Peroxisomes are single membrane-bounded organelles that participate in several metabolic pathways, including plasmalogen synthesis, β-oxidation of very long chain fatty acids, β-oxidation of branched fatty acids, and H₂O₂ metabolism (1, 2). These peroxisomal functions are essential for human development. Indeed, several diseases are caused by defects in peroxisomal activity, either the impaired function of a peroxisomal enzyme or defects in peroxisome biogenesis (3). The physiological significance of plasmalogens is demonstrated by the human peroxisomal disorders such as Zellweger syndrome and rhizomelic chondrodysplasia punctata (4). Plasmalogens are enriched in the central nervous system (5), and the metabolic stability of plasmalogens varies between different regions of the brain. For instance, plasmalogens are relatively stable in myelin, whereas there is a dynamic pool of plasmalogens in gray matter that is rapidly metabolized and has a half-life of less than an hour (6). The levels of plasmalogens are reduced in several diseases, including sporadic Alzheimer disease and Pelizaeus-Merzbacher disease (7–9). Therefore, it is important to elucidate the molecular mechanisms that regulate the synthesis and degradation of plasmalogens.

Plasmalogens are synthesized via a seven-step reaction pathway that starts with the conversion of dihydroxyacetonephosphate (DHAP)² to 1-acyl-DHAP by peroxisomal matrix dihydroxyacetonephosphate acyltransferase. Then, the formation of an ether bond is catalyzed by another peroxisomal matrix protein, alkyl-dihydroxyacetonephosphate synthase (ADAPS), which replaces the acyl chain of 1-acyl-DHAP with a long-chain fatty alcohol. The peroxisomal enzyme fatty acyl-CoA reductase 1 (Far1) is essential for the formation of fatty alcohols (10). We demonstrated previously that the cellular level of plasmalogens modulates the stability of Far1 (11). In this study, we investigated the biogenesis and topology of Far1 and characterized the region of Far1 that is required for its plasmalogen-dependent degradation.

**EXPERIMENTAL PROCEDURES**

**Biochemicals**—Restriction enzymes and DNA-modifying enzymes were purchased from Nippon Gene (Tokyo, Japan) and Takara (Kyoto, Japan). Fetal bovine serum, DMEM, and Ham’s F-12 medium were from Invitrogen. Anti-human Far2 antibody was raised in rabbits by injection of DNA coding for human Far2 protein or 1-acyl-DHAP. Rabbit antibodies against human Far1, rat 70-kDa peroxisomal integral membrane protein (PMP70) (12), Pex3p (13), Pex14p (15), peroxisomal targeting signal type 1 (PTS1) (16), GFP (MBL, Nagoya, Japan), and malate dehydrogenase (17, 18); the goat antibody against lactate dehydrogenase (19).
dorgenase (Rockland, Gilbertsville, PA); and mouse antibodies against actin (Millipore, Billerica, MA), GFP (Santa Cruz Biotechnology, Inc.), FLAG (catalog no. M2, Sigma), and influenza virus HA (catalog no. 16B12, Covance) were used. Plasmeneylethanolamine purified from bovine brain was purchased from Doosan Serdary Research Laboratories (Kyungki-Do, South Korea). [14C]palmitate and [14C]palmitoyl-CoA were purchased from Moravek Biochemicals Inc. (Brea, CA). The solvent for TLC and N-ethylmaleimide (NEM) were purchased from Nakarai Tesque (Kyoto, Japan). Stealth TM siRNA (Invitrogen) was used to knock down human PEX19 in HeLa cells and FAR2 in MCF7 cells. The target sequences of the siRNAs are as follows: human PEX19, 5′-GCCAGTGGTAACAGTGCTGATCA-3′; human FAR2-61, 5′-GGGAAGGTTCCTGCGGCA-TAA-3′; human FAR2-62, 5′-GACCTTCTCGAGGCCCA-GTGCTAA-3′; and human FAR2-63, 5′-GAGCATTCAAG-CACGCTCAAGTTA-3′.

