Peroxisomal Membrane Protein Pmp47 Is Essential in the Metabolism of Middle-chain Fatty Acid in Yeast Peroxisomes and Is Associated with Peroxisome Proliferation*

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Pmp47 of the methylotrophic yeast *Candida boidinii* belongs to a mitochondrial family of solute transporters and is localized in peroxisomal membranes. Its human homolog, Pmp34, is also known. In this study, we characterized the role of Pmp47 in fatty acid metabolism and peroxisome proliferation using the *pmp47*-deleted strain of *C. boidinii* (strain *pmp47Δ*). The wild-type strain grew well on a middle-chain fatty acid, laureate, as the single carbon source, and mild peroxisome proliferation was observed during its growth. The *pmp47Δ* strain could not grow on laureate but could grow on long-chain fatty acids including palmitate, myristate, and oleate. The levels of laureate oxidation activity in intact cells and in semi-permeabilized cells of strain *pmp47Δ* were lower than the respective level in the wild-type strain, although the level of laureate oxidation activity in the cell lysate and the level of lauroyl-CoA oxidation in semi-permeabilized cells of strain *pmp47Δ* were indistinguishable from the respective level in the wild-type strain. When lauroyl-CoA was provided in the cytosol of strain *pmp47Δ* through expression of *Saccharomyces cerevisiae* Faa2p (lauroyl-CoA synthetase) in which its peroxisome targeting signal was deleted, the growth of strain *pmp47Δ* on laureate was recovered to the level of growth of the wild-type strain. Laureate is converted to its CoA form in peroxisomes by the action of lauroyl-CoA synthetase. These results suggested that Pmp47 is involved in the transport of a small molecule (possibly ATP) required in the conversion of laureate to its CoA form in peroxisomes and that the absence of Pmp47 causes impairment of laureate metabolism, which results in the inability of *pmp47Δ* cells to grow on laureate. In addition, Pmp47 may be involved in peroxisome proliferation, because the *pmp47Δ* strain contained a reduced number of peroxisomes, as judged from the fluorescence analysis of cells expressing green fluorescent protein tagged with the peroxisome targeting signal 1 (GFP-AKL).

Peroxisome is a ubiquitous organelle in nearly all eukaryotic cells in which various kinds of oxidative metabolism occur through at least one H₂O₂-generating oxidase and an H₂O₂-decomposing enzyme, catalase (CTA)¹. Peroxisomal matrix enzymes are transported to peroxisomes by their cis-acting signal, i.e. peroxisome targeting signals (PTSs), PTS1, PTS2, and mPTS, and a subset of trans-acting proteins involved in peroxisomal protein transport called peroxins. Metabolism within the peroxisome requires not only proper enzyme localization but also proper transport (import and export) of various metabolites. The roles that various peroxisomal membrane proteins (PMPs) play in peroxisome assembly have begun to be elucidated; many peroxins, e.g. Pex1p, Pex3p, Pex10p, and Pex12p, are integral PMPs (1). On the other hand, knowledge regarding roles of PMPs in metabolic functions is limited. Biochemical experiments using purified peroxisomes have been difficult to perform because of the fragile nature of peroxisomes. Early studies led to the hypothesis that peroxisomal membranes are freely permeable to compounds of low molecular weight (2). However, some metabolites are unable to permeate the peroxisome membrane (3), and the peroxisomal ABC transporters, Pat1p/Pat2p, are involved in the transport of fatty acyl-CoA, which was revealed in genetic studies using *Saccharomyces cerevisiae* (4).

We have used the methylotrophic yeast *Candida boidinii* as the model organism to study the function of PMPs (5–8). The carbon source on which *C. boidinii* is grown induces the expression of a metabolic enzyme(s) that is specific to that particular carbon source; however, various peroxisome-inducing carbon sources induce the expression of two PMPs, Pmp47 and Pex11p (7, 9). These results suggest that Pmp47 and Pex11p are not metabolism-specific peroxisomal proteins but that they are general PMPs responsible for basic peroxisomal function. Also, both Pmp47 and Pex11p have putative homologs in mammalian cells (10–12). Pmp47 is a peroxisomal integral membrane protein that shows the highest similarity to a yeast mitochondrial ATP/ADP exchanger (13–15). Interestingly, the Pmp47 protein in the peroxisomal membrane is situated in the opposite orientation than that of the mitochondrial ATP/ADP exchanger, which suggests that the role of Pmp47 is to transport ATP from the cytosol into the peroxisome. In our previous study (6), we suggested that the low molecular weight compound transported by Pmp47 into the peroxisome is involved in

