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Environmental Response of Yeast Peroxisomes

Aspects of Organelle Assembly and Degradation

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ABSTRACT

Nutritional changes can affect either the assembly or disassembly of yeast peroxisomes. In the past decade, insights regarding the molecular mechanisms of peroxisome assembly have been gained chiefly through the cloning of the *PEX* genes obtained by complementation of corresponding *pex* mutants in several yeast strains and Chinese hamster ovary cell lines. Depletion of these peroxins (proteins encoded by *PEX* genes) by deletion of the corresponding genes affects peroxisomal protein import, biogenesis, or proliferation. To complement these studies in the field, the authors undertook an investigation of the functions of a subset of *Candida boidinii* peroxisomal membrane proteins (PMPs), Pex11, Pmp47, and Pmp20, by analyzing strains of *C. boidinii* in which the genes encoding these proteins were deleted. The authors' studies show that Pex11p is involved in peroxisome proliferation; Pmp47 plays a role in the translocation, folding, or assembly of dihydroxyacetone synthase; and Pmp20 is probably involved in methanol metabolism. In contrast to the studies on peroxisome assembly, the molecular mechanisms of peroxisome degradation remain poorly understood. To shed light on this problem, the authors isolated *Pichia pastoris* mutants defective in peroxisome autophagy (*pag* mutants). A novel, double-fluorescence method used for the characterization of wild-type cells and of *pag* mutants enabled us to dissect the microautophagic degradation of peroxisomes into several distinct stages. These studies show that specific *PAG* gene products are involved in multiple steps of the process. Future cloning and characterization of the functions of *PAG* genes will reveal the molecular basis of peroxisome degradation.

Index Entries: Peroxisomal membrane protein (PMP); autophagy; methylotrophic yeasts.

INTRODUCTION

Yeast peroxisomes respond to certain nutrients by altering their metabolic repertoire, their number, and their morphology. One of the merits of using methylotrophic yeasts, such as *Pichia pastoris* and *Candida boidinii*, for studies involving peroxisomes, is their ability to grow on multiple peroxisome-inducing carbon sources, e.g., methanol, oleate, and D-alanine. When glucose-grown cells are transferred to a medium containing one of these

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three carbon sources, peroxisome proliferation is observed. These environmental responses of yeast peroxisomes have been successfully exploited to study the genetics and molecular mechanisms of peroxisome assembly *(1,2)*.

In contrast to this peroxisome-induction response, when peroxisome-induced cells are transferred back to glucose medium, peroxisomes are degraded by autophagy *(3–8)*, a process whose mechanism remains largely unknown.

This article summarizes recent efforts using the environmental responses of yeast peroxisomes to understand the function of certain peroxisomal membrane proteins (PMPs) in *C. boidinii* and the mechanism of peroxisome degradation via autophagy in *P. pastoris*.

PMP ANALYSIS IN *C. BOIDINII*: LESSONS FROM PMP-KNOCK-OUT STRAINS

Regulation of Peroxisome Matrix and Membrane Proteins

C. boidinii is able to grow on three different carbon sources; methanol, oleate, and D-alanine *(9)*. Expression of matrix enzymes, excluding the peroxisomal marker enzyme, catalase, is generally in response to specific metabolic needs. For example, methanol-induced peroxisomes contain alcohol oxidase (AO) and dihydroxyacetone synthase (DHAS), which are necessary only for methanol metabolism; β-oxidation enzymes are induced only upon growth on oleate *(9)*. Recent studies show that D-alanine-induced peroxisomes are insensitive to glucose repression, but methanol- or oleate-induced peroxisomes are sensitive. Marker enzymes of D-alanine-induced peroxisomes (D-amino acid oxidase and catalase) and proliferation of the organelle are not repressed by the simultaneous presence of glucose *(9A)*.

In contrast to the regulation of these matrix enzymes, PMPs seem to behave quite differently. *C. boidinii* was the first yeast strain whose peroxisomal membrane was biochemically characterized *(10)*. The membrane fraction contains three major PMPs: PMP20, PEX11 (originally designated as PMP30), and PMP47. Among these proteins, PMP20 is induced specifically on methanol, and Pex11p and PMP47 are induced on all three peroxisome-inducing carbon sources *(9)*. These results strongly suggest that the function of PMP20 may be specific to methanol metabolism, but that of Pex11p and PMP47 is more general in peroxisomal functions.

PMP Gene Disruption in Haploid Strain of C. boidinii

Although three PMP genes were cloned from the *C. boidinii* genome, further research was hampered by the absence of a molecular breeding system for this nonconventional yeast strain. Although the original strain of *C. boidinii* ATCC32195 used by Goodman et al. *(11–13)* had two sets of genes per genome for each of the three PMPs, the authors' culture collection of *C. boidinii* included a haploid strain, S2, containing only one gene per genome for each of the three PMPs and other genes *(12,14)*. Using strain S2, the authors expected to develop a molecular breeding technique for *C. boidinii*, including sequential gene disruption, heterologous gene expression, and Autonomous Replicating Sequence (ARS)-based transformation *(15–18)*. As the first step to assess the function of these PMPs, three genes, coding for Pex11p, PMP47, and PMP20, were recloned from a haploid strain S2, and the corresponding knock-out strains, *pex11*Δ, *pmp47*Δ, and *pmp20*Δ, were derived and characterized.