Cell Culture—MCF7 cells were purchased from RIKEN BRC Cell Bank (Tsukuba, Japan). MCF7 and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum in 5% CO2 and 95% air. CHO-K1 and ZPEG251 cells (19) were cultured in DMEM supplemented with 10% fetal bovine serum in 5% CO2 and 95% air (19, 20). CHO-K1 cells stably expressing FLAG-FAR1, FLAG-FAR1 490FAR2, and ZPEG251 cells stably expressing FLAG-FAR1-HA2 were isolated by selection with Zeocin TM (250 μg/ml). Plasmalogen levels in ZPEG251 cells were restored by adding 10 μM 1-O-hexadecylglycerol (HG) to the culture media every 24 h for the indicated period (19). Plasmalogen levels in CHO-K1 cells were increased by adding bovine brain plasmalogenethanolamine (37.5 μg/ml) or ethanolamine (2 μM).

Lipid Analysis—Plasmalogen biosynthesis was assessed by labeling cells with [14C]palmitate for 5 h. Then, cells were subjected to alkaline methanolysis to obtain 1-alkyl-GPE, 1-alkeyl-GPE, and 2-acyl-GPE were resolved on TLC plates (silica gel 60, Merck KgaA) using a chloroform/methanol/acetic acid solution (v/v/v, 65/25/10). In vitro Far enzyme activity was determined using [14C]palmitoyl-CoA as described (11).

Construction of FAR1—To construct pcDNAZeo3.1FLAG-FAR1-HA2, pcDNAZeo3.1FLAG-FAR1 (11) was digested with BglII and ApaI, and pcDNA3.1/Zeo. To generate FLAG-FAR1, a NotI site was introduced at the 5′ end of FLAG-FAR1 by PCR using the KpnI-Fw and SpeI primers and FLAG-FAR2-HA2, a SpeI site was introduced at the 3′ end of FLAG-FAR2 by PCR using the FL-Fw and XhoI primers. A DNA fragment encoding amino acids 508–515 of Far1 was fused to EGFP using the CMV.Fw (5′-cgcaaatggccggttcg-3′) and EGFP-C8-ApaI-Rv (5′-ccggccccatctagcatctggctcg-3′) primers. The PCR product was digested with NheI and ApaI and was ligated between the Nhel and Apal sites of pcEGFP-C1.
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**FLAG-FAR1$_{507}$FAR2**—A DNA fragment encoding the eight amino acids at the C terminus of Far2 was amplified by PCR using the CMV.Fw and Far1/507-FAR2/508.Rv (5’-taggccccac-ttgagccattctggagagacgctgctgcttggc-3’) primers. The product was digested with PstI and ApaI and was ligated between the PstI and ApaI sites of pcDNA3.1/Zeo/FLAG-FAR1.

**FLAG-FAR1FAR2$_{491}$**—To generate FLAG-FAR1FAR2$_{491}$-507, a DNA fragment encoding amino acids 491–507 of Far2 and the eight amino acids at the C terminus of Far1 was amplified by PCR using the Far2/491/507/Far1.Rv (5’-gggggccctcgtac-tctagctgtctgcttgaagagag-3’) and CMV.Fw primers and FLAG-FAR1$_{496}$FAR2$_{2}$ as the template. The product was digested with BspEI and ApaI, and FLAG-FAR1 was digested with Nhel and BspEI. These fragments were ligated between the Nhel and Apal sites of pcDNA3.1/Zeo.

