Intracellular ATP Correlates with Mode of Pexophagy in *Pichia pastoris*

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The methylotrophic yeast *Pichia pastoris* can degrade peroxisomes selectively though two distinct pexophagic pathways, viz., micropexophagy and macropexophagy. These micro- and macropexophagy pathways are induced by adaptation of methanol-grown cells to glucose-containing and ethanol-containing media respectively. However, our understanding of the molecular signal(s) that determine which pathway is activated or repressed in response to environmental changes is limited. In this study, the determinant for these pathways was sought using cells undergoing pexophagy under a variety of conditions. Micropexophagy and macropexophagy were distinguished in living cells by fluorescence microscopy. Our results indicate that glucose and ethanol were not specific inducers of micro- and macropexophagy respectively. Micropexophagy was found to be more sensitive to ATP-depletion than macropexophagy, suggesting that the micropexophagic process requires a higher level of ATP than the macropexophagic process. From these and other results, we postulate that intracellular ATP levels play an important role in determining which pexophagic pathway is activated.

Key words: autophagy; peroxisome degradation; vacuole; methylotrophic yeasts

Cells must respond to environmental changes in order to survive and to differentiate. This cellular adaptation encompasses not only expression of various proteins required under new environmental conditions, but also degradation of proteins to recycle amino acids for protein synthesis as a form of post-translational regulation. While selective degradation of proteins occurs mainly through the ubiquitin/proteasome system, bulk degradation of cytosolic proteins and organelles is carried out by the lysosome/vacuole *via* a process known as autophagy.1-3 Furthermore, selective autophagy-related processes also exist, viz., the Cvt pathway and pexophagy. Pexophagy and the Cvt pathway are selective pathways for peroxisome degradation, and aminopeptidase I (ApeI) and α-mannosidase biosynthesis, respectively.4,5 As is true for general autophagy, there are two distinct modes of pexophagy, viz., micropexophagy (pexophagy through microautophagy) and macropexophagy (pexophagy through macroautophagy). The methylotrophic yeast *Pichia pastoris* executes the two pexophagic pathways depending on the carbon source used for induction. Micropexophagy is induced by glucose and macropexophagy by ethanol (Fig. 1).5,6 During micropexophagy, peroxisomes are incorporated directly into the lytic compartment by invaginated or septated vacuoles.5,7 In contrast, during macropexophagy, peroxisomes are sequestered primarily by inclusion within newly-formed membranes. The peroxisome-containing pexphagosome then fuses with the vacuole to deliver its cargo.6,8 Due to these differences in membrane dynamics, micro- and macropexophagic pathways can easily be discriminated by microscopic observation of fluorescent cells of *P. pastoris* double-labeled with green fluorescent protein tagged with a peroxisomal targeting signal (GFP-PTS1) and with FM 4-64 for delineating the vacuolar membrane.6,7

Recent studies on membrane dynamics and mechanisms in *P. pastoris* indicate that many ATG gene products, including the ubiquitin-like PpAtg8-system, share common functions in the formation of membrane structures, *e.g.*, the micropexophagic apparatus (MIPA) during micropexophagy and the pexophagosome during macropexophagy.1,9,10 The switching mechanism between micro- and macropexophagic pathways, however, remains to be clarified.

In the present study, the regulation of pexophagic pathways was examined by analysis of peroxisome degradation in cells induced under a variety of conditions. Our results suggest that the intracellular ATP level is one of the determinants regulating the pexophagic pathways, especially in the switching process between micro- and macropexophagy.

**Materials and Methods**

*Pichia strain and cultivation. Pichia pastoris* strain STW1 (*arg4, his4::pTW51 [P*AOX1*-GFP-SKL, HIS4]*)6 was used throughout this study. Cells were cultivated at 28°C in synthetic medium (0.67% yeast nitrogen base
without amino acid [YNB; Difco Becton Dickinson, Lincoln Park, NJ], supplemented with amino acids according to auxotrophic requirements) and a carbon source. The carbon sources used were 0.5% (v/v) methanol, 0.5% (v/v) ethanol, 2% (w/v) glucose, 2% (w/v) fructose, 2% (w/v) glycerol, 2% (w/v) 2-deoxyglucose, and 0.5% (w/v) sodium acetate. Cells were grown on methanol medium for 12 h to induce peroxisomes, and subsequently shifted to medium containing a different carbon source to initiate pexophagy.