C. boidinii *Pex11p (PMP30): Involvement in Peroxisome Proliferation*

Peroxisomal morphology of *C. boidinii* changes dramatically upon growth in certain carbon sources *(12,19)*. When the wild-type cells are grown on methanol, a spheroid 2 μm in diameter is present in each cell. In contrast, oleate-grown cells have about 5–12 small peroxisomes, ranging in diameter from 0.1 to 0.7 μm.

Pex11p is the only peroxin that was not cloned by complementing the corresponding mutant yeast strain, but by the information based on the primary structure of the purified protein *(12,20–22)*. Other *C. boidinii pex* mutants (e.g., *pex5*Δ deficient in the PTS1-receptor) are not able to grow on any of the peroxisome-inducing carbon sources (methanol, oleate, nor D-alanine). In contrast, *Cbpex11*Δ was still able to grow on plates containing these carbon sources, but the *pex11* disruption caused a small but reproducible inhibition of growth in oleate, and a dramatic inhibition of growth in methanol *(12)*.

Pex11p was found to be involved in the maintenance of normal peroxisome morphology. Methanol-induced *Cbpex11*Δ cells were characterized by the existence of one or two unusually spherical peroxisomes ranging in diameter from about 0.7 to 1.7 μm, although sometimes cells were also observed that contained normal-sized peroxisomes. The large, round, methanol-induced peroxisomes, which have not been observed previously in the wild-type strain, suggested that the fragmentation of the original peroxisome into smaller ones had not occurred in the mutant. A similar but more dramatic effect was observed with oleate-induced *Cbpex11*Δ cells, which had only one or two giant peroxisomes *(12)*.

Independent studies identified the *Saccharomyces cerevisiae* homolog of *Cbpex11 (20,21)*. The effect of deleting the *PEX11* gene in *S. cerevisiae* was similar to that observed in *Cbpex11*Δ. Furthermore, overexpression of Pex11p induced peroxisome proliferation *(21)*. To determine whether CbPex11p is a functional homolog of ScPex11p, mutual complementation experiments were performed in *pex11*Δ strains of both *S. cerevisiae* and *C. boidinii (12)*. Complementation, in terms of cell growth and organelle size, shape, and number, was successful in both directions. Because *S. cerevisiae* cannot utilize methanol, it is striking that ScPex11p can substitute for a protein that is important, albeit indirectly, for methanol utilization in *C. boidinii*. Furthermore, overproduction of Pex11p promoted peroxisome proliferation in methanol-induced peroxisomes of *C. boidinii* and oleate-induced peroxisomes of *S. cerevisiae*. Recently, a mammalian homolog of Pex11p has been described, and again, overexpression of this protein in CHO cells caused peroxisome proliferation *(23)*. These studies show that Pex11p is a conserved molecule that promotes peroxisome proliferation or peroxisome division.

The molecular mechanism by which Pex11p is involved in peroxisome division and its control is just beginning to be uncovered. It was postulated that the dimerization of Pex11p is related to the formation of tubular peroxisomes *(24)*. Unlike the yeast, Pex11p, the mammalian counterpart has the dilysine KXKXX motif near its carboxyl-terminus, which faces the cytosol. The guanosine 5′O-(3-thiotriphosphate) (GTP)-γS-dependent binding of adenosine diphosphate (ADP)-ribosylation factor and coatomer to the peroxisome membrane was observed, and the binding was proposed to be mediated by carboxy-terminal dilysine motif of Pex11p *(23)*. Coatomer was suggested to help the conversion of tubular peroxisomes into smaller spherical ones. The mechanism of peroxisomal division may become one of the exciting fields in the near future, especially in relation to the signal response to peroxisome proliferators.

C. boidinii *PMP47: Role in DHAS Assembly/Import*

C. boidinii PMP47 is an integral PMP related to the mitochodrial adenosine triphosphate (ATP)/ADP exchanger *(25,26)*, and is induced in all peroxisome-inducing carbon sources *(9)*. The sorting signal of Pmp47 resides within the loop present in the peroxisome matrix between two of the membrane-spanning regions *(27)*.

Depletion of PMP47 from strain S2 results in retarded growth on oleate and a complete loss of growth on methanol *(14)*. Electron microscopy observations reveal the presence of peroxisomes in methanol- and oleate-grown cells, and the presence of materials of high electron density in the cytoplasm in both cases.

Methanol-induced cells of *pmp47Δ* were investigated in detail *(14)*. The activity of one of the methanol-induced peroxisomal matrix enzymes, DHAS, was not detected in *pmp47Δ* cells. Further biochemical and immunocytochemical experiments revealed that the DHAS protein aggregated in the cytoplasm as an inclusion body; two other peroxisome matrix enzymes, AO and catalase, were active and were found in peroxisomes. Thus, PMP47-depletion enabled differentiation between protein transport of AO and DHAS, despite the fact that both these proteins have the C-terminal peroxisomal targeting signal (PTS)1 motif, -ARY *(28)* and -NHL, respectively, and are transported into peroxisomes in a Pex5p-dependent manner (Y. Sakai, unpublished results).

