Regulation of Phosphatidylcholine Metabolism in Chinese Hamster Ovary Cells by the Sterol Regulatory Element-binding Protein (SREBP)/SREBP Cleavage-activating Protein Pathway*

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Thomas A. Lagace, Margo K. Storey‡, and Neale D. Ridgway§

From the Atlantic Research Center and the Departments of Pediatrics and Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

Sterol regulation-defective (SRD) 4 cells expressing a mutant sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP D443N) and Chinese hamster ovary (CHO) cells stably expressing SCAP (CHO-SCAP) and SCAP D443N (CHO-SCAP-D443N) have increased cholesterol and fatty acid synthesis because of constitutive processing of SREBPs. We assessed whether constitutive activation of SREBPs also influenced the CDP-choline pathway for phosphatidylcholine synthesis. Relative to control CHO 7 cells, SRD 4 cells displayed increased PtdCho synthesis and degradation as indicated by a 4–6-fold increase in [3H]choline incorporation into PtdCho and 10–15-fold increase in intracellular [3H]glycerophosphocholine. [3H]Phosphocholine levels in SRD 4 cells were reduced by over 10-fold, suggesting enhanced activity of CTP:phosphocholine cytidylyltransferase α (CCTα). CHO-SCAP and CHO-SCAP D443N cells displayed modest increases in [3H]choline incorporation into PtdCho (2-fold) and only a 2-fold reduction in [3H]phosphocholine. Elevated PtdCho metabolism in SRD 4, compared with SCAP-overexpressing cells, was correlated with fatty acid synthesis. Inhibition of fatty acid synthesis by cerulenin resulted in almost complete normalization of fatty acid synthesis. In contrast to apparent activation of SRD 4 cells, indicating that fatty acids or a fatty acid-derived signal.

In vivo activity were therefore hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: National Jewish Medical and Research Center, Dept. of Medicine, 1400 Jackson St., Denver, CO 80206.
§ To whom correspondence should be addressed: Atlantic Research Center, Rm. C306, Clinical Research Center, 5849 University Ave., Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada. Tel.: 902-494-7133; Fax: 902-494-1394; E-mail: nridgway@ns.dal.ca.

1 The abbreviations used are: PtdCho, phosphatidylcholine; ACAT, acyl-CoA:cholesterol acyltransferase; CHO, Chinese hamster ovary; CPT, cholinephosphotransferase; CCT, CTP:phosphocholine cytidylyltransferase; GPC, glycerophosphocholine; SRD, sterol regulation-defective; SREBP, sterol regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; iPLA2, calcium-independent phospholipase A2.
In addition to regulation of cholesterol and fatty acid biosynthesis, SREBP(SCAP could potentially regulate the synthesis of phospholipids and sphingolipids by direct or indirect mechanisms. For example, SREBP 1 was shown to regulate the transcription of glycerol-3-phosphate acyltransferase in cultured cells (14) and transgenic mouse models (10). Glycerol-3-phosphate acyltransferase is the initial enzyme in the synthesis of phosphatidic acid and diglyceride, two important precursors of phospholipids. Other evidence suggests that SREBPs influence phospholipid synthesis by indirect mechanisms. PtdCho synthesis was reduced in sterol-regulatory-defective (SRD) cells 6 (15) that have reduced cholesterol and fatty acid synthesis as a consequence of defective SREBP processing (5, 16). The absence of active SREBPs in SRD 6 cells did not affect the activity of the first and last enzymes in the CDP-choline pathway, choline phosphotransferase (CPT) and choline kinase. Instead, the activity of CTTa, the rate-limiting enzyme in the CDP-choline pathway (17), was reduced as the result of insufficient activation by fatty acids or a related derivative (15). Sphingomyelin synthesis was also decreased in SRD 6 cells, but again the in vitro activity of biosynthetic enzymes in the pathway was unaffected (18). Reduced sphingomyelin synthesis in SRD 6 cells could have been secondary to a relative deficiency in PtdCho, which provides the phosphocholine headgroup to sphingomyelin. Collectively, these results suggest that SREBPs regulate PtdCho and sphingomyelin synthesis indirectly by modifying the supply of precursors or lipid activators for CCT.