Morphometric analysis of pexophagy. Observation of peroxisome-vacuole dynamics were made as described previously. Pexophagic pathways were visualized by labeling the cells with 0.93 µg/ml FM4-64 (Molecular Probes, Eugene, OR) during a 12-h incubation in methanol-containing medium starting with an OD₆₀₀ = 0.5. Pexophagy was observed with an IX70 fluorescence microscope (Olympus) equipped with a XF52 filter set (Omega Optical). Images were acquired with a Sensys™ Charged Coupled Device camera (Photometrics) and analyzed using MetaMorph imaging software (Universal Imaging, West Chester, PA) and Adobe® Photoshop 6.0.

After a 3-h adaptation, the mode of pexophagy was judged to be micropexophagy or macropexophagy by comparing the number of cells having an invaginated vacuole or the number of cells releasing peroxisomal GFP-PTS1 via diffusion into the lumen of spherical vacuoles. Approximately 500–800 fluorescent cells were counted per assay. Cells undergoing micropexophagy showed deeply invaginated vacuoles, whereas cells undergoing macropexophagy contained spherical vacuoles exhibiting diffuse GFP-PTS1 fluorescence within the lumen. GFP-PTS1 diffusion within the vacuole lumen through macropexophagic can be observed after 30–60 min of pexophagy-induction, whereas micropexophagy requires more than 90 min. The pexophagic processes identified by these fluorescent images were also confirmed by electron microscopic observation.

Determination of intercellular ATP. Cells adapted to pexophagy-inducing medium for 3 h were washed twice, re-suspended in 50 mM Tris–HCl (pH 7.5), and disrupted using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Intact cells and glass beads were removed by centrifugation, and the amount of ATP in the resultant supernatant was assayed. Determination of ATP was performed using a mixture of hexokinase (HK)- and glucose-6-phosphate dehydrogenase (G6PDH) (SIGMA, St. Louis, U.S.A.). The assay mixture contained 100 µmol of glucose, 0.25 µmol of NADP (pH 7.5), 10 µmol of MgCl₂, 0.2 and 0.1 U of HK and G6PDH respectively, 50 µmol of Tris–HCl (pH 7.5), and an appropriate amount of supernatant solution or authentic ATP solution as a standard. It was incubated at 25 °C for 30 min, and the amount of NADPH formed was

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**Fig. 1.** Two Distinct Modes of Pexophagy in *P. pastoris.*

*P. pastoris*, which has very large peroxisomes when grown on methanol as sole carbon source, undergoes two distinct pexophagic pathways depending on the adapted carbon source. Vac, vacuole; Ps, peroxisomes; MIPA, micropexophagic apparatus; Ppg, pexophagosome. A, Micropexophagy is induced in cells by adaptation to 2% glucose medium. Based on fluorescence microscopy, the vacular membrane (FM4-64) invaginates, septates, and directly engulfs a peroxisome cluster. A cup-shaped MIPA is newly synthesized and attached to a peroxisome on the far side of the invaginating vacuole. B, Macropexophagy occurs when the cells are adapted to 0.5% ethanol medium. A newly formed double membrane vesicle (pexophagosome: Ppg) enwraps a single peroxisome compartment and transports it to the vacuole and a peroxisomal GFP-PTS1 signal is released therein. An intact peroxisome cluster gives a strong signal near the vacuolar membrane in the early stages of membrane dynamics.
measured spectrophotometrically at 340 nm. Protein was determined by the Bradford method (Bio-Rad Protein Assay Kit, Bio-Rad) using bovine serum albumin as standard.

Results

Induction of pexophagy by adaptation to various carbon sources

In previous studies, micropexophagy was induced in methanol-grown cells adapted to 2% glucose-medium and macropexophagy in cells adapted to 0.5% ethanol-medium.5,6 Here, methanol-grown cells were adapted to various carbon sources to determine which pexophagic pathway was dominantly induced under each condition. The mode of pexophagy was determined using Pichia cells expressing GFP-PTS1 as peroxisomal fluorescent marker (strain STW1) co-stained with FM4-64 to delineate the vacuolar membrane. Macropexophagic cells exhibited release of GFP-PTS1 into the lumen of spherical vacuole without significant changes in vacuolar morphology. In contrast, micropexophagic cells exhibited deeply invaginated or septated vacuoles.6,7 Morphometric analysis was performed on 500–800 cells per carbon source after a 3-h adaptation. About 30% of the total cell population adapted to 2% glucose had invaginated vacuoles. In contrast, less than 7% of the total cell population adapted to 0.5% ethanol exhibited invaginated vacuoles, and more than half of the cells had GFP-PTS1 fluorescence within the vacuolar lumen (Fig. 2A and B). Cells adapted to media containing 2% glucose, 2% fructose, and 2% glycerol underwent micropexophagy. On the other hand, adaptation to media containing 0.5% ethanol or 0.5% sodium acetate induced macropexophagy. Cells adapted to 2% 2-deoxyglucose (glucose analog) did not undergo significant changes in...
fluorescent morphology, indicating that pexophagy was not induced within these cells. This suggest that glucose might not play a direct role as a signaling molecule for micropexophagy induction. These results also indicate that glucose and ethanol are not specific signaling molecules for induction of micro- and macropexophagy respectively.

