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Cell Biology

A fibroblast cell line defective in alkyl-dihydroxyacetone phosphate synthase: A novel defect in plasmalogen biosynthesis

(ether lipids/catalase/peroxisomal disorders/somatic cell mutants)

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ABSTRACT Using fluorescence-activated cytotoxicity selection, followed by colony autoradiographic screening of the surviving population, we have isolated a unique plasmalogen-deficient Chinese hamster ovary (CHO) cell line. The mutant, NZel-1, showed a dramatic (90%) reduction in the rate of biosynthesis and levels of plasmalogens, as determined using short- and long-term labeling with $^{32}P_i$. Enzymatic assays and lipid supplementation studies showed that NZel-1 was defective in a single step in the biosynthetic pathway for plasmalogens. This step, catalyzed by the peroxisomal enzyme, alkyl-dihydroxyacetone phosphate (DHAP) synthase, is responsible for the introduction of the ether bond found in plasmalogens. The activity of alkyl-DHAP synthase was reduced in whole-cell homogenates from NZel-1 to 18% of wild-type values. Unlike previously described plasmalogen-deficient mutants, NZel-1 contained peroxisomes, as confirmed by immunofluorescence microscopy and catalase release by digitonin. Peroxisomal functions, including the breakdown of very long-chain (>20 carbons) fatty acids, phytanic acid oxidation, and the acylation of DHAP, were normal. Cell fusion studies revealed that the mutation is recessive and belongs to a new complementation group. To our knowledge this is the first report describing the isolation and characterization of a mutant CHO cell line defective in plasmalogen biosynthesis which contains intact, functional peroxisomes. These cells will allow us to examine the role of ether lipids in cellular functions without complications associated with peroxisome deficiency.

Plasmalogens are a subclass of glycerophospholipids characterized by the presence of a vinyl ether linkage at the *sn*-1 position of the glycerol backbone instead of the acyl linkage (1). Plasmalogens are found in all mammalian tissues to varying extents, with particular enrichment in muscle, heart, and brain (1), where they can constitute a major portion of the ethanolamine and choline phospholipids. Their roles in cellular function are not understood. We have attempted to generate and isolate plasmalogen-deficient mutants in somatic cell lines, such as the Chinese hamster ovary (CHO) cell line in an effort to gain insight into factors that regulate plasmalogen biosynthesis and their function in animal cells. Previous attempts at mutant isolation have employed a selection procedure that involved exposure of the mutagenized population to a fluorescent fatty alcohol (P9OH) followed by irradiation to UV light. This procedure succeeded in the isolation of plasmalogen-deficient cell lines, but the primary lesion in all of these has always been a defect in peroxisome assembly (2–4). The first two steps in plasmalogen biosynthesis are catalyzed by the peroxisomal enzymes, peroxisomal dihydroxyacetone phosphate (DHAP)-acyltransferase (ATase), and alkyl-DHAP synthase. These activities are lost in peroxisome-deficient cells and, therefore, the loss of plasmalogen biosynthesis is a phenotype that invariably accompanies peroxisome-deficient cells.

Aperoxisomal mutants have been instrumental in the isolation of factors involved in peroxisome assembly and function (5). These point to the central role of peroxisomes in plasmalogen biosynthesis and have served as useful somatic cell models for exploring inborn human disorders of peroxisome biogenesis such as the Zellweger syndrome (3). However, the loss of an organelle that is important to lipid metabolism makes any conclusions concerning the role of plasmalogens in cell function, or in the pathophysiology of human aperoxisomal disorders, unclear.

The fact that survivors of the P9OH/UV selection were consistently aperoxisomal caused us to suspect that the crucial determinant for this selection was the ability or inability of a cell to assemble peroxisomes (4). However, the isolation of plasmalogen$^-$/peroxisome$^+$ mutants from another cell line using P9OH/UV technique demonstrated that this was not the case (6). These mutants, from the murine macrophage-like cell line, RAW 264.7, were defective in peroxisomal DHAP-ATase and plasmenylethanolamine desaturase activities, but contained functional peroxisomes (7). These results prompted us to perform a more rigorous search for plasmalogen$^-$/peroxisome$^+$ mutants from the CHO-K1 cell line. We report the isolation of a unique mutant from this line that is defective in plasmalogens due to a dramatic decrease in alkyl-DHAP synthase activity. This activity is responsible for the formation of the ether bond found in this class of phospholipids. Although this represented the loss of a peroxisomal activity, these mutants contained intact, functional peroxisomes.

EXPERIMENTAL PROCEDURES

Materials. 9-(1′-Pyrene)nonanol (P9OH) was purchased from Molecular Probes. [1-^{3}H]Ethanolamine (30 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham. [γ-^{32}P]ATP and EN^{3}HANCE spray were obtained from DuPont/NEN. Dihydroxyacetone [^{32}P]phosphate was synthesized by enzymatic phosphorylation of dihydroxyacetone using [γ-^{32}P]ATP and glycerol kinase (2, 8). 1-Acyl-DHAP and 1-alkyl-DHAP were synthesized according to Hajra *et al.* (9). Ecoscint A liquid

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Abbreviations: DHAP, dihydroxyacetone phosphate; ATase, acyltransferase; G3P, glycerol-3-phosphate; PE, phosphatidylethanolamine.

