Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5170–5174, July 1986
Genetics

Isolation of animal cell mutants deficient in plasmalogen biosynthesis and peroxisome assembly

(ether lipids/catalase/Zellweger syndrome/Chinese hamster ovary cells)

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Communicated by Eugene P. Kennedy, March 17, 1986

ABSTRACT **A rapid autoradiographic screening procedure has been developed for identifying Chinese hamster ovary cell mutants defective in the peroxisomal enzyme dihydroxyacetonephosphate (DHAP) acyltransferase. Ten mutants were found among 60,000 colonies grown from a stock of mutagen-treated cells, and 3 have been characterized with respect to their enzymology and phospholipid biosynthesis. All three contain 3% (or less) of the parental DHAP acyltransferase activity measured at pH 5.5, the optimum for the peroxisomal enzyme. When measured at pH 7.4, all three contained 70–85% of the wild-type activity, but it was sensitive to *N*-ethylmaleimide. Glycerol-3-phosphate acyltransferase activities were identical in mutant and parent strains. Two other peroxisomal enzymes, alkyl-DHAP synthase and particulate catalase, were also reduced by factors of 5–10 in all three mutants, suggesting that these strains are deficient in some aspect of peroxisome assembly, possibly like cells from patients with Zellweger syndrome. Short-term and long-term labeling with $^{32}P_i$ revealed that these mutants are grossly deficient in the *de novo* synthesis and content of plasmalogens. In parental cells the plasmalogen form of phosphatidylethanolamine constitutes 7.1% of the total phospholipid, but it is reduced to 0.7% in the mutants. This decrease is accompanied by a compensatory increase in the diacyl form of phosphatidylethanolamine. The results presented here support the view that there are two DHAP acyltransferases in animal cells and that the peroxisome is essential for the biosynthesis of plasmalogens.**

In animal cells, the acylation of dihydroxyacetonephosphate (DHAP) represents one of two biosynthetic pathways leading to phosphatidic acid, and it is thought to be an obligatory step in the production of ether lipids (1). It is generally accepted that the acylation of DHAP is catalyzed by a peroxisomal DHAP acyltransferase (acylTase) (1), but evidence suggests the presence of a second, microsomal isozyme (2, 3). The latter may represent a dual catalytic function of the microsomal glycerol-3-phosphate (Gro-3-*P*) acylTase and can be distinguished from the peroxisomal activity by its neutral pH optimum (pH 7.2 vs. pH 5.5–6.0) and its sensitivity to inhibition by *N*-ethylmaleimide (MalNEt) (2, 3). The existence of a second DHAP acylTase activity is considered controversial, since some studies attribute any activity found in the microsomal fraction to peroxisomal contamination (4, 5). Consequently, the number of DHAP acylTases and their roles in phospholipid biosynthesis remain uncertain.

In an effort to resolve these issues and to study the function of plasmalogens, we have designed a rapid colony screening procedure for identifying Chinese hamster ovary (CHO) cell mutants deficient in the peroxisomal DHAP acylTase. Initial characterization of three such CHO mutant strains supports a central role for peroxisomes in plasmalogen biosynthesis and also provides strong evidence for the existence of a second DHAP acylTase activity. Furthermore, these mutants may serve as useful somatic cell models for exploring Zellweger syndrome, a human inborn error in which peroxisomes (6) and plasmalogens (7) are deficient.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Palmitic acid (56.6 mCi/mmol; 1 Ci = 37 GBq), $^{32}P_i$, and [γ-^{32}P]ATP were purchased from New England Nuclear. [1-^{14}C]Hexadecanol was synthesized from [1-^{14}C]palmitic acid by the method of Davis and Hajra (8). [^{32}P]DHAP and [^{32}P]Gro-3-*P* were synthesized by the methods of Schlossman and Bell (3). Palmitoyl-DHAP was the gift of A. Hajra (University of Michigan). Palmitoyl-CoA was purchased from P-L Biochemicals. Titanyl sulfate ($TiOSO_4$) was obtained from Chemtech (Hayward, CA). All other reagents were obtained from Sigma.

