THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 12, pp. 8537–8542, March 19, 2010

Posttranslational Regulation of Fatty Acyl-CoA Reductase 1, Far1, Controls Ether Glycerophospholipid Synthesis*[S]◆

Received for publication, November 9, 2009, and in revised form, January 8, 2010 Published, JBC Papers in Press, January 13, 2010, DOI 10.1074/jbc.M109.083311

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Plasmalogens are a major subclass of ethanolamine and choline glycerophospholipids in which a long chain fatty alcohol is attached at the *sn*-1 position through a vinyl ether bond. This ether-linked alkyl bond is formed in peroxisomes by replacement of a fatty acyl chain in the intermediate 1-acyl-dihydroxyacetone phosphate with a fatty alcohol in a reaction catalyzed by alkyl dihydroxyacetone phosphate synthase. Here, we demonstrate that the enzyme fatty acyl-CoA reductase 1 (Far1) supplies the fatty alcohols used in the formation of ether-linked alkyl bonds. Far1 activity is elevated in plasmalogen-deficient cells, and conversely, the levels of this enzyme are restored to normal upon plasmalogen supplementation. Down-regulation of Far1 activity in response to plasmalogens is achieved by increasing the rate of degradation of peroxisomal Far1 protein. Supplementation of normal cells with ethanolamine and 1-*O*-hexadecylglycerol, which are intermediates in plasmalogen biosynthesis, accelerates degradation of Far1. Taken together, our results indicate that ether lipid biosynthesis in mammalian cells is regulated by a negative feedback mechanism that senses cellular plasmalogen levels and appropriately increases or decreases Far1.

Plasmalogens are a major subclass of choline and ethanolamine glycerophospholipids that link a long chain fatty alcohol at the *sn*-1 position by a vinyl ether bond. Plasmalogen synthesis is accomplished via a seven-step reaction pathway (1), which is initiated by the peroxisomal matrix protein, dihydroxyacetone phosphate acyltransferase (DHAPAT)[2] (2). Formation of the ether bond is catalyzed by another peroxisomal matrix enzyme, alkyl dihydroxyacetone phosphate synthase (ADAPS), which replaces the acyl chain of 1-acyl-DHAP with a long chain fatty alcohol (1, 3). In animal cells, fatty alcohols are primarily produced by reduction of the corresponding fatty acyl-CoAs (4) and are utilized as substrates for the synthesis of ether lipids and wax esters. Fatty acyl-CoA reductase (Far) converts a fatty acyl-CoA into a fatty alcohol and CoA-SH and is enriched in subcellular fractions containing catalase, a peroxisomal marker enzyme (4, 5). Tissues such as the brain and intestine mucosa synthesize large amounts of plasmalogens, and correspondingly, contain abundant Far activity. In contrast, Far activity is low in liver, which contains little ether lipid (6). Far activity is increased 4–5-fold in plasmalogen-deficient fibroblasts from patients with rhizomelic chondrodysplasia punctata (RCDP), apparently resulting in accumulation of fatty alcohol (7).

Recently, *FAR1* and *FAR2* cDNAs were isolated from human and mouse (8). Far1 prefers saturated and unsaturated fatty acyl-CoAs of 16–18 carbon atoms as substrates, consistent with the composition of fatty alcohols in the *sn*-1 position of plasmalogens, whereas Far2 prefers saturated fatty acyl-CoA substrates of 16–18 carbons. These studies and those mentioned above suggest that Far1 is responsible for supplying fatty alcohols for ether bond formation and that the activity of this enzyme may be up-regulated in the absence of plasmalogens. If so, Far1 activity may be down-regulated in the presence of plasmalogens.

In the present study, we investigate the expression of *FAR1* in plasmalogen-deficient and plasmalogen-replete cells. We show that Far1 is regulated by modulating the turnover of the enzyme in a plasmalogen-dependent fashion.

EXPERIMENTAL PROCEDURES

Lipid Analysis—Cells were metabolically labeled with [^{14}C]palmitate as described in the supplemental Experimental Procedures. For the alkaline methanolysis, lipids were extracted from cells by the Bligh and Dyer method (9), dissolved in 0.5 M sodium methoxide in anhydrous methanol, and incubated at 50 °C for 20 min. After neutralization with acetic acid, lipids were re-extracted by the Bligh and Dyer method (9). To detect plasmenylethanolamine (PlsEtn) by one-dimensional TLC, PlsEtn was converted to either 2-acyl-glycerophosphoethanolamine (GPE) by trichloroacetic acid treatment (3) or 1-alkenyl-GPE by alkaline methanolysis.

siRNA-mediated Knockdown of FAR1—FAR1 knockdown in HeLa cells was performed using Stealth™ siRNA. The primers used were: human FAR1 siRNA 05 (target sequence, 5′-CCACTTTCAAGAGGAATCCTCTCGA-3′), human FAR1 siRNA 06 (target sequence, 5′-GAGATGCTGTTCAGTTAAATGTGAT-3′), human FAR1 siRNA 07 (target sequence, 5′-GGCA-

* This work was supported in part by a CREST grant from the Science and Technology Agency of Japan (to Y. F.); grants-in-aid for scientific research (to M. H. and Y. F.) and a grant from the Global Center of Excellence Program from The Ministry of Education, Culture, Sports, Science, and Technology of Japan; a grant from the Japan Foundation for Applied Enzymology (to Y. F.).

