Mutants in a Macrophage-like Cell Line Are Defective in Plasmalogen Biosynthesis, but Contain Functional Peroxisomes*

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We have used a fluorescence-activated cytotoxicity protocol, 9-(1'-pyrene)nonanol (P90H)/UV selection (Morand, O. H., Allen, L.-A. H., Zoeller, R. A., and Raetz, C. R. H. (1990) Biochim. Biophys. Acta 1034, 132-141), to isolate a series of plasmalogen-deficient mutants in a murine, macrophage-like cell line, RAW 264.7. Three of these mutants, RAW 7, RAW 12, and RAW 108, displayed varying degrees of plasmalogen deficiency (48, 17, and 14% of wild-type levels, respectively), and all three mutants were deficient in peroxisomal dihydroxycetone phosphate (DHAP) acyltransferase activity (5% of wild-type). Unlike previously described Chinese hamster ovary (CHO) cell mutants, the RAW mutants contained intact, functional, peroxisomes and normal levels of alkyl-DHAP synthase activity, a peroxisomal, membrane-bound enzyme. In RAW 7 and RAW 108 cells, the loss of peroxisomal DHAP acyltransferase is the primary lesion. RAW 12 displayed not only a deficiency in the DHAP acyltransferase activity, but also a second lesion in the biosynthetic pathway, a deficiency in Δ1'-desaturase activity (plasmalogenethanolamine desaturase, EC 1.14.99.19), the final step in plasmalogenethanolamine biosynthesis. The deficiencies expressed in the mutants represent unique lesions in plasmalogen biosynthesis. The deficiencies expressed in the mutants represent unique lesions in plasmalogen biosynthesis. The deficiencies expressed in the mutants represent unique lesions in plasmalogen biosynthesis. The deficiencies expressed in the mutants represent unique lesions in plasmalogen biosynthesis.

In animal cells, each class of amino phosphoglyceride can be divided into three subtypes, distinguished by the substituent associated with the sn-1 carbon of the glycerol backbone. Fig. 1 illustrates the three subtypes of ethanolamine-linked phospholipids. These are: 1) 1,2-diacyl-sn-glycero-3-phosphoethanolamine (phosphatidylethanolamine); 2) 1-alkyl-2-acyl-sn-glycero-3-phosphoethanolamine (plasmalogenethanolamine); and 3) 1-alk-1'-enyl-2-acyl-sn-glycero-3-phosphoethanolamine (plasmalogenethanolamine). The latter two species are ether-linked lipids with the sn-1 position bearing a long chain fatty alcohol attached through an ether bond. Plasmalogens (the general name given to the plasmenyl form of a lipid class; e.g., plasmalogenethanolamine) have a cis-double bond between the first and second carbon of the fatty alcohol chain. In mammalian cells, plasmalogens are most commonly found as a subspecies of the ethanolamine phospholipids and can constitute a large portion of this class in certain cell types (1, 2). In general, stimulatable cells (macrophages, neutrophils, muscle, brain, and neural tissue) contain high levels of plasmalogens (1-3). In neutrophils, for example, 65% of the ethanolamine phospholipids are plasmalogenethanolamine (4). Macrophages show a similar profile, although not to as great an extent (2). Human heart muscle is unusual in that it contains very high levels of the plasmalogens form of choline head group species (plasmalogenylcholine) (1, 2).

Functional roles for plasmalogens are not known. Proposed functions include: 1) prostaglandin production and/or arachidonic acid metabolism (5); 2) membrane fusion-mediated events such as exocytosis and endocytosis (6); and 3) protection against active oxygen species such as singlet oxygen (7). The findings that the lipid portion of certain phosphatidylinositol-glycan-anchored proteins are ether-linked (8, 9), and that a murine lymphoma cell line, unable to express such a protein (Thy-1), is deficient in ether lipid biosynthesis (10), suggest that certain ether-linked lipids could be important for the proper localization of these proteins.

The murine macrophage-like cell line, RAW 264.7, responds to a variety of stimuli to release arachidonic acid and make prostaglandins (11), phagocytize zymosan particles (12, 13), and secrete proteins (13, 14). We have isolated a series of mutant derivatives from the RAW 264.7 cell line that are reduced in plasmalogen content in an effort to link plasmalogens with these, or other, cellular processes. These mutants are unique in that they contain intact, functional peroxisomes, unlike previously described mutants (15), and one of these mutants bears an as yet undescribed lesion in the biosynthesis of plasmalogenethanolamine, a deficiency in Δ1'-desaturase activity. Using these mutants, we can evaluate the role of plasmalogens in cellular function without considering the loss of peroxisomes as a contributing factor. In this report, we describe the selection and biochemical characterization of these mutant cells.