**FLAG-FAR2FAR1$_{491}$**—In amino acids 491–507, only the residues at positions 492, 494, 496, and 499 differ between Far1 and Far2. These four amino acids in Far2 were mutated to the corresponding residues of Far1. A DNA fragment encoding amino acids 491–507 of Far1 was amplified by PCR using the Far2/491/507/Far1.Rv (5’-caagatctcagatggctcggaatatctggacttcgtggtaagcctgtgg-3’) and BHG.Rv primers and pcDNA3.1/Zeo/FL-FLAG-FAR1$^{507}$ as the template. In a second PCR, this fragment and Far2/354Far1/355.Fw (5’-ctacagtctcagatggctcggaatatcttggacttcgtggataatctc-3’) and BHG.Rv primers and FLAG-FAR1$_{507}$FAR2$_{2}$ as the template. The product was digested with ScaI and ApaI, and FLAG-FAR1$_{496}$FAR2$_{2}$ was digested with EcoRI and ApaI and was ligated between the EcoRI and ApaI sites of pcDNA3.1/Zeo.

**FLAG-FAR2FAR1$_{355}$**—To generate FLAG-FAR1$_{355}$FAR2, a DNA fragment encoding amino acids 350–354 of Far2 and 161 amino acids of FLAG-FAR1$_{507}$FAR2 was amplified by PCR using the Far2/354Far1/355.Fw (5’-tcagatctcagatggctcggaatatctttggacttcgtggataatctc-3’) and BHG.Rv primers and FLAG-FAR1$_{507}$FAR2$_{2}$ as the template. The product was digested with ScaI and ApaI, and pcDNA3.1/Zeo/FLAG-FAR2 was digested with EcoRI and BamHI. These fragments were ligated between the EcoRI and XhoI sites of pcDNA3.1/Zeo.

**FLAG-FAR2FAR1$_{286}$**—To generate FLAG-FAR$_{286}$FAR1$_{507}$, a DNA fragment encoding 230 amino acids of FLAG-Far1$_{507}$Far2 was amplified by PCR using the Far1507FAR2 (5’-gtagatctcagatggctcggaatatcttcgtaagagag-3’) and CMV.Fw and pcDNA3.1/Zeo/FLAG-FAR2FAR1$_{491}$/507 as a template. The product was digested with EcoRI and XhoI and ligated between the EcoRI and XhoI sites of pcDNA3.1/Zeo.

**FLAG-FAR2FAR1$_{491}$**—The three amino acids from the C terminus of FLAG-FAR2FAR1$_{491}$/507 was mutated to the corresponding residues of Far1. A DNA fragment encoding amino acids of FLAG-FAR2FAR1$_{491}$/507 was amplified by PCR using the primers Far1507Rv Xho (5’-cctagatctcagatggctcggaatatcttcgtaagagag-3’) and CMV.Fw and pcDNA3.1/Zeo/FLAG-FAR2FAR1$_{491}$/507 as a template. This fragment and FLAG-FAR2FAR1$_{491}$/507 was digested with EcoRI and ApaI and ligated between the EcoRI and XhoI sites of pcDNA3.1/Zeo.

**Biochemical Analysis**—Subcellular fractionation and carbonate extraction were performed as described previously (22). For proteinase K treatment, postnuclear supernatant (PNS) was prepared in the absence of protease inhibitors, and 20 µg of PNS was digested with proteinase K (1 µg) on ice for 30 min in a 200-µl reaction volume. Tryptic digestion was performed likewise.

**In vitro transcription/translation reactions in a rabbit reticulocyte-lysate protein-synthesizing system were performed using the quick-coupled transcription/translation system (TNT T7, Promega) according to the instructions of the manufacturer.

**RESULTS**

**Far1 Is a Peroxisomal Integral Membrane Protein**—We demonstrated previously that endogenous Far1 is localized to peroxisomes in ZPEG251 cells, in which plasmalogen synthesis is defective (11). In this study, we used sodium carbonate extraction to analyze the integrity of Far1 in ZPEG251 and CHO-K1 cells (Fig. 1B). Subcellular fractionation demonstrated that Far1 was exclusively recovered in the membrane fraction (Fig. 1A). Far1 was not extracted by sodium carbonate treatment and was recovered in the membrane fractions of ZPEG251 and CHO-K1 cells. Pex3p, a peroxisomal membrane peroxin, was similarly present in the membrane pellet, whereas catalase, a peroxisomal matrix protein, was recovered in the soluble fraction (Fig. 1B). These results indicate that Far1 is an integral peroxisomal membrane protein.

