A simple estimation of peroxisomal degradation with green fluorescent protein – an application for cell cycle analysis

Kunizo Arai[a,*], Shoji Ohkuma[a], Toru Matsukawa[b], Satoru Kato[b]

[a] *Department of Molecular and Cellular Biology, Faculty of Pharmaceutical Sciences, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920-0946, Japan*

[b] *Department of Molecular Neurobiology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920-0840, Japan*

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Abstract When nutrients are depleted from the environment, mammalian cells begin to degrade their own cytosol and organelles. This bulk protein degradation is mediated by autophagy. In this study, peroxisomes in living CHO-K1 cells were visualized by targeting the green fluorescent protein (GFP) tagged with a type 1 peroxisomal targeting signal. The nutrient-starved condition induced a decay of GFP fluorescence in the peroxisomes and autophagic inhibitors such as 3-methyladenine suppressed the decay of GFP fluorescence (13–60% of starvation). By double labeling the nuclear DNA and peroxisomal GFP, the autophagy specifically occurred at the G1 phase of the cell cycle and the autophagic inhibitors suppressed the G1 arrest. The vital stain technique with GFP is a very simple and useful marker to quantitatively estimate or to further study peroxisomal degradation.

Key words: Autophagy; Peroxisome; Green fluorescein protein; Cell cycle

1. Introduction

In the last decade, much has been studied about the biogenesis of subcellular organelles, including the peroxisome. However, little is known about the selective degradation of organelles. The elucidation of the mechanism of organelle degradation is essential for an understanding of the process of organelle homeostasis, a process that is likely to ensure that a cell directs its energies optimally toward specific functions or for cell survival [1]. Protein degradation in eukaryotic cells occurs in the cytosol and in the subcellular compartments. Lysosomes are involved in two types of autophagic processes [2]. The first, called microautophagy, is the sequestration of small portions of cytoplasm by invagination of the lysosomal membrane. The second process, called macroautophagy, refers to the sequestration of organelles and cytosol by membranes probably derived from the endoplasmic reticulum to generate autophagosomes [3] that then fuse with the endosomes or lysosomes to release autophagic bodies that accumulate and are degraded, within the lysosomes [2]. These processes are present in virtually all eukaryotic cells and constitute the major pathway for the degradation and recycling of cellular proteins. The autophagic degradation of organelles has been well documented in yeasts, such as *Saccharomyces cerevisiae* and *Pichia pastoris* [4]. The autophagosomes involved in macroautophagy have been characterized ultrastructually in *S. cerevisiae* and shown to be induced by nutrient deprivation or by mutations in genes affecting lysosomal proteinases. In contrast to yeast, the autophagic process of organelles in mammalian cells is not thoroughly understood and is not suited to a genetic approach. Recently a new technique for the vital stain of subcellular organelles such as the endoplasmic reticulum, mitochondria and peroxisome has developed. The peroxisomes of mammalian cells were labeled with green fluorescent protein (GFP) tagged with a type 1 peroxisomal targeting signal (PTS1, consisting of the COOH-terminal peptide, serine–lysine–leucine [SKL]) for peroxisomes [5]. By using this labeling technique, they examined dynamic behavior and association with microtubules in the peroxisome. There is no report of using GFP to study autophagy in mammalian cells. In the present study, we describe the use of the GFP–PTS1 fusion protein to study peroxisomal degradation (pexophagy) in nutrient deprivation conditions. We also tested the relationship between pexophagy and the cell cycle by double labeling the nuclear DNA and peroxisomal GFP.

2. Materials and methods

2.1. Cell line and culture system

CHO-K1 cells were maintained in F-12 medium (Gibco BRL) with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin under a humidified atmosphere containing 5% CO_2 at 37°C.

2.2. GFP transformants of CHO-K1 cells

A GFP–PTS1 fusion construct was cloned in the mammalian expression vector pEGFP-C2 (Clonetech labs., MD, USA) which encodes an enhanced GFP. GFP–PTS1 was obtained using primers (5′-CCGGAATTCTCAAATTATAATAAGGACCGGT-3′, coding strand and 3′-GGTATTAAGAGGTTTAATATTTTCCTAGGG-CA-5′, non-coding strand). The primers were inserted between the *Eco*RI and *Bam*HI sites of the multiple cloning site of the vector pEGFP-C2 under control of the cytomegalovirus promoter, yielding the plasmids pEGFP-PTS1. The vector GFP was used just the pEGFP-C2. CHO-K1 cells were transfected with pEGFP and pEGFP-PTS1 using lipofectamine (Gibco BRL). GFP was expressed at detectable levels between 24 and 72 h after transfection. Cells were used for further experimentation 24 h after transfection. The subcel-

*Corresponding author. Fax: (81)-76-234 4493.
E-mail address: arai@dbs.p.kanazawa-u.ac.jp (K. Arai).

