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Quantitative monitoring of autophagic degradation

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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

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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

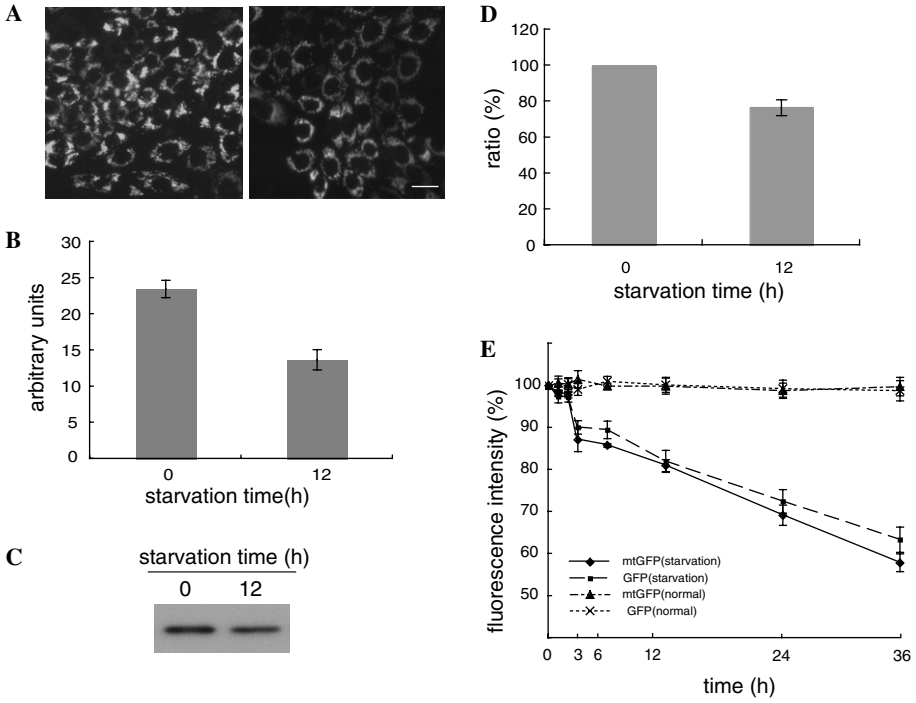

Fig. 1. Degradation of mtGFP after amino acid and serum starvation. (A) mtGFP-CHO cells were cultured in normal growth medium (left panel) or medium depleted of amino acids and serum (right panel) for 12 h and examined by a confocal microscope. A sliced image with the largest cytoplasmic area is shown. Bar = 20 μm. (B) The average fluorescent intensity in a cell was quantified as described in Materials and methods. The result shown is an average of three independent experiments. Error bars indicate SEM. (C) mtGFP-CHO cells were treated as in (A) and lysates were prepared, and analyzed by Western blotting using anti-GFP antibody as described in Materials and methods. The same amount of protein (30 μg) was loaded into each lane. A representative picture of three experiments is shown. (D) The bands in (C) were densitometrically quantified and the ratio of the starved cell sample to the control was calculated. The result shown is an average of three independent experiments. Error bars indicate SEM. (E) Cells were incubated with normal growth medium (normal) or starvation medium (starvation) for the indicated time, harvested, and lysed. The GFP fluorescence and the DNA content of the samples were analyzed using a fluorometer as described in Materials and methods. The fluorescent intensity of GFP per DNA content was calculated and expressed as the ratio to the non-treated control cell sample. mtGFP, mtGFP-CHO; GFP, CHO expressing GFP in the cytoplasm.

likely, by limitations in the morphological quantitation method.

To evaluate these possibilities and furthermore develop a simpler, less time-consuming, and quantitative method, we employed fluorometry developed in our laboratory previously [17]. Cells were incubated with normal growth medium or starvation medium for 1–36 h and the fluorescent intensity of GFP per DNA content, which approximated mean GFP fluorescence per cells, was analyzed. The fluorescence was unchanged after incubation with control normal medium for up to 36 h (Fig. 1E, mtGFP, normal). In contrast, starvation induced a gradual reduction in GFP fluorescence (Fig. 1E, mtGFP, starvation). The ratio of the reduction after 12 h starvation was about 80% and this was in good accordance with the level of mtGFP degradation determined by Western blotting (Figs. 1C and D). Therefore, we concluded that the degradation of GFP simultaneously proceeded with the quenching of the fluorescence so that the loss of fluorescence reflects the degradation of protein. Intriguingly, GFP fluorescence was almost unchanged for the first one to two hours of starvation. The fluorescence was then dramatically reduced after 3 h of starvation (less than 90% was left). As the induction of autophagy has been reported to peak after 2 h of starvation [13], it is probable that the latency in the reduction in mtGFP fluorescence reflected the time required for the formation of autolysosomes. A steady reduction in GFP fluorescence was observed with longer incubation, indicating the continuous degradation of mtGFP by starvation.