EXPERIMENTAL PROCEDURES

Materials—9-(1'-Pyrene)nonanol (P90H) was purchased from Molecular Probes, Inc. (Eugene, OR). [1-3H]Ethanolamine and [γ-

* The abbreviations used are: P90H, 9-(1'-pyrene)nonanol; DHAP, dihydroxycetone phosphate; CHO, Chinese hamster ovary; 1-HG, 1-[(hexadecyl)glycerol; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.

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Fig. 1. Ethanolamine head group species found in animal cells.

$^3$P{ATP were obtained from Amersham and Du Pont-New England Nuclear, respectively. Dihydroxyacetone $[^3]$Pphosphate and glycerol-3-phosphate were synthesized by enzymatic phosphorylation of dihydroxyacetone or glycerol using 1-ATP and glycerokinase (15, 16). 1-Acyl-DHAP was synthesized according to Hacra et al. (17). [2-3H]Dihydrophylitol (30 Ci/mmole) was custom-synthesized by Amersham using a method of TR3 hydrogenation of phytol. [2-3H]Phytanate (30 Ci/mmole) was synthesized from [2-3H]dihydrophytol using a minor modification of the method described by Patton and Benson (18). Bio-Safe II liquid scintillation fluid was obtained from RPI Corp. (Mount Prospect, IL). Silica Gel 60 thin layer chromatography plates (E. Merck) were purchased from American Scientific Products (McGaw Park, IL), while Silica Gel H and Silica Gel G plates were obtained from Analtech (Newark, DE). Titanyl sulfate was obtained from Chemtech (Hayward, CA). All other reagents, unless otherwise specified, were purchased from Sigma. Tissue culture dishes (Corning) were obtained from VWR Scientific (Boston, MA), and Petri dishes were purchased from Baxter Scientific (McGaw Park, IL).

Phospholipid Standards—Plasmenylethanolamine from bovine brain was purchased from Sigma and was actually a mixture of plasmenylethanolamine (60%) and phosphatidylethanolamine (40%). To produce lysophosphatidylethanolamine (1-alk-1'-enyl-2-lyso-sn-glycero-3-phosphoethanolamine), the brain lipids were subjected to mild base hydrolysis (0.5 M NaOH at 60 °C for 2 h) and extracted with chloroform after neutralization of this mixture. Lysophosphatidylethanolamine was purified by thin layer chromatography using Silica Gel H and chloroform: methanol:H2O (65:25:10) as the solvent system. Lysophosphatidylethanolamine was produced by PtO2 hydrogenation of the brain phosphatidylethanolamine mixture to reduce the vinyl ether double bond (19). Again, this was heavily contaminated with phosphatidylethanolamine. Lysophosphatidylethanolamine could be purified from this mixture by mild base hydrolysis, followed by TLC as described above for the purification of lysophosphatidylethanolamine. Egg yolk phosphatidylethanolamine (Sigma) was used as the diacyl species standard.

Cells and Culture Conditions—CHO.K1 (CCL61) and macrophage-like cell line, RAW 264.7 (ATCC TIB71), were obtained from the American Type Culture Collection. HR-82 is a peroxisome/plasmalolamine could be purified from this mixture by mild base hydrolysis, followed by TLC as described above for the purification of lysophosphatidylethanolamine. Egg yolk phosphatidylethanolamine (Sigma) was used as the diacyl species standard.

Selection of Either Lipid-deficient Mutants—Mutants were isolated from RAW 264.7 cells after three rounds of selection using the P90H/UV selection technique (20). Mutagenized RAW 264.7 cells (5 × $10^5$) were plated out in a 100-mm diameter tissue culture plate in 10 ml of medium and allowed to attach overnight. The following day, 5 ml of medium containing 30 μM P90H was added (final concentration of 10 μM P90H), the cells were incubated at 37 °C for 20 h, and the P90H-containing medium was then removed and replaced with 15 ml of P90H-free medium. The cells were incubated at 37 °C for another 5 h and then irradiated for 5 min with long-wavelength ultraviolet light. Irradiation was accomplished by placing the cells on a 1.5-mm thick glass plate which was suspended over a light source. The distance between the light source (Black-Ray UV lamp Model XX-15L; UVP, Inc., San Gabriel, CA) and the cells was adjusted to obtain an intensity of 2200 microwatts/cm2 at the surface of the tissue culture dish. The cells were allowed to grow out at 37 °C for 7–10 days after irradiation. The survivors from four plates were combined and placed through a second and third round of P90H/UV selection. In these subsequent rounds of selection, fewer cells were treated (106 cells). On the third round of P90H/UV selection, no cell killing was observed. Isolates from the P90H/UV-resistant population were obtained by limiting dilution.