Though *pmp47Δ* did not have any soluble or active DHAS protein, DHAS was in a cytoplasmically active and folded form in both *pex* mutants and *pex pmp47Δ* double mutants, including *PEX5*-derivatives *(14)*. These observations raise the question of how and where DHAS is folded into an active form.

Several studies show that some folded proteins are competent for peroxisomal transport *(29–31)*. However, these reports do not exclude the existence of proteins that are folded or oligomerized after their import into peroxisomes. Indeed, it was shown recently that AO is transported into peroxismes as monomers, and then assembles into octamers in the peroxisomes *(32)*. Although chaperones have been postulated in peroxisomes, there is only one report suggesting the presence of a 70-kDa heat shock protein in watermelon glyoxysomes *(33)*. The authors' observations suggest that DHAS normally folds/oligomerizes within peroxisomes, where it requires a peroxisomal factor. If the folding of DHAS could occur in the cytoplasm in wild-type cells, it is difficult to explain the phenotype of *pmp47Δ* mutants.

To explain these observed phenotypes, the authors hypothesize a relationship between the biochemical function of PMP47 and the translocation-folding process of DHAS (Fig. 1). At least four components are thought to be necessary for this process: DHAS, PMP47, the unknown solute transported by PMP47, and a peroxisomal factor necessary for DHAS folding. According to the model, observed phenotypes are explained as follows: Although DHAS and the solute transported by PMP47 are both mislocalized in the cytoplasm of *pmp47Δ*, the absence of the peroxisomal factor in the cytoplasm prohibits proper folding of DHAS. This problem might be aggravated by the large concentration of DHAS in the cytoplasm. However, *pex* mutant strains allow folding of DHAS in the cytoplasm, probably because the peroxisomal factor is also mislocalized there. The factor is likely to be a PTS1-containing protein, because a cytoplasmic, folded form of DHAS could be observed in *pex5* and *pex5 pmp47Δ* mutant strains. More recent experiments support this hypothesis: When DHAS without -NHL sequence was expressed in the *das1Δ* strain, inactive DHAS aggregates were observed in the cytosol, and soluble DHAS protein was not found, as observed in *pmp47Δ* cells; the PTS1 sequence of DHAS, the tripeptide -NHL, was sufficient to transport green fluorescence protein (GFP) to peroxisomes, not only in the wild-type strain, but also in the *pmp47Δ* strain, leading to the conclusion that the failure of peroxisomal transport and folding of DHAS resides not in the PTS1-sequence, but in another part of the coding region; and Bellion and Goodman *(34)* and Waterham et al. *(32)* suggested the possibility of co-transport of DHAS and AO. However, in *aod1Δ* cells, DHAS was imported and active in the peroxisome. Conversely, in *das1Δ* cells, AO was imported into the peroxisome and showed its activity. Experiments are now under way to see whether DHAS is transported as a dimer in a folded form, or is folded and/or oligmerized after its transport into peroxisomes.

What does PMP47 transport directly? Because the protein family to which PMP47 belongs is known to transport small solute compounds such as ATP/ADP, phosphate, tricarboxylate, 2-oxoglutarate/malate, and so on, the authors favor the hypothesis that

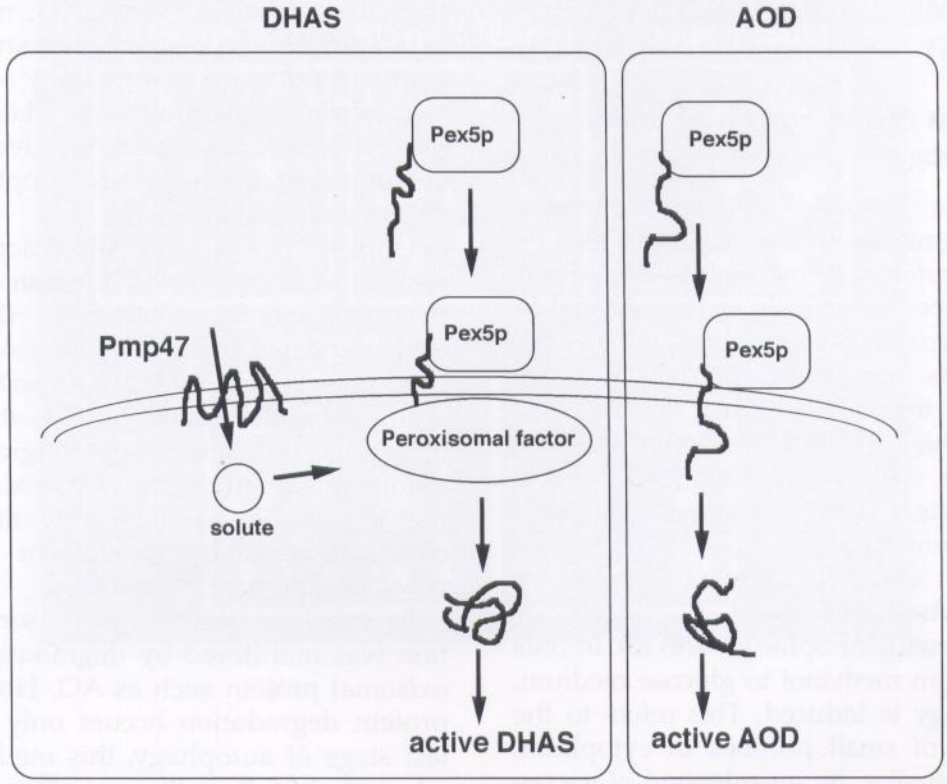

Fig. 1. Biochemical diversity of peroxisomal protein transport revealed by depletion of PMP47 in *C. boidinii*, and a working hypothesis for the mechanism of DHAS import and folding. Both DHAS and AO are assumed to be folded and oligomerized after transport into peroxisomes (14,32).

