Isolation of a Chinese hamster fibroblast variant defective in dihydroxyacetonephosphate acyltransferase activity and plasmalogen biosynthesis: use of a novel two-step selection protocol

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We have developed a two-step selection protocol to generate a population of Chinese hamster ovary (CHO) cell variants that are plasmalogen-deficient, but contain intact, functional peroxisomes (plasmalogen$^-$/peroxisome$^+$). This involved sequential exposures of a mutagenized cell population to photodynamic damage by using two different pyrene-labelled sensors, 9-(1′-pyrene)nonanol and 12-(1′-pyrene)dodecanoic acid. By this procedure we generated several isolates, all except one of which displayed a severe decrease in plasmalogen biosynthesis. Further characterization of one of the plasmalogen-deficient isolates, NRel-4, showed that it contained intact, functional peroxisomes. Whole-cell homogenates from NRel-4 displayed severely decreased dihydroxyacetone phosphate acyltransferase, which catalyses the first step in plasmalogen biosynthesis. NRel-4 and another, recently described, plasmalogen-deficient cell line, NZel-1 [Nagan, Hajra, Das, Moser, Moser, Lazarow, Purdue and Zoeller (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 4475–4480] were hypersensitive to singlet oxygen, supporting the notion of plasmalogens as radical oxygen scavengers. Wild-type-like resistance could be conferred on NRel-4 upon restoration of plasmalogen content by supplementation with a bypass compound, *sn*-1-hexadecylglycerol. NRel-4 and other plasmalogen$^-$/peroxisome$^+$ strains will allow us to examine further the role of ether lipids in cellular functions without complications associated with peroxisome deficiency, and might serve as an animal cell model for certain forms of the human genetic disorder rhizomelic chondrodysplasia punctata.

INTRODUCTION

Plasmalogens are a subclass of glycerophospholipids characterized by a vinyl ether linkage at the *sn*-1 position of the glycerol backbone [1]. In mammalian cells, plasmalogens are most commonly found as a subspecies of ethanolamine or choline phospholipids [1–4]. Plasmalogens constitute 18% of the total phospholipid mass in humans [1], yet we know little about their physiological function(s) and the mechanisms used to regulate their levels. We have attempted to isolate plasmalogen-deficient variants from well-characterized somatic cell lines to identify cell functions that are dependent on these phospholipids and to obtain information about the regulation of the biosynthetic pathway [5].

The 9-(1′-pyrene)nonanol (P9OH)/UV procedure was developed to allow the selective survival of plasmalogen-deficient cells [6]. This involved exposure of the cells to P9OH followed by irradiation with long-wavelength UV. P9OH/UV selections, with the hamster fibroblast-like cell line Chinese hamster ovary (CHO)-K1, have resulted in plasmalogen-deficient survivors, but peroxisome-deficient cells have always heavily dominated the populations [6]. The first two steps in plasmalogen biosynthesis are catalysed by peroxisomal membrane proteins, dihydroxyacetone phosphate acyltransferase (DHAPAT) and alkyl-dihydroxyacetone phosphate synthase, and a loss of peroxisomes is always accompanied by a loss of these two activities and a loss of plasmalogens [6–8]. The peroxisome deficiency makes analysing the cellular role of plasmalogens complicated. We recently reported the isolation of plasmalogen$^-$/peroxisome$^+$ variants from mutagenized CHO populations [5,9]. These mutants were minor representatives of the selected population and additional labour-intensive screening procedures were required to identify them.

We have developed a protocol that selects against peroxisome-deficient cells. This selection was developed on the basis of findings that peroxisome-deficient (plasmalogen$^-$/peroxisome$^-$) cells were more sensitive than their plasmalogen$^-$/peroxisome$^+$ counterparts to exposure to a pyrene-labelled fatty acid, 12-(1′-pyrene)dodecanoic acid (P12), followed by irradiation with UV (P12/UV selection). We used this protocol, in concert with the P9OH/UV selection technique, to rapidly isolate a population of plasmalogen-deficient, peroxisome-competent variants from a mutagenized population of CHO cells. From this population we isolated and characterized a new plasmalogen-deficient CHO variant, NRel-4, that displays a singular defect in DHAPAT and a resulting loss of plasmalogens.

EXPERIMENTAL

Materials

P9OH and P12 were purchased from Molecular Probes (Eugene, OR, U.S.A.). [1-^{3}H]Ethanolamine (specific radioactivity 30 Ci/mmol) was obtained from Amersham (Arlington Heights,

Abbreviations used: CHO, Chinese hamster ovary; DHAP, dihydroxyacetone phosphate; DHAPAT, dihydroxyacetone phosphate acyltransferase; G3PAT, glycerol-3-phosphate acyltransferase; HG, *sn*-1-hexadecylglycerol; P12, 12-(1′-pyrene)dodecanoic acid; P9OH, 9-(1′-pyrene)nonanol; VLCFA, very-long-chain fatty acids.