We next analyzed the membrane topology of Far1 in CHO-K1 cells by expressing FLAG-Far1-HA$_{2}$, which encodes both N- and C-terminally epitope-tagged Far1. The accumulation of hexadecanol was evident when FLAG-Far1-HA$_{2}$ was expressed in ZPEG251 cells (Fig. 1C, lanes 3 and 5), and this accumulation was reduced when plasmalogen levels were restored by the addition of HG (lane 2). This implies that FLAG-Far1-HA$_{2}$ is functional. CHO-K1 cells expressing FLAG-Far1-HA$_{2}$ were permeabilized with Triton X-100 and colabeled...
with anti-FLAG and anti-HA antibodies or anti-FLAG and anti-PTS1 antibodies. Colocalization indicated that FLAG-Far1-HA2 was localized in peroxisomes (Fig. 1D, a–d). When CHO-K1 cells expressing FLAG-Far1-HA2 were permeabilized with 25 μg/ml digitonin under conditions in which plasma membranes are selectively permeabilized and intraperoxisomal proteins are inaccessible to exogenous antibodies (25, 26), labeling with an anti-FLAG antibody was punctate (Fig. 1D, e and g), whereas labeling with anti-HA and anti-PTS1 antibodies generated no signal (h and j). FLAG-Far1-HA2 showed the same membrane topology in ZPEG251 and CHO-K1 cells (data not shown).

We further assessed the topology of endogenous Far1. When a PNS fraction prepared from ZPEG251 cells was treated with proteinase K, Far1 was resistant to protease treatment, whereas it was completely digested in the presence of NEM (Fig. 1E, lanes 2 and 5). NEM treatment inactivated Far1 (Fig. 1F), as demonstrated previously (27). Interestingly, Far1 migrated slightly slower in NEM-treated cells than in untreated cells (Fig. 1E, lanes 3 and 4). We speculate that this indicates that a catalytic domain of Far1 is tightly packed and that the structure of this domain is modified by NEM treatment so that it becomes accessible to proteases. Taken together, these results suggest that the C terminus of Far1 is exposed to the peroxisome matrix, whereas a large catalytic domain in its N terminus is located outside of peroxisomes.

Far1 contains 515 amino acids. The NADPH-binding domain corresponds to amino acids 15–285. Hydropathy analysis according to a Doolittle and Kyte plot predicted that the transmembrane segment of Far1 is located in its C terminus region (amino acids 466–483). To assess the topogenesis of Far1, we examined whether several deletion mutants of Far1 localize to peroxisomes (Fig. 2A). FLAG-Far1Δ507, which lacks the final eight amino acids at the C terminus, was resistant to carbonate extraction (supplemental Fig. S1) and localized to peroxisomes, as assessed by colocalization with the peroxisomal membrane protein Pex14p (Fig. 2B, c and d). By contrast, FLAG-Far1Δ90 and FLAG-Far1Δ467 were localized in the mitochondrion and cytosol, respectively (Fig. 2B). These results suggest that the C terminus of Far1 is important for its peroxisomal localization.
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**A**

| Protein         | Localization | Efficiency |
|-----------------|--------------|------------|
| Flag-Far1       | NADPH        | Ps 90±5    |
| Flag-Far1_507   | NADPH        | Ps 86±5    |
| Flag-Far1_490   | NADPH        | Mt 94±2    |
| Flag-Far1_467   | NADPH        | Cyt 100    |

