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LC3B-II deacetylation by histone deacetylase 6 is involved in serum-starvation-induced autophagic degradation

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ABSTRACT

Autophagy is a conserved mechanism for controlling the degradation of misfolded proteins and damaged organelles in eukaryotes and can be induced by nutrient withdrawal, including serum starvation. Although differential acetylation of autophagy-related proteins has been reported to be involved in autophagic flux, the regulation of acetylated microtubule-associated protein 1 light chain 3 (LC3) is incompletely understood. In this study, we found that the acetylation levels of phosphotidylethanolamine (PE)-conjugated LC3B (LC3B-II), which is a critical component of double-membrane autophagosome, were profoundly decreased in HeLa cells upon autophagy induction by serum starvation. Pretreatment with lysosomal inhibitor chloroquine did not attenuate such deacetylation. Under normal culture medium, we observed increased levels of acetylated LC3B-II in cells treated with tubacin, a specific inhibitor of histone deacetylase 6 (HDAC6). However, tubacin only partially suppressed serum-starvation-induced LC3B-II deacetylation, suggesting that HDAC6 is not the only deacetylase acting on LC3B-II during serum-starvation-induced autophagy. Interestingly, tubacin-induced increase in LC3B-II acetylation was associated with p62/SQSTM1 accumulation upon serum starvation. HDAC6 knockdown did not influence autophagosome formation but resulted in impaired degradation of p62/SQSTM1 during serum starvation. Collectively, our data indicated that LC3B-II deacetylation, which was partly mediated by HDAC6, is involved in autophagic degradation during serum starvation.

1. Introduction

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved process that plays an important role in the turnover of misfolded or aggregated proteins and damaged organelles in eukaryotic cells [1]. Such self-degradation not only provides nutrients for the maintenance of cellular functions, but also serves as an adaptive mechanism for protecting the organism against various pathologies, such as neurodegeneration, infection, cancer, aging, and heart diseases [2]. Thus, autophagy acts as a pro-survival mechanism for coping with various metabolic stresses, including energy deficiency, nutrient starvation, and growth factor withdrawal [3].

Numerous studies have reported that acetylation plays a significant role in autophagy regulation. The clearance of mutant huntingtin through autophagic degradation can be regulated by the acetylation at its Lys444 residue [4]. In cells deprived of growth factors, glycogen synthase kinase-3 (GSK3) activates acetyltransferase TIP60 through phosphorylating TIP60-Ser86. The activated TIP60 directly acetylates and thereby stimulates the protein kinase ULK1, which is required for autophagy induction [5]. Genetic analysis of *Saccharomyces cerevisiae* also identified Esa1 as a histone acetyltransferase required for autophagy [6]. Thus, the acetylation of autophagy-related proteins plays an important role in regulating autophagic flux. It has reported that the acetyltransferase p300 can acetylate Atg5, Atg7, Atg8 (the yeast homolog of the mammalian LC3 gene product) and Atg12 proteins; acetylation by p300 inhibits autophagy, whereas silencing of p300 increases autophagic flux [7]. Deacetylation of Atg8 is regulated by Sirt1, a well-known histone deacetylase [8]. LC3 is a core component of autophagosome and functions as an adaptor for delivering the cargoes to autophagosomes. Newly synthesized LC3 precursor was processed to form a soluble LC3 (LC3-I) with a C-terminal glycine residue [9]. Upon autophagy induction, LC3-I is conjugated with phosphatidyzlethanolamine (PE) through its C-terminus to form PE-conjugated LC3 (also known as LC3-II) that is tightly associated with the

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autophagosomal membrane [10]. LC3-PE mediates the degradation of autophagosome contents by their fusion with lysosomes. However, such a process requires cleavage and removal of LC3-PE by Atg4 prior to the fusion, at least in yeast. Inability to carry out the second cleavage confines LC3-PE at the outer surface of the autophagosome, which prevents the release of LC3 from PE and the fusion of autophagosomes with lysosomes thus limiting autophagic degradation of their contents [9,11]. Thus, protein modification of LC3 may regulate the final completion of autophagic degradation.

HDAC6 is a member of histone deacetylases (HDACs), which are potential regulators for autophagy. There are four classes of HDACs: class I (HDACs 1–3 and 8), class II (HDACs 4–7, 9 and 10), class III (the Zn-independent, NAD-dependent deacetylases Sirts 1–7), and class IV (HDAC11) [12]. By deacetylating α-tubulin, HDAC6 modulates retrograde transport of aggregate-containing inclusion bodies to be degraded via autophagy [13]. Parkin-mediated clearance of damaged mitochondria also requires the participation of HDAC6 [14]. It is also reported that HDAC6 recruits and deacetylates cortactin, thereby promoting F-actin remodeling important for autophagosome–lysosome fusion and protein aggregate clearance [15]. In the present study, we observed that LC3B-II, which is essential for autophagy, was markedly deacetylated during serum-starvation-induced autophagy in HeLa cells. High levels of LC3B-II acetylation, upon inhibiting the deacetylase activity of HDAC6 by tubacin or siRNA knockdown, were correlated with impaired degradation of p62/SQSTM1 during serum starvation, suggesting a linkage between LC3B-II deacetylation and autophagic degradation.