PMP47 is an ATP/ADP exchanger, based on the following observations: The highest sequence similarity of PMP47 among the nearly 40 transporters in the family is with the mitochondrial ATP/ADP exchanger *(25,26)*. The inverted topology of PMP47 with respect to the mitochondrial ATP/ADP exchanger *(27)* suggests an opposite direction of the exchange of substrates, which is consistent with the function of the two organelles. ATP within mitochondria is required for translocation *(35)*; there is a cytoplasmic ATP requirement for peroxisomal import *(36,37)*. Peroxisome proliferation may well require energy in the form of ATP. ATP is present in the cytoplasm and could enable DHAS folding in the PTS1-receptor-deficient strain. The development of a direct transport assay with purified peroxisomes has been hindered by the extreme fragility of peroxisomes. However, a detailed analysis with the *pmp47Δ* cells grown in oleate will give us a key to answer this question. Such an effort is now under way.

C. boidinii *Pmp20: Involvement in Methanol Metabolism?*

In contrast to Pex11p and PMP47, PMP20 is induced specifically on methanol medium, suggesting that Pmp20 is related directly to methanol metabolism *(10,13)*. This is supported by the observations that *pmp20Δ* cells do not grow on methanol, but retain normal growth on oleate and D-alanine, and the fact that peroxisomes of methanol-induced *pmp20Δ* cells show normal morphology.

PEROXISOME DEGRADATION VIA AUTOPHAGY

P. pastoris *as Model System to Study Microautophagy*

Yeast cells respond to environmental changes, not only by synthesizing, but also by degrading, proteins, or organelles. When peroxisome-induced yeast cells are transferred to a carbon source that does not require peroxisomes for its utilization, peroxisomes are degraded in the lysosome-like vacuole via autophagy. This type of degradation has been demonstrated with *S. cerevisiae* and methylotrophic yeasts, *C. boidinii, Hansenula polymorpha*, and *P. pastoris (3–5,7)*.

P. pastoris exhibits different modes of autophagy when transferred to glucose or ethanol after methylotrophic growth *(8)*. In cells transferred from methanol to glucose medium, microautophagy is induced. This refers to the sequestration of small portions of cytoplasm, including organelles, by invagination of vacuolar membrane. On the other hand, ethanol adaptation induces macroautophagy, which involves the sequestration of organelles and cytosol by membranes of unknown origin to generate autophagosomes, which then fuse with the vacuolar membrane. These two types of autophagic processes are conserved in virtually all eukaryotic cells and constitute a major degradative pathway for cellular proteins.

Morphological, and more recent genetic, analyses have begun to elucidate the molecular basis of macroautophagy in *S. cerevisiae*. Autophagosomes have been characterized ultrastructurally in *S. cerevisiae*, and has been shown to be induced by nutrient deprivation, and inhibited by protease inhibitors or by mutations in genes affecting vacuolar proteinases *(38,39)*. The distinct morphology of the autophagic bodies that accumulate under nutrient deprivation conditions has been used to isolate *S. cerevisiae* mutants deficient in the accumulation of these structures. The resulting mutants fall into 14 distinct complementation groups *(40–43)*. In *S. cerevisiae*, a vacuolar protein, aminopeptidase I, which is imported from the cytosol to the vacuolar matrix by the cytoplasm-to-vacuole pathway, shares components used for macroautophagy *(44–46)*. In *H. polymorpha*, peroxisome degradation-deficient (*pdd*) mutants have been isolated, and these are impaired in the macroautophagic degradation of peroxisomes *(6)*.

Unlike the process of macroautophagy, which has been examined in some detail in *S. cerevisiae* and *H. polymorpha*, the microautophagic degradation of peroxisomes has not been investigated in depth. *P. pastoris* gives us a unique model system for this study because it exhibits both macroautophagic and microautophagic degradation of peroxisomes, making it possible to determine the extent of overlapping gene involvement in the two types of autophagic processes.

In previous studies, peroxisome degradation was monitored by degradation of a peroxisomal protein such as AO. However, since protein degradation occurs only at the very last stage of autophagy, this method was not adequate to follow the early stages of peroxisome degradation. To overcome this problem, both the peroxisome and the vacuole of *P. pastoris* cells were labeled with vital strains, i.e., GFP-SKL for peroxisomes, and a red styryl dye, FM4-64, for the vacuolar membrane *(47)*. Using this double-fluorescence labeling technique, microautophagy was followed at its early stages, and this process was dissected into several intermediate steps. Mutants defective in peroxisome degradation by microautophagy were isolated and classified according to their morphological phenotypes.