Cell Culture. CHO-K1 cells were obtained from the American Type Culture Collection (CCL-61) and maintained in an atmosphere of 5% CO_2/95% air in Ham's F12 growth medium (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum (GIBCO). Cells were passaged using trypsin.

All mutageneses were performed using ethyl methanesulfonate (EtMes) as described (9). Mutagenized cells were grown out for several generations at 33°C and stored frozen in liquid nitrogen. Each mutagenized stock was tested for frequency of ouabain-resistant colonies prior to use in screenings. Only those mutageneses that showed a frequency of resistance to 1 mM ouabain of greater than 1 in 10^4 were used.

Screening for Mutants Deficient in Peroxisomal DHAP AcylTase. The screening procedure is described in Fig. 1. Whatman no. 50 filter papers were washed by repeated boiling in 100% ethanol, prior to drying and sterilization. Glass beads were prepared as described (9).

Assays. Cells were typically harvested prior to confluence after growth at 33°C. Medium was removed, the cells were washed three times with 10 ml of phosphate-buffered saline (PBS; 0.137 M NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 6.48 mM Na_2HPO_4, pH 7.4) and scraped in PBS with a rubber policeman. After centrifugation at 1000 × *g* for 10 min, the cells were resuspended in 5 vol of 0.25 M sucrose, 25 mM Tris·HCl (pH 7.4), 0.5 mM dithiothreitol, 1.0 mM EDTA, and 0.02% (wt/vol) sodium azide. The cell suspension was briefly sonicated (10 sec) using a sonic probe (Heat System/Ultrasonics, Plainview, NY, model W 185 F) equipped with a microtip, at a setting of 4. The sonicated cell pellets were stored at −20°C prior to use. Assays of DHAP and Gro-3-*P* acylTases were performed using a modification of the methods of Schlossman and Bell (3). The assay of alkyl-DHAP synthase was performed as described by Davis and Hajra (8).

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Abbreviations: EtMes, ethyl methanesulfonate; MalNEt, *N*-ethylmaleimide; DHAP, dihydroxyacetonephosphate; Gro-3-*P*, glycerol-3-phosphate; acylTase, acyltransferase; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine.

Assay conditions were optimized for CHO cells. Catalase assays were performed by the modified method of Peters *et al.* (10) on the subcellular fractions described below. Protein was determined using the method of Lowry *et al.* (11).

Preparation of Subcellular Fractions. Approximately 2×10^8 cells were grown and harvested as described above. Cells were homogenized in 5 ml of sucrose buffer without sodium azide and then homogenized with 10 strokes of a Teflon–glass homogenizer. After centrifugation at $1000 \times g$ for 10 min, the supernatant was diluted to 10 ml with homogenization buffer and centrifuged for 90 min at $100,000 \times g$. The supernatant (cytosol) was removed. The pellet (particulate fraction) was resuspended in 1.0 ml of PBS, pH 7.4, using a Teflon–glass homogenizer and subjected to a brief sonication (see above) to assure complete dispersion. All procedures were performed at 0–4°C. Samples were stored at −80°C prior to use.

RESULTS

Isolation of Mutants Defective in Peroxisomal DHAP AcylTase. Mutants were isolated by the colony autoradiography technique developed by Esko and Raetz (9). It was initially thought that the desired mutation might be lethal. Consequently, the screening procedure was designed to include temperature-sensitive conditional mutants. Cells that had been treated with the mutagen EtMes were grown clonally into filter papers for 21 days at 33°C. Next, the filters containing the immobilized colonies were placed at 40°C for 12 hr and then subjected to freeze–thawing to lyse the cells. The colonies were assayed for their ability to convert [^{32}P]DHAP into acid-precipitable product (acyl-[^{32}P]DHAP) dependent on the presence of the cosubstrate, palmitoyl-coenzyme A. Assays were performed at pH 5.5 and in the presence of MalNEt to assure that only the peroxisomal enzyme was being detected. Mutants defective in DHAP acylTase were identified by comparing the colony autoradiogram (Fig. 1*B*) to the pattern on the filter stained with Coomassie blue (Fig. 1*A*). Using this technique, we were able to isolate 10 putative mutants. These were purified by subjecting each mutant to two additional cycles of screening. Three of these mutants, ZR-78, ZR-82, and ZR-87, each derived from a separate mutagenesis, were used for further characterizations.

