved in vitro by hAtg4B and that overexpression of hAtg4B influences MAP1LC3B lipidation in vivo (12) and that the carboxyl-terminal Gly120 of rat LC3 is essential for its modification (3). Since there is significant identity between MAP1LC3B and rat LC3 (Fig. 1A), we expected that carboxyl-terminal cleavage of MAP1LC3B would expose Gly120. However, using a recombinant Myc-MAP1LC3B-His protein, in which MAP1LC3B was fused to the Myc epitope at its amino terminus and to the His8 tag at its carboxyl terminus, it was reported that the carboxyl terminus of human MAP1LC3B is not cleaved and that it is modified at Lys122, but not at Gly120 (13). If Gly120 is not exposed, the subsequent E1 and E2 reactions could not occur.

To minimize any artificial effect of an amino-terminal tag on the LC3 modification, we first investigated carboxyl-terminal cleavage of MAP1LC3B using a series of mutant MAP1LC3B-3xFLAG proteins, in which MAP1LC3B was fused to three repeats of the FLAG epitope at its carboxyl terminus (Fig. 1B). MAP1LC3B-3xFLAG was expressed in HEK293 cells; total cell lysate proteins were separated on 12.5% SDS-polyacrylamide gels, blotted with anti-FLAG antibody, and incubated with anti-MAP1LC3B antibody. Two bands corresponding to MAP1LC3B and MAP1LC3B-PL were recognized by anti-MAP1LC3B antibody (Fig. 1C, left panel, WT), but not by anti-FLAG antibody (right panel, WT). Expression of mutant MAP1LC3B-G120A-3xFLAG, in which Gly120 of MAP1LC3B was changed to Ala, resulted in a single band (of slower mobility compared with wild-type MAP1LC3B) that was recognized by anti-MAP1LC3B antibody (Fig. 1C, G120A).

We next investigated the effect of MAP1LC3B Lys122 on the cleavage reaction. Expression of MAP1LC3B-K122A-3xFLAG, in which Lys122 of MAP1LC3B was changed to Ala, resulted in

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**AF087871** using a small interfering RNA target finder program on the Ambon web site (www.ambion.com/techlib/misc/siRNA_Finder.html), we introduced a pair of oligonucleotides, MAP1LC3B-RNAV (5’-GATCCCGGGGAGAACAGAGAGACGAAGAGGAG-3’) and MAP1LC3B-RNAirv (5’-AGGTCCCCCAAAAACCGACACGAGGATGCAGTCGGAATTCGCCCACGTT-3’), into the pSilencer-3.0-H1 vector according to the manufacturer’s protocol. A BLAST search of the sequence for MAP1LC3B RNA interference on the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov/BLAST/) indicated that the sequence is less homologous than the hAtg4 sequences of human MAP1LC3A (GenBank™/EBI accession number AF276658) and MAP1LC3C (accession number AF276659).

**Cell Culture and Transfection—**HEK293 and HeLa cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Cells were transfected with the indicated constructs using LipofectAMINE 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. After 48 h, the cells were harvested for further analyses.

**Antibodies—**Anti-MAP1LC3B (hLC3) antibody was prepared as described (12); this antibody shows little cross-reactivity with human GABARAP and GATE-16. Anti-Myc antibody was purchased from Cell Signaling Technology (Danvers, MA); anti-TRX antibody was from Sigma; anti-His antibody was from Molecular & Biological Laboratories Co. Ltd. (Nagoya, Japan); and anti-TRX antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

**Other Techniques—**Our in vitro assay for cleavage of the carboxyl terminus of TRX-MAP1LC3B-Myc has been described (12).
two bands corresponding to MAP1LC3B and MAP1LC3B-PL, both of which were recognized by immunoblotting with anti-MAP1LC3B antibody, but not with anti-FLAG antibody (Fig. 1C, K122A versus WT), indicating that Lys122 has no effect on the carboxyl-terminal cleavage of MAP1LC3B. We obtained similar results for MAP1LC3BG120A and MAP1LC3BK122A fused to the GFP protein at the carboxyl terminus and expressed in HeLa and HEK293 cells (data not shown). These results were consistent with our hypothesis derived from biochemical evidence (9, 10, 12, 15).

