Stimulation of phosphatidylcholine biosynthesis in mouse MLE-12 type-II cells by conditioned medium from cortisol-treated rat fetal lung fibroblasts

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Treatment of murine adult MLE-12 type-II and fetal-rat type-II cells with fetal-rat-fibroblast-conditioned medium (FFCM) resulted in a 2-fold stimulation of [^{14}C]choline incorporation into phosphatidylcholine. Soluble CTP:phosphocholine cytidylyltransferase (CT) activity was increased approx. 3-fold in FFCM-treated fetal-rat type-II cells but was not changed in MLE-12 cells. Neither choline kinase nor cholinephosphotransferase activities were affected by treatment of MLE-12 cells with FFCM. Long-term labelling of MLE-12 cells with [^{14}C]choline, followed by a 14–18 h chase with FFCM, resulted in a 2.5-fold decrease in [^{14}C]phosphocholine levels relative to controls, suggesting that CT was being activated. In contrast, oleate treatment increased CT activity in the particulate fraction in both cells. Western blots indicate that soluble CT undergoes dephosphorylation in response to FFCM, but no translocation to the particulate fraction was noted. Treatment with oleate stimulated a marked translocation. Tryptic phosphopeptide maps from FFCM-treated cells revealed only minor alterations in the phosphorylation pattern. It is concluded that FFCM and oleate activate CT through different mechanisms. The results are consistent with FFCM activating CT in MLE-12 as well as fetal type-II cells. However, the reason why this activation cannot be detected *in vitro* is not known.

INTRODUCTION

Pulmonary surfactant is synthesized and secreted by type-II pneumocytes and functions by reducing the surface tension at the air/liquid interface [1, 2]. Surfactant is composed of about 10% protein (mainly the surfactant proteins SP-A, SP-B, SP-C and SP-D) and 90% lipid, predominantly phosphatidylcholine [1–3]. The CDP-choline pathway is the primary pathway for *de novo* phosphatidylcholine synthesis in type-II cells [1–4]. The rate-limiting enzyme in the CDP-choline pathway is CTP:phosphocholine cytidylyltransferase (CT, EC 2.7.7.15), a 42 kDa phosphoprotein that catalyses the conversion of phosphocholine into CDP-choline, the activated form of choline [4–7].

Cytidylyltransferase is an amphitropic enzyme in that it is associated with both the soluble and particulate cellular compartments [7]. Activation of CT is coincident with the association of the enzyme with lipid, either through the translocation of all or part of the soluble enzyme to a membrane fraction, or by the adsorption of membrane lipid by the inactive soluble form (designated the 'L-form') converting it into a soluble, but active, high-molecular-mass lipoprotein complex ('H-form') [7–10]. The H-form is similar to the membrane-associated form of CT [8–10], and therefore conversion of the L-form into the H-form probably occurs via a mechanism similar to that used in CT translocation. Considerable evidence exists suggesting an inherent role for phosphorylation–dephosphorylation in regulating CT activity [11,12]. The inactive form of the enzyme is highly phosphorylated, whereas the active form is largely, but not completely, dephosphorylated [12,13]. The phosphorylation sites of rat liver CT have been identified, and some of these sites appear within consensus sequences for proline-directed protein kinases [14]. Phosphopeptide maps of rat liver and Chinese-hamster-ovary (CHO) cells are identical, indicating conservation of phosphorylation sites between species [14].

During late gestation in the rat (18–22 days) increased CT activity and phosphatidylcholine (PC) synthesis by fetal type-II cells is correlated with an increase in the membrane-associated form of CT [15] and in the soluble H-form [2,16]. After birth and into adulthood CT activity in the lung remains very high [2,16]. In the fetal lung, fibroblasts influence CT activity in type-II cells by the production of fetal pneumocyte factor (FPF) [17,18]. The precise molecular nature of FPF is not known, and in fetal cells its effect is more differentiative than proliferative [18]. Under experimental conditions the production of FPF in conditioned medium may be augmented by treatment of isolated 19–20-day fetal-lung fibroblasts or explants with glucocorticoids such as hydrocortisol [17,18]. The mechanism of FPF-mediated CT activation is not known, although some evidence indicates that the process is dependent on fatty acid synthesis [19]. It has been established that FPF does not directly enhance CT gene expression, as treatment of fetal lung explants with dexamethasone has no effect on CT mRNA or enzyme levels [20,21].

In contrast with fetal lung, little is known about the interactions between fibroblasts and type-II cells in adult lung and about the signals which act on adult type-II cells to maintain levels of highly active CT. Studies on the regulatory aspects of surfactant synthesis have been hampered by the lack of a model system in which various manipulations can be performed; however, some promising cell lines have recently become available. In the present study we have used the murine cell line MLE-12 [22] to study the regulation of CT in an 'adult-like' type-II cell.