We have now examined PtdCho synthesis in SRD 4 cells (19, 20), and CHO cell lines expressing wild-type or a sterol-resistant SCAP mutant (D443N). SRD 4 cells express a SCAP allele with a point mutation (D443N) in the putative "sterol sensing" domain that renders it insensitive to suppression by sterols, resulting in constitutive proteolysis of SREBP 1 and SREBP-2 to the mature transcription factors (20, 23). As expected, these cell lines had elevated cholesterol and fatty acid synthesis but also displayed a 2–6-fold increase in PtdCho synthesis because of increased CTTa activity in vivo. In SRD 4 cells, activation of PtdCho synthesis (6-fold) was correlated with increased fatty acid synthesis and CTTa localization to the nuclear envelope. The modest increase in PtdCho and fatty acid synthesis in SCAP transfected cells (2-fold) was not accompanied by changes in CTTa expression or localization. Nuclear envelope localization of CTTa in control cells was reproduced by exogenous oleate, suggesting that elevated synthesis of this CTTa activator (or a derivative thereof) stimulated PtdCho synthesis in sterol regulation-defective cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**[methyl-3H]Choline, CDP-[methyl-3H]choline, [1-14C]acetate, [32P]ATP, [32P]Phosphate, and [methyl-3H]Phosphocholine were purchased from NEN Life Science Products. Choline, CDP-choline, phosphocholine, and ceruloenin were from Sigma. Lovastatin was provided by Merck. Silica gel 60 TLC plates were from BDH. Mono- and glycolaldehyde against HSV and T7 epitope tags. Mock transfected cells were selected after growth in G418. Four transfected cell lines expressing epitope-tagged SCAP, SCAP D443N, or empty vector were selected for further characterization. All cultures of SCAP transfected cells were maintained in medium A with 300 µg/ml G418 and 0.3 µg/ml 25-hydroxycholesterol and were subcultured for experiments in medium A without G418 or 25-hydroxycholesterol. Analysis of Labeled Phospholipids, Choline Metabolites, and Steroids—After labeling with [3H]choline (see figure legends for specific conditions), cells were rinsed once with cold PBS and scraped in 1 ml of methanol-water (5:4, v/v). The culture dish was rinsed with 1 ml of methanol-water, and the extracts were combined in a glass screw cap tube. [3H]PtdCho and aqueous [3H]choline metabolites were separated by extraction with chloroform as described previously (15). Labeled PtdCho was resolved by TLC in chloroform-methanol-water (65:25:4, v/v/v), whereas aqueous metabolites were separated in a solvent system of chloroform: methanol: water (65:25:4, v/v). In some experiments, [3H]PtdCho was measured by scintillation counting of an aliquot of the chloroform phase (>98% of the radioactivity was in PtdCho). Sterol and fatty acid synthesis was measured by [14C]acetate labeling (24). Briefly, cells were incubated with 5 µCi/ml [14C]acetate for 2 h, cell monolayers were dissolved in 1 ml of 0.5 N NaOH, transferred to screwcap tubes, and saponified in 3 ml of ethanol and 0.5 ml of 50% (w/v) KOH for 1 h at 60 °C. The sterol fraction was extracted with 4 ml of hexane and resolved by TLC in petroleum ether-diesthyl ether-acetic acid (60:40:1, v/v/v). The zone corresponding to cholesterol was scraped into vials, and radioactivity was measured by scintillation counting. Fatty acids were extracted from the hydrolysate with 4 ml of hexane after acidification (pH <3) with HCl. Radioactivity in an aliquot was measured by scintillation counting (>99% of the radioactivity in this fraction was in fatty acids). Total cellular phospholipids and sphingolipids were determined by thin layer chromatography and phosphate analysis (15).

