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Research Paper

Lysosomal Turnover, but Not a Cellular Level, of Endogenous LC3 is a Marker for Autophagy

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KEY WORDS

autophagy, Atg8/Apg8/Aut7, autophagosome, LC3, lysosome, lysosomal protease, cathepsin, E64d, pepstatin A

ABBREVIATIONS

CBB	Coomassie brilliant blue
DMEM	Dulbecco's modified Eagle medium
GFP	green fluorescent protein
LC3	microtubule-associated protein 1 light chain 3
LC3-I	soluble unlipidated form of LC3
LC3-II	membrane-bound LC3-phospholipid conjugate modified by endogenous Atg7 and Atg3
PAGE	polyacrylamide gel electrophoresis
PVDF	polyvinylidene fluoride
S6RP	S6 ribosomal protein
SDS	sodium dodecyl sulfate

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ABSTRACT

During starvation-induced autophagy in mammals, autophagosomes form and fuse with lysosomes, leading to the degradation of the intra-autophagosomal contents by lysosomal proteases. During the formation of autophagosomes, LC3 is lipidated, and this LC3-phospholipid conjugate (LC3-II) is localized on autophagosomes and autolysosomes. While intra-autophagosomal LC3-II may be degraded by lysosomal hydrolases, recent studies have regarded LC3-II accumulation as marker of autophagy. The effect of lysosomal turnover of endogenous LC3-II in this process, however, has not been considered. We therefore investigated the lysosomal turnover of endogenous LC3-II during starvation-induced autophagy using E64d and pepstatin A, which inhibit lysosomal proteases, including cathepsins B, D and L. We found that endogenous LC3-II significantly accumulated in the presence of E64d and pepstatin A under starvation conditions, increasing about 3.5 fold in HEK293 cells and about 6.7 fold in HeLa cells compared with that in their absence, whereas the amount of LC3-II in their absence is cell-line dependent. Morphological analyses indicated that endogenous LC3-positive puncta and autolysosomes increased in HeLa cells under starvation conditions in the presence of these inhibitors. These results indicate that endogenous LC3-II is considerably degraded by lysosomal hydrolases after formation of autolysosomes, and suggest that lysosomal turnover, not a transient amount, of this protein reflects starvation-induced autophagic activity.

INTRODUCTION

Autophagy, the bulk degradation of intracellular components including organelles, is induced in cells under starvation conditions.[1-4] In mammals, autophagy shows significant associations with neurodegenerative diseases, cancer, cardiomyopathies, aging, type II programmed cell death, bacterial invasion, MHC class II antigen-presentation, and early neonatal stage, as well as cellular maintenance.[3,5-13] During autophagy, cup-shaped preautophagosomes (or phagophores) enwrap cytoplasmic components, including organelles, and become enclosed, forming autophagosomes, also known as 'autophagic vesicles containing undegraded cytoplasm' (Avi) (Fig. 1, Step 1).[14-19] Autophagosomes fuse with lysosomes to form autolysosomes, the degradative form of autophagic vacuoles (Avd) (Fig. 1, Step 2), in which intra-lumenal components are degraded by lysosomal hydrolases (Fig. 1, Step 3). Under starvation conditions in perfused rat liver, the populations of Avi and Avd attain a steady state within about 20 min.

The autophagy-related genes, *ATG/APG/AUT/CVT* genes, have been isolated and characterized in yeast and mammals.[2,20,21] In mammalian cells, LC3, one of the homologues of yeast Atg8/Apg8/Aut7, is modified via a ubiquitylation-like system.[22,23] The carboxyl terminal region of LC3 is cleaved, generating a soluble form known as LC3-I and exposing a carboxyl terminal glycine essential for further reactions.[22,24,25] LC3-I, in turn, is modified to a membrane-bound form, LC3-II (a LC3-phospholipid conjugate), by mammalian Atg7/Apg7/Cvt2/Gsa7 and Atg3/Apg3/Aut1 homologues, which are E1- and E2-like enzymes, respectively[22,25-27] to localize to autophagosomes and autolysosomes.[22] Thus, the amount of LC3-II in mammalian cells is a good early marker for the formation of autophagosomes (Fig. 1, Step 1).[22] Thereafter, autophagosomes fuse with lysosomes to degrade enwrapped cytosolic components, forming autolysosomes (Fig. 1, Step 2). Therefore, it is probable that the degradation of intra-lumenal LC3-II will occur (Fig. 1, Step 3). However, there is little information available regarding the degradation of endogenous LC3-II in the autolysosomes during starvation-induced autophagy.

Several lines of evidence have indicated that intra-autophagosomal components are rapidly degraded by the lysosomal proteolytic system after formation of autophagosomes during autophagy. Morphological studies have indicated that, in primary cultured hepatocytes, only a few autophagosomes and autolysosomes are observed under starvation-conditions without inhibition of lysosomal hydrolases,[18,19,28,29] probably because the lysosomal fusion and degradation of autophagosomes occurs rapidly under starvation conditions. Therefore, autophagy, including pexophagy (the degradation of peroxisomes by the autophagic machinery), has been studied morphologically and biochemically by inhibiting lysosomal function using agents that inhibit cathepsins or lysosomal acidification.[18,19,28,30] Autolysosomes are easily isolated from leupeptin-treated starved rat liver, but not from untreated starved rat liver. These results have indicated that lysosomal degradation of intra-autophagosomal components occurs rapidly after fusion of autophagosomes with lysosomes to form autolysosomes.