◆ This article was selected as a Paper of the Week.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures" and Figs. 1–6.

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[2] The abbreviations used are: DHAPAT, dihydroxyacetone phosphate acyltransferase; ADAPS, alkyl dihydroxyacetone phosphate synthase; Etn, ethanolamine; PE, phosphatidylethanolamine; GPE, glycerophosphoethanolamine; PlsCho, choline plasmalogen; PlsEtn, plasmenylethanolamine; Far, fatty acyl-CoA reductase; HA, influenza virus hemagglutinin; HG, 1-*O*-hexadecylglycerol; RCDP, rhizomelic chondrodysplasia punctata; CHO, Chinese hamster ovary; siRNA, small interfering RNA.

GCCTGGTATTCCGGAGTTAAT-3′), and Stealth™ RNAi negative control medium GC duplex 2 (Invitrogen).

Far Enzyme Assay—Confluent 60-mm dishes of fibroblasts were washed with phosphate-buffered saline three times and cultured for 18 h in Dulbecco's modified Eagle's medium supplemented with 33 μM palmitic acid and 2.4 μM [^{14}C]palmitate (8), both conjugated with fatty acid-free bovine serum albumin. Lipids were extracted from aliquots (100 μg of protein) of cell lysates according to Folch *et al.* (10). ^{14}C-Labeled hexadecanol was separated from other lipids by TLC on a 150 Å silica-gel plate (Whatman) using two solvent systems (8) and detected by autoradiography using a FLA-5000 imaging analyzer (Fuji Film, Tokyo, Japan). *In vitro* Far enzyme activity was determined as described (7). We homogenized cells in 50 mM Tris-Cl, pH 8.0, 0.25 M sucrose by passaging several times through a 27-gauge needle and prepared a postnuclear supernatant fraction by centrifugation (11, 12). Aliquots (50 μg each) of this fraction were added to reaction tubes (final volume = 0.2 ml) containing 50 mM sodium phosphate, pH 7.2, 2 mM $MgCl_2$, 1 mg/ml fatty acid-free bovine serum albumin, 1.25 mM NADPH, and 0.1 μCi of [^{14}C]palmitoyl-CoA and subsequently incubated for 20 min at 37 °C. The reaction was terminated with 4 volumes of chloroform:methanol (2:1, v/v). [^{14}C]Hexadecanol was extracted and detected as described above.

RESULTS

Far1 Is Essential for Plasmalogen Synthesis—Ether-linked alkyl bond formation in ether lipids is catalyzed by ADAPS and requires fatty alcohols that are converted from fatty acids by reduction of fatty acyl-CoAs. This reaction is catalyzed by Far. Recently, two types of Far cDNAs, *FAR1* and *FAR2*, were cloned from human and mouse, and their peroxisomal localization was demonstrated by expressing epitope-tagged Far proteins (8). Therefore, Far1 and/or Far2 are believed to be the enzymes responsible for the production of fatty alcohols. Because *FAR1* expression was detected in many mouse tissues (8), we first analyzed the expression level of *FAR1* and *FAR2* in several cell lines. Reverse transcription-PCR of total RNA indicated that *FAR1* was predominantly expressed in human fibroblasts and that *FAR1* was exclusively expressed in HeLa and CHO-K1 cells (supplemental Fig. 1). Based on these findings, we used HeLa and CHO-K1 cells to investigate the physiological function of Far1. We attempted to detect PlsEtn with ^{14}C-labeled palmitic acid, which should be incorporated into PlsEtn at the *sn*-1 position upon reduction to [^{14}C]hexadecanol by Far1. [^{14}C]Palmitate was indeed incorporated efficiently into various phospholipids (Fig. 1*A*, *lanes 1*, *5*, and *9*). Upon alkaline methanolysis, fatty acids were liberated from the *sn*-1 and *sn*-2 positions of glycerophospholipids. In contrast, sphingomyelin (*SM*) was resistant to this alkaline treatment (*lanes 3*, *7*, and *11*). Under these conditions, PlsEtn was converted to 1-alkenyl-GPE in CHO-K1 and HeLa cells (Fig. 1*A*, *lanes 7* and *11*) but not in ZPEG251 cells (*lane 3*). The latter cell line lacks the ADAPS enzyme and thus cannot synthesize ether lipids. Moreover, the vinyl ether bond of 1-alkenyl-GPE was completely hydrolyzed by subsequent treatment with trichloroacetic acid (3), thereby indicating that [^{14}C]palmitate was linked to glycerol via an ether linkage, consistent with incorporation into the *sn*-1 position of PlsEtn (Fig. 1*A*, *lanes 8* and *12*).

