Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 351 (2006) 71–77
BBRC
www.elsevier.com/locate/ybbrc

Quantitative monitoring of autophagic degradation

Akinori Kawai, Syuichi Takano, Nobuhiro Nakamura, Shoji Ohkuma *

Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192, Japan

Received 21 September 2006
Available online 12 October 2006

Abstract

We developed a quantitative method for analyzing the induction of autophagy using a CHO-K1 cell line stably expressing a green fluorescent protein (GFP) in mitochondrial matrix (mtGFP-CHO). When mtGFP-CHO cells were incubated with a medium depleted of amino acids and serum, the GFP fluorescence was decreased concomitant with degradation of the protein. Biochemical and morphological analyses strongly suggested the degradation of mtGFP was mediated by bulk and non-selective degradation of mitochondria by autophagy. Quantitative measurement of the mtGFP degradation was performed by measuring the GFP fluorescence and DNA content by a fluorometric method and calculating the relative GFP intensity of DNA content, which approximated mean GFP fluorescence per cell. Using this method, we showed for the first time that different inducers, such as amino acids and serum starvation or rapamycin treatment, promote autophagy with different kinetics. This method is easy, relatively quick, and may be easily adapted to high throughput screening for novel drugs that enhance or inhibit autophagy, and also for genes that regulate or modulate autophagy.

Keywords: Macroautophagy; Starvation; Kinetics; GFP; Mitochondrial degradation

Macroautophagy, usually referred to simply as autophagy, is an intracellular bulk degradation system that is induced when cells are forced to restructure their components or correct redundancies under nutrient starvation, hormonal stimulation or cell differentiation, and development (for reviews, see [1–6]). When cells are starved, they induce autophagy, degrade, and reuse non-essential parts of the components to enable energy production and material supplies, such as amino acids, for maintaining the essential cellular functions required for survival [2]. In autophagy, double membrane structures, known as limiting membranes, are formed *de novo* in the cytoplasm, which enclose and capture a portion of the cytoplasm including organelles, such as mitochondria, to form autophagosomes. The autophagosomes then fuse with endosomes or lysosomes that contain degradative enzymes and become autolysosomes, commencing the degradation of their contents [4]. It has been thought that the degradation by autophagy is non-selective and that constitutive basal level autophagy accounts for the turnover of long-lived proteins [2,3,7]. However, growing evidence indicates that autophagy can also participate in selective degradation of cytoplasmic materials including protein aggregates, impaired mitochondria, peroxisomes, and even invading pathogens (for reviews, see [5,8,9]).

Autophagy has been traced by biochemical and morphological methods (reviewed in [10]). Generally, biochemical methods are quantitative but rather time-consuming and it is inherently difficult to distinguish autophagy from other intracellular degradation pathways, such as the ubiquitin-proteasome pathway and microautophagy [8,11,12]. On the other hand, morphological methods are simple, and less time-consuming, although they are rather qualitative and have difficulty in quantitation. Using GFP fused LC3, a mammalian Atg8 orthologue, the formation of autophagosomes in mammalian cells can be monitored in live, enabling morphological quantitation [13,14]. However, as LC3 dissociates from autophagosomes after fusion with lytic compartments [13,14], precise quantitation of autophagosome formation or induction of autophagy is

* Corresponding author. Fax: +81 76 234 4462.
E-mail address: ohkuma@kenroku.kanazawa-u.ac.jp (S. Ohkuma).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.09.168

not possible. Furthermore, morphological methods are difficult to apply for high throughput screening. Under these circumstances, we attempted to establish an alternative quantitative monitoring system for autophagy. We have chosen mitochondria as a marker because mitochondria are a well-established and easily traceable cytoplasmic component in autophagic degradation [1,3,15]. In addition, as mitochondria are large organelles and are thought to be degraded mainly by macroautophagy [15,16], they are able to serve as a specific marker for macroautophagy.

Materials and methods

Reagents. Chloroquine diphosphate, ammonium chloride, bafilomycin A_1, leupeptin, pepstatin, and rapamycin were purchased from Wako Fine Chemicals (Osaka, Japan). 3-Methyladenine, protease inhibitor cocktail, and lactacystin were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). Cycloheximide and DMSO were purchased from Nacalai Tesque (Kyoto, Japan). Anti-ubiquitin antibody and chymostatin were purchased from Calbiochem (San Diego, CA, USA). Antipain was purchased from Bachem AG (Bubendorf, Switzerland).

Cell line and culture conditions. CHO-K1 cells (a Chinese hamster ovary cell line) were maintained in Ham's F-12 medium (Gibco-BRL) supplemented with 10% FBS (growth medium) containing 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C under 5% CO_2. Medium depleted of serum was Ham's F-12 medium without supplements. Amino acids depleted medium was formulated with the recipe of Ham's F-12 medium without amino acids.

Transfection and production of a stable cell line. pCAGGS-GFP (GFP expressing in the cytoplasm) and pCAGGS-pOTC-GFP (mtGFP) were kindly donated by Dr. Masataka Mori (Kumamoto University, Japan). pGFP-LC3 was kindly donated from Dr. Tamotsu Yoshimori (Osaka University, Japan). CHO-K1 cells were transfected with the plasmids using Effectene (Qiagen, Hilden, Germany). Stable transfectants were selected and maintained with growth medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G418 (Sigma–Aldrich).