Abbreviations: GFP, green fluorescent protein; 3-MA, 3-methyladenine; PTS1, type 1 peroxisomal targeting signal; PP2A, protein phosphatase 2A; PI3K, phosphatidylinositol 3-kinase; TOR, target of rapamycin

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lular localization of these proteins was observed by fluorescence microscopy. Stable transformants were selected with 20 μg/ml hygromycin.

2.3. Immunohistochemistry

The cells expressing GFP–PTS1 or GFP were fixed with 4% paraformaldehyde in Dulbecco's modified PBS for 30 min. Then the cells were incubated three times with 0.1% Triton X-100 in PBS for 10 min. After washing the cells with high salt PBS (0.5 M NaCl, 0.1% Tween 20 in PBS), the cells were incubated with anti-peroxisomal membrane protein antibody [6] diluted 1:500 in low salt PBS (0.05% Tween 20 in PBS) containing 0.1% BSA overnight at 4°C. After washing, the cells were incubated with tetramethyl rhodamine isothiocyanate (TRITC) labeled secondary anti-rabbit IgG. Washed three times, the cells were observed by a Carl-Zeiss fluorescence microscope (Axio vert 100 M).

2.4. A fluorometric assay of GFP in CHO-K1 cells

GFP intensity of CHO-K1 cells during nutrient deprivation or after the addition of autophagic inhibitors was assayed by fluorometry. Cells cultured in various conditions were washed twice with PBS and then resuspended with 0.1% SDS in sodium phosphate buffer. The cells were sonicated for 15 s on ice. The DNA content in the cell homogenate was measured according to the method of Labarea and Paigen [7]. Briefly, 3 ml of Hoechst 33258 (1 μg/ml in 0.05 M PBS) containing 2 M NaCl and 2 mM EDTA was added and mixed to 100 μl of cell homogenate. DNA was fluorometrically measured at 358 nm for excitation and 458 nm for emission using calf thymus DNA as a standard. The GFP fluorescence was measured by Hitachi F-4500 fluorimeter at 490 nm for excitation and 525 nm for emission.

2.5. A flow cytometric assay

The subconfluent cells were treated for 16–24 h under serum free medium with various drugs. The cells were harvested with trypsin/EDTA, washed twice with PBS, fixed with 30% methanol and then processed by enzymic digestion of RNA by RNase I. Cell nuclei were stained with propidium iodide for DNA. Analysis was performed on a FACScan flow cytometer (Becton Dickinson). The data were plotted against DNA distribution and GFP fluorescence intensity. If the GFP fluorescence intensity at different phase of the cell cycle was required, a gate of each cell cycle (G0–G1, S and G2–M phase) was created.

3. Results

3.1. Stable transformants with GFP in the peroxisomes

The plasmid vector (pEGFP-PTS1) was transfected into CHO-K1 cells and the stable clones were further selected by hygromycin. 24 h after transfection, a punctuate fluorescent signal could be seen (Fig. 1A). The fusion protein, GFP–PTS1, was localized only in the peroxisomes as indicated by its colocalization of peroxisomal protein with an antibody against the peroxisomal membrane protein (Fig. 1B). The