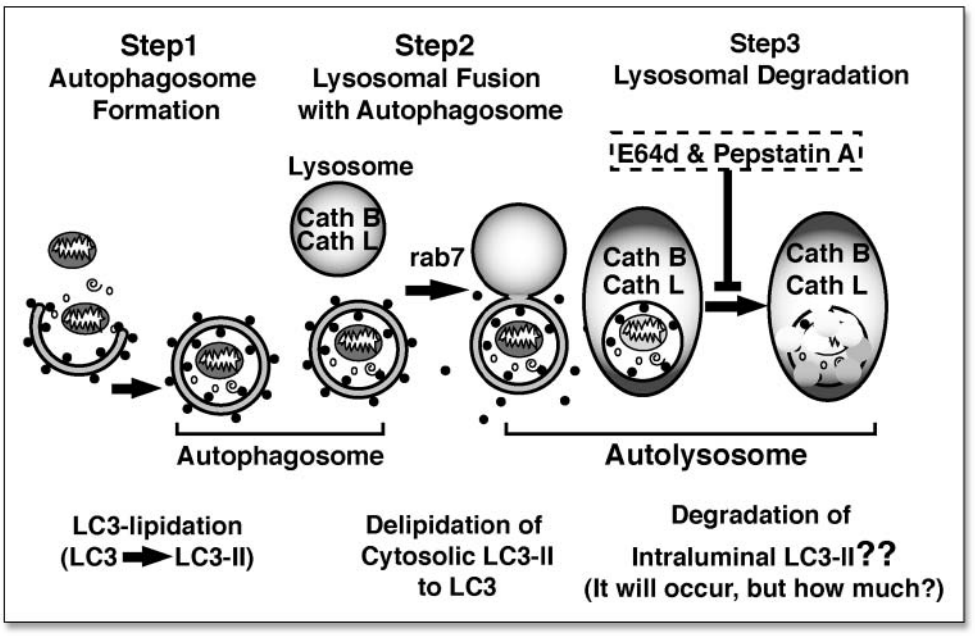

Figure 1. Schematic representation of the relationship between formation of autophagosomes and autolysosomes and the amount of LC3-II during mammalian autophagy. Under starvation conditions, a cup-shaped preautophagosome (or isolation membrane) is formed and engulfs cytosolic compartments including organelles (Step 1). The preautophagosome finally becomes enclosed, forming an autophagosome as a double membrane structure. During this step, cytosolic LC3 (closed dot) is lipidated, becoming localized on autophagosomes as an LC3-phospholipid conjugate (LC3-II) on both the cytosolic and intra-lumenal faces. After the formation of the autophagosome, it fuses with a lysosome to form a autolysosome (Step 2). This fusion is mediated by a small GTPase, rab7. Following this fusion, cytosolically oriented LC3-II on the autophagosome is delipidated by human Atg4B and released into the cytosol. After the formation of the autolysosome, intra-autophagosomal components are rapidly degraded by lysosomal hydrolases, including cathepsins B, D and L (Step 3). Intra-autophagosomal LC3-II is also degraded at the same time. When E64d and pepstatin A are added to the medium, the proteolytic activities of these cathepsins are inhibited, and autolysosomes containing cytosolic components accumulate.

Furthermore, in the yeast *Sachharomyces cerevisiae*, the vacuolar proteolytic system contributes considerably to the degradation of engulfed compartments during autophagy.[20,31-34] Accumulation of autophagic bodies in the vacuoles (yeast vacuoles containing autophagic bodies correspond to mammalian autolysosomes) is observed under starvation conditions in a *pep4* or *prb1* mutant, or following treatment with the serine protease inhibitor phenylmethylsulfonyl fluoride, but not in wild type yeast.[31] Pep4 (proteinase A) is a vacuolar aspartic protease required for the posttranslational precursor-maturation of vacuolar proteinases,[35-37] whereas Prb1 (proteinase B) is a vacuolar serine protease involved in protein degradation in the vacuoles.[38,39] Therefore, these results indicate that the degradation of intra-autophagosomal components in the vacuoles occurs very rapidly during starvation-induced autophagy in yeast. Atg8-PE, a yeast counterpart of LC3-II, is an autophagosomal marker localized on autophagosomes during both the cytosolic and lumenal faces.[32] Atg8-positive autophagic bodies accumulate in the vacuoles of *pep4* mutant cells under starvation conditions, while little accumulates in the vacuoles of wild type yeast.[31,32,40] Taken together, these results indicate that, in wild type yeast, intra-autophagosomal Atg8-PE is dramatically degraded in the vacuoles during autophagy.

Recently, the cellular level of LC3-II has been regarded as an indicator that reflects the activation of autophagy, without considering the lysosomal degradation of LC3-II. If the lysosomal degradation of LC3-II will be much slower than the formation of autophagosomes during autophagy, the cellular level of LC3-II reflects an activation of autophagy. However, considering the contribution of the lysosomal/vacuolar proteolytic system during autophagy in mammals and yeast as described above, we hypothesized that intra-autophagosomal LC3-II will be rapidly degraded by lysosomal proteases during autophagy following formation of autolysosomes. At present, little work is known about the lysosomal turnover of endogenous LC3-II under starvation conditions. We therefore examined the contribution of lysosomal turnover of endogenous LC3-II during starvation-induced autophagy using the protease inhibitors E64d and pepstatin A, and found that its lysosomal turnover dramatically occurs after autophagosome-formation during starvation-induced autophagy.