To clarify in a straightforward manner whether the carboxyl terminus of Myc-MAP1LC3B-His is cleaved in vivo, we con-

**Fig. 1.** Cleavage of the carboxyl terminus of MAP1LC3B in *vivo* and demonstration of the essentiality of its Gly120 for cleavage. A, shown is a comparison of amino acid sequences of rat LC3 and hLC3/MAP1LC3B using the ClustalW program. Asterisks indicate identical amino acids, and dots indicate similar amino acids. Note that residues 1–120 of human LC3 are 98.3% identical to those of rat LC3. B, shown is a schematic presentation of wild-type and mutant MAP1LC3B-3xFLAG (hLC3 WT-3xFLAG), MAP1LC3BG120A-3xFLAG (hLC3 G120A-3xFLAG), and MAP1LC3BK122A-3xFLAG (hLC3 K122A-3xFLAG). C, the three proteins in B were expressed in HEK293 cells under the control of the cytomegalovirus promoter. Cells were harvested and lysed by sonication, and 10 μg of total protein of each lysate were separated on 12.5% SDS-polyacrylamide gel, followed by immunoblotting with anti-MAP1LC3B (WB:anti-LC3; left panel) and anti-FLAG (WB:anti-FLAG; right panel) antibodies. hLC3–3xFLAG, non-cleaved MAP1LC3BG120A-3xFLAG protein; hLC3, cytosolic form of MAP1LC3B; hLC3-PL, MAP1LC3B-PL conjugate. Note that only MAP1LC3BG120A-3xFLAG was recognized by immunoblotting with anti-FLAG antibody. D, shown is a schematic presentation of wild-type and mutant Myc-MAP1LC3B-His proteins: Myc-MAP1LC3B-His (myc-hLC3 WT-His), Myc-MAP1LC3BG120A-His (myc-hLC3 G120A-His), Myc-MAP1LC3BK122A-His (myc-hLC3 K122R-His), and Myc-MAP1LC3BK122A-His (myc-hLC3 K122A-His). E, the four proteins in D were expressed in HEK293 cells under the control of the cytomegalovirus promoter. The negative control (−) was pCMV-Tag3B. Cells were harvested and lysed by sonication, and 10 μg of total protein of each lysate were separated on 12.5% SDS-polyacrylamide gel, followed by immunoblotting with anti-Myc (WB:anti-myc; upper panel) and anti-His (WB:anti-6xHis; lower panel) antibodies. myc-hLC3, cytosolic form of MAP1LC3B; myc-hLC3–6xHis, non-cleaved Myc-MAP1LC3B G120A-His protein; myc-hLC3-PL, MAP1LC3B-PL conjugate. Note that only Myc-MAP1LC3BG120A-His was recognized by immunoblotting with anti-His antibody.
expressed in E. coli with anti-Myc (WB:anti-myc was added; the samples were boiled for 5 min; and total proteins were at 37 °C for the indicated times (0, 30, and 60 min), SDS sample buffer.

Gly120 of Human LC3/ MAP1LC3B Is Essential for LC3 Lipidation

Fig. 2. Gly120 of MAP1LC3B is essential for carboxyl-terminal cleavage in vitro. A, schematic presentation of wild-type TRX-MAP1LC3B-Myc (WT) and mutants TRX-MAP1LC3BG120A-Myc (G120A) and TRX-MAP1LC3BG120A-Myc (ΔG120). B, in vitro assay for the carboxyl-terminal cleavage of MAP1LC3B. The three proteins in A were expressed in E. coli, and the FLAG-tagged cysteine protease hAtg4B was expressed in HEK293 cells as described (15). Following incubation at 37 °C for the indicated times (0, 30, and 60 min), SDS sample buffer was added; the samples were boiled for 5 min; and total proteins were separated on 10% SDS-polyacrylamide gel, followed by immunoblotting with anti-Myc (WB:anti-myc; upper panel), anti-TRX (WB:anti-TRX; middle panel), and anti-FLAG (WB:anti-FLAG; lower panel) antibodies. TRX-LC3, carboxyl-terminally cleaved form of TRX-MAP1LC3B-Myc; input +, the initial amount of FLAG-hAtg4B in each reaction.