B

**CHO-K1**

- αFlag
- a Pex14p
- c Pex14p
- d Pex14p
- h MDH
- g Pex14p

C

**CHO-K1**

- EGFP-Far1_451-515
- EGFP-Far1_469-515
- EGFP-Far1_490-515
- EGFP-Far1_508-515

**FIGURE 2.** C-terminal portion of Far1 is important for its peroxisome localization. A, Far1 deletion mutant constructs. NADPH indicates the predicted NADPH-binding domain of Far1, and the hatched box indicates the predicted transmembrane domain of Far1. The intracellular localizations of these constructs are indicated in the center column. Ps, peroxisomes; Mt, mitochondria; Cyt, cytosol. The numbers in the right column show the percentages of cells showing the organelles targeted by the respective Far1 truncated mutants. Data were collected by counting more than 100 cells expressing Far1 variants from three independent experiments and are represented by the mean ± S.D. B, intracellular localizations of these Far1 mutants were examined in CHO-K1 cells. Cells were stained with antibodies against Flag (a, c, e, and g), Pex14p (b, d, and h), and malate dehydrogenase (i). Scale bar = 5 μm.

**FIGURE 3.** The C-terminal portion of Far1 is sufficient for its peroxisome localization. A, schematic representation of the EGFP fusion proteins used to search for the minimum region of Far1 that is required for the localization of this protein to peroxisomes. The peroxisomal targeting activity of each fusion protein is represented by percentage. Data were collected by counting more than 100 cells expressing EGFP fused Far1 mutants from three independent experiments and are represented by the mean ± S.D. Note that EGFP-Far1_469-515 was localized to both peroxisomes and mitochondria. B, each construct was expressed in CHO-K1 cells for 14 h and detected by monitoring GFP fluorescence. 

Localization. We further assessed the minimum region required for the peroxisomal targeting of Far1 using EGFP as a reporter protein. The 65 and 47 amino acids at the C terminus of Far1 were attached to the C terminus of EGFP to generate EGFP-Far1_451-515 and EGFP-Far1_469-515, respectively. These fusion proteins were localized to peroxisomes, suggesting that Far1 associates with peroxisomes via its C-terminal region (Fig. 3B). Further deletion of the predicted hydrophobic region of Far1 abrogated its peroxisomal localization, as demonstrated by analysis of the EGFP-Far1_490-515 and EGFP-Far1_508-515 fusion proteins. By contrast, EGFP-Far1_451-507 was localized to peroxisomes in a manner resistant to carbonate extraction (Fig. 3, B and C), consistent with the results that indicated that the final eight amino acids at the C terminus of Far1 are not essential for its peroxisomal localization (Fig. 2). EGFP-Far1_469-507 contains the predicted transmembrane domain and 22 amino acids of the luminal domain of Far1. This protein less efficiently bound...
to Pex19p and localized to peroxisomes and mitochondria, suggesting that amino acids 451–468 are required for the efficient binding to Pex19p. These results indicate that amino acids 451–507 in the C-terminal region of Far1 are sufficient for the targeting and integration of Far1 into peroxisomal membranes, which strongly implies that Far1 is a peroxisomal tail-anchored protein.

**Far1 Is Localized to Peroxisomes in a Pex19p-dependent Manner**—Mammalian Pex26p is a peroxisomal tail-anchored protein, and Pex19p is required for the correct targeting of this protein (28). To assess whether Far1 is localized to peroxisomes in a Pex19p-dependent manner, we first analyzed the interaction between Pex19p and EGFP-tagged Far1 proteins. HA-Pex19p and various EGFP-Far1 fusion proteins were expressed in pex19 ZP119 cells, and these fusion proteins were immunoprecipitated using an anti-GFP antibody. Pex19p was coimmunoprecipitated with EGFP-tagged Far1 fusion proteins that localized to peroxisomes, strongly suggesting that Far1 is transported to peroxisomes in a Pex19p-dependent manner (Fig. 4A). We further verified the requirement of Pex19p for the peroxisomal localization of Far1 by reducing Pex19p expression in HeLa cells and by using the PEX19-deficient CHO cell line pex19 ZP119 (24). Expression of Pex19p was efficiently reduced in HeLa cells by transfection of dsRNA against PEX19 (Fig. 4C). When HA-Pex12p and EGFP-Far1 were coexpressed in HeLa cells, both proteins colocalized with labeling of an anti-PTS1 antibody, indicating that they localized to peroxisomes (Fig. 4B, a–c). By contrast, HA-Pex12p had a mitochondrion-like localization in HeLa cells treated with dsRNA against PEX19, indicating that expression of Pex19p was efficiently reduced in these cells. EGFP-Far1 was also not targeted to peroxisomes in these cells, despite peroxisomes remaining intact as indicated by the punctate labeling of the anti-PTS1 antibody. Scale bar = 5 μm. C, depletion of Pex19p expression was confirmed by immunoblotting. Actin was used as a loading control.
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**A** adaps ZPEG251
Flag-Far1  Na2CO3
Flag-Far2
αFlag
αPex3p
αCatalase