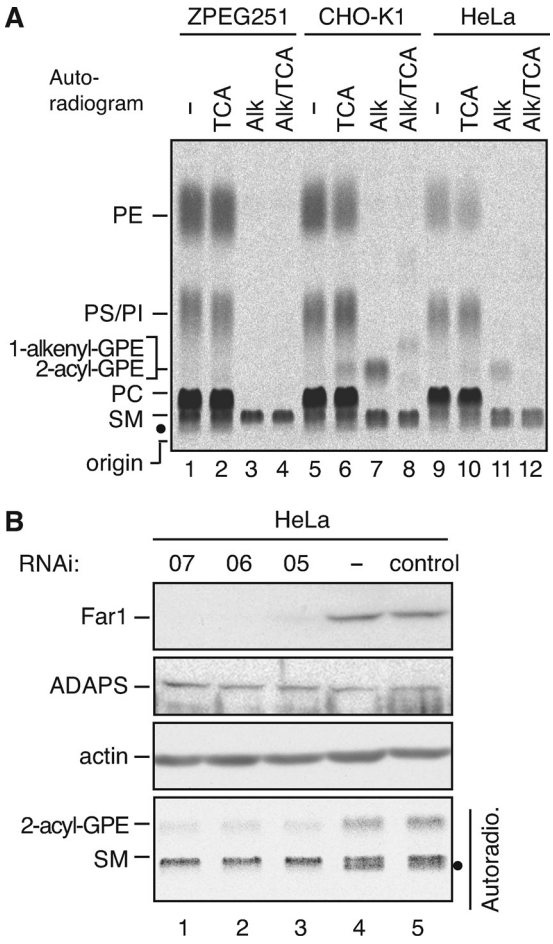

FIGURE 1. **Essential enzyme Far1 in plasmalogen biosynthesis.** *A*, detection of ethanolamine ether lipids with [^{14}C]palmitic acid. *ADAPS*-deficient *adaps* ZPEG251 (*lanes 1–4*), CHO-K1 (*lanes 5–8*), and HeLa (*lanes 9–12*) cells were metabolically labeled with [^{14}C]palmitate for 18 h. Lipids were extracted (−), treated with trichloroacetic acid (*TCA*) prior to extraction, and subjected to alkaline methanolysis (*Alk*) or alkaline methanolysis plus trichloroacetic acid (*Alk/TCA*). An autoradiogram of a representative TLC plate taken on a FLA-5000 imaging analyzer is shown. Note that sphingomyelin (*SM*) and 1-alkenyl-GPE remained after alkaline methanolysis and vinyl-ether bond of 1-alkenyl-GPE was cleaved with trichloroacetic acid. *Solid circle*, an unidentified lipid migrating just below sphingomyelin on TLC plate. *PC*, phosphatidylcholine; *PS*, phosphatidylserine; *PI*, phosphatidylinositol. *B*, Far1 is essential for plasmalogen synthesis. Ether lipid synthesis was verified in HeLa cells that had been treated for 3 days without (−) (*lane 4*) or with a control double-stranded RNA (*lane 5*) or three different double-stranded RNAs (*07*, *06*, and *05*) (*lanes 1–3*) against *FAR1*. Cells were metabolically labeled with [^{14}C]palmitate in the last 18 h during the knockdown of *FAR1*. Expression of Far1 and ADAPS was assessed by immunoblotting with antibodies to Far1 and ADAPS, respectively. Lipids were extracted from the respectively treated cells (100 μg of protein), subjected to alkaline methanolysis, separated by TLC, and analyzed by autoradiography (*autoradio.*). Actin was used as a loading control. The *solid circle* indicates an unidentified lipid migrating just below sphingomyelin on TLC plate, as in *A*. *RNAi*, RNA interference.

Next, we verified whether Far1 is essential for plasmalogen synthesis. *FAR1* knockdown specifically gave rise to impaired plasmalogen biosynthesis (Fig. 1*B*). Taken together, these results indicate that Far1 is responsible for supplying long chain fatty alcohols for the formation of ether lipids in these cell types.

Restoring Plasmalogen Levels Reduces Accumulation of Hexadecanol in Plasmalogen-deficient Mutant Cells—The rate-limiting step of plasmalogen biogenesis has not been elu-

