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LC3- and p62-based biochemical methods for the analysis of autophagy progression in mammalian cells

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ABSTRACT

Autophagy is an intracellular degradation system that delivers cytoplasmic materials to the lysosome or vacuole. This system plays a crucial role in various physiological and pathological processes in living organisms ranging from yeast to mammals. Thus, an accurate and reliable measure of autophagic activity is necessary. However, autophagy involves dynamic and complicated processes that make it difficult to analyze. The term "autophagic flux" is used to denote overall autophagic degradation (i.e., delivery of autophagic cargo to the lysosome) rather than autophagosome formation. Immunoblot analysis of LC3 and p62/SQSTM1, among other proteins, has been widely used to monitor autophagic flux. Here, we describe basic protocols to measure the levels of endogenous LC3 and p62 by immunoblotting in cultured mammalian cells.

1. Introduction

Autophagy comprises all processes in which intracellular materials are degraded within the lysosome or vacuole. There are at least three major types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy [1,2]. Among these, macroautophagy (hereafter referred to as autophagy), which is distinguished by the formation of a unique double-membrane organelle called the autophagosome [3], is the most extensively studied. As autophagy is involved in various physiological and pathological processes [1,4,5], an exact measure of autophagic activity is necessary.

As yet, more than 30 autophagy-related (*Atg*) genes have been identified in yeast, and many of these are conserved among higher eukaryotes [6]. Among known *Atg*-encoded proteins, only microtubule-associated protein 1 light chain 3 (LC3), a mammalian orthologue of yeast Atg8, can localize to all types of autophagic membranes, including the phagophore (the immature autophagosome, also known as the isolation membrane), the autophagosome, and the autolysosome (a hybrid organelle formed by fusion of the autophagosome and lysosome) [7,8]. Nascent LC3 (proLC3) is processed by Atg4-family proteins, which are cysteine proteases, into LC3-I immediately after synthesis [7]. During autophagy, cytosolic LC3-I is conjugated to phosphatidylethanolamine (PE) to become LC3-II by the activating enzyme Atg7 [9] and the conjugating enzyme Atg3 [10] (Fig. 1). The conjugation of LC3 to PE is also facilitated by the Atg12–Atg5 conjugate along with Atg16L1 [11–13] (Fig. 1). LC3-II is then recruited to autophagosomal membranes. Finally, LC3 is released from LC3-PE by a second Atg4-dependent cleavage [14], while LC3-II in the autolysosomal lumen is degraded by autophagy [15]. Thus, LC3 conversion (LC3-I to LC3-II) and lysosomal degradation of LC3-II reflect the progression of autophagy, and detecting LC3 by immunoblot analysis is often used to monitor autophagic activity.

However, the number of autophagic organelles at a given moment is regulated by both the on-rate (autophagosome formation) and off-rate (degradation upon fusion with lysosomes). Thus, although the amount of LC3-II correlates with the number of autophagosomes, its amount at a certain time point does not necessarily indicate the degree of autophagic flux, a term used to indicate overall autophagic degradation (i.e., delivery of autophagic cargo to the lysosome) rather than autophagosome formation [16,17]. Furthermore, not all LC3-II is present on autophagic membranes. A significant amount of LC3-II can still be detected in cells deficient in some of the upstream Atg factors (e.g., FIP200, Atg13, Atg9, Vps34, Beclin-1, Atg14, and Atg2) (Fig. 1) [16,18–22], suggesting that some population of LC3-II may be ectopically generated in an autophagy-independent manner. LC3 can also be recruited directly to bacteria-containing phagosome membranes in a process

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termed LC3-associated phagocytosis [23]. Thus, it is important to measure the amount of LC3-II delivered to the lysosomes by comparing LC3-II amounts in the presence and absence of bafilomycin A_1 (a vacuolar H^+-ATPase inhibitor), lysosomal protease inhibitors (e.g., E64d and pepstatin A), or lysosomotropic agents (e.g., chloroquine) to inhibit lysosomal degradation of LC3-II (Fig. 2).

Another widely used autophagy marker, p62, also called sequestosome 1 (SQSTM1), binds directly to LC3 and GABARAP (Atg8 orthologues) family proteins via a short LC3 interaction region (LIR). This may serve as a mechanism to deliver selective autophagic cargo for degradation by autophagy. The p62 protein is itself degraded by autophagy and serves as a marker to study autophagic flux [24–26]. When autophagy is inhibited, p62 accumulates, while when autophagy is induced, p62 quantities decrease (Fig. 1).

Here, we outline detailed methodologies for LC3- and p62-based biochemical analysis of autophagy progression. We also discuss possible pitfalls and important cautions regarding LC3 and p62 immunoblot analysis.

2. Materials

2.1. Cell lines

- HeLa cells.
- Mouse embryonic fibroblasts (MEFs).

2.2. Reagents

- Dulbecco's modified eagle's medium (DMEM), high glucose (Sigma-Aldrich, cat. no. D6546).
- Fetal bovine serum (FBS), sterile filtered (Equitech-Bio, lot. no. SFBM30-2566).
- L-Glutamine, 200 mM solution (Life technologies, cat. no. 25030-081).
- Penicillin-streptomycin, liquid (Life technologies, cat. no. 15070-063).
- Dulbecco's phosphate-buffered saline (DPBS, 10×) (Life technologies, cat. no. 14200-075).
- D-MEM (high glucose) with sodium pyruvate, without amino acids (Wako, cat. no. 048-33575).
- Hydrochloric acid (HCl, Wako, cat. no. 080-01066).