Morphometric Studies on Microautophagy Using Fluorescence Microscopy

During the switch of *P. pastoris* cells from methanol to glucose medium, a series of distinct morphological intermediates could be distinguished, and they were confirmed by electron microscopy, particularly at the earliest stages of microautophagy. Just prior to the activation of microautophagy, most methanol-grown cells have one large, spherical vacuole per cell (Fig. 2A, stage 0). Within 5–10 min

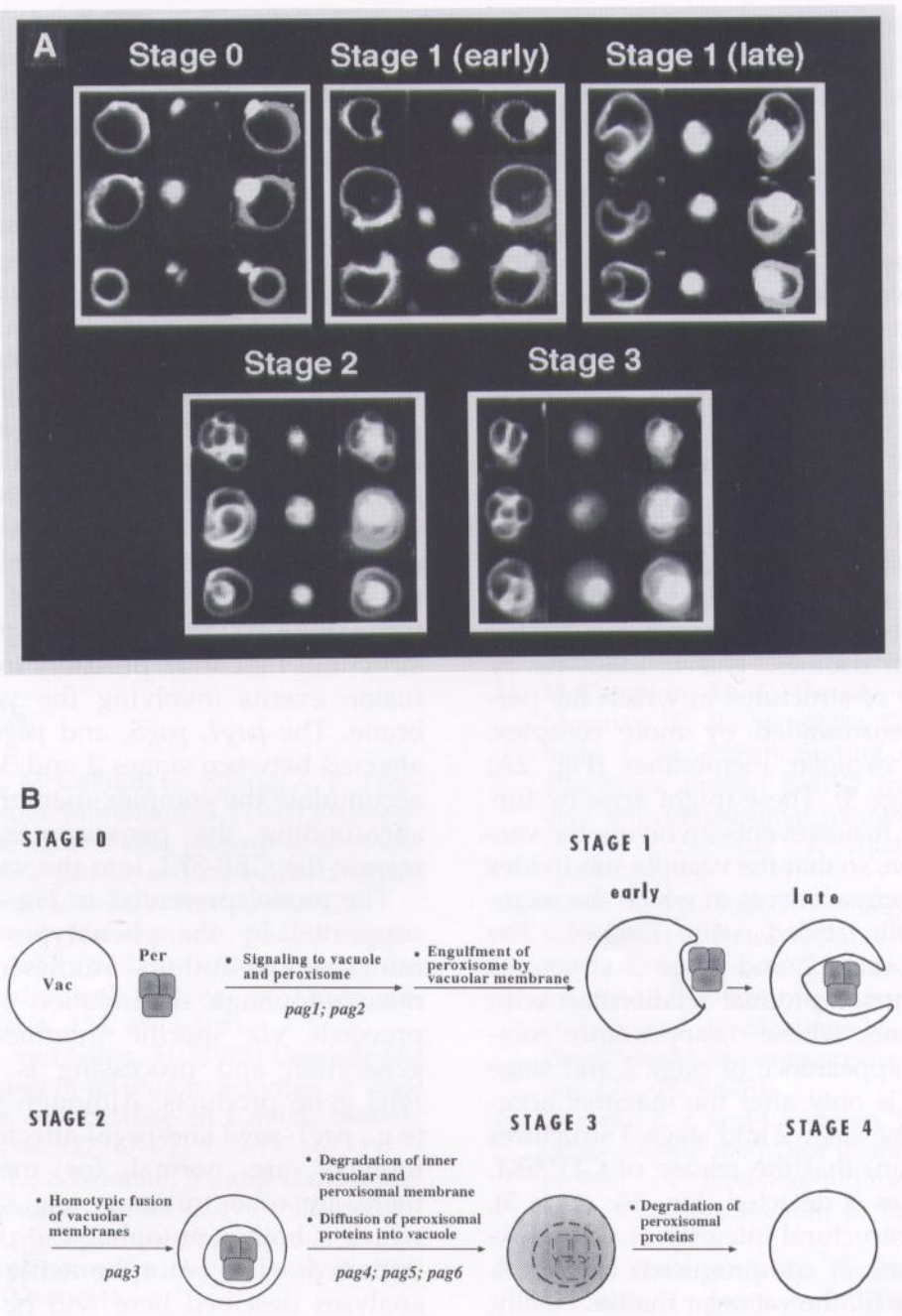

Fig. 2. **(A)** Fluorescence images showing various stages during microautophagic degradation of peroxisomes. (Left) FM4-64; (center) GFP-SKL; (right) superimposed FM4-64 and GFP-SKL signals. **(B)** Schematic model of microautophagic degradation of peroxisomes in *P. pastoris*. The process is dissected into four stages, depending on the morphology and the appearance and disappearance of intermediates in the process. Per, peroxisomes; Vac, vacuole.

after the shift to glucose, cells respond to the environmental change by invagination of vacuolar membranes (Fig. 2A; stage 1). The appearance of these vacuoles, with slight or deep invaginations of the vacuolar membranes, is accompanied by the simultaneous disappearance of spherical vacuoles, reminiscent of a precursor–product relationship between the two types of structures.