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scintillation fluid was obtained from National Diagnostics. Silica gel 60 TLC plates (Merck) were purchased from American Scientific Products (McGaw Park, IL). Titanyl sulfate was obtained from Chemtech (Hayward, CA). Tissue culture dishes (Corning) were obtained from VWR Scientific. Polyester cloth (17-μm mesh) was purchased from Tetko (Elmsford, NY). Phosphatidylethanolamine (PE) standard from bovine brain was purchased from Doosan/Serdary Chemicals (Englewood Cliffs, NJ), and total bovine heart lipids were purchased from Avanti Polar Lipids. All other reagents, unless specified, were purchased from Sigma.

Cells and Culture Conditions. CHO-K1 cells were obtained from the American Type Culture Collection. ZR-82 is a peroxisome$^-$/plasmalogen$^-$ derivative of CHO-K1 (2). Mutageneses were performed using ethyl methanesulfonate at 300 μg/ml for 20 h at 37°C. Following mutagenesis, the cells were allowed to grow for 7 days before selection. All cell lines were maintained in Ham's F-12 or RPMI 1640 medium (BioWhittaker) supplemented with 10% fetal bovine serum (BioWhittaker), 1 mM glutamine, 100 units/ml penicillin G, and 75 units/ml streptomycin. For all experiments, selection, and mutagenesis, CHO cells were plated into tissue culture dishes as adherent monolayers and maintained in an atmosphere of 5% CO_2/95% air.

Selection of Ether Lipid-Deficient Mutants. Mutants were selected from a mutagenized population of CHO-K1 cells using the P9OH/UV selection technique (4). Mutagenized CHO-K1 cells were plated out in a 100-mm-diameter tissue culture dish (5×10^5/dish) in 10 ml of medium and allowed to attach overnight at 33°C. The following day, 5 ml of medium containing 60 μM P9OH was added (final concentration, 20 μM). After 3 h at 37°C, the P9OH-containing medium was removed and replaced with 15 ml of P9OH-free medium. The cells were incubated at 37°C for another 1 h and then irradiated for 5 min with long wavelength UV light. Irradiation was accomplished by placing the dishes on a 1.5-mm-thick glass plate that was suspended ≈10–15 cm over the UV source (Black-Ray UV lamp, model XX-15L; Ultraviolet Products, San Gabriel, CA). This resulted in a UV intensity of 2,200 $\mu W/cm^2$ at the surface of the glass plate. The survivors from six dishes were combined and placed through a second (10^4 cells/dish; 20 μM P9OH; 5′ UV) and a final more stringent (10^4 cells/dish; 20 μM P9OH; 10′ UV) round of P9OH/UV selection. The surviving population was screened for peroxisomal DHAP-ATase activity using colony autoradiography as described in Fig. 1.

Enzymatic Assays. Glycerol-3-phosphate (G3P)-ATase, DHAP-ATase, alkyl-DHAP synthase, and alkyl/acyl-DHAP reductase activities were all measured in whole-cell homogenates. Cells were grown to near confluence in 100-mm-diameter tissue culture dishes, and harvested in ice-cold PBS using a rubber policeman. Following centrifugation, the pellet was resuspended in 1 ml of 0.25 M sucrose and 1 mM EDTA (pH 7.4) and frozen at −80°C. Cell suspensions were thawed on ice and resuspended using a Teflon-glass tissue homogenizer. Peroxisomal and microsomal DHAP-ATase and G3P-ATase activities were measured as described by Jones and Hajra (10). Alkyl-DHAP synthase was assayed as described by Davis and Hajra (11). 1-Acyl-DHAP/1-alkyl-DHAP reductase was assayed by the method of LaBelle and Hajra (12). Protein content was determined using the method of Lowry *et al.* (13).

Cell Fusions. Cells were fused using polyethylene glycol (PEG) (M_r 3350) by the method of Kennett (14) as modified (3). In each fusion pair, one of the cell lines bore the secondary lesions, hypoxanthine phosphoribosyltransferase-deficiency and ouabain-resistance. Hybrid cells were grown in hypoxanthine/aminopterin/thymidine (HAT)/ouabain selection medium [aminopterin (0.65 μM), hypoxanthine (100 μM), thymidine (15 μM), and ouabain (1 mM)] for 2 weeks before use in experiments.

RESULTS

P9OH/UV Selection. Approximately 3×10^6 mutagenized CHO-K1 cells were treated with the pyrene-labeled long-chain fatty alcohol, P9OH, followed by exposure to long-wavelength (>300 nm) UV light. Studies by Morand *et al.* (4) have shown that this fluorescent lipid is taken up by cells and is incorporated into ether lipids, as either the fatty alcohol or as the fatty acid following oxidation. Cells are killed upon UV irradiation, presumably due to the generation of singlet oxygen (15). Plasmalogen-deficient cells take up much less P9OH than the wild-type cells and are, therefore, less susceptible to UV irradiation that follows treatment with this compound. In this study the mutagenized population of cells were subjected to three rounds of P9OH/UV selection. Under these conditions, >99% killing was observed during the initial round of selection. Subsequent rounds of selection resulted in much less killing to the point that the third round killed few of the mutant cells while wild-type cells did not survive (data not shown).