**Enzyme Assay—**Cells were harvested by scraping in cold PBS, sedimented at 2,000 × g for 5 min, and homogenized in 20 µl Tris-HCl (pH 7.4), 10 µl EDTA, 5 µl dithiothreitol, and 0.1 m phenylmethylsulfonyl fluoride by 20 passages through a 23-gauge needle. The homogenate was centrifuged at 100,000 × g for 60 min at 4 °C. The sterol fraction was collected, and the particulate (total membrane) fraction was resuspended in the same buffer containing 250 µM sucrose. CCT activity in the membrane and soluble fractions was measured by conversion of [3H]phosphocholine to CDP-[3H]choline in the presence or absence of PtdCho-olente (1:1, mol/mol) vesicles as described previously (15, 25). CPT activity in membranes was measured by conversion of CDP-[3H]choline to [3H]PtdCho in the presence of 1 mM deoxylglycerol/0.15% Tween-20 (26). Protein was measured by the method of Lowry et al. (27).

**Immunoblotting and Immunoprecipitation of CCTa—**Soluble and total membrane fractions from cells were prepared as described above. Membranes were treated with 1% (v/v) Nonidet P-40 on ice for 15 min, and the detergent-soluble fraction was isolated by centrifugation at 15,000 × g for 15 min. A total cell Nonidet P-40 soluble fraction was prepared in a similar manner. Equivalent amounts of protein from Nonidet P-40-solubilized cells, Nonidet P-40 solubilized membranes, and cytosol were resolved by SDS-10% PAGE and transferred to nitrocellulose. The filter was incubated with a polyclonal antibody against 45 amino acids of the C-terminal phosphorylation domain of CCTa (provided by Martin Post, Hospital for Sick Children, Toronto, Canada; Ref. 28), followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase, and developed by the chemiluminescence method according to the manufacturer’s instructions (Amersham Pharmacia Biotech). All antibody incubations were in 20 µl Tris-HCl (pH 7.4), 150 mM NaCl, 5% (w/v) skim milk powder, and 0.1% (v/v) Tween-20. This antibody does not cross-react with the CCTb isoforms because of sequence divergence in the C-terminal phosphorylation domain (29, 30).

Phosphorylation of CCTa was measured by labeling cells for 15 h in phosphate-free medium A containing 25 µCi/ml [32P]Phosphate. Soluble and total membrane fractions were prepared in buffer A as described previously (15). The membrane fraction was treated with 0.3% Triton X-100 on ice for 15 min, and the detergent-soluble fraction was recovered after centrifugation at 400,000 × g for 20 min at 4 °C. [32P]CCTα...
was immunoprecipitated from cytosol and solubilized membranes (50 μg) in buffer A containing 1% Triton X-100 with a 1:400 dilution of an antibody against the membrane binding region of CCTα (kindly provided by Rosemary Cornell, Simon Fraser University, Vancouver, Canada; Ref. 31) for 1 h at 4 °C. Protein A-Sepharose was added for 45 min followed by 6–8 washes with 0.5 ml of PBS containing 1% Triton X-100. Samples were boiled in SDS-PAGE sample buffer and separated by SDS-PAGE in 10% gels. Dried gels were exposed to film at −70 °C.

mRNA Quantitation—CCTα mRNA was quantitated by S1 nuclease protection assays using a [α-32P]dATP-labeled antisense probe corresponding to a 92-base pair HindIII–EcoRI fragment of the rat cDNA as described previously (18). The CCTα S1 probe is against the 5′ end of the mRNA and will not hybridize to CCTβ mRNA because of limited sequence similarity in that region (29, 30). Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as an internal load control.

