Role of dihydroxyacetonephosphate acyltransferase in the biosynthesis of plasmalogens and nonether glycerolipids

Dailan Liu,* Narasimhan Nagan,[1,*] Wilhelm W. Just,[†] Claus Rodemer,[†] Thanh-Phuong Thai,[†] and Raphael A. Zoeller[2,*]

Department of Physiology and Biophysics,* Boston University School of Medicine, Boston, MA 02118; and Biochemie-Zentrum,[†] Universitat Heidelberg, D-69120 Heidelberg, Germany

Abstract **The variant CHO-K1 cell line, NRel-4, is unable to synthesize plasmalogens because of a severe reduction in dihydroxyacetonephosphate acyltransferase (DHAPAT) activity (Nagan, N., A. K. Hajra, L. K. Larkins, P. Lazarow, P. E. Purdue, W. B. Rizzo, and R. A. Zoeller. 1998. Isolation of a Chinese hamster fibroblast variant defective in dihydroxyacetonephosphate acyltransferase activity and plasmalogen biosynthesis: use of a novel two-step selection protocol. *Biochem. J.* 332: 273–279). Northern analysis demonstrated that the loss of this activity was attributable to a severe reduction in mRNA levels for DHAPAT. Transfection of NRel-4 cells with a plasmid bearing the human DHAPAT cDNA recovered DHAPAT activity and plasmalogen biosynthesis. Examination of clonal isolates from the transfected population showed that recovery of as little as 10% of wild-type DHAPAT activity restored plasmalogen levels to 55% of normal, whereas in one isolate, NRel-4.15, which overexpressed DHAPAT activity by 6-fold over wild-type cells, plasmalogen levels were returned only to wild-type values. Although the rate of plasmenylethanolamine biosynthesis was restored in NRel-4.15, the biosynthesis of nonether glycerolipids was either decreased or unaffected, suggesting that peroxisomal DHAPAT does not normally contribute to nonether glycerolipid biosynthesis. These data demonstrate that a defect in the gene that codes for peroxisomal DHAPAT is the primary lesion in the NRel-4 cell line and that the peroxisomal DHAPAT is essential for the biosynthesis of plasmalogens in animal cells.**—Liu, D., N. Nagan, W. W. Just, C. Rodemer, T-P. Thai, and R. A. Zoeller. **Role of dihydroxyacetonephosphate acyltransferase in the biosynthesis of plasmalogens and nonether glycerolipids. *J. Lipid Res.* 2005.** 46: **727–735.**

Supplementary key words glycerol-3-phosphate acyltransferase • variant • Chinese hamster ovary cells

Plasmalogens represent a subset of phospholipids that differ from other glycerolipids in that, attached to the *sn*-1 position of the glycerol backbone is an alkyl chain attached through a vinyl ether linkage (1). The vinyl ether gives plasmalogens their unique physical and chemical properties. Every mammalian cell contains plasmalogens. In most cells, plasmalogens are represented as a subclass of ethanolamine phospholipids, in which they can represent a major portion of that head group class (2). Typically less abundant are choline plasmalogens, although in certain tissues, such as heart and muscle, they are found at relatively high levels. Plasmalogens have been proposed to be important for the stimulated release of arachidonic acid from phospholipid pools (3–5), the fusion of biological membranes (6), and the protection against reactive oxygen species (7–10).

The mechanism for the maintenance of plasmalogen levels and the rate-limiting factor(s) for plasmalogen biosynthesis have not been identified. Although the pathway for plasmenylethanolamine biosynthesis has been established, the exact biosynthetic pathway for plasmenylcholine has not. Unlike plasmenylethanolamine, the saturated ether lipid species, plasmanylcholine is not a precursor for plasmenylcholine (11). Plasmenylcholine appears to be synthesized from plasmenylethanolamine through the replacement of ethanolamine with choline (11, 12) or possibly through the methylation of plasmenylethanolamine (13). Our lack of knowledge in this area is attributable, in part, to the lack of molecular tools for studying the system. Only recently have any of the enzymes that participate in the biosynthetic pathway for plasmalogens been purified or the genes that code for them cloned (14, 15).

Abbreviations: DHAP, dihydroxyacetonephosphate; DHAPAT, dihydroxyacetonephosphate acyltransferase; Gro-3-P, glycerol-3-phosphate; Gro-3-PAT, glycerol-3-phosphate acyltransferase; Pi, inorganic phosphate; PTS1, peroxisome targeting signal type 1; SSC, saline-sodium citrate buffer.

[1] Present address of N. Nagan: Athena Diagnostics, Inc., Four Biotech Park, 377 Plantation Street, Worcester, MA 01605.

[2] To whom correspondence should be addressed.
e-mail: rzoeller@bu.edu

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Although early steps in any biosynthetic pathway are commonly candidates as control points, this paradigm may not apply to plasmalogen biosynthesis. The regulation of plasmalogen biosynthesis is likely to be complex and linked to the biosynthesis of nonether glycerolipids, because later steps in the pathway are also used in the synthesis of diacyl phospholipids and triglycerides (1). Recent evidence also suggests that dihydroxyacetonephosphate acyltransferase (DHAPAT), which catalyzes the first step in plasmalogen biosynthesis, may play a significant role in nonether glycerolipid biosynthesis as well; this activity is increased several-fold during the differentiation of 3T3-L1 cells to adipocytes (16). Therefore, the role of DHAPAT in the synthesis of this latter group of glycerolipids (nonether) remains to be determined.