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IL, U.S.A.). [^{32}P]ATP and EN^{3}HANCE spray were obtained from DuPont–NEN (Boston, MA, U.S.A.). ^{32}P-labelled dihydroxyacetone phosphate (DHAP) was synthesized by enzymic phosphorylation of dihydroxyacetone with [^{32}P]ATP and glycerol kinase [10]. [2,3-^{3}H]Phytanic acid was synthesized from [2,3-^{3}H]dihydrophytol (Amersham) as described [11]. 1-Acyl-DHAP and 1-alkyl-DHAP were synthesized by the method of Hajra et al. [12]. Ecoscint A liquid-scintillation fluid was obtained from National Diagnostics (Atlanta, GA, U.S.A.). Silica gel 60 TLC plates (Merck) were purchased from American Scientific Products (McGaw Park, IL, U.S.A.). Titanyl sulphate was obtained from Chemtech (Hayward, CA, U.S.A.). Tissue culture dishes (Corning) were obtained from VWR Scientific (Boston, MA, U.S.A.). Polyester cloth (17 μm mesh) was purchased from Tetko (Elmsford, NY, U.S.A.). Phosphatidylethanolamine standard from bovine brain was purchased from Doosan/Serdary Chemicals (Englewood Cliffs, NJ, U.S.A.), and total bovine heart lipids were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). All other reagents, unless otherwise specified, were purchased from Sigma (St. Louis, MO, U.S.A.).

Cell culture and conditions

CHO-K1 cells were obtained from the American Type Culture Collection. ZR-82 and NZel-1 are peroxisome$^-$/plasmalogen$^-$ and peroxisome$^+$/plasmalogen$^-$ derivatives of CHO-K1 respectively [7,9]. Mutagenesis was performed and cells were maintained as described previously [9]. Cells were fused by using poly(ethylene glycol) (3350 Da) by the method of Kennett [13] as modified [8].

Selection of plasmalogen$^-$/peroxisome$^+$ variants

A P9OH/UV-resistant population of CHO cells was obtained as described previously [9]. The P9OH/UV-resistant cells were put through two rounds of P12/UV selection [14] as described below. Cells were plated into 24-well tissue culture plates at a density of 10^3 cells per well in 0.5 ml of medium. The next day, 0.5 ml of medium containing 4 μM P12 (final concentration 2 μM) was added, the cells were incubated at 37 °C for 20 h, the P12-containing medium was removed and replaced with 1 ml of P12-free medium per well followed by irradiation for various periods from 0 to 10 min with long-wavelength UV (more than 300 nm). Irradiation was accomplished by placing the dishes on a glass plate 1.5 mm thick suspended 10–15 cm over the UV source (Black-Ray UV lamp, model XX-15L; Ultraviolet Products, San Gabriel, CA, U.S.A.). This resulted in a UV intensity of 1500 μW/cm^2 at the surface of the glass plate. The cells were allowed to grow at 37 °C for 7–10 days after irradiation. The survivors from 6 min of exposure to UV were pooled, expanded and put through a second round of exposure to P12/UV as before. The survivors from an 8 min and a 10 min exposure to UV in this round of selection were pooled and used to generate clonal isolates by limiting dilution.

Enzymic assays

The P12/UV-resistant isolates were initially screened for the presence or absence of DHAPAT activity by colony autoradiography [15] as modified below. A 20 μl aliquot of cell suspension (10^6 cells/ml) from each isolate was spotted on 100 mm diameter tissue culture dishes and allowed to attach for 1.5 h, after which 15 ml of medium was added. These 'artificial colonies' were overlaid with a sterile polyester cloth and left undisturbed at 37 °C for 2 days to permit the growth of the colonies into the polyester. The polyester was removed, rinsed three times by immersion in a bath of ice-cold PBS and placed at −80 °C to lyse the cells. The polyester was thawed and used in the autoradiographic detection of DHAPAT at pH 5.7 [15].

Glycerol-3-phosphate acyltransferase (G3PAT), DHAPAT, alkyl-DHAP synthase, and acyl/alkyl-DHAP reductase activities were all measured with whole-cell homogenates, which were prepared as described previously [9]. DHAPAT and G3PAT activities were measured as described by Jones and Hajra [16]. Alkyl-DHAP synthase was assayed as described by Davis and Hajra [17]. Acyl/alkyl-DHAP reductase was assayed by the method of Hajra et al. [18]. Protein content was determined with the method of Lowry et al. [19].

Determination of relative plasmenylethanolamine content with [^{3}H]ethanolamine labelling

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 (2 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 chloroform/methanol/PBS (5:10:4, by vol.) containing 200 μg of a carrier lipid (beef brain phosphatidylethanolamine). After transfer to test tubes, 1 ml of chloroform and 1 ml of PBS were added to form a two-phase Bligh and Dyer system [20] and the lower (organic) phase was collected after centrifugation. Solvent was removed with a stream of nitrogen and the labelled phospholipids were separated by two-stage one-dimensional TLC [15]. The labelled species were detected by autoradiography at −80 °C after the plates had been sprayed with EN^{3}HANCE.