**B** CHO-K1/Flag-Far2-HA3
Flag
TX-100
Digitonin

**C** MCF7
PلسEtn
False-Far2 SHORT exp.
False-Far2 long exp.
αPex14p
αactin
2-acyl-GPE-

**D** MCF7
Flag-Far1
Flag-Far2
HG
αFlag
αPex14p
αactin
2-acyl-GPE-

**E** CHO-K1
1 Flag-Far1
1 15
286 406 515
NADPH
*50±2* +

2 Flag-Far2
1 15
100±10 n.d.

3 Flag-Far1490Far2
1
100±10 n.d.

4 Flag-Far1490Far2
1
NADPH
90±3 +

5 Flag-Far1507Far2
1
NADPH
*60±10* +

6 Flag-Far1507Far2
1
NADPH
100±20 n.d.

7 Flag-Far2Far491507
1
NADPH
100±15 n.d.

8 Flag-Far2Far491507
1
NADPH
100±15 n.d.

9 Flag-Far2Far166515
1
100±20 n.d.

10 Flag-Far2Far1355507
1
NADPH
100±10 n.d.

11 Flag-Far2Far1286507
1
286 507 515
NADPH
90±2 n.d.

FIGURE 5. The transmembrane-flanking region of Far1 is required for its plasmalogen-dependent degradation. A, FLAG-tagged Far1 and Far2 were expressed in ZPEG251 cells, and their membrane insertion was assessed by carbonate extraction as described in Fig. 1B. P and S are membrane pellet and soluble fractions, respectively. B, the membrane topology of FLAG-Far2-HA3 in CHO-K1 cells was assessed by differential membrane permeabilization with digitonin as described in Fig. 1D. Scale bar = 5 μm. C, MCF7 cells were cultured for 2 days in the presence (+) or absence (−) of plasmalogenethanolamine (PясEtn). The expression levels of endogenous Far1 (solid arrowhead) and Far2 (open arrowhead), Pex14p, and actin were assessed by immunoblotting with antibodies to Far2, Pex14p, and actin, respectively. Note that anti-Far2 antibody recognized both Far1 and Far2. An in vitro-translated Far2 was loaded in the same SDS-PAGE. The same blot for Far1 and Far2 at a longer exposure is also shown (second panel). The plasmalogen level was detected as 2-acyl-GPE and is shown in the fifth panel. Dots indicate a nonspecific band. D, FLAG-Far1 and FLAG-Far2 were transfected to MCF7 cells. Cells were divided into two dishes and cultured for 2 days in the presence (+) or absence (−) of HG. Proteins were detected by Western blotting with the antibodies indicated on the left. FLAG-tagged Far proteins were quantified by a LAS-4000 mini lumino image analyzer. Relative expression levels of FLAG-Far1 and FLAG-Far2 are shown, where the levels of respective Far proteins in untreated MCF7 cells were designated as 100. Scale bars represent the mean ± S.D. of three experiments. *, p < 0.05; Student’s t test versus untreated MCF7 cells. E, schematic of Far1 and Far2 chimeric fusion proteins. Plasmalogen-dependent degradation and enzyme activity of these fusion proteins are summarized on the right. n.d., not done. The relative expression levels of Far1/Far2 chimera proteins were determined, where the expression levels of respective Far1/Far2 chimera proteins in plasmalogen-deficient ZPEG251 cells were designated as 100. Numbers represent the mean ± S.D. of three experiments. *, p < 0.05; Student’s t test versus untreated ZPEG251. F, ZPEG251 cells were transfected with FLAG-Far1, FLAG-Far2, or the chimeric fusion proteins and then cultured in the presence (+) or absence (−) of HG for 48 h. The levels of each of the fusion proteins, PMP70, and actin were assessed by labeling with their corresponding antibodies. The plasmalogen level is shown in the bottom panel. G, PNS prepared from ZPEG251 cells expressing FLAG-Far1 or FLAG-Far1490Far2 was digested with trypsin. The digestion was terminated by the addition of trichloroacetic acid, and the protease sensitivities of FLAG-Far1 and FLAG-Far1490Far2 were assessed by immunoblotting with antibodies against Far1 and Pex14p. Solid and open arrowheads indicate FLAG-tagged and endogenous Far proteins, respectively.