MATERIALS AND METHODS

Cell culture. HeLa and HEK293 cell lines, purchased from the ATCC (Manassas, VA), were cultured in 60-mm dishes in DMEM (Invitrogen Corp., Carlsbad, CA, USA) containing 10% fetal calf serum (FCS; Invitrogen) in a humidified 5% CO_2 atmosphere at 37°C. For starvation, cells were incubated in Krebs-Ringer medium at 37°C for the indicated times.[29,41]

Materials, biochemical and molecular biological techniques. E64d and pepstatin A were purchased from the Peptide Institute (Osaka, Japan). Molecular biological and biochemical techniques were performed as described previously.[42] Coomassie brilliant blue staining of proteins on a polyvinylidene fluoride (PVDF) membrane after immunoblotting was performed using a Quick CBB kit (Wako, Tokyo, Japan). Total lysates of human cell lines cultured in DMEM containing 10% FCS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Antibodies. Antiserum against thioredoxin-human LC3 was raised in rabbits, and anti-human LC3 antibody was purified by affinity chromatography on a glutathione-S-transferase-immobilized human LC3-Sepharose column.[42]

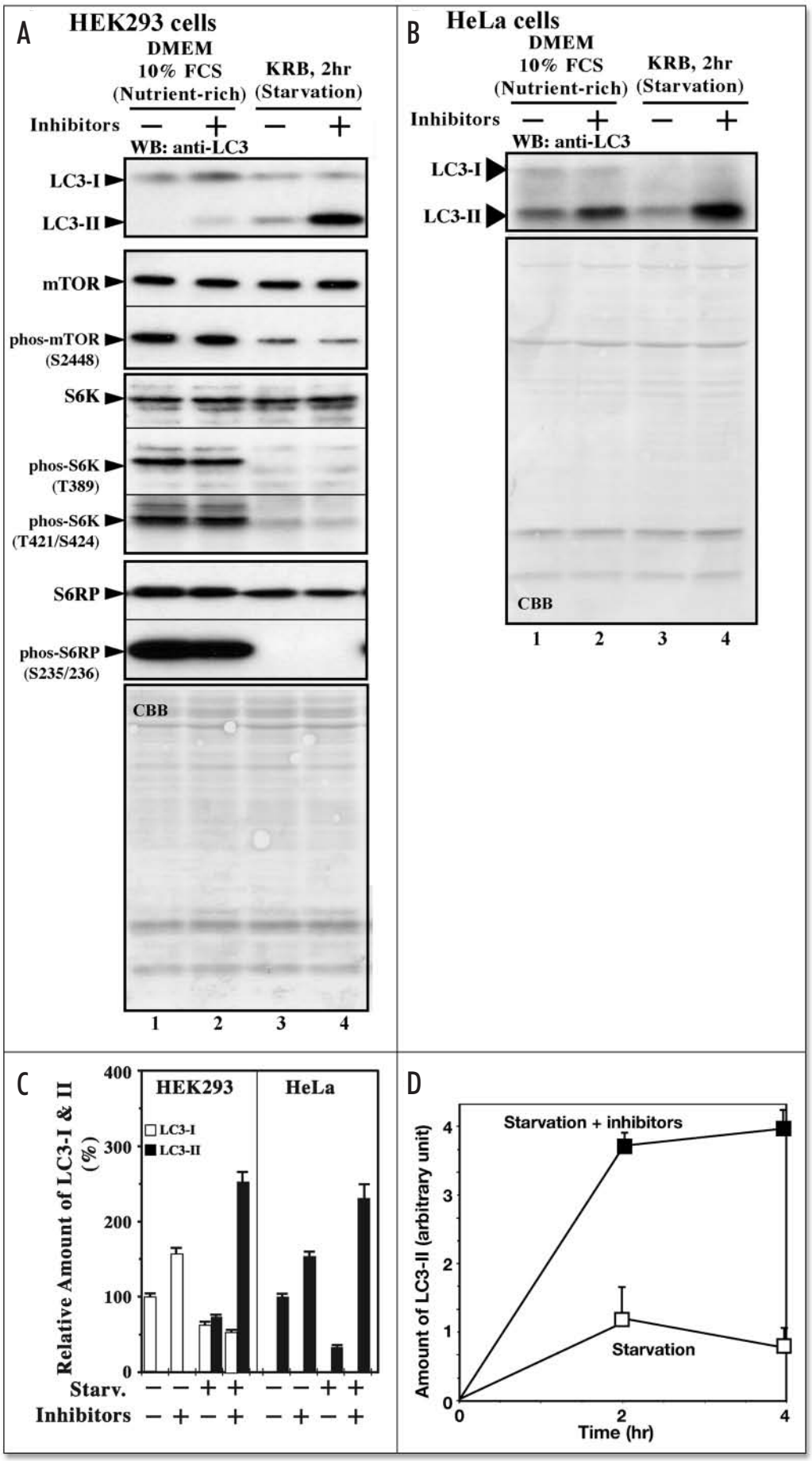

Figure 2. Lysosomal degradation of endogenous LC3 during autophagy. Endogenous LC3 in (A). HEK293 or (B) HeLa cells during autophagy. For Nutrient-rich conditions, cells were cultured in DMEM medium containing 10% FCS. Where indicated, cells were treated with the protease inhibitors, E64d (10 μg/ml) and pepstatin A (10 μg/ml) (Inhibitors +) for 4 h, or, as a negative control (Inhibitors -), with the solvent, dimethylsulfoxide. For Starvation conditions, cells were incubated for 4 hrs in Krebs-Ringer medium (KRB) in the presence (+) or absence (-) of protease inhibitors. The cells were lysed, total proteins (10 μg per lane) were separated by SDS-PAGE, and endogenous LC3 in the lysates was recognized by immunoblotting with an anti-LC3 antibody (WB: anti-LC3). LC3-I, soluble form of LC3; LC3-II, membrane-bound form of LC3. Antisera to phosphorylated and dephosphorylated forms of Tor, S6K and S6RP were used to monitor Tor activity. (C) Densitometric analyses of endogenous LC3-I and LC3-II in HEK293 (A) and HeLa (B) cells. The relative amounts of LC3-I (open bars) and LC3-II (closed bars) are shown. Bars indicate standard errors. D. Time course of the amount of endogenous LC3-II under starvation conditions in the absence (open box) or presence (closed box) of E64d (10 μg/ml) and pepstatin A (10 μg/ml) in HEK293 cells.

