Partial Phenotypic Suppression of a Peroxisome-deficient Animal Cell Mutant Treated with Aminoglycoside G418*

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Certain enzymes normally associated with peroxisomes, such as the dihydroxyacetone phosphate (DHAP) acyltransferase involved in plasmalogen biosynthesis, are present at low levels in peroxisome-deficient mutants of Chinese hamster ovary (CHO) cells. We now show that the aminoglycoside G418 increases the residual DHAP acyltransferase in mutant ZR-82 by 60-fold. This is accompanied by a dose- and time-dependent restoration of the plasmalogen content. G418 treatment of ZR-82 also increases residual peroxisomal β-oxidation activity by 3.8-fold. G418 does not affect wild-type CHO cells (CHO-K1) or a different peroxisome-deficient mutant, ZR-78.1. The effects of G418 on ZR-82 are transient, since plasmalogens and DHAP-acyltransferase decline to basal levels 5 days after G418 withdrawal. Other aminoglycosides and lysosomotropic agents do not alter plasmalogen levels in ZR-82. The subcellular distribution of catalase (an enzyme of the peroxisomal matrix which is present in normal amounts in peroxisome-deficient mutants but is mislocalized in the cytosol) is unaffected by G418 treatment of ZR-82, demonstrating that G418 does not restore peroxisomes. Localization of catalase by immunofluorescence microscopy confirms a total absence of intact peroxisomes in ZR-82, either before or after exposure to G418. This study is the first to demonstrate that some peroxisome-deficient mutants can be induced to accumulate functional DHAP acyltransferase and other peroxisomal enzymes, usually missing in the absence of peroxisomes. G418 may have some therapeutic value in selected patients with inborn errors of peroxisome assembly, such as Zellweger syndrome.

A key function of peroxisomes is the detoxification of hydrogen peroxide by catalase within the peroxisomal matrix (de Duve and Baudhuin, 1966). In addition, peroxisomes play a unique role in mammalian lipid metabolism. Peroxisomes contain dihydroxyacetone phosphate (DHAP) acyltransferase and alkyl-DHAP synthase, the first two enzymes of plasmalogen (ether lipid) biosynthesis (Hajra and Bishop, 1982), and they also possess a unique β-oxidation pathway for degrading very long chain fatty acids that are not metabolized by mitochondria (Lazarow, 1987).

All peroxisome-deficient enzymes and proteins studied thus far are synthesized on free polyribosomes and imported, after translation, into existing peroxisomes (Borst, 1986). In peroxisome-deficient cells from patients with Zellweger syndrome (Suzuki and Hashimoto, 1986; Suzuki et al., 1987; Santos et al., 1988a, 1988b; Wiemer et al., 1989; Schram et al., 1986), peroxisomal proteins are synthesized normally, but most are rapidly degraded. Presumably, the accelerated turnover of peroxisomal enzymes accounts for their reduced activity in these mutant cells.

We have reported previously the isolation of Chinese hamster ovary (CHO) cell mutants lacking intact peroxisomes (Zoeller and Raetz, 1986; Zoeller et al., 1989). These mutants are deficient in multiple peroxisomal enzyme activities, including DHAP acyltransferase (Zoeller and Raetz, 1986), and they provide a somatic cell model of the human aperoxisomal diseases (Schutgens et al., 1986; Moser, 1987). In the CHO mutants, DHAP acyltransferase activity is reduced 100-fold (Zoeller and Raetz, 1986; Allen et al., 1989), plasmalogen content is reduced 10-fold (Zoeller and Raetz, 1986), and β-oxidation of very long chain fatty acids is reduced 25–100-fold (Allen et al., 1990).

G418 is a neomycin analog widely used in transfection experiments to select for eukaryotic cells that have acquired neomycin resistance genes (Santerre et al., 1984; Danielson et al., 1989). G418, like other aminoglycosides, affects cell growth (Vazquez, 1978), protein synthesis (Vazquez, 1978; Eustice and Wilhelm, 1984a), and phospholipid turnover (Aubert-Tulkens et al., 1979) in eukaryotes. G418 also induces misreading of mRNAs by reducing the fidelity of aminoacyl-tRNA selection (Eustice and Wilhelm, 1984a, 1984b). This loss of ribosomal accuracy causes cells to accumulate misread, abnormal proteins (Buchanan et al., 1987). Total protein synthesis is also partially inhibited (Eustice and Wilhelm, 1984b). Further, aminoglycosides accumulate in lysosomes and raise lysosomal pH, thereby inhibiting sphingomyelinase and certain phospholipases (Aubert-Tulkens et al., 1979; Mingeot-Leclercq et al., 1988).

Tartakoff and co-workers (Gupta et al., 1988) reported that

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1 The abbreviations used are: DHAP, dihydroxyacetone phosphate; CHO, Chinese hamster ovary; neo, neomycin; PBS, phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; TES, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.
**Peroxisomal Enzyme and Plasmalogen Accumulation Induced by G418**

G418 restores transient surface expression of the T-cell surface antigen Thy-1 in class F Thy-1-deficient T-cell lymphoma mutants. The molecular basis for this effect is unclear. Recent work (Stevens and Raetz, 1990) has demonstrated that the class F mutants are deficient in peroxisomal DHAP acyltransferase activity and ether lipids, although they retain intact peroxisomes. These data imply that ether lipid synthesis or some other peroxisomal function might be required for surface expression of Thy-1.

In view of these findings, we examined the effects of G418 on peroxisome-deficient CHO cell mutants. We now show that G418 treatment increases DHAP acyltransferase activity, plasmalogen synthesis, and peroxisomal β-oxidation activity in the peroxisome-deficient CHO cell mutants ZR-82 and S3 but not in mutant ZR-78.1. The G418-treated mutants do not regain intact peroxisomes. The effects of G418 are transient and cannot be duplicated by other aminoglycosides or lysosomotropic agents. These data imply that G418 may have therapeutic value in some patients with peroxisome-deficiency diseases.