EXPERIMENTAL

Materials

All solvents were of reagent grade from BDH chemicals. Minimal essential medium (MEM), phosphate-free MEM, fetal-bovine

Abbreviations used: FFCM, fetal-rat-fibroblast-conditioned medium; CT, CTP:phosphocholine cytidylyltransferase; FBS, fetal-bovine serum; MEM, minimal essential medium; Tos-Phe-CH_2Cl-trypsin, tosyl-phenylalanylchloromethane ('TPCK')-treated trypsin; DTT, dithiothreitol; CHO, Chinese-hamster ovary; PC, phosphatidylcholine; FPF, fetal pneumocyte factor; SP, surfactant protein.

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serum (FBS) and gentamicin sulphate were from Gibco–Life Technologies. Carrier-free [^{32}P]orthophosphate (400–800 mCi/ml) was from ICN, [*methyl*-^{14}C]choline (55.4 mCi/mmol), [*methyl*-^{14}C]CDP-choline (55.4 mCi/mmol) and ^{125}I-anti-(rabbit IgG) antiserum were from Amersham International. Microcrystalline cellulose TLC plates were from Kodak, and silica-gel LK6 thin-layer plates were from Whatman. Proteinase inhibitors and all other chemicals were from Sigma or Boehringer-Mannheim. A polyclonal antibody generated against the peptide MDAQSSAKVNSRKRRKEV corresponding to residues 1–18 of rat liver cytidylyltransferase (N-antibody; [11]) and a polyclonal antibody generated against whole recombinant CT expressed in baculovirus-infected insect cells [23] were gifts from Dr. Claudia Kent, University of Michigan, Ann Arbor, MI, U.S.A.

Maintenance of cell lines

MLE-12 cells were obtained from Dr. Jeffrey Whitsett, University of Cincinatti, Cincinatti, OH, U.S.A., and were maintained in MEM supplemented with 5% FBS at 37 °C in an CO_2/air (1:19) atmosphere. The MLE-12 cell line was derived from a lung adrenocarcinoma expressing the SP-C promoter/simian-virus-40 large T antigen chimaeric gene. The cells do not express SP-A, but do express SP-B and SP-C and contain microvilli and multivesicular bodies typical of adult type-II cells [22].

Isolation of primary type II cells

Type II cells from 20-day-old fetal rats were obtained as described by Caniggia et al. [18]. Briefly, the dams were killed by CO_2 asphyxiation and the pups were removed and placed on ice. The lungs were excised, washed with ice-cold Hanks balanced salt solution, and the heart and major airways removed. The lung tissue was then minced with fine scissors and digested with trypsin and DNase I for 20 min at 37 °C. The digested material was then resuspended in MEM containing 5% FBS, filtered, centrifuged at 1500 rev./min in a Beckman J6B preparative centrifuge and resuspended in serum-free MEM. To the cell suspension was added collagenase and DNase I, and the suspension was incubated at 37 °C with gentle stirring for 15 min. The cell suspension was then centrifuged as described above, washed with MEM, centrifuged again and the cells resuspended in MEM containing 5% FBS. To remove the fibroblasts the cells were plated in 75 cm^2 flasks and incubated at 37 °C for 1 h, after which the medium, containing non-adhering fibroblasts and epithelial cells, was removed to a second set of 75 cm^2 culture flasks and incubated again at 37 °C for 1 h. Fresh MEM containing 5% FBS was added to the original flasks and these were returned to the incubator. At the conclusion of the second incubation period the medium was removed from the second set of flasks and fresh medium was added. The medium removed from these flasks, containing epithelial cells, was plated in MEM containing 5% FBS.

Preparation of fetal-fibroblast-conditioned medium (FFCM) as a source of FPF

The fetal fibroblasts were allowed to grow to confluency (overnight) and were then washed extensively with MEM. They were then incubated in MEM for 24 h at 37 °C, after which the medium was removed and replaced with MEM containing 10^{-7} M hydrocortisol. The fibroblasts were incubated for a further 24 h after which the conditioned medium was collected and divided into aliquots and stored at −20 °C. Occasionally the medium collected before exposure of the fibroblasts to hydrocortisol was also stored at −20 °C and was called 'pre-conditioned medium'.

Cell labelling with [^{14}C]choline

Type II and MLE-12 cells were trypsin-treated and plated at high density [(1–2) × 10^7 cells/60 mm^2 dish]. The cells were allowed to adhere overnight in MEM/FBS before being washed with MEM. Cells were incubated in MEM for 2 h, after which the medium was removed and replaced with either a 1:1 dilution of MEM/FFCM [18,24] or a 1:1 dilution MEM/MEM containing 10^{-7} M hydrocortisol (in both cases the final volume of medium added was 2.0 ml). In some instances the medium was replaced with a 1:1 dilution of MEM/pre-conditioned medium. The cells were incubated at 37 °C for 14–18 h. At 1–2 h before the incubation was to be terminated, 1 μCi of [*methyl*-^{14}C]choline was added to each dish (the final choline concentration was 16 μM). Incubations were terminated by removal of the medium and washing the cells with ice-cold PBS. The cells were scraped into 1 ml of ice-cold PBS, and half of this was extracted into chloroform by the method of Bligh and Dyer [25], the remainder being saved for protein determination.