We have described the isolation of variants of the fibroblast-like cell line CHO-K1 that are deficient in plasmalogens as a result of the loss of various steps along the biosynthetic pathway (17–19). These cell lines can be useful in identifying the factors that determine plasmalogen levels as well as in defining the relationship between ether lipids and diacyl lipid synthesis. The mutant CHO cell line NRel-4 displays greatly reduced plasmalogen levels because of a loss of DHAPAT (19). In this report, we show that the loss of this activity is attributable to reduced levels of the message for DHAPAT, making NRel-4 a useful null cell model for examining the role of DHAPAT in the regulation of plasmalogen levels. We have expressed the human DHAPAT gene in NRel-4 cells to demonstrate that this can recover plasmalogen biosynthesis in these cells and to examine what effect varied levels of DHAPAT activity have on the synthesis of both ether and nonether glycerolipids.

MATERIALS AND METHODS

Materials

[1-^{3}H]ethanolamine (30 Ci/mmol; 1 Ci = 37 gigabecquerel) was obtained from Amersham (Arlington Heights, IL). [γ-^{32}P]ATP, [^{32}P]inorganic phosphate (^{32}Pi), [9,10-^{3}H]oleic acid, and EN^{3}HANCE spray were obtained from Perkin-Elmer/New England Nuclear (Boston, MA). ^{32}P-labeled dihydroxyacetonephosphate (DHAP) and glycerol-3-phosphate (Gro-3-P) were synthesized by enzymatic phosphorylation of dihydroxyacetone or glycerol using [γ-^{32}P]ATP and glycerol kinase (20). Ecoscint A liquid scintillation fluid was obtained from National Diagnostics (Atlanta, GA). Silica gel 60 TLC plates (Merck) were purchased from American Scientific Products (McGaw Park, IL). Tissue culture dishes (Corning) were obtained from VWR Scientific (Boston, MA). All other reagents, unless specified, were purchased from Sigma (St. Louis, MO).

Cell lines and culture conditions

CHO-K1 cells were obtained from the American Type Culture Collection (Manassas, VA). Plasmenylethanolamine is the dominant plasmalogen species, constituting ~35–40% of the ethanolamine phospholipids; there is almost no plasmenylcholine. NRel-4 is a plasmalogen-deficient CHO-K1-derived cell line with a severe deficiency in DHAPAT activity (19). NZel-1 is a plasmalogen-deficient variant of CHO-K1 defective in alkyl-DHAP synthase (18). Cells were grown in Ham's F12 medium supplemented with 10% fetal bovine serum (Atlas Biologicals, Denver, CO) at 37°C in 5% CO_2.

Construction of an expression vector containing human DHAPAT

The human DHAPAT cDNA (14) was removed from the pBluescript SK phagemid vector and inserted into the shuttle vector pBK-CMV (Stratagene, La Jolla, CA) using the *Eco*RI (5′) and *Xho*I (3′) restriction sites. The construct was used to transform XL1-Blue MRF′, which was then grown on kanamycin-containing agar to form colonies. Selected white colonies were expanded and cDNA preparations were analyzed by restriction analysis, and those strains whose cDNA demonstrated the appropriate agarose electrophoresis patterns were further expanded for large-scale DNA preparation using an endotoxin-free plasmid kit supplied by Qiagen (Valencia, CA). The cDNA inserts were sequenced to ensure that DHAPAT cDNA was unaltered and properly inserted. NRel-4 cells were transfected using SuperFect transfection reagent (Qiagen), and the transfected populations were grown for 6 weeks in G418 (500 μg/ml) to develop a stably transfected population.

To generate clonal isolates, a cell suspension was diluted to a concentration of 2 cells/ml, and 200 μl aliquots were transferred to the wells of a 96-well plate. After 10 days of growth at 37°C, wells were examined for the formation of a single colony. The cells in these wells were harvested with trypsin and expanded for further study. Conditioned medium (medium in which cells had been previously grown) was filtered using a sterile 0.22 μm filter and used for the dilutions and transfer to the 96-well plates. This was necessary to ensure that the cells would grow at very low density.

Enzyme assays

Glycerol-3-phosphate acyltransferase (Gro-3-PAT) and DHAPAT activities were measured using whole-cell homogenates that were prepared as described previously (18). DHAPAT and Gro-3-PAT activities were measured as described by Jones and Hajra (21) using palmitoyl-CoA and either ^{32}P-labeled Gro-3-P or DHAP as the acyl acceptor. Protein content was determined using the method of Lowry et al. (22). Detection of DHAPAT in immobilized colonies (colony autoradiography) was performed as described previously (23). Briefly, transfected cells were plated onto a 100 mm diameter tissue culture dish at 300 cells per dish and allowed to attach overnight. Cells were then overlaid with a sterile polyester cloth. The cells were allowed to grow and form colonies within the polyester matrix for 8–10 days at 37°C. The polyester bearing the colonies was removed from the dish, washed once with PBS, and placed at −20°C overnight. The polyester cloth was thawed and placed in a 100 mm tissue culture dish containing 3 ml of DHAPAT assay mix at pH 5.5 (4 μCi [^{32}P]DHAP/ml) and incubated for 15 min at 37°C. The reaction was stopped by the addition of 3 ml of ice-cold 20% TCA. The cloth was then placed on a Buchner funnel and rinsed five times with 25 ml portions of ice-cold 3% TCA to remove TCA-soluble substrate; the TCA-insoluble product, acyl-[^{32}P]DHAP, remained associated with the colony. After drying for 1 h, the cloth was exposed to X-ray film overnight at −80°C. To visualize the colonies, the cloth was placed in 0.5% (w/v) Coomassie blue in methanol-water-acetic acid (45:45:10, v/v) for 30 min followed by destaining with methanol-water-acetic acid (45:45:10, v/v).

