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LC3B is indispensable for selective autophagy of p62 but not basal autophagy

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ABSTRACT

Autophagy is a unique intracellular protein degradation system accompanied by autophagosome formation. Besides its important role through bulk degradation in supplying nutrients, this system has an ability to degrade certain proteins, organelles, and invading bacteria selectively to maintain cellular homeostasis. In yeasts, Atg8p plays key roles in both autophagosome formation and selective autophagy based on its membrane fusion property and interaction with autophagy adaptors/specific substrates. In contrast to the single Atg8p in yeast, mammals have 6 homologs of Atg8p comprising LC3 and GABARAP families. However, it is not clear these two families have different or similar functions. The aim of this study was to determine the separate roles of LC3 and GABARAP families in basal/constitutive and/or selective autophagy. While the combined knockdown of LC3 and GABARAP families caused a defect in long-lived protein degradation through lysosomes, knockdown of each had no effect on the degradation. Meanwhile, knockdown of LC3B but not GABARAPs resulted in significant accumulation of p62/Sqstm1, one of the selective substrate for autophagy. Our results suggest that while mammalian Atg8 homologs are functionally redundant with regard to autophagosome formation, selective autophagy is regulated by specific Atg8 homologs.

1. Introduction

Macroautophagy (hereafter referred to as autophagy) is an evolutionally conserved intracellular bulk degradation system. Single membrane sac called isolation membrane or phagophore elongates and sequesters cytoplasmic components including entire organelles randomly. Subsequently, the edge of isolation membrane fuses with each other to form a double membrane structure called autophagosome. Eventually, the contents in autophagosome are degradated via the fusion with lysosome [1]. This pathway is highly inducible and serves as a supplier of molecular building blocks under starved conditions and also contributes to cellular renovation during cell differentiation [2]. Besides such fundamental role, increasing evidence points to the selectivity of autophagy in sorting of vacuolar enzymes and removal of aggregate-prone proteins, unwanted organelles, and invading bacteria [3]. Such selectivity allows diverse cellular regulation, similar to the ubiquitin proteasome pathway.

Taking advantage of yeast genetics and biochemical analysis, 36 *ATG* (autophagy-related) genes have been identified so far [1]. The molecular function of each Atg protein is almost conserved in eukaryotes from yeasts to mammals. Among them, Atg8 and its mammalian homolog microtubule-associated protein 1 light chain 3/MAP1LC3 (LC3), whose structures comprise ubiquitin fold and N-terminal two α-helices, play a crucial role in autophagosome formation [4,5]. Atg4, a cysteine protease, processes the C-terminal amino acid of Atg8/LC3 to expose a glycine residue immediately

Abbreviations: GABARAP, gamma-aminobutyrate receptor-associated protein; GEC-1, glandularepithelial cell protein 1; GATE-16, golgi-associated ATPase enhancer of 16 kDa; ITC, isothermal titration calorimetry; LC3, microtubule-associated protein 1 light chain 3/MAP1LC3; LIR, LC3-interacting region; PE, phosphatidylethanolamine.

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after its synthesis [1]. Thereafter, matured Atg8/LC3 is activated by Atg7 (E1) in an ATP-dependent manner and transferred to Atg3 (E2) [1]. Subsequently, the C-terminal glycine of Atg8/LC3 conjugates to the amino group of phosphatidylethanolamine (PE) in an Atg12–Atg5 Atg16-dependent manner [1]. Atg8-PE/LC3-PE is localized in the inner and outer membranes of the isolation membrane/phagophore [1]. Atg8-PE/LC3-PE localized on the outer membrane is re-cleaved by Atg4 following completion of autophagosome formation and released Atg8/LC3 is recycled, whereas Atg8-PE/LC3-PE present on the inner membrane is degraded together with other cellular constituents by vacuolar/lysosomal proteases [1]. The Atg8-PE/LC3-PE is essential for biogenesis and closure of autophagosomal membrane [6–8].

In addition to the importance of both Atg8 and its homologs in autophagosome formation, their interaction with specific proteins plays a crucial role in selective autophagy [3]. p62/Sqstm1 (hereafter referred to as p62), which is the best characterized autophagy-substrate in mammals, localizes at the autophagosome formation site and directly interacts with Atg8 homologs through the LC3-interacting region (LIR) [9–11], and it is incorporated subsequently into the autophagosome and finally degraded. Impaired autophagy is accompanied by the accumulation of p62, followed by the formation of aggregates positive for p62 and ubiquitinated proteins because of the nature of both self-oligomerization and the ubiquitin-binding capacity of p62 [12]. This protein serves as a signaling hub for several signal transductions and as an autophagy-adaptor for ubiquitinated cargos [13].

