Decreased phosphatidylcholine biosynthesis and abnormal distribution of CTP:phosphocholine cytidylyltransferase in cholesterol auxotrophic Chinese hamster ovary cells

Margo K. Storey, David M. Byers, Harold W. Cook, and Neale D. Ridgway[1]

The Atlantic Research Centre and Departments of Pediatrics and Biochemistry, Dalhousie University, 5849 University Avenue, Halifax, Nova Scotia, Canada B3H 4H7

Abstract Phosphatidylcholine (PtdCho) biosynthesis was examined in the sterol regulatory defective (SRD) Chinese hamster ovary (CHO) cell line SRD 6. SRD 6 cells do not display transcriptional activation of sterol-regulated genes and are cholesterol auxotrophs. Compared to CHO 7 cells (parental line from which the SRD cells were derived), incorporation of [^{3}H]choline during a 2-h pulse into PtdCho and sphingomyelin was reduced 3- and 4.5-fold, respectively, in SRD 6 cells grown with or without cholesterol. SRD 6 cells grown in cholesterol-free medium for 24 h had 8% less phosphatidylcholine (PtdCho) mass compared to CHO 7 cells. Consistent with impaired CTP:phosphocholine cytidylyltransferase activity, [^{3}H]choline-labeled SRD 6 cells had elevated [^{3}H]phosphocholine and delayed conversion to [^{3}H]PtdCho during a 2-h chase period. Compared to CHO 7 cells, cytosolic cytidylyltransferase activity was elevated 2- to 3-fold in SRD 6 cells grown in the absence of cholesterol, but activity in the total membrane fraction was normal. Immunoblot analysis confirmed that cytidylyltransferase mass was increased 2-fold in SRD 6 total cell extracts and cytosol, but not membranes. The amount of [^{32}P]phosphate-labeled cytidylyltransferase in cytosol and membranes of SRD 6 cells were similar to controls. Likewise, cytidylyltransferase mRNA levels were not significantly different between SRD 6 and CHO 7. The defect in PtdCho synthesis in SRD 6 cells could be overcome by treatment with 150 μM oleate, such that after 5 h [^{3}H]choline incorporation into PtdCho and phosphocholine in SRD 6 and CHO 7 cells was similar. Cholesterol auxotrophic SRD 6 cells display reduced PtdCho mass and synthesis and elevated levels of cytosolic cytidylyltransferase, defects that were only partially corrected by growth in exogenous cholesterol. These results indicate a requirement for normal cholesterol regulation and synthesis in the maintenance PtdCho levels and activity of cytidylyltransferase.—**Storey, M. K., D. M. Byers, H. W. Cook, and N. D. Ridgway.** Decreased phosphatidylcholine biosynthesis and abnormal distribution of CTP:phosphocholine cytidylyltransferase in cholesterol auxotrophic Chinese hamster ovary cells. *J. Lipid Res.* 1997. **38:** 711–722.

Supplementary key words phosphatidylcholine • cholesterol regulation • CTP:phosphocholine cytidylyltransferase • sterol regulatory defective CHO cells

Strict control of the intracellular concentration of cholesterol is maintained by an elaborate feed-back mechanism that regulates the transcription of cholesterol biosynthetic enzymes and the LDL receptor, stability of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase and cholesterol esterification (1). Regulation of cholesterol synthesis, esterification, and uptake is probably only one component of a more complex regulatory pathway that integrates signals for the maintenance of appropriate levels and ratios of membrane phospholipids and sterols. The cholesterol:phospholipid ratio is an important determinant in membrane fluidity and packing (2) and influences the activity of membrane-associated enzymes and transporters (3).

Coordinate changes in cholesterol and phospholipid metabolism have been observed in several systems. Sphingomyelin and cholesterol are known to co-localize intracellularly (4, 5), their levels are positively correlated under different pathological and experimental conditions (6, 7), and there is evidence of coordinate metabolism (8–10). Phosphatidylcholine (PtdCho), the major phospholipid in eukaryotic cells, has also been shown to be affected by alterations in cellular cholesterol and cholesteryl ester levels. Treatment of L_6 cells with compactin or 25-hydroxycholesterol resulted in a

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; CHO, Chinese hamster ovary; CT, CTP:phosphocholine cytidylyltransferase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HMG, 3-hydroxy-3-methylglutaryl; LDL, low density lipoprotein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; SRD, sterol regulatory defective; SM, sphingomyelin; SREBP, sterol regulatory element binding protein.

[1]To whom correspondence should be addressed.

50% reduction in PtdCho synthesis that appeared to be secondary to inhibition of sterol synthesis (11). These treatments caused a 20–30% inhibition of CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme in PtdCho synthesis, in the soluble and membrane fractions of these cells (11). In contrast, cholesterol loading of macrophages increased PtdCho synthesis by stimulating the membrane activity of cytidylyltransferase (12) by enzyme dephosphorylation (13). In all these studies the activities of choline kinase and choline phosphotransferase were not affected. These results suggest that changes in cholesterol and PtdCho synthesis occur in parallel, thus maintaining a constant ratio of these two components in cellular membranes.

Previous studies have used acute treatment regimes that rapidly alter cholesterol homeostasis in cultured cells (11–13). In the present study we have examined PtdCho metabolism in the CHO sterol regulatory defective (SRD) 6 cell line (14). SRD 6 cells are cholesterol auxotrophs due to an inability to activate transcription of sterol-regulated genes and cholesterol synthesis, the result of incomplete proteolytic cleavage and activation of the endoplasmic reticulum-bound transcription factors SREBP-1 and 2 (15). SRD 6 cells provide a unique model system to study how perturbations in cholesterol homeostasis affect phospholipid metabolism. Results indicate that SRD 6 cells have a pronounced defect in PtdCho synthesis that results from reduced cytidylyltransferase activity and abnormal distribution of enzyme activity and protein.

MATERIALS AND METHODS

Cholesterol complexed with methyl β-cyclodextrin, methyl β-cyclodextrin, oleic acid, 1,2-dioleolyl-*sn*-glycerol, mevalonate, choline, phosphocholine, CDP-choline, and PtdCho were purchased from Sigma Chemical Co. 25-Hydroxycholesterol was purchased from Steraloids. [Methyl-^{3}H]choline, [methyl-^{14}C]CDP-choline, [methyl-^{3}H]phosphocholine, and [α^{32}P]dATP were from DuPont-NEN. Silica Gel 60 thin-layer chromatography plates were from E. Merck. Kits for determining cellular cholesterol were purchased from Boehringer-Mannheim. Silica Gel G plates were from Fisher Scientific. S1 nuclease (from *Aspergillus oryzae*) was purchased from Gibco-BRL. Delipidated fetal calf serum was prepared by centrifugation at a density of 1.21 g/ml (16) and dialyzed against phosphate-buffered saline (PBS). All other chemicals were of reagent grade.