Ethanolamine labeling of cellular phospholipid

Cells (2.5×10^{5}) were grown for 18 h in sterile glass scintillation vials at 37°C in growth medium containing [1-^{3}H]ethanolamine (1 μCi/ml). The medium was removed, the cells were washed once with 2 ml of PBS, and the lipids were extracted in 3.8 ml of $CHCl_3$/methanol/PBS (1:2:0.8) containing 300 μg of a carrier lipid (beef heart lipids). After transferring to a test tube, 1 ml of $CHCl_3$ and 1 ml of PBS were added to form a two-phase Bligh and Dyer system (24), and the lower (organic) phase was collected after centrifugation. Solvent was removed using a stream

of nitrogen, and the labeled phospholipids were separated by two-stage, single-dimension TLC (23). The labeled species were visualized by exposure to X-ray film at −80°C after spraying the plates with EN^{3}HANCE. Labeled bands were scraped into scintillation vials containing 1 ml of methanol, 8 ml of scintillation fluid was added, and radioactivity was quantitated by liquid scintillation spectrometry.

Northern analysis

A 454 bp fragment of the 5′ end of CHO-K1 DHAPAT was generated by polymerase chain reaction using primers 5′-ATGGACGTTCCTAGCTCCTN-3′ for the 5′ end and 5′-AGCAGGACTACAGGGTGCTC-3′ for the 3′ end. The primers were designed based on the nucleotide sequence of the CHO-K1 DHAPAT gene (D. Liu, unpublished data). Probe (100 ng) and 50 ng of pTRI-B-Actin-Mouse (Ambion) were labeled with [α-^{32}P]ATP using the RadPrime kit (Invitrogen, Carlsbad, CA). Total RNA was isolated from cells using Trizol reagent (Invitrogen). RNA was fractionated using a denaturing 0.8% agarose-formaldehyde gel, transferred to nylon membranes (Schleicher and Schuell, Keene, NH), air-dried, and fixed by ultraviolet cross-linking at 1,200 joules. Nylon membranes were prehybridized in Church-Gilbert hybridization buffer (0.5 M sodium phosphate, pH 7.4, 7% sodium dodecylsulfate, 1% BSA, and 1.0 mM EDTA) for 4 h at 62°C. The radioactive probes were denatured by boiling for 5 min and added to the hybridization buffer. After overnight hybridization at 62°C, the blots were washed sequentially as follows: 2× saline-sodium citrate buffer [1× saline-sodium citrate buffer (SSC) = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0] containing 0.5% SDS for 15 min at 42°C, 0.5× SSC and 0.5% SDS at 42°C for 15 min, and 0.1× SSC and 0.5% SDS for 30 min at 42°C. The blots were then exposed to Kodak X-AR film at −80°C for 2 days.

Western analysis

Immunoblotting using the anti-DHAPAT antibody was carried out as described previously (14). Briefly, confluent monolayers were scraped and pelleted, solubilized in Solu-CHAPS (1 mM dithiothreitol, 1 mM EDTA, 15 mM CHAPS, 50 mM Tris-HCl, and 150 mM NaCl, pH 7.0), and proteins were precipitated using 10% TCA. Proteins were separated using 12% SDS-PAGE and subsequently transblotted into polyvinylidene difluoride membranes. After blocking with PBS and 5% nonfat dry milk, blots were processed with anti-DHAPAT antibody. Horseradish peroxidase-conjugated secondary antibody and chemiluminescence (ECL kit; Amersham, San Francisco, CA) were used to visualize the bands.

Subcellular localization of catalase and DHAPAT

Immunofluorescence microscopy was carried out as described previously (25). Briefly, cells were grown on glass coverslips, washed, and fixed with 3% paraformaldehyde. After 5 min of permeabilization in 1% Triton X-100, cells were washed and incubated for 1 h with anti-catalase (25) and anti-DHAPAT (14) antibodies that had been raised in rabbits. After washing, secondary goat anti-rabbit antibodies (Sigma) coupled to either FITC (for DHAPAT) or tetramethyl rhodamine isothiocyanate (for catalase) were used to detect the proteins. Cells were then mounted on slides using moviol/paraphenylendiamine, and images were taken with a Zeiss Axiovert microscope.

Phospholipid composition, biosynthesis, and turnover

Cells were plated into sterilized glass scintillation vials and grown for 72 h at 37°C in medium containing ^{32}Pi (2.5 μCi/ml) to label the phospholipids to constant specific activity. The medium was removed, the cell monolayer was washed once with PBS, and cellular lipids were extracted as described above. Phospholipids were separated using two-dimensional TLC (26). Between dimensions, the region containing lipids was sprayed with 10 mM $HgCl_2$ in glacial acetic acid to cleave the vinyl ether bond of the plasmenyl species, allowing for the separation of the resulting *sn*-1-lysophospholipid and the unaffected diacyl species (27). Plates were exposed to GBX-2 X-ray film at −80°C after preflash. ^{32}Pi-labeled phospholipids were scraped from the TLC plates directly into scintillation vials for quantitation by liquid scintillation spectrometry. For short-term labeling, cells were incubated for 3 h in medium containing ^{32}Pi (20 μCi/ml) and samples were processed as above.