Cells that do not contain intact peroxisomes do not properly express the peroxisomal form of the enzyme, DHAP-ATase (pDHAPAT), and we reasoned that any cell expressing pDHAPAT would contain intact peroxisomes. Therefore, the P9OH/UV-resistant population was screened for isolates that expressed this enzyme, using colony autoradiography (2, 16). We identified 3 positive colonies in a field of ≈150 colonies (Fig. 1). These were recovered from the master dish, and clonal isolates were generated from each colony using limiting dilution. Since hybridization analyses showed that all three isolates

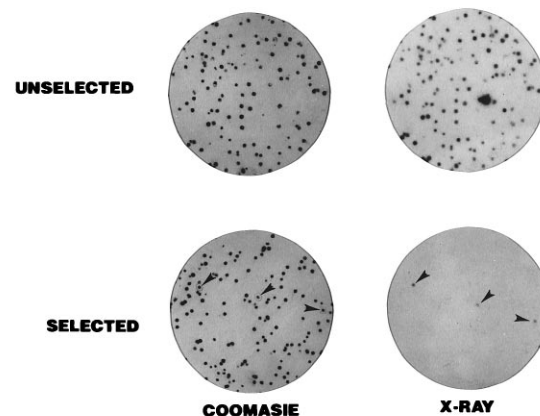

FIG. 1. Screening of the P9OH/UV-resistant population for peroxisomal DHAP-ATase activity by colony autoradiography. Cells were plated out into 100-mm-diameter tissue culture dishes at a concentration of 400 cells/dish and allowed to attach overnight. The cells were overlaid with a sterile polyester cloth (17) and left undisturbed at 37°C for 9 days to allow for the formation of colonies of cells both on the master dish and the polyester. The polyesters were removed, rinsed three times in 100 ml ice-cold PBS, and placed at −80°C to lyse the cells. The master dishes were fed with fresh medium and placed at 28°C to keep the colonies viable. Each polyester was thawed and placed in 3 ml of a solution containing 100 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 100 mM Mes (pH 5.5), 100 μM palmitoyl-CoA, 1.5 mM [^{32}P]DHAP (4–6 μCi/μmol), 8 mM NaF, 5 mM $MgCl_2$, 50 mM KCl, 2 mM KCN, and 2 mg/ml bovine serum albumin (2, 8). After 15 min at 40°C, 3 ml 20% trichloroacetic acid (TCA) was added to precipitate the radioactive product (1-acyl-DHAP). The polyesters were washed three times with 50 ml 3% TCA and exposed to GBX-2 x-ray film following preflash. Following autoradiography, the colonies were visualized by staining with Coomassie blue (18). All of the colonies from the unselected population were DHAPAT$^+$, as indicated by the corresponding signal on the x-ray film. Only three colonies yielded a signal in the P9OH/UV-selected population.

Table 1. DHAP and G3P ATase activities in cell homogenates

	DHAP-ATase, pmol/min per mg		G3P-ATase, nmol/min per mg
Cell line	pH 5.5	pH 7.4	pH 7.5
CHO-K1	283 ± 16	451 ± 20	8.9 ± 0.25
ZR-82	19 ± 2	167 ± 17	6.4 ± 0.27
NZel-1	278 ± 9	365 ± 14	7.6 ± 0.37

Whole-cell homogenates were prepared and ATase activities were assayed as described. DHAP and G3P ATase activities represent the average ± SD of three samples. Assays at pH 5.5 were performed in the presence of 5 mM *N*-ethylmaleimide.

were within the same complementation group (data not shown), one isolate, designated NZel-1, was used for further characterizations.

Whole-cell homogenates from NZel-1 were assayed for DHAP-ATase activity (2) (Table 1). Both the peroxisomal (measured at pH 5.5) and the microsomal (measured at pH 7.4) forms of DHAP-ATase, as well as the G3PAT (G3P-ATase) activities, were normal in NZel-1 when compared with the wild-type cells. Homogenates from the previously described, plasmalogen/peroxisome-deficient CHO mutant, ZR-82 (2), showed only 7% of the parental activity at pH 5.5, consistent with previous results using this strain (2). The loss of activity at pH 7.4 in ZR82 homogenates was due to the loss of the peroxisomal form, which contributes to the activity at this pH (2).