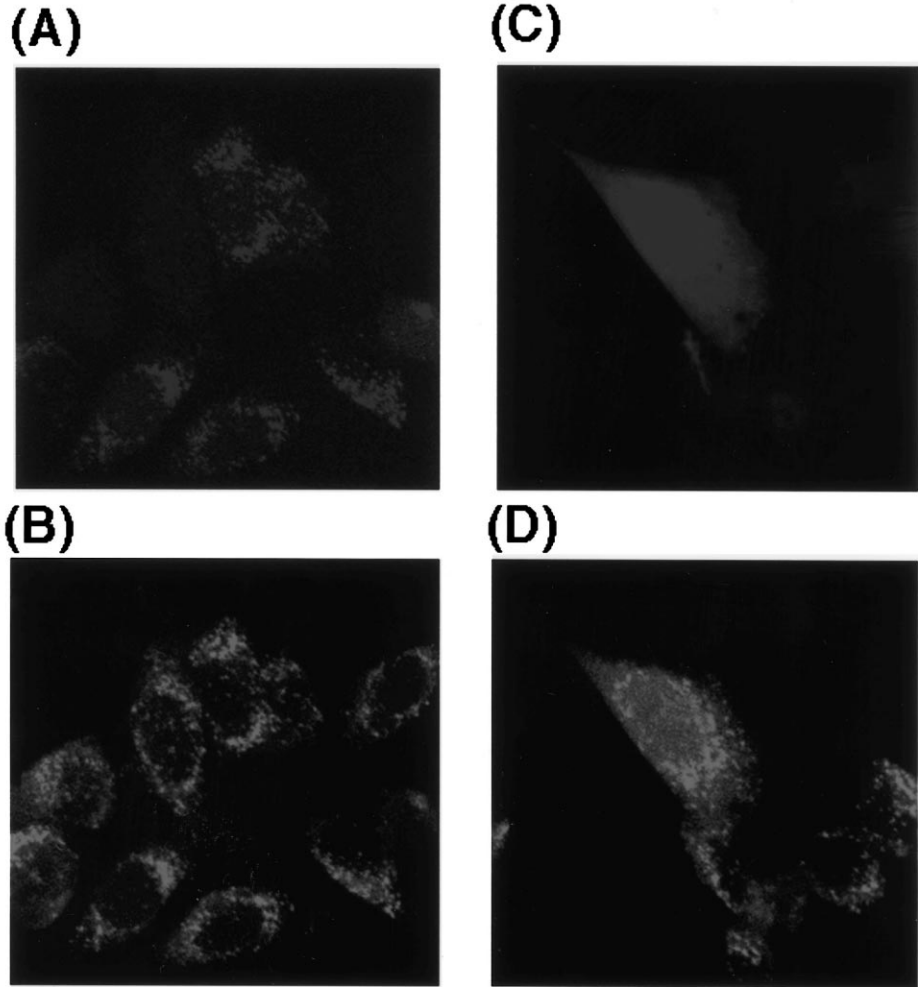

Fig. 1. Subcellular localization of GFP and GFP–PTS1 in CHO cells. CHO cells were transfected with pEGFP and pEGFP-PTS1. After 24 h the cells were examined directly for GFP–PTS1 (A,B) or GFP (C,D) expression. Alternatively, pEGFP-PTS1-transfected CHO cells were fixed, permeabilized, and processed for immunofluorescence. In a double labeling experiment, the cells were incubated with rabbit anti-peroxisome, and subsequently with rhodamine conjugated goat anti-rabbit IgG. The cells were monitored for the subcellular localization of GFP fluorescence (A,C) in the FITC channel and peroxisome (B,D) in the rhodamine channel.