Determination of the phospholipid composition

Cells (5×10^5) were grown for 72 h in 75 cm^2 tissue culture flasks at 37 °C in medium containing [^{32}P]P_i (5 μCi/ml), with or without 20 μM *sn*-1-hexadecylglycerol (HG), to label the phospholipids to constant specific radioactivity. The medium was removed and the cells were harvested with trypsin, pelleted by centrifugation at 600 ***g*** for 7 min and then resuspended in 0.8 ml of PBS. The lipids were extracted [20] after the addition of 300 μg of carrier lipid (total lipid from bovine heart). Phospholipids were separated by two-dimensional TLC [21]. Plates were exposed to GBX-2 X-ray film at −80 °C after pre-flash. [^{32}P]P_i-labelled phospholipid species were scraped from the TLC plates directly into scintillation vials for quantification by liquid-scintillation spectrometry [5].

Release of catalase from digitonin-permeabilized cells

Cells were suspended in PBS at 10^7 cells/ml; 0.3 ml aliquots were added to an equal volume of PBS containing 0, 50 or 600 μg/ml digitonin (0, 25 or 300 μg/ml final digitonin concentrations). After 10 min at room temperature the suspensions were centrifuged for 40 s at 13000 ***g*** in a Microfuge (Beckman); the supernatant was used immediately for catalase activity determinations by the modified method of Peters et al. [22]. Supernatants from cells incubated with 0 and 300 μg/ml digitonin were used as 0 % and 100 % release values respectively.

Immunofluorescence microscopy

Cells were grown on coverslips in serum-supplemented RPMI 1640 medium and fixed with 4 % (w/v) paraformaldehyde. Fixed cells were permeabilized with 1 % (v/v) Nonidet P40 in PBS and subjected to immunofluorescence microscopy as described [8]

with anti-(bovine liver catalase) [23] followed by fluorescein-conjugated donkey anti-rabbit IgG.

Quantification of very-long-chain fatty acids (VLCFAs) and oxidation of phytanic acid

For VLCFA determinations, total cellular lipids were extracted by the method of Bligh and Dyer [20] and transesterified with 2% (v/v) H_2SO_4 in methanol [24], and the methyl esters were analysed by capillary GLC [24]. Oxidation of phytanic acid was assayed by measuring the cell-mediated release of water-soluble radioactivity from [2,3-^{3}H]phytanic acid [11,25]. Cells were grown to confluence in culture dishes 35 mm in diameter, and the medium was changed to 2 ml of Dulbecco's modified Eagle's medium containing 10% (v/v) lipid-free fetal bovine serum 1 day before study. Medium was replaced with 2 ml of medium containing 1% (v/v) fetal bovine serum and 3.0 μCi of [2,3-^{3}H]phytanic acid (final phytanate concentration of 50 nM), and cells were incubated for 24 h at 37 °C before work-up.

Quantification of P12 uptake by the wild-type and mutant cell lines

Cells (2×10^5) were grown overnight in sterile glass scintillation vials at 37 °C, in medium containing 2 μM P12. After 20 h at 37 °C, medium was removed and the cells were washed with 3 ml of serum-containing medium followed by 3 ml of ice-cold PBS. The control vials lacking P12 were washed similarly and the cells solubilized in 1 ml of 0.5 M NaOH before protein determination [19]. Lipids were extracted as described above. The solvent was evaporated with a stream of nitrogen, the dried lipids were resuspended in ethanol and the P12 content was determined by measuring fluorescence in a Sim-Aminco Bowman Series 2 luminescence spectrometer with an excitation wavelength of 344 nm and an emission wavelength of 378 nm. Absolute values were obtained by comparison of the sample values with those of P12 standard curves.

RESULTS

P9OH/UV selects for a CHO population dominated by peroxisome-deficient cells

We exposed a mutagenized population of CHO-K1 cells (3×10^6) to three rounds of P9OH/UV selection. This technique was developed to select for plasmalogen-deficient animal cell mutants [6]. P9OH is a pyrene-containing long-chain fatty alcohol. Cells that take up pyrene become sensitive to long-wavelength (more than 300 nm) UV irradiation owing to the generation of cell-damaging singlet oxygen. Because a large portion of the P9OH that enters a cell is incorporated into plasmalogens, plasmalogen biosynthesis mutants accumulate substantially less P9OH and are less sensitive to UV [6]. Under the conditions chosen (see the Experimental section) the first round of selection resulted in the killing of more than 90% of the cells, whereas most of the cells survived the second round (results not shown). The population that survived three rounds of selection, designated P9R, was considerably more resistant to P9OH/UV treatment than the parent CHO-K1 cells many generations after selection (Figure 1A). This population was much less able to synthesize plasmenylethanolamine, the major plasmalogen species in CHO-K1 cells (Figure 1B). Whereas 40.0±1.1% (S.D., $n = 3$) of the lipid-associated radioactivity in [1-^{3}H]ethanolamine-labelled cells was found in plasmenylethanolamine (the plasmalogen), only 7.8±0.2% ($n = 3$) of the label was associated with plasmenylethanolamine in the selected population.