**Far1 Is Specifically Degraded in Response to Cellular Plasmalogen Levels**—We demonstrated previously that the stability of Far1 is regulated in response to plasmalogen levels (11). The amino acid sequence of human Far2 has 59% identity and 78% similarity to that of human Far1. Far1 and Far2 both localize to peroxisomes and catalyze fatty alcohol formation. The substrate specificities of the proteins slightly differ because Far2 only catalyzes the formation of saturated C16 and C18 fatty
lamine, whereas the expression level of Far2 was not altered significantly (Fig. 5C). When FLAG-tagged Far2 was expressed in MCF7, FLAG-Far2 was relatively more stable than FLAG-Far1 upon elevating the plasmalogen level by supplementing with HG (Fig. 5D). Taken together, these results suggest that Far1 stability is more dynamically regulated than Far2 in MCF7 cells.

To elucidate the molecular mechanism underlying plasmalogen-dependent degradation of Far1, we took advantage of the similarities in the domain structures of Far1 and Far2 and constructed several chimeric proteins (Fig. 5E). In FLAG-Far1466Far2 and FLAG-Far1490Far2, the 49 and 25 amino acids at the C terminus of Far1 were replaced with the corresponding region of Far2, respectively. Both chimeric proteins were efficiently targeted to peroxisomes (supplemental Fig. S3). Neither chimeric protein was degraded in ZPEG251 cells when plasmalogen levels were restored. This suggests that the C-terminal region of Far1 that is exposed to the peroxisome matrix is required for plasmalogen-dependent degradation of Far1 (Fig. 5F).

Next, the C-terminal region of Far1 was divided into two parts, and these fragments were replaced with the corresponding fragments of Far2 (Fig. 5F). Substitution of the final eight amino acids of Far1 with those of Far2 did not abrogate plasmalogen-dependent degradation of Far1. By contrast, degradation of FLAG-Far1Far2491/507, in which 17 amino acids in the transmembrane-flanking region of Far1 were replaced with the corresponding residues of Far2, was not stimulated in ZPEG251 cells when plasmalogen levels were restored. This indicates that these 17 amino acids in the transmembrane-flanking region of Far1 are necessary for plasmalogen-dependent degradation of Far1.

Finally, we investigated whether these 17 amino acids in the transmembrane-flanking region of Far1 are sufficient for the plasmalogen-dependent degradation of this protein. To this end, the transmembrane-flanking region of Far2 was replaced with the corresponding region of Far1 to generate FLAG-Far2Far1491/507. This protein was efficiently targeted to peroxisomes. However, its expression level was not reduced when plasmalogen levels were restored in ZPEG251 cells. This indicates that amino acids 491–507 in the transmembrane-flanking region of Far1 are necessary but not sufficient for the degradation of Far1 in response to plasmalogen levels. In addition, FLAG-Far2Far1491/515 and FLAG-Far2Far1466/515 were not degraded, suggesting that the C-terminal 8 amino acids of Far1 do not influence its plasmalogen-dependent degradation. Moreover, FLAG-Far2Far1355/507 and FLAG-Far2Far1285/507 were not degraded in a plasmalogen-dependent manner. Together, these data indicate that almost the entire sequence of Far1 is needed for its plasmalogen-dependent degradation.