RESULTS

PtdCho Synthesis in SRD 4- and SCAP-overexpressing Cells—Our previous results showing reduced PtdCho synthesis in cholesterol auxotrophic SRD 6 cells prompted an analysis of PtdCho synthesis in SRD 4 cells, which display elevated cholesterol synthesis that is resistant to down-regulation by 25-hydroxycholesterol (19, 20, 23). PtdCho synthesis and turnover were measured in SRD 4 cells, as well as parental CHO 7 cells, by pulse labeling with [3H]choline for 1 h followed by 4 h of chase in medium containing unlabeled choline (Fig. 1). Following a 1-h pulse, choline incorporation into PtdCho was 4-fold higher in SRD 4 cells relative to controls. The increase in PtdCho synthesis in SRD 4 cells after the 1-h choline pulse was accompanied by a 12-fold decrease in [3H]phosphocholine and 3-fold increase in [3H]CDP-choline, indicative of increased CCT activity. [3H]Phosphocholine was gradually lost from CHO 7 cells, because of conversion to PtdCho, and by 4 h was similar to SRD 4 cells. Phosphocholine pool size in CHO 7 and SRD 4 cells were similar (6.9 ± 1.2 versus 4.6 ± 0.7 nmol/mg protein (n = 3), respectively), indicating that diminished precursor pool size in SRD 4 cells cannot account for rapid synthesis and turnover of radiolabeled PtdCho. In SRD 4 cells, [3H]glycerophosphate (GPC), a product of PtdCho catabolism by phospholipase A, was elevated over 10-fold at the end of the pulse and increased during the chase period. Elevated [3H]GPC in SRD 4 cells during the chase period was accompanied by progressive decay of [3H]PtdCho. However, the loss of radioactivity from PtdCho in SRD 4 cells from 0 to 4 h was not entirely accounted for by increased intracellular GPC. The difference (approximately 500 dpm/mg protein × 10⁻³) was recovered in the cell culture medium as [3H]choline (results not shown).

[3H]Choline pulse experiments showed that SRD 4 cells had elevated PtdCho synthesis and degradation (Fig. 1). Consistent with this observation, PtdCho mass in SRD 4 cells was not significantly different than CHO 7 controls (60.9 ± 4.7 nmol/mg protein in CHO 7 cells (n = 3) versus 53.4 ± 8.1 nmol/mg protein in SRD 4 cells (n = 3)). The mass of phosphatidyethanolamine, phosphatidylserine, and sphingomyelin in SRD 4 cells was also similar to controls.

In addition to the SCAP D443N mutation, SRD 4 cells have a single point mutation in the ACAT gene that renders the enzyme inactive (32). Although chronic ACAT inhibition by 58–035 in CHO 7 or SCAP transfected CHO cells (see below) did not affect PtdCho synthesis (results not shown), we could not rule out the possibility that reduced ACAT activity or another mutation in the SRE 4 cells contributed to the results shown in Fig. 1. To address this question, CHO 7 cells were stably transfected with the cDNA for wild-type SCAP or the SCAP D443N mutant and PtdCho synthesis was examined. Initially, four clones resistant to killing by 0.5 μg of 25-hydroxycholesterol/ml and expressing epitope-tagged SCAP or SCAP D443N (hereafter referred to as CHO-SCAP or CHO-SCAP D443N) were isolated, and PtdCho synthesis was measured by a 1-h [3H]choline pulse. The average rate of PtdCho synthesis in four mock transfected cell lines was 122.5 ± 32.4 dpm/μg protein, compared with 221.1 ± 22.8 and 206.2 ± 22.0 dpm/μg protein in four CHO-SCAP and CHO-SCAP D443N cell lines, respectively. Increased PtdCho synthesis in SCAP transfected cells was also accompanied by a 2–3-fold reduction in [3H]phosphocholine.