DHAP AcylTase Activities in Parental and Mutant Strains. DHAP acylTase activities were measured at 33°C in whole-cell lysates of parental and mutant strains that had been grown at 33°C. The activity measured at pH 5.5 should reflect only the peroxisomal enzyme, since the putative microsomal enzyme is totally inactive at pH 5.5 (12). Table 1 shows that the activity of the mutant strains at pH 5.5 is only 3% of the parental activity. Thus, the peroxisomal enzyme is either missing or nonfunctional at pH 5.5 in the mutants. The residual activity is not temperature sensitive. All three mutants grow normally at 40°C, 37°C, and 33°C.

When measured at pH 7.4, considerable residual DHAP acylTase (70–85% of the parental activity) is detected in all mutants (Table 1). Although the pH optimum for the peroxisomal DHAP acylTase is 5.5, this enzyme demonstrates a rather broad pH optimum in extracts of rat liver, including some residual activity at pH 7.4 (12). The results of Table 1 could be explained by postulating that the mutants have an altered peroxisomal DHAP acylTase with a higher pH optimum than wild type. In the parental cells, however, the activity observed at pH 7.4 is only inhibited 40% by the addition of 5 mM MalNEt, while the activity remaining in the three mutants at pH 7.4 is almost completely inhibited by MalNEt (Table 1). These data support the conclusion that the mutant strains have lost a MalNEt-insensitive DHAP acylTase activity with a pH optimum of 5.5, while still containing a MalNEt-sensitive activity, active at neutral pH.

Gro-3-*P* AcylTase Activities in Parent and Mutant Strains. Examination of the Gro-3-*P* acylTase activities in parental and mutant strains shows that there is little, if any, difference (Table 2). Mammalian cells contain at least two Gro-3-*P*