For chase experiments, cells were plated as described above, with the exception that 1 μCi of [*methyl*-^{14}C]choline was included in the medium (final choline concn. 16 μM). The cells were incubated for 36–48 h, after which they were washed and the medium replaced with 3 ml of serum-free MEM containing 1 μCi of [*methyl*-^{14}C]choline. The cells were then preincubated as described above for 2–3 h, after which the medium was removed, the cells washed three or four times with serum-free MEM and chased with FFCM as described above. No difference in total [^{14}C]choline incorporation was noted in cells labelled for 36 or 48 h.

For stimulation of choline incorporation with oleic acid, cells were plated as described above, washed with MEM and incubated with MEM containing 0.33% fatty-acid-free BSA and 1 μCi of [*methyl*-^{14}C]choline (final choline concn. 16 μM). The cells were then challenged with 0.5 mM oleic acid for 1 h, after which they were processed as above.

For labelling with [^{32}P]phosphate, cells were plated as above, washed with phosphate-free MEM and incubated for 3 h in 1 ml of the same medium containing 5 mCi of carrier-free [^{32}P]phosphate. At the conclusion of the incubation period the cells were challenged by the addition of either 1 ml of FFCM or 1 ml of MEM containing 10^{-7} M cortisol. The cells were then incubated overnight.

Immunoprecipitations

Cells labelled with [^{32}P]phosphate were washed three times with PBS and partitioned into soluble and particulate fractions by the digitonin permeabilization method as previously described [11,12]. Cytidylyltransferase was immunoprecipitated and blotted on to Immobilon-P poly(vinylidene difluoride) membranes as previously described [11]. All buffers contained a proteinase-inhibitor cocktail consisting of 5 μg/ml leupeptin, 2 μg/ml pepstatin, 0.2 mM Pefabloc (Boehringer-Mannheim) and 0.5 mM benzamidine.

Two-dimensional phosphopeptide mapping

The ^{32}P-labelled cytidylyltransferase band was excised from the immobilon membrane, washed several times with distilled water and then incubated for 30 min at 37 °C in 0.5% polyvinylpyrrolidone-40 in 100 mM acetic acid. The immobilon membrane

was then washed several times again with distilled water and several times with 50 mM NH_4HCO_3. The membrane was then incubated in 50 mM NH_4HCO_3 with freshly prepared Tos-Phe-CH_2Cl ('TPCK')-treated trypsin as described in [14]. The digests were evaporated to dryness *in vacuo* and the phosphopeptides resolved on microcrystalline cellulose TLC plates as described in [14].

Immunoblotting

Cells challenged with oleate and FFCM were separated into soluble and particulate fractions by digitonin permeabilization as previously described [12]. The proteins were separated by SDS/PAGE and transferred to Immobilon-P. Immunoblotting for cytidylyltransferase was performed using the N-antibody [11,23] as the primary and ^{125}I-anti-rabbit IgG as the secondary antibody. Cytidylyltransferase was then revealed by autoradiography.

Cell fractionation and enzyme assays

MLE-12 and fetal type II cells in 60 mm culture dishes were incubated as above in the presence or absence of FFCM or oleate, washed and scraped gently into 1 ml of ice-cold lysis buffer [10 mM Tris/HCl, pH 7.5, containing 15 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol (DTT) and proteinase inhibitors as described above]. The cells were lysed at 4 °C with approx. 30 strokes in a Dounce homogenizer and the homogenate centrifuged for 30 min at 100000 *g*. The supernatant was removed and the pellet was resuspended in 1 ml of cold lysis buffer. CTP:phosphocholine cytidylyltransferase, choline kinase and choline phosphotransferase were assayed as previously described [26–28]. In some cases cells were fractionated using the digitonin-permeabilization method, in which case the cell ghosts were scraped into 0.5 ml of 50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 mM EDTA/2 mM DTT containing proteinase inhibitors.

Miscellaneous procedures

Protein was determined by the procedure of Bradford [29] using the reagent supplied by Bio-Rad, with BSA as the standard. SDS/PAGE was performed as described by Laemmli [30].