P90H/UV Sensitivity Assays—Cells were plated out in 24-well tissue culture plates at a density of 500 cells/well in 0.5 ml of medium. After adjustment at 37 °C overnight, 0.25 ml of medium containing 3 times the final desired P90H concentration was added, and the cells were placed through the same P90H/UV selection protocol as described above, with the exception that the final concentration of P90H was varied. The colonies which developed from the surviving cells were visualized after 7 days by staining with 0.1% Coomassie Brilliant Blue in methanol:H2O-acetic acid (45:45:10).

Enzymatic Assays—1-2-acyltransferase, glycerol-3-phosphate acyltransferase, alkyl-DHAP synthase, and alkyl/acyl-DHAP reductase activities were all measured in whole cell homogenates. Cells were grown as suspension cultures to near confluence, harvested by centrifugation for 7 min at 600 × g, and washed once with Tris-buffered saline (Sigma). The cells were resuspended in 0.05 M Tris-HCl, pH 7.4, and frozen at −80 °C. Cell suspensions were thawed once and resuspended using a Teflon-glass tissue homogenizer. Peroxosomal and microsomal DHAP acyltransferase and glycerol-3-phosphate acyltransferase activities were measured as described by Jones and Hacra (21). Alkyl-DHAP synthase was assayed as described by Davis and Hajra (22). Alkyl-DHAP/1-alkyl-DHAP reductase was assayed by the method of LaBelle and Hacra (23). Protein content was determined using the method of Lowry et al. (24).

Assay of Δ1′-Desaturase Activity—The Δ1′-desaturase assays were performed on postnuclear supernatants. Cells were harvested from suspension culture, pelleted by centrifugation for 7 min at 600 × g, and resuspended in 0.1 M Tris-HCl, pH 7.4. The cell suspension was sonicated with a microprobe (Branson Sonic Power Co.) while on ice for 30 s (using 3-10-a bursts at a setting of 4). The lysate was centrifuged for 40 s, at 12,000 × g, in a Beckman Microfuge, and the supernatant was used for the assays. The Δ1′-desaturase assays were performed as described by Abraham et al. (19). The enzyme contained 0.1 M Tris-HCl, pH 7.4, 2 mM NADH, 10 units of catalase, human catalase, and [1-3H]glycerol-3-phosphate (200 cpm) in a total volume of 1 ml. Reactions were started by the addition of NADH. After 30 min at 37 °C, the reaction was stopped by the addition of acetic acid, and 2.5 ml of chloroform and 1.0 ml of PBS were added to form a 2-phase Bligh and Dyer system. The lower organic phase was removed, the upper phase was washed once with an equal volume of pre-equilibrated lower phase, and the lower phases were combined. This was dried under N2 and developed on TLC using Si gel H and the double development, single dimension system described by Merend et al. (26). Briefly, samples were developed for 5 cm in chloroform:methanol:acetic acid:water (25:15:3:5). This separated the lyso-substrate from the acylated products. After drying for 5 min, the plate was sprayed with 10 mM HgCl2 in acetic acid, cleaving any vinyl ether double bonds which may have been formed (27). After drying the plate for 20 min in a fume hood, the plate was redeveloped in the same dimension for 20 cm using the same solvent system to separate newly generated 1-lyso-2-acyl product. The radioactive products were localized by fluorography at −80 °C for 6 days after spraying the TLC plates with ENVANCE (Du Pont-New England Nuclear). The bands of interest were scraped into liquid scintillation vials containing 1 ml of methanol, and the radioactivity was measured at the addition of 8 ml of scintillation mixture.