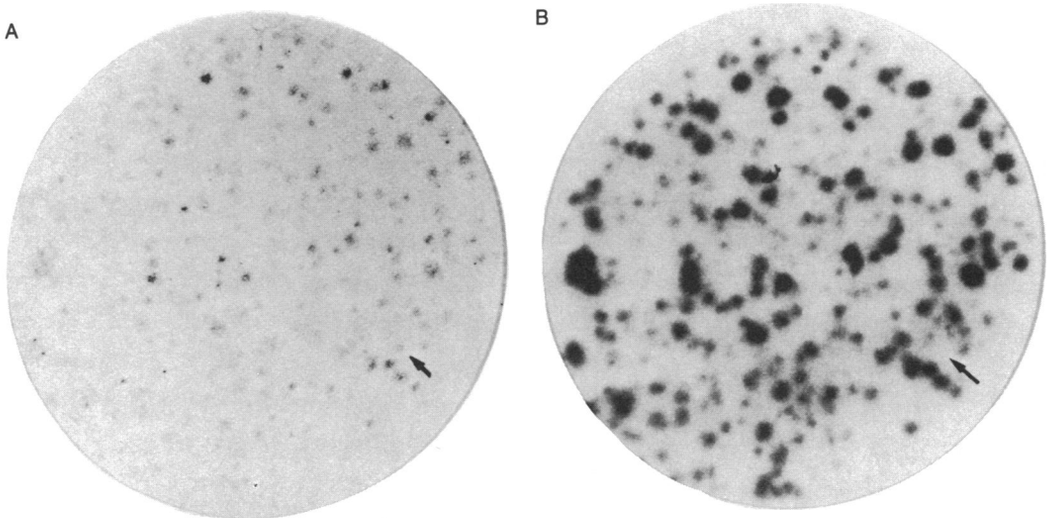

FIG. 1. Isolation of mutants defective in peroxisomal DHAP acylTase. Cells that had been treated with EtMes (9) were plated out in 100-mm-diameter tissue culture dishes to achieve a density of approximately 200 colonies per dish. After overnight incubation at 33°C, the cells were overlaid with Whatman no. 50 filter paper as described (9). Colonies were allowed to grow into the filter papers for 21 days at 33°C, with medium changes every 4 or 5 days. After 21 days, the beads were decanted, and the filter papers containing the colonies were incubated for 12 hr, at 40°C in 10 ml of fresh medium. Fresh medium was also added to each of the master dishes that were then placed at 28°C until putative mutants could be identified and isolated. Filter papers containing the colonies were washed in PBS, pH 7.4, blotted to remove excess moisture, and frozen to break the cells (9). Next, the filter papers were thawed, blotted, and placed in a Petri dish with 0.8 ml of an assay mixture containing 100 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 100 mM Mes (pH 5.5), 100 μM palmitoyl-CoA, 1 mM [^{32}P]DHAP (4–6 μCi/μmol), 8 mM NaF, bovine serum albumin at 2 mg/ml, 5 mM $MgCl_2$, 50 mM KCl, 5 mM MalNEt, and 2 mM KCN. After 15 min at 40°C, 2 ml of 20% (wt/vol) trichloroacetic acid was added to each dish to stop the reaction. The filters were washed three times on a Buchner funnel with 30 ml of 2.5% (wt/vol) trichloroacetic acid. After drying, the filters were autoradiographed and stained with Coomassie blue (9). Mutants were identified by comparison of the autoradiogram and the protein staining patterns on the filter paper. Putative mutants were retrieved from the master dishes and carried through two more cycles of colony screening to achieve complete purification of each strain before use.

Table 1. DHAP acylTase activities in parent and mutant strains at pH 5.5 and pH 7.4

	Specific activity, pmol per min per mg of protein		
	pH 5.5	pH 7.4	
Cells	(−)	(−)	(+)
CHO-K1	472	529	297
ZR-78	16	377	47
ZR-82	13	452	47
ZR-87	13	423	54

Cells were grown to near confluence at 33°C, harvested, and lysed. Whole-cell lysates were assayed at 33°C in the absence (−) or presence (+) of 5 mM MalNEt. All assay mixtures consisted of 100 mM Mes, 100 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (pH 5.5 or 7.4), 100 μM palmitoyl-CoA, 1.5 mM [^{32}P]DHAP (2–4 μCi/μmol), 8 mM NaF, 5 mM $MgCl_2$, 50 mM KCl, 2 mM KCN, bovine serum albumin at 2 mg/ml, and cell protein in a total volume of 300 μl. All values represent the average from duplicate cultures and vary by less than 10%.

acylTases, one microsomal and the other mitochondrial (13). Although both enzymes have optimal activity at pH 7.4, they can be distinguished by their respective sensitivities to MalNEt; the microsomal enzyme is inhibited by MalNEt, while the mitochondrial enzyme is unaffected. Extracts of both parent and mutant strains show similar levels of a MalNEt-sensitive Gro-3-*P* acylTase (Table 2). The MalNEt-resistant mitochondrial activity is low in CHO cells. If we assume a 95% inhibition of the microsomal activity, using MalNEt (14), we calculate that the mitochondrial enzyme accounts for 6–10% of the overall Gro-3-*P* acylTase activity. These results are consistent with the work of Stern and Pullman (14), who have found that cultured cells generally have low levels of the mitochondrial enzyme.