RESULTS

Treatment of MLE-12 and fetal rat type-II cells with conditioned medium enhances phosphatidylcholine synthesis

Enhanced production of FPF may be obtained by treatment of confluent, late-gestational fetal fibroblasts with 10^{-7} M cortisol in serum-free medium [18]. Without further purification the source of FPF was simply called FFCM. Type II cells isolated from 20-day-old fetal rats and mouse MLE-12 type II cells respond to treatment with FFCM by increasing choline incorporation into PC by 50–100% (Table 1). In contrast, HeLa cells showed no response to treatment with FFCM (Table 1). In some experiments we treated cells with medium taken from fibroblasts prior to treatment with cortisol (preconditioned medium). Cells treated with preconditioned medium incorporated [^{14}C]choline into PC at a rate that was about 20–30% higher than untreated controls (results not shown), consistently lower than the 50–100% increase observed in cells treated with cortisol-conditioned medium. That choline incorporation in response to FFCM was increased in MLE-12 cells was somewhat surprising, as this cell line is derived from an adult murine adrenocarcinoma cell line [22].

Table 1 Effect of FFCM or oleic acid on the incorporation of [^{14}C]choline into PC in MLE-12, fetal-rat type-II and HeLa cells

Results are means ± S.D. for *n* separate experiments (in parentheses) performed in triplicate. Abbreviation: N.D., not determined.

	Incorporation (d.p.m./μg of protein)		
	MLE-12	Fetal-rat type-II	HeLa
Control	341.8 ± 79.9 (6)	127.4 ± 17.2 (6)	70.1 ± 17.8 (4)
FFCM	590.1 ± 172.3 (6)*	197.1 ± 38.0 (6)†	70.1 ± 14.1 (4)
Oleic acid	2936.4 ± 267.3 (4)	822.5 ± 156.2 (4)	N.D.

* Significantly different from controls ($P < 0.01$); significance was determined by Student's *t* test.

† Significantly different from controls ($P < 0.01$).

The possibility existed that the FFCM-induced increase in [^{14}C]choline incorporation into PC could be due to a difference in the rate at which choline is taken up by FFCM-treated MLE-12 cells. However, an analysis of the water-soluble choline metabolites after FFCM treatment revealed very little difference between treated versus untreated, with [^{14}C]phosphocholine levels being virtually the same in both cases and very little radioactivity in either choline or CDP-choline (results not shown).

FFCM treatment stimulates CT activity in fetal-rat type-II cells but no increase is observed in MLE-12 cells

Consistent with previous work [24] we observed a twofold stimulation of CT activity in FFCM-treated fetal type II cells; however, no increase in CT activity, either in the presence or absence of activating lipids, was observed in MLE-12 cell extracts (Table 2). The increase in specific CT activity in FFCM-treated fetal type-II cells was confined to the soluble enzyme and was most evident when CT was assayed in the absence of activating lipid, suggesting a conversion of L-form into H-form. The lack of a discernable effect of FFCM on CT activity in MLE-12 cells was puzzling, so as a control we examined the effect of oleic acid on CT activation in fetal type II and MLE-12 cells. Oleic acid is a well-documented activator of CT activity in HeLa cells [12,31]. Treatment with 0.5 mM oleate increased choline in-

Table 2 Effect of FFCM on cytidylyltransferase activity in total homogenates and subcellular fractions from MLE-12 and fetal-rat type-II cells

Results are means ± S.D. for four separate experiments performed in triplicate.

	Specific activity (nmol of CDP-choline/min per mg of protein)			
	MLE-12		Fetal rat type-II	
Fraction	−Lipid	+Lipid*	−Lipid	+Lipid*
Homogenate	2.2 ± 1.0	6.0 ± 2.1	2.4 ± 0.4	5.4 ± 1.7
Homogenate FFCM	2.3 ± 1.0	6.9 ± 2.5	4.8 ± 0.2	6.1 ± 2.3
Cytosol	1.6 ± 0.6	14.2 ± 3.9	1.2 ± 0.3	7.9 ± 2.4
Cytosol, FFCM	1.2 ± 0.4	15.6 ± 5.1	3.7 ± 0.4†	8.0 ± 1.6
Membranes	2.5 ± 0.5	5.0 ± 0.5	1.4 ± 0.4	2.3 ± 0.6
Membranes, FFCM	2.4 ± 0.7	4.9 ± 0.4	1.5 ± 0.4	2.2 ± 0.3

* Cytidylyltransferase assayed in the presence of phosphatidylcholine/oleic acid vesicles.

† Significantly different from control cytosol ($P < 0.001$); significance was determined by Student's *t* test.

Table 3 Effect of oleic acid on cytidylyltransferase activity in total homogenates and subcellular fractions from MLE-12 and fetal rat type-II cells

Results are means ± S.D. for three separate experiments performed in triplicate.