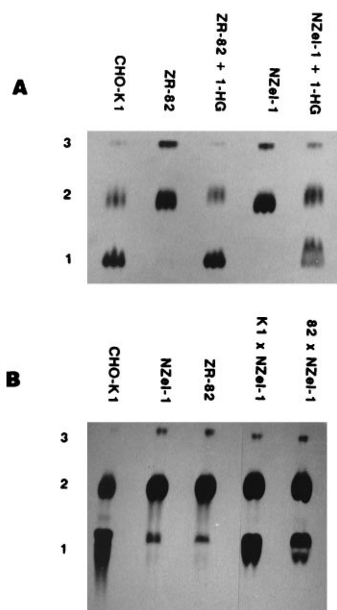

FIG. 2. Subspecies patterns of ethanolamine phospholipids in wild-type cells, mutants, and fusates. Cells (2.5×10^5) were grown for 18 h in sterile glass scintillation vials at 37°C, in medium containing [1-^{3}H]ethanolamine (2 μCi/ml) with or without 20 μM 1-hexadecylglycerol (1-HG) (*A*). The medium was removed, the cells were washed once with 2 ml PBS, and the lipids were extracted in 3.8 ml $CHCl_3$/methanol/PBS (1:2:0.8) containing 200 μg of a carrier lipid (beef brain PE). After transfer to test tubes, 1 ml $CHCl_3$ and 1 ml PBS were added to form the two-phase Bligh and Dyer system (19) 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 (14). The labeled species were visualized by autoradiography at −80°C after spraying the plates with EN^{3}HANCE. Hybrid cell lines (*B*) were generated as described. The first cell line listed in the hybrid pairings were the lines that carried the secondary mutations required for hybrid selection. Band 1, plasmenylethanolamine (R_f = 0.3); band 2, PE (R_f = 0.5); band 3, unknown (R_f = 0.9).

NZel-1 Cells Are Plasmalogen-Deficient and Belong to a New Complementation Group. NZel-1 cells were much less able to synthesize plasmenylethanolamine, the major plasmalogen species in CHO-K1 cells (2), as measured by incorporation of [1-^{3}H]ethanolamine into this phospholipid (Fig. 2*A*). In wild-type CHO cells, label was equally distributed between the plasmenylethanolamine and the PE bands. Very little of the label was found in plasmenylethanolamine in NZel-1 cells, similar to the mutant ZR-82. The plasmenylethanolamine labeling could be restored to a wild-type-like pattern in both NZel-1 and ZR-82 by supplementation with *sn*-1-hexadecylglycerol, which enters the biosynthetic pathway downstream of the third step as 1-alkyl-2-lyso-*sn*-G3P (20).

The genetic lesion responsible for the plasmalogen deficiencies found in NZel-1 and ZR-82 were different. Hybrid populations resulting from the fusion of the two cell lines displayed a wild-type-like [^{3}H]ethanolamine labeling pattern (Fig. 2*B*), demonstrating that the cell lines are in different complementation groups. These results, along with the fact that fusion of NZel-1 with wild type, resulted in plasmalogen$^+$ hybrids showed that the mutation is recessive.

Long-term labeling of the phospholipids with $^{32}P_i$ confirmed a drastic reduction in plasmenylethanolamine in NZel-1 cells. Quantitation of the $^{32}P_i$-labeled phospholipids (Table 2) showed that, while the plasmalogen species makes up ≈9.5% of the total phospholipid in the wild-type cells, it is reduced by a factor of 10 in the mutant strain NZel-1. Interestingly, the overall level of ethanolamine phospholipids in the mutant strain remained constant. The relative amounts of other major phospholipid head group species also remained unaffected. These results were consistent with the results obtained from the short-term $^{32}P_i$ - labeling of the phospholipids, in which the only differences between the parental and mutant strains were a dramatic (>80%) decrease in the rate of plasmenylethanolamine biosynthesis and a corresponding increase in the synthesis of the diacyl form, PE (data not shown).

NZel-1 Is Defective in Alkyl-DHAP Synthase. Peroxisomal DHAP-ATase activity, catalyzing the first step in plasmalogen biosynthesis, was normal in NZel-1 cells (Table 1). Also, *sn*-1-hexadecylglycerol was able to bypass the plasmalogen deficiency (Fig. 2), demonstrating that events downstream of

Table 2. Phospholipid composition of parental and mutant cell lines

	Phospholipid composition, % total					
Cell	SPH	PC	PI	PS	pPE	PE
CHO-K1*	7.3	67.0	2.3	1.7	9.5	10.5
NZel-1†	6.6	68.0	2.6	2.1	0.95	19.0

Cells were grown for 72 h in 75-cm^2 tissue culture flasks at 37°C in medium supplemented with $^{32}P_i$ (5 μCi/ml) to label the phospholipids to constant specific activity. The medium was removed, the cells were harvested using trypsin, pelleted by centrifugation at $600 \times g$ for 7 min, and resuspended in 0.8 ml PBS. This suspension was added to 3 ml $CHCl_3$/methanol (1:2) and the lipids were extracted twice by the method of Bligh and Dyer (19), following the addition of 300 μg of carrier lipid (total lipid from bovine heart). Phospholipids were extracted using two-dimensional TLC as described (21). Plates were exposed to GBX-2 x-ray film at −80°C following preflash. $^{32}P_i$-labeled phospholipid species were scraped from the TLC plates directly into scintillation vials containing 1 ml methanol. After the addition of 8 ml scintillation fluid the radioactivity was quantitated by liquid scintillation spectrometry. All values represent the percent of the total radioactivity found in phospholipids. Other lipid species, which represented 5–10% of the chloroform soluble counts were not included in the calculations and did not vary significantly. SPH, sphingomyelin; PC, phosphatidylcholine-diacyl form; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine-diacyl form; pPE, phosphatidylethanolamine-plasmalogen form.

*Values represent the average of two samples and vary by <10%.