After the invagination of the vacuolar membrane around the peroxisomes (or peroxisome clusters), the vacuolar membrane engulfs the peroxisomes (or peroxisome clusters) completely, so that a double membrane forms around the peroxisomes (Fig. 2A, stage 2 and stage 3). The outer of these two membranes is derived from the vacuolar membrane. These two-membrane structures could form by homotypic fusion of the vacuolar membrane that engulfs the peroxisomes. This is followed by the appearance of structures in which the peroxisomes are surrounded by more complex, and multiple, vacuolar membranes (Fig. 2A; stage 2 and stage 3). These might arise by further homotypic fusion events involving the vacuolar membrane, so that the vacuole subdivides into multiple compartments in which the membranes are still labeled with FM4-64. The appearance of stage 2 and stage 3 structures exhibits a precursor–product relationship with stage 1 structures, whose disappearance coincides with the appearance of stage 2 and stage 3 structures. It is only after the maximal accumulation of these stage 2 and stage 3 structures (at about 60 min) that the release of GFP-SKL into the vacuoles is detected (Fig. 2A; stage 3). In stage 3, the structural integrity of the peroxisome membrane is compromised, and GFP-SKL leaks out to fill the vacuolar matrix. Finally, the vacuolar proteases degrade the GFP-SKL, and its fluorescence disappears.

Mutants Defective in Microautophagy Support Model Proposed for this Process

The authors used a strategy, similar to the ones used previously in *P. pastoris (7)* and *H. polymorpha (6)*, for the isolation of the *pag* mutants deficient in peroxisome microautophagy. However, the ability to distinguish the morphological and kinetic steps in autophagy allowed classification of these mutants and use of them to lend additional support for the model proposed in Fig. 2B. Based on this analysis, the *pag1* and *pag2* mutants are impaired in the transition from stage 0 to stage 1, a process that requires the peroxisomes and the vacuole to respond to glucose signaling. These mutants could therefore be affected either in the signal-transduction pathway that senses or responds to glucose in the environment (as in the *gsa1* mutant of *P. pastoris* described by Yuan et al. *[48]*). Part of this response mechanism must reside on the organelles themselves, perhaps on the membranes. The *pag3* mutant is capable of proceeding to late stage 1, but fails to form the double- and complex-membrane structures that arise, probably from homotypic fusion events involving the vacuolar membrane. The *pag4*, *pag5*, and *pag6* mutants are affected between stages 2 and 3, because they accumulate the complex-membrane structures surrounding the peroxisomes, but fail to release the GFP-SKL into the vacuolar matrix.

The model presented in Fig. 2B is strongly supported by the phenotypes of these *pag* mutants. The authors' studies reveal that the microautophagic degradation of peroxisomes proceeds via specific intermediates, whose generation and processing is controlled by *PAG* gene products. Although some mutants (e.g., *pag1–pag3* and *pag6*) affected in microautophagy are normal for macroautophagy, there are other mutants (e.g., *pag4*, *pag5*) in which both autophagic processes are impaired. The morphometric and kinetic analyses descibed here will be useful in the characterization of these and additional mutants, as well in the elucidation of the functions of the gene products involved.

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REFERENCES

1. Gould, S. J., McCollum, D., Spong, A. P., Heyman, J. A., and Subramani, S. (1992) Development of the yeast *Pichia pastoris* as a model organism for a genetic and molecular analysis of peroxisome assembly. *Yeast* **8,** 613–628.
2. Liu, H., Tan, X., Veenhuis, M., McCollum, D., and Cregg, J. M. (1992) An efficient screen for peroxisome-deficient mutants of *Pichia pastoris*. *J. Bacteriol.* **174,** 4943–4951.
3. Borman, C. and Sahm, H. (1978) Degradation of microbodies in relation to activities of alcohol oxidase and catalase in *Candida boidinii*. *Arch. Microbiol.* **117,** 67–72.
4. Chiang, H.-L., Schekman, R., and Hamamoto, S. (1996) Selective uptake of cytosolic, peroxisomal, and plasma membrane proteins into the yeast lysosome for degradation. *J. Biol. Chem.* **271,** 9934–9941.
5. Veenhuis, M., Douma, A., Harder, W., and Osumi, M. (1983) Degradation and turnover of peroxisomes in the yeast *Hansenula polymorpha* induced by selective inactivation of peroxisomal enzymes. *Arch. Microbiol.* **134,** 193–203.
6. Titorenko, V. I., Keizer, I., Harder, W., and Veenhuis, M. (1995) Isolation and characterization of mutants impaired in the selective degradation of peroxisomes in the yeast *Hansenula polymorpha*. *J. Bacteriol.* **177,** 357–363.
7. Tuttle, D. L., Lewin, A. S., and Dunn, W. A. J. (1993) Selective autophagy of peroxisomes in methylotrophic yeasts. *Eur. J. Cell Biol.* **60,** 283–290.
8. Tuttle, D. L. and Dunn, W. A. (1995) Divergent modes of autophagy in methylotrophic yeast *Pichia pastoris*. *J. Cell Sci.* **108,** 25–35.
9. Goodman, J. M., Trapp, S. B., Hwang, H., and Veenhuis, M. (1990) Peroxisomes induced in *Candida boidinii* by methanol, oleic acid and D-alanine vary in metabolic function but share common integral membrane proteins. *J. Cell Sci.* **97,** 193–204.