	Specific activity (nmol of CDP-choline/min per mg of protein)			
	MLE-12		Fetal rat type-II	
Fraction	−Lipid	+Lipid*	−Lipid	+Lipid*
Homogenate	2.9 ± 0.3	10.0 ± 1.7	1.6 ± 0.3	7.4 ± 1.5
Homogenate, oleate	7.7 ± 0.7†	10.6 ± 1.0	4.8 ± 0.9‡	8.1 ± 2.3
Cytosol	1.7 ± 0.8	12.7 ± 4.8	1.4 ± 0.6	9.8 ± 2.7
Cytosol, oleate	2.2 ± 1.0	10.5 ± 3.3	1.8 ± 0.6	10.3 ± 2.2
Membranes	2.2 ± 0.2	3.7 ± 0.6	1.7 ± 0.5	2.5 ± 1.1
Membranes, oleate	14.1 ± 6.3	17.6 ± 8.2	7.3 ± 1.1	9.2 ± 3.4

* Cytidylyltransferase assayed in the presence of phosphatidylcholine/oleic acid vesicles.
† Significantly different from control homogenate ($P < 0.001$).
‡ Significantly different from control homogenate ($P < 0.01$); significance was determined by Student's t test.

corporation and CT activity in both fetal type II and MLE-12 cells (Tables 1 and 3). As expected, the increase in choline incorporation with oleate was considerably higher than that observed with FFCM (Table 1). In addition, a specific increase in membrane-associated CT activity was observed in both oleate-treated fetal type-II and MLE-12 cells. Thus, oleate treatment induces a parallel activation of membrane-associated CT in fetal type-II and MLE-12 cells.

The two other enzymes in the CDP-choline pathway for PC synthesis are choline kinase and choline phosphotransferase. Although CT is generally accepted as the regulatory enzyme in the pathway, some evidence exists suggesting that choline kinase may under certain conditions also be regulatory [32–34], and so the effect of FFCM on this enzyme and choline phosphotransferase in MLE-12 cells was examined. However, we saw no change in the activities of choline kinase (5.8 ± 1.2 nmol/min per mg of protein in control versus 5.3 ± 1.3 nmol/min per mg of protein in treated) or choline phosphotransferase (4.3 ± 1.5 nmol/min per mg of protein and 4.4 ± 1.1 nmol/min per mg of protein in treated and untreated respectively) in FFCM-treated and control MLE-12 cells. In addition, we found no differences in 1,2-diacylglycerol levels between treated and untreated MLE-12 cells (0.29 nmol of diacylglycerol/mg of lipid phosphorus and 0.33 nmol/mg of lipid phosphorus in treated and untreated respectively. Results are averages from two separate experiments and represent values obtained after an 18 h incubation with FFCM). Thus the increase in choline incorporation seen in treated MLE-12 cells could not be attributed to altered diacylglycerol levels.

FFCM treatment of MLE-12 cells does not enhance PC turnover

The fact that [^{14}C]choline incorporation was enhanced in FFCM treated MLE-12 cells without an apparent increase in biosynthetic enzyme activity may suggest that the effect of FFCM was due to an increase in the rate of PC turnover. The effect of FFCM on the distribution of [^{14}C]choline in water-soluble metabolites was examined. To achieve isotopic equilibrium, cells were labelled with [^{14}C]choline for 36–48 h, chased in the presence of FFCM, and the choline-containing metabolites examined. The amount of ^{14}C found in phosphocholine was considerably lower in treated cells than untreated, while that found in glycerophosphocholine was essentially the same (Table 4). Very little ^{14}C radioactivity was present in choline in both treated and untreated cells, indicating that the increase in phosphocholine levels seen in FFCM-treated MLE-12 cells is not simply due to increased phosphatase-catalysed breakdown. The amount of [^{14}C]choline found in PC was slightly higher in treated cells, but this was not found to be statistically significant (Table 4). No radioactivity was observed in CDP-choline. Only small amounts of [^{14}C]choline, [^{14}C]phosphocholine and [^{14}C]glycerophosphocholine were found extracellularly, and there was no difference between treated and untreated cells (not shown). In addition, extracellular ^{14}C-labelled PC was identical for treated and untreated cells (results not shown).

Table 4 Effect of FFCM on long-term [^{14}C]choline incorporation into phosphatidylcholine and water-soluble metabolites in MLE-12 cells

Results are means ± S.D. for three separate experiments performed in triplicate; levels of CDP-choline were undetectable and are therefore not included.

	Incorporation (d.p.m./μg of protein)		
Treatment	Phosphatidylcholine	Phosphocholine	Glycerophosphocholine
Control	1707.1 ± 286.5	102.2 ± 15.2	204.6 ± 24.3
FFCM	1897.6 ± 217.2*	40.1 ± 11.8†	197.1 ± 46.8

* Not significantly different.
† Significantly different ($P < 0.01$); significance was determined by Student's t test.