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¹ The abbreviations used are: CTA, catalase; MCFA(s), middle-chain fatty acid(s); LCFA(s), long-chain fatty acid(s); DHAS, dihydroxyacetone synthase; GFP, green fluorescent protein; MES, 2-(N-morpholinooxy)ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; PTS(s), peroxisome targeting signal(s); PMPs, peroxisomal membrane proteins; YPD, yeast extract/peptone/dextrose.
we investigated the metabolism of fatty acids of various lengths in the pmp47Δ cells to determine the role of Pmp47 in peroxisome function. In S. cerevisiae, laurate, a middle-chain fatty acid (MCLA), is converted to lauroyl-CoA in the peroxisome by lauroyl-CoA synthetase (Faa2p); on the other hand, long-chain fatty acids (LCFA)s, e.g. oleate, can be converted to their CoA form outside the peroxisome, and the CoA form is subsequently transported into the peroxisome by the ABC transporters, Pat1p/Pat2p (4). Therefore, if Pmp47 is involved in the transport of ATP from the cytosol into the peroxisome, the reaction catalyzed by lauroyl-CoA synthetase is expected to be inhibited in strain pmp47Δ. In this paper, we show both genetic and biochemical evidence that laurate metabolism in strain pmp47Δ is inhibited at the step in which laurate is converted to its CoA form. Furthermore, we found that the absence of Pmp47 affects peroxisome proliferation.

EXPERIMENTAL PROCEDURES

Microorganisms and Growth Conditions—C. boidinii wild-type strain GC (16), strain pmp47Δ (8), and strain pca5Δ (7) were grown on various fatty acids, and their growth was assayed. C. boidinii strain pmp47Δura3 was constructed as described previously (6, 17). Briefly, a pSP-derived URA3-cassette was inserted between the flanking sequences of pMP47, yielding the PMP47-disruption vector, pMP47SPr. pMP47SPr was introduced into strain TK62 (ura3) (18), and the inserted URA3 cassette was deleted by 5-fluoroorotic acid selection (17). This strain was named strain D47-U. Proper gene disruption was confirmed by Southern analysis. The green fluorescent protein (GFP) tagged with PTS1 (GFP-ALK) expression plasmid, pGFP-ALK, was introduced into strain D47-U, yielding strain GFP-ALK/D47 (7). Strain GFP-ALK/uvt (7) was used as the wild type for comparison of peroxisome morphology.

Synthetic MI medium was used as the culture medium in which C. boidinii was cultured (19). One or more of the following were used as the carbon source in each experiment: 1% glucose (w/v), 1% methanol (v/v), 0.5% lauric acid (v/v), 0.5% myristic acid (v/v), 0.5% palmitic acid (v/v), or 0.5% oleic acid (v/v). Tween 80 was added to the fatty acid medium at a concentration of 0.05% (v/v). The initial pH value of the medium was adjusted to 6.0. The complex yeast extract/potato medium containing 2% bactopeptide and 1% yeast extract (Difco) was also used as the basal medium in some experiments. YPcontains 2% glucose, yeast extract/potato/methanol/glycerol contains 0.5% methanol and 0.5% glycerol, and yeast extract/potato/oleic acid/Tween 80 contains 0.5% oleic acid and 0.05% Tween 80 as the carbon sources.

The C. boidinii were incubated aerobically at 28 °C under reciprocal shaking, and the growth of the yeast was followed by measuring the optical density at 610 nm (OD610). The experiment in which the pmp47Δ strain was grown on oleate was performed by transferring YPD-grown cells to oleate/MI medium at an OD610 of 0.5, and incubating the cells for 16 h. Escherichia coli DH5α (20) was routinely used for plasmid propagation.

Enzyme Assays—The level of acyl-CoA synthetase activity was determined by the method of Ichihara and Shibasaki (21). The amount of protein quantitation was performed using the method of Bradford (24).