**EXPERIMENTAL PROCEDURES**

**Materials—**G418 (Geneticin) was obtained from Gibco. [1-14C]Lignoceric acid (1.70 GBq/mmol) and Biosafe II liquid scintillation mixture were purchased from Research Products International Corp. (Mount Prospect, IL). [2-14C]Ethanolamine (1.96 GBq/mmol) and [γ-32P]ATP ([γ-32P]orthophosphate and 1-[32P]methionine were obtained from ICN (Costa Mesa, CA). Dihydroxyacetone phosphate was synthesized enzymatically according to the procedure of Schlossman and Bell (1976). Titanyl sulfate was obtained from Chemtech (Hayward, CA). All other reagents were purchased from Sigma. Somatostatin and rabbit anti-rat liver phospholipid antibodies (EM Science) were purchased from Baxter (McGraw Park, IL).

**Cells and Culture Conditions—**All cells were grown in Ham's F-12 medium containing l-glutamine (Whittaker Biorproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), penicillin G (100 units/ml), and streptomycin (100 μg/ml) in an atmosphere of 5% CO2 in air. Cells were grown up, with or without 50 μg/ml G418, for 3 days at 37 °C as described above and then incubated for an additional day at 37 °C, as described above. Cells were harvested using 0.5 mM EDTA from 100-mm dishes containing 0.3 M mannitol, 50 mM MOPS (pH 7.4), 10 mM ATP, 1 mM CO2, and 100 μM NAP. After 15-20 min at 30 °C, reactions were stopped by the addition of 3 ml of chlorofrom/methanol (1:2, v/v). 0.5 ml of water, 400 μg of rat liver phospholipids, and 50 μl of concentrated HCl were also added. After a 10-min incubation at 25 °C, 1 ml of chloroform and 1 ml of water were added to form a two-phase system. The phases were separated by centrifugation, and the lower phase was washed twice with 3 ml of preagitated acidic upper phase (Bligh and Dyer, 1959). The final lower phases were collected, evaporated to dryness in glass scintillation vials, and counted in 10 ml of Biosafe II liquid scintillation mixture.

**Assay of Peroxisomal β-Oxidation—**Cells were grown in 100-mm tissue culture dishes at 37 °C in medium containing 0.2 μCi/ml [3-14C]linoleic acid (46 mCi/mmol) and 50-200 μg/ml cell protein in a total volume of 0.3 ml. After 1 h at 37 °C, reactions were stopped, and the amount of [14C]acetate produced was quantified essentially as described by Wanders et al. (1986).

**Peroxisome Fractionation—**Six 100-mm dishes of each cell type were plated into five new dishes in F-12 containing 0.2 μCi/ml [2-14C]ethanolamine (0.2 μCi/ml). Half of the dishes also received 50 μg/ml G418. After 3 days at 37 °C, the medium was removed, and the cells were rinsed with 4 ml of PBS. Cells were detached from each dish at 25 °C by a 10-min incubation in 5 ml of PBS containing 0.5 mM EDTA, harvested using 0.5 ml of 0.25 M sucrose (pH 7.4), 250 mM sucrose, and 1 mM EDTA. Cells were resuspended in the same buffer (0.5 ml/100-mm dish) and frozen at −20 °C to break the cells. All cells were thawed and kept on ice prior to assay (~30 min). DHAP acyltransferase activity was assayed as described by Schlossman and Bell (1976) with the following modifications. Reaction mixtures contained 50 mM KCl, 100 mM MES, and 100 mM TES (pH 5.5 or 7.4, respectively), 5 mM MgCl2, 8 mM NaF, 2 mM KCN, 2 mg/ml bovine serum albumin, 0.1 mM palmityl-CoA, 0.8 mM [32P]DHAP (34 μCi/μmol), and 50-100 μg of cell protein in a total volume of 0.3 ml. Some reactions also contained 0.63 mg/ml N-ethylmaleimide. After 15-20 min at 30 °C, reactions were stopped by the addition of 3 ml of chloroform/methanol (1:2, v/v). 0.5 ml of water, 400 μg of rat liver phospholipids, and 50 μl of concentrated HCl were also added. After a 10-min incubation at 25 °C, 1 ml of chloroform and 1 ml of water were added to form a two-phase system. The phases were separated by centrifugation, and the lower phase was washed twice with 3 ml of preagitated acidic upper phase (Bligh and Dyer, 1959). The final lower phases were collected, evaporated to dryness in glass scintillation vials, and counted in 10 ml of Biosafe II liquid scintillation mixture.

**Peroxisomal Enzyme and Plasmalogen Accumulation Induced by G418**

In this study, we examined the effects of G418 on peroxisomal function in CHO cell mutants. We observed that G418 treatment increases DHAP acyltransferase activity, plasmalogen synthesis, and peroxisomal β-oxidation activity. These findings suggest that G418 may have therapeutic value in patients with peroxisomal dysfunction.