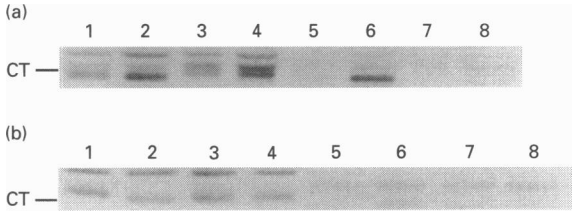

Figure 1 Western-blot analysis of the soluble and particulate forms of CT from MLE-12 and fetal rat type-II cells incubated in the presence or absence of FFCM or 0.5 mM oleic acid

MLE-12 type-II or fetal-rat type-II cells were treated with FFCM or 0.5 mM oleic acid, fractionated into soluble and particulate compartments, separated on an SDS/10%-PAGE gel, blotted and probed with the N-antibody as described in the Experimental section. (**a**) MLE-12 cells; (**b**) fetal type-II cells. Lane 1, FFCM-treated, soluble; lane 2, oleate-treated, soluble; lane 3, FFCM control, soluble; lane 4, oleate control, soluble; lane 5, FFCM-treated, particulate; lane 6, oleate-treated, particulate; lane 7, FFCM control, particulate; lane 8, oleate control, particulate. The upper band in the soluble fractions is non-specific and may represent the same 50 kDa protein recognized by this antibody in HeLa cell extracts [12].

FFCM treatment affects the phosphorylation state of CT in MLE-12 cells

The results obtained in Table 4 suggested that the increase in [^{14}C]choline incorporation into PC in FFCM-treated MLE-12 cells could be due to an alteration in CT activity. Cytidylyltransferase is a phosphoprotein, and the degree of phosphorylation has been correlated to the activity and intracellular localization of the enzyme [11,12]. Alterations in the phosphorylation state of CT in response to various treatments can be monitored by SDS/PAGE as changes in the electrophoretic mobility of the enzyme [11,12]. MLE-12 cells were

treated with either FFCM or oleate and the soluble and particulate fractions separated by digitonin permeabilization and probed with an antibody generated against a peptide corresponding to the first 18 amino acids of rat liver CT. In the controls almost all the immunoreactive CT was found in the soluble fraction (Figure 1a). Moreover, the soluble CT appeared to be present in multiple phosphorylated forms as deduced by the multiplicity of bands (Figure 1a, lanes 1–4). Treatment of MLE-12 cells with FFCM (Figure 1a, lanes 1 and 3) or oleate (Figure 1a, lanes 2 and 4) resulted in the appearance of a predominant high-mobility form of CT which is not present in controls. Commensurate with oleate treatment was the appearance of highly mobile immunoreactive CT in the particulate fraction (Figure 1a, lane 6). A faint band corresponding to CT may be seen in the particulate fraction of FFCM-treated MLE-12 cells; however, as no enhancement in CT activity could be shown for this fraction (Table 2), it is difficult to ascribe this band to translocated CT (Figure 1a, lane 5). Similar results were obtained for fetal-rat lung type-II cells (Figure 1b).

To analyse possible changes in the phosphorylation state of CT more directly, we generated phosphopeptide maps from ^{32}P-labelled CT immunoprecipitated from MLE-12 and fetal rat type-II cells which had been incubated in the presence and absence of FFCM or oleate. Although the results in Figure 1 suggested that soluble CT was being dephosphorylated in response to FFCM or oleate, soluble CT was still highly phosphorylated. The amounts of ^{32}P immunoprecipitable radioactivity associated with soluble CT were similar in both treated and untreated cells and two-dimensional phosphopeptide maps revealed little difference between FFCM-treated and control MLE-12 cells (Figure 2). Indeed, the relative intensities of CT phosphopeptides 1 and 10 appeared to increase in FFCM-treated MLE-12 cells (Figures 2a and 2b). Oleate stimulation of MLE-12 cells resulted in a reduction in the intensity of phosphopeptide 4, but an increase in the intensity of phosphopeptides 5 and 6 (Figures 2c and 2d). FFCM treatment of fetal type-II cells failed to produce any noticeable change in the phosphopeptide pattern of CT other than the appearance of phosphopeptide 6 and a slight increase in the intensity of phosphopeptide 5 (results not shown). No ^{32}P-labelled CT was observed in the particulate fraction.