Cell culture

CHO 7 and SRD 6 cells (kindly provided by James Metherall, University of Utah) were grown in monolayers at 37°C in an atmosphere of 5% CO_2 (14, 17). CHO 7 cells were maintained in Dulbecco's modified Eagle's medium containing 5% delipidated fetal calf serum (medium A). SRD 6 cells were maintained in Dulbecco's modified Eagle's medium with 5% delipidated calf serum, 1 mM mevalonate, and 2 μg cholesterol/ml complexed with cyclodextrin (medium B). Cells were subcultured in 60-or 100-mm dishes in 3 or 8 ml medium, respectively. On day 3, cells received medium B (+cholesterol) or medium A containing 42 μg cyclodextrin/ml (−cholesterol) and experiments were started 18–24 h later (refer to figure legends for specific details).

Extraction and analysis of phospholipids and choline metabolites

Cells were rinsed once with ice-cold PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4), scraped in 1 ml methanol–water 5:4 (v/v), and transferred to screw-cap tubes. Each culture dish was then rinsed with 1 ml of methanol–water, and extracts from two dishes were combined and sonicated on ice for 30 s. After an aliquot was taken for protein determination (18), 4 ml of chloroform was added to each tube and phases were separated by centrifugation at 2,000 *g* for 5 min. The organic phase was extracted twice with 2 ml methanol–0.58% NaCl–chloroform 45:47:3 (v/v) and dried over anhydrous sodium sulfate. Lipids were separated by thin-layer chromatography on silica gel 60 plates in a solvent system of chloroform–methanol–water 65:25:4 (v/v) and visualized by exposure to iodine vapor. PtdCho and sphingomyelin were scraped from plates into vials and radioactivity was quantitated by scintillation counting.

Aqueous choline metabolites were separated by thin-layer chromatography on silica G plates in ethanol–water–ammonia 48:95:6 (v/v). Choline (R_f = 0.1), phosphocholine (R_f = 0.5), glycerophosphocholine (R_f = 0.6), and CDP-choline (R_f = 0.8) were visualized by staining with 1% phosphomolybdic acid in chloroform–ethanol 1:1 (v/v) and 1% stannous chloride in 3 N HCl, and scraped into vials with 0.5 ml water for scintillation counting.

Separation and detection of phospholipids for mass analysis was by thin-layer chromatography as described above. Cellular phosphocholine mass was quantitated by thin-layer chromatography as previously described (19). Samples were scraped into glass tubes and phosphorus was quantitated using the method of Rouser, Siakatos, and Fleisher (20).

Enzyme assays

Cytosol and membrane fractions for the assay of PtdCho biosynthetic enzymes were prepared as follows. CHO 7 and SRD 6 cells were harvested in cold PBS,

sedimented at 2,000 *g* for 5 min, and homogenized in 20 mM Tris-HCl (pH 7.4), 0.1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM EDTA, and 5 mM dithiothreitol (buffer A) by 40 strokes in a glass dounce and two 5-s bursts with a needle probe sonicator (40% power setting). Homogenates were centrifuged for 1 h at 130,000 *g*, the supernatant (cytosol) was collected and the pellet (total membranes) was resuspended in buffer A containing 0.25 M sucrose.

Choline kinase assays of cell cytosol were for 10 min at 37°C (21). [^{3}H]phosphocholine was isolated by thin-layer chromatography as described above. CTP:phosphocholine cytidylyltransferase activity in cytosol and membranes was assayed by measuring conversion of [^{3}H]phosphocholine to [^{3}H]CDP-choline for 25 min at 37°C both in the presence and absence of PtdCho–oleic acid 1:1 (mol/mol) vesicles (22). Choline phosphotransferase activity in membranes was assayed by measuring conversion of [^{14}C]CDP-choline to [^{14}C]PtdCho for 15 min at 37°C (23).

Immunoblotting and immunoprecipitation

Cytosol and membrane fractions from CHO 7 and SRD 6 cells were isolated as described above. Membranes were treated in buffer A containing 1% (v/v) Nonidet NP-40 and the soluble fraction was collected by centrifugation for 15 min at 15,000 *g*. Total cell extracts were prepared by solubilization of cell pellets in buffer A containing 1% Nonidet NP-40 and the soluble fraction was collected after centrifugation as described above. Equivalent amounts of protein from cytosol, solublized membranes, and total cell extracts were separated by 10% SDS-PAGE and transferred to a nitrocellulose filter. The filter was probed with a rabbit anti-rat cytidylyltransferase antibody (24) raised against the full-length protein (kindly provided by Suzanne Jackowski, St. Jude Children's Research Hospital, Memphis, TN) in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 5% (w/v) skim milk powder and 0.1% (v/v) Tween-20 for 2 h. The filter was subsequently incubated with goat anti-rabbit antibody conjugated to horseradish peroxidase for 45 min and developed by the chemiluminescence procedure as described by the manufacturer (DuPont NEN).

SRD 6 and CHO 7 cells were labeled for 12 h in phosphate-free medium A containing 25 μCi/ml [^{32}P]phosphate. Cytosol and membrane fractions were isolated as described above in buffer A containing okadaic acid (100 nM) and sodium vanadate (100 μM). Triton X-100 was added to membrane and cytosol fractions to a final concentration of 0.3% (w/v) and samples were subjected to centrifugation for 15 min at 10,000 *g*. The soluble fractions were recovered and incubated with a 1/200 dilution of rabbit anti-rat cytidylyltransferase antibody at 4°C for 1 h, followed by protein A-Sepharose for 45 min. Sepharose beads were washed 6–7 times with PBS containing 1% (w/v) Triton X-100, boiled in SDS-PAGE sample buffer, and ^{32}P-labeled cytidylyltransferase was resolved by 10% SDS-PAGE.

mRNA analysis

Cytidylyltransferase was quantitiated using a single-stranded antisense probe prepared from M13 mp19 containing a 86 bp Hind III-Eco RI fragment of the rat liver cDNA (25). Cytidylyltransferase and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probes for quantitative S1 nuclease protection assays were purified by 7 M urea–6% polyacrylamide gel electrophoresis (26). Total RNA for cytidylyltransferase (30 μg) and G3PDH (10 μg) were hybridized in 90% formamide buffer with an excess of single-stranded DNA probe (0.6×10^9 dpm/μg) at 90°C followed by slow cooling to 37°C and incubation at this temperature for 16 h. Hybridization reactions were digested with S1 nuclease, separated on 7 M urea–6% polyacrylamide gels, and exposed to film at −70°C. Digestion products were quantitated from autoradiograms by image analysis on a Macintosh Apple ONE Scanner using the NIH Image software package (version 1.55).