Preparation of Tritiated 1-Alkyl-2-lyso-sn-glycerol-3-phosphoethanolamine—RAW 12 cells were cultured out as a suspension culture in 100-mm diameter Petri dish at a density of 5 × $10^5$ cells/dish in 5 ml of medium containing 20 μM 1-hexadecylglycerol and 100 μCi of [1-3H] ethanolamine. Under these conditions, the RAW 12 cells accumulate plasmanylethanolamine (see Fig. 5). After 20 h at 37 °C, the cells...
were harvested, pelleted, and resuspended in 0.8 ml of PBS. This was added to 3 ml of chloroform:methanol (1:2). After 10 min at room temperature, 1 ml of chloroform and 1 ml of PBS were added to form 2 phases, and the lower phase was recovered after centrifugation. The upper phase was washed once with 2 ml of chloroform, and the 2 lower phases were dried and redissolved in N2. The lipids were applied to TLC, and the plate was sprayed with phosphatidylethanolamine and radioactivity were visualized by spraying the plate with water. The sample was then hydrolyzed by incubation in 0.1 N NaOH in chloroform:methanol (1:4) for 1 h at 40 °C, completely deacetylating phosphatidylethanolamine and removing the acyl group from the 2-position of the plasmenylethanolamine. After neutralization of the hydrolysis mixture, the resulting 1-alkyl-2-lys-sn-glycero-3-phosphoethanolamine was extracted by the method of Bligh and Dyer (25) purified by development on Silica Gel H using chloroform:methanol:acetic acid:H2O (25:15:3:1.5) and recovered from the silica gel using chloroform:methanol (1:1). Approximately 8 µCi of this substrate could be recovered from a preparation (containing 5 x 108 RAW.12 cells) at an approximate activity of 6.4 Ci/µmol.

Catalase Latency in Digitonin-permeabilized Cells—Cells were harvested from near confluent suspension cultures, pelleted by centrifugation, washed once with 0.1% Triton X-100.

Determinations of Plasmenylethanolamine Content—As a rapid, qualitative way to determine the relative plasmenylethanolamine content, RAW cells were grown for 48 h, fixed with 4% paraformaldehyde, incubated with primary rabbit antiserum, and subsequently with a fluorescein-conjugated goat anti-rabbit IgG (30). Phorbol 12-myristate 13-acetate (1 µM) was added to the cell cultures for the last 12 h prior to fixation in order to induce a state of catalase latency known to result from the generation of active oxygen species such as singlet oxygen. Immunofluorescence microscopy was performed by the modified method of Peters et al. (28). Lactate dehydrogenase was assayed as described previously (29). For a 100% release control, 1 aliquot of cells was treated with 0.1% Triton X-100.

RESULTS

Isolation of a P90H/UV-resistant Population of RAW Cells—A method to select for ether lipid-deficient Chinese hamster ovary cell mutants has been described (20). This involves treating cultured cells with a pyrene-labeled long chain fatty alcohol (9-(1'-pyrene)nonanol, P90H) followed by exposure to long-wavelength UV light. The fluorescent compound is taken up by the cells, incorporated into complex lipids as either the fatty alcohol or as the fatty acid (following oxidation by long chain fatty alcohol:NAD+ oxidoreductase). Pyrene-treated cells are killed during irradiation, presumably due to the generation of active oxygen species such as singlet oxygen. Plasmenoglycerol-deficient cells take up less P90H than wild-type cells (20) and therefore are less susceptible to UV irradiation.

Mutagenized populations of wild-type RAW cells (RAW 264.7) were subjected to three rounds of P90H/UV selection, resulting in the development of populations of P90H/UV-resistant cells (Fig. 2). Wild-type cells were killed using 5 µM P90H, whereas at least a portion of the resistant population survived using 30 µM P90H. P90H/UV Mutants Are Reduced in Ether Lipid Content—Thirteen clonal strains were isolated from resistant populations, and each was rapidly screened for its ability to synthesize plasmenylethanolamine by measuring the ratio of incor-

![Fig. 2. Sensitivity of RAW 264.7 and a P90H/UV-resistant population to P90H/UV treatment. Cells were plated out in 24-well tissue culture plates at 250 cells/well in 0.5 ml of medium. After overnight attachment, 0.25 ml of medium containing 0, 15, 30, 45, 60, and 90 µM P90H was added, and the cells were incubated for 20 h at 37 °C. The medium was then removed, the cells were incubated for an additional 5 h at 37 °C in 1 ml of fresh P90H-free medium, followed by UV irradiation for 5 min (see "Experimental Procedures"). Surviving cells were allowed to grow for 7 days to form colonies and then were stained with Coomassie Blue (0.1% in methanol:H2O:acetic acid, 45:45:10).](https://www.jbc.org/content/259/3/8301/F2.large.jpg)
poration of $[^1\text{H}]$ethanolamine into plasmeneylethanolamine and phosphatidylethanolamine (Table I). In wild-type RAW cells, approximately 60% of the label was found in plasmenylethanolamine while the remainder was in the phosphatidylethanolamine/plasmenylethanolamine band. Relatively less ethanolamine was incorporated into the plasmencyl fraction in all of the P90H/UV-resistant isolates, although there was a great deal of variation between strains. Three isolates, RAW.7, RAW.12, and RAW.108 were chosen for more detailed analyses. RAW.7 and RAW.12 were from the same P90H/UV population, while RAW.108 was isolated from a second mutagenesis stock.

