6

Autophagic Proteolysis of Long-Lived Proteins in Nonliver Cells

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Summary

Autophagy is a cellular homeostasis pathway used to sustain cellular anabolic needs during times of nutrient or energy deprivation. Autophagosomes sequester cytoplasmic constituents, including macromolecules such as long-lived proteins. Upon fusion of autophagosomes with lysosomes, the engulfed cargo is degraded. The proteolysis of long-lived proteins by macroautophagy is a standard, specific measure of autophagic degradation and represents an end-point assay for the pathway. The assay is based on a pulse-chase approach, whereby cellular proteins are radiolabeled by an isotopically marked amino acid, the short-lived, rapidly turned over, proteins are allowed to be degraded during a long chase period, and then the remaining, stable radiolabeled proteins are subjected to autophagic degradation. The classical application of this method has been in hepatocytes, but the recent growth of interest in autophagy has necessitated adaptation of this method in nonliver cells. Here we describe a protocol to quantify autophagic degradation of long-lived proteins in macrophages. This chapter details the method of analyzing autophagic proteolysis in RAW264.7 mouse macrophages.

Key Words: Autophagy; proteolysis; long-lived proteins; macrophage.

1. Introduction

Autophagy is an intracellular bulk degradation mechanism beginning with the engulfment of cytoplasmic constituents including proteins into double-membrane autophagosomes and ending with fusion of autophagosomes with lysosomes for final degradation of the sequestered macromolecules ***(1–3)***. Fusion of autophagosomes with lysosomes to form autolysosomes is important for delivery of lysosomal hydrolases and completion of the autophagic

From: *Methods in Molecular Biology, vol. 445: Autophagosome and Phagosome*
Edited by: V. Deretic © Humana Press, Totowa, NJ

pathway via degradation of cytoplasmic components. Among the cytoplasmic constituents targeted for autophagolysosome degradation are stable, long-lived proteins within the cell. A simple radiolabeled amino acid–based pulse chase assay can be used to assess the function of the autophagic pathway in cells by quantitatively monitoring the turnover of long-lived proteins during induction of autophagy or during its inhibition ***(2–4)***. This assay was traditionally used to examine autophagy in hepatocytes, since liver cells are robust in their autophagic activity ***(3,4)***. Since autophagy is now studied in many different cell types, it is necessary to adapt and optimize original methods developed for liver cells ***(2–4)*** to other individual cell types. This chapter describes the methodologies used to examine the autophagic proteolysis of long-lived proteins in nonhepatic cell types and details the quantification of autophagic proteolysis in macrophages.

The majority of analyses of proteolysis under stimulation of autophagy have utilized a variation of the method developed by Codogno and colleagues in 1996 ***(5)*** when they examined the role of G_{i3} protein in autophagy in HT-29 cells (a human colon carcinoma cell line). This method uses L-(^{14}C)valine for the radiolabeled incorporation of amino acid into de novo synthesized proteins (pulse). The radioisotope is subsequently chased by incubating cells in media containing cold valine to allow the degradation of short-lived proteins (chase). It is only after this period of short-lived protein degradation that the autophagic induction of long-lived protein degradation can be specifically examined. Upon a period of autophagic induction, the analysis becomes a simple calculation of the ratio of soluble radioactivity present in the culture supernatant to total cellular radioactivity that reflects the rate of stable protein degradation ***(5,6)***. Several derivatives of this method have been used depending on the experimental situation and have included using higher concentrations of radioisotope in mouse embryonic fibroblasts (MEFs) and HeLa cells ***(7,8)***, different methods of cellular lysis (MEFs and HeLa) ***(7,8)***, and various degradation measurements such as percent degradation over a time course rather than single time point measurement as performed in MEFs and human fibroblasts ***(9,10)***. One deviation from the method of Ogier-Denis ***(5)*** has been the use of tritiated leucine as the amino acid of choice for examining the autophagic proteolysis of long-lived proteins. This is probably a simple issue of preference and availability rather than advantage since it has been shown that, at least in human fibroblasts, L-(^{3}H)leucine- or L-(^{3}H)valine-labeled cells lose protein radioactivity at a similar rate, making these amino acids equally useful in evaluating the degradation of stable proteins ***(9)***.