RESULTS

Altered phosphatidylcholine biosynthesis in SRD 6 cells

[^{3}H]choline incorporation into PtdCho and sphingomyelin was measured at 2 and 4 h in SRD 6 cells and control CHO 7 cells. Cells were grown in the absence or presence of cholesterol for 24 h prior to addition of [^{3}H]choline (**Fig. 1**). These growth conditions did not appreciably alter total cholesterol content of CHO 7 cells (17–22 μg cholesterol/mg protein). SRD 6 cells grown in the absence or presence of cholesterol for 24 h had 11.1 ± 2.7 and 23.4 ± 3.4 μg cholesterol/mg protein, respectively (mean ± SEM of three determinations). Compared to CHO 7 cells, incorporation of the radiolabeled precursor choline into PtdCho in SRD 6 cells was reduced by 70% and 50% at 2 and 4 h, respectively. [^{3}H]choline incorporation into sphingomyelin was also reduced, probably as a result of lower specific activity in its precursor PtdCho. The inclusion of cholesterol in SRD 6 or CHO 7 medium for 24 h prior to labeling did not significantly affect isotope incorporation into PtdCho.

To ensure that the SRD 6 mutation did not have a pleiotropic effect on phospholipid synthesis, [^{3}H]se-

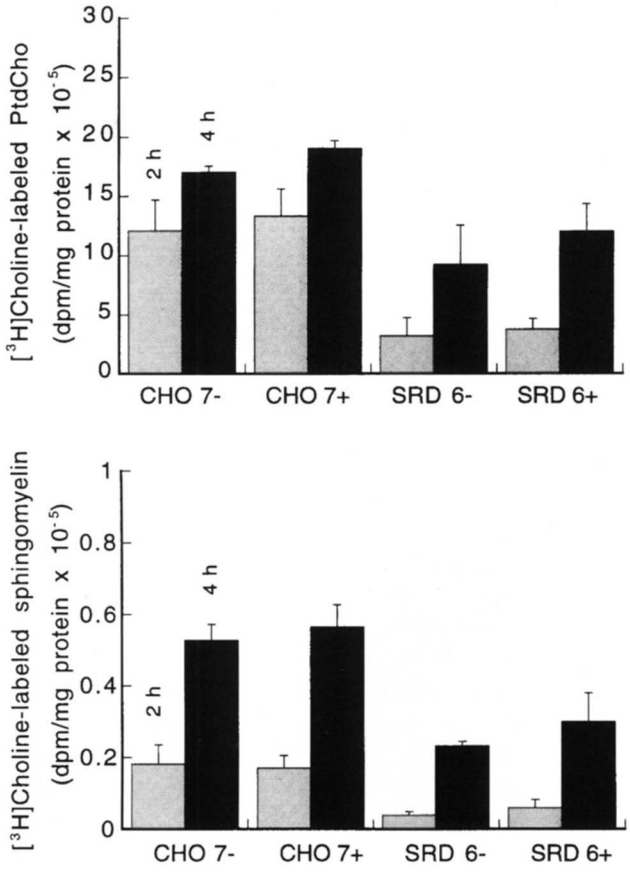

Fig. 1. Incorporation of [^{3}H]choline into PtdCho and sphingomyelin in SRD 6 and CHO 7 cells. Cells were grown in medium A (42 μg cyclodextrin/ml, CHO 7− and SRD 6−) or medium B (2 μg cholesterol/ml complexed with cyclodextrin with 1 mM mevalonate, CHO 7+ and SRD 6+) for 24 h prior to a medium change for 3 ml of choline-free medium A ± 2 μg cholesterol/ml complexed to cyclodextrin. After 1 h, cells received 3 ml of the same media containing 2 μCi [^{3}H]choline/ml for 2 (gray bars) or 4 h (solid bars). Cells were harvested and the amount of labeled PtdCho and SM was quantitated. Results are the mean ± SEM for three separate experiments.

rine and [^{3}H]ethanolamine labeling of phospholipids was analyzed. [^{3}H]ethanolamine incorporation into PtdEtn in SRD 6 cells grown in cholesterol was reduced by only 25%, and [^{3}H]serine labeling of PtdSer and PtdEtn was not significantly different compared to CHO 7 cells (results not shown).

The PtdCho content of cells is usually regulated within a narrow range, probably as the result of coordination between rates of synthesis and degradation (27, 28). We measured phospholipid levels in SRD 6 and CHO 7 cells to see whether reduced PtdCho synthesis was reflected by reduced PtdCho mass. SRD 6 cells depleted of cholesterol for 24 h exhibited a significant 8% reduction in PtdCho mass compared to CHO 7 cells (**Fig. 2**). Incubation of SRD 6 cells with cholesterol increased PtdCho such that it was no longer significantly different from CHO 7 cells grown under the same conditions. Reduced PtdCho in cholesterol-minus SRD 6 cells was compensated for by slight elevations in other phospholipid classes, but only in the case of PtdEtn was this significant. There was no gross alteration in total phospholipid:protein ratio in SRD 6 compared to CHO 7 cells (Fig. 2, inset).

To determine which step in the CDP-choline pathway was inhibited in SRD 6 cells, we examined the distribution of water-soluble metabolites after incubation with [^{3}H]choline for 2 and 4 h (**Fig. 3**). In SRD 6 cells grown in the absence of cholesterol, the levels of [^{3}H]choline were elevated compared to CHO 7 cells, but the most obvious difference was a >10-fold increase in [^{3}H]phosphocholine. [^{3}H]CDP-choline was a minor component in both cell lines and its levels were elevated only slightly in SRD 6 cells. Incubation of SRD 6 cells with cholesterol (2 μg/ml) for 24 h did not affect [^{3}H]choline uptake by cells or incorporation into the three major metabolites shown in Fig. 3. Total [^{3}H]choline uptake and