We quantitated the relative mass of the three subspecies of choline and ethanolamine phospholipids in the wild-type and the mutant cell lines (Table II). In wild-type cells, the ethanolamine phospholipids consisted of 36% plasmenylethanolamine, and there was little, or no, plasmencyl ethanolamine.

**TABLE I**

**Distribution of $[^1\text{H}]$ethanolamine into plasmeneylethanolamine in wild-type and mutant RAW strains**

The cells were labeled for 48 h in medium containing $[^1\text{H}]$ethanolamine. The values represent the percentage of chloroform-soluble label that was associated with viable cells after that time. The remainder of the chloroform-soluble label was found primarily as phosphatidyl- and ethanolamine phospholipids in the wild-type and mutant RAW strains. In wild-type cells, the etha-

| Strain  | Percent of chloroform-soluble label in plasmenylethanolamine |
|--------|---------------------------------------------------------------|
| RAW 264.7 | 59.8 |
| RAW 1 | 42.1 |
| RAW 2 | 29.3 |
| RAW 3 | 30.6 |
| RAW 4 | 15.1 |
| RAW 5 | 37.8 |
| RAW 6 | 35.3 |
| RAW 7 | 42.3 |
| RAW 8 | 20.6 |
| RAW 9 | 42.5 |
| RAW 10 | 31.5 |
| RAW 11 | 30.6 |
| RAW 12 | 12.1 |
| RAW 108 | 7.0 |

**TABLE II**

**Quantitation of phosphatidyly- , plasmanyl- , and plasmenyl- species in ethanolamine and choline phospholipids**

The phospholipid head group species were isolated, and the individual subspecies were converted to the benzyolated derivatives as described under “Experimental Procedures.” The derivatives were separated by high performance liquid chromatography and quanti-
tated by the method of Blank et al. (51). Values represent duplicates and did not vary by more than 3%. Experiments using thin layer chromatography to separate the benzyolated species (see “Experimental Procedures”) yielded very similar results. All values represent the percent mass of that head group species.

| Strain  | Percent of ethanolamine phospholipids | Percent of choline phospholipids |
|--------|---------------------------------------|---------------------------------|
|        | Phosphatidyl | Plasmanyl | Plasmenyl | Phosphatidyl | Plasmanyl | Plasmenyl |
| 264.7  | 61.1         | 2.8       | 36.1      | 89.9         | 10.1      | —        |
| 7      | 81.5         | 1.1       | 17.4      | 94.9         | 5.1       | —        |
| 12     | 88.2         | 5.7       | 6.1       | 95.4         | 4.6       | —        |
| 108    | 35.1         | —         | 64.9      | 98.2         | 1.8       | —        |

*— , the value represented ≤1% of the total.

RAW.7 was 50% reduced in plasmenylethanolamine content, while the plasmenyl species in RAW.12 and RAW.108 was more severely reduced (3–6% of the ethanolamine phospholipid was plasmenylethanolamine). In RAW.12, approximately 6% of the ethanolamine phospholipid was found as plasmenylethanolamine. This accumulation of plasmenylethanolamine was interesting since even wild-type cells contained only 2.8% of this subspecies. There was no change in overall ethanolamine-containing phospholipid in the RAW mutants. Instead, the loss of plasmenylethanolamine was associated with an increase in phosphatidylethanolamine levels (not shown).

The choline phospholipid fraction in wild-type cells consisted of lesser amounts of ether-linked lipid with plasmenylcholine making up 10% of the choline phospholipids and plasmenycholine representing only 1% in wild-type cells. RAW.7 and RAW.12 were only partially reduced, while RAW.108 displayed very little of either of the ether lipid subspecies.

Peroxisomal DHAP Acyltransferase Activity Is Reduced in the RAW Mutants—We measured the activity of the first three reactions in plasmenylethanolamine biosynthesis in whole cell homogenates. Acylation of DHAP in peroxisomes is the first step in ether lipid biosynthesis (34). We were able to detect two DHAP acyltransferase activities in wild-type RAW cell homogenates; the peroxisomal form (measured at pH 5.5), as well as a DHAP acyltransferase which was active at pH 7.4. This latter activity was similar to that reported for ether-deficient CHO cell mutants (15) and was probably due to the lack of specificity of the microsomal glycerol-3-phosphate acyltransferase for acyl acceptor. All three mutants showed severely reduced peroxisomal DHAP acyltransferase activity (Table III). RAW.7 and RAW.12 displayed a small amount of activity (5%), and no activity could be detected in RAW.108. There was only a moderate reduction (30%) in microsomal activity in all of the mutants (not shown). Glycerol-3-phosphate acyltransferase activity was unaltered in the mutant cells.