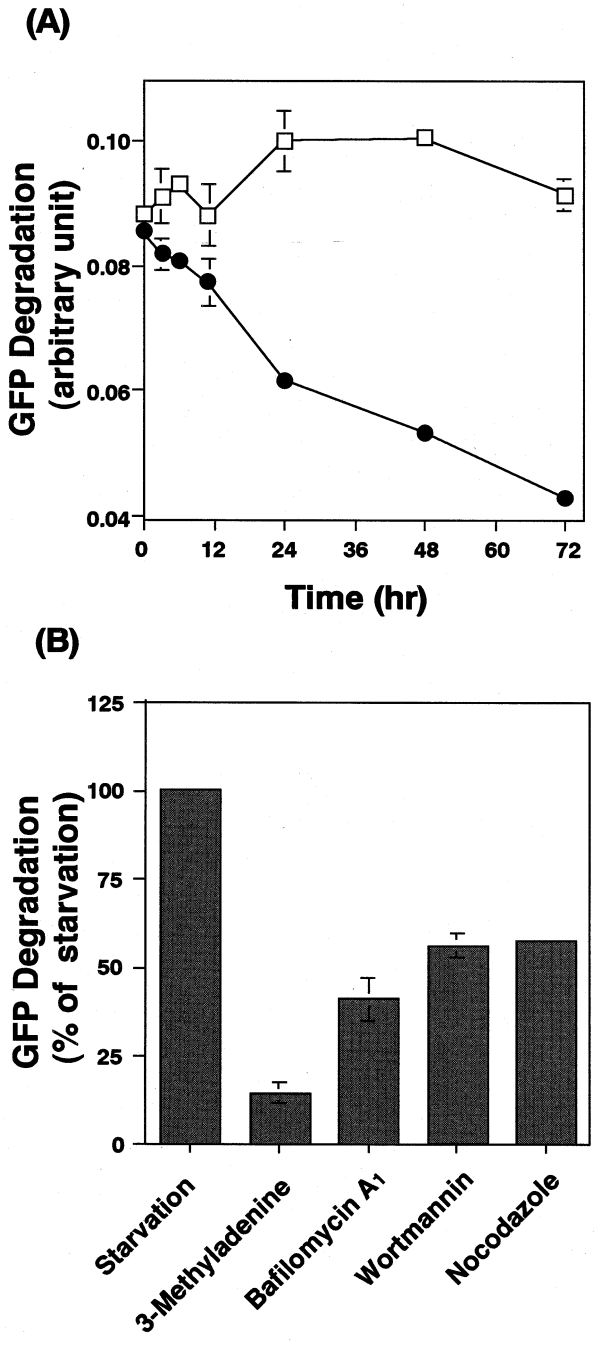

Fig. 2. GFP degradation on GFP–PTS1-transfected CHO cells under serum free medium. A: Time-course of GFP degradation. Subconfluent CHO cells were cultured under standard (□) or serum free (●) medium and GFP intensity was analyzed at the indicated times using a spectrofluorometer as described in Section 2. B: Effect of autophagic inhibitors on GFP degradation. CHO cells were cultured in the serum free medium in combination with 10 mM 3-MA, 100 nM bafilomycin A1, 100 nM wortmannin or 30μM nocodazole. After 18 h of culture, GFP intensity was measured. All experiments were performed three times ($n=3$).

fluorescent pattern or number of the labeled peroxisomes with GFP and anti-peroxisomal protein antibody was similar. In control cells expressing only vector GFP, a diffuse cytosolic fluorescent signal was observed (Fig. 1C,D). Thus, we can get stable transformants of CHO cells with high fidelity and efficiency of imported GFP–PTS1 to peroxisomes.

3.2. *Peroxisomal degradation monitoring GFP fluorescence by spectrofluorimetry*

In order to promote the autophagic process of the peroxisomes, the CHO-K1 cells with GFP were incubated in serum free medium. Fig. 2A shows a time-course of decay in GFP–PTS1 fluorescence intensity under standard (□) or serum free (●) conditions measured by spectrofluorimetry. The fluorescent intensity of GFP in the peroxisomes was unchanged in the standard medium, while the fluorescent intensity was decreased to 75% or 50% during 24 h or 72 h of starvation, respectively. Four drugs, including 3-methyladenine (3-MA) which is well known as an inhibitor of autophagic processes [8], all inhibited the decay of GFP fluorescence induced by nutrient deprivation conditions as shown in Fig. 2B. 3-MA is the most potent (13% of starvation) and after 3-MA bafilomycin A1, wortmannin and nocodazole followed in order (40–60% of starvation). In the present study the proxisomal GFP fluorescence was decreased in starved condition and the decrease was blocked by autophagic inhibitors. These results strongly suggest that increment and decay of GFP–PTS1 fluo-