Preparation of Spheroplasts and Organellar Fractionation—Oleate-induced cells were centrifuged at 1,000 × g at 30 °C for 10 min, and the cell pellet was resuspended in 20 ml of a solution of 100 mM Tris-HCl, pH 7.5, 50 mM EDTA and 0.9% b-mercaptoethanol, and incubated for 15 min at 30 °C. The cells were again centrifuged at 1,000 × g at 30 °C for 10 min and then washed once in 20 ml of a solution of 1 M sorbitol and 20 mM KH2PO4, pH 7.5, and resuspended in 20 ml of a solution of 1 M sorbitol and 20 mM KH2PO4, pH 7.5. Zymolyase 100T (Seikagaku Co., Tokyo, Japan) was added at 1 mg/150 OD610 units of cells. The cells were incubated for 60 min at 30 °C.

The spheroplasts were lysed according to the method of Goodman et al. (25). Briefly, the spheroplasts were harvested by centrifugation at 1,000 × g at 4 °C for 10 min, and the pelleted cells were suspended in a solution of 550 mM sucrose, 5 mM MgCl2, 2 mM KCN, 0.1 mM FAD, 1 mM NAD, 0.005% Triton X-100 (w/v), 1 milliunits/ml-acyl-CoA synthetase (Research Molecular Biological Chemicals), and 2 mM [1-14C]palmityl-CoA (60 mCi/mmol) (NEN Life Science Products) was added. The reaction was allowed to proceed for 10 min at 30 °C. The level of 14C-labeled acid-soluble radioactivity was determined as described above.

To measure the level of β-oxidation of laurate and palmitate in cell lysates, spheroplasts were centrifuged at 1,000 × g at 4 °C for 20 min, and the collected cells were lysed by the addition of 5 mM MOPS, pH 7.5, containing 0.1% Triton X-100 (w/v), 1 mM EDTA, and 1 mM PMSF. An aliquot of 25 µl (5–10 µg of protein) of cell extracts was mixed with 0.2 ml of a solution of 150 mM Tris-HCl, pH 8.5, 5.5 mM ATP, 5 mM MgCl2, 2 mM KCN, 0.1 mM FAD, 1 mM NAD, 1 mM CoA, 0.005% Triton X-100 (w/v), 1 milliunits/ml-acyl-CoA synthetase (Research Molecular Biological Chemicals), and 2 mM [1-14C]palmityl-CoA (60 mCi/mmol). The reaction was allowed to proceed for 10 min at 30 °C. The level of 14C-labeled acid-soluble radioactivity was determined as described above.

To measure the level of β-oxidation of laurate, lauroyl-CoA, palmitate, and palmitoyl-CoA in semi-permeabilized cells, 25 µl of spheroplasts (OD610 of 1.0–2.0) were incubated with 10 min with 25 µl of digoxigenin-11-oxime (0–200 µg/ml) in spheroplast buffer (1.4 M sorbitol and 20 mM potassium phosphate buffer, pH 7.5). Subsequently, 0.2 ml of spheroplast buffer containing 5 mM ATP, 5 mM MgCl2, 2 mM KCN, 0.1 mM FAD, 1 mM NAD, 1 mM CoA, and 0.2 nmol [1-14C]laurate, [1-14C]palmitate, [1-14C]lauroyl-CoA (55 mCi/mmol), or [1-14C]palmitoyl-CoA (60 mCi/mmol) (NEN Life Science Products) was added. The reaction was allowed to proceed for 10 min at 30 °C. The level of 14C-labeled acid-soluble radioactivity was determined as described above.

Preparation of [1-14C]Lauroyl-CoA—[1-14C]Lauroyl-CoA was synthesized as described previously by Yamashita et al. (27). Briefly, [1-14C]lauroyl chloride was prepared by incubating 70 µCi of [1-14C]laurate (55 mCi/mmol, Moravek Biochemicals) with oxaly chloride (2 ml) in spheroplast buffer (1.4 M sorbitol and 20 mM potassium phosphate buffer, pH 7.5). Digestion of the incubation, the pH value in the solution was maintained at 8 by the addition of NaOH. [1-14C]Lauroyl-CoA was purified by sequential chromatographies using Sep-Pak Plus C18 cartridges ( Waters Associates, Milford, MA) and thin-layer chromatography (Silica gel 60, Merck). The purity of [1-14C]Lauroyl-CoA was over 97%.