The second step in ether lipid biosynthesis is catalyzed by another peroxisomal, membrane-bound enzyme, alkyl DHAP synthase (22). This activity was normal in the whole cell homogenates from all of the RAW mutant strains. Another activity associated with ether lipid biosynthesis, acyl/alkyl DHAP reductase (the third step in the biosynthesis of ether lipids), was also found to be normal in the mutants.

**Intact Peroxisomes Exist in the RAW Mutants**—The previous use of the P90H/UV selection technique on populations of CHO cells resulted in the production of mutants in which the primary lesion was peroxisomal deficiency (20). This resulted in the loss of the peroxisomal activities, DHAP acyltransferase and alkyl-DHAP synthase. The fact that the RAW mutants maintained normal levels of alkyl-DHAP synthase suggested that the mutants possessed peroxisomes.

Catalase is found primarily within the peroxisome, but in peroxisome-deficient cells this activity is found in the cytosol (15, 35, 36). The subcellular distribution of catalase was determined in peroxisome-containing and peroxisome-deficient CHO cell lines, as well as in the RAW strains, using digitonin permeabilization (Fig. 3) (36). Treatment of CHO cells with low levels of digitonin (10 µg/ml) causes disruption of the plasma membrane due to its high cholesterol content, resulting in the permeabilization of the cells and the release of soluble cytosolic proteins, such as lactate dehydrogenase (Panel A). Much higher levels of digitonin (150–300 µg/ml) were required to release soluble peroxisomal proteins, such as catalase, from peroxisome-containing, wild-type CHO cells.
TABLE III
Activities of enzymes involved in the early steps of plasmalogen biosynthesis
Assays were performed on whole cell homogenates as described under "Experimental Procedures." All values represent the average of two experiments and vary by less than 10%.

| Strain       | Peroxisomal DHAP acyltransferase | Glycerol-3-phosphate acyltransferase | Alkyl-DHAP synthase | Alkyl/acyl-DHAP reductase |
|--------------|----------------------------------|--------------------------------------|---------------------|--------------------------|
| RAW 264.7    | 623                              | 1,096                                | 179                 | 6,860                    |
| RAW.7        | 22                               | 1,073                                | 163                 | 6,430                    |
| RAW.12       | 12                               | 922                                  | 210                 | 7,480                    |
| RAW.108      | <5                               | 1,033                                | 229                 | 8,760                    |

Fig. 3. Catalase latency in CHO and RAW cell lines. Cells were incubated for 10 min in the presence of various concentrations of digitonin and centrifuged, and the supernatants were assayed for catalase and lactate dehydrogenase activity as described under “Experimental Procedures.” Panel A: ●, CHO.K1; ○, ZR-82. Panel B: ●, RAW.264.7; ○, RAW.7; △, RAW.12; ▲, RAW.108. The solid line represents catalase activity. The dotted line represents lactate dehydrogenase activity released by only the wild-type strains (CHO.K1 and RAW.264.7), although greater than 90% of the lactate dehydrogenase activity was released at 10 μg/ml digitonin in all cell lines examined. Each value represents the average of two separate experiments and did not vary by more than 10%.

In a peroxisome-deficient CHO mutant ZR-82 (15), catalase was released from the cells along with lactate dehydrogenase (Panel A), indicating that catalase was cytosolic in those cells. In wild-type and all of the mutant RAW strains (Panel B), catalase was released only when high levels of digitonin were used, suggesting that catalase was still localized in the peroxisomes. Also, in studies using classical differential centrifugation techniques (15), the catalase distribution in the RAW mutants was similar to the wild-type strain (data not shown); 80-90% of the catalase activity was associated with subcellular organelles.

The catalase localization was confirmed using immunofluorescent microscopy. In wild-type cells, and all three RAW mutant strains, immunofluorescence micrographs using antibodies against catalase showed a concentration of catalase in discrete organelles, presumably peroxisomes (Fig. 4). Again, this is unlike peroxisome-deficient mutants which displayed no such subcellular concentration of catalase (15, 20, 37).