9A. Sakai, Y., Yurimoto, H., Matsuo, H., and Kato, N. (1998) Regulation of peroxisomal proteins and organelle proliferation by multiple carbon sources in the methylotrophic Yeast, *Candida boidinii*. *Yeast* **14,** 1175–1187.

10. Goodman, J. M., Maher, J., Silver, P. A., Pacifico, A., and Sanders, D. (1986) The membrane proteins of the methanol-induced peroxisomes of *Candida boidinii*. *J. Biol. Chem.* **261,** 3464–3468.
11. McCammon, M. T., Dowds, C. A., Orth, K., Moomaw, C. R., Slaughter, C. A., and Goodman, J. M. (1990) Sorting of peroxisomal membrane protein PMP47 from *Candida boidinii* into peroxisomal membranes of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265,** 20,098–20,105.
12. Sakai, Y., Marshall, P. A., Saiganji, A., Takabe, K., Saiki, H., Kato, N., and Goodman, J. M. (1995) *Candida boidinii* peroxisomal membrane protein PMP30 has role in peroxisomal proliferation and is functionally homologous to Pmp27 from *Saccharomyces cerevisiae*. *J. Bacteriol.* **177,** 6773–6781.
13. Garrard, L. J. and Goodman, J. M. (1989) Two genes encode the major membrane-associated protein of methanol-induced peroxisomes from *Candida boidinii*. *J. Biol. Chem.* **264,** 13,929–13,937.
14. Sakai, Y., Saiganji, A., Yurimoto, H., Takabe, K., Saiki, H., and Kato, N. (1996) The absence of Pmp47, a putative yeast peroxisomal transporter, causes a defect in transport and folding of a specific matrix enzyme. *J. Cell Biol.* **134,** 37–51.
15. Sakai, Y., Kazarimoto, T., and Tani, Y. (1991) Transformation system for an asporogenous methylotrophic yeast, *Candida boidinii*: cloning of orotidine-5′-phosphate decarboxylase gene (*URA3*), isolation of uracil auxotrophic mutants, and use of mutants for integrative transformation. *J. Bacteriol.* **173,** 7458–7463.

16. Sakai, Y. and Tani, Y. (1992) Directed mutagenesis in an asporogenous methylotrophic yeast: cloning, sequencing, and one-step gene disruption of the 3-isopropylmalate dehydrogenase gene (*LEU2*) of *Candida boidinii* to derive doubly auxotrophic marker strains. *J. Bacteriol.* **174,** 5988–5993.
17. Sakai, Y., Goh, T. K., and Tani, Y. (1993) High-frequency transformation of a methylotropic yeast, *Candida boidinii,* with autonomously replicating plasmids which are also functional in *Saccharomyces cerevisiae. J. Bacteriol.* **175,** 3556–3562.
18. Sakai, Y., Akiyama, M., Kondoh, H., Shibano, Y., and Kato, N. (1996) High-level secretion of fungal glucoamylase using *Candida boidinii* gene expression system. *Biochim. Biophys. Acta* **1308,** 81–87.
19. Sulter, G. J., Waterham, H. R., Goodman, J. M., and Veenhuis, M. (1990) Proliferation and metabolic significance of peroxisomes in *Candida boidinii* during growth on D-alanine or oleic acid as the sole carbon source. *Arch. Microbiol.* **153,** 485–489.
20. Erdmann, R. and Blobel, G. (1995) Giant peroxisomes in oleic acid-induced *Saccharomyces cerevisiae* lacking peroxisomal membrane protein Pmp27p. *J. Cell Biol.* **128,** 509–524.
21. Marshall, P. A., Krimkevich, Y. I., Lark, R. H., Dyer, J., Veenhuis, M., and Goodman, J. M. (1995) Pmp27 promotes peroxisomal proliferation. *J. Cell Biol.* **129,** 345–355.
22. Moreno, M., Lark, R., Campbell, K. L., and Goodman, J. M. (1994) Peroxisomal membrane proteins of *Candida boidinii*: Gene isolation and expression. *Yeast* **10,** 1447–1457.
23. Passreiter, M., Anton, M., Lay, D., Frank, R., Harter, C., Wieland, F., Gorgas, K., and Just, W. W. (1998) Peroxisome biogenesis: involvement of ARF and coatomer. *J. Cell Biol.* **141,** 373–383.
24. Marshall, P. A., Dyer, J. M., Quick, M. E., and Goodman, J. M. (1996) Redox-sensitive homodimerization of Pex11p: a proposed mechanism to regulate peroxisomal division. *J. Cell Biol.* **135,** 123–137.
25. Jank, B., Habermann, B., Schweyen, R. J., and Link, T. A. (1993) PMP47, a peroxisomal homologue of mitochondrial solute carrier proteins. *Trends Biochem. Sci.* **18,** 427–428.
26. Kuan, J. and Saier, M. H. J. (1993) The mitochondrial carrier family of transport proteins: structural, functinal, and evolutionary relationships. *Crit. Rev. Biochem. Mol. Biol.* **28,** 209–233.
27. McCammon, M. T., McNew, J. A., Willy, P. J., and Goodman, J. M. (1994) An internal region of the peroxisomal membrane protein PMP47 is essential for sorting to peroxisomes. *J. Cell Biol.* **124,** 915–925.
28. Sakai, Y. and Tani, Y. (1992) Cloning and sequencing of the alcohol oxidase-encoding gene (*AOD1*) from the formaldehyde-producing asporogenous methylotrophic yeast, *Candida boidinii* S2. *Gene* **114,** 67–73.
29. Glover, J. R., Andrews, D. W., and Rachubinski, R. A. (1994) *Saccharomyces cerevisiae* peroxisomal thiolase is imported as a dimer. *Proc. Natl. Acad. Sci. USA* **91,** 10,541–10,545.
30. McNew, J. A. and Goodman, J. M. (1994) An oligomeric protein is imported into peroxisomes in vivo. *J. Cell Biol.* **127,** 1245–1258.
31. Walton, P. A., Hill, P. E., and Subramani, S. (1995) Import of stably folded proteins into peroxisomes. *Mol. Biol. Cell* **6,** 675–683.
32. Waterham, H. R., Russell, K. A., de Vries, Y., and Cregg, J. M. (1997) Peroxisomal targeting, import, and assembly of alcohol oxidase in *Pichia pastoris. J. Cell Biol.* **139,** 1419–1431.
33. Wimmer, B., Lottspeich, F., van der Klei, I., Veenhuis, M., and Gietl, C. (1997) The glyoxysomal and plastid molecular chaperones (70-kDa heat shock protein) of watermelon cotyledons are encoded by a single gene. *Proc. Natl. Acad. Sci. USA* **94,** 13,624–13,629.
34. Bellion, E. and Goodman, J. M. (1987) Proton ionophores prevent assembly of a peroxisomal protein. *Cell* **48,** 165–173.
35. Cyr, D. M., Stuart, R. A., and Neupert, W. (1993) A matrix ATP requirement for presequence translocation across the inner membrane of mitochondria. *J. Biol. Chem.* **268,** 23,751–23,754.
36. Imanaka, T., Small, G. M., and Lazarow, P. B. (1987) Translocation of acyl-CoA oxidase into peroxisomes requires ATP hydrolysis but not a membrane potential. *J. Cell Biol.* **105,** 2915–2922.
37. Wendland, M. and Subramani, S. (1993) Cytosol-dependent peroxisomal protein import in a permeabilized cell sytem. *J. Cell Biol.* **120,** 675–685.
38. Baba, M., Takeshige, K., Baba, N., and Ohsumi, Y. (1994) Ultrastructural analysis of autophagic process in yeast: detection of autophagosomes and their characterization. *J. Cell Biol.* **124,** 903–913.
39. Baba, M., Osumi, M., and Ohsumi, Y. (1995) Analysis of membrane structures involved in