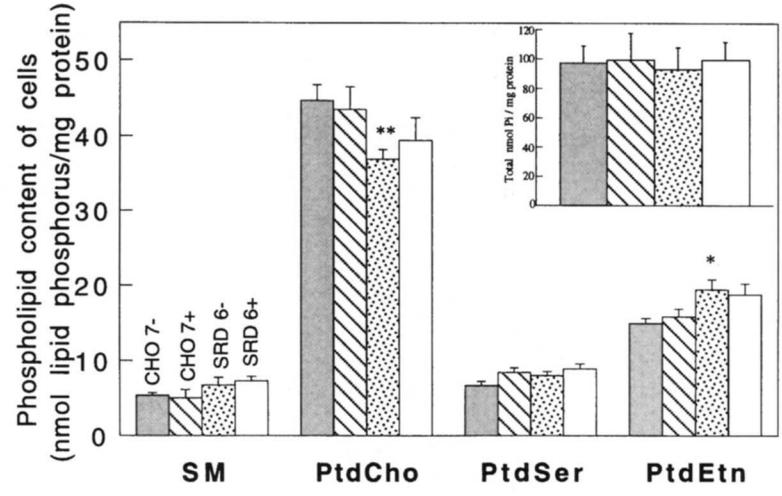

Fig. 2. Phospholipid composition of SRD 6 and CHO 7 cells. Cells were cultured in medium A with (+) or without (−) cholesterol for 24 h as described in the legend to Fig. 1, harvested, and phospholipids were isolated and quantitated. Insert: total phospholipid/protein ratio in SRD 6 and CHO 7 cells grown with or without cholesterol. Results are the mean ± SEM for four separate experiments. Significance was determined using two-tailed *t*-test compared to CHO 7 cells incubated without cholesterol (* = $P < 0.05$, ** = $P < 0.025$).

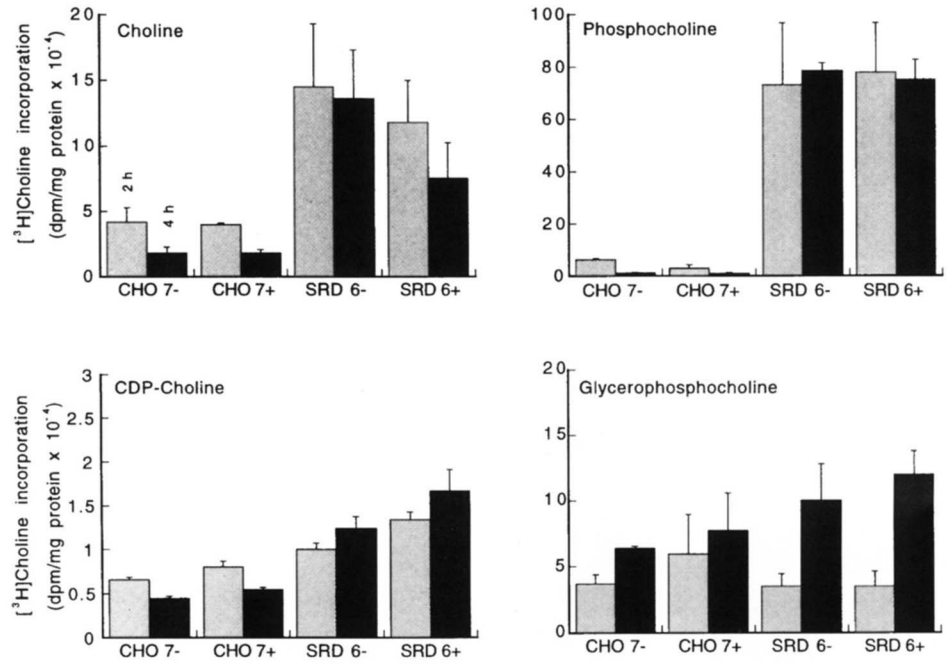

Fig. 3. [^{3}H]choline-labeled metabolites in SRD 6 and CHO 7 cells. Cells were grown in medium A with or without cholesterol and labeled with [^{3}H]choline for 2 h (gray bars) or 4 h (solid bars) as described in the legend to Fig. 1. Choline metabolites were extracted, separated by thin-layer chromatography, and quantified as described in Materials and Methods. Results are the mean ± SEM for three separate experiments.

incorporation into lipid and water-soluble metabolites was identical between SRD 6 cells and CHO 7 (growth either in the presence or absence of cholesterol) suggesting that reduced PtdCho synthesis is due to decreased conversion of phosphocholine to CDP-choline in SRD 6 cells.

The turnover of [^{3}H]choline metabolites in SRD 6 and CHO 7 cells is shown in pulse-chase experiments in **Fig. 4.** [^{3}H]choline levels were rapidly reduced in both cell lines 30 min after removal of isotope. In CHO 7 cells, [^{3}H]phosphocholine was reduced to very low levels (70% of initial values) at the end of the 2-h chase period. In contrast, [^{3}H]phosphocholine in SRD 6 cells was greatly elevated at the end of the pulse and 40% of radioactivity was lost by the end of the chase period. While this represented a substantial 4-fold greater loss of radioactivity compared to controls, it should be noted that [^{3}H]phosphocholine levels were initially lower in control cells and appeared to have reached a minimum by the end of the chase period. Comparison of [^{3}H]choline incorporation into PtdCho and phosphocholine at the start of the pulse indicated that accumulation of radioactivity in phosphocholine largely accounts for reduced incorporation into PtdCho in SRD 6 cells. The excess [^{3}H]phosphocholine in SRD 6 cells appears to be slowly incorporated into [^{3}H]PtdCho, which unlike control cells increased steadily during the chase period. Also, reduced production of [^{3}H]glycerophosphocholine in SRD 6 cells indicated a substantial delay in [^{3}H]PtdCho degradation compared to controls. With the exception of SRD 6 cells grown without cholesterol, release of total radioactivity into the medium by SRD 6 and CHO 7 cells was similar. [^{3}H]choline accounted for 80% and 70% of medium radioactivity in CHO 7 and SRD 6 cells, respectively. The remainder was found in [^{3}H]phosphocholine (results not shown).

Consistent with elevated labeling of phosphocholine and slowed conversion to PtdCho, phosphocholine mass in SRD 6 cells grown in the presence or absence of cholesterol was increased relative to CHO 7 cells. CHO 7 cells had phosphocholine levels of 6.9 ± 1.2 and 7.2 ± 0.7 nmol/mg cell protein (mean ± SEM of three determinations) when grown in the absence or presence of cholesterol, respectively, whereas phosphocholine values in SRD 6 cell were 12.6 ± 1.7 and 11.9 ± 1.9 nmol/mg cell protein (mean ± SEM of three determinations) when grown under the same conditions.