Mitophagy, the specific degradation of mitochondria by autophagy, was reported to be induced by mitochondrial damage [21–25]. Thus, we tried to evaluate whether mtGFP was degraded specifically or non-specifically by the classical bulk degradation mechanism of autophagy [26]. For this purpose, we generated CHO cells stably expressing GFP in the cytoplasm and quantified the reduction in the GFP fluorescence by the same fluorometric method. As shown in Fig. 1E, the kinetics of the decrease in fluorescence were almost the same as for mtGFP, strongly suggesting that the degradation of mtGFP was mediated by a bulk degradation mechanism by autophagy under amino acid and serum starvation. As the degradation of mtGFP was thought to be coincident with the disruption of mitochondria in autolysosomes (further discussed in the next section) and more specifically to reflect macroautophagy, only mtGFP-CHO was analyzed hereafter.

mtGFP is degraded by autophagy

We took several approaches to confirm that the degradation of mtGFP was caused by autophagy and not by another cytoplasmic degradation mechanism, such as the ubiquitin-proteasome system.

First, we carried out a morphological analysis to look for evidence of autophagic degradation of mitochondria. If mitochondria are enclosed in autophagosomes and fused with the lysosomal compartment, co-localization of lysosomes and mitochondria has to be observed. To label lysosomes, mtGFP-CHO cells were loaded with Texas Red dextran as described in Materials and methods. The cells were then starved and the distribution of mitochondria and lysosomes analyzed by confocal microscopy. To better preserve the autophagocytosed mitochondria, cells were treated with protease inhibitors. When control cells were analyzed, the distribution of mitochondria and lysosomes was clearly distinct and virtually no co-localization was observed (Fig. 2A, top panels). In contrast, apparent close apposition of mitochondria and lysosomes was observed after 6 h of starvation (Fig. 2A, bottom panels and B, yellow spots). Careful inspection of the cells, by reconstructing 3D images of the cells, revealed actual overlapping of mitochondria and lysosomes suggesting the engulfment of mitochondria in autolysosomes (Figs. 2B and C). In most of the cells, one or two of these structures were observed. The low number of these structures may reflect rapid breakdown of mitochondrial integrity and dispersal of mtGFP in autolysosomes and/or partial degradation of mtGFP that was not completely inhibited by added protease inhibitors. In any case, the observation of mitochondria in lysosomes suggests that the mitochondria are autophagocytosed and delivered to autolysosomes.

Second, we tested whether compounds that inhibit autophagy affect the degradation of mtGFP. Chloroquine diphosphate and ammonium chloride are basic compounds that have been reported to raise the pH in an acidic compartment [27]. Bafilomycin A_1 is a selective inhibitor of V-ATPase and also raises the pH in an acidic compartment [28]. These compounds have been shown to inhibit the formation of autolysosomes, probably by inhibiting the fusion