†Values represent the average of three samples and vary by <10%.

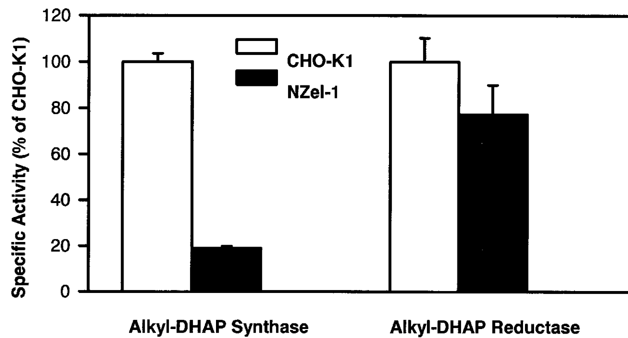

FIG. 3. Alkyl-DHAP synthase and alkyl-DHAP reductase activities in CHO-K1 and NZel-1. Assays were performed on whole cell homogenates as described. All values represent the averages ± SD of three experiments.

the third step in the pathway were not affected. These findings suggested that either alkyl-DHAP synthase or alkyl-DHAP reductase activity, which catalyze the second and third steps, respectively, had been affected. Examination of these activities in whole-cell homogenates revealed that alkyl-DHAP synthase was reduced in NZel-1 to 18% of the wild-type values, while the activity of alkyl-DHAP reductase was normal (Fig. 3).

NZel-1 Cells Contain Intact Functional Peroxisomes. The loss of alkyl-DHAP synthase, a peroxisomal protein, in NZel-1 cells prompted us to see if these cells contained functional peroxisomes. Digitonin release (22) was used to determine the subcellular distribution of catalase. Catalase is a soluble enzyme found primarily within the peroxisome, but in mutants deficient in the assembly of peroxisomes, this activity is found in the cytosol (2, 5, 22). Low levels of digitonin (10 μg/ml) cause a selective disruption of the plasma membrane (due to its high cholesterol content), resulting in the release of soluble cytosolic proteins, such as lactate dehydrogenase. In the peroxisome-deficient mutant, ZR-82, all of the catalase activity was released along with the lactate dehydrogenase, indicating that catalase was cytosolic in this cell line (Fig. 4*A*). Much higher levels of digitonin (150–300 μg/ml) were required to release catalase from both the wild-type CHO-K1 cells and the mutant strain NZel-1. The presence of intact peroxisomes was confirmed by immunofluorescence microscopy (Fig. 4*B*). Both the wild-type CHO-K1 and the mutant NZel-1 cells showed a clear concentration of catalase in discrete organelles, presumably peroxisomes, while ZR-82 showed a diffuse labeling pattern, indicative of cytosolic catalase.

The breakdown of very long-chain fatty acids (>20 carbons in length) within the cells is attributed to the presence of a unique peroxisomal β-oxidation system (26). Loss of this system, resulting from a loss of peroxisome assembly, results in an accumulation of very long-chain fatty acids (27, 28). Measurements of 26:0 and 26:1 levels revealed that NZel-1 cells displayed only slightly elevated levels compared with wild-type cells while peroxisome-deficient ZR-82 cells contained 37 times the normal levels of very long-chain fatty acids (Table 3). Phytanic acid oxidation, another function that is defective in peroxisome-deficient cells, was found to be at least as high in NZel-1 cells as in wild-type cells (Table 3).

DISCUSSION

Although the distrubution of plasmalogens in animal cell tissues has been well documented (1, 31–33), the mechanism by which cells regulate plasmalogen levels and the role these phospholipids serve in cell function remain unclear. A series of inherited disorders, in which the patients' tissues lack plasmalogens, have been described (34, 35). These patients are