Regulation and distribution of cytidylyltransferase in SRD 6 cells is altered

Conversion of phosphocholine to CDP-choline, catalyzed by the rate-limiting enzyme CTP:phosphocholine

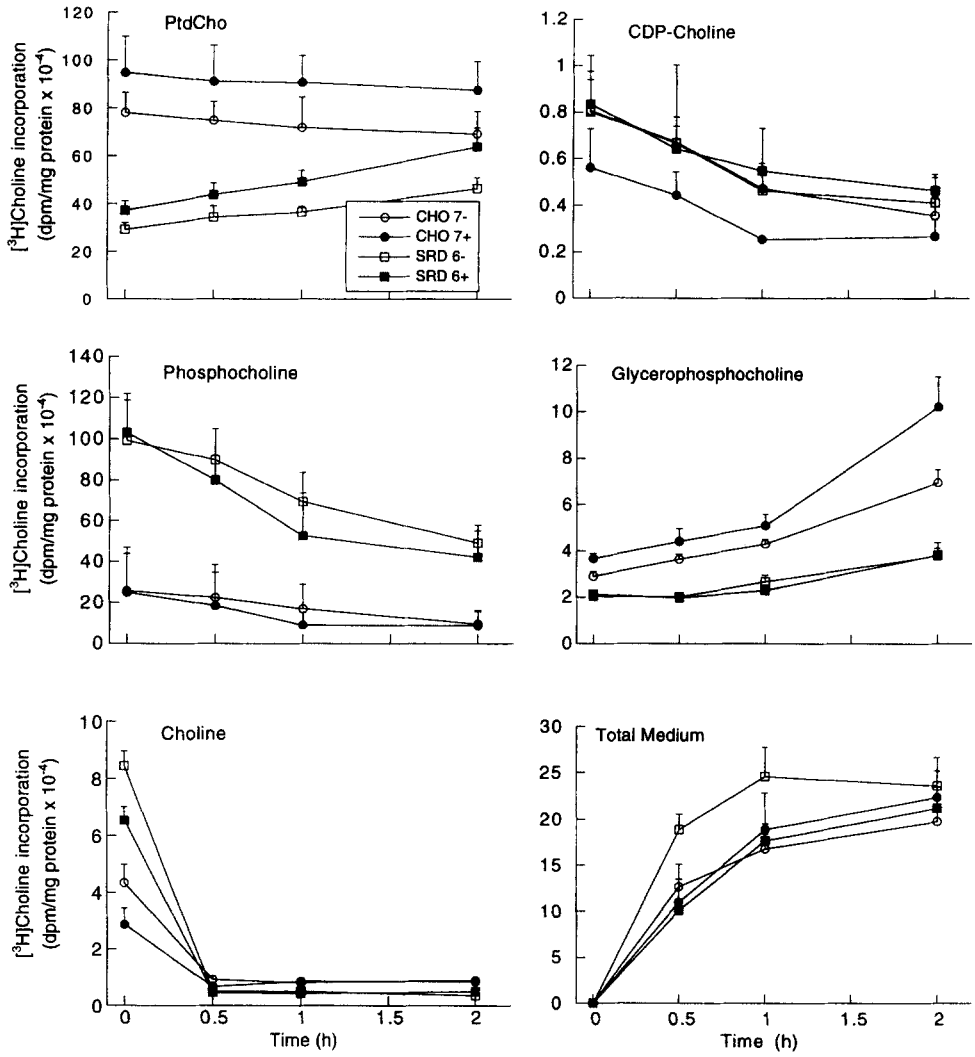

Fig. 4. Turnover of [^{3}H]choline-labeled metabolites in SRD 6 and CHO 7 cells. Cells were grown in medium A with or without cholesterol and described in Fig. 1. SRD 6 and CHO 7 cells were pulse-labeled for 1 h with 2 μCi [^{3}H]choline/ml in medium A, followed by medium A without isotope, and at the indicated times cells were harvested and labeled choline metabolites and PtdCho were quantitated. Results are the mean ± SEM of three experiments.

cytidylyltransferase (29), appears to be compromised in SRD 6 cells. Cytidylyltransferase is regulated by translocation of an inactive cytosolic form to the endoplasmic reticulum or nuclear envelope (29, 30). Translocation to membranes is regulated by fatty acids, diglyceride, and protein phosphorylation (29). To determine whether the activities of cytidylyltransferase or of the other two enzymes of the CDP-choline pathway were altered by cholesterol auxotrophy, the in vitro activities for these three enzymes were assayed in SRD 6 and CHO 7 cells (**Table 1**). The activities of choline kinase and choline phosphotransferase were not significantly different between the SRD 6 and CHO 7 cells. Cytosolic cytidylyltransferase activity assayed in the presence or absence of lipid activators (oleate/PtdCho vesicles) was increased 2- and 3-fold, respectively, in SRD 6 cells grown without cholesterol. Cytidylyltransferase activity was also elevated 2-fold in cytosol from SRD 6 cells grown with cholesterol, but this result was only significant when assayed with oleate/PtdCho vesicles. In contrast to cytosolic activity, the total membrane fraction from SRD 6 and CHO 7 cells had similar cytidylyltransferase activity whether assayed in the presence or absence of PtdCho/oleate vesicles.

Immunoblot analysis of cytidylyltransferase in total cell lysates, cytosol, and membrane fractions is shown in **Fig. 5.** It is evident that immunoreactive enzyme protein in detergent extracts of SRD 6 cells (Fig. 5A) is in-

TABLE 1. Activity of phosphatidylcholine biosynthetic enzymes in CHO 7 and SRD 6 cells

	Choline Kinase	Choline Phosphotransferase	CTP:Phosphocholine Cytidylytransferase			
			Cytosol −Oleate/PtdCho	Cytosol +Oleate/PtdCho	Membrane −Oleate/PtdCho	Membrane +Oleate/PtdCho
	nmol/min/mg protein		*nmol/min/mg protein*			
CHO 7−	0.40 ± 0.08	0.56 ± 0.08	0.44 ± 0.08	1.28 ± 0.11	0.85 ± 0.10	1.06 ± 0.26
CHO 7+	0.37 ± 0.05	0.57 ± 0.08	0.40 ± 0.05	1.15 ± 0.14	1.02 ± 0.10	1.14 ± 0.12
SRD 6−	0.35 ± 0.05	0.54 ± 0.05	1.24 ± 0.32[b]	2.66 ± 0.34[a]	1.16 ± 0.13	1.42 ± 0.16
SRD 6+	0.44 ± 0.09	0.64 ± 0.14	0.70 ± 0.13	2.17 ± 0.32[b]	1.30 ± 0.12	1.46 ± 0.28

Enzyme activities were assayed in cytosol or membranes isolated from cells cultured in medium A containing cyclodextrin (no cholesterol, CHO 7−, SRD 6−) or medium B (+cholesterol, CHO 7+, SRD 6+) for 24 h. Results are the mean ± SEM for duplicate determinations from 3–8 separate experiments. Significance was determined using a two-tailed *t*-test and compared to the corresponding activity for CHO 7 cells cultured without cholesterol for 24 h ([a]$P < 0.005$; [b]$P < 0.05$).

creased approximately 2-fold compared to similar extracts from CHO 7 cells. Consistent with results for cytidylyltransferase activity shown in Table 1, immunoreactive enzyme mass is increased in SRD 6 cell cytosol (Fig. 5B), but not the membrane fraction (Fig. 5C). We consistently observed that cytidylyltransferase was resolved into 2 or 3 bands due to altered mobility in SDS-PAGE as the result of extensive phosphorylation (31, 32). The distribution of phosphorylated isoforms of cytidylyltransferase was similar between SRD 6 and CHO 7 cells in total extracts; however, cytosolic cytidylyltransferase in SRD 6 cells appeared to have less of the higher mobility isoform compared to controls.