Human has 6 genes coding for Atg8 homologs, and gene products can be grouped into two subfamilies; (1) the LC3 subfamily containing LC3A, LC3B, and LC3C, and (2) the gamma-aminobutyrate receptor-associated protein (GABARAP) subfamily containing GABARAP, GABARAPL1/GEC-1, and GABARAPL2/GATE-16. While previous studies showed that these proteins are all conjugated to PE [14], they appear to have complex non-redundant functions in membrane biogenesis of autophagosomes and in preferential binding to adaptors/targets for selective autophagy [5,8,15–17]. Herein we demonstrate that LC3 and GABARAP families are both dispensable for basal autophagy in HEK293T cells and that among of the 6 Atg8 homologs, LC3B is responsible for selective degradation of p62 through autophagy.

2. Materials and methods

2.1. Cell culture and transfection

HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 5 U/ml penicillin and 50 μg/ml streptomycin. At subconfluence, HEK293T cells were transfected with the indicated 25 nM siGENOME SMART pool siRNAs (MAP1LC3B siRNA Pool: M012846, GABARAP siRNA Pool: M012368, GABARAPL1 siRNA Pool: M014715, GABARAPL2 siRNA Pool: M006853, ULK1 siRNA Pool: M-005049, ATG3 siRNA Pool: D-015375, Non-targeting siRNA Pool: D-001206, Thermo Scientific) using DharmaFECT 1 transfection reagent according to the manufacturer's protocol (Thermo Scientific). Cells were analyzed at 48 h after transfection. The immortalized MEFs were transfected with GFP-LC3, GFP-GABARAP, GFP-GABARAPL1 or GFP-GABARAPL2 using the retrovirus vector system, and then cultured with medium in the presence of 2 μg/mL puromycin to select stable transformants.

2.2. RT-PCR

cDNA was synthesized from 1 μg of DNase I-treated total RNA using the SuperScript First-Strand Synthesis System (Gibco BRL) and oligo $(dT)_{12-18}$ primers. Specific primers for each gene were indicated in Supplementary Table 2.

2.3. Droplet digital-PCR

Using the Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science), cDNA was synthesized from 1 μg of total RNA. Digital PCR was was performed using the QX100 Droplet Digital PCR system (BioRad) according to the manufacturer's instructions [18]. Specific primer pairs were designed by ProbeFinder software (Roche). Data from absolute quantification are shown.

2.4. Immunological analysis

Samples were separated using the NuPAGE system (Invitrogen) on 12% Bis-Tris gels in MOPS-NuPAGE buffer, and then transferred to polyvinylidene difluoride (PVDF) membranes. The antibodies for LC3B (#2775, Cell Signaling Technology), GABARAP (PM037, MBL), GABARAPL1 (ab86497, Abcam), GABARAPL2 (PM038, MBL), Atg3 (PM034, MBL), ULK1 (H-240, Santa Cruz), p62 (GP62-C, Progen), and actin (MAB1501, Millipore) were purchased from the indicated suppliers. Band intensities were quantified by ImageJ 1.42q software (NIH, Bethesda, MD). Statistical significance was calculated by two-tailed Student's t test. A p value less than 0.05 denoted the presence of significant difference. For LC3- or GABARAP- and p62-staining, cells were fixed and stained with anti-LC3B (4E12, MBL) or anti-GABARAP (PM037, MBL) and anti-p62 (GP62-C, Progen) antibodies, respectively, as described previously [10]. Images were acquired with a laser scanning confocal microscope (FV1000-D, Olympus). After image acquisition, image contrast and brightness were adjusted using Photoshop CS4 (Adobe).

2.5. Long-lived protein degradation assay

The assay was performed essentially as described previously [19].

2.6. Computer-based structure modeling

Structural models of GABARAP-LIR and GABARAPL2-LIR complex were created based on those of the rat GABARAP [20] and bovine GABARAPL2/GATE-16 [21], respectively, using the MOE program (version 2005.06; Chemical Computing Group, Montreal, Canada). The models shown in Fig. 2A were generated by PyMOL.

2.7. Isothermal titration calorimetry

GST-tagged LC3 and GABARAPL2 were purified as described previously [10]. LIR peptide (*N-acetyl*-GGDDDWTHLS-amide) was purchased from Toray Research Center. ITC experiments were performed in PBS at 25 °C on a MicroCal-iTC$_{200}$ system (GE Healthcare). In each run, 40 μl of 1.0 mM LIR was injected 39 times at 2 min intervals from a stirring syringe (1000 rpm) into the sample cell containing 200 μl of 0.1 mM LC3 or GABARAPL2/GATE-16. Binding data were analyzed using Origin 5.0 (MicroCal Inc.) and standard deviations were derived from three separate runs.

2.8. Pull down analysis

HEK293T co-expressing One-Strep-FLAG-p62 and GFP-LC3, GFP-GABARAP, or GFP-GABARAPL2 cells were lysed in TNE buffer and centrifuged at 15,000 rpm for 5 min at 4 °C. The resulting supernatants were incubated with Strep-Tactin Sepharose (IBA) at 4 °C for 1 h. The protein complexes were washed three times with lysis buffer, and eluted with elution buffer (50 mM Tris–HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, and 2.5 mM D-desthiobiotin [Sigma–Aldrich]).