**Conclusion**

G418 restores transient surface expression of Thy-1 in peroxisome-deficient CHO cell mutants. This effect may be due to increased DHAP acyltransferase activity, plasmalogen synthesis, and peroxisomal β-oxidation activity. These findings suggest that G418 may have therapeutic value in patients with peroxisomal dysfunction.
Peroxosomal Enzyme and Plasmalogen Accumulation Induced by G418

RESULTS

Colonies Autoradiography of Peroxisomal DHAP Acyltransferase—DHAP acyltransferase is a peroxisomal enzyme that catalyzes the first step in the plasmalogenic biosynthetic pathway (Hajra and Bishop, 1982; Lazarow, 1987; Zoeller et al., 1988). CHO cell mutants lacking morphologically identifiable peroxisomes (Zoeller et al., 1989) are deficient in DHAP acyltransferase activity and contain reduced levels of plasmalogens relative to wild-type CHO cells (Zoeller and Raetz, 1986; Allen et al., 1990). CHO cells overlaid with polyester cloth discs proliferate upward and form colonies in the polyester cloth (Raetz et al., 1982). The immobilized colonies can then be assayed for DHAP acyltransferase using colony autoradiography (Zoeller and Raetz, 1986). By this assay, peroxisome-deficient CHO cells lack detectable DHAP acyltransferase activity (Zoeller and Raetz, 1986; Zoeller et al., 1989; Allen et al., 1989). When the aminoglycoside G418 was included in the medium during the last 3 days of the polyester overlay, the colonies of the peroxisome-deficient mutant ZR-82 regained measurable DHAP acyltransferase activity (Fig. 1, column A), although the level of activity was reduced somewhat relative to CHO-K1. All of the colonies on the disc responded to G418 treatment. Similar data were obtained for a spontaneous peroxisome-deficient mutant, S3 (data not shown), isolated by a photosensitization-based selection method (Morand et al., 1990). However, not all peroxisome-deficient mutants responded to G418, as illustrated for strain ZR-78.1 (Fig. 1, column A).

Plasmalogenethanolamine Content of G418-treated Cells—The recovery of DHAP acyltransferase activity in ZR-82 suggested that plasmalogen levels might also increase in response to G418. Plasmalogenethanolamine is the major ether lipid in CHO cells and normally comprises ~50% of the ethanolamine-linked phospholipid (Zoeller and Raetz, 1986). In peroxisome-deficient cells, plasmalogenethanolamine levels are reduced, and phosphatidylethanolamine content increases, but the total amount of ethanolamine-containing phospholipid remains constant (Zoeller and Raetz, 1986). To assess the effect of G418 on plasmalogen levels, cells were labeled with [2-14C]ethanolamine in the presence or absence of 50 μg/ml G418. The lipids were extracted, and ethanolamine-containing phospholipid species were separated using the two-step, one-dimensional TLC described under “Experimental Procedures.” Lipid species were visualized using autoradiography and quantified by liquid scintillation counting. Solid bars, controls; hatched bars, +G418. Error bars indicate the range of duplicate cultures.

FIG. 1. Autoradiographic detection of peroxisomal DHAP acyltransferase in colonies treated with G418. Cells were grown into HD-17 polyester cloth discs for 8–10 days at 37°C, with medium changes every 4 days, as described previously (Zoeller et al., 1989). Some dishes received 50 μg/ml G418 during the final 3 days of polyester overlay. Polyester cloths were harvested, rinsed with PBS (pH 7.4), air dried, and frozen at ~20°C to lyse the cells. The immobilized colonies were assayed for peroxisomal DHAP acyltransferase at pH 5.5 in the presence of N-ethylmaleimide as described previously (Zoeller and Raetz, 1986; Allen et al., 1989). Polyester cloths were exposed to x-ray film for ~24 h at ~80°C. Following autoradiography, the polyester cloths were stained with Coomasie Blue to visualize the colonies. A and C, colony autoradiography of peroxisomal DHAP acyltransferase; B and D, the corresponding polyester cloths stained with Coomasie Blue. A and B, +G418; C and D, controls.

FIG. 2. Effect of G418 on the plasmalogenethanolamine content of CHO cells. Cells were grown for 3 days at 37°C in medium containing 0.2 μCi/ml [2-14C]ethanolamine, in the presence or absence of 50 μg/ml G418. The lipids were extracted, and ethanolamine-containing phospholipid species were separated using the two-step, one-dimensional TLC described under “Experimental Procedures.” Lipid species were visualized using autoradiography and quantified by liquid scintillation counting. Solid bars, controls; hatched bars, +G418. Error bars indicate the range of duplicate cultures.

Sugar, and 1 mM EDTA and then resuspended in 5 ml of the same buffer. Nitrogen cavitation was used to break the cells (200 ps.i., 10 min). Homogenates were centrifuged for 10 min at 400 × gpara, and the postnuclear supernatants were held on ice. The nuclear pellets were resuspended in 1 ml of buffer and centrifuged at 400 × gpara, for 10 min. The two postnuclear supernatants were pooled and then recentrifuged at 400 × gpara, for 10 min to remove residual nuclei and intact cells. The final postnuclear supernatant fractions were centrifuged in a 50-Ti rotor (Beckman) at 100,000 × gpara, for 90 min in a Beckman ultracentrifuge. The supernatants (soluble fractions) were collected, and the pellets (particulate fractions) were resuspended in 0.5 ml of buffer. Catalase was assayed in all fractions prior to freezing.

Macromolecular Synthesis—Cells were plated at 3 × 10^6 cells/60-mm dish in 3 ml of medium, in the presence or absence of 50 μg/ml (nontransfected cells) or 500 μg/ml (transfected cells) G418. After 3 days at 37°C, cells were labeled with [methyl-3H]-thymidine or L-[35S]methionine as follows. For [methyl-3H]-thymidine labeling, cells were incubated in 2.5 ml of fresh medium containing 1 μCi/ml [methyl-3H]-thymidine. After 2 h at 37°C, the medium was removed, each dish was rinsed with 2 ml of PBS, and [3H]thymidine incorporation was evaluated as described previously by Mosley et al. (1981). To assess [35S]methionine incorporation, another set of dishes received 2.5 ml of [35S]methionine-containing medium containing 1 μCi/ml [35S]methionine. After 2 h at 37°C, the medium was removed, and the cells were rinsed with 2 ml of PBS/dish. The cells were scraped into 1 ml of PBS, and 1 ml of ice-cold 20% (w/v) trichloroacetic acid was added. After a 1 h incubation on ice, the solutions were filtered through Whatman GF/B filters, and each filter was washed with 3 ml of ice-cold 10% trichloroacetic acid. For some experiments, cells were incubated for 3 days at 37°C with 50 μg/ml G418 and then reincubated for 24 h in the absence of G418 before labeling with [3H]thymidine or [35S]methionine. Parallel dishes were used for protein determinations.