Table 2. Gro-3-*P* acylTase activities in parent and mutant cells

	Specific activity, nmol per min per mg of protein	
Cells	(−)	(+)
CHO-K1	2.66	0.45
ZR-78	2.65	0.29
ZR-82	3.00	0.33
ZR-87	3.20	0.40

Cells were grown to near confluence at 33°C, harvested, and lysed. Whole-cell lysates were assayed at 33°C and pH 7.4, in the absence (−) or presence (+) of 5 mM MalNEt. The assay mixtures were identical to those described in Table 1, with the exception that 1.5 mM [^{32}P]Gro-3-*P* (2–4 μCi/μmol) was used as the acyl acceptor. All values represent the average from two cultures and do not vary by more than 10%.

Plasmalogen Content and Synthesis. The role of the peroxisomal enzyme in maintaining the phospholipid profile and in the production of plasmalogens was examined by long-term labeling of the phospholipids with $^{32}P_i$ followed by two-dimensional TLC (Fig. 2). This analysis revealed a drastic reduction in the plasmalogen-form of phosphatidylethanolamine (PtdEtn) in all three mutants. Quantitation of the radiolabeled phospholipids (Table 3) showed that, in the parent, the plasmalogen species makes up approximately 40% of the total PtdEtn, but it is reduced by at least a factor of 10 in the mutants. A reduction is also observed in the plasmalogen–phosphatidylcholine (PtdCho) content (Table 3). It is not certain whether the residual, plasmalogen-like material actually is plasmalogen. It may represent some other, as yet unknown, substance. Interestingly, the overall level of plasmalogen plus diacyl-PtdEtn in the mutants did not decrease as a result of the loss of the plasmalogen species.