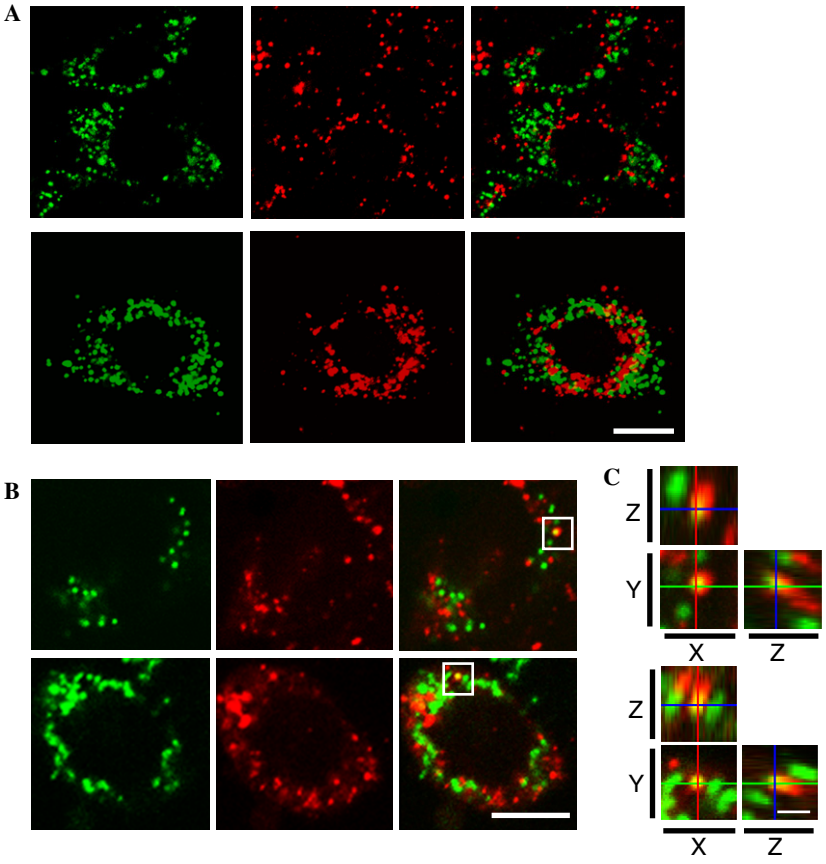

Fig. 2. Co-localization of mitochondria with lysosomes in starved cells. mtGFP-CHO cells were loaded with Texas Red dextran and then incubated in normal growth medium (A, top panels) or medium depleted of amino acids and serum (A, bottom panels, B and C) for 6 h in the presence of protease inhibitors. Cells were fixed and observed using a confocal microscope. (A) A sliced image with the largest cytoplasmic area is shown. Left panels, mtGFP; center panels, Texas Red dextran; right panels, merged pictures. Bar = 10 μm. (B) Left gallery; representative sliced images with clear co-localization of mitochondria with lysosomes. Panels are in the same arrangement as in (A). Bar = 10 μm. (C) Areas marked by white squares in merged pictures of (B) were enlarged and presented with sliced images of the *z*-axis of the corresponding areas. Top, *x*–*z* images; bottom left, *x*–*y* images; bottom right, *y*–*z* images. Green, blue, and red lines in the panels indicate the planes for *x*–*z*, *x*–*y*, and *y*–*z* images. Bar = 2 μm.

Table 1
Effect of autophagy inhibitors on mtGFP degradation

	The rate of inhibition (%) ($n = 3$)
Chloroquine	40.0 ± 4.0
Ammonium chloride	47.1 ± 6.7
Bafilomycin A_1	49.3 ± 7.8
3-Methyladenine	53.2 ± 4.8

mtGFP-CHO cells were cultured in a medium deprived of amino acids and serum in the presence of the indicated inhibitors for 6 h. The cell lysates were fluorometrically analyzed as described in Fig. 1.

between autophagosomes and lysosomes [29,30]. 3-Methyladenine is an inhibitor of phosphatidylinositol-3-kinase (PI3K), which specifically inhibits macroautophagy [31]. When mtGFP-CHO cells were incubated with amino acid- and serum-free starvation medium for 6 h in the presence of any of these compounds, the degradation of mtGFP was inhibited by 40–50% (Table 1). These results suggest that the degradation of mtGFP is mediated by autophagy.

Third, the induction of autophagy was confirmed biochemically. LC3 is a mammalian orthologue of yeast Atg8p and known to be converted to a higher mobility isoform and localized on newly formed autophagosomes in response to the induction of autophagy [13]. Incubation of mtGFP-CHO cells in amino acid and serum depleted medium strongly induced the conversion of LC3-I to a faster migrating LC3-II isoform (unpublished observation and Fig. 3A) suggesting the efficient induction of autophagy in our experimental system.