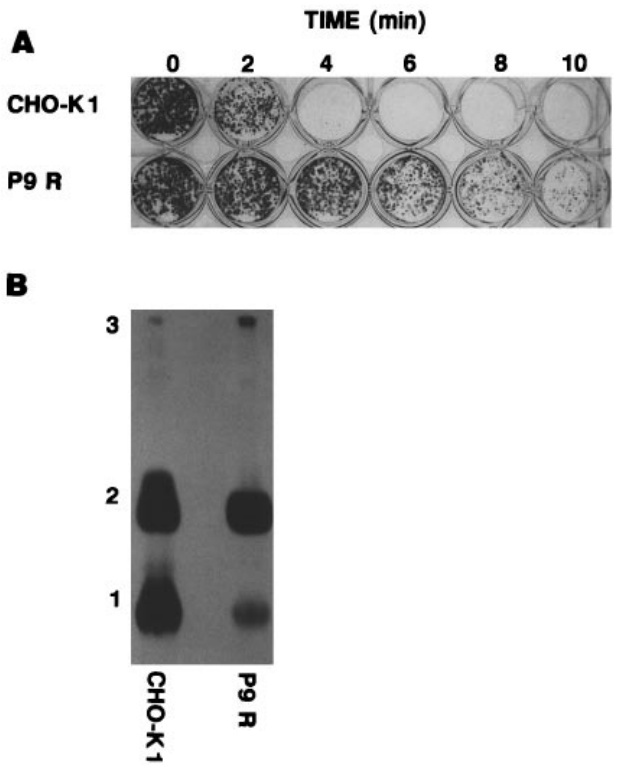

Figure 1 P9R population is resistant to P9OH/UV treatment and is plasmalogen-deficient

(**A**) P9OH/UV resistance. Cells were plated into 24-well tissue culture plates at 10^3 cells per well in 0.5 ml of medium. After attachment overnight, 0.5 ml of medium containing 40 μM P9OH (final concentration 20 μM) was added and the cells were incubated for 3 h at 37 °C. The medium was then removed, the cells were incubated for an additional 1 h at 37 °C in 1 ml of fresh P9OH-free medium, followed by UV irradiation for the indicated periods (see the Experimental section). Surviving cells were allowed to grow for 3 days and then stained with Coomassie Blue [0.1% in methanol/water/acetic acid (45:45:10, by vol.)]. (**B**) Subspecies patterns of ethanolamine phospholipids. Cells were grown for 18 h in medium containing [1-^{3}H]ethanolamine (2 μCi/ml). The ethanolamine-labelled phospholipids were isolated and separated by TLC as described in the Experimental section. Band 1, plasmenylethanolamine (R_F 0.3); band 2, phosphatidylethanolamine (R_F 0.5); band 3, unknown (R_F 0.9).

Previously, the primary lesion associated with isolates obtained from P9OH/UV selection of CHO cells has always been a defect in peroxisome assembly [6]. Catalase, normally located in the peroxisomal matrix, is found in the cytosol in these mutants and can be released from the cells by disruption of the plasma membrane with relatively low concentrations of digitonin. By using 25 μg/ml digitonin, 91±1% ($n = 3$) of the catalase activity was released by the peroxisome-deficient CHO cell line ZR-82, whereas only 18±4% ($n = 3$) of the catalase activity was released from wild-type cells, owing to catalase's primary location within the peroxisomes. The pattern of catalase release by the P9R population was similar (81±5%; $n = 3$) to that of ZR-82, demonstrating that a majority of this population was peroxisome-deficient. Catalase release was slightly lower ($P = 0.05$) in the P9R population, suggesting that a small proportion of the population might contain peroxisomes.

P12/UV treatment selects against peroxisome-deficient CHO cells

CHO cells incorporate the pyrene-labelled fatty acid P12 into membrane lipids [26]. As with P9OH/UV treatment, cells that accumulate more P12 are more sensitive to UV irradiation [14].