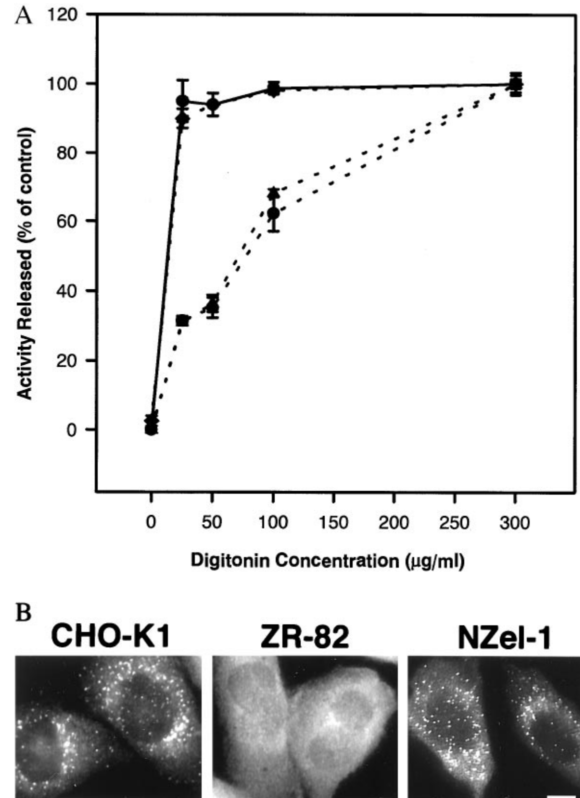

FIG. 4. Localization of catalase using digitonin release and immunofluorescence microscopy. (*A*) Catalase latency in digitonin-permeabilized cells. Cells were suspended in PBS at 1×10^7 cells/ml, and 0.3 ml aliquots were added to an equal volume of digitonin-containing PBS. After 10 min at room temperature, the suspensions were centrifuged for 40 s at $12,000 \times g$ in a microfuge (Beckman) and the supernatant used immediately for catalase and lactate dehydrogenase activity determinations. Catalase and lactate dehyrogenase activities were determined as described (23, 24). The solid line represents lactate dehydrogenase activity released by CHO-K1 cells (identical values were obtained from the other cell lines). The dotted line represents catalase activities. ●, CHO-K1; ■, NZel-1; ▲, ZR-82. All values represent the percent of total cellular catalase activity that could be released at maximal digitonin concentration (300 μg/ml). All values represent the average ± SD of three samples. (*B*) Localization of catalase using immunofluorescence microscopy. Cells were grown on coverslips in serum-supplemented RPMI 1640 medium and fixed with 4% paraformaldehyde. Fixed cells were permeabilized with 1% Nonidet P-40 in PBS and subjected to immunofluorescence microscopy as described (3) using anti-bovine liver catalase (25) followed by fluorescein-conjugated donkey anti-rabbit IgG (25).

severely mentally retarded, display a lack of muscle tone and multiple congenital anomalies, and often do not survive beyond the second year (34, 35). These disorders can be

Table 3. Very long-chain fatty acid levels and phytanic acid oxidation

Strain	C26:0 + C26:1, μg/mg protein	Phytanic acid oxidation, pmol/min per mg protein
CHO-K1	0.045	20.2
NZel-1	0.146	38.4
ZR-82	1.64	1.1

Total cellular lipids were extracted according to Bligh and Dyer (19), transesterified using 2% H_2SO_4 in methanol and the methyl esters, were analyzed by capillary gas-liquid chromatography (29). The oxidation of phytanic acid was assayed by measuring the release of water-soluble radioactivity from [2,3-^{3}H]phytanic acid-labeled cells (30). All values represent averages of duplicate cultures and vary by <10%.

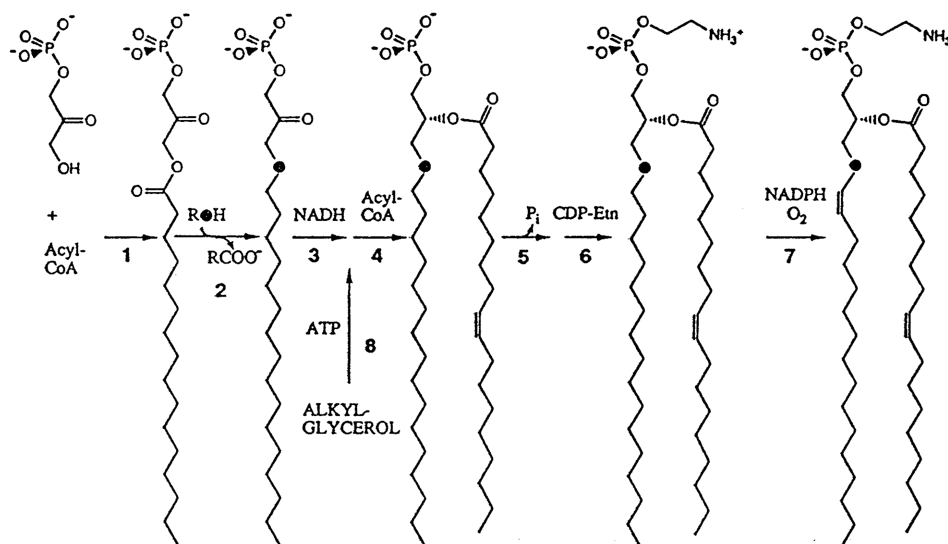

FIG. 5. Biosynthesis of plasmenylethanolamine in animal cells. The enzymatic activities that catalyze each step are as follows: step 1, peroxisomal DHAP-ATase (pDHAPAT); step 2, alkyl-DHAP synthase; step 3, acyl/alkyl-DHAP reductase; step 4, 2-lyso-phosphatidate-ATase; step 5, phosphatidate phosphohydrolase; step 6, CDP-ethanolamine/diglyceride ethanolamine phosphotransferase; step 7, plasmenylethanolamine desaturase ($\Delta 1'$-desaturase); step 8, 1-alkylglycerol kinase. Steps 1 and 2 are peroxisomal activities while the remaining activities can be found in microsomes.

subdivided into three clinical entities of Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease. In these disorders, the organelle fails to be formed normally and there are a number of biochemical abnormalities, including the inability to break down very long-chain fatty acids and eicosanoids (26, 36), alterations in cholesterol biosynthesis (37), and the redistribution of soluble peroxisomal enzymes such as catalase into the cytosol (3). The second catagory has the clinical phenotype of rhizomelic chondrodysplasia punctata (RCDP) and is associated with shortening of limbs and skeletal abnormalities. Here, peroxisome structure is usually intact, but plasmalogen synthesis and phytanic acid oxidation are severely impaired and peroxisomal 3-oxoacyl-CoA thiolase fails to be processed normally. Other human peroxisomal disorders involve a defect of a single peroxisomal protein (38). Wanders *et al.* (39) have reported a patient who exemplifies this third catagory of peroxisomal disorders. This patient showed the full range of multiple clinical defects associated with RCDP, but biochemical abnormalities were confined to a defect in DHAP-ATase activity and a resulting lack of plasmalogens. These findings suggest a critical role for plasmalogens or other ether lipids in proper neuromuscular function or development, but that role is undefined.