Because all four SCAP- and SCAP D443N-overexpressing cell lines appeared to have a similar phenotype with respect to increased PtdCho synthesis, one cell line from each group was examined in detail. The expression of epitope-tagged and endogenous SCAP and SCAP D443N in the two cell lines is shown in Fig. 2. Immunoblotting with a HSV monoclonal antibody detected a protein doublet of approximately 140–150 kDa in the Nonidet P-40 extracts of CHO-SCAP and CHO-SCAP D443N cell membranes but not in mock transfected controls (Fig. 2A). It appeared that higher expression of wild-type SCAP compared with the D443N mutant was required to maintain 25-hydroxycholesterol resistance. The cell extracts from Fig. 2A, as well as those from CHO 7 and SRD 4, were also probed with antibody R-139 to detect both endogenous and overexpressed SCAP (21). This antibody also detected a 140–150-kDa protein doublet in all cells. As expected, expression was highest in the CHO-SCAP and CHO-SCAP D443N cells relative to mock, CHO 7, and SRD 4 cells (Fig. 2B).
Irrespective of differences in expression, both CHO-SCAP and CHO-SCAP D443N cells displayed a 2-fold increase in $[{14C}]$acetate incorporation into cholesterol relative to mock transfected controls (Fig. 3). Cholesterol and fatty acid synthesis in SRD 4 cells was increased 3-fold above CHO 7 cells. Unlike that of SRD 4 cells, $[{14C}]$acetate incorporation into fatty acids in the SCAP-overexpressing cell lines was not significantly increased.

PtdCho synthesis was examined in the SCAP-overexpressing cells by $[{3H}]$choline labeling followed by 4-h chase in medium containing unlabeled choline (Fig. 4). At the end of the 1-h $[{3H}]$choline pulse (0 h) and throughout the 4-h chase, PtdCho synthesis was increased 1.5–2.5-fold in CHO-SCAP and CHO-SCAP D443N cells. Increased PtdCho synthesis in these cells was accompanied by a 2-fold reduction in $[{3H}]$phosphocholine levels, a 2–3-fold increase in $[{3H}]$CDP-choline, and a 2-fold elevation in $[{3H}]$GPC production.

Our previous results in SRD 6 cells showed that decreased PtdCho synthesis was not correlated with changes in cellular cholesterol levels but rather with the availability of fatty acids (16). However, these studies did not rule out the possibility that de novo synthesis of cholesterol and PtdCho are somehow coupled because cholesterol synthesis in SRD 6 cells is virtually absent because of aberrant SREBP processing (16, 35). The relationship between cholesterol synthesis and PtdCho metabolism was examined in SRD 4 and SCAP-transfected cells using lovastatin to inhibit HMG-CoA reductase activity and cholesterol synthesis (Table I). As expected, PtdCho synthesis was significantly elevated in SRD 4, CHO-SCAP, and CHO-SCAP D443N cells (as measured by $[{3H}]$choline incorporation). Treatment of cells with lovastatin for 8–10 h did not affect $[{3H}]$choline incorporation into PtdCho in control, SRD 4, CHO-SCAP, or CHO-SCAP D443N cell lines. Under these conditions, $[{14C}]$acetate incorporation into cholesterol was reduced by >90%, and fatty acid labeling was unaffected.

Next, we examined the relationship between elevated fatty acid and PtdCho synthesis by selectively reducing fatty acid synthesis in SRD 4 cells to the level of control cells and examining $[{3H}]$choline incorporation into PtdCho and choline metabolites (Fig. 5). SRD 4 cells were treated with cerulenin, a potent irreversible inhibitor of mammalian and bacterial fatty acid synthase (34), for up to 4 h and pulse labeled for the final hour with either $[{3H}]$choline or $[{14C}]$acetate to measure PtdCho or fatty acid synthesis, respectively. Treatment of SRD 4 cells with cerulenin for 2–4 h was sufficient to reduce $[{14C}]$acetate incorporation into fatty acids to the level of untreated CHO 7 cells (Fig. 5, closed circles at 0 h). Treatment of SRD 4 cells for
4 h with cerulenin did not significantly influence radiolabeling of cholesterol (172 ± 8 versus 182 ± 29 dpm [14C]acetate incorporated/mg protein in untreated and cerulenin-treated SRD cells, respectively). However, cerulenin suppressed choline incorporation into PtdCho and GPC by 70 and 85%, respectively, relative to controls. Cerulenin also dramatically increased radiolabeled phosphocholine (9-fold) in SRD 4 cells, indicative of reduced flux through the CDP-choline pathway as a consequence of decreased CCT activity.