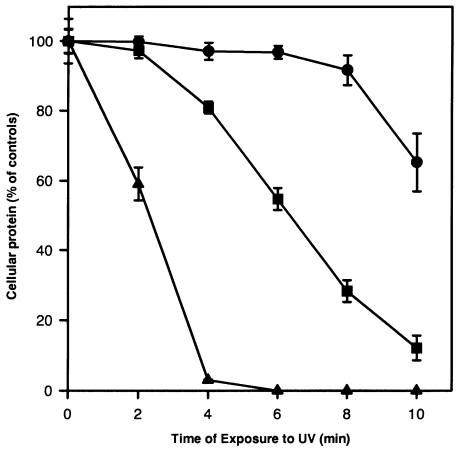

Figure 2 Sensitivity of the plasmalogen-deficient mutants to P12-mediated photodynamic damage (P12/UV)

Cells (10^4 per well) were plated out in 24-well tissue culture plates in 0.5 ml of growth medium. After attachment overnight, 0.5 ml of medium containing 4 μM P12 was added (2 μM final P12 concentration) and the cells were incubated for 18 h at 37 °C. Medium was removed and replaced with 1 ml per well of P12-free medium. This was followed immediately by irradiation for the indicated periods as described in the Experimental section. Surviving cells were allowed to grow for 3 days. Cellular protein in each well was determined after removal of medium and a rinse of the cell monolayers with PBS. Values are expressed as percentages of the protein in non-irradiated wells and represent means $\pm$ S.D. for three wells. Non-irradiated wells contained similar protein levels in all cell lines. Symbols: ●, CHO-K1; ■, NZel-1; ▲, ZR-82.

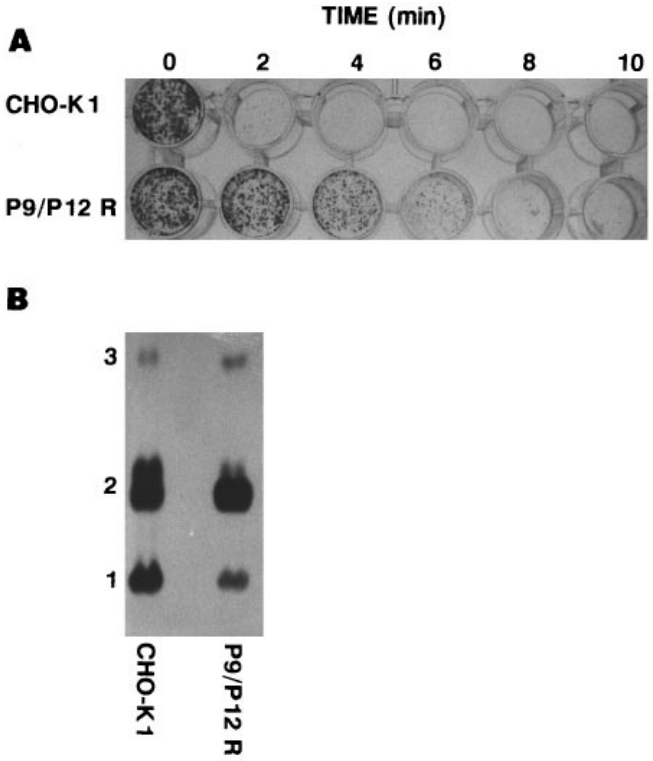

Figure 3 P9/P12R population remains P9OH/UV-resistant and is plasmalogen-deficient

(**A**) P9OH/UV resistance. Cells were plated into 24-well tissue culture plates at 10^3 cells per well in 0.5 ml of medium. The experiment was performed as detailed in Figure 1 and in the Experimental section. (**B**) Subspecies patterns of ethanolamine phospholipids. Cells were grown for 18 h in medium containing [1-^{3}H]ethanolamine (2 μCi/ml). The ethanolamine-linked phospholipids were isolated and separated by TLC as described in the Experimental section and the bands were detected by exposure to X-ray film. Band 1, plasmenylethanolamine (R_F 0.3); band 2, phosphatidylethanolamine (R_F 0.5); band 3, unknown (R_F 0.9).

It has been reported that peroxisome-deficient CHO mutants were more sensitive to P12/UV treatment [14,27]. This hypersensitivity was due to two factors: (1) the loss of the peroxisomal β-oxidation system resulted in an accumulation of P12, which results in the additional generation of reactive oxygen on UV irradiation, and (2) the loss of plasmalogens, proposed scavengers of reactive oxygen species, left the mutants more susceptible to the generation of reactive oxygen species during P12/UV treatment.

Recently we reported the isolation of a plasmalogen-deficient CHO mutant, NZel-1, that contained peroxisomes, including a functional peroxisomal β-oxidation system [9]. NZel-1 was more sensitive to P12/UV exposure than were the wild-type cells, whereas the peroxisome-deficient cell line ZR-82 was more sensitive than either of the other cell lines (Figure 2). The loss of peroxisomes in the ZR-82 cells was responsible for the 3-fold increase in P12 levels over wild-type cells (5.85 ± 0.14 compared with 2.08 ± 0.48 nmol/mg of cell protein; $n = 3$) after exposure to 2 μM P12 for 20 h. This increased accumulation was probably an important determinant in the extreme sensitivity of ZR-82 to P12/UV treatment. P12 levels in the NZel-1 cells (1.52 ± 0.11 nmol/mg of cell protein; $n = 3$) were similar to, or slightly lower than, those in wild-type cells (although not significantly lower; $P = 0.4$), but the loss of plasmalogens rendered them more sensitive to reactive oxygen species.