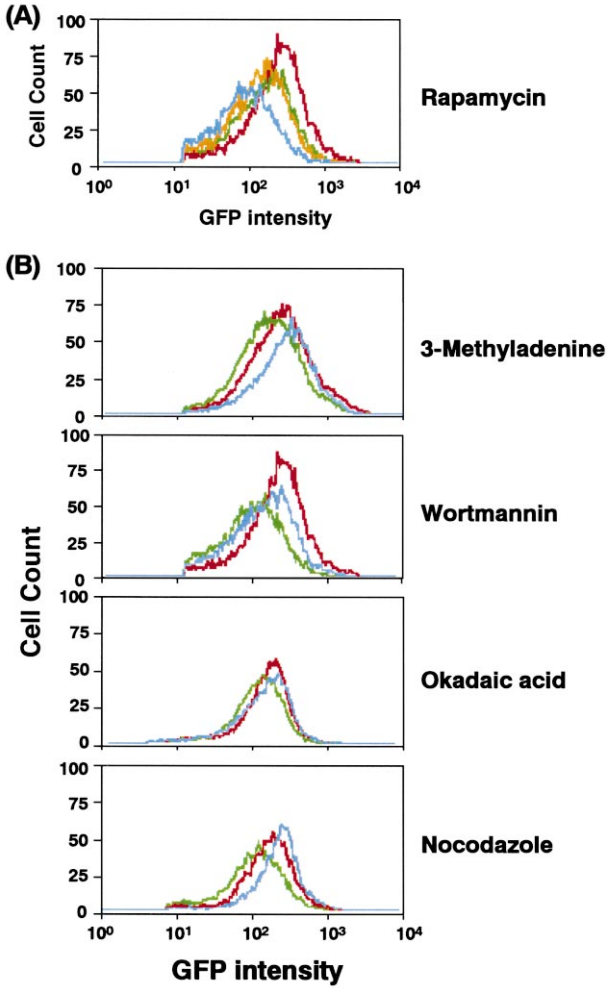

Fig. 3. Effect of various autophagy-modifying agents on GFP degradation. Subconfluent CHO cells cultured in the complete medium (red line), serum free medium (green line), complete medium with an autophagy-modifying agent (brown line), and serum free medium with an autophagy-modifying agent (blue line), were analyzed with a flow cytometer. GFP intensity vs. cell count were plotted in histograms.

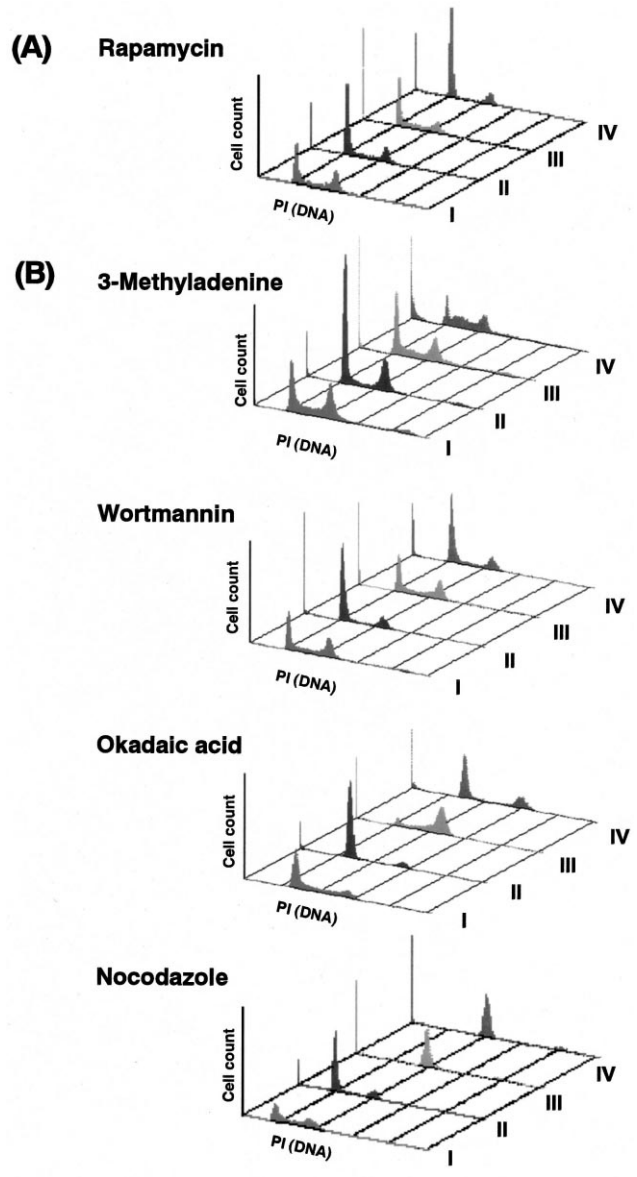

Fig. 4. Effect of various autophagy-modifying agents on the cell cycle. DNA–propidium iodide binding of CHO cells at the culture conditions in the complete medium (column I), serum free medium (column II), complete medium containing an agent (column III), and serum free medium containing an agent (column IV), were analyzed with a flow cytometer.

rescence is a useful marker for peroxisomal degradation and it allows us for the first time to quantitatively estimate the pexophagy in mammalian cells.