Recently, autophagy has been linked to innate immune defense against several pathogens ranging from RNA viruses, like poliovirus ***(11)***, gram-positive bacteria, like *Streptococcus* ***(12)***, gram-negative bacteria, like *Shigella*

(13), and even *M. tuberculosis* in infected macrophages ***(14,15)***. Autophagy has been implicated in antigen presentation, which can occur in various cell types, but is best achieved by macrophages and dendritic cells ***(16)***. Because of this, quantification of autophagic processes is of interest in antigen-presenting cells in general and specifically in macrophages. Macrophages represent a challenging experimental system while analyzing autophagy because they are robust in phagolysosome biogenesis, free-radical production, and cytokine secretion and signaling. Therefore, establishing reproducible techniques to evaluate autophagic processes in macrophages is of paramount importance to enable researchers to probe the role of autophagy in innate and adaptive immunity. Since the autophagic proteolysis of long-lived proteins is a gold standard method to examine autophagy, we have optimized this assay for autophagic proteolysis in macrophages (**Fig. 1**). The following methods describe a protocol for examining the autophagic proteolysis of long-lived proteins in the easily transfectable mouse macrophage cell line RAW264.7.

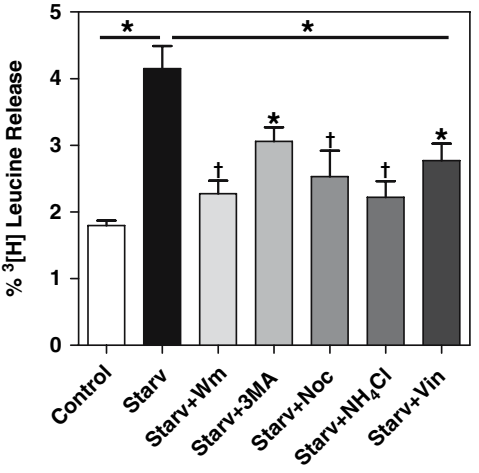

Fig. 1. Proteolysis of long-lived proteins in macrophages. RAW264.7 macrophages were examined for autophagic degradation of long-lived proteins using Earle's balanced salt solution (Starv). Starvation enhances proteolysis twofold ($*p < 0.05$) over that in complete DMEM (Control). Classical inhibitors of autophagy were examined for their efficacy in preventing proteolysis (Wm = wortmannin [100 n*M*]; 3MA = 3-methyladenine [10 m*M*]; Noc = nocodazole [20 μ*M*]; NH_4Cl = ammonium chloride [10 m*M*]; Vin = vinblastine [50 μ*M*]). All inhibitors exerted a significant reduction in levels of autophagic proteolysis when compared to starvation. Individual statistics showed that not all inhibitors reduced proteolysis to control levels ($*p < 0.05$; $^{\dagger}p > 0.05$).

2. Materials

2.1. Cell Culture, Media, and Reagents

1. RAW264.7 cells (ATCC TIB-71) are grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone, Ogden, UT) and 4 m*M* L-glutamine (BioWhittaker, Walkersville, MD) (complete DMEM).
2. Leucine-free DMEM (Chemicon International, Temecula, CA) created using the formulation from Gibco/BRL (Bethesda, MD).
3. 100% trichloroacetic acid.
4. 10% bovine serum albumen (BSA) in 1X phosphate-buffered saline (PBS) pH 7.2.
5. lysis buffer: 10 m*M* Tris HCl (pH 8.0), 150 m*M* NaCl, , 2 m*M* EDTA, 0.5% deoxycholate, 2% NP-40 in dH_2O **(Note 1)**.
6. "cold" L-leucine (Sigma, St. Louis, MO).
7. Autophagy reagents: Earle's Balanced Salt Solution (EBSS), wortmannin (Sigma), 3-methyladenine (Sigma), Nocodazole (Sigma), vinblastine sulfate salt (Sigma).
8. 1.7-mL Ultraclear graduated microcentrifuge tubes (GENEMate, Kaysville, UT) **(Note 2)**.

2.2. Radioactive Reagents and Supplies

1. Radioactivity: 1 μCi/mL L-(4,5-^{3}H)leucine (73.0 Ci/mmol) (Amersham, Waltham, MA).
2. Scintillation fluid: Optiphase HiSafe 3 Scintillation cocktail (PerkinElmer, Waltham, MA).
3. Scintillation vials: Borosilicate glass vial with urea cap (22mm) with foil liner (Wheaton, Millville, NJ).
4. Packard Tri-Carb 2100TR liquid scintillation analyzer, (PerkinElmer, Waltham, MA).

3. Methods

3.1. Pharmacological Inhibitor Preparation

1. Wortmannin (FW = 484.4 g/mol): use at 100 n*M* final.
   a. Dilute 1mg (supplied in a single vial) in 2.064 mL 100% DMSO to yield 1 m*M* stock.
   b. Perform a 1:10 dilution in 1X PBS to yield 100 μ*M* stock (working solution).
   c. Dilute 1:1000 (1 μL in 1 mL) for final use = 100 n*M* final.
2. 3-Methyladenine (FW = 149.16 g/mol): use at 10 m*M* final.
   a. Prepare 100 m*M* stock by dissolving 0.014916 g in 1 mL of dH_2O. Heat between 60 and 80°C/10 min in a heating block with cap-lock.
   b. Add 100 μL of 100 m*M* stock per 1 mL of media to achieve 10 m*M* final.