Expression of the S. cerevisiae FAA2ΔPTS1—The S. cerevisiae FAA2ΔPTS1 expression plasmid consisted of the C. boidinii PMP47...
Role of C. boidinii Pmp47 in β-Oxidation of Laureate

Pmp47 Is Necessary for the Growth of C. boidinii on Laureate but Is Not Necessary for Growth on Myristate or Palmitate—Wild-type C. boidinii grew well when grown on the three media containing laureate (C12:0), myristate (C14:0), or palmitate (C16:0), and PMP47 expression in the wild-type strain grown on each of the three media was confirmed (data not shown). During their growth, peroxisome proliferation was observed more in the cells cultured in laureate than in those cultured in glucose (Fig. 1, A and B). On the other hand, the pmp47Δ strain had no ability to grow on the medium where laureate was the sole carbon source (Fig. 1C), whereas the pmp47Δ strain showed growth comparable with that of the wild-type strain when grown on medium where myristate or palmitate was used as the sole carbon source. Expression of the PMP47 gene restored the ability of strain pmp47Δ to grow on laureate (data not shown). The pex5Δ strain that lacks the PTS1 receptor (Pex5p) could not grow on any of the fatty acids tested, confirming that normal protein transport to peroxisomes is essential for C. boidinii to grow on MCFA (laureate) and on LCFAs (myristate and palmitate). These results show that proper peroxisome assembly is necessary for C. boidinii to grow on MCFA and on LCFAs. Pmp47 is indispensable for C. boidinii to grow on MCFA. However, Pmp47 is not indispensable for C. boidinii to grow on LCFAs.

β-Oxidation of Fatty Acids in Intact Cells and Cell Lysates—The step in MCFA metabolism that strain pmp47Δ lacks was investigated by quantification of [1-14C]labeled β-oxidation products. In intact pmp47Δ cells and wild-type cells incubated with laureate or palmitate, the quantity of labeled acid-soluble degradation products derived from laureate and that derived from palmitate increased linearly up to 10 min in both strains. Thereafter, the level of laureate β-oxidation in the intact pmp47Δ cells decreased, and the amount of [1-14C]labeled β-oxidation products in the intact pmp47Δ cells was 34% of that in the intact wild-type cells, whereas the level of β-oxidation of palmitate in intact pmp47Δ cells remained essentially the same as that in the intact wild-type cells (Fig. 2A). Next, the level of β-oxidation of laureate and palmitate in the cell lysate of the pmp47Δ and wild-type strains in which the peroxisomes had been disrupted by adding 0.1% Triton X-100 was compared. In these experiments, the levels of β-oxidation of both laureate and palmitate in the pmp47Δ strain were slightly higher than the respective level in the wild-type strain (Fig. 2B). These results showed that Pmp47 is essential for laureate metabolism in intact cells but that the total activity of β-oxidation involved in laureate metabolism was unaffected by the absence of Pmp47.

β-Oxidation of Fatty Acids in Semi-permeabilized Cells—Next, the level of β-oxidation of laureate, lauroyl-CoA, palmitate, and palmitoyl-CoA in digitonin-permeabilized wild-type and pmp47Δ cells was measured using [14C]-labeled substrates. Under our experimental conditions, approximately 50% of the CTA activity was recovered in the cell pellet at a digitonin concentration of 25–50 μg/ml, and 100% release of CTA required 200 μg/ml of digitonin (data not shown). As shown in Fig. 3, the level of β-oxidation of laureate in pmp47Δ cells was markedly lower than that in the wild-type cells. On the other hand, the levels of β-oxidation of lauroyl-CoA, palmitate, and palmitoyl-CoA in pmp47Δ cells were essentially the same as the respective level in the wild-type. These biochemical results suggested that whereas the transport of lauroyl-CoA is not affected by the absence of Pmp47, the conversion of laureate to lauroyl-CoA is impaired in strain pmp47Δ.

Overexpression of ScFAA2 in the Cytosol Restored the Ability of Strain pmp47Δ to Grow on Laureate—In S. cerevisiae, Faa2p, the only acyl-CoA synthetase that catalyzes the conversion of laureate to lauroyl-CoA, is exclusively localized within peroxisomes, whereas the other acyl-CoA synthetases are not peroxisomal enzymes (4, 28). Next, we tried to induce the production of ScFaa2p in the cytosol of strain pmp47Δ to prove that the conversion of laureate to lauroyl-CoA is the only defective step that underlies the inability of strain pmp47Δ to grow on laureate. Expression of the ScFaa2 gene in pmp47Δ strain restored the ability of strain pmp47Δ to grow on laureate (Fig. 4).
grow on laureate. If the lauroyl-CoA produced in the cytosol of the constructed strain (strain D47-FAA2 PTS1) is transported into peroxisomes, the growth defect of pmp47 should be corrected.