Selection of plasmalogen$^-$/peroxisome$^+$ cells from the P9R population

The P9R population was exposed to P12 and UV under conditions known to be selectively toxic to the peroxisome-deficient mutants. This resulted in the death of more than 90% of the cells (results not shown). A second round of selection with P12/UV resulted in a population of cells, designated P9/P12R, that were still resistant to P9OH/UV treatment (Figure 3A) and deficient in plasmalogen biosynthesis (Figure 3B). Again, only $10.3 \pm 1.2\%$ ($n = 3$) of the lipid-associated radioactivity from [1-^{3}H]ethanolamine was found in plasmenylethanolamine, whereas $41.5 \pm 6.8\%$ ($n = 3$) of the label was plasmenylethanolamine-associated in wild-type cells. Only $25.4 \pm 4.1\%$ ($n = 3$) of the total catalase was released from these cells when exposed to low levels (25 μg/ml) of digitonin. This was similar to values from CHO-K1, suggesting that they contained intact peroxisomes.

Fifteen isolates were generated from the P9/P12R population. All except one of the isolates was plasmalogen-deficient, as judged by ethanolamine labelling, and 10 were defective in DHAPAT activity as judged by colony autoradiography [15]. We chose four DHAPAT-deficient isolates, designated NRel-1 to NRel-4, for further analysis. Fusion analysis revealed that all four isolates belonged to the same complementation group and expressed a recessive mutation (results not shown). One of these isolates, NRel-4, was used for further characterizations.

Plasmalogens are decreased in NRel-4

Long-term labelling of the phospholipid pools with [^{32}P]P_i confirmed that the NRel-4 cells were plasmalogen-deficient (Table 1). Whereas the plasmalogen species make up 11% of the total phospholipid in the wild-type cells, they are decreased to

Table 1 Phospholipid composition of CHO-K1 and NRel-4 cells

Cells were grown for several generations in medium containing [^{32}P]P_i (5 μCi/ml) with or without 20 μM HG to label the phospholipids to constant specific radioactivity. Phospholipids were isolated and analysed by two-dimensional TLC as described in the Experimental section. All values are means ± S.D. for three cultures and are expressed as percentages of the total radioactivity found in phospholipids. Abbreviations: SPH, sphingomyelin; PC, phosphatidylcholine, diacyl form; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine, diacyl form; pPE, phosphatidylethanolamine, plasmalogen form (plasmenylethanolamine). Other lipids include cardiolipin and phosphatidylglycerol.

	Phospholipid composition (% of chloroform-soluble radioactivity)						
Cells	SPH	PC	PS	PI	PE	pPE	Other lipids
CHO-K1	8.5 ± 0.1	54.8 ± 0.3	5.3 ± 0.2	6.7 ± 0.1	10.0 ± 0.6	11.4 ± 0.5	3.3 ± 0.1
NRel-4	10.7 ± 0.4	54.0 ± 0.4	6.4 ± 0.1	7.2 ± 0.4	17.6 ± 0.4	0.8 ± 0.1	3.3 ± 0.3
NRel-4 (+HG)	10.0 ± 0.3	54.2 ± 0.3	6.4 ± 0.2	8.0 ± 0.3	9.6 ± 0.3	8.9 ± 0.2	2.9 ± 0.5

Table 2 Specific activities of enzymes involved in plasmalogen biosynthesis

Whole-cell homogenates were prepared and assays were performed as described in the Experimental section. Unless noted otherwise, all values are means ± S.D. for three separate whole-cell homogenates from two sets of experiments.

	Specific activity (nmol/min per mg of cell protein)				
	DHAPAT				
Cells	pH 5.7	pH 7.4	G3PAT	Alkyl-DHAP synthase	Acyl/alkyl-DHAP reductase
CHO-K1	0.78 ± 0.1	0.58 ± 0.02	1.49 ± 0.15	0.04 ± 0.008	0.30 ± 0.07
NRel-4	0.04 ± 0.01	0.16 ± 0.01	2.70 ± 0.35	0.05 ± 0.007	0.31 ± 0.08

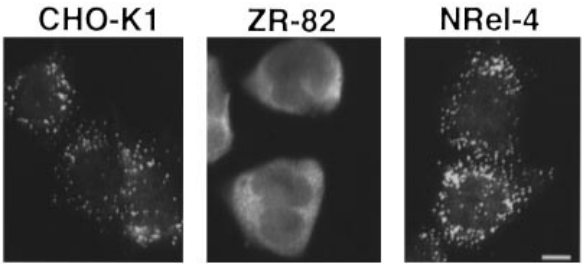

Figure 4 Localization of catalase by immunofluorescence microscopy

Cells were grown on coverslips, fixed with 4% (w/v) paraformaldehyde and subjected to immunofluorescence microscopy as described in the Experimental section. Scale bar, 10 μm.

less than 1% in NRel-4 cells. As in previous studies with plasmalogen-deficient mutants [7,9], the phospholipid headgroup composition was unaffected. A loss of plasmenylethanolamine was accompanied by increased levels of the diacylated species phosphatidylethanolamine.