FIG. 2. Effect of G418 on the plasmalogenethanolamine content of CHO cells. Cells were grown for 3 days at 37°C in medium containing 0.2 μCi/ml [2-14C]ethanolamine, in the presence or absence of 50 μg/ml G418. The lipids were extracted, and ethanolamine-containing phospholipid species were separated using the two-step, one-dimensional TLC described under “Experimental Procedures.” Lipid species were visualized using autoradiography and quantified by liquid scintillation counting. Solid bars, controls; hatched bars, +G418. Error bars indicate the range of duplicate cultures.
with [2-14C]ethanolamine and increasing concentrations of G418. Plasmenylethanolamine levels were measured after 1–5 days. Fig. 3 shows that G418 caused a dose- and timedependent increase in plasmenylethanolamine in ZR-82, with plasmenylethanolamine content approaching wild-type levels after 5 days in 50 μg/ml G418. Similar data were obtained for mutant S3 (not shown).

Experiments by Tartakoff and co-workers (Gupta et al., 1988) revealed that the ability of G418 to restore surface Thy-1 in the class F lymphoma mutants is transient. Therefore, we investigated the stability of plasmenylethanolamine levels in CHO cells exposed to G418. Wild-type and peroxisome-deficient cells were treated with 50 μg/ml G418 for 3 days at 37 °C and then reincubated for up to 6 days in medium lacking G418. Cells were labeled with [2-14C]ethanolamine throughout the course of the experiment, and plasmenylethanolamine content was determined using TLC. Care was taken to ensure that all cells remained in logarithmic growth throughout the course of the experiment. As shown in Fig. 4, plasmalogen levels increased in ZR-82 and S3 in the presence of G418 and continued to rise for 1–2 days after G418 removal. Plasmalogen levels then declined, reaching basal levels ~5 days after G418 was removed. Again, mutant ZR-78.1 was unaffected. Plasmenylethanolamine levels also increased slightly in CHO-K1 after G418 removal (Fig. 4).

Specificity for G418—In another set of experiments, other aminoglycosides were tested for their ability to induce plasmalogen synthesis in ZR-82. Cells were grown for 3 days in the presence of [2-14C]ethanolamine and 50 μg/ml G418, or the maximum nontoxic dose of paromomycin (500 μg/ml), hygromycin B (5 μg/ml), or gentamycin (200 μg/ml). As shown in Fig. 5, none of the other aminoglycosides altered the plasmenylethanolamine content of ZR-82. This is interesting in view of the fact that gentamycin is nearly identical to G418 in structure (Gupta et al., 1988). In addition, all of these aminoglycosides inhibit protein synthesis (Vazquez, 1978) and lysosome function (Mingeot-Leclercq et al., 1988); and paromomycin and G418 promote misreading of mRNAs (Eustice and Wilhelm, 1984b; Buchanan et al., 1987). Lysosomotropic agents, such as chloroquine or ammonium chloride (at their maximum nontoxic doses), and low concentrations of cycloheximide also had no effect on the plasmalogen content of ZR-82 (Fig. 5).

Plasmenylethanolamine Synthesis in ZR-82 Transfectants

![Fig. 3. Dose- and time-dependent recovery of plasmenylethanolamine in mutant ZR-82 treated with G418.](image)

**Fig. 3.** Dose- and time-dependent recovery of plasmenylethanolamine in mutant ZR-82 treated with G418. ZR-82 was grown in 60-mm culture dishes at 37 °C in medium containing 10, 25, or 50 μg/ml G418 and 0.2 μCi/ml [2-14C]ethanolamine. After 1, 2, 3, or 5 days at 37 °C, the lipids were extracted, and the amount of plasmenylethanolamine was quantified as described under "Experimental Procedures." In control incubations ZR-82 was labeled with [2-14C]ethanolamine for 3 days in the absence of G418. Each data point represents an individual culture.

![Fig. 4. The effect of G418 on plasmalogen levels in ZR-82 is transient.](image)

**Fig. 4.** The effect of G418 on plasmalogen levels in ZR-82 is transient. Cells were treated with 50 μg/ml G418 for 3 days at 37 °C in medium containing 0.2 μCi/ml [2-14C]ethanolamine as described under "Experimental Procedures." At the end of this period a portion of each culture was used for lipid extraction and TLC. The rest of each sample was plated into five new dishes in medium containing 0.2 μCi/ml [2-14C]ethanolamine (but no G418) and reincubated at 37 °C for 1, 2, 3, 5, or 6 days (as indicated by the arrow) prior to lipid extraction and TLC. Day 0 values indicate cultures labeled with [2-14C]ethanolamine for 3 days in the absence of G418. The arrow indicates the end of the G418 treatment period. Error bars indicate the range of duplicate cultures.

![Fig. 5. Plasmenylethanolamine content of mutant ZR-82 treated with various agents.](image)

**Fig. 5.** Plasmenylethanolamine content of mutant ZR-82 treated with various agents. ZR-82 was grown for 3 days at 37 °C in medium containing 0.2 μCi/ml [2-14C]ethanolamine and the indicated amounts of various compounds: lane 1, no additions; lane 2, 50 μg/ml G418; lane 3, 500 μg/ml paromomycin; lane 4, 5 μg/ml hygromycin B; lane 5, 200 μg/ml gentamycin; lane 6, 0.05 μM cycloheximide; lane 7, 50 μM chloroquine; lane 8, 1 mM ammonium chloride. At the end of the incubation period the lipids were extracted using neutral Bligh-Dyer conditions (Esko and Raetz, 1980), containing lipid species were separated by TLC, and the amount of plasmenylethanolamine was quantified as described under "Experimental Procedures." Error bars indicate the range of duplicate cultures.