The antibody did not cross-react with GATE-16 or GABARAP, the other mammalian Atg8 homologues. Affinity purified polyclonal rabbit antibodies against human Atg7 and Atg3 have been described.[26,27] Antiserum against glutathione-S-transferase-human beclin 1[43] was raised in rabbits, and the antibodies were purified by affinity chromatography on a thioredoxin-beclin 1-immobilized Sepharose column. Monoclonal mouse anti-green fluorescent protein (GFP) antibody was purchased from BD Biosciences (Palo Alto, CA). Polyclonal rabbit antibodies against mTOR, phospho-mTOR (Ser2448), p70 S6 kinase, phospho-p70 S6 kinase (Thr389), phospho-p70 S6 kinase (Thr421/Ser424), S6 ribosomal protein, and phospho-S6 ribosomal protein (Ser235/236) were purchased from Cell Signaling (Beverly, MA).

Immunoblot analyses. Protein concentrations were determined using the BCA protein assay reagent (Pierce, Rockford, IL). Immunoblot analyses were carried out according to standard protocols using a chemiluminescent method with SuperSignal West Dura

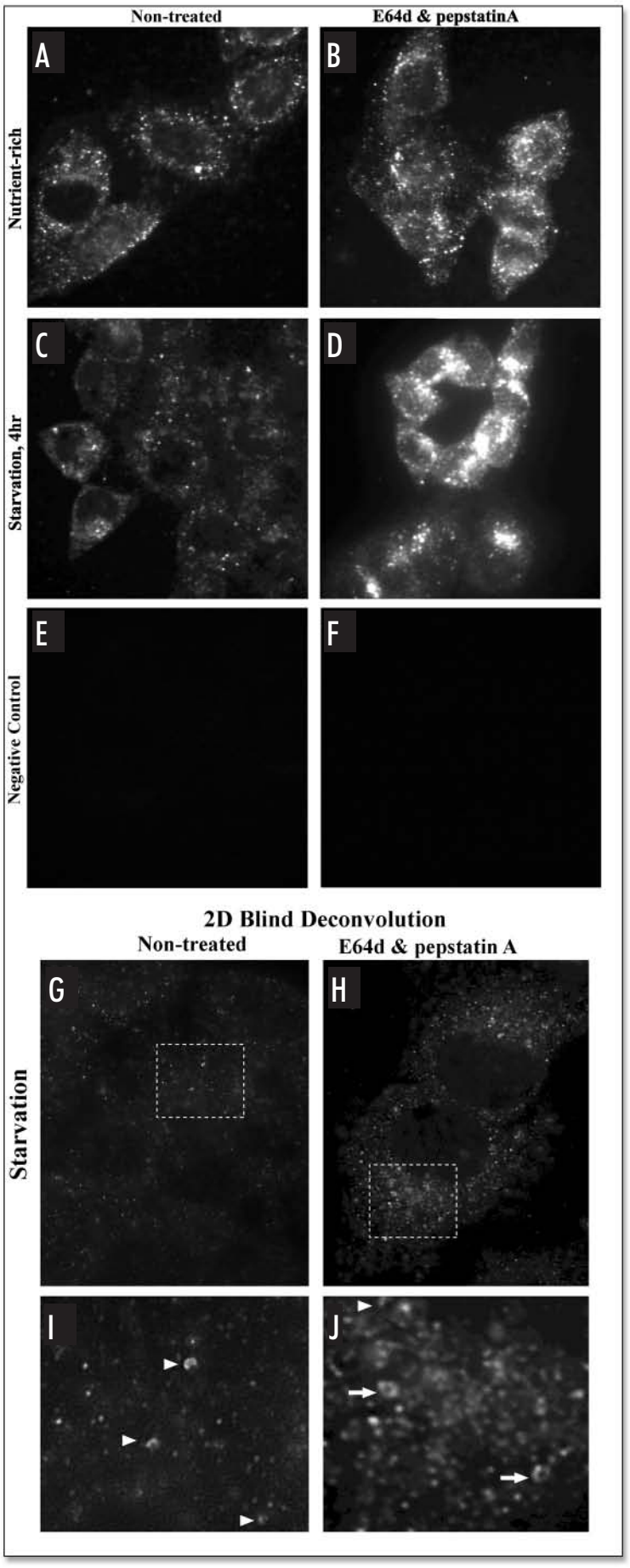

Figure 3. Accumulation of endogenous LC3-positive puncta under starvation conditions in the presence of E64d and pepstatin A in HeLa cells. HeLa cells were cultured in DMEM containing 10% FCS (Nutrient-rich, A and B) or transferred to KRB medium for 4 h (Starvation, C, D and G–J). "E64d & pepstatin A" (B, D, F, H and J) indicates that cells were incubated for 4 h with these protease inhibitors, whereas "Non-treated" (A, C and E) indicates addition of a solvent instead of these inhibitors. The cells were fixed and permeabilized with digitonin, and endogenous LC3 was recognized using Alexa Flour594-conjugated anti-human LC3 antibody. Intracellular fluorescence of endogenous LC3 in HeLa cells was observed. As a negative control, rabbit IgG was employed instead of anti-human LC3 antibody. A series of Z-scanned fluorescent images was deconvoluted by a program for 2D blind deconvolution with Aqua C-imaging software (Hamamatsu Photonics) (G–J). Images (I and J) are 4 fold magnifications of the images shown in boxes with dotted lines in (G and H), respectively. An arrowhead indicates a cup-shaped preautophagosome, and an arrow indicates an autophagosome/autolysosome.