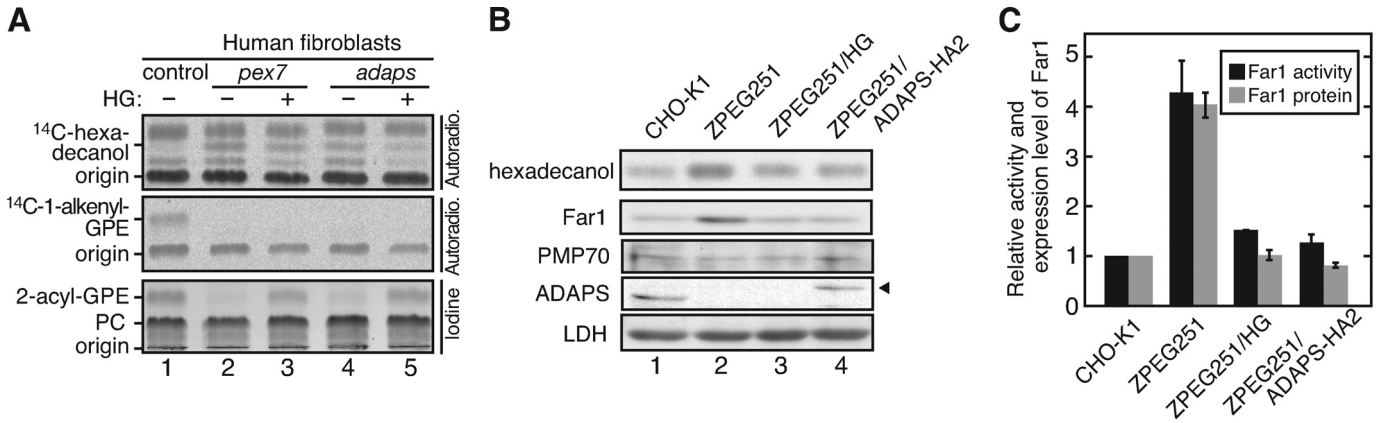

FIGURE 2. **An elevated level of Far1 in plasmalogen-deficient cell mutant is normalized upon restoring plasmalogen synthesis.** *A*, human fibroblasts from a normal control (*lane 1*), a *PEX7*-deficient (*pex7*) patient (*lanes 2* and *3*), and an *ADAPS*-deficient (*adaps*) patient (*lanes 4* and *5*) were cultured in the presence of ethanol (−) or 10 μM HG (+) for 30 h and were then metabolically labeled with [^{14}C]palmitate for another 18 h in the presence of ethanol (−) or HG (+). Cells were divided into three tubes, and cellular lipids were extracted according to Folch *et al.* (10) (*upper panel*) or subjected to alkaline methanolysis (*middle panel*) or trichloroacetic acid treatment (*lower panel*). [^{14}C]Hexadecanol (*upper panel*) and 1-[^{14}C]alkenyl-GPE (*middle panel*) were detected by a FLA-5000 imaging analyzer. The total amount of plasmalogens was detected by exposure to iodine vapor (*lower panel*). Note that ^{14}C-labeled 1-alkenyl-GPE was not detectable in *pex7* and *adaps* cells, even in the presence of sufficient amount of plasmalogens synthesized by bypassing the first three steps of plasmalogen biogenesis with HG. *PC*, phosphatidylcholine; *Autoradio.*, autoradiogram. *B*, postnuclear supernatant was prepared from CHO-K1, ZPEG251, ZPEG251 cultured with HG for 48 h, and ZPEG251/*ADAPS-HA*$_2$. Far1 activity was determined with [^{14}C]palmitoyl-CoA as a substrate (*upper panel*). Expression level of Far1 was also assessed by immunoblotting using anti-Far1 antibody and a LAS-4000 mini lumino image analyzer. 70-kDa peroxisomal integral membrane protein (*PMP70*), ADAPS, and lactate dehydrogenase (*LDH*) for a loading control were detected with respective specific antibodies. The *arrowhead* indicates ADAPS-HA$_2$. *PC*, phosphatidylcholine. *C*, activity (*solid bar*) and expression level of Far1 (*gray bar*) in respective cells were represented by taking those in CHO-K1 as 1. Each bar represents the mean ± ranges from two independent experiments.

cidated. Far is likely to be a key enzyme in this regard based on the finding that Far activity is highest in tissues that contain large amounts of ether glycerophospholipids (5, 6). Moreover, an accumulation of fatty alcohols, possibly due to elevated Far activity, was reported in fibroblasts from patients with an inherited defect in plasmalogen synthesis (7). Thus, we suspected that Far activity might be regulated in response to cellular levels of plasmalogens.

To test this hypothesis, we first verified whether fatty alcohols accumulate in plasmalogen-deficient cell lines, including fibroblasts from patients with RCDP of the *PEX7*-deficient type 1 (13) and *ADAPS*-defective type 3. In control fibroblasts, [^{14}C]palmitate was efficiently incorporated into PlsEtn, whereas PlsEtn was undetectable in plasmalogen-deficient fibroblasts, which instead accumulated hexadecanol as reported previously (7) (Fig. 2*A*, *lanes 1*, *2*, and *4*). In contrast, in both types of plasmalogen-deficient RCDP fibroblasts, the accumulation of hexadecanol was significantly reduced when plasmalogen levels were restored by supplementation of the medium with 10 μM 1-*O*-hexadecylglycerol (HG) (Fig. 2*A*, *lanes 3* and *5*). In the *ADAPS*-CHO cell mutant, ZPEG251 (3), restoration of plasmalogens with HG led to a similar reduction in hexadecanol (supplemental Fig. 2). Together with the data showing that only *FAR1* is expressed in CHO cells, these results strongly suggest that Far1 activity is down-regulated in response to the plasmalogen level in ZPEG251 cells.