DISCUSSION

In the present study we show that choline incorporation into PC is increased in murine MLE-12 type-II cells treated with cortisol-

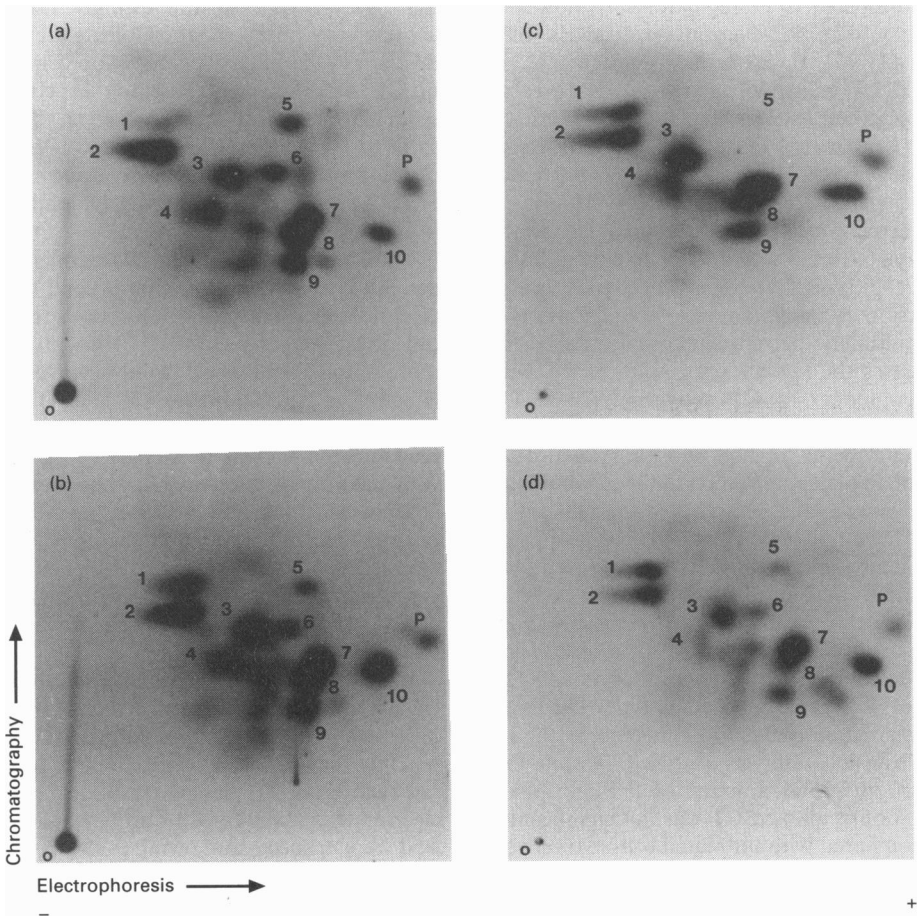

Figure 2 Phosphopeptide mapping of ^{32}P-labelled CT from MLE-12 cells incubated in the presence or absence of FFCM or oleate

Cytidylyltransferase was immunoprecipitated from the soluble fraction of MLE-12 cells incubated in the presence (**a**) and absence (**b**) of FFCM or the presence (**c**) and absence (**d**) of 0.5 mM oleic acid, separated on a 10% gel and blotted on to Immobilon. The ^{32}P-labelled CT band was excised, digested with trypsin as described in the Experimental section, and two-dimensional phosphopeptide maps were generated to resolve the various phosphopeptides. Phosphopeptides were numbered arbitrarily from left to right. Only distinct, reproducible spots were considered in the numbering scheme.

conditioned medium obtained from 20-day-old fetal fibroblasts. Although an increase in the specific activity of CT was not observed in *in vitro* assays, two lines of evidence are suggestive of a possible role of CT in the observed phenonenon: (1) the phosphocholine level was considerably lower in FFCM-treated as compared with untreated cells and (2) there was an apparent change in the mobility of CT on SDS/PAGE upon treatment with FFCM. Insofar as the activities of choline kinase and choline phosphotransferase remain unchanged in treated as compared with untreated cells, the decrease in phosphocholine levels in treated cells appears significant in light of the generally recognized role of CT as the rate-limiting enzyme in the *de novo* pathway of PC synthesis [1–7]. In contrast with MLE-12 cells, fetal type-II cells treated with FFCM showed a 2–3-fold stimulation in the specific activity of soluble CT, thus confirming earlier observations [24]. The enhanced activity seemed to be related to an activation process, since similar activities were observed when CT was assayed in the presence of PC/oleic acid vesicles.

The two other enzymes in the CDP-choline pathway for PC biosynthesis are choline kinase and choline phosphotransferase. Under certain conditions choline kinase demonstrates regulatory properties, where increases in the activity of this enzyme mirrors increases in PC synthesis [32–34]. Choline phosphotransferase, which catalyses the transfer of choline from CDP-choline to 1,2-diacylglycerol, is not considered to be regulatory [7]; however, some evidence indicates that 1,2-diacylglycerol levels may influence PC synthesis [35–37]. It has been suggested that, in phorbol-ester-treated HeLa cells [36] and fatty-acid-treated hepatocytes [37], diacylglycerol levels regulate CT activity. Treatment of MLE-12 cells with FFCM did not result in an increase in cellular diacylglycerol levels relative to untreated controls.