Finally, we investigated the involvement of the proteasomal degradation system in mtGFP degradation. The inhibition of proteasomes can be monitored by accumulation of ubiquitinated proteins, which is detected as smear bands with lower mobility, by Western blotting of a cell lysate with anti-ubiquitin antibody [32]. mtGFP-CHO cells were incubated with starvation medium in the presence or absence of lactacystin, a proteasome inhibitor [33], lysed, and analyzed using anti-ubiquitin antibody (Fig. 3B). There was no significant accumulation of ubiquitinated proteins in mock treated starved cells (Fig. 3B, DMSO). In striking contrast, massive accumulation of ubiquitinated proteins was detected in lactacystin treated starved cells clearly indicating inhibition of the proteasomal degradation system (Fig. 3B, Lact). In contrast, accumulation of LC3-II was not affected, indicating that inhibition of the proteasomal degradation system did not interfere with the induction of autophagy (Fig. 3A, compare DMSO and Lact). Under these conditions, no significant inhibition of the degradation of mtGFP was observed (Fig. 3C). Therefore, the proteasomal degradation system appears not to be involved in the degradation of mtGFP.

Taken together, these results strongly suggested that in our experimental system the degradation of mtGFP was mostly caused by autophagy, probably by bulk engulfment of mitochondria in autophagosomes and consecutive formation of autolysosomes.

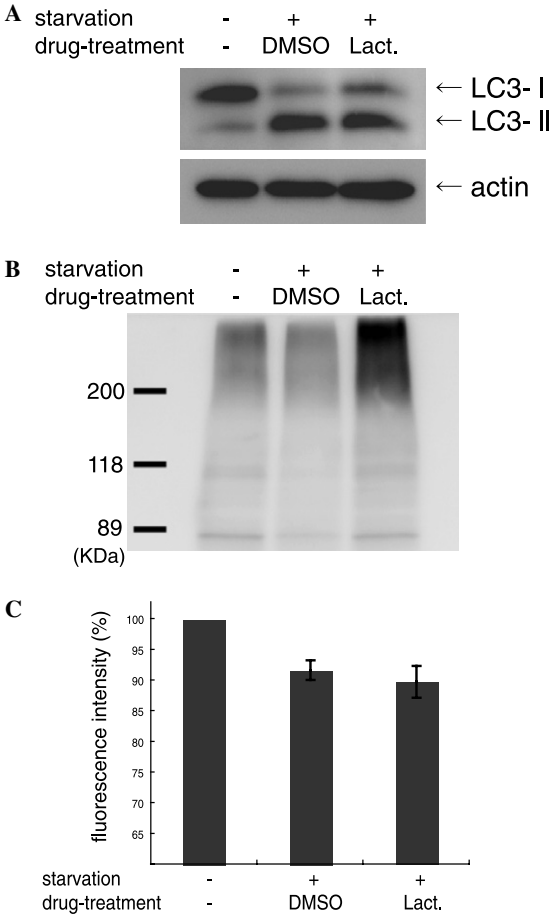

Fig. 3. Effect of proteasome inhibitors on mtGFP degradation. mtGFP-CHO cells were cultured in normal growth medium or medium depleted of amino acids and serum-containing lactacystin (Lact) or mock carrier solvent (DMSO) for 6 h. (A,B) The cell lysates were subjected to immunoblotting analysis using an antibody against LC3 and actin (A) or ubiquitin (B). (C) GFP fluorescence of the cell lysates was measured as in Fig. 1E.

Kinetic analysis of autophagy

The fluorometric assay was relatively easy and less time-consuming and was well suited for the kinetic analysis of autophagy. Using this method, detailed quantitative measurement of autophagic activity is possible. To start, we challenged the cells with several different conditions that have been shown to induce autophagy and analyzed the kinetics of autophagic induction. We compared rapamycin treatment and amino acid or serum starvation, which have all been shown to induce autophagy but by activation of different points in the autophagic signal transduction machinery (see reviews for [5,34]). As mentioned in Fig. 1E, dramatic induction of autophagic degradation was observed under double depletion of amino acids and serum after 3 h of treatment. In contrast, depletion of only amino acids or only serum showed only weaker induction of autophagic degradation at the same time point (Fig. 4A). The continuous and gradual induction kinetics were very similar for sole depletion of amino acids or