**Pexophagy induction in 2% glucose in the presence of sodium azide**

To determine how intracellular energy level affects induction of pexophagy, the pexophagic process was monitored in cells adapted to 2% glucose medium containing inhibitors of the mitochondrial respiratory chain, sodium azide (200 μM), antimycin A (1.0 μM) or oligomycin (oligomycin A + B + C mixture, 100 μg/ml). All of these inhibitors blocked both modes of pexophagy at these concentrations (data not shown). However, exposure to lower concentrations of sodium azide (10–50 μM) induced macropexophagy (Fig. 3).

To confirm energy depletion in the sodium azide-treated cells, the intracellular ATP level (nmol/mg protein) was determined prior to and after induction of pexophagy. Under azide-free conditions, the intracellular ATP level increased about 6.1-fold after a 3-h adaptation to 2% glucose. As expected, the increase in sodium azide caused a decrease in the resulting intracellular ATP level. Although both modes of pexophagy require the same ATP-dependent, ubiquitin-like PpAtg8-modification pathways, the micropexophagic process was found to be more sensitive to energy depletion.

The intracellular ATP level was found to correlate well with the mode of pexophagy in sodium azide-treated cells, i.e., micropexophagy was induced at higher ATP levels and macropexophagy at lower ATP levels. This observation was also consistent with the results shown in Fig. 2. Micropexophagy-induced cells, viz., 2% glucose-, 2% fructose-, and 2% glycerol-adapted cells, showed remarkably higher ATP levels (4- to 6.1-fold higher) than methanol-grown cells. On the other hand, macropexophagy-induced cells, i.e., cells adapted to 0.5% ethanol or 0.5% sodium acetate, showed low levels of ATP, similar to those of methanol-grown cells (Fig. 2).

**Pexophagy induction by adaptation to glucose-limiting conditions**

Next we tried to modulate the intracellular ATP level by limiting glucose concentration during pexophagy induction, thereby minimizing the effects of changes in metabolic pathways. As shown in Fig. 4, macropexophagy occurred at lower concentrations of glucose (0.01–0.5%), while higher concentrations of glucose (0.1–5.0%) induced micropexophagy. Intracellular ATP was found to decrease with decreases in glucose concentration, and reached a level comparable to that measured in methanol-grown cells adapted to 0.05% glucose, when macropexophagy became dominant.

**Discussion**

Thus far, switching of autophagic pathways has been examined mainly in terms of selectivity, i.e., N-starvation induced non-selective autophagy as opposed to selective autophagy-related pathways (Cvt pathway and pexophagy). In contrast, the mechanism of switching between micro- and macroautophagic pathways has not been considered in detail. Our present results indicate that glucose and ethanol are not specific inducers of micro- and macropexophagy respectively, and that intracellular ATP levels correlate well with the induced mode of pexophagy. This suggests that ATP is one of the key determinants controlling the mode of the two pexophagic pathways.

Both micro- and macropexophagic pathways require many common ATG gene products for formation of MIPA and pexophagosomes and thus both require ATP for activation of the PpAtg8-modification pathway. Indeed, high concentrations of respiratory chain inhibitors block both pexophagic pathways concomitant with ATP-depletion. However, the minimum concentration of inhibitors required to cause these inhibitory effects differed between micropexophagy and macropexophagy. The micropexophagic process was found to be more sensitive to sodium azide, suggesting that micropexophagy requires a higher intracellular ATP level than macropexophagy (Fig. 2). On the other hand, nitrogen starvation-induced macroautophagy was not affected by the microtubule-depolymerizing drug nocodazole. Treatment of 2%-glucose-adapted *Pichia* cells with an inhibitor of actin-depolymerization Latrunculin A inhibited micropexophagy but induced macropexophagy, indicating that micropexophagy requires intracellular redistribution of actin and that a higher level of ATP is required for micropexophagy (Oku and Sakai, unpublished data). In many pexophagy-defective mutants, including *Ppatg7*, vacuole engulfs peroxisomes and ceased micropexophagy just prior to sequestration of peroxisomes from the cytosol (Stage 1c). Therefore, at least in these Stage 1C *atg* mutants, the micropexophagic process started after glucose-adaptation. This suggests that ATP consumption through the *Atg7*-dependent ubiquitin-like cascade does not contribute to the switching mechanism between micro- and macropexophagy. From these results, cells appear to be able to select the pexophagic pathway as a function of intracellular ATP.