Resistant to G418—G418 is "slowly toxic" to eukaryotic cells, i.e. it accumulates in cells over time, eventually causing cell death. Although there was no apparent cytotoxicity caused by G418 in any of the experiments described above, we wanted to assess more clearly whether cytotoxicity was responsible for the elevated plasmalogen content of ZR-82. CHO cells transfected with a neomycin resistance gene (Santerre et al., 1984; Danielson et al., 1989) acquire the ability to grow indefinitely in concentrations of G418 that would kill nontransfected cells. The neomycin resistance gene encodes a phosphotransferase that catalyzes the transfer of the γ-phosphate from ATP to G418 (Danielson et al., 1989). The phosphorylated G418 product is nontoxic (Danielson et al., 1989). This suggests that the concentration of unmodified G418 in transfected cells should be lower than in nontransfected cells.
CHO cells cotransfected with pKOneo and the bone-liver-kidney form of alkaline phosphatase (designated K1nAP2.2, 82nAP8.3, and 78.1nAP1.5, respectively) can be propagated indefinitely in 0.5 mg/ml G418 (data not shown). The transfecants were labeled for 3 days with [2-14C]ethanolamine in the presence or absence of 0.5 mg/ml G418, and their plasmalogen content was measured as described above. Plasmalogen levels in K1nAP2.2 and 78.1nAP1.5 were unchanged after growth in G418 (Fig. 6). On the other hand, plasmenylethanolamine levels in 82nAP8.3 increased to ~15-16% of the ethanolamine phospholipid pool in the presence of 0.5 mg/ml G418 (Fig. 6). The magnitude of the plasmalogen increase in 82nAP8.3 was proportional to the concentration of G418 present in the medium (data not shown), as was shown for ZR-82 (Fig. 3). However, higher concentrations of G418 were required to elevate plasmalogens in the transfecants. This is consistent with the idea that the effective intracellular concentration of G418 is lower in the transfected cells. The fact that the transfecants can be grown indefinitely in medium containing 0.5 mg/ml G418 renders unlikely the possibility that cytotoxicity was responsible for the plasmalogen increase in the mutant strains. It is also unlikely that the alkaline phosphatase gene present in the transfecants contributed to the plasmalogen increase because similar data were obtained for ZR-82 transfected with only the vector.2

Macromolecular Synthesis—Previous studies have shown that G418 partially inhibits protein synthesis and slows the growth of eukaryotic cells (Vazquez, 1978). To evaluate the sensitivity of CHO cells to these effects of G418, CHO-K1, ZR-82, ZR-78.1, and their corresponding transfecants were grown for 3 days in 50 or 500 μCi/ml G418, respectively. Protein and DNA synthesis were measured after a subsequent 2-h incubation, as described under “Experimental Procedures.” G418 treatment inhibited protein synthesis as much as 50% (Table I) and DNA synthesis up to 40%. There was no apparent correlation between increased plasmalogen levels (Figs. 2 and 6) and the degree of inhibition of protein and DNA synthesis (Table I), as revealed by comparing data for ZR-82 and 82nAP8.3, or ZR-78.1 and 78.1nAP1.5. These results suggest that the effects of G418 on macromolecular synthesis are not related to its effects on other lipid synthesis. In addition, the effects of G418 on protein and DNA synthesis were readily reversible (Table I). One day after G418 removal, macromolecular synthesis in ZR-82 and CHO-K1 was indistinguishable from control cells.

Phospholipid Composition—Because G418 increased plasmalogen levels in ZR-82, we thought that G418 might also alter the levels of other phospholipids in CHO cells. CHO-K1 and ZR-82 were labeled for 3 days with [32P]orthophosphate, in the presence or absence of 50 μCi/ml G418, and phospholipid species were separated by two-dimensional TLC as described under “Experimental Procedures.” Phospholipids were visualized by autoradiography. Parallel dishes were set up with and without G418 for protein determination. Panel A, ZR-82; panel B, ZR-82 + G418; panel C, CHO-K1; panel D, CHO-K1 + G418. Lipid species: 1, sphingomyelin; 2, phosphatidylethanolamine; 3, phosphatidylserine; 4, phosphatidylinositol; 5, phosphatidylglycerol; 6, lyso phosphatidylethanolamine; 7, unidentified.

were readily reversible (Table I). One day after G418 removal, macromolecular synthesis in ZR-82 and CHO-K1 was indistinguishable from control cells.

**Table I**

| Strain     | [35S]Methionine incorporated | [3H]Thymidine incorporated |
|------------|-----------------------------|---------------------------|
|            | Control + G418 | + G418 | + wash | Control + G418 | + G418 | + wash |
| CHO-K1     | 8.81 ± 0.19 | 7.93 ± 0.19 | 10.10 ± 0.19 | 9.8 ± 0.17 | 6.4 ± 0.17 | 9.7 ± 0.17 |
| ZR-82      | 6.23 ± 0.19 | 3.15 ± 0.19 | 5.56 ± 0.19 | 11.6 ± 0.17 | 7.0 ± 0.17 | ND     |
| ZR-78.1    | 5.67 ± 0.19 | 5.64 ± 0.19 | ND      | 8.2 ± 0.17 | 7.3 ± 0.17 | ND     |
| S3         | 11.70 ± 0.19 | 8.20 ± 0.19 | ND      | 8.6 ± 0.17 | 5.4 ± 0.17 | ND     |
| K1nAP2.2   | 8.52 ± 0.19 | 4.72 ± 0.19 | ND      | 14.3 ± 0.17 | 12.9 ± 0.17 | ND     |
| 82nAP8.3   | 9.18 ± 0.19 | 8.92 ± 0.19 | ND      | 15.7 ± 0.17 | 14.7 ± 0.17 | ND     |
| 78.1nAP1.5 | 5.15 ± 0.19 | 3.38 ± 0.19 | ND      | 13.5 ± 0.17 | 10.7 ± 0.17 | ND     |