We have attempted to isolate mutants, from established cell lines such as CHO-K1, whose primary defect is a severe reduction in plasmalogen biosynthesis. We describe here the isolation of a unique, plasmalogen-deficient CHO variant, NZel-1, with a singular enzymatic defect in the biosynthetic pathway. Alkyl-DHAP synthase is a peroxisomal membrane-associated enzyme; however, NZel-1 contained intact, functional peroxisomes. We have previously described the isolation of plasmalogen-deficient (peroxisome^{+}) mutants using the murine, macrophage-like cell line RAW 264.7 as the parent cell line (6). These mutants were defective in peroxisomal DHAP-ATase, which catalyzes the first step in plasmalogen biosynthesis, and plasmenylethanolamine desaturase, which catalyzes the insertion of the vinyl ether double bond (Fig. 5) (7). Alkyl-DHAP synthase catalyzes the crucial second step in the pathway, forming the ether bond found in plasmalogens (Fig. 5) (40). To our knowledge, this is the first report of any somatic cell mutant defective in this activity.

The availability of mutants such as NZel-1 and the RAW variants, in which plasmalogen biosynthesis is the primary lesion, allows us to evaluate plasmalogen function without having to consider the loss of peroxisomes. For example, it has been proposed, that plasmalogens, or ether phospholipids, play an important role in the stimulated formation of eicosanoids (41), membrane fusion-mediated events such as exocytosis and endocytosis (42), and protection against active oxygen species such as singlet oxygen (43). Because peroxisomes are involved in eicosanoid (36, 44) and oxygen (45) metabolism, their loss in plasmalogen/peroxisome-deficient mutants, such as ZR-82, must be considered as a contributing factor.

The availability of plasmalogen-deficient mutants in different cell types, such as CHO (fibroblast) and RAW (macrophage), allows us to determine if a plasmalogen function is cell type-specific or common to all cell types. Mutants derived from the CHO-K1 cell line are particularly important for further genetic and biochemical analysis of plasmalogen biosynthesis in animal cells. The CHO-K1 cell line is genetically well characterized and a number of protocols have been developed and used to generate stable transfectants at a high frequency (46, 47). Peroxisome-deficient CHO mutants have been used to isolate genes that code for peroxisome assembly factors (48) through gene-mediated complementation. Similarly, mutants such as NZel-1 will allow for the isolation of structural and regulatory genes involved in plasmalogen biosynthesis and will also be useful for *in vivo* functional assays using modified gene constructs.

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1. Horrocks, L. A. & Sharma, M. (1982) in *Phospholipids*, eds. Hawthorne, J. N. & Ansell, G. B. (Elsevier, Amsterdam), p. 51.
2. Zoeller, R. A. & Raetz, C. R. H. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5170–5174.
3. Zoeller, R. A., Allen, L.-A. H., Santos, M. J., Lazarow, P. B., Hashimoto, T., Tartakoff, A. M. & Raetz, C. R. H. (1989) *J. Biol. Chem.* **264,** 21872–21878.
4. Morand, O. H., Allen, L.-A. H., Zoeller, R. A. & Raetz, C. R. H. (1990) *Biochim. Biophys. Acta* **1034,** 132–141.
5. Tsukamoto, T., Yokata, S. & Fujiki, Y. (1990) *J. Cell Biol.* **110,** 651–660.