The truncated form of FAA2, in which the PTS1 sequence, glutamate-lysine-leucine, was deleted from its carboxyl-terminal, was placed under the PMP47 promoter and introduced into C. boidinii strain D47-U. The level of lauroyl-CoA synthetase activity in the cell lysate of strain D47-FAA2 PTS1 (0.95 ± 0.12 units/mg protein) was considerably higher than that in the cell lysate of strain pmp47 (0.12 ± 0.08 units/mg protein). The cytosolic portion of lauroyl-CoA synthetase activity in strain D47-FAA2 PTS1 was 87%. These results confirmed that FAA2 PTS1 was expressed in the cytosol of this C. boidinii strain. Next, the ability of the FAA2PTS1 strain to grow on laureate was examined. Although strain pmp47 could not grow on laureate, the growth of strain D47-FAA2PTS1 on a laureate medium was comparable with that of the wild-type strain (Fig. 4). This genetic experiment confirmed the results of the biochemical experiments that, although strain pmp47 cannot metabolize laureate, it can metabolize lauroyl-CoA if lauroyl-CoA is provided in the cytosol.

Localization of Acyl-CoA Synthetases in C. boidinii in the Wild-type Strain and in the Pmp47Δ Strain—Our results indi-
cated that the reaction catalyzed by lauroyl-CoA synthetase is inhibited in strain pmp47D. Because the absence of Pmp47 induces aggregation of the inactive form of a peroxisomal matrix enzyme, DHAS, in the cytosol of methanol-induced cells (6), inhibition of the conversion of laureate to lauroyl-CoA in strain pmp47D may be due to inactivation of the lauroyl-CoA synthetase enzyme. The other possibility is that although lauroyl-CoA synthetase is active, the import of one of the substrates required in the enzyme reaction into the peroxisome is inhibited. If we could show that lauroyl-CoA synthetase is localized exclusively in the peroxisomes in the wild-type strain and that the enzyme is in the active form in the peroxisomes of strain pmp47D, the former possibility could be excluded.

First, the localization of lauroyl- and oleoyl (C18:1)-CoA synthetase activity was analyzed by subjecting each strain to differential centrifugation, which separated the cells into a cytosolic supernatant (S) and an organelle-pellet fraction (P) consisting mainly of peroxisomes and mitochondria (Fig. 5). In the wild-type cells, nearly 90% of the lauroyl-CoA synthetase activity was localized in the organelle-pellet fraction (Fig. 5A). On the other hand, more than half of the oleoyl-CoA synthetase activity was localized in the supernatant fraction (Fig. 5A). Isopionic centrifugation on a continuous Nycodenz gradient revealed that the intracellular localization of lauroyl-CoA synthetase activity coincided with the localization of peroxisomal CTA activity and was distinct from the localization of mitochondrial cytochrome c oxidase (Fig. 5B).

In strain pmp47D, a significant proportion of the intracellular lauroyl-CoA synthetase and CTA activities were in the organelle-pellet fraction (Fig. 5C). Again, lauroyl-CoA synthetase in the organelle-pellet fraction from strain pmp47D was fractionated further by Nycodenz equilibrium density gradient centrifugation. Top panel, lauroyl-CoA synthetase activity; middle panel, oleoyl-CoA synthetase activity; bottom panel, activity of marker enzymes. Closed circle, CTA; open circle, mitochondrial cytochrome c oxidase.

In the pex5D strain (7), more than 90% of each of lauroyl-CoA synthetase activity, oleoyl-CoA synthetase activity and peroxisomal CTA activity was found in the cytosolic supernatant (Fig. 5E). Under the same conditions, 55% of the thiolase activity (PTS2-enzyme) in the cells was pelletable, which indicates that the PTS2 enzyme is not impaired in strain pex5D. From these results, we hypothesize that Pmp47 functions as a transporter of some reaction substrate from the cytosol to the matrix of peroxisomes (presumably ATP; see “Discussion”) that is needed for lauroyl-CoA synthetase activity.