Next, we determined the Far1 activity in CHO-K1, *adaps* ZPEG251, ZPEG251 supplemented with HG, and ZPEG251 cells stably expressing a human ADAPS tagged with tandem influenza virus hemagglutinin (ADAPS-HA$_2$) epitopes (3). In ZPEG251 cells, [^{14}C]hexadecanol was detectable at about a 4-fold higher level than that in CHO-K1 cells (Fig. 2*B*, *lanes 1* and *2*). Moreover, upon restoring plasmalogen levels in the ZPEG251 cells either by supplementation with HG or by expression of *ADAPS-HA*$_2$, [^{14}C]hexadecanol levels were decreased to those found in untreated CHO-K1 cells (Fig. 2*B*, *lanes 3* and *4*). This result implied that Far1 activity had been reduced to the level typically found in wild-type CHO-K1 cells. In additional experiments with ZPEG251 cells, the expression level of Far1 protein was elevated ~4-fold as compared with CHO-K1 cells (Fig. 2*B*, *second panel*, *lanes 1* and *2*). Upon restoration of plasmalogens in ZPEG251 cells, Far1 protein levels were decreased to those of CHO-K1 cells(Fig. 2*B*, *second panel*, *lanes 3* and *4*), in good agreement with the changes in Far1 enzymatic activity (Fig. 2*C*). These results indicated that Far1 activity is regulated at the protein level in a manner dependent on a cellular levels of plasmalogens.

Degradation of Far1 Is Enhanced by Restoring Plasmalogen Levels in Plasmalogen-deficient Cells—To explore the mechanism underlying the regulation of Far1 activity, we first measured *FAR1* mRNA levels. Semiquantitative reverse transcription-PCR showed that restoration to a normal level of plasmalogens in *ADAPS*-deficient fibroblasts and ZPEG251 cells did not alter the cellular levels of *FAR1* mRNA (supplemental Fig. 3, *A* and *B*). These results suggested that Far1 activity is regulated at a post-translational stage as opposed to regulation at the transcriptional level. To confirm this suggestion, we established a cell line, ZPEG251/*FLAG-FAR1*, which was derived from ZPEG251 by stable transfection of a cDNA expressing FLAG-Far1. Both recombinant FLAG-Far1 and endogenous Far1 were detected in peroxisomes (supplemental Fig. 4*A*). In ZPEG251/*FLAG-FAR1* cells, FLAG-Far1 expression level was reduced by HG supplementation or by expression of ADAPS-HA$_2$ (supplemental Fig. 4, *B* and *C*), as was observed for endogenous Far1 in ZPEG251 cells (Fig. 2).

Next, we investigated the turnover of Far1 in the presence of plasmalogens. ZPEG251/*FLAG-FAR1* cells were cultured for 48 h in the presence or absence of HG, pulse-labeled with

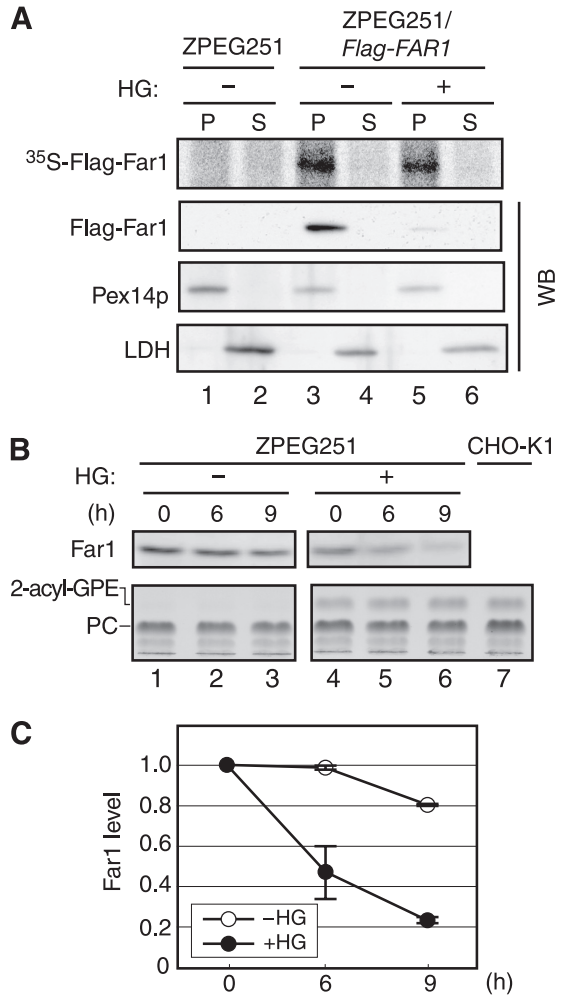

FIGURE 3. **Far1 activity is regulated at the protein level.** *A*, ZPEG251 (*lanes 1* and *2*) and ZPEG251/*FLAG-FAR1* (*lanes 3–6*) were cultured for 48 h in the presence (+) or absence (−) of HG and then labeled with [^{35}S]Met and [^{35}S]Cys for 1 h. Organelle (*P*) and cytosolic (*S*) fractions were assessed by Western blotting (*WB*) for FLAG-Far1, peroxisomal membrane peroxin Pex14p, and lactate dehydrogenase (*LDH*). [^{35}S]FLAG-Far1 was immunoprecipitated using FLAG-agarose. Note that newly synthesized FLAG-Far1, [^{35}S]FLAG-Far1, was efficiently targeted to the membrane even upon supplementation of plasmalogens with HG. *B*, ZPEG251 was cultured for 13 h in the presence of 20 μM HG (+) (*lanes 4–6*) or ethanol (−) (*lanes 1–3*) and further for the indicated time in the same medium but containing 10 μg/ml cycloheximide. Cell lysates were subjected to lipid analysis (100 μg each) and SDS-PAGE and immunoblotting (10 μg each) with anti-Far1 antibody. Detection was done by a LAS-4000 mini lumino image analyzer. Plasmalogen level in CHO-K1 was taken as a reference (*lane 7*). *C*, the relative amount of Far1 in the absence (*open circle*) or presence (*filled circle*) of HG at each time point was represented by taking as 1 that at the time point of cycloheximide addition. All values are given as the mean ± ranges from two independent experiments.