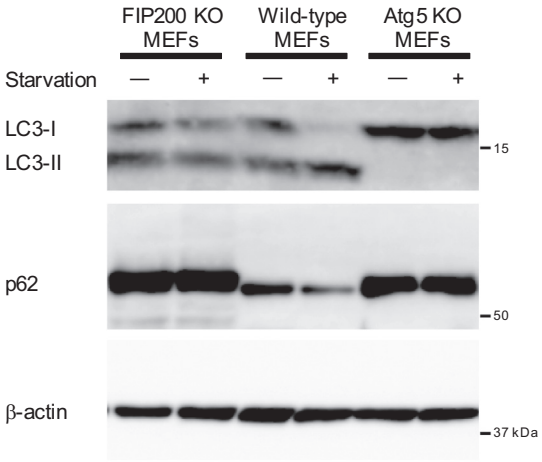

Fig. 1. Expression levels of LC3 and p62 during starvation. Wild-type, Atg5 knockout (KO), or FIP200 KO mouse embryonic fibroblasts (MEFs) were cultured in regular DMEM or DMEM without amino acids and serum (starvation medium) for 2 h. Cell lysates were subjected to immunoblot analysis using anti-LC3 or p62, and β-actin antibodies.

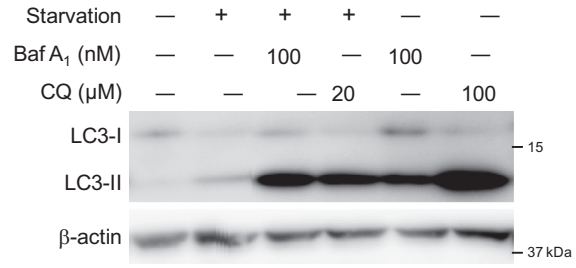

Fig. 2. Endogenous expression patterns of LC3. MEFs were cultured in regular DMEM (lane 1), DMEM without amino acids and serum (starvation medium, lane 2), starvation medium containing 100 nM bafilomycin A_1 (lane 3), starvation medium containing 20 μM chloroquine (lane 4), regular DMEM containing 100 nM bafilomycin A_1 (lane 5), or regular DMEM containing 100 μM chloroquine (lane 6) for 2 h. Cell lysates were subjected to immunoblot analysis using anti-LC3 and β-actin antibodies.

- NaCl (Wako, cat. no. 191-01665).
- Na_2HPO_4 $12H_2O$ (Wako, cat. no. 196-02835).
- KCl (Wako, cat. no.163-03545).
- KH_2PO_4 (Wako, cat. no. 169-04245).
- 0.05% Trypsin-EDTA (1×), phenol red (Life technologies, cat. no. 25300-062).
- Triton X-100 (Nacalai tesque, cat. no. 35501-15).
- 2NA(EDTA.2Na) (Dojindo, cat. no. 345-01865).
- Phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich, cat. no. P7626).
- 2-Propanol (Wako, cat. no. 166-04836).
- Na_3VO_4 (Wako, cat. no. 198-09752).
- cOmplete, EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics, cat. no. 11873580001).
- Tris(hydroxymethyl)aminomethane, nuclease and protease tested (Nacalai tesque, cat. no. 35434-34).
- Sodium lauryl sulfate granular (SDS, Nacalai tesque, cat. no. 02873-75).
- 30% acrylamide/bis solution, 29:1 (Bio-Rad, cat. no. 161-0156).
- *N,N,N′,N′*-tetramethylethylenediamine (TEMED, Wako, cat. no. 205-06313).
- Ammonium peroxodisulfate (APS, Wako, cat. no. 016-08021).
- Glycine (Wako, cat. no. 073-00737).
- Methanol (Wako, cat. no. 137-01823).
- Polysorbate 20 (MP Biomedicals, cat. no. 103168).
- Glycerol (Wako, cat. no. 075-00616).
- Bromophenol blue (Wako, cat. no. 021-02911).
- (+/−)-Dithiothreitol (DTT, Wako, cat. no. 048-29224).
- Albumin standard, 2.0 mg/ml (Thermo Scientific, cat. no. 23209).
- BCA protein assay reagent A (Thermo Scientific, cat. no. 23228).
- BCA protein assay reagent B (Thermo Scientific, cat. no. 23224).
- Precision plus protein dual color standards (Bio-Rad, cat. no. 161-0374).
- Whatman 3MM Chr chromatography paper (GE Healthcare, cat. no. 3030917).
- Immobilon-P transfer membrane (Millipore, cat. no. IPVH00010).
- Ponceau S (MP Biomedicals, cat. no. 190644).
- Trichloroacetic acid (TCA, Wako, cat. no. 204-02405).
- 5-Sulfosalicylic acid dihydrate (Wako, cat. no. 190-04572).
- Skim milk powder (Wako, cat. no. 198-10605).
- Rabbit polyclonal antibody against LC3 (NM1) was described previously [27]. Other LC3 antibodies (e.g., anti-LC3 pAb, MBL, cat. no. PD014 and PM036) can be used instead.
- Anti-p62 (SQSTM1) pAb (MBL, cat. no. PM045).
- Monoclonal anti-β-actin antibody produced in mouse (Sigma-Aldrich, cat. no. A5316).

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