Extended Duration Substrate or SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunofluorescence analysis. Endogenous LC3 in HeLa cells was stained using Alexa Flour594-conjugated anti-LC3 antibody as previously described,[44] and fluorescence was monitored with a Zeiss Axioplan2 fluorescence microscope (Carl Zeiss, Thornwood, NY) and ORCA-ER CCD camera (Hamamatsu Photonics, Tokyo, Japan). For 2D blind deconvolution of Z-scanned images, a series of fluorescent images were obtained with a Zeiss Axioplan2 fluorescence microscope (Carl Zeiss) and Aqua C-imaging system (Hamamatsu Photonics).

Electron microscopy analysis. HeLa cells cultured on cover glass were prefixed in 4% glutaraldehyde in PBS at 4°C, treated with 1% OsO_4 for 2 h at 4°C, dehydrated in a graded series of ethanol, and flat embedded in epon. Ultra-thin sections were doubly stained with uranyl acetate and observed using a JEOL JEM2000EX electron microscope at 100kV (Peabody, MA).

Other techniques. Quantification of the images was performed using Aqua C-imaging (Hamamatsu Photonics) and NIH image (Dr. W. Raeband, National Institute of Health, Bethesda, MD, USA) programs on Endeavor (EPSON direct, Tokyo, Japan) and Macintosh G4 (Apple Computer, NY) computers, respectively.

RESULTS

Endogenous LC3-II markedly accumulates in the presence of E64d and pepstatin A during starvation-induced autophagy in HEK293 and HeLa cells. During autophagy, intra-autophagosomal components are degraded by lysosomal hydrolases following the formation of autophagosomes and their fusion with lysosomes to form autolysosomes. Therefore, a significant amount of endogenous LC3-II on autophagosomes may be rapidly degraded by lysosomal hydrolases. To investigate the lysosomal turnover of intra-autophagosomal LC3-II, we employed two protease inhibitors, E64d, a membrane-permeable inhibitor of cathepsins B, H, and L,[45-47] and pepstatin A, an inhibitor of cathepsins D and E.[29,47,48] Preliminary experiments with HEK293, HeLa, P19EC, C2C12, MPC, and B16-F1 cells and rat primary cultured hepatocytes treated with 10 μg/ml of E64d and 10 μg/ml of pepstatin A indicated that these inhibitors have little effect on cell viability, morphology and growth under nutrient-rich conditions for at least 24 h.

Using E64d (10 μg/ml) and pepstatin A (10 μg/ml), we sought to determine the contribution of lysosomal degradation of LC3-II under starvation conditions. Under nutrient-rich conditions in HEK293 cells, endogenous LC3 is mainly present as the cytosolic form (LC3-I) (Fig. 2A, lane 1). Following the incubation of HEK293 cells in starvation medium, the amount of endogenous LC3-I decreased while that of LC3-II increased (Fig. 2A lane 3 vs. lane 1), indicating that LC3-lipidation