2. Materials and methods

2.1. Reagents

Chloroquine (CQ), rapamycin, tubacin and Hoechst33342 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Rapamycin was dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich) and kept at −20 °C. The final concentration of DMSO never exceeded 0.2%, which had no cytotoxicity on cells. Dulbecco's modified Eagle's medium (DMEM), antibiotics, fetal bovine serum (FBS), and Lipofectamine RNAi MAX were purchased from Invitrogen (Carlsbad, CA, USA). Polyvinylidene difluoride (PVDF) membranes (Hybond-P) and Protein A-Sepharose were purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA). Enhanced chemiluminescence (ECL) kit was obtained from Beyotime (Haimen, China). Antibodies against acetyl-lysine, acetyl-α-tubulin, α-tubulin, LC3B, p62/SQSTM1, HDAC6, and HRP-conjugated sheep anti-rabbit IgG were all obtained from Cell Signaling Technology (Danvers, MA, USA). DyLight 488-conjugated LC3B polycolonal antibody was purchased from Pierce (Rockford, IL, USA). HDAC6 siRNA was purchased from Abgent (San Diego, CA, USA).

2.2. Cell culture and transfection

HeLa cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and maintained in DMEM supplemented with 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2. HeLa cells were seeded in 6-well plates for 24 h. Then cells were treated with HDAC6 siRNA in DMEM with Lipofectamine RNAi MAX for transfection. After 72 h, cells were used for experiments.

2.3. Immunofluorescence microscopy

Immunofluorescence was performed essentially as previously reported [16]. In brief, cells were fixed with 4% paraformaldehyde and permeabilized with 100% methanol. The permeabilized cells were incubated with appropriate primary antibodies at 4 °C overnight. After PBS wash, cells were incubated with CF488-conjugated goat-anti-mouse IgG or CF568-conjugated goat-anti-rabbit IgG (Biotium, Hayward, CA, USA) at room temperature for 1 h. Nuclei were revealed by Hoechst33342 staining. Fluorescence images were collected under a Leica DMIRB fluorescent microscope (Leica Microsystems, Wetzlar, Germany) armed with a Spinning Disk Confocal Microscopy system (UltraView cooled CCD; Perkin Elmer, Waltham, MA, USA).

2.4. Protein extraction

Cells were washed thoroughly with ice-cold PBS and lysed with RIPA lysis buffer (Beyotime) for assaying degradation of proteins and immunoprecipitation [17]. Protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Samples were subjected to Western blotting or immunoprecipitation.

2.5. Immunoprecipitation and Western blotting

Protein lysates (200 μg) were prepared from HeLa cells and mixed with the indicated antibodies (4 °C overnight) followed by incubation with 30 μl of Protein A-Sepharose (Cell Signaling Technology) at 4 °C for 2 h. Immune complexes were washed five times with lysis buffer (Beyotime). Then samples were boiled in 2× loading buffer and were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto PVDF membranes. After incubation in blocking buffer (50 mM Tris-buffered saline (pH7.4) containing 5% non-fat milk and 0.1% Tween-20), the membranes were probed with indicated antibodies, followed by a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. Bands were revealed by ECL and recorded on X-ray films (Kodak, Xiamen, China). Images were acquired by using FluorChem 8000 (AlphaInnotech, San Leandro, CA, USA).

3. Results

3.1. Serum starvation induced autophagy in HeLa cells

As serum-deprivation is an effective stimulus to induce autophagy in HeLa cells [18], we used this cellular model to study serum-starvation-induced autophagy. To monitor autophagy induction, we assayed the accumulation of LC3B-II and the formation of LC3B puncta in HeLa cells cultured under serum-deprived medium. Western blot analysis revealed that serum starvation time-dependently increased the level of LC3B-II compared to control (0 h), and culminated in its peak level at 8 h (Fig. 1A). Moreover, serum deprivation in the presence of chloroquine (CQ), a lysosomal inhibitor that blocks autophagic degradation, induced a higher level of LC3B-II compared to serum deprivation alone (Fig. 1B). These results confirmed an increased autophagic flux of LC3B-II during serum starvation. In support of this, we observed a robust formation of LC3B puncta in serum-deprived cells compared to control, and more LC3 puncta in the presence of CQ (Fig. 1C). These data indicated that 8 h serum starvation induced a marked autophagy in HeLa cells, thus this time point was adopted for the following experiments.

3.2. Acetylation levels of LC3B-II were decreased during serum-starvation-induced autophagy

To evaluate the involvement of LC3 acetylation during autophagy, we next tested whether the acetylation levels of LC3B are regulated upon autophagy induction by serum starvation. The