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 [32P]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, phytic 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, but have been employed in 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.

A peroxisomal mutant has 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 peroxisomal mutant was used 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 RAW264.7, were defective in peroxisomal DHAP-ATase and plasmalylolamine 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. [1-1H]Ethanolamine (30 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham. [γ-32P]ATP and ENHANCE spray were obtained from DuPont/NEN. Dihydroxyacetone [32P]phosphate was synthesized by enzymatic phosphorylation of dihydroxyacetone using [γ-32P]ATP and glycerol kinase (2, 8). 1-Acyl-DHAP and 1-alkyl-DHAP were synthesized according to Hajra et al. (9). Ecoscint A liquid contains 4% Emulsion A, 5% PPO, and 1% DROPCOUNT (Alltech Associates) is used. 

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 specified, were purchased from Sigma. P9OH; 5.5), 100 mM Mes (pH 7.4), 100 mM NaF, 5 mM MgCl₂, 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 precipitates 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.

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-aminomethanesulfonic acid, 100 mM Mes (pH 5.5), 150 mM palmitoyl-CoA, 0.5 mM [³²P]DHAP (4–6 Ci/mmol), 8 mM NaF, 5 mM MgCl₂, 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 precipitates 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.

RESULTS

P9OH/UV Selection. Approximately 3 × 10⁶ 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). Peroxisomal-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

midine (15 μM), and ouabain (1 mM) for 2 weeks before use in experiments.

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cies were visualized by autoradiography at pH 5.5 and 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%.
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). 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. 4B). 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 C26:0 and C26:1 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). Phytic 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 distribution 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 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>
<thead>
<tr>
<th>Strain</th>
<th>C26:0 + C26:1, μg/mg protein</th>
<th>Phytic acid oxidation, pmol/min per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-K1</td>
<td>0.045</td>
<td>20.2</td>
</tr>
<tr>
<td>NZel-1</td>
<td>0.146</td>
<td>38.4</td>
</tr>
<tr>
<td>ZR-82</td>
<td>1.64</td>
<td>1.1</td>
</tr>
</tbody>
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Total cellular lipids were extracted according to Bligh and Dyer (19), transesterified using 2% H2SO4 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 [2,3-3H]phytanic acid-labeled cells (30). All values represent averages of duplicate cultures and vary by <10%.
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 plasmalogens and phytic 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 plasmalogens 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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