**CCTα and CPT Activity in SRD 4 and CHO-SCAP Cells**—We examined various parameters of CCTα expression and regulation to determine the cause of increased CCTα activity in SRD 4- and SCAP-overexpressing cells. Initially, we measured CCTα mRNA levels by S1 nuclease protection assays in SRD 4 and CHO-SCAP or CHO-SCAP D443N cells. As shown in Fig. 6, CCTα mRNA was reduced by 30% in SRD 4 cells and was unaffected in SCAP- and SCAP D443N-overexpressing cells. The primary mechanism for CCTα regulation is via post-transcriptional mechanisms involving phosphorylation and translocation to the endoplasmic reticulum or nuclear envelope (17). To assess whether in vivo activation of CCTα was reflected in changes in enzyme distribution or activation in vitro, activity was measured in the soluble and total membrane fraction of cells, either in the presence or absence of PtdCho:oleate vesicles. In vitro CCTα activity in SRD 4 cells was reduced 3- and 2-fold in the soluble and membrane fractions, respectively, relative to controls (Table II). However, residual soluble and membrane CCTα activity from SRD 4 cells was activated by PC:oleate vesicles to a similar extent as controls. CPT activity in SRD 4 cells was also reduced by 30%. In vitro CCTα activity in soluble and membrane fractions was also measured in CHO-SCAP and CHO-SCAP D443N cells (Table III). CCTα activity, measured with and without PC:oleate vesicles, from control and the two SCAP transfected cells was not significantly different. The one exception was significant increase (60%) in unstimulated soluble CCTα activity in CHO-SCAP D443N cells. CPT activity in CHO-SCAP cells was not significantly different from controls.

Next, we compared enzyme activity with the levels of CCTα protein in soluble, membrane, and total homogenates of SRD 4- and SCAP-overexpressing cells by immunoblotting with polyclonal antibody against the N-terminal phosphorylation domain of CCTα (28). Consistent with enzyme activity measurements in Table II, CCTα protein was reduced in both the soluble and membrane fractions of SRD 4 cells relative to
Controls (Fig. 7). CCTα protein mass in total cell homogenates was also reduced by approximately 2-fold in SRD 4 cells. In CHO-SCAP and CHO-SCAP D443N cells, CCTα protein expression was similar to mock transfected cells in all fractions. Again, this finding agreed with enzyme activity measurements in Table III. Similar results to those shown in Fig. 7 were also obtained using a polyclonal antibody against the membrane-phosphorylation domain of CCTα (results not shown).

Finally, we determined whether the phosphorylation status of CCTα was altered in SRD 4 or SCAP transfected cells (Fig. 8). In these experiments, CCTα was labeled with [32P]phosphate in vivo, immunoprecipitated from soluble and membrane fractions and analyzed by SDS-PAGE. Phosphorylation of soluble and particulate CCTα from SRD 4 cells was reduced relative to CHO 7 controls, consistent with reduced protein expression and enzyme activity in both these fractions (Fig. 7). There were no apparent changes in the phosphorylation of CCTα in CHO-SCAP or CHO-SCAP D443N cells.

Localization of CCTα in SRD 4 and SCAP Transfected Cells—CCTα in CHO and HeLa cells was shown to strongly localize to the nucleus and translocate to the nuclear envelope in response to phospholipase C-mediated degradation of PtdCho and exogenous oleate (35–37). This raised the possibility that CCTα activities shown in Table II and III did not accurately reflect the distribution of CCTαs in intact cells because of trapping in the nucleus or disruption of CCTαs association with membranes during fractionation. To circumvent these potential problems, we analyzed the intracellular distribution of CCTα by indirect immunofluorescence (Fig. 9). As reported for CHO-K1 cells (37), CCTα in CHO 7 cells was exclusively localized in the nucleus, but with no evidence of nuclear envelope staining (Fig. 9A and D). In contrast, SRD 4 cells displayed reduced CCTα immunofluorescence in the interior of the nucleus and a prominent fluorescent ring around the periphery corresponding to the nuclear envelope (Fig. 9B). This staining pattern could be reproduced in CHO 7 cells treated with 0.1 mM oleate for 1 h (Fig. 9C). CCTα also localized to structures within the nucleus of SRD 4 and oleate-treated CHO 7 cells that did not appear to be associated with the nuclear envelope. CCTα in