Effect of the Absence of Pmp47 on Peroxisome Assembly and Proliferation—To examine whether the general PTS1 pathway is impaired in strain pmp47D, the pGFP-AKL harboring GFP

![Fig. 5. Subcellular localization of lauroyl-CoA and oleoyl-CoA synthetase activity in C. boidinii strains. A and B, wild-type strain; C and D, strain pmp47D; E, strain pex5D. Each strain was grown on oleate synthetic medium; the cells were then gently lysed and fractionated into the organelle-pellet (P) and cytosolic supernatant (S) fractions. A, C, and E, the proportion of lauroyl-CoA synthetase (C12:0), oleoyl-CoA synthetase (C18:1), and catalase (CTA) activities in the cytosolic supernatant and in the organelle-pellet fractions. B and D, the organelle-pellet fraction from the wild-type strain (B) and the organelle-pellet fraction from strain pmp47D (D) were fractionated further by Nycodenz equilibrium density gradient centrifugation. Top panel, lauroyl-CoA synthetase activity; middle panel, oleoyl-CoA synthetase activity; bottom panel, activity of marker enzymes. Closed circle, CTA; open circle, mitochondrial cytochrome c oxidase.](http://www.jbc.org/)

Role of C. boidinii Pmp47 in β-Oxidation of Laureate

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Role of C. boidinii Pmp47 in β-Oxidation of Laureate

Regulation of peroxisomal membrane elongation by C. boidinii Pmp47

Fig. 6. Absence of Pmp47 reduces the number of peroxisomes formed. Fluorescent images of GFP-AKL (upper panels) and Nomarski images (lower panels). A, C, and E, strain GFP-AKL/wt. B, D, and F, strain GFP-AKL/pmp47Δ. A and B, cells were grown on oleate-medium. C and D, cells were grown on methanol plus glycerol. E and F, cells were grown on YPD. Complex YP medium was used as the basal medium.

D

and

strain GFP-AKL/D47.

As shown in Fig. 6, GFP-AKL expression in strain GFP-AKL/D47 (as the type strain and 1.46

membrane elongation in strain pmp47

6

the wild-type strain (7.28

D

wild-type strain (2.22

0.11 in the wild-type strain grown on palmitate was lower than that in the

strain grown on palmitate was lower than that in

strain (4), we speculated that Pmp47 is involved in the reaction in which laureate is converted to lauroyl-CoA.

Laureate β-oxidation in vivo was inhibited in strain pmp47Δ, although the level of β-oxidation activity in the cell lysate of the wild-type strain and that in the cell lysate of the pmp47Δ strain were similar (Fig. 2). However, when lauroyl-CoA was provided to semi-permeabilized cells, the level of β-oxidation in strain pmp47Δ recovered to that in the wild-type strain (Fig. 3). Also, our complementation experiments by overexpression of ScFAA2APTS1 indicated that laureate is converted to its CoA form in peroxisomes in vivo and that the absence of the conversion of laureate to lauroyl-CoA in strain pmp47Δ is responsible for the growth defect of this strain (Fig. 4). These biochemical and genetic results showed that Pmp47 plays an essential role in the conversion of laureate to lauroyl-CoA. Our data also suggested that in C. boidinii most of the laureate is not converted to its CoA form in the cytosol in vivo and that if, in fact, a small amount of laureate is converted to lauroyl-CoA in the cytosol, the resultant lauroyl-CoA would be oxidized with equal efficiency in the pmp47Δ and wild-type strains, but it would not be sufficient for the pmp47Δ strain to grow. Lauroyl-CoA could be transported into peroxisomes possibly by ABC-transporter family proteins (4).

The active form of lauroyl-CoA synthetase is present in the peroxisomes of strain pmp47Δ (Fig. 5D), and Pmp47 plays an essential role in the conversion of laureate to lauroyl-CoA. Furthermore, our genetic and biochemical data showed that Pmp47 plays an essential role in the conversion of laureate to lauroyl-CoA. Our data also suggested that in C. boidinii most of the laureate is not converted to its CoA form in the cytosol in vivo and that if, in fact, a small amount of laureate is converted to lauroyl-CoA in the cytosol, the resultant lauroyl-CoA would be oxidized with equal efficiency in the pmp47Δ and wild-type strains, but it would not be sufficient for the pmp47Δ strain to grow. Lauroyl-CoA could be transported into peroxisomes possibly by ABC-transporter family proteins (4).