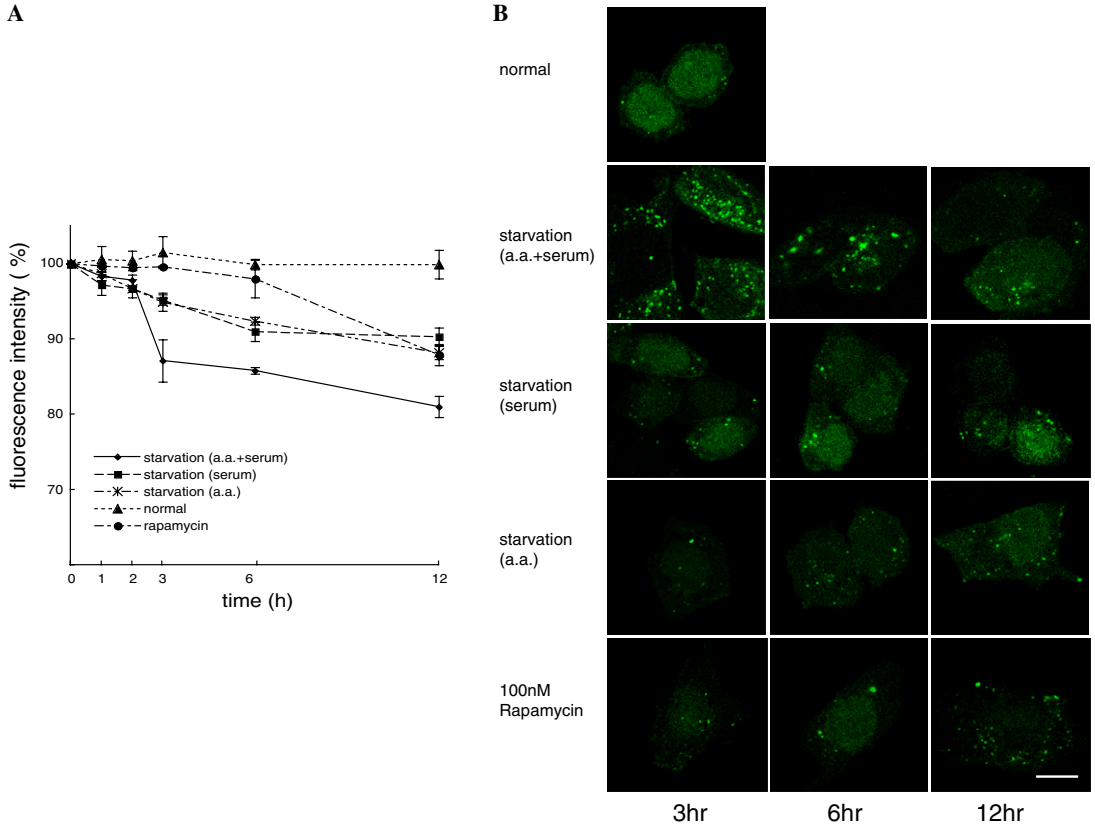

Fig. 4. Kinetic analysis of autophagy induction by different autophagic inducers. (A) mtGFP-CHO cells were cultured with normal growth medium with rapamycin or medium depleted of amino acids, serum or both for the indicated time. GFP fluorescence of the cell lysates was measured as in Fig. 1E. (B) CHO cells expressing LC3-GFP were treated as in (A). The cells were fixed and examined by confocal laser scanning microscopy. Bar = 10 μm.

serum (Fig. 4A). Interestingly, the induction of autophagic degradation by rapamycin was further slower compared to the depletion of amino acids, serum or both. Significant induction was only observable after 12 h of treatment (Fig. 4A).

To confirm that the kinetic difference was actually reflecting that of autophagy induction, we morphologically analyzed the kinetics of the activation of autophagy by LC3 activation, GFP-tagged LC3 rapidly accumulates on cytoplasmic spots during the early stage of autophagosome formation [10,14]. A CHO cell line stably expressing LC3-GFP was produced and treated with rapamycin or was starved of amino acids or serum (Fig. 4B). Amino acid and serum starvation strongly induced the accumulation of LC3-GFP on numerous cytoplasmic spots after 3 h of treatment (Fig. 4B). The induction of LC3-GFP dots remained at a similar level after 6 h of treatment and slightly decreased after 12 h. This correlated well with the induction of autophagic degradation (Fig. 4A). As expected, the number of LC3-GFP dots was lower with the sole starvation of amino acids or serum after 3 h of treatment and remained at a similar level with longer incubation (Fig. 4B). The induction of LC3 dots was even lower under rapamycin treatment after 3 and 6 h, and then significantly increased after 12 h of treatment. These results were in good agreement with the kinetic analysis (Fig. 4B) and strongly support the idea that the kinetics of mtGFP degradation reflects the kinetics of autophagy induction.