To measure the turnover of total phospholipid pools, cells were labeled for 72 h with ^{32}Pi (2.5 μCi/ml). Cells were harvested with trypsin and plated into sterile glass scintillation vials (10^5 cells/vial) in 1 ml of medium containing ^{32}Pi (2.5 μCi/ml) and allowed to attach overnight. Labeling medium was removed and replaced with 2 ml of unlabeled medium. Cellular phospholipids were extracted at 0, 24, and 48 h after removal of the labeled medium. Solvent was removed by evaporation under a nitrogen stream, samples were resuspended in $CHCl_3$, and an aliquot was transferred to a scintillation vial. After allowing the solvent to evaporate, 1 ml of methanol was added, followed by 8 ml of scintillation fluid, and the amount of chloroform-soluble radioactivity was determined using liquid scintillation spectrometry.

[9,10-^{3}H]oleate labeling of cellular lipids

Cells were plated into sterilized glass scintillation vials and allowed to attach overnight. Medium was removed and replaced with 1 ml of growth medium containing 2 μM [9,10-^{3}H]oleic acid. After 3 h at 37°C, medium was removed, the cell monolayer was washed once with 3 ml of growth medium, and the cellular lipids were extracted as described above. An aliquot of each sample was run on silica gel 60 using *n*-hexane-ethyl ether-acetic acid (70:30:1, v/v) to separate neutral lipids; another aliquot was separated on silica gel 60 using chloroform-methanol-acetic acid-water (25:15:3:1.5, v/v) to separate phospholipid head group classes. Plates were exposed to GBX-2 X-ray film at −80°C after spraying with EN^{3}HANCE. Labeled lipids were scraped from the TLC plates directly into scintillation vials containing 1 ml of methanol followed by the addition of 8 ml of liquid scintillation cocktail. Radioactivity was quantitated by liquid scintillation spectrometry.

Analysis of subclass distribution of ethanolamine and choline phospholipids by analysis of diradyl species

To determine the relative levels of the different subclasses (diacyl, plasmanyl, and plasmenyl) within a head group class, lipids were extracted from unlabeled cells as above and isolated by development on silica gel G plates using chloroform-methanol-acetic acid-water (25:15:3:1.5, v/v). The choline and ethanolamine phospholipids were recovered from the plate and treated with phospholipase C followed by benzoylation of the resulting diradylglycerols (28). Diacyl, alkylacyl, and alkenylacyl benzoylated derivatives were separated by TLC (silica gel G) using benzene-hexane-ethyl ether (50:45:5, v/v), visualized by spraying the TLC plate with water, and recovered from the plate using chloroform-methanol (2:1). Solvent was removed by evaporation under a nitrogen stream, and samples were resuspended in 100% ethanol and quantitated by absorbance at 230 nm.

Statistical analysis

Multiple groups were analyzed by ANOVA, followed by Tukey's honestly significant difference multiple comparison procedure. The data in Table 2 were analyzed with Student's *t*-test.

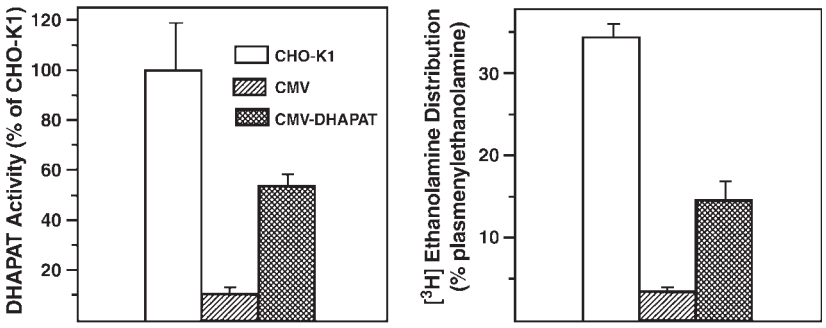

Fig. 1. Dihydroxyacetonephosphate acyltransferase (DHAPAT) activities and plasmenylethanolamine levels are partially recovered in an NRel-4 population transfected with DHAPAT cDNA. Cells were transfected with the pBK-CMV vector bearing the DHAPAT cDNA. Transfected populations were used after selection in G418 for 6 weeks. DHAPAT activity was measured in whole-cell homogenates at pH 5.5, as described in Materials and Methods. DHAPAT activity in CHO-K1 homogenates was 540 pmol/min/mg. For ethanolamine labeling, cells were labeled for 24 h with [1-^{3}H]ethanolamine, cellular lipids were extracted, phosphatidylethanolamine and plasmenylethanolamine were separated using a two-stage, single-dimension TLC system, and radioactivity associated with each subclass was determined as described in Materials and Methods. Any plasmanylethanolamine that may be labeled was not separated from the phosphatidylethanolamine using this procedure. All values represent averages ± SD of three determinations within one representative experiment.

RESULTS

Transfection and expression of DHAPAT in NRel-4 cells

The variant strain NRel-4, derived from the CHO-K1 cell line, is severely reduced in DHAPAT activity, resulting in a 90% decrease in plasmalogen levels (19). We inserted the human DHAPAT gene into the pBK-CMV vector downstream of the constitutive CMV promoter and used this to transfect NRel-4 cells. Analysis of the stably transfected population showed that DHAPAT activity and plasmenylethanolamine levels were partially recovered (**Fig. 1**). When this population was screened, using colony autoradiography, for DHAPAT activity (**Fig. 2**), ~15% of the colonies displayed a strong signal, indicating recovery of activity. A control population, transfected with pBK-CMV vector alone, displayed no increase in plasmalogen content, and we were unable to identify any DHAPAT-positive colonies by colony autoradiography (data not shown).