[^{35}S]Met and [^{35}S]Cys for 1 h, and examined for FLAG-Far1 expression (Fig. 3*A*). In immunoblots, FLAG-Far1 was barely detectable in HG-supplemented ZPEG251/*FLAG-FAR1* cells (supplemental Fig. 4, *B* and *C*). Strikingly, nearly the same amount of newly synthesized [^{35}S]FLAG-Far1 was recovered in organelle fractions containing peroxisomes isolated from both ZPEG251/*FLAG-FAR1* and HG-supplemented ZPEG251/*FLAG-FAR1* cells (Fig. 3*A*, *lanes 3* and *5*). This result indicated that newly synthesized Far1 was efficiently transported to peroxisomes and subsequently degraded in cells replete with plasmalogens. To assess the fate of Far1 after targeting to peroxisomes, ZPEG251 cells were cultured in the presence or absence of 20 μM HG for 13 h and then further incubated for 6 and 9 h in the presence cycloheximide (Fig. 3*B*). Far1 was stable when ZPEG251 cells were cultured in the absence of plasmalogens (Fig. 3*B*, *lanes 1–3*), whereas a large portion of Far1 was degraded in cells supplemented with 20 μM HG (*lanes 4–6*), indicating that the rate of Far1 protein turnover was accelerated in the presence of plasmalogens (Fig. 3, *B* and *C*). Taking these results together, we think it is most likely that the activity of Far1 is regulated by modulating Far1 stability in response to the level of plasmalogens. Moreover, supplementation of ZPEG251 cells with PlsEtn, but not phosphatidylethanolamine (PE) and 2-acyl-GPE, increased the degradation of Far1 (supplemental Fig. 5), indicating that the vinyl ether bond at the *sn*-1 position of PlsEtn is important for sensing plasmalogen content.

Far1 Activity Is Regulated by Plasmalogens in Wild-type Cells—We next asked whether Far1 was similarly regulated by plasmalogens in wild-type CHO-K1 cells. Upon elevation of cellular PlsEtn level by supplementation, Far1 protein level and the synthesis of PlsEtn were coordinately reduced by ~50% in this cell type (Fig. 4*A*, *lanes 1* and *2*). In contrast, PE supplementation did not alter plasmalogen synthesis or Far1 stability (Fig. 4*A*, *lane 3*). We attempted to increase plasmalogen levels in CHO-K1 cells by supplementation with ethanolamine (Etn), HG, or a combination of these two precursors. When CHO-K1 cells were cultured in the presence of Etn for 6 h, PlsEtn and PE, both biosynthetically labeled with [^{14}C]palmitate, were significantly elevated as compared with untreated controls (Fig. 4*B*, *lanes 1* and *2*), implying that the amount of Etn in cells under normal culture conditions is not sufficient for the synthesis of PlsEtn and PE. HG supplementation slightly elevated the biosynthetic labeling of PlsEtn with [^{14}C]Etn (Fig. 4*B*, *lanes 3* and *4*). Conversely, lowering the PE level had an opposite effect (Fig. 4*B*, *lanes 3* and *4*). By adding both Etn plus HG, PlsEtn was more distinctly increased (~2-fold), and Far1 levels were substantially reduced (Fig. 4*C*). Taken together, these results again suggest that Far1 activity is regulated via modulation of Far1 stability in response to cellular levels of plasmalogens.

Peroxisomal Matrix Proteins Are Not Required for the Degradation of Far1—We next tested whether peroxisomal matrix proteins were required for Far1 regulation. We used a *PEX1*-deficient CHO cell mutant, ZP107, as a representative cell line defective in matrix protein import (14). Upon restoring plasmalogen levels in *pex1* ZP107, Far1 was largely eliminated, just as was observed in ZPEG251 cells (supplemental Fig. 6, *lanes 2* and *4*). Thus, neither peroxisomal matrix proteins nor Pex1p were required for degradation of Far1.

DISCUSSION

In the present study, we demonstrate that Far1 is an essential enzyme for supplying fatty alcohols for ether bond formation in ether glycerophospholipid synthesis (Fig. 1*B*). Two Far isozymes, Far1 and Far2, synthesize long chain fatty alcohol by reducing fatty acyl-CoA (8). *FAR1* mRNA is detectable in many types of tissues and is therein presumably responsible for a majority of fatty alcohol production. In contrast, *FAR2* mRNA is largely restricted to eyelid, skin, and brain (8). Currently, it