We previously showed that MAP1LC3B forms an E2-substrate intermediate (Fig. 3A). When MAP1LC3B-His protein was expressed in HEK293 cells, two bands corresponding to the cytosolic and lipidated forms of Myc-MAP1LC3B were recognized by immunoblotting with anti-Myc antibody, but not with anti-His antibody (Fig. 1E, WT). Similar results were obtained when Myc-MAP1LC3B-K122A-His and Myc-MAP1LC3BG120A-His were expressed in HEK293 cells (Fig. 1E, K122A and K122A). Expression of mutant Myc-MAP1LC3BG120A-His resulted in a single band (of faster mobility compared with wild-type Myc-MAP1LC3B-His on SDS-polyacrylamide gel) that was recognized by both anti-Myc and anti-His antibodies (Fig. 1E, G120A). Mutation of Gly120 to Ala showed that Hisα at the carboxyl terminus affects the mobility of non-cleaved mutant Myc-MAP1LC3BG120A-His on SDS-polyacrylamide gel. These results indicate that cleavage of the carboxyl terminus of MAP1LC3B in HEK293 cells requires Gly120, but not Lys122.

Gly120 Is Essential for the Carboxyl-terminal Cleavage of MAP1LC3B by hAtg4B in Vitro—We further investigated the significance of Gly120 for carboxyl-terminal cleavage using an in vitro assay for cleavage of MAP1LC3B by hAtg4B (Fig. 2). We prepared three recombinant proteins: TRX-MAP1LC3B-Myc (in which MAP1LC3B was fused to TRX-Hisα at its amino terminus and to the Myc epitope at its carboxyl terminus), TRX-MAP1LC3BG120A-Myc, and TRX-MAP1LC3BG120A-Myc (in which Gly120 was deleted) (Fig. 2A). When wild-type TRX-MAP1LC3B-Myc was incubated with FLAG-hAtg4B at 37 °C for 30 and 60 min, a band corresponding to the protein, which was recognized by immunoblotting with anti-TRX antibody, had a faster mobility on SDS-polyacrylamide gel than before incubation (Fig. 2B, lanes 1–3), and this faster band was not recognized by anti-Myc antibody. When TRX-MAP1LC3BG120A-Myc and TRX-MAP1LC3BG120A-Myc were used as substrates for FLAG-hAtg4B, they were recognized by both anti-TRX and anti-Myc antibodies, even after incubation for 60 min (Fig. 2B, lanes 4–9), and both showed little shift in mobility. In each reaction mixture, FLAG-hAtg4B was recognized after incubation for 60 min (Fig. 2B, lanes 10–15). These results indicate that Gly120 of MAP1LC3B is essential for its carboxyl-terminal cleavage by FLAG-hAtg4B in vitro.

Gly120, but Not Lys122, of MAP1LC3B Is Essential for Formation of E1- and E2-Substrate Intermediates—We previously showed that when the mammalian Atg7(C572S) active-site mutant is expressed together with GFP-MAP1LC3B, the two proteins form a stable E1-substrate intermediate (15). In addition, mutant hAtg7(C572S) (in which the active-site Cys572 for the E1-like reaction was changed to Ser) forms stable intermediates with the other Atg8 orthologs, human GATE-16 and GABARAP, with Gly116 in the latter two proteins being essential for the E1 reaction (11). It was not clear, however, whether Gly120 (or Lys122) of MAP1LC3B is essential for the formation of the hAtg7(C572S)-MAP1LC3B (E1-substrate) intermediate. To avoid any effect of an amino-terminal tag on MAP1LC3B in its E1 and E2 reactions with hAtg7 and hAtg3, we constructed a series of mutant MAP1LC3B proteins without any tag (Fig. 3A) and expressed these proteins together with hAtg7(C572S) in HEK293 cells (Fig. 3B, lanes 6–10). Total cell lysate proteins were separated on 7% SDS-polyacrylamide gel, and MAP1LC3B and hAtg7 were recognized by immunoblotting with their respective antibodies. When wild-type MAP1LC3B and hAtg7(C572S) were expressed, both the hAtg7(C572S)-MAP1LC3B (E1-substrate) intermediate and the free proteins were recognized (Fig. 3B, lane 6 versus lane 1). E1-substrate intermediates were also observed when hAtg7(C572S) was expressed together with MAP1LC3B-TF, in which the five carboxyl-terminal amino acids (MKLSV) were deleted to expose Gly120, or MAP1LC3B-K122A (Fig. 3B, lanes 8 and 10). When MAP1LC3B, MAP1LC3B-TF, or MAP1LC3B-K122A alone was expressed, however, little intermediate was observed (Fig. 3B, lanes 1, 3, and 5). Similarly, there was little intermediate when hAtg7(C572S) was expressed together with MAP1LC3B-TF, in which the six carboxyl-terminal amino acids (GMKLSV) were deleted (Fig. 3B, lane 7 versus lane 8), or with MAP1LC3B-K122A (lane 9). Similar results were obtained when a series of mutant FLAG-MAP1LC3B and GFP-MAP1LC3B proteins were expressed instead of MAP1LC3B (data not shown). These findings indicate that MAP1LC3B can react with hAtg7 after carboxy-terminal cleavage of the latter and that exposure of Gly120 is essential for the E1-like reaction mediated by hAtg7, whereas Lys122 contributes little to it.