Three clonal isolates, designated 4.1, 4.2, and 4.15, were generated from this transfected population. Whole-cell homogenates from these transfectants displayed varying amounts of DHAPAT activity when measured at pH 5.5, the optimal pH for DHAPAT (**Table 1**). Although isolates 4.1 and 4.2 had recovered only a small fraction of wild-type DHAPAT levels, isolate 4.15 displayed almost six times the level of DHAPAT activity compared with CHO-K1 cells.

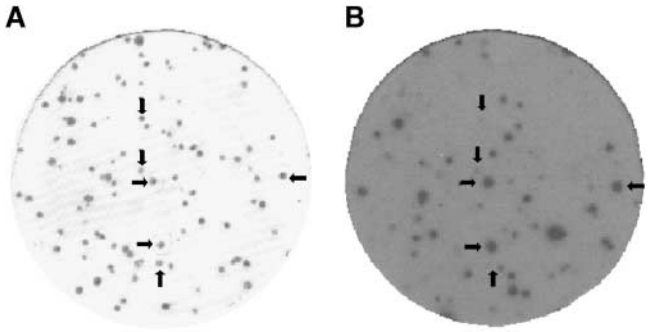

Fig. 2. Colony autoradiography shows that a fraction of the transfectants are DHAPAT-positive. Cells were allowed to grow and form colonies within polyester cloth. DHAPAT assays were performed at pH 5.5, exposed to X-ray film, and then stained with Coomassie blue to visualize the colonies, as described in Materials and Methods. A: Coomassie blue-stained colonies. B: X-ray film showing DHAPAT activity associated with each colony. Horizontal arrows indicate DHAPAT-positive colonies, and vertical arrows indicate colonies displaying no DHAPAT activity.

TABLE 1. DHAPAT activities and ethanolamine phospholipid content in CHO strains

Strain	DHAPAT Activity	Relative Phospholipid Level	
		Plasmenyl	Diacyl
	pmol/min/mg	*% of total*	
CHO-K1	418 ± 78	9.0 ± 0.4	12.1 ± 0.7
NRel-4[a]	ND[b]	0.8 ± 0.1[b]	21.5 ± 0.6[b]
Isolate 4.1	78 ± 14[b]	3.6 ± 0.5[b]	19.0 ± 0.6[c]
Isolate 4.2	41 ± 13[b]	4.9 ± 0.2[b]	18.3 ± 0.3[c]
Isolate 4.15	2,454 ± 151[b]	9.1 ± 0.5	10.6 ± 0.5[c]

Dihydroxyacetonephosphate acyltransferase (DHAPAT) was assayed at pH 5.5 using whole-cell homogenates, as described in Materials and Methods. The relative levels of phosphatidylethanolamine (diacyl) and plasmenylethanolamine (plasmenyl) were determined by steady-state labeling (72 h) with inorganic phosphate (^{32}Pi), as described in Materials and Methods; values represent the percentage of total phospholipid-associated radioactivity. The phosphatidylethanolamine fraction contains small amounts of plasmanylethanolamine. All values represent averages ± SD of three samples assayed in one experiment. ND, <10 pmol/min/mg.

[a] NRel-4 cells were transfected with CMV vector with no gene insert as a control.

[b] Value (or group of closely clustered values) is significantly different ($\alpha < 0.01$) from the value obtained for CHO-K1 with Tukey's honestly significant difference (HSD) multiple comparison procedure.

[c] Value (or group of closely clustered values) is significantly different ($\alpha < 0.05$) from the value obtained for CHO-K1 with Tukey's HSD multiple comparison procedure.

TABLE 2. Subclass distribution within the ethanolamine and choline phospholipids in CHO-K1 and isolate 4.15

	Ethanolamine Phospholipids			Choline Phospholipids		
Strain	Plasmenyl	Plasmanyl	Diacyl	Plasmenyl	Plasmanyl	Diacyl
	% of head group class					
CHO-K1	45.8 ± 2.0	5.6 ± 2.9	48.6 ± 4.0	0.4 ± 0.5	25.4 ± 0.6	74.2 ± 1.2
Isolate 4.15	41.8 ± 1.5	4.3 ± 3.0	53.9 ± 4.4	ND	17.5 ± 0.7[a]	82.5 ± 0.7[a]

Cellular lipids were extracted and choline and ethanolamine phospholipids were isolated as described in Materials and Methods. Phospholipids were treated with phospholipase C, and the resulting diradylglycerols were benzoylated using benzoic anhydride. Benzoylated derivatives of the different radyl species, diacyl, alkylacyl (plasmanyl), and alkenylacyl (plasmenyl), were separated and quantitated as described in Materials and Methods. All values represent averages ± SD of three samples within one experiment. ND, not detected.

[a] Value versus CHO-K1 ($P \leq 0.001$) as determined by Student's *t*-test. All other comparisons yielded *P* values >0.05.