Cytidylyltransferase has 16 phosphorylation sites on serine residues and is the substrate for multiple protein kinases (25, 33). The soluble, hyperphosphorylated form of cytidylyltransferase is dephosphorylated upon membrane binding, but dephosphorylation alone appears not to be sufficient to promote translocation (25). We examined the [^{32}P]phosphate content of cytidylyltransferase by immunoprecipitation to determine whether changes in phosphorylation status in SRD 6 cells could be related to the increased cytosolic distribution of the enzyme or reduced activity of the membrane-bound form (**Fig. 6**). [^{32}P]phosphate incorporation into cytosolic cytidylyltransferase was similar in control and SRD 6 cells grown in the absence of cholesterol. However, as enzyme mass is increased in SRD 6 cytosol (Fig. 5), it would appear that cytidylyltransferase in these mutant cells is slightly dephosphorylated relative

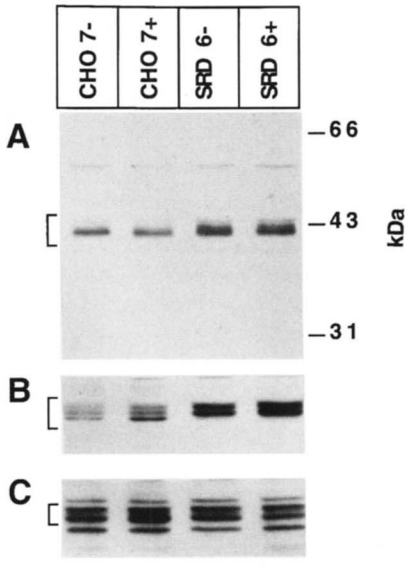

Fig. 5. Immunoblot analysis of cytidylyltransferase in SRD 6 and CHO 7 cells. Cells were grown in medium A with or without cholesterol for 24 h as described in the legend to Fig. 1. Total cell lysates (A), cytosol (B), and membranes (C) were prepared and 15 μg of protein from each fraction was subjected to 10% SDS-PAGE and immunoblotting as described in Materials and Methods. Similar results were seen in three other experiments. The position of cytidylyltransferase on the immunoblot is indicated by brackets.

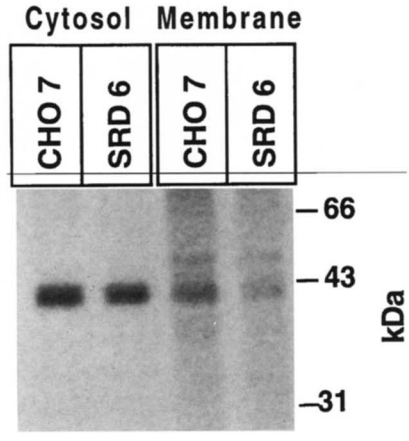

Fig. 6. Phosphorylation of cytidylyltransferase in SRD 6 and CHO 7 cells. Cytidylyltransferase in SRD 6 and CHO 7 cells (grown for 24 h in medium A, without cholesterol) was labeled with [^{32}P]phosphate and immunoprecipitated from 50 μg of cytosol and membrane protein as described in Materials and Methods. Immunoprecipitates were separated by 10% SDS-PAGE and the dried gel was exposed to Kodak Bio Max film for 48 h at −70°C. Similar results were seen in two other experiments.

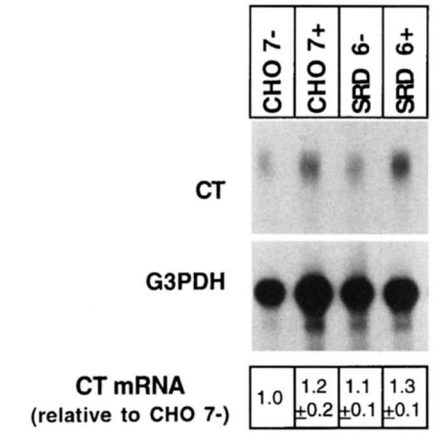

Fig. 7. Cytidylyltransferase mRNA expression in SRD 6 and CHO 7 cells. Cells were grown in medium A with or without cholesterol for 24 h as described in the legend to Fig. 1. Cytidylyltransferase mRNA levels were normalized to G3PDH mRNA and expressed relative to CHO 7 cells grown in the absence of cholesterol (CHO 7−). Results are the mean ± SEM for three separate experiments.

to CHO 7 cells. Phosphorylation of the membrane-bound form of the enzyme was reduced in both cell lines compared to the soluble enzyme, and cytidylyltransferase from SRD 6 membranes appeared to be slightly dephosphorylated relative to controls.

Cytidylyltransferase mRNA levels were quantitated in SRD 6 and CHO 7 cells by S1 nuclease protection analysis to test whether increased mRNA could account for elevated cytidylyltransferase activity and protein in SRD 6 cells (**Fig. 7**). Results clearly show that cytidylyltransferase mRNA levels in SRD 6 and CHO 7 cells are not significantly different, and growth in cholesterol for 24 h did not affect mRNA expression in either cell line.