Starvation. 2×10^5 cells were seeded in a 6-cm dish and cultured with growth medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G418 for 48 h. Cells were washed twice with experimental medium and cultured in the same medium for the indicated time. Where indicated, 100 μM chloroquine diphosphate, 40 mM ammonium chloride, 10 mM 3-methyladenine, 100 nM Bafilomycin A_1 (with 0.01% DMSO), 100 nM rapamycin (with 0.01% DMSO), 10 μM lactacystin (with 0.1% DMSO) or 0.1% DMSO was added to the medium.

Fluorescent microscopy. For labeling lysosomes, mtGFP-CHO cells were pre-incubated with a growth medium containing antibiotics and 500 μg/ml of Texas Red dextran (Molecular Probes, Eugene, OR, USA) for 16 h. Cells were incubated with a growth medium or starvation mediums containing protease inhibitors (pepstatin, leupeptin, antipain, and chymostatin) for the indicated time at 37 °C, washed 3× with phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde. Cells were observed under a laser scanning confocal microscope (LSM510, Carl Zeiss, Jena, Germany). For quantitation, average fluorescent intensities of the cells were measured by ImageJ (NIH, USA). A cell area was manually enclosed using freehand line selection and the mean intensity then measured. For each image, 20 cells were randomly selected and the mean calculated. This was then subtracted from the mean background obtained by the measurement of three randomly selected areas with no cells in the same image.

Fluorometric analysis. This was performed as described previously with slight modifications [17]. Briefly, cells were washed and lysed with SDS-containing buffer and sonicated. GFP fluorescence was measured using a fluorometer (F-4500, Hitachi, Tokyo, Japan) at 480 nm for excitation and 510 nm for emission. The DNA content was measured using Hoechst 33258 dye [18].

Western blotting. mtGFP-CHO cells were washed with PBS and lysed in lysis buffer (0.1 M Tris–HCl [pH 6.7], 4% SDS). The lysate was sonicated (Sonifier 250D, Branson, Danbury, CT, USA) and protein concentration was determined by BCA protein assay kit (Pierce Chemical Company, Rockford, MI, USA). This was then adjusted to 0.1 M DTT, 0.2 M sucrose, and 0.02% BPB, and analyzed by SDS polyacrylamide gels electrophoresis (15% or 12.5% polyacrylamide gels). Proteins were then electrophoretically transferred to polyvinylidene difluoride membrane (Immobilon, Millipore Corp., Bedford, MA, USA) using semi-dry transfer blotter. The membranes were then incubated with the rabbit anti-GFP polyclonal antibody (Santa Cruz Biotechnology, CA, USA), mouse anti-actin monoclonal antibody (Chemicon international, CA, USA), rabbit anti-ubiquitin polyclonal antibody (Calbiochem) or rabbit anti-LC3 antibody (kindly donated by Dr. Tamotsu Yoshimori, Osaka University), and then anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (1:5000) (Santa Cruz). The reaction was visualized using the ECL detection system (Amersham Pharmacia Biotech, Uppsala, Sweden) with a luminescence analyzer (LAS-1000, Fuji Film Corp., Tokyo, Japan). Densitometry was performed with Image Gauge (Fuji Film Corp.).

Results and discussion

Monitoring the degradation of mitochondrial matrix marker protein (mtGFP)

To monitor mitochondrial degradation, CHO-K1 cells stably expressing a green fluorescent protein (GFP) in mitochondrial matrix (mtGFP-CHO) were produced using a GFP fused with a pre-sequence of mitochondrial ornithine transcarbamylase (mtGFP) [19]. When mtGFP-CHO cells were examined for the localization of GFP under a fluorescent microscope, cytoplasmic dots or thread-like structures, typical of mitochondria, were observed (Fig. 1A, right panel). These structures were precisely co-stained with MitoTracker Red CMXRos (Invitrogen), which specifically and vitally stains mitochondria, (unpublished observation) indicating that the mtGFP was properly targeted to mitochondria and suitable for further analyses.

First, we tried amino acid starvation, which has been widely used to induce autophagy in cultured cells [13,20]. When mtGFP-CHO cells were treated with a medium depleted of serum and amino acids for 12 h, fixed, and observed under a confocal microscope, the fluorescence of the mitochondria was significantly lower than the control cells, although the structure and distribution of mitochondria was not significantly affected (Fig. 1A). The average intensity of fluorescence per cells was reduced to about 60% (Fig. 1B). Western blotting analysis was performed to confirm that the reduction was caused by the degradation of mtGFP. As expected, starvation apparently reduced the amount of mtGFP (Fig. 1C). The reduction was about 80% when quantified by densitometry (Fig. 1D). This result indicated that the reduction in the GFP fluorescence was mostly caused by degradation of the protein. However, the rate of fluorescence reduction was significantly higher compared to that of the protein amount. This may be caused by quenching of GFP fluorescence preceding degradation, or more