autophagy in yeast by freeze-relica method. *Cell Struct. Funct.* **20,** 465–471.

40. Tsukada, M. and Ohsumi, Y. (1993) Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett.* **333,** 169–174.
41. Thumm, M., Egner, R., Koch, B., Schlumpberger, M., Straub, M., Veenhuis, M., and Wolf, D. H. (1994) Isolation of autophagocytosis mutants of Saccharomyces cerevisiae. *FEBS Lett.* **349,** 275–280.
42. Noda, T., Matsuura, A., Wada, Y., and Ohsumi, Y. (1995) Novel system for monitoring autophagy in the yeast *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **210,** 126–132.
43. Harding, T. M., Morano, K. A., Scott, S. V., and Klionsky, D. J. (1995) Isolation and characterization of yeast mutants in cytoplasm to vacuole protein targeting pathway. *J. Cell Biol.* **131,** 591–602.
44. Harding, T. M., Hefner, G. A., Thumm, M., and Klionsky, D. J. (1996) Genetic and phenotypic overlap between autophagy and cytoplasm to vacuole protein targeting pathway. *J. Biol. Chem.* **271,** 17,621–17,624.
45. Scott, S. V., Hefner, G. A., Morano, K. A., Noda, T., Ohsumi, Y., and Klionsky, D. J. (1996) Cytoplasm-to-vacuole targeting and autophagy employ the same machinery to deliver proteins to the yeast vacuole. *Proc. Natl. Acad. Sci. USA* **93,** 12,304–12,308.
46. Scott, S. V., Baba, M., Ohsumi, Y., and Klionsky, D. J. (1997) Aminopeptidase I is targeted to vacuole by nonclassical vesicular mechanism. *J. Cell Biol.* **138,** 37–44.
47. Sakai, Y., Koller, A., Rangell, L. K., Keller, G. A., and Subramani, S. (1998) Peroxisome degradation by microautophagy in *Pichia pastoris*: Identification of specific steps and morphological intermediates. *J. Cell Biol.* **141,** 625–636.
48. Yuan, W., Tuttle, D. L., Shi, Y. J., Ralph, G. S., and Dunn, W. J. (1997) Glucose-induced microautophagy in *Pichia pastoris* requires the alpha-subunit of phosphofructokinase. *J. Cell Sci.* **110,** 1935–1945.