The Peroxisomal β-Oxidation System Is Functional in RAW Mutants—The peroxisome has a unique β-oxidation system which is responsible for the breakdown of very long chain fatty acids, such as 26:0. Every peroxisome-deficient tissue or cell line examined to date has displayed elevated levels of very long chain fatty acids, due to the loss of this system (38, 39). This is true for peroxisome-deficient CHO mutants (Table IV) which contained 5-20 times the wild-type levels of 26:0. Very long chain fatty acid levels in the RAW mutants, however, were not elevated in comparison to the wild-type strain (264.7), suggesting a functional peroxisomal β-oxidation system. Phytanic acid oxidation, another peroxisomal function which is defective in peroxisomal-deficient cells, is normal in all the RAW strains (Table IV), further demonstrating the presence of functional peroxisomes.

Bypass of the Plasmalogen Deficiency Using 1-Hexadecylglycerol—Supplementation of the growth medium with 1-hexadecylglycerol (1-HG) bypasses the plasmalogen deficiency in human (40) and CHO (7) mutant cell lines. The 1-HG is phosphorylated by 1-alkylglycerol kinase (34) and enters the biosynthetic pathway downstream of the enzymatic lesions (as 1-alkyl-2-lyso-sn-glycerol-3-phosphate). Although 1-HG supplementation restored the plasmalylethanolamine content of RAW.7 (not shown) and RAW.108 (Fig. 5) to levels...
TABLE IV

| Strain         | Hexadecanoyl (26:0) levels* | Pytanate oxidation† |
|----------------|-----------------------------|---------------------|
| Peroxisome* cells | 0.078 ± 0.021 | 2.9 ± 0.5 |
| Peroxisome* cells | 0.460 ± 0.190 | ≥0.1 |
| RAW 264.7      | 0.046 ± 0.009 | 1.4 ± 0.6 |
| RAW 7          | 0.030 ± 0.012 | 1.5 ± 0.5 |
| RAW 12         | 0.028 ± 0.005 | 2.9 ± 0.5 |
| RAW 108        | 0.029 ± 0.010 | 1.5 ± 0.6 |

* Values for 26:0 levels in peroxisome-containing (peroxisome*) and peroxisome-deficient (peroxisome-) cultured cells were obtained using CHO cell lines CHO-K1 (n = 3) and ZR-82 (n = 3), respectively. ZR-82 is a peroxisome-deficient mutant derived from the wild-type strain, CHO-K1.

† Values for pytanate oxidation in peroxisome* and peroxisome- cultured cells were obtained from normal human fibroblasts (n = 9) and fibroblasts from patients suffering from Zellweger syndrome (n = 4), respectively.

observed in RAW 264.7 cells, the plasmalogen ethanolamine deficiency could not be fully bypassed in RAW.12 cells. Instead, the immediate biosynthetic precursor of plasmalognanolamine, plasmalogenethanolamine, accumulated. During supplementation with 20 µM 1-HG, this latter species represented 55% of the total ethanolamine-containing phospholipid mass (data not shown). Plasmalogenethanolamine did not accumulate in RAW.108 or RAW.7, even when supplemented with 1-HG.

RAW.12 Is Deficient in Δ1'-Desaturase Activity—The inability to bypass the plasmalogen deficiency with 1-HG and the accumulation of plasmalogenethanolamine suggested a loss of Δ1'-desaturase activity in RAW.12 cells. This enzyme (the final step in plasmalogenethanolamine biosynthesis) converts plasmalogenethanolamine to plasmalogenethanolamine by introducing the cis double bond between the first and second carbon of the alcohol moiety to produce the HgCl₂-cleavable vinyl ether linkage. When tritium-labeled 1-alkyl-2-lyso-sn-glycero-3-phosphoethanolamine was incubated with a postnuclear supernatant from RAW 264.7, two products were formed (Fig. 6). These co-migrated with standards plasmalogenethanolamine (acylated only; band 3) and plasmalogenethanolamine (acylated and desaturated; band 2). This profile of product formation is similar to that reported for Δ1'-desaturase assays using microsomes from other cultured cell lines (19). Postnuclear supernatants from RAW.12 cells were able to acylate the substrate (to form plasmalogenethanolamine), but they were not able to desaturate (form the vinyl ether double bond). This activity was reduced by at least 90% in RAW.12 (Fig. 7). When equivalent amounts of protein from wild-type and RAW.12 supernatants were mixed and used for these assays (not shown), intermediate activity was observed suggesting that no soluble, trans-acting inhibitor was present in the mutants.