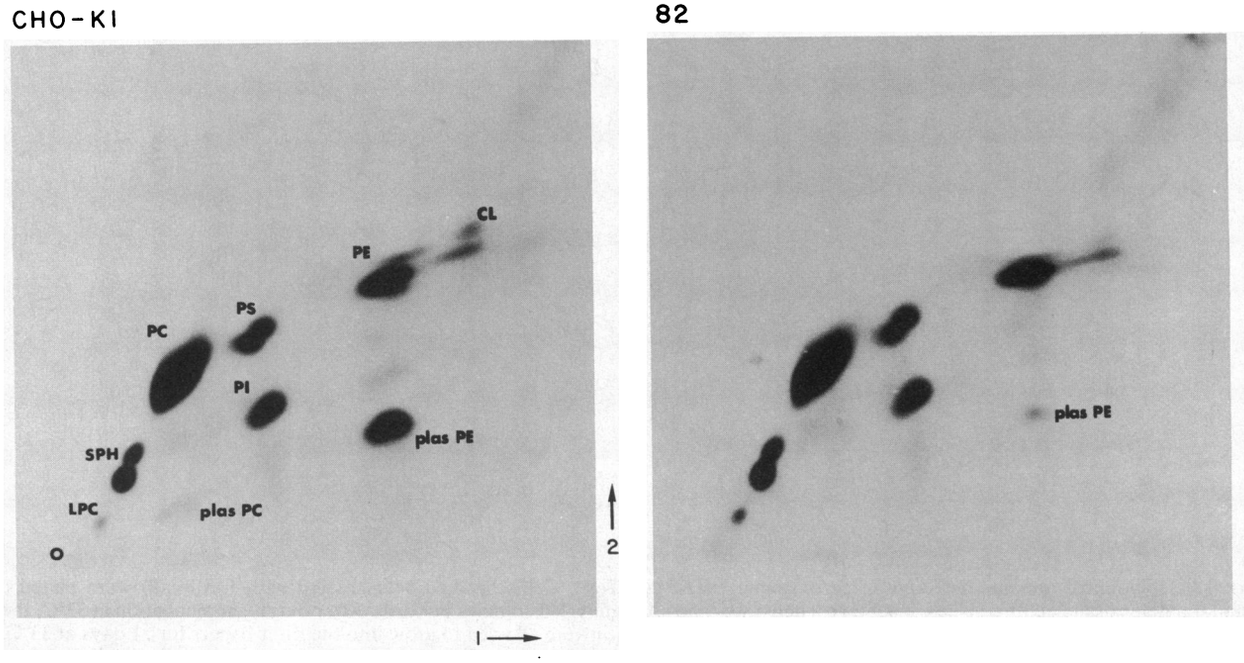

FIG. 2. Phospholipid composition of parent and mutant cells. Cells were grown for several generations at 37°C in medium supplemented with $^{32}P_i$ (5 μCi/ml) to label the phospholipids to constant specific radioactivity. Cells were harvested in PBS and extracted twice by the method of Bligh and Dyer (15). The upper phase was acidified with 0.1 M HCl and 600 μg of carrier lipid (from mouse liver) was added prior to extraction. The lower phases were combined and washed once with 0.1 M HCl. Phospholipids were analyzed using two-dimensional TLC as described (16). Between dimensions, the sample lane was treated with 10 mM $HgCl_2$ (17) and dried for 20 min to cleave the alk-1-enyl moiety. The plasmalogen-form of the phospholipid (now converted to lysoPtdEtn) could be separated from the still intact diacyl and 1-alkyl-2-acyl species. Mild alkaline methanolysis (18) revealed that the latter fraction contained only minor amounts (<5%) of the 1-alkyl-2-acyl species in both parent and mutant cells. LCP, lysoPtdCho; PC, PtdCho-diacyl form; plas PC, PtdCho-plasmalogen form; SPH, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PE, PtdEtn-diacyl form; plas PE, PtdEtn–plasmalogen form; CL, cardiolipin.

Table 3. Phospholipid compositions of parental and mutant cells

Cells	Phospholipid composition, % total								
	LPC	SPH	plasPC	PC	PI	PS	plasPE	PE	CL
CHO-K1	0.4	9.9	0.7	57.4	6.3	5.2	7.1	11.2	1.8
ZR-78	0.9	13.0	0.2	53.5	6.6	5.5	0.7	17.0	2.6
ZR-82	0.5	11.5	0.2	56.3	6.7	5.7	0.7	16.7	2.0
ZR-87	0.9	11.9	0.1	54.1	5.1	5.5	0.7	20.1	1.6

Cells were grown for several generations in medium containing $^{32}P_i$ (5 μCi/ml). Phospholipids were isolated and analyzed by two-dimensional TLC as described in Fig. 2. The labeling of individual phospholipid species was quantitated by scraping each into a scintillation vial containing 2 ml of methanol, followed by the addition of 10 ml of Patterson and Green scintillation cocktail (19). All values represent the average of three cultures and vary by less than 10%. All values were calculated to total phospholipids, 100%. Other lipid species, which represented less than 5% of the chloroform-soluble ^{32}P, were not included in the above calculations and did not vary significantly. The abbreviations for the lipid species are described in the legend of Fig. 2.

Fig. 3 shows a short-term $^{32}P_i$ labeling of the phospholipids. There is little difference between the parental and mutant strains with respect to their overall phospholipid synthetic rates (Fig. 3*A*). The relative rates of PtdCho biosynthesis (Fig. 3*B*) were also unaltered. As expected for a biosynthetic lesion, the rate of plasmalogen–PtdEtn formation was greatly reduced (Fig. 3*C*) in the mutants, but the labeling of the diacyl form (Fig. 3*D*) was increased. This explains the increased content of the diacyl species in the mutants.