To our knowledge, this is the first study to demonstrate that different inducers promote autophagy with different kinetics. These kinetic differences may reflect the different steps of the autophagic signal transduction pathway that rapamycin and amino acid or serum starvation stimulate [5,34,35].

We conclude that mtGFP serves as a quantitative marker to analyze the kinetics of autophagy induction. The quantitative method we have developed would be easily adapted to high-throughput screening for novel drugs that enhance or inhibit autophagy, as well as for genes that regulate or modulate autophagy. Future application of this analytical method will greatly benefit the understanding of autophagy and development of new drugs and treatments of autophagy-related disorders, including cancer [36–38].

Acknowledgments

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References

[1] H. Glaumann, J.L. Ericsson, L. Marzella, Mechanisms of intralysosomal degradation with special reference to autophagocytosis and heterophagocytosis of cell organelles, Int. Rev. Cytol. 73 (1981) 149–182.
[2] G.E. Mortimore, A.R. Poso, Intracellular protein catabolism and its control during nutrient deprivation and supply, Annu. Rev. Nutr. 7 (1987) 539–564.
[3] W.A. Dunn Jr., Autophagy and related mechanisms of lysosome-mediated protein degradation, Trends Cell Biol. 4 (1994) 139–143.
[4] D.J. Klionsky, Y. Ohsumi, Vacuolar import of proteins and organelles from the cytoplasm, Annu. Rev. Cell Dev. Biol. 15 (1999) 1–32.
[5] A.J. Meijer, P. Codogno, Regulation and role of autophagy in mammalian cells, Int. J. Biochem. Cell Biol. 36 (2004) 2445–2462.
[6] F. Reggiori, D.J. Klionsky, Autophagosomes: biogenesis from scratch? Curr. Opin. Cell Biol. 17 (2005) 415–422.
[7] P.O. Seglen, P. Bohley, Autophagy and other vacuolar protein degradation mechanisms, Experientia 48 (1992) 158–172.
[8] J. Kim, D.J. Klionsky, Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells, Annu. Rev. Biochem. 69 (2000) 303–342.
[9] M. Ogawa, C. Sasakawa, Bacterial evasion of the autophagic defense system, Curr. Opin. Microbiol. 9 (2006) 62–68.
[10] N. Mizushima, Methods for monitoring autophagy, Int. J. Biochem. Cell Biol. 36 (2004) 2491–2502.
[11] M. Hochstrasser, Ubiquitin-dependent protein degradation, Annu. Rev. Genet. 30 (1996) 405–439.
[12] A. Hershko, A. Ciechanover, The ubiquitin system, Annu. Rev. Biochem. 67 (1998) 425–479.
[13] Y. Kabeya, N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, T. Yoshimori, LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing, EMBO J. 19 (2000) 5720–5728.
[14] N. Mizushima, A. Yamamoto, M. Matsui, T. Yoshimori, Y. Ohsumi, In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker, Mol. Biol. Cell. 15 (2004) 1101–1111.
[15] C. De Duve, R. Wattiaux, Functions of lysosomes, Annu. Rev. Physiol. 28 (1966) 435–492.
[16] E. Holtzman III, Turnover of organelles and intracellular macromolecules in eukaryotes, in: M. Alfert, W. Beermann, G. Rudkin, W. Sandritter, P. Sitte (Eds.), Lysosomes: A Survey, Springer Werlag, Wien, New York, 1976, pp. 129–152.
[17] K. Arai, S. Ohkuma, T. Matsukawa, S. Kato, A simple estimation of peroxisomal degradation with green fluorescent protein—an application for cell cycle analysis, FEBS Lett. 507 (2001) 181–186.
[18] C. Labarca, K. Paigen, A simple, rapid, and sensitive DNA assay procedure, Anal. Biochem. 102 (1980) 344–352.