![Fig. 7. Expression of CCTα protein in SRD 4 and SCAP-transfected CHO cells.](http://www.jbc.org/)

**TABLE II**

|                   | Choline phosphotransferase | CTP:phosphocholine cytidylyltransferase |
|-------------------|----------------------------|----------------------------------------|
|                   | nmol/min/mg protein        | nmol/min/mg protein                    |
| **CHO 7**         | 1.53 ± 0.49                | 2.64 ± 0.46                            |
| **SRD 4**         | 1.10 ± 0.18                | 0.72 ± 0.19a                           |

*p < 0.005 versus CHO 7 controls.

**TABLE III**

|                   | Choline phosphotransferase | CTP:phosphocholine cytidylyltransferase |
|-------------------|----------------------------|----------------------------------------|
|                   | nmol/min/mg protein        | nmol/min/mg protein                    |
| **Mock**          | 1.52 ± 0.33                | 1.75 ± 0.28                            |
| **CHO-SCAP**      | 1.72 ± 0.24                | 1.81 ± 0.70                            |
| **CHO-SCAP D443N**| 1.47 ± 0.38                | 2.86 ± 0.18a                           |

*p < 0.05 versus mock transfected cells.
overexpression of SCAP or a sterol insensitive SCAP mutant (D443N) results not only in increased sterol and fatty acid synthesis but also increased synthesis and catabolism of PtdCho. This provides further evidence that sterol, fatty acid, and PtdCho synthesis are coordinately controlled via the activity of the SREBP/SCAP regulatory pathway. PtdCho synthesis was not regulated at the transcriptional level, as shown for other enzymes of sterol and fatty acid synthesis but rather by altering the availability of fatty acids or a related lipid activator of CCTα, the rate-limiting enzyme in the CDP-choline pathway.

PtdCho metabolism was examined in three cell lines that had increased sterol and fatty acid synthesis because of manipulation of SCAP expression. SRD 4 cells and CHO 7 cells stably transfected with epitope-tagged versions of SCAP and SCAP D443N had significantly elevated cholesterol synthesis. However, SRD 4 cells had a 3-fold increase in fatty acid synthesis, compared with only a 25–30% increase in CHO-SCAP and CHO-SCAP D443N cells. This is a significant variation in phenotype that may explain differences in PtdCho metabolism and CCTα activity and localization. All three cell lines displayed alterations in PtdCho synthesis and metabolite profiles, but the changes in SRD 4 cells were much greater compared with CHO-SCAP and CHO-SCAP D443N cells. In SRD 4 cells, there was rapid flux of [3H]choline through the biosynthetic pathway such that virtually no phosphocholine accumulated and PtdCho was the major biosynthetic product at the end of a 1-h pulse. In comparison, the majority of the [3H]choline incorporated into control and SCAP-transfected cells was confined to phosphocholine at the end of the 1-h pulse. Taking into account the high level of GPC in SRD 4 cells at the end the pulse period, we conclude that as much as 30% of newly made PtdCho was degraded during this time. If degradation is factored in, PtdCho synthesis in SRD 4 cells is actually elevated by approximately 6-fold relative to controls at the end of the 1-h pulse.

Also consistent with a role of fatty acids in elevated CCTα activity was the localization of CCTα to the nuclear envelope in SRD 4 cells (Fig. 9). CCTα translocation to the nuclear envelope and membrane/particulate fraction was previously observed in response to exogenous fatty acids in numerous cell models (17, 36, 39). Exogenous oleate in the medium of CHO 7 cells also stimulated CCTα translocation to the nuclear envelope, suggesting that elevated fatty acid synthesis in SRD 4 cells is responsible for CCTα activation, membrane localization, and increased PtdCho synthesis. CCTα in CHO-SCAP and CHO-SCAP D443N cells was not localized to the nuclear envelope, consistent with poor stimulation of fatty acid synthesis relative to SRD 4 cells, possibly related to high level SCAP and SCAP D443N expression in a wild-type background.