What kind of molecules are responsible for recognizing the intracellular ATP level and transmitting a signal to determine the mode of pexophagy? One of the candidate molecules is the α subunit of phosphofructokinase, Pfk1. Eukaryotic phosphofructokinase is a heterooctamer composed of 4α-subunits and 4β-subunits encoded by *PFK1* and *PFK2* respectively, in *Saccharomyces cerevisiae*. *Yuan et al.* showed *PFK1* is necessary for micropexophagy but not for macropexophagy, and that it is involved in an early signaling...
Fig. 3. Effect of Sodium Azide on Mode of Pexophagy.
Methanol-grown *Pichia* cells were adapted to 2% glucose medium in the presence of various amounts of the respiratory chain inhibitor, sodium azide (NaN₃). A, Morphometric analysis. B, Fluorescent images of 2% glucose-adapted cells undergoing macropexophagy in the presence of 20 μM sodium azide. C, The intracellular ATP level is shown as a percentage of the ATP level in methanol-grown cells (71.8 ± 15.2 nmol/mg protein) prior to induction of pexophagy. The figures to the right of the bar show the determined values (nmol/mg protein).

Fig. 4. Induction of Macropexophagy by Adaptation to Glucose-Limited Conditions.
Methanol-grown *Pichia* cells were adapted to a medium containing the indicated amounts of glucose. A, Morphometric analysis. B, Fluorescent images of 0.05% glucose-adapted cells undergoing macropexophagy. C, Intracellular ATP level. The intracellular ATP level is shown at the relative value (%) to the ATP level in methanol-grown cells (71.8 ± 15.2 nmol/mg protein) before pexophagy induction, and the figures right of the bar are the measured values in nmol/mg protein.
event in micropexophagy. Furthermore, because the catalytically inactive form of PFK1 can complement the pfk1 defect in micropexophagy, the glycolytic metabolite downstream of the phosphofructokinase-catalyzed reaction is excluded as the direct signaling molecule. This notion was also supported by our present results that 2% glycerol, a downstream metabolite, induced micropexophagy. More importantly, phosphofructokinase is a highly regulated protein with several conserved binding motifs for ATP, AMP, GTP, citrate, and fructose bisphosphate, and has been shown to bind to actin. Because micropexophagy but not macro-pexophagy requires an ATP-dependent actin rearrangement (Oku and Sakai, unpublished data), ATP might regulate the cytoskeletal rearrangement necessary for vacuolar invagination through binding to Pfk1 during micropexophagy.

The Atg1-complex, consisting of Atg1, Atg11, Atg13, Atg17, and Vac8, is the regulatory machinery regulating nonselective macropexophagy, which is induced by nitrogen starvation under conditions of low ATP. The selective Cvt pathway occurs in nutrient rich medium under conditions of high ATP through protein association and phosphorylation. Previously, PpATG1, PpATG11, and PpVAC8, were shown to be necessary for micropexophagy, and recently, we showed that a sorting nexin, PpAtg24, which co-localizes with PpVac8 and PpAtg17, regulates vacuolar membrane dynamics but not membrane formation.

Because the Atg1 complex also includes these cytoskeletal molecules, it might be a regulator of both vacuolar dynamics and de novo membrane formation, including the switching between micro- and macropexophagy. Tor, which is suggested to be an ATP sensor in mammalian cells, might also be involved in regulation of the Atg1 complex.

Methanol-induced peroxisomes contain a large amount of alcohol oxidase (nearly 40% of total soluble protein), the first enzyme necessary for both assimilation and dissimilation of methanol. Therefore, peroxisomes, which are reactive oxygen species- (ROS-) generating organelles, play an important role in methanol metabolism and must achieve appropriate homeostasis through regulation of their synthesis and degradation. During macropexophagy, peroxisomes are degraded one by one so that cells can modulate their number. In contrast, peroxisomes are incorporated into the vacuole as a cluster during micropexophagy. No methanol-induced peroxisomes were found to be excluded from this process. What, then, is the physiological significance of the switch between micro- and macropexophagy? Under conditions of high ATP, which induced macropexophagy, cells perhaps make a commitment to degrade all peroxisomes (i.e., ROS-generating organelles) completely. Under conditions of low ATP, which induced macropexophagy, cells may still prepare for a return to methanol-containing medium. Such carbon source shifts can occur in nature, because methylotrophic yeasts are often found in pectin-rich environments (containing methyl ester compounds), such as fruit surfaces. Our present results indicate that the micro- and macropexophagy pathways are not independently regulated, but are mutually dependent pathways affected by intracellular ATP levels. Selection of the appropriate pexophagic pathway is likely to be a function of the balance between energetics and the risks associated with harboring ROS-generating organelles under new environmental conditions.

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