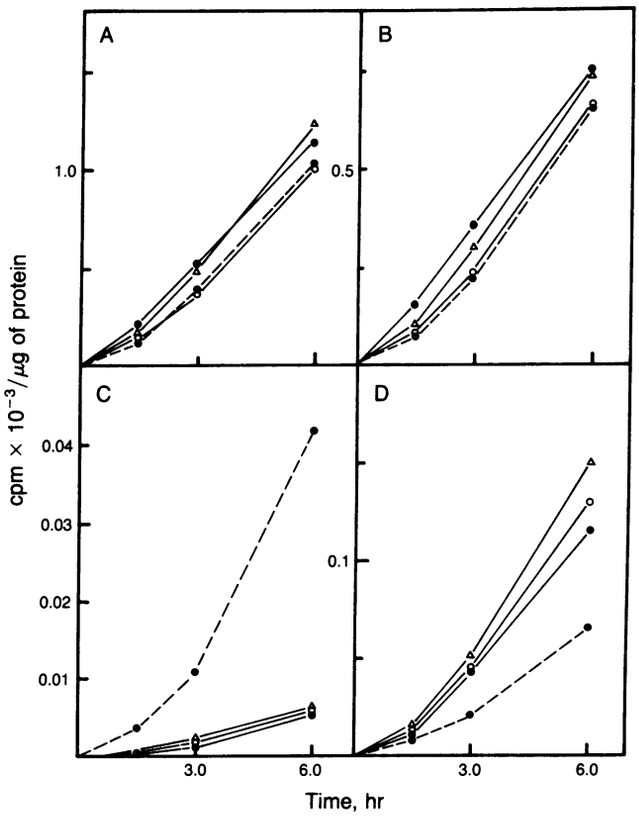

FIG. 3. Phospholipid synthesis in parent and mutant cells. Cells were placed into 60-mm diameter tissue culture dishes in 5 ml of growth medium at a density of 5×10^5 cells per dish and allowed to attach overnight. Medium was removed, and 3.0 ml of medium containing $^{32}P_i$ (50 μCi/ml) was added. Cells were harvested at 1.5, 3.0, and 6.0 hr by scraping. Phospholipids were extracted, and the different species were isolated as described in Fig. 2. The labeling of phospholipid species was quantitated by scraping appropriate regions of the plate directly into a scintillation vial containing 2 ml of methanol. Patterson and Green fluid (19) was added prior to liquid scintillation counting. *A*, Total phospholipid; *B*, PtdCho (diacyl form); *C*, PtdEtn (plasmalogen form); *D*, PtdEtn (diacyl form). ●—●, CHO-K1; ○—○, ZR-78; ■—■, ZR-82; △—△, ZR-87. Triplicate, unlabeled cultures were scraped and used for protein determinations. Values represent the average of duplicate radiolabeled cultures and vary less than 10%.

Other Peroxisomal Enzymes. Examination of two other peroxisomal enzymes, alkyl-DHAP synthase (20) and particulate (peroxisomal) catalase (21), strongly suggests that the lesion in all three mutants is not in one enzyme but rather is a defect in some aspect of peroxisomal assembly (Table 4). The three mutants were deficient in both alkyl-DHAP synthase and particulate catalase. Whole-cell lysates of mutants 78 and 82 contained 20%, while mutant 87 showed only 12%, of the parental alkyl-DHAP synthase activity. Catalase activity, which is found both in the particulate fraction and in the cytosolic fraction in the parental cells, was no longer associated with the particulate fraction in the mutants. The cytosolic activity, however, was increased by 2- to 2.5-fold compared to the parental cells.

DISCUSSION

The data presented in this paper provide strong evidence that there are at least two distinct DHAP acylTases functioning in animal cells. We have isolated mutants deficient in the MalNEt-insensitive activity with a pH optimum of 5.5 (Table 1). The mutants retained a second DHAP acylTase activity, similar to that described by Schlossman and Bell (2, 3), that

Table 4. Alkyl-DHAP synthase and catalase activities in parent and mutant strains

Cells	Alkyl-DHAP synthase, pmol per min per mg	Particulate catalase, unit(s)/mg	Cytosolic catalase, units/mg
CHO-K1	148	18.4 ± 3.1	8.0 ± 1.8
ZR-78	30	1.0 ± 0.5	19.2 ± 4.8
ZR-82	29	0.2 ± 0.3	16.9 ± 2.7
ZR-87	15	0.4 ± 0.4	18.2 ± 2.5