Plasmalogen deficiency in NRel-4 is due to a singular defect in DHAPAT activity

Plasmalogens could be restored to nearly wild-type levels in NRel-4 by supplementation of the growth medium with HG (Table 1). This compound readily enters cells, is phosphorylated by a kinase and enters the biosynthetic pathway downstream of the third step as 1-alkyl-2-lyso-*sn*-glycerol 3-phosphate [28]. The ability to bypass the plasmalogen deficiency with HG demonstrated that the pathway downstream of the third step was functional in NRel-4.

The first three steps in plasmalogen biosynthesis are catalysed by DHAPAT (step 1), alkyl-DHAP synthase (step 2), and acyl-alkyl-DHAP reductase (step 3) [9]. These activities were measured in whole-cell homogenates from NRel-4 (Table 2). DHAPAT activity, when measured at pH 5.7, was only 5% of the wild-type levels in NRel-4, although there was significant residual activity at pH 7.4 (28% of CHO-K1). G3PAT activity in NRel-4 cells was increased 1.8-fold compared with the control (CHO-K1) fibroblasts. Alkyl-DHAP synthase and acylalkyl-DHAP reductase activities were normal in NRel-4 as well. These results demonstrated that the loss of DHAPAT activity is the only defect in the biosynthetic pathway.

NRel-4 contains intact, functional peroxisomes

The loss of DHAPAT activity, a peroxisomal protein, in NRel-4 cells prompted us to see whether these cells contained functional peroxisomes. The detection of normal alkyl-DHAP synthase activity (a peroxisomal activity) and the resistance of the cells to digitonin-mediated catalase release (results not shown) suggested that these cells contained intact peroxisomes. The presence of intact peroxisomes was confirmed by immunofluorescence microscopy (Figure 4). Both the wild-type CHO-K1 and the mutant NRel-4 cells showed a concentration of catalase in discrete organelles, characteristic of peroxisome-containing cells. The peroxisome-deficient ZR-82 cells showed a diffuse labelling pattern, indicative of cytosolic catalase.

The breakdown of VLCFAs (more than C_{20}) within the cells is attributed to the presence of a unique peroxisomal β-oxidation system [29]. As observed previously in peroxisome-deficient cells [7], the loss of this system in the peroxisome-deficient CHO mutant cell line ZR-82 resulted in an accumulation of VLCFAs, including $C_{26:0}$ (0.81 ± 0.02 μg/mg of cell protein; $n = 4$). $C_{26:0}$ levels in NRel-4 cells (0.02 ± 0.01 μg/mg of cell protein; $n = 3$) were comparable to those found in wild-type cells (0.03 ± 0.01 μg/mg of cell protein; $n = 4$). Oxidation of phytanic acid, another function that is lost in peroxisome-deficient cells, was found to be comparable in NRel-4 (1.21 ± 0.28 pmol/h per mg of cell protein; $n = 7$) and wild-type cells (1.45 ± 0.24 pmol/h per mg of cell protein; $n = 8$), whereas it was undetectable in the ZR-82 cells.

Plasmalogen deficiency in NRel-4 is associated with a hypersensitivity to P12/UV exposure

As observed with the NZel-1 cells (Figure 2), the NRel-4 cells were hypersensitive to P12/UV exposure compared with the wild-type cells (Figure 5). The amount of cell-associated P12 in NRel-4 under these conditions was 1.62 ± 0.11 nmol/mg of

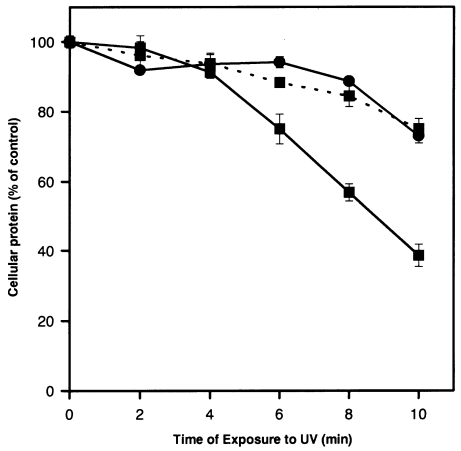

Figure 5 Hypersensitivity of NRel-4 cells to P12/UV treatment and rescue by supplementation with HG