The phospholipid composition was determined using steady-state ^{32}Pi labeling. Although plasmenylethanolamine, the predominant plasmalogen species in CHO cells, was reduced in NRel-4, levels were returned to normal in NRel-4.15 (Table 1). Isolates 4.1 and 4.2, which displayed only minor increases in DHAPAT activity, recovered plasmenylethanolamine to 45–55% of wild-type levels. With the exception of the ethanolamine subclass levels, there were no other significant changes in head group composition regardless of DHAPAT activity (data not shown).

The possibility existed that increased DHAPAT activity would result in the accumulation of the alkylacyl (plasmanyl) subclass. These are not separated from the diacyl subclass using the two-dimensional TLC system described above. Therefore, a more detailed analysis of the subclass distribution of the ethanolamine and choline phospholipids was performed (**Table 2**). Although plasmenylethanolamine (alkenylacyl) was the predominant ether-linked species in the ethanolamine phospholipids, there was little or none of this form in the choline phospholipids; plasmanylcholine (alkylacyl) was the dominant ether-linked species in this head group class. There was no increase in the amount of any ether-linked phospholipid species over wild-type levels in isolate 4.15.

Northern analysis (**Fig. 3**) using a probe directed to the 5′ end of the message for CHO-K1 revealed a band in CHO-K1 corresponding to ~2 kb, the size of the published sequences from mouse (29) and human (14). This band is greatly reduced or missing in NRel-4. NRel-4.15 displayed a band with a somewhat higher molecular mass (as a result of the β-galactosidase sequence associated with the initial transcript from the vector). Western analysis, using antibody against human DHAPAT, revealed a faint band of ~72 kDa in CHO-K1 and another plasmalogen-deficient CHO strain, NZel-1 (**Fig. 4**). This was close to the known molecular mass (77 kDa) of DHAPAT. The antibody raised against the human protein did not cross-react well with the Chinese hamster protein, and it was impossible to determine if DHAPAT levels were reduced in NRel-4. Isolate 4.15, expressing the human DHAPAT, displayed a very strong signal that ran adjacent to these bands. Immunofluorescence microscopy (**Fig. 5**) showed that DHAPAT in isolate 4.15 colocalized with catalase, another peroxisomal protein.

Measurement of phospholipid biosynthesis revealed a modest increase in the synthesis rates of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin, whereas phosphatidylinositol biosynthesis remained unchanged in NRel-4 cells compared with CHO-K1 cells (**Table 3**). The reduction of plasmenylethanolamine labeling was offset by an increase in labeling of the nonether phospholipid, phosphatidylethanolamine. Biosynthesis rates were returned to wild-type values in isolate 4.15 with the exception of phosphatidylinositol, which was somewhat depressed. Plasmenylethanolamine synthesis was also returned to nearly normal rates.

Overall phospholipid turnover was examined by monitoring the loss of label from phospholipid pools in cells that had been prelabeled with ^{32}Pi as described previously (17). The loss of label was identical in CHO-K1 and NRel-4

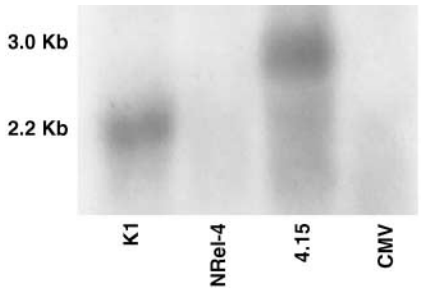

Fig. 3. DHAPAT loss in NRel-4 is attributable to a reduction in mRNA levels. Total RNA was separated and probed for DHAPAT message as described in Materials and Methods. Northern analysis, using probes for β-actin, gave similar signals in all lanes (not shown).

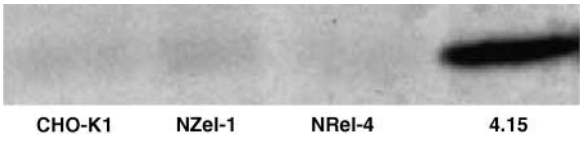

Fig. 4. Western blot analysis shows overexpression of DHAPAT in isolate 4.15. Proteins were loaded and separated on a 12% SDS-PAGE gel and blotted onto polyvinylidene difluoride membranes. Blots were processed with anti-DAPAT antibody followed by horseradish peroxidase-conjugated secondary antibody and chemiluminescent reagents to visualize the bands, as described in Materials and Methods. NZel-1 is another plasmalogen-deficient variant of CHO-K1 with normal levels of DHAPAT activity (18). Plasmalogen loss in this cell line is attributable to a deficiency in alkyl-DHAP synthase.

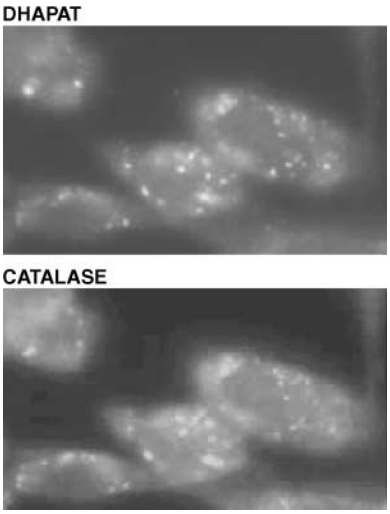

Fig. 5. Immunofluorescence microscopy shows colocalization of DHAPAT with catalase in isolate 4.15. DHAPAT and catalase were visualized using antibody and secondary antibody labeled with FITC (DHAPAT) or tetramethyl rhodamine isothiocyanate (catalase), as described in Materials and Methods.

cells, with the loss of ~50% of the label after 48 h (data not shown).