These results led us to examine whether replacement of the transmembrane-flanking region of Far1 induces a conformational change in the catalytic domain of the protein that abrogates plasmalogen-dependent degradation of mutant Far1 proteins. To address this issue, PNS fractions prepared from ZPEG251 cells expressing FLAG-Far1 and FLAG-Far1490Far2 were treated with trypsin (Fig. 5G). FLAG-Far1 was largely resistant to trypsin digestion and was partially digested upon incubation with a large amount of trypsin. Pex14p was effi-

![Figure 5—continued](image-url)
Far1 was sensitive to proteinase K in the presence of NEM. Far1 has six Cys residues, and the Cys residue at position 500, which is conserved in several species, including human, rat, and mouse, is located on the peroxisomal matrix side. This suggests that at least one Cys residue in the catalytic domain of Far1 remains accessible to NEM modification. Notably, Far activity is abolished by sulphydryl reagents, and the active site of the enzyme was suggested to contain an essential thiol residue (27).

The mammalian proteins Pex26p, Far1, mitochondrial fission protein 1 (Fis1), and mitochondrial fission factor are peroxisomal tail-anchored proteins, and Fis1 and mitochondrial fission factor localize to both mitochondria and peroxisomes (28, 32–34). Of these proteins, the peroxisomal localizations of Pex26p and Fis1 are mediated by Pex19p. Pex26p has two neighboring Pex19p-binding sites in its transmembrane domain and luminal C terminus. The luminal Pex19p-binding site is essential for correct targeting of full-length Pex26p to peroxisomes (28). By contrast, the Pex19p-binding site of Fis1 is located in the region of the C terminus that contains the transmembrane domain (33). Common immunoprecipitation of Far1 with Pex19p revealed that the Pex19p-binding site of Far1 overlaps with its transmembrane domain. Consistent with our experimental findings, a potential Pex19p-binding site of Far1 is predicted in its transmembrane domain by mPTS Predictor (35). Positively charged residues in the transmembrane domain that flanks the C terminus were suggested recently to be important for Pex19p-mediated peroxisome localization of tail-anchored proteins (36). Far1 has five basic amino acids in its C-terminal luminal region. Interestingly, FLAG-Far1_{490p} which has arginine residues at positions 485 and 490, interacted with Pex19p but was mislocalized to mitochondria in a carbonate-resistant manner (supplemental Fig. S1). This suggests that the luminal region of Far1 might participate in the integration of Far1 into peroxisomal membranes.

In this study, we showed that the N terminus and C terminus of Far2 are oriented toward the cytosolic and luminal faces of peroxisomes, respectively (Fig. 5B). The C-terminal region of Far2 is sufficient for its peroxisomal localization, as demonstrated by experiments with FLAG-Far1_{490p}Far2. In this construct, the C-terminal region of Far2, including the transmembrane domain, was fused to a Far1 mutant lacking the region necessary for peroxisomal localization. Together with the results of these experiments, the similarities in the domain structures of Far1 and Far2 strongly suggest that Far2 is also a peroxisomal C-tail-anchored protein.

### Regulation of Far1 Expression Levels

Two Far isozymes, Far1 and Far2, localize to peroxisomes and synthesize long-chain fatty alcohols by reducing fatty acyl-CoA with slightly different substrate specificities (10). Interestingly, we showed that the stability of Far1, but not Far2, is regulated in response to the level of plasmalogens in CHO-K1 cells and that the transmembrane-flanking region of Far1 is necessary for this plasmalogen-dependent degradation. Together with the topological analysis of Far1, these observations suggest that peroxisomal membrane or matrix proteins participate in the regulation of Far1 stability. However, we suspect that it is more likely that a peroxisomal membrane protein is involved because Far1 was efficiently degraded when plasmalogen levels were restored in...
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