[19] M. Yano, M. Kanazawa, K. Terada, C. Namchai, M. Yamaizumi, B. Hanson, N. Hoogenraad, M. Mori, Visualization of mitochondrial protein import in cultured mammalian cells with green fluorescent protein and effects of overexpression of the human import receptor Tom20, J. Biol. Chem. 272 (1997) 8459–8465.
[20] D.B. Munafo, M.I. Colombo, Induction of autophagy causes dramatic changes in the subcellular distribution of GFP-Rab24, Traffic 3 (2002) 472–482.
[21] L. Xue, G.C. Fletcher, A.M. Tolkovsky, Mitochondria are selectively eliminated from eukaryotic cells after blockade of caspases during apoptosis, Curr. Biol. 11 (2001) 361–365.
[22] S.P. Elmore, T. Qian, S.F. Grissom, J.J. Lemasters, The mitochondrial permeability transition initiates autophagy in rat hepatocytes, FASEB J. 15 (2001) 2286–2287.
[23] I. Kissova, M. Deffieu, S. Manon, N. Camougrand, Uth1p is involved in the autophagic degradation of mitochondria, J. Biol. Chem. 279 (2004) 39068–39074.
[24] M. Priault, B. Salin, J. Schaeffer, F.M. Vallette, J.P. di Rago, J.C. Martinou, Impairing the bioenergetic status and the biogenesis of mitochondria triggers mitophagy in yeast, Cell Death Differ. 12 (2005) 1613–1621.
[25] S. Rodriguez-Enriquez, I. Kim, R.T. Currin, J.J. Lemasters, Tracker dyes to probe mitochondrial autophagy (mitophagy) in rat hepatocytes, Autophagy 2 (2006) 39–46.
[26] J. Kopitz, G.O. Kisen, P.B. Gordon, P. Bohley, P.O. Seglen, Nonselective autophagy of cytosolic enzymes by isolated rat hepatocytes, J. Cell Biol. 111 (1990) 941–953.
[27] S. Ohkuma, B. Poole, Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents, Proc. Natl. Acad. Sci. USA 75 (1978) 3327–3331.
[28] T. Yoshimori, A. Yamamoto, Y. Moriyama, M. Futai, Y. Tashiro, Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells, J. Biol. Chem. 266 (1991) 17707–17712.
[29] A. Yamamoto, Y. Tagawa, T. Yoshimori, Y. Moriyama, R. Masaki, Y. Tashiro, Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells, Cell Struct. Funct. 23 (1998) 33–42.
[30] P. Boya, R.A. Gonzalez-Polo, N. Casares, J.L. Perfettini, P. Dessen, N. Larochette, D. Metivier, D. Meley, S. Souquere, T. Yoshimori, G. Pierron, P. Codogno, G. Kroemer, Inhibition of macroautophagy triggers apoptosis, Mol. Cell Biol. 25 (2005) 1025–1040.
[31] P.O. Seglen, P.B. Gordon, 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes, Proc. Natl. Acad. Sci. USA 79 (1982) 1889–1892.
[32] E.G. Mimnaugh, P. Bonvini, L. Neckers, The measurement of ubiquitin and ubiquitinated proteins, Electrophoresis 20 (1999) 418–428.
[33] D.H. Lee, A.L. Goldberg, Proteasome inhibitors: valuable new tools for cell biologists, Trends Cell Biol. 8 (1998) 397–403.
[34] D.A. van Sluijters, P.F. Dubbelhuis, E.F. Blommaart, A.J. Meijer, Amino-acid-dependent signal transduction, Biochem. J. 351 (Pt 3) (2000) 545–550.
[35] G.E. Mortimore, M. Kadowaki, Autophagy: its mechanism and regulation, in: A.J. Ciechanover, A.L. Schwartz (Eds.), Cellular Proteolytic Systems, Wiley Liss, New York, 1994, pp. 65–87.
[36] T. Schmelzle, M.N. Hall, TOR, a central controller of cell growth, Cell 103 (2000) 253–262.
[37] J.J. Lum, D.E. Bauer, M. Kong, M.H. Harris, C. Li, T. Lindsten, C.B. Thompson, Growth factor regulation of autophagy and cell survival in the absence of apoptosis, Cell 120 (2005) 237–248.
[38] G. Kroemer, M. Jaattela, Lysosomes and autophagy in cell death control, Nat. Rev. Cancer 5 (2005) 886–897.