Phosphatidylcholine synthesis in SRD 6 and CHO 7 cells in response to oleate

SRD 6 cells appear to have sufficient in vitro cytidylyltransferase activity in membranes to maintain PtdCho synthesis, yet the accumulation of cytosolic enzyme, elevated phosphocholine mass and [^{3}H]phosphocholine, and attenuated PtdCho production points to an obvious defect in the reaction catalyzed by cytidylyltransferase. These results seem to indicate reduced translocation to membranes or impaired in vivo activity of the membrane-bound enzyme. To determine whether cytidylyltransferase in SRD 6 cells responds normally to a stimulus known to promote translocation and increase PtdCho synthesis, cells were treated with oleate and [^{3}H]choline incorporation into PtdCho and soluble metabolites was measured (**Fig. 8**). Treatment of CHO

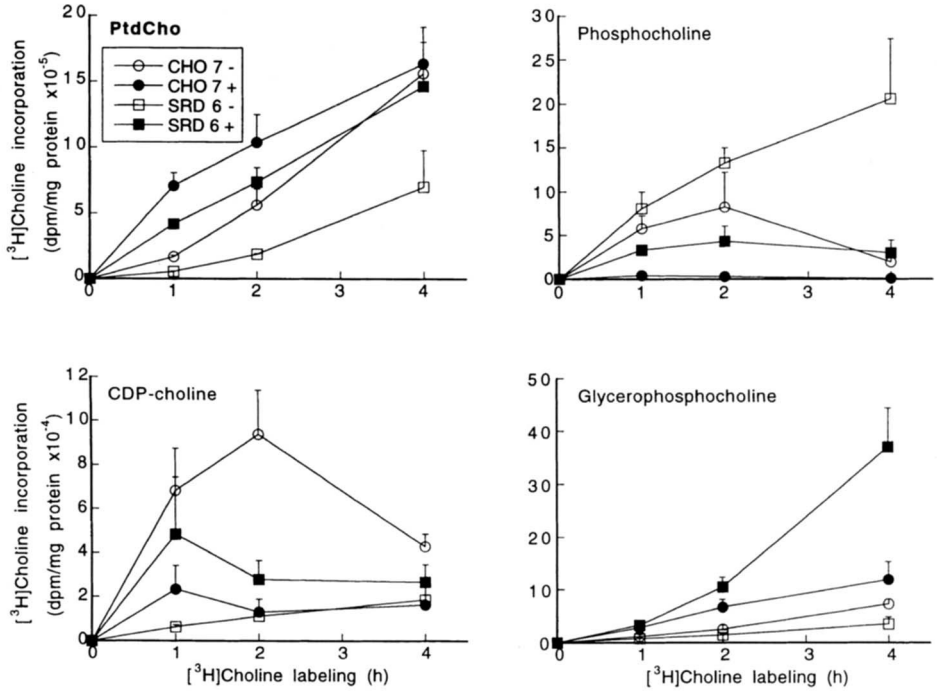

Fig. 8. Stimulation of phosphatidylcholine synthesis by oleate in SRD 6 and CHO 7 cells. CHO 7 and SRD 6 cells were grown for 24 h in medium A (minus cholesterol) prior to receiving choline-free medium A with (+) or without (−) 150 μM oleate complexed to bovine serum albumin. After 1 h, [^{3}H]choline (2 μCi/ml) was added directly to each dish and at the indicated times cells were harvested for quantitation of PtdCho and the soluble metabolites phosphocholine, CDP-choline and glycerophosphocholine. Results are the means of four determinations ± SEM.

7 cells with 150 μM oleate caused a 2- to 3-fold increase in [^{3}H]PtdCho synthesis at 1 and 2 h after [^{3}H]choline addition, and markedly reduced the lag in [^{3}H]PtdCho synthesis observed in untreated cells. As shown in previous figures, [^{3}H]PtdCho synthesis was significantly reduced in untreated SRD 6 cells compared to control. Oleate treatment stimulated synthesis in SRD 6 cells such that by 4 h, [^{3}H]choline incorporation into PtdCho was similar to oleate-treated and untreated CHO 7 cells. We also examined the production of labeled phosphocholine, CDP-choline, and glycerophosphocholine in oleate-treated SRD 6 and CHO 7 cells. Oleate-treated CHO 7 and SRD 6 cells had significantly reduced [^{3}H]phosphocholine compared to their untreated counterpart at 1 and 2 h of the [^{3}H]choline pulse. By 4 h, the level of [^{3}H]phosphocholine in oleate-treated SRD 6 cells was similar to that in oleate-treated and untreated CHO 7 cells. Enhanced conversion of phosphocholine to CDP-choline in oleate-treated SRD 6 cells resulted in an initial elevation in [^{3}H]CDP-choline, but levels of [^{3}H]CDP-choline in treated and untreated SRD 6 and CHO 7 cells reached a similar level by 4 h. In addition to enhanced synthesis of [^{3}H]PtdCho, oleate treatment of SRD 6 and CHO 7 cells also stimulated [^{3}H]glycerophosphocholine production. Interestingly, [^{3}H]glycerophosphocholine levels were elevated to the greatest extent in oleate-treated SRD 6 cells indicating enhanced catabolism of newly synthesized PtdCho.

DISCUSSION

A greater understanding of how cholesterol and phospholipid syntheses are coordinately regulated has important implications for diseases that are caused by aberrant cholesterol or phospholipid homeostasis. For example, it is well established that PtdCho, sphingomyelin, and cholesterol accumulate in atherosclerotic lesions and foam cells (12, 34–36). In the Niemann-Pick sphingolipid and cholesterol storage disorders, cholesterol, sphingolipids, and phospholipids accumulate irrespective of whether the defect is in cholesterol or sphingomyelin metabolism (6). However, little is known of the effect of reduced cholesterol synthesis on metabolism of phospholipids. Using cholesterol auxotrophic SRD 6 cells, we have demonstrated a relationship between PtdCho and cholesterol metabolism in which reduced cholesterol synthesis results in decreased production of PtdCho via the CDP-choline pathway.

Studies on CHO cell mutants have provided important information on the mechanism and control of cholesterol regulation (14, 15, 17, 37) and trafficking (38), and phospholipid biosynthesis (39). The SRD 6 cells used in this study have a well-characterized defect in cholesterol regulation and response to regulatory sterols such as 25-hydroxycholesterol. SRD 6 cells have constitutively low and unregulated levels of mRNA for HMG-CoA reductase, HMG-CoA synthase, and LDL receptor, and sterol synthesis is 15% of normal, but post-transcriptional regulation of ACAT and HMG-CoA reductase is normal (14). As a result these cells are cholesterol auxotrophs. It was recently demonstrated that the defect in SRD 6 cells results from incomplete proteolytic cleavage of SREBP-1 and 2 to the mature nuclear-localized transcription factor (15). SREBP-1 and 2 are membrane proteins of the endoplasmic reticulum that are cleaved in a two-step process by sterol-regulated and constitutive proteases (15) to mature, soluble transcription factors that migrate to the nucleus and activate transcription of HMG-CoA synthase and the LDL receptor (40, 41).