We previously showed that MAP1LC3B forms an E2-substrate intermediate with hAtg3(C264S), in which the active-site Cys264 was changed to Ser, in the presence of wild-type hAtg7 (10). It was unclear, however, whether Gly120 (or Lys122) of MAP1LC3B is essential for formation of the hAtg3(C264S)-MAP1LC3B (E2-substrate) intermediate. To determine whether Gly120 is essential for formation of this intermediate, we expressed a series of mutant MAP1LC3B proteins together with wild-type hAtg7 and mutant GFP-hAtg3(C264S) (Fig. 3B, lanes 11–15). After separation of total cell lysate proteins on 7% SDS-polyacrylamide gel, MAP1LC3B, hAtg7, and GFP-hAtg3 were recognized by immunoblotting using specific antibodies. When MAP1LC3B-TF or MAP1LC3B-K122A was expressed together with MAP1LC3B-TF, in which the five carboxyl-terminal amino acids (MKLSV) were deleted to expose Gly120, but not Lys122, A, schematic presentation of wild-type TRX-MAP1LC3B-Myc (WT) and mutants TRX-MAP1LC3BG120A-Myc (G120A) and TRX-MAP1LC3BG120A-Myc (ΔG120). B, in vitro assay for the carboxyl-terminal cleavage of MAP1LC3B. The three proteins in A were expressed in E. coli, and the FLAG-tagged cysteine protease hAtg4B was expressed in HEK293 cells as described (15). Following incubation at 37 °C for the indicated times (0, 30, and 60 min), SDS sample buffer was added; the samples were boiled for 5 min; and total proteins were separated on 10% SDS-polyacrylamide gel, followed by immunoblotting with anti-Myc (WB:anti-myc; upper panel), anti-TRX (WB:anti-TRX; middle panel), and anti-FLAG (WB:anti-FLAG; lower panel) antibodies. TRX-LC3, carboxyl-terminally cleaved form of TRX-MAP1LC3B-Myc; input +, the initial amount of FLAG-hAtg4B in each reaction.
MAP1LC3B is Essential for LC3 Lipidation

Our experiments indicate that MAP1LC3B is essential for the lipidation of LC3 in vivo. We have observed that MAP1LC3B hypertrophied in the absence of Atg7, similar to the results observed with wild-type MAP1LC3B (Fig. 3B, upper and lower panels, lanes 11, 13, and 15). In contrast, when MAP1LC3B-TF or MAP1LC3B\(^{G120A}\) was expressed together with hAtg7 and GFP-hAtg3\(^{2364S}\), little E2-substrate intermediate was detected by immunoblotting (Fig. 3B, lanes 12 and 14). These results indicate that MAP1LC3B can react with hAtg3 and that the exposure of Gly\(^{120}\) is essential for this E2 reaction, whereas Lys\(^{122}\) contributes little to it.

In contrast to a report that the carboxyl terminus of Myc-MAP1LC3B-His is not cleaved (13), we found that the carboxyl terminus of wild-type Myc-MAP1LC3B-His was cleaved in vivo (Fig. 1E). If the Gly\(^{120}\) of Myc-MAP1LC3B-His is exposed after carboxyl-terminal cleavage, Myc-MAP1LC3B-His will form E1- and E2-substrate intermediates with hAtg7\(^{C572S}\) and hAtg3\(^{2364S}\), respectively. We therefore investigated whether Myc-MAP1LC3B-His can react with hAtg7\(^{C572S}\) and hAtg3\(^{2364S}\). As shown for untagged MAP1LC3B (Fig. 3B), Myc-MAP1LC3B-His formed these intermediates, whereas Lys\(^{122}\) was not essential for this reaction (Fig. 3C).