DISCUSSION

Although several functions have been proposed for plasmalogens, a definitive proof for any of there has not been forthcoming. We’ve chosen to isolate animal cell lines that are deficient in plasmalogens and determine if any cellular processes are affected. Previous attempts, using the Chinese hamster ovary cell line, CHO.K1 (15, 20, 35), have resulted in the isolation of ether lipid-deficient mutants, but the primary lesion has been the loss of peroxisomes. In addition, all
of the human genetic disorders which involve plasmalogen deficiency also involve a deficiency in peroxisomes and/or peroxisomal functions such as peroxisomal β-oxidation and phytanic acid oxidation (41). Interpretation of results with respect to plasmalogen or ether lipid function using fibroblasts from these patients or the CHO cell mutants is complicated by the possibility that the loss of the peroxisomes or peroxisomal function may be a contributing factor. Unlike any of the P90H/UV-resistant CHO strains, the RAW mutants possessed intact, functional peroxisomes as indicated by: the latency of catalase in digitonin permeabilization studies, 2) the localization of catalase using immunofluorescence microscopy, 3) the normal levels of very long chain fatty acids, and 4) normal phytic acid oxidation rates.

The only biochemical lesion associated with plasmalogen biosynthesis in RAW.7 and RAW.108 was a loss of peroxisomal DHAP acyltransferase activity. The enzymes catalyzing the next two steps in plasmalogen biosynthesis (alkyl-DHAP synthase and alkyl-DHAP reductase) were active. Also, supplementation of the growth medium with 1-hexadecylglycerol (which enters the pathways just after the reduction of 1-alkyl-DHAP) restored normal plasmanylethanolamine levels, indicating that subsequent steps are not affected. The lesion in RAW.108 appears to be quite stringent, in that there is little residual ether-linked ethanolamine or choline phospholipid, and the peroxisomal DHAP acyltransferase was virtually undetectable in our assays. RAW.7 cells were leaky with respect to plasmalogen biosynthesis. This may have been the result of residual peroxisomal DHAP acyltransferase activity.

RAW.12 displayed a deficiency in 2 steps of the pathway. Like the other two mutants, peroxisomal DHAP acyltransferase activity is greatly reduced; however, 1-alkylglycerol supplementation only partially restored plasmanylethanolamine levels. Instead, the cells accumulated its immediate precursor, plasmanylethanolamine. Even without 1-hexadecylglycerol supplementation, there is a higher than normal level of this lipid species. These data, and the inability of RAW.12 lysates to convert this plasmaly- intermediate to a HgCl$_2$-sensitive lipid in the presence of NADH, strongly suggest that RAW.12 is deficient in Δ1'-desaturase activity. Although the lesion appears to be quite severe (5% of wild-type activity), the presence of increased amounts of endogenous plasmanylethanolamine in the RAW.12 membranes makes an accurate determination of specific activities impossible using these assay conditions. However, since plasmanylethanolamine levels in RAW.12 cells are only 2-3-fold higher than wild-type cells (Table II), endogenous substrate levels cannot account for the 20-fold decrease in Δ1'-desaturase activity. The fact that plasmanylethanolamine accumulates in cells which are restricted in an early step of plasmanylethanolamine biosynthesis (peroxisomal DHAP acyltransferase) suggests that the Δ1'-desaturase deficiency is quite severe. Further kinetic analysis using detergent-solubilized systems should aid in this determination. Also, the Δ1'-desaturase system is a membrane-bound, multicomponent, system consisting of an electron transport component (cytochrome $b_5$ and cytochrome $b_6$ reductase) and a terminal, cyanide-sensitive protein (42). Further analysis is required to determine if any of these components are defective, and whether other enzyme systems, which utilize the cytochrome $b_5$-cytochrome $b_6$ reductase electron transport system (e.g., the stearoyl-CoA desaturase system; Ref. 43) are affected.

Presently, we do not know whether the two lesions associated with RAW.12 are due to the alteration in one gene or two. A deficiency in Δ1'- desaturase activity should not prevent the incorporation of fatty alcohol into ether-linked ethanolamine phospholipids, and therefore, the P90H/UV protocol should not select for such mutants. It seems more likely that the loss of DHAP acyltransferase and Δ1'- desaturase activities are due to the alteration in one genetic locus which affects both activities. We are also unable to explain why the P90H/UV selection technique generates peroxisome-deficient CHO mutants (20) while the first three P90H/UV-resistant RAW mutants that we have examined contain functional peroxisomes.

In summary, we have described the isolation of somatic cell mutants which display at least two unique lesions with respect to plasmalogen biosynthesis, demonstrating that the P90H/UV selection technique can be used to isolate mutants with defects specifically in the plasmalogen biosynthetic process. Rigorous examination of other P90H/UV-resistant cell lines, especially conditionally lethal mutants, may yield mutations in other steps of plasmalogen biosynthesis. Some of these activities are shared with diacylphospholipid biosynthetic pathway.

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