In addition to defective cholesterol synthesis and regulation, SRD 6 cells have reduced PtdCho synthesis. Reduced PtdCho mass could be partially corrected by inclusion of cholesterol in the culture medium. This could indicate that cholesterol supplementation reduced PtdCho catabolism, thus partially compensating for defective synthesis. However, [^{3}H]choline labeling studies indicated that PtdCho synthesis and choline metabolites were unaffected by the addition of cholesterol to SRD 6 cells. There are several possible explanations for the apparent lack of effect of cholesterol on PtdCho synthesis. First, a product of the isoprenoid biosynthetic pathway, and not the end product cholesterol, may be required for full restoration of PtdCho synthesis. For example, farnesyl and geranylgeranyl groups are added post-translationally to numerous proteins involved in signal transduction and growth regulation (42), and could be involved directly or indirectly in the regulation of the CDP-choline pathway. Second, active cholesterol synthesis in the endoplasmic reticulum could be a prerequisite for PtdCho synthesis. The lipid composition of the endoplasmic reticulum and activity of enzymes in this organelle may be dependent on active cholesterol synthesis and transport. Third, sterols could affect expression of another gene(s) required for PtdCho synthesis. Cytidylytransferase mRNA levels or the activity of the other two enzymes in the CDP-choline pathway are not regulated by sterols. However, sterols have been shown to suppress transcription of the genes for two key enzymes in fatty acid biosynthesis, fatty acid synthase (43) and acetyl CoA carboxylase (44). The promoters for these two genes have binding sites for SREBP, which appear to be required for sterol-dependent regulation of reporter gene constructs. SREBP also appears to be important in determination and differentiation of cul-

tured preadipocytes (45). Thus, SREBP regulates the expression of enzymes in both cholesterol and fatty acid biosynthesis that have key roles in regulating membrane composition and biogenesis. This relationship may extend to PtdCho biosynthesis as fatty acids are constituents of PtdCho and other phospholipids, and fatty acid availability will affect phospholipid synthesis. It should be stressed that only PtdCho synthesis and mass were affected in SRD 6 cells, and other phospholipids were relatively resistant to the effects of cholesterol auxotrophy. Fatty acids and diglyceride are also known to activate PtdCho synthesis by promoting the translocation of cytidylyltransferase to membranes (29). Thus, when fatty acid and diacylglycerol are reduced, a mechanism exists for down-regulating CDP-choline production by maintaining cytidylyltransferase in a soluble, inactive form. Whether the absence of a lipid activator or altered membrane composition is responsible for altered cytidylyltransferase in SRD 6 cells will require further investigation.

[^{3}H]choline pulse-chase studies showed that SRD cells accumulated [^{3}H]phosphocholine and had delayed conversion to [^{3}H]PtdCho. This was consistent with defective activity of the rate-limiting enzyme CTP:phosphocholine cytidylyltransferase. While cytidylyltransferase activity appeared to be reduced in intact SRD 6 cells, it was normal or elevated when assayed in vitro in total membranes or cytosol, respectively. This could indicate that the membrane-bound form is relatively inactive and the soluble enzyme is restricted in its capacity to translocate to membranes in SRD 6 cells. This block in cytidylyltransferase activation could be overcome by the addition of oleate, suggesting that lack of a specific lipid activator or abnormal lipid environment in the endoplasmic reticulum or nuclear membrane could be responsible for reduced PtdCho synthesis in these mutant cells. Cytidylyltransferase has been shown to translocate to membranes in response to fatty acids (46), phospholipase C treatment (47) or choline deficiency (48–50). Interaction of cytidylyltransferase with membranes is mediated by amino acids 239–298 (51, 52). This region appears to be an inhibitory domain, and its removal or association with lipids results in enzyme activation (52). The precise role of lipid environment and activators in controlling cytidylyltransferase interaction with native membranes is not known.

Phosphorylation of cytidylyltransferase appears to be another important mechanism for regulation of activity. Although cytidylyltransferase is potentially phosphorylated on 16 serines, recent mutagenesis studies indicate that deletion of these sites or conversion to a constitutive phosphorylation phenotype (by substitution of negatively charged amino acids) does not completely override membrane/cytosol localization or seriously affect enzyme activity (25). Instead, phosphorylation may have a subtle effect on the cooperative activation of the enzyme by oleate/PtdCho vesicles (53). Dephosphorylation of membrane cytidylyltransferase was recently proposed to account for stimulation of PtdCho synthesis in cholesterol-loaded macrophages (13). In this model, enzyme mass was similar in membranes and cytosol from control and cholesterol-loaded macrophages, but cytidylyltransferase was substantially dephosphorylated in membranes relative to controls resulting in increased catalytic activity (13). Contrary to results in cholesterol-loaded macrophages, we did not observe differences in phosphorylation status between SRD 6 and CHO 7 cells that would account for altered in enzyme activity. Differences in cytidylyltransferase activity in SRD 6 cells (relative to CHO 7 cells) could be accounted for solely on the basis of increased enzyme mass. Indeed, it was noted that [^{32}P]phosphate labeling of cytidylyltransferase was slightly reduced in SRD 6 cells. This may indicate that dephosphorylation of cytidylyltransferase is favored in an attempt to overcome a block in enzyme activation in SRD 6 cells. These differences in the regulation of cytidylyltransferase in CHO cells and macrophages were not surprising as it was reported that CHO cells do not increase cytidylyltransferase activity and PtdCho synthesis when forced to accumulate cholesterol (13). We have similarly observed that 25-hydroxycholesterol-resistant SRD 2 cells, which have elevated and unregulated cholesterol synthesis (17), did not have increased PtdCho synthesis or altered cytidylyltransferase activity (M. K. Storey and N. D. Ridgway, unpublished results). Thus, regulation of cytidylyltransferase in macrophages and CHO cells may differ in responses to changes in intracellular cholesterol. However, our results on cytidylyltransferase activity and PtdCho synthesis in SRD 6 cells offer some interesting correlations to findings in the cholesterol-loaded macrophages (12) and livers of cholesterol-fed rats (54) regarding cholesterol availability and PtdCho synthesis. In these two systems where PtdCho synthesis was increased, the proportion of cytidylyltransferase activity in membranes was also elevated. The opposite was true in SRD 6 cells, where the proportion of cytidylyltransferase activity and mass was increased in the inactive cytoplasmic pool, and PtdCho mass and synthesis were reduced. The large pool of cytosolic cytidylyltransferase in SRD 6 cells is suggestive of an inability to translocate to membranes. As mentioned above, this could be related to the lack of an appropriate lipid activator or abnormal composition of intracellular membranes.

In summary, cholesterol auxotrophy due to lack of transcriptional induction of sterol-regulated genes results in reduced PtdCho synthesis and mass in mutant SRD 6 cells. This finding provides a more complete pic-

ture of how cellular PtdCho synthesis is integrated with cholesterol homeostasis through regulation of cytidylyltransferase activity and localization.

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