Overexpression of MAP1LC3B Increases the MAP1LC3B-PL Conjugate, and the Conjugate Fractionates into the Pellet—We have shown that, in HeLa cells, endogenous MAP1LC3B-PL is a lipidated form of endogenous MAP1LC3B (12), and the lipidated protein fractionated into the 100,000 \(\times\) g pellet (Fig. 4A). If recombinant MAP1LC3B is lipidated by a ubiquitylation-like reaction after its carboxyl-terminal cleavage, overexpression of MAP1LC3B would result in increases in the lipidated and cytosolic forms of MAP1LC3B. We therefore investigated whether the mobility of recombinant MAP1LC3B-PL is similar to that of endogenous MAP1LC3B-PL on SDS-polyacrylamide gel. When we expressed untagged MAP1LC3B under the control of the elongation factor 1 \(\alpha\) promoter in HeLa cells, recombinant and endogenous proteins showed similar mobility (Fig. 4B, lanes 1 and 3 versus lanes 2 and 4). After subcellular fractionation by centrifugation at 100,000 \(\times\) g for 1 h, a band corresponding to MAP1LC3B-PL was observed in the pellet (Fig. 4B, lanes 6 and 7), suggesting that recombinant MAP1LC3B-PL is a lipidated form, similar to the endogenous protein, and that this band does not represent a degradation product of MAP1LC3B, findings in agreement with our previous observations (10–12, 16, 17). Similar results were observed when MAP1LC3B-TFG and MAP1LC3B\(^{K122A}\) were expressed in HeLa cells (data not shown).

We have shown that FLAG-MAP1LC3B, in which MAP1LC3B was fused to the FLAG epitope at its amino terminus, is recognized as two bands in HEK293 cells (11, 16) and that a lipidated form of FLAG-MAP1LC3B (FLAG-MAP1LC3B-PL) is fractionated into the pellet in HEK293 cells (11). To remove the possibility that the lipidation of FLAG-MAP1LC3B is specific to HEK293 cells, we investigated whether FLAG-MAP1LC3B-PL is fractionated into the pellet in HeLa cells. When FLAG-MAP1LC3B was expressed in HeLa cells, FLAG-MAP1LC3B-PL showed faster mobility compared with the unmodified forms and was fractionated into the pellet in a manner similar to that of the endogenous protein (Fig. 4C), in agreement with our previous findings in HEK293 cells (11, 16). We have also shown that GFP-MAP1LC3B, in which MAP1LC3B was fused to GFP at its amino terminus, forms E1- and E2-substrate intermediates with mammalian Atg7 and Atg3, respectively (10, 16). However, it is unclear whether GFP-MAP1LC3B would be recognized as two bands (cytosolic

\[ \text{hAtg7CS-myCL3, E1-substrate intermediate of Myc-MAP1LC3B with hAtg7}^{C572S}, \text{GFP-hAtg3CS-myLC3, E2-substrate intermediate of Myc-MAP1LC3B with GFP-hAtg3}^{2364S}, \text{myLC3, Myc-MAP1LC3B, myLC3-PL, Myc-MAP1LC3B-PL. The asterisk indicates the E1-substrate intermediate of endogenous LC3 with hAtg7}^{C572S}. \]
GFP-MAP1LC3B and membrane-bound GFP-MAP1LC3B-PL fractionates into the pellet. When GFP-MAP1LC3B was expressed in HeLa cells and probed with anti-GFP antibody, two bands were observed (Fig. 4D). These bands were of closer mobility than the two bands observed with FLAG-MAP1LC3B because the GFP tag is larger than the FLAG tag. Subcellular fractionation indicated that GFP-MAP1LC3B-PL was in the pellet, along with the lipidated forms of the endogenous and other recombinant proteins (Fig. 4D, Sup and Ppt). These results indicate that, like the endogenous protein, recombinant MAP1LC3B is lipidated in vivo.

**RNA Interference of hLC3/MAP1LC3B Inhibits Expression of**
**Endogenous LC3 in HEK293 Cells.—** Endogenous MAP1LC3B and MAP1LC3B-PL were recognized by immunoblotting with anti-MAP1LC3B antibody. However, this antibody may show significant cross-reaction with MAP1LC3A or MAP1LC3C. To determine whether this is the case, we used a vector expressing MAP1LC3B-specific short hairpin RNA under the control of a human H1 promoter (pSi-hLC3) to interfere with MAP1LC3B mRNA. In the presence of this hairpin, bands recognized by anti-MAP1LC3B antibody and corresponding to endogenous MAP1LC3B and MAP1LC3B-PL will be simultaneously decreased. Furthermore, the employed hairpin sequence shows little identity to the DNA sequences of MAP1LC3A and MAP1LC3C, and a BLAST search found no other identical sequences. When we transfected HEK293 cells with pSi-hLC3, cultured the cells for 48 h, and immunoblotted the total cell lysates with anti-MAP1LC3B antibody, we found that the quantities of both endogenous MAP1LC3B and MAP1LC3B-PL were significantly decreased compared with control cells (Fig. 5). Since RNA interference specifically inhibits mRNA containing 100% identical sequence, these results indicate that endogenous MAP1LC3B-PL and MAP1LC3B are derived from authentic hLC3/MAP1LC3B mRNA and not from MAP1LC3A or MAP1LC3C.