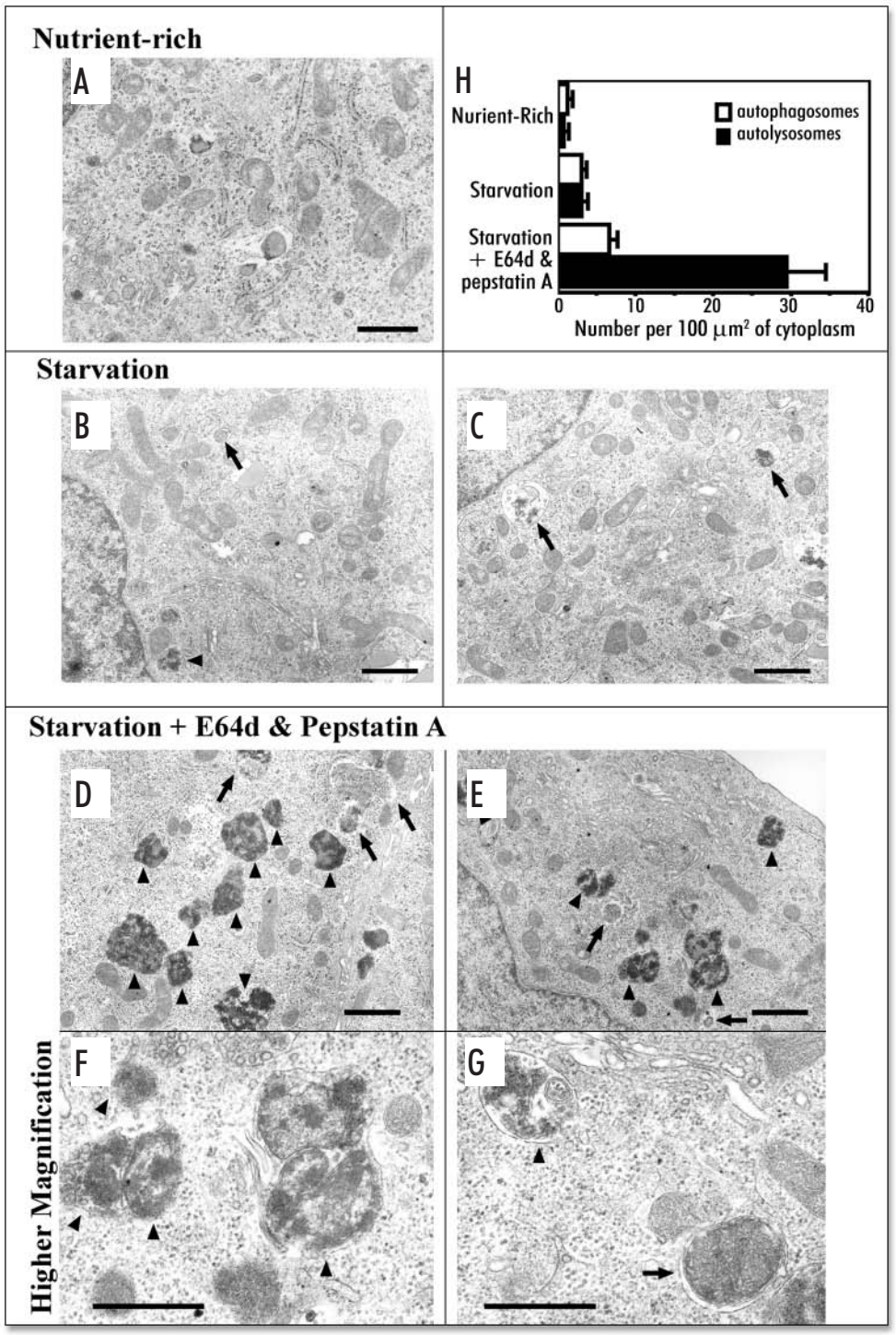

Figure 4. Accumulation of autolysosomes under starvation conditions in the presence of protease inhibitors in HeLa cells. Representative electron microscopic images of the cytoplasmic regions of cells are shown. HeLa cells were cultured in DMEM containing 10% FCS (Nutrient-rich, A), or incubated in KRB medium in the absence (B and C) or presence (D–G) of E64d and pepstatin A. Bar indicates 500 nm. An arrowhead indicates an autolysosome, and an arrow indicates an autophagosome. Double-membrane-structures, representative of autophagosomes, were observed in (B and C). Electron-dense structures, representative of autolysosomes, were accumulated in (D–G). Autophagosomes and autolysosomes under these conditions were quantified (H).

is facilitated under these conditions. LC3-II also markedly accumulated in the presence of E64d and pepstatin A under the same conditions (Fig. 2A, lane 3 vs. lane 4). Densitometric analysis indicated that, under starvation conditions, the amount of LC3-II in the presence of these inhibitors was about 3.5 fold higher than that in their absence in HEK293 cells (Fig. 2C, left panel). Time-course experiments showed that, in the absence of E64d and pepstatin A, the amount of endogenous LC3-II increased 2 hrs after the induction of starvation, but slightly decreased at 4 hrs (Fig. 2D, KRB). In the presence of E64d and pepstatin A, endogenous LC3-II dramatically increased in a time-dependent manner (Fig. 2D, KRB$^+$ inhibitors). While the Tor kinase-signaling pathway is inactivated under starvation conditions, these inhibitors have no effect on the Tor kinase-signaling pathway related to autophagy (Fig. 2A). These results suggested that endogenous LC3-II is rapidly degraded in the lysosomes after lipidation during starvation-induced autophagy in HEK293 cells.

In HeLa cells, endogenous LC3 is mainly present as the membrane-bound form (LC3-II) (Fig. 2B, lane 1). Under starvation conditions, LC3-II markedly accumulated in the presence of E64d and pepstatin A, whereas it decreased in the absence of these inhibitors (Fig. 2B, lane 4 vs. lane 3), suggesting that LC3-II is degraded by lysosomal hydrolases soon after lipidation. Under starvation conditions, the amount of LC3-II in the presence of these inhibitors was about 6.7 fold higher than that in their absence in HeLa cells (Fig. 2C, right panel). These results suggested that LC3-II is dramatically degraded by lysosomal hydrolases under starvation conditions in both HeLa and HEK293 cells.

LC3-positive puncta, indicative of autolysosomes, accumulate in the presence of E64d and pepstatin a under starvation conditions in HeLa cells. When expressed under the control of a constitutive CMV (or CAG) promoter in HeLa cells, GFP-rat LC3 has been reported to localize to punctate-structures (autophagosomes) under starvation conditions.[49-51] Although the expression of endogenous human LC3-II increases under starvation conditions,[22] little is known of its intracellular localization in the presence of these inhibitors under starvation conditions. Using immunofluorescence microscopy and an anti-human LC3 antibody, we assayed the intracellular localization of endogenous LC3 in HeLa cells during starvation-induced autophagy in the presence or absence of E64d and pepstatin A. We hypothesized that, if these inhibitors inhibit the lysosomal degradation of intra-autophagosomal components during autophagy, LC3-positive puncta would increase in their presence under starvation conditions. Even under nutrient-rich conditions, we observed immunofluorescence of puncta of endogenous LC3 in either the presence or absence of E64d and pepstatin A (Fig. 3A and B). When HeLa cells were transferred to starvation conditions for 4 h, a decrease in LC3-puncta fluorescence was observed (Fig. 3C). This decrease coincides with the decrease in endogenous LC3-II observed by

immunoblotting with anti-human LC3 antibody. When E64d and pepstatin A were added to the medium under starvation conditions, endogenous LC3-positive puncta accumulated (Fig. 3D).

We further analyzed Z-scanned fluorescent images of LC3-positive puncta in HeLa cells using a program for 2D blind deconvolution (Fig. 3G–J). Under starvation conditions, and in the absence of E64d and pepstatin A, cup-shaped structures, representative of preautophagosomes, were observed (Fig. 3G and I). In the presence of E64d and pepstatin A under starvation conditions, both ring-shaped structures, representative of autophagosomes/autolysosomes, and cup-shaped structures were observed (Fig. 3H and J). Together with our finding that the amount of endogenous LC3-II increased under starvation conditions in the presence of E64d and pepstatin A (Fig. 3B), these results indicate that this increase in LC3-II correlates with an increase in LC3-positive puncta.