3.3. Cell cycle analysis of peroxisomal degradation by flow cytometry

It is well known that nutrient deprivation not only induces autophagy but also arrests cells at early G1 phase. In order to study the relationship between the cell cycle and autophagy, we applied the present GFP–PTS1 fluorescence technique to double labeling the DNA with propidium iodide. Thus, we can easily analyze the cell population, DNA distribution and GFP intensity by a flow cytometer. Fig. 3A shows the effect of rapamycin on GFP fluorescence. Rapamycin potently inhibits downstream signaling from the target of rapamycin (TOR) protein which blocks the formation of autophagosomes [9]. Rapamycin induced a decrease of GFP fluorescent intensity even under nutrient-rich conditions (brown line) like starvation (green line) as compared with standard condition (red line). Rapamycin in serum free medium further enhanced the decrease of GFP fluorescence (blue line). The present data confirm that rapamycin induces pexophagy through the TOR pathway. Fig. 3B shows the effect of autophagic inhibitors on the GFP–PTS1 fluorescence intensity under starvation. All four types of inhibitors (3-MA, wortmannin, okadaic acid and nocodazole) clearly suppressed the starvation-induced pexophagy (blue line). Fig. 4A illustrates the effect of rapamycin on DNA distribution. Starvation induced an increase in cell population of the G1 phase as compared with normal conditions. Rapamycin induced an increase of cell population at G1 phase even in nutrient-rich conditions. Rapamycin under starvation further enhanced the increase of cell population at G1 phase more than starvation or rapamycin in the standard medium alone. Fig. 4B shows the effect of autophagic inhibitors on DNA distribution. 3-MA in the starved condition induced a suppression of cell population at G1 phase and an increase of cell population at S phase (column IV). Okadaic acid induced a suppression as compared with normal condition (column I) of cell population at G1 phase. Treatment with nocodazole induced an increase of cell population at G2–M phase (column IV). In contrast to other inhibitors wortmannin did not influence the cell cycle (cf. columns II and IV). Next, we investigated the GFP–PTS1 fluorescence intensity at each cell cycle stage (G0–G1, S, G2–M phase). Each cell cycle stage was determined by setting the gate in the DNA distribution. The decay of GFP fluorescence (peroxisomal degradation) specifically occurred at the G1 phase in both starvation or rapamycin conditions but did not occur at other phases (Fig. 5).

4. Discussion

4.1. Peroxisomal autophagy and its inhibition monitored with GFP–PTS1 fusion protein

The ability to target GFP–PTS1 to peroxisomes provides a vital stain for this compartment. We have used GFP-labeled peroxisomes to study the degradation of peroxisomes by autophagy. The increment and decay of GFP fluorescence correlated with the peroxisomal degradation (pexophagy) induced by starvation or rapamycin and its blockage induced by autophagic inhibitors, respectively. Among autophagic inhibitors, 3-MA was a potent inhibitor of autophagy (13% of starvation), but the other inhibitors were partial (40–60% of starvation). This indicates that the peroxisomal autophagy in CHO-K1 cells is composed of 3-MA-sensitive and other inhibitor partially -insensitive processes. Macroautophagy is defined as several steps: the first step is the formation of autophagosomes, the second step is the acidification of autophagosomes by V-ATPase and the third step is the fusion of autophagosomes with lysosomes [2]. In this study 3-MA is an inhibitor of the first step. Bafilomycin A1 inhibits the V-ATPase [10]. Nocodazole is an inhibitor of the fusion of autophagosomes with lysosomes [11]. In mammalian cells, phosphorylation of ribosomal protein S6 (p70S6) strongly correlates with inhibition of macroautophagy [12]. This system promotes the cells towards protein synthesis. The TOR proteins are key molecules for the regulation of protein synthesis