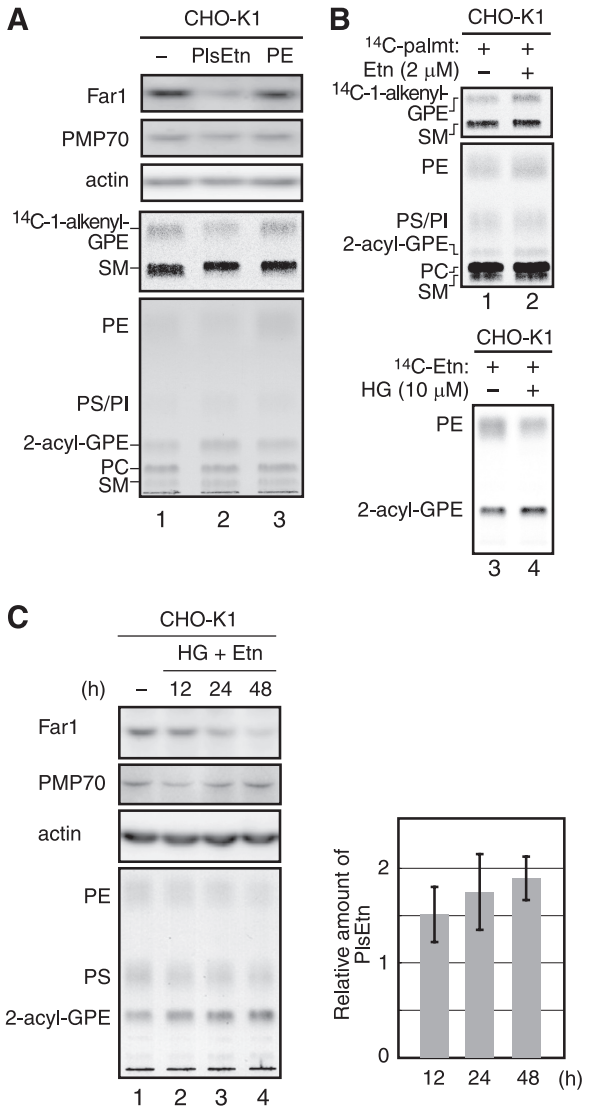

FIGURE 4. **Stability of Far1 in CHO-K1 cells.** *A*, CHO-K1 cells were cultured for 48 h in the presence of PlsEtn or PE and assessed for the expression level of Far1 and the synthesis of ether lipids by labeling cells with [^{14}C]palmitate added at 45 h of lipid supplementation. *SM*, sphingomyelin; *PS*, phosphatidylserine; *PI*, phosphatidylinositol; *PC*, phosphatidylcholine. *B*, CHO-K1 cells were cultured for 6 h for labeling with [^{14}C]palmitate (*^{14}C-palmt.*) in the presence (+) and absence (−) of 2 μM Etn (*upper panel*). CHO-K1 cells were likewise labeled with [^{14}C]Etn (*^{14}C-Etn*) in the presence (+) and absence (−) of 10 μM HG (*lower panel*). Synthesis of ether lipids (*upper top panel*) was assessed by alkaline methanolysis. Synthesis of phospholipids (*upper bottom panel*) and plasmalogens (*lower panel*) was verified by trichloroacetic acid hydrolysis. Note that supplementation of Etn significantly increased the synthesis of ether lipids. *C*, CHO-K1 cells (*lane 1*) were cultured in the presence of Etn and HG (*lanes 2–4*) for the indicated periods of time. Expressed levels of Far1, 70-kDa peroxisomal membrane protein (*PMP70*), and actin were analyzed with respective antibodies. Lipids were detected with ninhydrin. 2-Acyl-GPE and PS were quantified using an image analysis software (Multi Gauge, Fuji Photo Film), normalized in a ratio to PS at each time point, and represented by taking as 1 that at the time point starting cell culture. Each bar represents the means ± ranges of two independent experiments.

remains unknown whether Far2 supplies fatty alcohols for ether bond formation in these tissues.

Fatty alcohols are utilized as substrates for the synthesis of ether lipids and wax esters, and excess amounts of fatty alcohol are oxidized to fatty acid (15). Accumulation of fatty alcohols has been reported in several cell lines defective in plasmalogen biosynthesis (7, 16). Given these findings, it seems likely that the enzymes involved in metabolism of fatty alcohols are coordinately regulated to make these substrates available depending on the biosynthetic needs of the cell. For example, brain contains higher amounts of plasmalogens and a correspondingly higher activity of Far, whereas fatty alcohol oxidase activity of this tissue is low (17). In contrast, the converse is true in liver, where plasmalogens are less abundant (6) and fatty alcohol oxidase activity is high. The latter enzyme activity is not altered in plasmalogen-deficient cells including a peroxisome assembly-defective CHO cell mutant, *PEX2*-deficient ZR-82 (7, 16), implying that the accumulation of fatty alcohols in these cells is conferred by a combination of impaired incorporation of fatty alcohols in plasmalogen biosynthesis and elevated stability of Far1.

We herein provide several lines of evidence that Far1 activity is regulated by modulating Far1 protein stability in response to the cellular levels of plasmalogens. Currently, we do not know the molecular mechanism behind Far1 degradation. It is also noteworthy that Far1 is degraded apparently in a plasmalogen-dependent manner after targeting to peroxisomes (Fig. 3). Moreover, peroxisomal matrix proteins and at least Pex1p do not seem to be required for this degradation process (supplemental Fig. 6).