For assays of alkyl-DHAP synthase, cells were grown to near confluence at 33°C, harvested, and lysed. Alkyl-DHAP synthase was assayed in whole-cell homogenates at 33°C. Each assay contained 75 μM Tris·HCl (pH 8.3), 40 mM NaF, 100 μM acyl-DHAP, 200 μM [1-^{14}C]hexadecanol (3.0×10^6 cpm per assay), 0.1% Triton X-100, and cell protein (40–100 μg) in a total volume of 300 μl. Values for alkyl-DHAP synthase represent the average of duplicate cultures and do not vary by more than 5%. For assays of catalase activity, cells were grown to near confluence at 33°C, harvested, and the particulate and cytosolic fractions were isolated. Catalase assays contained 10 mM imidazole·HCl (pH 7.2), 0.5% bovine serum albumin, 0.1% Triton X-100, 8.8 mM H_2O_2, and 15–30 μg of protein in a total volume of 200 μl. Reactions were performed at 25°C for 30 sec and stopped by the addition of 2.0 ml of the titanyl sulfate solution. One unit of catalase activity is defined as the amount of enzyme required to degrade 90% of the H_2O_2 in 1 min (21). Values represent the mean ± SD of three separate preparations.

is active at neutral pH and is inhibited by MalNEt. This second activity may represent an alternate catalytic activity of the microsomal Gro-3-*P* acylTase (2, 3), but the data do not exclude the possibility of yet another enzyme. The peroxisomal DHAP acylTase activity is apparently not required for diacyl-phospholipid synthesis (Fig. 3). The essential function of either of the DHAP acylTase activities for ether lipid biosynthesis could not be determined unequivocally, since at least one other enzyme involved in the ether lipid pathway (the alkyl-DHAP synthase) is also deficient. It is apparent, however, that the peroxisome is somehow crucial to normal plasmalogen synthesis.

Decreased plasmalogen biosynthesis had little effect upon the phospholipid headgroup composition (Table 3). Although plasmalogen PtdEtn was reduced by a factor of 10, the overall level of PtdEtn remained constant. This was due to a compensatory increase in the synthesis of the diacyl form (Fig. 3). The mechanisms by which these cells increase diacyl-PtdEtn synthesis may be of interest since PtdEtn can be synthesized in animal cells by two routes. The pathway described by Kennedy and Weiss (22) involves the condensation of CDP-ethanolamine and diglyceride. A second pathway involves the decarboxylation of phosphatidylserine (23). It is possible that only one of these pathways is involved in the synthesis of the plasmalogen-form of PtdEtn, while the other is responsible for the synthesis of the diacyl form.

The availability of mutants defective in the biosynthesis of plasmalogens may allow us to examine the function(s) of these lipids *in vivo* and in isolated membranes. Although plasmalogens are found in virtually every mammalian cell type, the functions of these molecules are not known (1). Plasmalogens are especially abundant in electrically active tissue, such as brain (24, 25), nerve (17), and myocardium (26). Because of these observations and the unique physical properties of plasmalogens (26, 27), a functional role for plasmalogens in the transmembrane movement of ions has been hypothesized (26). This notion is given some support by studies involving human patients suffering from Zellweger syndrome (28). The tissues of Zellweger patients are deficient in peroxisomes (6) and contain extremely low levels of plasmalogens when compared to normal individuals (7). The disease is also characterized by a lack of muscle tone and mental retardation. Although a causal link has not been established, it is conceivable the lack of plasmalogens may be responsible for these symptoms.

Fibroblasts from Zellweger patients are also deficient in peroxisomal DHAP acylTase, alkyl-DHAP synthase (29), and peroxisomal catalase activities (30). Considering the many similarities between our mutants and Zellweger fibroblasts, our mutants may serve as somatic cell models with which to further characterize the human disease. Perhaps, all possible genetic loci required for peroxisome assembly could be identified among a large collection of CHO mutants deficient in DHAP acylTase. Consequently, our mutants may yield new information regarding the biogenesis of peroxisomes and the targeting of specific enzymes to peroxisomes. The phenotypic and functional consequences of plasmalogen deficiency also requires further study.

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