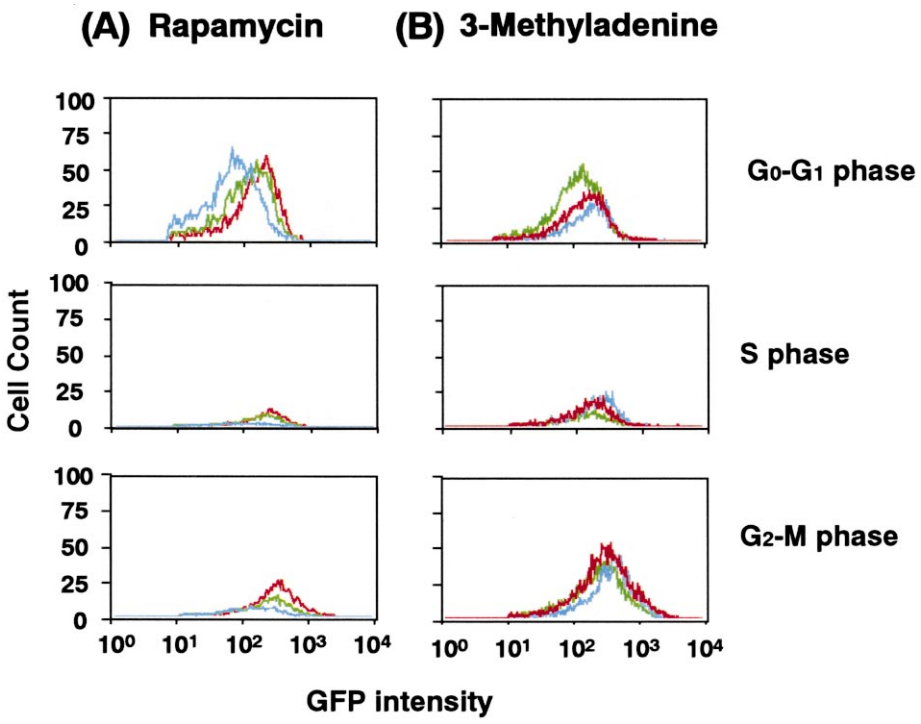

Fig. 5. Effect of various autophagy-modifying agents on GFP intensity at each phase of the cell cycle. Subconfluent CHO cells cultured in the complete medium (red line), serum free medium (green line), and serum free medium with an autophagy-modifying agent (blue line), were analyzed with a flow cytometer. GFP intensity vs. cell count were plotted in histograms. Representative histograms of cell frequency vs. GFP intensity at each phase of the cell cycle were shown.

and degradation in living cells through the p70S6 kinase-TOR kinase system [12]. Rapamycin inhibits TOR kinase-induced autophagy even under nutrient-rich conditions. In yeast cells, TOR phosphorylates TAP42 to inhibit protein phosphatase 2A (PP2A), and negatively regulates induction of autophagy [13]. Although the TAP42 has not been identified in mammalian cells, the inhibition of PP2A by okadaic acid leads to the inhibition of autophagy under starved conditions. Phosphatidylinositol 3-kinase (PI3K) is involved at least in the first and third steps of the autophagic processes. Wortmannin, a famous inhibitor of PI3K thus inhibited the peroxisomal degradation under starvation in this study. Blommaart [14] showed the inhibitory effect of 3-MA or wortmannin on the autophagic processes determined by measurement of [^{14}C]sucrose sequestration. Both the inhibitors suppressed autophagy with a similar potency. It disagrees with our results in which 3-MA is more potent than wortmannin (Fig. 3B). The difference can be explained by two possibilities: (i) a difference of experimental procedure with GFP fluorescence decay and [^{14}C]sucrose sequestration; (ii) a difference between organelle (peroxisomal) degradation and cytosolic protein degradation. Future studies are needed to elucidate this process.

4.2. A relationship between the cell cycle and pexophagy

TOR, a phosphatidylinositol kinase-related kinase, is known to be involved in the signaling pathway from nutrient starvation to G1 arrest in yeast [15]. In the present study nutrient starvation or rapamycin induced autophagy and arrested cells at G1 phase (an increase of cell population at G1 phase), whereas all autophagic inhibitors except wortmannin suppressed the increase of cell population at G1 phase. It is thought that 3-MA or wortmannin blocks PI3K and inhibits autophagy. However, our cell cycle analysis reveals that 3-MA affects the cell cycle (decrease of cell population at G1 phase and increase of cell population at S phase) while wortmannin does not affect the cell cycle at all, indicating different pathways between both these inhibitors. Nocodazole induced a decrease of cell population at G1 phase and an increase of cell population at G2–M phase as compared with the starved condition. In most eukaryotic cells, organelle structure is markedly changed at the onset of mitotic prophase. Endoplasmic reticulum fragments, Golgi complex vesiculates and nuclear envelopes all disassemble themselves, allowing equal partition of their membrane components from dividing cell to daughter cells. These events accompany the cessation of transport through endocytic and secretory pathways. The mechanism underlying the cessation in vesicular transport and organelle fragmentation is thought to be a transient inhibition of active fusion machinery [16]. A similar cessation of formation and maturation of autophagosomes may occur in cell mitosis. Although the present study does not show consistent effects of various autophagic inhibitors on the cell cycle, the peroxisomal degradation specifically occurs at G1 phase. Our simple GFP fluorescence method would contribute to further understanding of this field.

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