Results from labeling of the cellular lipids with [9,10-^{3}H] oleic acid were somewhat different from those observed with ^{32}Pi labeling (**Table 4**). There was increased labeling in only the ethanolamine phospholipid fraction in NRel-4 (versus CHO-K1); however, overexpression of DHAPAT in isolate 4.15 resulted in reduced uptake of oleate into all phospholipids except the phosphatidylinositol/phosphatidylserine fraction. As expected, labeling of alkyldiacylglycerols was dramatically reduced in NRel-4, and this was reversed in isolate 4.15. NRel-4 displayed increased labeling in the cholesterol ester fraction, with a return to wild-type levels in the transfectant. Triglyceride labeling was unaffected in NRel-4 but slightly reduced in the transfectant.

DHAPAT activity can be measured over a very broad pH range (30). All three cell lines displayed DHAPAT activity when measured at pH 7.4 (**Table 5**). There was significant residual DHAPAT activity in mutant homogenates when measured at this pH, although this was reduced compared with values from CHO-K1. As expected, activity in the transfectant was increased ~4-fold over the wild-type activity. When Gro-3-PAT activity was measured, NRel-4 cells displayed a 60% increase in activity compared with CHO-K1 cells. Gro-3-PAT activity was returned to levels similar to those in CHO-K1 in isolate 4.15.

DISCUSSION

Mammalian DHAPAT is a peroxisomal protein that is targeted to the peroxisome through the peroxisome targeting signal type 1 (PTS1) (31). A number of human genetic disorders have been described in which the primary lesion is a disruption in the targeting of peroxisomal proteins such as DHAPAT (32). This results in a loss of DHAPAT activity and decreased plasmalogen biosynthesis, as in NRel-4 cells. However, when we initially described the isolation of the NRel-4 cell line, we reported that alkyl-DHAP synthase activity (another peroxisomal protein), very long chain fatty acid (e.g., C26) levels, and phytanic acid oxidation were normal (19). Also, immunofluorescence microscopy showed catalase [which also uses the PTS1 system (33)] to be localized in discrete organelles, and digitonin-mediated release of catalase activity from the cells was latent. All of these data led us to the conclusion that NRel-4 cells have normal, functional peroxisomes and that the targeting peroxisomal systems were functional. The primary lesion in NRel-4 appeared to be a defect in the structural gene for DHAPAT. The data presented here confirm this. Northern analysis demonstrated a severe reduction in message for DHAPAT and transfection of NRel-4 with the human DHAPAT resulted in recovered DHAPAT activity, recovered plasmalogen biosynthesis, and colocalization of DHAPAT with catalase. The latter results also confirmed a functional PTS1 system. The NRel-4 cell line, therefore, can be used to examine the biosynthesis and functions of ether lipids without the complications associated with the loss of other peroxisomal functions.

TABLE 3. Short-term ^{32}P labeling of cellular phospholipids

	^{32}Pi Labeling of Phospholipid Classes					
Strain	CerPCho	PtdCho	PtdIns	PtdSer	PtdEtn	PlsEtn
	cpm/μg cellular protein					
CHO-K1	4.1 ± 0.3	200.7 ± 10.0	96.9 ± 6.1	5.4 ± 0.4	60.4 ± 3.2	54.0 ± 3.4
NRel-4[a]	7.8 ± 1.0[b]	325.2 ± 13.5[c]	100.7 ± 4.1	10.6 ± 0.6[c]	130.2 ± 5.0[c]	10.0 ± 0.7[c]
Isolate 4.15	4.0 ± 0.2	231.0 ± 20.0	69.6 ± 7.0[c]	3.8 ± 0.5[b]	52.6 ± 3.0	43.3 ± 5.9

Cells were incubated in growth medium containing ^{32}Pi (20 μCi/ml) at 37°C for 3 h. Cellular lipids were extracted and separated, and the amount of radioactivity associated with each phospholipid class was quantitated as described in Materials and Methods. All values represent averages ± SD of three samples within one representative experiment. CerPCho, sphingomyelin; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PlsEtn, plasmenylethanolamine.

[a] NRel-4 cells were transfected with CMV vector with no gene insert as a control.

[b] Value (or group of closely clustered values) is significantly different ($\alpha < 0.05$) from the value obtained for CHO-K1 with Tukey's HSD multiple comparison procedure.

[c] Value (or group of closely clustered values) is significantly different ($\alpha < 0.01$) from the value obtained for CHO-K1 with Tukey's HSD multiple comparison procedure.

TABLE 4. Short-term uptake of [9,10-^{3}H]oleate into lipid fractions

Strain	[9,10-^{3}H]Oleate Incorporated over 3 h					
	PtdCho	PtdIns/PtdSer	PtdEtn	TG	ADG	CE
	cpm/μg protein					
CHO-K1	569 ± 87	85 ± 3	73 ± 4	458 ± 70	28 ± 5	133 ± 4
NRel-4[a]	682 ± 62	84 ± 13	117 ± 18	484 ± 27	4 ± 1[b]	208 ± 36[c]
Isolate 4.15	448 ± 34	79 ± 18	59 ± 6[b]	411 ± 11	31 ± 3	99 ± 6

Cells were incubated in growth medium containing 2 μM [9,10-^{3}H]oleate (5 μCi/ml) at 37°C for 3 h. Cellular lipids were extracted and separated, and the amount of radioactivity associated with each lipid class was quantitated as described in Materials and Methods. All values represent averages ± SD of three samples in one representative experiment. PtdCho, phosphatidylcholine, PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdEtn, ethanolamine phospholipids; TG, triglycerides; ADG, alkyldiacylglycerols; CE, cholesteryl esters.