2 T. Kobayashi and C. Raetz, unpublished observation.
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Table II

| Lipid     | CHO-K1 | K1 + G418 | ZR-82 | 82 + G418 |
|-----------|--------|-----------|-------|----------|
| % of total phospholipid |        |           |       |          |
| SM        | 8.6 ± 1.1 | 8.1 ± 0.8 | 9.8 ± 0.5 | 8.2 ± 0.6 |
| PC        | 45.9 ± 0.2 | 49.1 ± 0.6 | 44.4 ± 0.7 | 48.7 ± 1.2 |
| PS        | 8.3 ± 0.3 | 7.3 ± 0.4 | 7.2 ± 0.2 | 7.0 ± 0.4 |
| PI        | 7.2 ± 0.5 | 7.9 ± 0.4 | 8.0 ± 0.4 | 7.8 ± 0.4 |
| PE        | 17.2 ± 0.4 | 15.2 ± 0.2 | 24.5 ± 0.1 | 19.1 ± 0.2 |
| PlasE     | 9.0 ± 0.5 | 8.3 ± 0.4 | 1.0 ± 0.2 | 5.0 ± 0.3 |
| Unidentified | 4.0 ± 0.1 | 4.2 ± 0.3 | 5.0 ± 0.3 | 4.3 ± 0.5 |

Table III

DHAP-acyltransferase activities in cells treated with G418

| Strain     | pH 5.5 | pH 7.4 |
|------------|--------|--------|
|            | Control | + NEM | Control | + NEM |
| CHO-K1     | 503 ± 11 | 557 ± 14 | 833 ± 19 | 155 ± 9 |
| K1 + G418  | 516 ± 13 | 546 ± 15 | 776 ± 38 | 122 ± 17 |
| ZR-82      | 2 ± 1 | 1 ± 1 | 717 ± 39 | 0.3 ± 0.3 |
| 82 + G418  | 122 ± 3 | 112 ± 7 | 576 ± 36 | 38 ± 5 |
| ZR-78.1    | 11 ± 1 | 7 ± 1 | 775 ± 50 | 13 ± 2 |
| 78.1 + G418 | 8 ± 1 | 3 ± 1 | 604 ± 13 | 8 ± 2 |

Table IV

Effect of G418 on peroxisomal β-oxidation of lignoceric acid

Cells were grown up at 37 °C in the presence or absence of 50 μg/ml G418 and broken by sonication as described under "Experimental Procedures." Reaction mixtures contained 0.3 M mannitol, 50 mM MOPS (pH 7.4), 10 mM ATP, 1 mM NAD, 0.1 mM FAD, 0.2 mM CoA, 5 mM MgCl₂, 5 mM KCN, 10 mM [1-14C]lignoceric acid (46 mCi/mmol), and 50-200 μg of cell protein in a total volume of 0.2 ml. After 1 h at 37 °C, reactions were stopped, and the amount of [14C]acetate produced was quantified essentially as described (Wanders et al., 1986). All assays contained KCN to inhibit mitochondrial β-oxidation. Data are the mean ± S.D. of three determinations.

| Strain     | 14C Acetate pmol/min/mg protein |
|------------|-------------------------------|
| CHO-K1     | 2.48 ± 0.94 | 1.72 ± 0.06 |
| S3         | 0.30 ± 0.02 | 1.13 ± 0.20 |
| ZR-82      | 0.25 ± 0.06 | 0.23 ± 0.04 |
| ZR-78.1    | 0.13 ± 0.08 | 0.59 ± 0.14 |
| 82 + G418  | 0.30 ± 0.02 | 1.13 ± 0.20 |

Menylenethanolamine levels five-fold in ZR-82 to ~5% of the total phospholipid, consistent with the data obtained using [2-14C] ethanolamine labeling (Figs. 2–5). The phospholipid composition of CHO-K1 was not altered by G418 (Fig. 7 and Table II). However, G418 induced a generalized phospholipidosis in CHO cells, resulting in a 30–50% increase in the phospholipid/protein ratio in CHO-K1 and ZR-82 (data not shown). This is consistent with previous studies (Aubert-Tulkens et al., 1979) which demonstrated that G418 and other aminoglycosides cause a generalized phospholipidosis in rat fibroblasts without affecting phospholipid composition.

Quantitation of Peroxisomal Enzyme Activities in Cells Treated with G418—The ability of G418 to restore plasmalogens (Fig. 7) and DHAP acyltransferase activity (Fig. 1) in ZR-82 suggested that G418 might affect the activity of additional peroxisomal enzymes. To address this question, we first quantified the amount of DHAP acyltransferase activity in cells grown in the presence or absence of G418. CHO cells contain two DHAP acyltransferase activities (Ballas et al., 1984; Webber et al., 1987). The peroxisomal form has an acidic pH optimum and is relatively resistant to N-ethylmaleimide. The microsomal form has a neutral pH optimum and is inhibited by N-ethylmaleimide. As shown in Table III, G418 treatment increased peroxisomal DHAP acyltransferase activity in ZR-82 more than 60-fold, whereas ZR-78.1 and CHO-K1 were unaffected. G418 did not perturb microsomal DHAP acyltransferase activity in any of the strains (Table III).