3. Nocodazole (FW = 301.32 g/mol): use at 20 μ*M* final.
   a. Dissolve 2 mg (supplied in a single vial) in 0.332 mL 100% DMSO to yield 20 m*M* stock.
   b. Dilute 1:1000 (1 μL in 1 mL) for final use = 20 μ*M* final.
4. Vinblastine sulfate salt (FW = 909.05 g/mol): use at 50 μ*M* final.
   a. Dissolve 1 mg (supplied in a single vial) in 0.044 mL 100% DMSO to yield 25 m*M* stock.
   b. Dilute 1:500 (50 μL in 1 mL) for final use = 50 μ*M*.
5. Ammonium chloride (FW = 53.49 g/mol): use 10 m*M* final.
   a. Dissolve 0.5349 g in 10 mL dH_2O to achieve a 1 *M* stock.
   b. Dilute 1:100 (10 μL in 1 mL) for final use = 10 m*M*.

3.2. Proteolysis Assay

1. Scrape a 2-day-old subculture of RAW macrophages (70–80% confluency) into 12 mL complete DMEM and count cells (10 μL) using trypan blue staining (5 μL) in a hemocytometer.
2. Dilute cells to 7×10^4/mL in complete DMEM and dispense 1 mL per well into 12-well plate. Incubate at 37°C, 5% CO_2 overnight.
3. Remove media and place 1 mL DMEM with 1 μCi/mL ^{3}H-leucine adjusted to 62.4 μ*M* final onto cells and incubate overnight at 37°C, 5% CO_2.
4. Remove tritiated media into radioactive liquid waste container and wash once with 0.5 mL/well cold DMEM containing 62.4 μ*M* L-leucine (adjusted DMEM). Dispose wash in radioactive liquid waste container.
5. Incubate macrophages in 1 mL/well adjusted DMEM overnight (typically 16 h) at 37°C, 5% CO_2 to chase out short-lived proteins.
6. Remove media from wells and dispose in radioactive liquid waste container. Wash wells destined for starvation once with 1 mL 37°C EBSS and add 0.5 mL EBSS/well. Incubate at 37°C/10 min. Repeat two times and add 0.5 mL EBSS (or EBSS + inhibitor)/well **(Note 3)**. Wash other wells with 1 mL adjusted DMEM and add 0.5 mL DMEM for control. Incubate samples at 37°C, 5% CO_2 for 4 h.
7. Remove sample media to a microcentrifuge tube (*see* **Note 2**). Add 50 μL of fresh 10% BSA in 1X PBS followed by 100 μL of room temperature 100% trichloroacetic acid to each tube and incubate on ice at least 10 min.
8. Spin samples at 5,500× gh/10 min at room temperature. The supernatant represents the soluble radioactivity from samples, which contain released tritiated leucine.
9. Add 0.5 mL of lysis buffer to cells remaining in the wells and place on shaker 10 min at room temperature to aid in lysis. Pipet several times to ensure total cell lysis. These samples represent the total cellular radioactivity.

10. Aliquot soluble radioactivity samples and total cellular radioactivity samples into scintillation vials containing 3–5 mL of scintillation fluid and read in a scintillation counter using 1 min read times. The percent leucine release is calculated by dividing the soluble radioactivity cpm by the total cellular radioactivity sample cpm.

4. Notes

1. Lysis buffer: Lysis buffer can be substituted by 0.1*M* NaOH or cold, sterile dH_2O (hypotonic lysis).
2. Microcentrifuge tubes: GENEMate cat. no. C-3218-1 Ultraclear microcentrifuge tubes resist leakage, preventing radioactive contamination.
3. Repeated washes for starvation: Repeated 10-min washes with EBSS are necessary to activate the starvation response in RAW264.7 macrophages. This may be the result of, besides the simple carryover of amino acids, a sufficient efflux of free amino acids that will then repress autophagic signal induction. A similar technique was used to enhance amino acid starvation in MCF7 and HeLa cells using hourly replacement of starvation media to discard secreted amino acids ***(17)***.

Acknowledgments

This work was supported by the New York Community Trust's Heiser Program for Research in Tuberculosis and Leprosy Postdoctoral Fellowship to E.A.R. and by grants AI45148 and AI069345 from the National Institutes of Health.

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