Autolysosomes are dramatically increased under starvation conditions by inhibiting lysosomal hydrolases. We previously showed that endogenous LC3-II accumulates in autolysosomes purified from leupeptin-treated rat liver.[22] Previous morphological studies of autophagosomes and autolysosomes suggest that the increase of LC3-positive puncta observed here are reflective of an increase of autophagosomes or autolysosomes. We therefore investigated whether autophagosomes or autolysosomes accumulated in HeLa cells in the presence of E64d and pepstatin A under starvation conditions by electron microscopy (Fig. 4). In the absence of E64d and pepstatin A, a few autolysosomes were observed in HeLa cell under both nutrient-rich (Fig. 4A and H) and starvation (Fig. 4B, C, and H) conditions. In the presence of these inhibitors, however, electron-dense structures corresponding to autolysosomes accumulated in these cells under starvation conditions (Fig. 4D–G and H). Higher magnification of the images showed that these electron-dense structures contain cytosolic components, including mitochondria and vesicular like structures (Fig. 4F and G). Measurement of the number of autolysosomes per 100 μm^2 of cytoplasmic area indicated that, under starvation conditions, the number of autolysosomes in the presence of these inhibitors is about 7.3 fold higher than that in their absence (Fig. 4H, autolysosomes). Taken together with the increase in LC3-II (Fig. 2B) and the accumulation of LC3-positive puncta (Fig. 3D) under the same conditions in HeLa cells, these results suggest that the increase of LC3-II is a marker for an increase in the number of autolysosomes under starvation conditions in the presence of protease inhibitors.

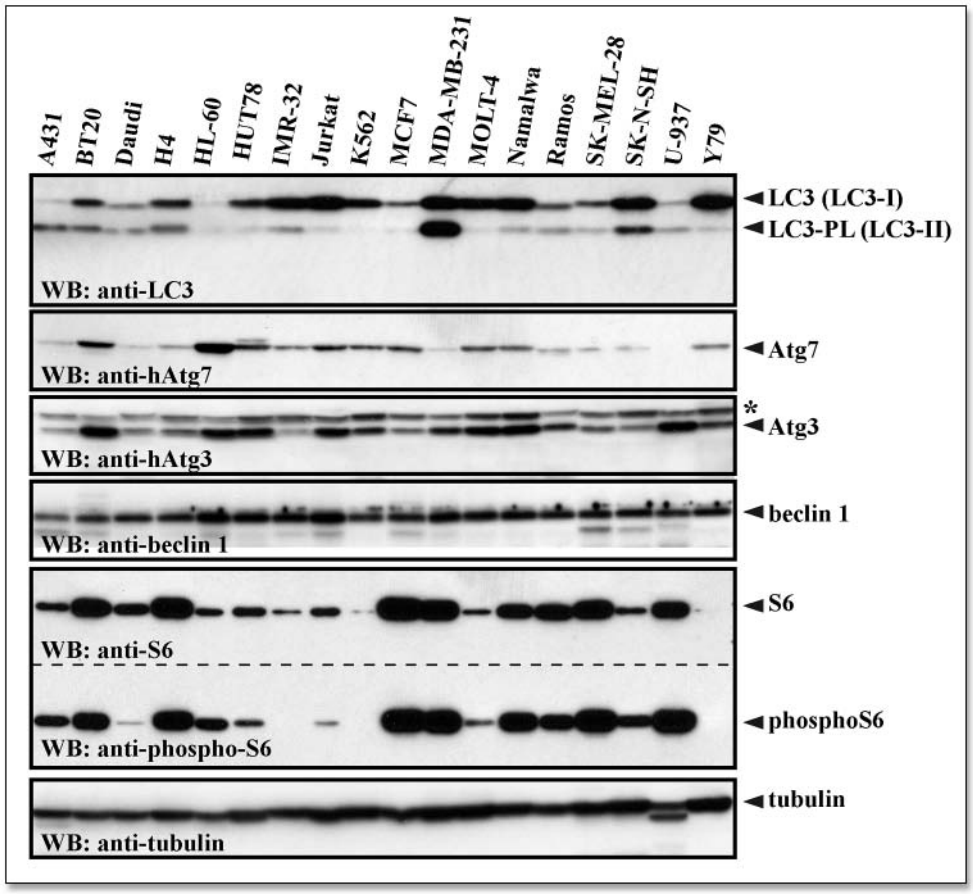

Figure 5. Endogenous LC3-I and LC3-II in human cell lines under nutrient-rich conditions. Expression of endogenous Atg proteins in human cell lines. Total proteins (10 μg per lane) of each cell lysate from cells grown in DMEM containing 10% FCS were separated by SDS-PAGE, and endogenous human LC3 (LC3-I and LC3-II), Atg7, Atg3, and beclin 1 were recognized by immunoblotting using anti-human LC3, Atg7, Atg3, and beclin 1 antibodies, respectively. Tor kinase-signaling pathway inactivation was determined by assaying S6 ribosomal protein (S6RP) and phosphorylated S6 ribosomal protein (phosphoS6RP) using anti-S6RP and anti-phosphoS6RP antibodies. As an internal control, tubulin was assayed using an anti-tubulin antibody.

Endogenous LC3 is present as LC3-II under nutrient-rich conditions independent of inactivation of the Tor kinase-pathway in human cell-lines. In HeLa cells, endogenous LC3 is present as LC3-II even under nutrient-rich conditions. While it has been reported that LC3-I is rapidly lipidated to LC3-II under starvation conditions, it is possible that, in other human cell lines in addition to HeLa cells, endogenous LC3-II is present even under nutrient-rich conditions. We then investigated whether the cytosolic form of LC3, LC3-I, is the major form of endogenous LC3 under nutrient-rich conditions in human cell lines (Fig. 5). Endogenous LC3-II accumulates in several human cell lines, especially, A423, MDA-MB-231, and SK-N-SH cells, even under nutrient-rich conditions. The amount of endogenous LC3-II is not dependent on the expression of endogenous human Atg7 (E1-like enzyme for LC3-lipidation),[26] human Atg3 (E2-like enzyme for LC3-lipidation)[27] or beclin 1 (mammalian Vps30/Apg6/Atg6 homolog).[43]

One explanation for these results is that Tor kinase may be inactivated in cells in which endogenous LC3-II accumulates under nutrient-rich conditions. We therefore assayed S6RP phosphorylation as a downstream marker of the Tor kinase pathway. We observed considerable S6RP phosphorylation in A431, MDA-MB-231, and SK-N-SH cells, all cell lines in which LC3-II accumulates (Fig. 5, S6RP vs. phospho-S6RP), indicating that, in these cell lines, endogenous LC3-II is present even under nutrient-rich conditions and also suggested that the cellular level of LC3-II is not a simple marker for autophagic activity.