In another set of experiments, the effect of G418 on peroxisomal β-oxidation was evaluated. The peroxisomal β-oxidation pathway can degrade palmitate, as well as very long chain fatty acids (>C22) which are not substrates for the mitochondrial pathway (Lazarow, 1987; Singh et al., 1984). Another distinguishing feature of the peroxisomal pathway is its resistance to inhibition by potassium cyanide (Manns et al., 1979). We found that G418-treated ZR-82 and S3 regained the ability to metabolize lignoceric acid in vitro (Table IV) via the peroxisomal β-oxidation pathway. Taken together, these data suggest that G418 was able to restore the activity of at least five peroxisomal enzymes in mutant ZR-82 (DHAP acyltransferase and the four enzymes of the peroxisomal β-oxidation pathway) (Lazarow, 1987). Peroxisomal β-oxidation activity was not increased in ZR-78.1 after G418 treatment (Table IV).

Peroxisome Content of G418-treated Cells—The simultaneous recovery of multiple enzyme activities normally associated with peroxisomes in G418-treated ZR-82 suggested that G418 might transiently restore intact peroxisomes, i.e. cause a temporary suppression of the mutant phenotype. In peroxisome-deficient cells catalase, in contrast to the peroxisomal enzymes discussed thus far, remains active but is located in the cytosol rather than the peroxisomal matrix (Zoeller and Raetz, 1986; Zeoller et al., 1989; Allen et al., 1989). Therefore, we investigated the ability of G418 to restore intact peroxisomes in ZR-82, using the localization of catalase as an indicator of peroxisome content. CHO-K1 and ZR-82 were grown up at 37 °C, lysed using nitrogen cavitation, and separated into soluble and particulate fractions as described under "Experimental Procedures." As shown in Table V, the specific activity of catalase was similar in CHO-K1 and ZR-82. However, the activity was primarily associated with the particulate fraction of wild-type cells but the cytosolic fraction of ZR-82. Although a small amount of the catalase activity was associated with the particulate fraction in ZR-82, this activity did not show any latency, strongly suggesting that some of the enzyme was adsorbed onto cellular membranes rather than being contained within the peroxisomal matrix. Cells were also cultured in G418 using conditions that restored nearly normal amounts of plasmalogens in ZR-82 (Fig. 4, day 4), i.e. 3 days
TABLE V

| Strain       | Catalase | Latency | Particulate | Soluble | Recovery |
|--------------|----------|---------|-------------|---------|----------|
| CHO-K1       | 15.0 ± 0.9 | 51 ± 2 | 71 ± 2 | 29 ± 2 | 96 ± 9   |
| K1 + G418    | 14.4 ± 1.2 | 49 ± 1 | 70 ± 4 | 30 ± 4 | 95 ± 5   |
| ZR-82        | 14.3 ± 0.9 | 1 ± 1  | 16 ± 2 | 84 ± 2 | 96 ± 5   |
| 82 + G418    | 12.8 ± 1.4 | 4 ± 2  | 18 ± 5 | 82 ± 5 | 93 ± 6   |

Fig. 8. Peroxisome content of CHO-K1 and ZR-82 treated with G418. CHO cells were grown for 3 days at 37 °C in the presence or absence of 50 μg/ml G418, and the subcellular localization of catalase was determined using immunofluorescence microscopy as described previously (Zoeller et al., 1988; Santos et al., 1988b). A, CHO-K1; B, CHO-K1 with G418; C, ZR-82; D, ZR-82 with G418.

at 37 °C in 50 μg/ml G418 followed by 1 day at 37 °C in the absence of G418 prior to cellular fractionation. Under these conditions, G418 did not markedly alter the distribution or latency of catalase in ZR-82 or CHO-K1 (Table V). The inability of G418 to redistribute catalase into the particulate fraction of ZR-82 suggested that these cells had not regained intact peroxisomes. Similar data were obtained for CHO-K1 and ZR-82 treated with G418 for only 3 days (Fig. 4, day 3) or for K1nAP2.2 and 82nAP8.3 grown continuously in medium containing 500 μg/ml G418 (data not shown).

Since a small number of intact peroxisomes might not be detected in subcellular fractionation experiments, we used immunofluorescence microscopy (Santos et al., 1988b; Wieder et al., 1989) to assess the subcellular location of catalase more closely in cells treated with G418. By this assay (Fig. 8), CHO-K1 contained numerous punctate fluorescent structures, i.e. peroxisomes, whereas ZR-82 lacked these structures altogether. Similarly, no peroxisomes were detected in ZR-82 after 3 days of growth in the presence of 50 μg/ml G418 (Fig. 8) or in 82nAP8.3 grown continuously in medium supplemented with 0.5 mg/ml G418 (data not shown). The peroxisome content of CHO-K1 was not affected by G418 (Fig. 8). These results are consistent with the subcellular fractionation data and demonstrate that G418 treatment does not restore intact peroxisomes in mutant ZR-82.

DISCUSSION

Although the aminoglycoside G418 is used extensively in transfection experiments to select for eukaryotic cells that have acquired neomycin resistance genes (Santerre et al., 1984; Danielson et al., 1989), the effects of G418 on cell physiology are complex and not fully characterized. We now report that G418 treatment restores plasmalogen biosynthesis and multiple peroxisomal enzyme activities in some peroxisome-deficient CHO cell mutants. The effects of G418 are time- and concentration-dependent. Plasmalogen levels in mutant ZR-82 approximate those of wild-type cells after a 5-day incubation in medium containing 50 μg/ml G418 (Fig. 3). Similarly, in extracts of G418-treated ZR-82 cells, DHAP acyltransferase is increased up to 60-fold (Table III), and peroxisomal β-oxidation is increased ~4 fold (Table IV). Every colony derived from a population of ZR-82 cells appears to respond to G418 (Fig. 1), and the effects of G418 are transient (Fig. 4). Stable revertants are not induced. The independently isolated CHO mutants used in this study differ considerably in their ability to increase plasmalogens and peroxisomal enzyme activities in response to G418. After 3 days in G418, plasmalylethanolamine levels increase ~5-fold in ZR-82 and ~2-fold in S3 but are unchanged in ZR-78.1 and CHO-K1 (Fig. 2). Similar results are obtained with DHAP acyltransferase (Table III) and peroxisomal β-oxidation activities (Table IV). The molecular basis for this difference is not obvious given that all the mutants appear to belong to a single complementation group (Zoeller et al., 1989; Allen et al., 1990). We are currently examining the sequence of the gene encoding the 35-kDa membrane protein of peroxisome assembly, recently cloned from rat liver (Tsukamoto et al., 1991), in ZR-82 and ZR-78, since we have found recently that the rat cDNA encoding this protein corrects all aspects of the peroxisome deficiency phenotype of ZR-82 and ZR-78.3.