We show that degradation of Far1 is accelerated upon restoring plasmalogen levels by supplementing deficient cells with PlsEtn or HG (Fig. 3 and supplemental Fig. 5). The vinyl ether bond seems to be important for the recognition of intracellular plasmalogen levels based on the finding that Far1 is not degraded by supplementation with PE or 2-acyl-GPE (supplemental Fig. 5). At present, we do not know whether the level of choline plasmalogen (PlsCho) functions as the signal for intracellular plasmalogen content or whether some other plasmalogen accomplishes this role. In CHO-K1 cells, only small amounts of PlsCho are detected (18). Supplementation of PlsCho to ZPEG251 cells does not lead to Far1 degradation, perhaps because of the higher activity of head group conversion from choline to ethanolamine in these cells (data not shown). PlsCho is in general less abundant in many tissues, except for heart and muscle (1), which suggests that the amount of PlsEtn is more likely key for monitoring intracellular plasmalogen levels. In cells containing a large amount of PlsCho, the regulatory significance of this lipid remains to be defined.

At the last step in the biosynthesis of PlsEtn and PE, phosphoethanolamine is transferred to alkylacyl- and diacylglycerol, respectively, via the CDP-ethanolamine pathway (1). If cells are unable to synthesize PlsEtn due to the defect in the process of plasmalogen biogenesis in peroxisomes, then the amount of PE is increased (3, 19, 20). Conversely, restoring PlsEtn by supplementing HG to such mutant cells reduces the level of PE (3, 20). Interestingly, enhanced synthesis of PE through the CDP-ethanolamine pathway requires a corresponding increase in diacylglycerol (21). When this result is considered with the finding that HG supplementation eliminates PE synthesis (Fig. 4), we suggest that alkylacyl- and diacylglycerol may be regulatory metabolites in the synthesis of PlsEtn and PE, respectively.

Based on the findings that supplementation of HG does not increase the level of PlsEtn, Nagan and Zoeller (1) suggested that the rate-limiting step of plasmalogen biosynthesis may

reside downstream of the first three steps in the pathway; however, the four enzymes responsible for these terminal steps have not yet been studied at the molecular level. Accordingly, the regulation of plasmalogen biosynthesis at the later steps has yet to be fully understood. An agent, 1-*O*-[9′-(1″-pyrenyl)]nonyl-*sn*-glycerol, may be useful for isolating mutant cell lines deficient in the terminal steps of plasmalogen biosynthesis (22).

In the present study, we were successful in showing that supplementation with Etn and HG elevated PlsEtn levels in CHO-K1 cells, thereby suggesting that plasmalogen biogenesis is more likely regulated within the first three steps of the pathway, which occur in peroxisomes as opposed to the later steps occurring in the endoplasmic reticulum. In peroxisomes, DHAPAT is an essential enzyme, catalyzing the biosynthesis of an ether lipid precursor, 1-acyl-DHAP. We believe that DHAPAT is less likely to be a regulatory enzyme in plasmalogen biogenesis based on the finding that overexpression of this enzyme in a *dhapat* CHO mutant, NRel-4, restores plasmalogen content to a level similar to that found in wild-type cells (18). Similarly, increased DHAPAT activity is noted under certain conditions such as the conversion of 3T3-L1 preadipocytes to adipocytes, whereas the plasmalogen level is not elevated during differentiation (23). Moreover, plasmalogen deficiency does not alter the activity of DHAPAT in *ADAPS*-defective cells (24). Together, these findings imply that DHAPAT activity is not modulated in response to the cellular level of plasmalogens. Similarly, the enzyme activity of ADAPS and acyl/alkyl-DHAP reductase catalyzing the third step of plasmalogen synthesis is not altered in plasmalogen-deficient cells such as the *dhapat* CHO mutant, NRel-4, or in fibroblasts from patients with *DHAPAT*-deficient RCDP type 2 (20, 25). These reports suggest that the activities of DHAPAT, ADAPS, and acyl/alkyl-DHAP reductase are not regulated in response to plasmalogen levels.

It is of interest to note that ADAPS requires long chain alcohols of 16 or 18 carbons but will not utilize 14-carbon fatty alcohols (26). Given the current findings suggesting that the level of fatty alcohols regulates ether glycerophospholipid synthesis, it is conceivable that hexadecanol- or octadecanol-containing ether lipids are indicators of cellular plasmalogen levels.

We and other groups earlier showed that PlsEtn is not localized to peroxisomes where Far1 resides (3, 27, 28). Given these data, we propose that the plasmalogen-dependent regulation of Far1 involves at least three steps: recognition of the PlsEtn level in the endoplasmic reticulum or post-Golgi compartments, transfer to peroxisomes of the signal indicating plasmalogen levels, and finally, modulation of Far1 stability. Feeding PlsEtn or alkylglycerol to rats failed to increase plasmalogen levels in most tissues with the exceptions of plasma and liver (29, 30). Moreover, dynamic pools of ether lipids were found in the gray matter of the brain (31). Therefore, regulation of plasmalogen biosynthesis by modulating Far1 stability may play a pivotal role in plasmalogen homeostasis in the living organism.

Acknowledgments—We thank S. Okuno for technical assistance, M. Nishi for preparing figures, and the other members of our laboratory for discussion.

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