6. Zoeller, R. A., Rangaswamy, S., Herscovitz, H., Rizzo, W. B., Hajra, A. K., Das, A. K., Moser, H. W., Moser, A., Lazarow, P. B. & Santos, M. J. (1992) *J. Biol. Chem.* **267,** 8299–8306.
7. Rangaswamy, S. & Zoeller, R. A. (1994) *Biochim. Biophys. Acta* **1211,** 79–84.
8. Schlossman, D. M. & Bell, R. M. (1976) *Arch. Biochem. Biophys.* **182,** 732–742.
9. Hajra, A. K., Saraswathi, T. V. & Das, A. K. (1983) *Chem. Phys. Lipids* **33,** 179–193.
10. Jones, C. L. & Hajra, A. K. (1980) *J. Biol. Chem.* **255,** 8289–8295.
11. Davis, P. A. & Hajra, A. K. (1981) *Arch. Biochem. Biophys.* **211,** 20–29.
12. LaBelle, E. F. & Hajra, A. K. (1974) *J. Biol. Chem.* **249,** 6936–6944.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265- 275.
14. Kennett, R. H. (1979) *Methods Enzymol.* **58,** 345–359.
15. Morand, O. M., Zoeller, R. A. & Raetz, C. R. H. (1988) *J. Biol. Chem.* **263,** 11597–11606.
16. Raetz, C. R. H., Wermuth, M. M., McIntyre, T., Esko, J. D. & Wing, D. C. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3223–3227.
17. Zoeller, R. A. & Raetz, C. R. H. (1992) *Methods Enzymol.* **209,** 34–51.
18. Esko, J. D. & Raetz, C. R. H. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1190–1193.
19. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917.
20. Snyder, F. (1992) *Methods Enzymol.* **209,** 211–215.
21. Esko, J. D. & Raetz, C. R. H. (1980) *J. Biol. Chem.* **255,** 4474–4480.
22. Wanders, R. J. A., Kos, M., Roest, B., Meijer, A. J., Schrakamp, G., Heymans, H. S. A., Tegelaers, W. H. H., van den Bosch, H., Schutgens, R. B. H. & Tager, J. M. (1984) *Biochem. Biophys. Res. Commun.* **123,** 1054–1061.
23. Peters, T. J., Muller, M. & De Duve, C. (1972) *J. Exp. Med.* **136,** 1117–1137.
24. Bergmeyer, H. U., ed (1980) *Methods of Enzymatic Analysis* (Academic, Orlando, FL), Vol. 1, pp 481–482,.
25. Santos, M. J., Imanaka, T., Shio, H., Small, G. M. & Lazarow, P. B. (1988) *Science* **239,** 1536–1538.
26. Lazarow, P. B. & De Duve, C. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2043–2046.
27. Schutgens, R. B. H., Heymans, H. S. A., Wanders, R. J. A., van den Bosch, H. & Tager, J. M. (1986) *Eur. J. Pediatr.* **144,** 430–440.
28. Moser, A. E., Singh, I., Brown, F. R., Solish, G. I., Kelley, R. I., Benke, P. J. & Moser, H. W. (1984) *N. Engl. J. Med.* **310,** 1141–1146.
29. Moser, H. W., Moser, A. B., Kawamura, N., Murphy, J., Suzuki, K., Schaumburg, H. & Kishimoto, Y. (1979) *Ann. Neurol.* **7,** 542–549.
30. Rizzo, W. B., Watkins, P. A., Philips, M. W., Cranin, D., Campbell, B. & Avigan, J. (1986) *Neurology* **36,** 357–361.
31. Sugiura, T. & Waku, K. (1987) in *Platelet Activating Factor and Related Lipid Mediators*, ed. Snyder, F. (Plenum, New York), pp. 55–85.
32. Tence, M., Jouvin-Marche, E., Bessou, G., Record, M. & Beneviste, J. (1985) *Thromb. Res.* **38,** 207–214.
33. Mueller, H. W., O'Flaherty, J. T., Greene, D. G., Samuel, M. P. & Wykle, R. (1984) *J. Lipid Res.* **25,** 383–388.
34. Kelly, R. I. (1983) *Am. J. Med. Genet.* **16,** 503–517.
35. Lazarow, P. B. & Moser, H. W. (1994) in *The Metabolic Basis of Inherited Disease*, eds. Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D. (McGraw Hill, New York), 3rd Ed., pp. 2287–2324.
36. Gordon, J. A., Zoeller, R. A. & Spector, A. A. (1991) *Biochim. Biophys. Acta* **1085,** 21–28.
37. Hodge, V. J., Gould, S. J., Subramani, S., Moser, H. W. & Krisans, S. K. (1991) *Biochem. Biophys. Res. Commun.* **181,** 537–541.
38. Subramani, S. (1993) *Annu. Rev. Cell Biol.* **9,** 445–478.
39. Wanders, R. J. A., Schumacher, H., Heikoop, J., Schutgens, R. B. & Tager, J. M. (1992) *J. Inherited Metab. Dis.* **15,** 389–391.
40. Hajra, A. K., Jones, C. L. & Davis, P. (1978) in *Enzymes of Lipid Metabolism*, eds. Gatt, S., Freysz, L. & Mandell, P. (Plenum, New York), pp. 369–378.
41. Horrocks, L. A. & Fu, S. C. (1978) *Adv. Exp. Med. Biol.* **101,** 397–406.
42. Lohner, K., Balgavy, P., Hermetler, A., Paltauf, F. & Laggner, P. (1991) *Biochim. Biophys. Acta* **1061,** 132–140.
43. Zoeller, R. A., Morand, O. H. & Raetz, C. R. H. (1989) *J. Biol. Chem.* **264,** 21872–21878.
44. Lazaro, P. B. (1995) *J. Neuropathol. Exp. Neurol.* **54,** 720–725.
45. Mannaerts, G. P. & VanVeldhoven, P. P. (1993) *Biochimie* **75,** 147–158.
46. Wigler, M., Silverstein, S., Lee, L. S., Pellicer, A., Cheng, Y. C. & Axel, R. (1977) *Cell* **11,** 223–232.
47. Hasan, M. T., Subbaroyan, R. & Chang, T. Y. (1991) *Somatic Cell Genet.* **17,** 513–517.
48. Tsukamoto, T., Miura, S. & Fujiki, Y. (1991) *Nature (London)* **350,** 77–81.