Although G418 restores the activities of multiple peroxisomal enzymes in ZR-82, the cells do not appear to regain intact peroxisomes. Under conditions in which the plasmalylethanolamine content of ZR-82 reaches ~75% of the wild-type value (Fig. 4, day 4), catalase latency and distribution are not significantly altered (Table V). Similarly, when catalase is visualized using immunofluorescence microscopy, CHO-K1 containing numerous fluorescent peroxisomes, whereas ZR-82 lacks these structures, either before or after G418 treatment (Fig. 8). These data show that G418 does not cause a complete suppression of the mutation in ZR-82, but we cannot eliminate the possibility that G418 restores peroxisomes devoid of catalase.

An important implication of these data is that the peroxisome-deficient CHO cell mutant ZR-82 retains the ability to synthesize peroxisomal proteins even though it lacks intact peroxisomes (Zoeller et al., 1989). All peroxisomal proteins studied thus far are synthesized on free polyribosomes and imported after translation into preexisting peroxisomes (Borst, 1986). In the absence of peroxisomes peroxisomal proteins are synthesized normally but are rapidly degraded (Schram et al., 1986; Santos et al., 1988a), presumably because they cannot be targeted to peroxisomes. It is likely that this...
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rapid turnover of peroxisomal proteins accounts for the low activity of peroxisomal enzymes in these cells.

Two lines of evidence support the conclusion that the effects of G418 on peroxisomal enzymes in ZR-82 are not related to gross cytotoxicity. (i) G418 (0.5 mg/ml) increases plasmeneylethanolamine levels ~3-fold in ZR-82 stably transfected with pKOneo (Fig. 6). These cells can be grown indefinitely in this concentration of G418. (ii) Although G418 partially inhibits protein and DNA synthesis in all of the CHO strains, including CHO-K1 (Table I), these effects are readily reversible within 24 h of G418 removal, and there is no apparent correlation between the degree of inhibition of protein synthesis and the plasmalogen content of the CHO mutants (Table I, data for ZR-82 and 82nAP8.3 or ZR-78.1 and 78.1nAP1.5). In addition, although all aminoglycosides inhibit protein synthesis (Eustice and Wilhelm, 1984a) and lysosome function (Aubert-Tulkens et al., 1979) in eukaryotes, the other aminoglycosides and lysosomal agents tested fail to mimic the effects of G418 on plasmalogen synthesis in ZR-82 (Fig. 5).

G418 (but not other aminoglycosides or lysosomal agents) has been shown previously to restore transient, partial surface expression of Thy-1 antigen in cell F, B, and A Thy-1-deficient T-cell lymphoma mutants (Gupta et al., 1988). These mutants lack surface expression of Thy-1 because they cannot properly assemble the glycosyl phosphatidylinositol anchor of Thy-1 (Hyman, 1989; Stevens and Raetz, 1991). Thy-1 protein is synthesized normally, but its turnover is accelerated (Gupta et al., 1988; Hyman, 1989). The common features of the Thy-1-deficient and the peroxisome-deficient mutants suggest that G418 somehow slows the turnover of proteins that cannot be targeted to their proper locations. If this were the only explanation for the G418 effect, however, some response should have been observed with mutant ZR-78.1.

Although the mechanism of action of G418 is unknown, one interesting possibility is that a heat shock response is induced in G418-treated cells. Recent work by Buchanan et al. (1987) suggests that heat shock proteins are produced in human fibroblasts treated with G418 but not in fibroblasts treated with other aminoglycosides. Therefore, it is tempting to hypothesize that heat shock proteins are synthesized in G418-treated ZR-82 cells and that the heat shock proteins increase the activity of peroxisomal enzymes by promoting their proper folding and assembly in the absence of peroxisomes. Correctly folded peroxisomal proteins might have a longer half-life, even if they are not contained in intact peroxisomes. To explain the difference between mutants ZR-82 and ZR-78.1, one would have to postulate that the latter does not accumulate heat shock proteins in response to G418, perhaps because of a secondary mutation.

Given that peroxisomes are not present in the CHO mutants treated with G418, it will be necessary to establish the location of the accumulated peroxisomal proteins in these cells. Santos et al. (1988b) discovered that in cells lacking peroxisomes, such as fibroblasts from patients with Zellweger syndrome, peroxisomal integral membrane proteins are not cytosolic but are found in small, apparently empty, vesicles termed “peroxisomal ghosts.” Although ghosts are present in other peroxisome-deficient cells (Wiener et al., 1989), including our CHO mutants (Zoeller et al., 1989), peroxisomal ghosts have not been isolated, and their exact composition is unknown.

Our findings indicate that one must be very careful when using G418/neoe as a selectable marker in transfection experiments because phenotypic suppression may arise from G418 treatment which is unrelated to the cotransfected DNA.

Regardless of the mechanism of action of G418, this study is the first demonstration that peroxisome-deficient cells can, at least transiently, regain substantial activity of peroxisomal enzymes and the ability to synthesize plasmalogens. If cultured skin fibroblasts from patients with Zellweger syndrome, or any of the other human peroxisome-deficiency diseases, also regain plasmalogens when grown in the presence of G418, it may be possible to use G418 or G418 analogs to alleviate some of the symptoms associated with these diseases.

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