DISCUSSION

While the lysosomal contribution during autophagy has been well studied, there have been few studies of the lysosomal turnover of endogenous LC3-II during autophagy. We have shown here that a significant amount of LC3-II is inevitably degraded by lysosomal hydrolases during starvation-induced autophagy. Using E64d and pepstatin A, we next showed that a significant amount of

intra-autophagosomal LC3-II is degraded by lysosomal hydrolases. During autophagy in HEK293 and HeLa cells, the amount of endogenous LC3-II in the presence of E64d and pepstatin A is about 3.5 and 6.7 fold higher, respectively, than in their absence, indicating that a significant amount of LC3-II is degraded by lysosomal hydrolases during starvation-induced autophagy. Immunofluorescent and electron microscopy analyses suggested that LC3-positive puncta and autolysosomes accumulate in the presence of E64d and pepstatin A under starvation conditions. We further attempted to investigate the relationship between LC3 and autolysosomes in the presence of these inhibitors by immunoelectron microscopy using an anti-human LC3 antibody, but unfortunately, this antibody was not suitable for immunoelectron microscopic analysis. However, several lines of evidence suggest that LC3-positive puncta, i.e., autolysosomes, highly accumulate in the presence of E64d and pepstatin A under starvation conditions, indicating that LC3-II is dramatically degraded in autolysosomes/lysosomes during starvation-induced autophagy. We next showed that, in some cell lines, including A431, MDA-MB-231, and SK-N-SH cells, a significant amount of LC3-II is present under nutrient-rich conditions, independent of the inactivation of Tor kinase, which occurs under starvation conditions. These results are in agreement with a series of previous morphological and biochemical studies of autophagy. Furthermore, these results indicated that the amount of LC3-II does not accurately reflect the activation of starvation-induced autophagy: however, monitoring LC3-II levels in the presence and absence of inhibitors can be used to estimate lysosomal degradation during autophagy.

Even without considering lysosomal turnover of LC3-II during autophagy, an increase in the amount of LC3-II has been regarded as marker for autophagy. In some cell lines, however, LC3-II remains present under nutrient-rich conditions without inactivation of Tor kinase. Furthermore, in HeLa cells, LC3-II decreases under starvation conditions in the absence of E64d and pepstatin A, while it increases under the same conditions in HEK293 cells. In the presence of these inhibitors, LC3-II dramatically accumulates under starvation conditions in both HeLa and HEK293 cells, as do LC3-positive puncta and autolysosomal structures. These results indicate that the lysosomal degradation of intra-autophagosomal components (Fig. 1, Step 3) significantly affects the amount of endogenous LC3-II after the formation of autophagosomes. Furthermore, inhibiting the fusion of autophagosomes with lysosomes using rab7 RNAi led to the accumulation of LC3-II, indicating that during the impairment of autophagosome-lysosome fusion LC3 is lipidated and LC3-II accumulates.[52] Therefore, without assaying the lysosomal turnover of LC3-II, the cellular level of LC3-II itself is far from conclusive regarding the activation of autophagy.

Using GFP-tagged LC3, it has been reported that an increase of GFP-positive puncta and LC3-II may be a marker of autophagy. However, an increase in the amount of endogenous LC3-II can occur under three conditions: (1) lipidation of LC3-I to form LC3-II during autophagosome-formation, (2) inhibition of the fusion of autophagosomes with lysosomes, and (3) inhibition of lysosomal degradation. Under the first condition, an increase in endogenous LC3-II may be regarded as a marker for autophagy. Under the latter two conditions, however, an increase in endogenous LC3-II is indicative of the inhibition of autophagic processes (Fig. 1, Steps 2 and 3). Thus, to distinguish the first condition from the other two, it is necessary to assay lysosomal turnover of endogenous LC3-II using E64d and pepstatin A. When we compared the lysosomal degradation of endogenous LC3 with that of GFP-human LC3, we found that GFP-human LC3 tended to accumulate less than endogenous LC3 in the presence of E64d and pepstatin A under starvation conditions (Tanida I, Ueno T, Kominami E, unpublished results Therefore, measurement of endogenous LC3 is better for estimating lysosomal degradation.

In some cell lines, including HeLa, A431, MDA-MB-231, and SK-N-SH cells, endogenous LC3-II is present even under nutrient-rich conditions without inactivation of Tor kinase. LC3-II may have two possible functions under nutrient-rich conditions in these cells. The first is that basal autophagic activity in these cells may be higher than in other cells. It has been reported that, even under nutrient-rich conditions, a basal level of autophagic activity is present in rat primary cultured hepatocytes.[53] If the basal level of autophagic activity is high under nutrient-rich conditions in these cells, LC3 would likely be present as LC3-II. Another possibility is that a pathway analogous to the yeast Cvt (Cytoplasm-to-vacuole targeting)-like pathway is activated in these cells.[54] In yeast, aminopeptidase I and α-mannosidase are synthesized in the cytosol and are directly transported to the vacuoles, the yeast counterparts of mammalian lysosomes.[55,56] This Cvt pathway requires most of the yeast Atg proteins,[21] indicating that autophagy and the Cvt-pathway use a common molecular machinery to some extent. At present, there is no evidence for a Cvt-like pathway in mammalian cells, but, if present, this pathway may be activated in these cell lines.

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