Cells (10^4 per well) were plated into 24-well tissue culture plates in 0.5 ml of medium, with or without 20 μM HG. After attachment overnight, 0.5 ml of medium containing 4 μM P12 was added (2 μM final P12 concentration) and the cells were incubated for 18 h at 37 °C. Medium was removed and replaced with 1 ml per well of P12-free medium. This was followed immediately by irradiation with long-wavelength UV for the indicated periods as described in the Experimental section. Surviving cells were allowed to grow for 3 days. Cellular protein in each well was determined after the removal of medium and a rinse of the cell monolayers with PBS. In the HG-treated cells, HG was present before and during the 18 h P12-labelling period, but not during UV exposure or the 3-day recovery period. Values are expressed as percentages of the protein in non-irradiated wells and represent means ± S.D. for three wells. Non-irradiated wells contained similar protein levels in all cell lines. Symbols: ●, CHO-K1; ■, NRel-4; dotted line, NRel-4 plus HG.

protein, which was very close to that of NZel-1 and wild-type cells. Restoration of plasmalogens to NRel-4, through supplementation of the growth medium with HG, restored wild-type-like resistance to P12/UV treatment consistent with the role of plasmalogens as endogenous antioxidants [14,27]. Similar results were obtained with NZel-1 (results not shown).

DISCUSSION

We have endeavoured to isolate plasmalogen-deficient mutants that have normal peroxisomal function to permit the examination of plasmalogen function. The current selection procedure (P9OH/UV) generates populations that are heavily dominated by peroxisome-deficient variants. Although plasmalogen-deficient, these mutants presented problems in the interpretation of results. It was reported that plasmalogen-deficient CHO mutants were more sensitive to conditions that generated reactive oxygen species than the parent cells [14,27]. To generate reactive oxygen, the cells were incubated with a pyrene-labelled fatty acid, P12, followed by excitation of the pyrene moiety with long-wavelength UV. At that time we were unable to attribute the mutants' hypersensitivity solely to a lack of plasmalogens because these cells were also deficient in peroxisomes (plasmalogen$^-$/peroxisome$^-$). These cells accumulated more pyrene-fatty acid than their wild-type counterparts owing to the loss of a functional peroxisomal β-oxidation system [26,30]. By using the plasmalogen-deficient, peroxisome-competent CHO variant, NZel-1, we showed that the P12/UV hypersensitivity phenotype observed in the peroxisome-deficient cells was indeed due to both the loss of peroxisomes and the loss of plasmalogens. By the two-step selection we rapidly generated a plasmalogen-deficient isolate, NRel-4, that displayed a singular defect in DHAPAT activity yet contained functional peroxisomes. NRel-4 cells, like NZel-1 cells, were hypersensitive to the photodynamic generation of singlet oxygen with P12/UV, supporting the possibility that plasmalogens serve to protect cells against reactive oxygen species.

Although DHAPAT activity was almost completely deficient (5% of wild-type) when measured at pH 5.7, there was residual DHAPAT activity when measured at pH 7.4. This behaviour was consistent with results obtained from the peroxisome-deficient CHO mutants [6,7]. This residual activity could be the result of G3PAT, as suggested by Schlossman and Bell [10]. Because the DHAPAT activity in NRel-4 cells at pH 7.4 was only 6% of the cellular G3PAT activity, it points to a very low specificity of G3PAT towards DHAP. Alternatively, the residual activity could be due to altered targeting and redistribution of this activity to other membranes. Within peroxisomal membranes, DHAPAT is active at pH 5.7 [16,31,32], but after detergent solubilization and purification there is a shift in pH optimum to 7.4 [16]. It is possible that DHAPAT, within other membrane environments such as the endoplasmic reticulum, displays a pH optimum of 7.4.

Plasmalogen content is decreased in a number of human genetic diseases in which the patients' cells display some degree of peroxisomal dysfunction, varying from a complete lack of the organelle to the loss of a specific subset of proteins or a specific peroxisomal function [33,34]. A common feature of these disorders is the lack of plasmalogens in the tissues of the patients. A new group of patients have been described whose fibroblasts display a biochemical phenotype very similar to NRel-4 [34,35], a defect in DHAPAT with functional peroxisomes. These patients displayed some of the features of the more general peroxisomal disorders including rhizomelia, cataracts and hypotonia. This was the first evidence relating the pathogenesis of certain peroxisomal disorders to the inability to synthesize ether-phospholipids, including plasmalogens.

NRel-4 and NZel-1 might be useful in the classification of human complementation groups through fusion analysis with patient's fibroblasts displaying similar biochemical lesions. Also, because CHO cells are easily transfected, NRel-4 will be useful in the isolation of a gene that is important in the expression of DHAPAT (structural or regulatory) through gene-mediated complementation. Once the gene has been isolated, these cells can be used for targeting studies with modified gene constructs.

We thank Andrew Lake for many helpful discussions during the course of this work. This work was supported by National Institutes of Health Grants GM 50571 (R.A.Z.), and NS 15747 (A.K.H.).

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Received 27 November 1997/12 February 1998; accepted 20 February 1998