Contrary to the immunofluorescence localization of CCTα to the nuclear envelope in SRD 4 cells, CCTα activity and/or mass was unchanged or reduced in the particulate/membrane fraction of these cells. CCTα translocation to membranes was previously shown to be required for activation and increased PtdCho synthesis (reviewed in Refs. 17 and 38). However, translocation in many of these studies was initiated by the acute addition of high concentrations of exogenous fatty acids or manipulation of PtdCho content with phospholipase C (36, 40). Why CCTα association with the nuclear envelope was not detected in the particulate fraction of SRD 4 cells in vitro is unclear. It is possible that CCTα in SRD 4 cells is weakly associated with the nuclear envelope and dissociated during cell homogenization or that the cell homogenization conditions caused significant trapping of CCTα in the nucleus that would confound measurements of the membrane-bound enzyme. The latter seems unlikely because we did not

FIG. 8. Phosphorylation of CCTα was not influenced by SCAP or SCAP D443N overexpression. CCTα phosphorylation was assessed by [32P]phosphate labeling and immunoprecipitation with a rabbit polyclonal antibody against the membrane binding region of CCTα (26) as described under “Experimental Procedures.” [32P]CCTα was separated by 8% SDS-PAGE, and the dried gel was exposed to film for 2 days at -70 °C. Similar results were seen in two other experiments.

FIG. 9. Immunofluorescence localization of CCTα in SRD 4 and SCAP-transfected cells. Cells were cultured in medium A on glass coverslips for 3 days prior to the start of experiments. CHO 7 cells (A), SRD 4 cells (B), CHO 7 cells treated with 100 μM oleate for 1 h (C), mock-transfected cells (D), CHO-SCAP cells (E), and CHO-SCAP D443N cells (F) were fixed in 3% (v/v) formaldehyde for 15 min at 20 °C and permeabilized in 0.05% Triton X-100 for 10 min at -20 °C. Coverslips were then incubated in PBS with 1% (w/v) bovine serum albumin and an antibody against the C terminus of CCTα (1/4,000 dilution) for 12 h at 4 °C, followed by a secondary goat anti-rabbit fluorescein isothiocyanate-labeled antibody for 45 min at 20 °C. CCTα was visualized by laser confocal microscopy (Zeiss LSM 510), and images were processed using Adobe Photoshop software.

has a central role by virtue of association with the lipid bilayer, that regulates membrane sterol and lipid composition. In this study we demonstrate that uncoupling this regulatory loop by

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observe increased particulate CCTα in SRD 4 cells using different cell fractionation conditions but have observed a shift of CCTα activity and mass to the particulate fraction in oleate-treated CHO 7.2 The possibility that the CCTα isoform (29, 30) is interfering with activity measurements seems unlikely. The expression of total CCT activity in cytosol and membranes closely paralleled the expression of CCTα protein in these fractions. As well, we have not detected expression in our CHO cells using a β-isofrom-specific antibody.

Although exogenous fatty acids promote rapid membrane translocation and activation of CCTα in intact cells, resulting in elevated PtdCho synthesis (17, 39), there was little evidence that fatty acids regulate PtdCho synthesis in vivo. Our finding that inhibition of fatty acid synthesis by cerulenin results in decreased radiolabeling of PtdCho is compelling evidence of a regulatory role for in vivo fatty acid synthesis. Reduction of fatty acid synthesis by cerulenin appeared to parallel increase in both synthesis and degradation of radiolabeled PtdCho in SRD 4 cells (17, 39), there was little evidence that cerulenin was not deemed to have a direct regulatory role based on lack of effect of cholesterol supplementation on PtdCho synthesis in SRD 4 cells. Based on these findings, iPLA2 is likely involved in increased particulate CCTα activity and mass to the particulate fraction in oleate-treated CHO 7.2 T. A. Lagace, M. K. Storey, and N. D. Ridgway, unpublished results.

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Thomas A. Lagace, Margo K. Storey and Neale D. Ridgway

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