[a] NRel-4 cells were transfected with CMV vector with no gene insert as a control.

[b] Value (or group of closely clustered values) is significantly different ($\alpha < 0.01$) from the value obtained for CHO-K1 with Tukey's HSD multiple comparison procedure.

[c] Value (or group of closely clustered values) is significantly different ($\alpha < 0.05$) from the value obtained for CHO-K1 with Tukey's HSD multiple comparison procedure.

The factors that regulate the production and levels of ether-linked phospholipids, including plasmalogens, in animal cells are not well understood. Previous studies argue that early steps in the pathway for plasmalogen biosynthesis are not involved in regulating plasmalogen levels; attempts to increase plasmalogen levels in both cultured cells (34) and animal tissues (35, 36) by media supplementation or feeding with alkylglycerols, which enter the biosynthetic pathway downstream of the first three steps in the pathway, failed to significantly increase plasmalogen levels, even though these compounds were incorporated into the plasmalogen pools. Recently, however, we demonstrated a 1.6- to 2-fold increase in plasmalogens in human endothelial cells when grown in alkylglycerol-supplemented medium (37). This was attributable, primarily, to a large increase in plasmenylcholine (although plasmenylethanolamine levels were also increased significantly). The question, therefore, remained of whether enzymes catalyzing the earlier steps in the pathway, such as DHAPAT, could be rate-limiting in the synthesis of plasmalogens.

The data presented here demonstrate that peroxisomal DHAPAT is absolutely required for plasmalogen biosynthesis. This is in agreement with evidence presented by Ofman, Lajmir, and Wanders (38) and Rodemer et al. (39). The evidence presented here also argues against this activity as a controlling factor in plasmalogen biosynthesis. First, the recovery of only a small amount of activity in isolates 4.1 and 4.2 resulted in substantial increases in plasmenylethanolamine content. These data agree with data from a DHAPAT-deficient variant of the murine macrophage cell line, 264.7. This variant strain, RAW.7, displayed 3.5% of parental DHAPAT activity, but plasmalogen levels were reduced by only 55% (40). Second, overexpression of peroxisomal DHAPAT activity in the NRel-4.15 transfectant did not result in increases in plasmalogen biosynthesis or increase their levels above normal. These results are in agreement with the observation that plasmalogen levels are not increased in differentiating 3T3-L1 cells, in which DHAPAT is induced several-fold (16).

Our findings here demonstrate that significant changes in peroxisomal DHAPAT activity do not influence the production of diacylated glycerophospholipids or triacylglycerols. Loss or overexpression of this activity had little or no effect on triacylglycerol production, whereas loss of DHAPAT activity actually resulted in a modest increase in glycerophospholipid biosynthesis. These findings appear to be in contrast with those of Hajra et al. (16), who reported induction of peroxisomal DHAPAT activity in differentiating 3T3-L1 cells. However, acyl/alkyl-DHAP reductase is also upregulated several-fold in these cells. It is possible that this latter activity, required for the reduction of the ketone at the *sn*-2 position and the formation of lysophosphatidic acid, is rate-limiting in animal cells and must also be induced for DHAPAT to contribute to the synthesis of nonether glycerolipids. The reductase may also be important for the transport of lysophosphatidic acid produced in the peroxisome to the endoplasmic reticulum for further metabolism.

It is not clear why Gro-3-PAT activity increased in NRel-4. It does not appear to be related to the loss of ether lipids; other ether lipid-deficient strains have not demonstrated a similar increase (18). We thought that it may be related to a loss of peroxisomal DHAPAT message, because recovery of peroxisomal DHAPAT activity in isolate 4.15 re-

TABLE 5. DHAPAT and Gro-3-PAT activities in whole-cell homogenates at pH 7.4

Strain	Specific Activities	
	DHAPAT	Gro-3-PAT
	pmol/min/mg	
CHO-K1	423 ± 9	2,240 ± 20
NRel-4[a]	168 ± 3[b]	3,560 ± 40[b]
Isolate 4.15	1,565 ± 50[b]	2,000 ± 24[b]

DHAPAT and glycerol-3-phosphate acyltransferase (Gro-3-PAT) were assayed at pH 7.4 using whole-cell homogenates as described in Materials and Methods. All values represent averages ± SD of three samples within one representative experiment.

[a] NRel-4 cells were transfected with CMV vector with no gene insert as a control.

[b] Value (or group of closely clustered values) is significantly different ($\alpha < 0.01$) from the value obtained for CHO-K1 with Tukey's HSD multiple comparison procedure.

stored Gro-3-PAT activity to a wild-type-like level. However, of three additional, independently isolated CHO strains deficient in peroxisomal DHAPAT activity, all within the same complementation group as NRel-4 and all displaying greatly reduced message for peroxisomal DHAPAT (none detected), only one showed a significant increase in Gro-3-PAT (A. G. Karthik, unpublished observations).

In summary, we have shown that acyl-DHAP, produced by peroxisomal DHAPAT, is absolutely required for plasmalogen biosynthesis in animal cells and that this activity does not appear to be a limiting factor in plasmalogen biosynthesis in CHO cells. We have also shown that this activity does not contribute, independently of other changes, to the synthesis of diacyl glycerolipids.

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