**DISCUSSION**

We have shown here that the carboxyl terminus of MAP1LC3B is cleaved *in vivo* and *in vitro* and that its Gly to leucine is essential for cleavage. Similarly, the carboxyl termini of MAP1LC3B*K122A*, 3xFLAG, Myc-MAP1LC3B-His, Myc-MAP1LC3B*K122A-His*, and Myc-MAP1LC3B*K122A*His are also cleaved. In contrast to He et al. (13), who reported that Myc-MAP1LC3B-His protein is modified at Lys122 without any carboxyl-terminal cleavage, we have shown here that Gly to leucine is essential for the cleavage of Myc-MAP1LC3B-His as well as MAP1LC3B-3xFLAG.

Following cleavage, both the cytosolic and lipidated forms of Myc-MAP1LC3B-His were recognized as two bands by immunoblotting with anti-Myc antibody, but not with anti-His antibody. Both Myc-MAP1LC3B-His and Myc-MAP1LC3B*K122A-His* formed E1- and E2-substrate intermediates with hAtg7*G572S* and hAtg3*G264S* respectively, as previously shown for untagged MAP1LC3B, FLAG-MAP1LC3B, and GFP-
MAP1LC3B (Fig. 3 and Ref. 10). RNA interference of MAP1LC3B mRNA showed that anti-MAP1LC3B antibody recognized endogenous MAP1LC3B, but not MAP1LC3A and MAP1LC3C. In addition, this antibody recognized endogenous MAP1LC3B as two bands corresponding to the cytosolic and membrane-bound forms and recognized the endogenous lipidated form of MAP1LC3B in 19 other human cell lines.² These results demonstrate that the carboxyl terminus of Myc-MAP1LC3B-His is cleaved to expose Gly120 for further lipidation in a manner similar to that observed with endogenous MAP1LC3B. Considering the biochemical properties of MAP1LC3B, hAtg4B (a cysteine protease that cleaves MAP1LC3B at its carboxyl terminus, exposing Gly120), hAtg7 (an E1-like enzyme for MAP1LC3B), and hAtg3 (an E2-like enzyme for MAP1LC3B) shown here and previously (9–12, 15–18), it is reasonable that the carboxyl-terminal cleavage of recombinant MAP1LC3B, even Myc-MAP1LC3B-His, occurs prior to ubiquitylation-like modification in a manner similar to that shown for endogenous MAP1LC3B.

Mutants MAP1LC3B-TF and MAP1LC3B K122A and wild-type MAP1LC3B can form E1- and E2-substrate intermediates with hAtg7 C572S and hAtg3 C264S, respectively, whereas mutant MAP1LC3B-TF cannot, suggesting that MAP1LC3B is cleaved at Met121 to expose Gly120. We obtained similar results using a series of Myc-MAP1LC3B-His proteins. Since Gly120 is exposed after carboxyl-terminal cleavage, it is not likely that Lys122 contributes to the E1 and E2 reactions because, in vivo, little of the remaining cytosolic MAP1LC3B would contain carboxyl-terminal residues 121–125 (MKLSV). When wild-type MAP1LC3B was overexpressed in HeLa cells, the mobility of recombinant MAP1LC3B and MAP1LC3B-PL was similar to that of the endogenous proteins, and recombinant MAP1LC3B-PL proteins were fractionated into the pellet. These findings indicate that the carboxyl terminus of MAP1LC3B is cleaved to expose Gly120 and that this residue, and not Lys122, is essential for further ubiquitylation-like modifications mediated by hAtg7 and hAtg3. Finally, MAP1LC3B is modified to MAP1LC3B-PL, i.e. hLC3/MAP1LC3B is an authentic modifier, as is rat LC3. Therefore, even if Lys122 of MAP1LC3B is modified prior to carboxyl-terminal cleavage, it could not contribute to the modifier function of MAP1LC3B or to its activity as a promising autophagosomal marker.

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