The active form of lauroyl-CoA synthetase is present in the peroxisomes of strain pmp47Δ (Fig. 5D), and the enzyme activity is responsible for β-oxidation of laureate (Fig. 2B). Therefore, it seems that disruption of Pmp47 impairs the transport of a substrate that is required for synthesis of lauroyl-CoA in peroxisomes across the peroxisomal membrane. For the synthesis of lauroyl-CoA in peroxisomes, ATP, laurate, and CoA need to be transported into the peroxisomes.

We herein hypothesize that ATP is the most plausible candidate of the solute that is transported by the Pmp47 protein for the following reasons. 1) Pmp47 shows the highest sequence similarity to the yeast mitochondrial ATP/ADP exchanger, and its inverted topology against the cytosol fits with its function as

type of peroxisomal transporter seems to be conserved among these higher and lower eukaryotes. We previously reported that C. boidinii that lack Pmp47 cannot grow on methanol and lack DHAS enzyme activity (6). However, DHAS, an enzyme responsible for fixation of formaldehyde to cell constituents in the methylotrophic yeast (19), has not been shown to be present or play a role in mammalian peroxisomes. If a novel knock-out effect of the absence of Pmp47 in C. boidinii could be found under non-methylotrophic conditions, it might give new insight into the physiological and biochemical role of Pmp47. In addition, Pmp47 is more abundant in the peroxisome membranes of oleate-grown cells than in the peroxisome membranes of methanol-grown cells (7, 9). Taking these facts into consideration, we investigated the fatty acid metabolism in strain pmp47Δ and found that the pmp47Δ strain retained the ability to grow on LCFA but had lost the ability to grow on laurate, an MCF.

In yeasts, β-oxidation of all fatty acids takes place inside peroxisomes (29). The only known metabolic difference between the β-oxidation of laureate and that of palmitate is the intracellular site at which each substrate is converted to its CoA form by acyl-CoA synthetase. In S. cerevisiae, four isozymes of acyl-CoA synthetase have been identified in the genome project, and these isozymes differ in substrate specificity against specific fatty acids (4). Of the four isozymes, only Fa2p acts on laureate and is a peroxisomal enzyme. In this study, the intracellular site at which laureate activation occurs in C. boidinii was limited to peroxisomes (Fig. 5). Because the C. boidinii pmp47Δ strain has lost the ability to grow on laureate, similar to the S. cerevisiae faa2Δ strain (4), we speculated that Pmp47 is involved in the reaction in which laureate is converted to lauroyl-CoA.

Laureate β-oxidation in vivo was inhibited in strain pmp47Δ, although the level of β-oxidation activity in the cell lysate of the wild-type strain and that in the cell lysate of the pmp47Δ strain were similar (Fig. 2). However, when lauroyl-CoA was provided to semi-permeabilized cells, the level of β-oxidation in strain pmp47Δ recovered to that in the wild-type strain (Fig. 3). Also, our complementation experiments by overexpression of ScFAA2APTS1 indicated that laureate is converted to its CoA form in peroxisomes in vivo and that the absence of the conversion of laureate to lauroyl-CoA in strain pmp47Δ is responsible for the growth defect of this strain (Fig. 4). These biochemical and genetic results showed that Pmp47 plays an essential role in the conversion of laureate to lauroyl-CoA. Our data also suggested that in C. boidinii most of the laureate is not converted to its CoA form in the cytosol in vivo and that if, in fact, a small amount of laureate is converted to lauroyl-CoA in the cytosol, the resultant lauroyl-CoA would be oxidized with equal efficiency in the pmp47Δ and wild-type strains, but it would not be sufficient for the pmp47Δ strain to grow. Lauroyl-CoA could be transported into peroxisomes possibly by ABC-transporter family proteins (4).

The active form of lauroyl-CoA synthetase is present in the peroxisomes of strain pmp47Δ (Fig. 5D), and the enzyme activity is responsible for β-oxidation of laureate (Fig. 2B). Therefore, it seems that disruption of Pmp47 impairs the transport of a substrate that is required for synthesis of lauroyl-CoA in peroxisomes across the peroxisomal membrane. For the synthesis of lauroyl-CoA in peroxisomes, ATP, laurate, and CoA need to be transported into the peroxisomes.

We herein hypothesize that ATP is the most plausible candidate of the solute that is transported by the Pmp47 protein for the following reasons. 1) Pmp47 shows the highest sequence similarity to the yeast mitochondrial ATP/ADP exchanger, and its inverted topology against the cytosol fits with its function as
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an ATP transporter from the cytosol into the peroxisomes (13–15). 2) The reduced number of peroxisomes observed in strain pmp47Δ is considered to be due to some deficiency in organelle proliferation. Organelle proliferation is accompanied by membrane dynamics (e.g. membrane fusion and budding), and ATP might be required for proliferation of peroxisomes. 3) Previously, the solute transported by Pmp47, together with some protein factor, was suggested to be necessary for the folding of DHAS in peroxisomes (6). Protein-folding reactions often require energy in the form of ATP. A similar charged, small molecule-compound, NAD⁺, is impermeable to yeast peroxisomal membrane (3). Therefore, ATP may be impermeable, and an ATP transporter may exist in the peroxisomal membrane. 4) It is unlikely that Pmp47 is involved in the transport of either CoA or laurate for the following reasons: β-oxidation of fatty acids requires CoA as a substrate in thiolase-catalyzed reactions, and a continuous supply of CoA in peroxisomes is required for the β-oxidation reaction to proceed. If Pmp47 is a transporter of CoA, the level of β-oxidation activity of palmitate should be reduced in strain pmp47Δ. However, the level of palmitate oxidation in intact cells and the levels of lauroyl-CoA and palmitoyl-CoA oxidation in semi-permeabilized cells of the pmp47Δ strain did not differ from the respective level in the wild-type strain (Figs. 2 and 3). Membrane uptake of fatty acids could be mediated by two different mechanisms, passive diffusion or a protein-facilitated process. MCFAs and short-chain fatty acids, but not LCFA, are transferred rapidly across lipid membranes (30, 31). Laureate in the cis leaflet may easily diffuse (flip-flop) to the trans leaflet of the peroxisomal membrane and may be converted to lauroyl-CoA via the peroxisomal Faa2p. In the case of a protein-facilitated process, three fatty acid transporters, CD36 (32), plasma membrane fatty acid-binding protein (33), and fatty acid transport protein (34) have been identified, but there is no sequence similarity between each of these proteins and Pmp47. Furthermore, the pleiotropic effect of the absence of Pmp47 on organelle proliferation and protein folding that was observed in strain pmp47Δ cannot be explained by intraperoxisomal deficiency of either laurate or CoA.

Another important point addressed by the present study is that the absence of Pmp47 did not affect the transport of GFP-AKL but caused a reduction in the number of peroxisomes in the oleate-, palmitate-, and methanol-glycerol-grown cells. We think that this is because of direct involvement of a solute (ATP) in organelle proliferation and not to a secondary effect of the metabolic deficiency in strain pmp47Δ for the following reasons. 1) The growth of strain pmp47Δ was comparable with that of the wild-type strain under the examined conditions. 2) LCFA metabolism was not impaired in strain pmp47Δ. 3) The number of peroxisomes having PTS2 protein was not reduced in the pex5Δ strain, in which metabolism of melatonin and oleate is completely inhibited (35) (data not shown).

Because the absence of Pmp47 causes a defect in the β-oxidation pathway and in peroxisome proliferation, the recently described human homolog of Pmp47, HsPmp34, may be involved in one of the peroxisomal diseases (12). If branched-chain fatty acids and very long-chain fatty acids are converted to their CoA forms in peroxisomes by branched-chain acyl-CoA synthetase (36) and very long-chain acyl-CoA synthetase (37), respectively, a mutation in Pmp34 might result in impairment of metabolism of such fatty acids in peroxisomes.

In this study we found that Pmp47 is involved in the conversion of a MCFAs into its CoA form in peroxisomes and that Pmp47 is associated with peroxisome proliferation. This is the first step toward understanding the molecular function of Pmp47 and has shed light on a novel biological and physiologic-
Peroxisomal Membrane Protein Pmp47 Is Essential in the Metabolism of Middle-chain Fatty Acid in Yeast Peroxisomes and Is Associated with Peroxisome Proliferation

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