In many cell types, activation of CT is accompanied by the translocation of the enzyme from a soluble state to a membrane-bound state [7]. In adult rat type-II and HepG2 cells, CT is also activated by the conversion of a delipidated soluble L-form to a high-molecular-mass multimeric lipoprotein complex called the H-form [8–10]. In all cases the known association of CT with lipid is an integral component of CT activation [23,38–43]. Membrane-bound CT is largely dephosphorylated, whereas the soluble form of the enzyme is highly phosphorylated [12], suggesting a role for protein phosphorylation in the regulation of CT. The nature of the kinases and phosphatases which act in concert to regulate CT have not been identified, but it appears that cyclic AMP-dependent protein kinase is not involved [44]. Changes in phosphorylation have not been reported to occur in the conversion of the L-form into the H-form; however, as H-form is analogous to the membrane-associated form of CT, one might assume that H-form is largely dephosphorylated. The threefold stimulation of CT activity in fetal type-II cells in response to FFCM was apparently due to an increase in the H-form, since no enzyme translocation was observed. Despite this increase in the H-form, the total amount of [^{32}P]phosphate incorporated into immunoprecipitated CT was not significantly different in treated as compared with untreated cells. Moreover, phosphopeptide maps of CT immunoprecipitated from treated and untreated cells did not reveal any major differences; indeed several phosphopeptides appeared to increase in intensity in the treated cells as opposed to untreated. It is possible that the observed activation of CT was due to a small amount of enzyme being converted into the H-form in response to treatment with FFCM and, as both the L-form and the H-form are soluble, any direct effect on the phosphorylation of CT by FFCM would be masked by the preponderance of the L-form. Although multiple CT bands were observed on the Western blots (Figure 1), it was difficult to delineate multiple [^{32}P]CT bands owing to the diffuse nature of the autoradiographic signal of the immunoprecipitates. Thus the small amount of CT converted into the H-form may be masked by a preponderance of ^{32}P-labelled L-form. The minor differences in the phosphopeptide patterns alluded to above may also be due to incomplete digestion by trypsin. The phosphopeptide maps generated with rat fetal type-II and MLE-12 cells are very similar to those previously published for mutant CHO cells expressing rat liver CT [14], indicating that the same sites are phosphorylated. Many of the phosphorylation sites in CT are on serine residues adjacent to tryptic cleavage sites, and it has been reported that trypsin cleaves at these sites only inefficiently [14].

Despite these caveats it is considered likely that the changes in electrophoretic mobility of the soluble forms of CT observed in FFCM-treated MLE-12 and fetal type-II cells are related to alterations in the phosphorylation state of CT [11,12]. Such alterations must be subtle, since no significant change in the phosphorylation pattern was observed. It is apparent from the more profound effects of oleate on CT activity that FFCM treatment results in the activation of only a small pool of the total CT available in type-II cells. CT activation through oleate treatment is mediated by the translocation of enzyme from the soluble fraction to the particulate fraction in both fetal and MLE-12 type-II cells. Interestingly, no decrease in soluble CT activity was observed upon oleate treatment. In addition, no decrease in the amount of immunoprecipitatable ^{32}P radioactivity in CT was noted in labelling experiments involving oleate stimulation, a surprising result given that the membrane-bound form of CT was dephosphorylated. Oleate-mediated activation of CT has been well documented in many cell types, including HeLa cells [12,31], hepatocytes [45], adult primary type-II cells [46,47] and HepG2 cells [10].

The observation that FFCM treatment results in increased CT activity in the soluble fraction of fetal type-II cells, while oleate treatment elevated membrane-associated CT activity with both cell types, suggests a difference in activation mechanisms. The more mobile, soluble CT band observed with FFCM treatment (Figure 1a, lane 1; Figure 1b, lane 1) probably represents the partially dephosphorylated CT H-form, and the decrease in phosphocholine radioactivity after FFCM treatment is consistent with enhanced CT activity [7]. Thus we are at a loss to explain the absence of increased CT activity in MLE-12 cell extracts. One possible explanation is that the activation process requires an interaction with cellular lipids which is disrupted during homogenization or permeablization with digitonin. Activation of CT in fetal type-II cells must therefore be more resistant to cell fractionation.

It must be stressed that the enhanced mobility observed in Figure 1 was not correlated with CT activation, which was only observed in FFCM-treated fetal type-II cells. Therefore it remains possible that the electrophoretic shift in soluble CT mobility is not related to CT phosphorylation. Attempts to attenuate the electrophoretic shift of CT during FFCM challenge with okadaic acid were unsuccessful, owing to the extreme cytotoxic effects of the molecule.

The stimulation of choline incorporation into PC in FFCM-treated MLE-12 cells shows some similarity with phorbol-ester treatment of HeLa cells [28,36]. Watkins and Kent [28] observed a 5-fold increase in [^{14}C]choline incorporation into PC in HeLa cells after a 1 h treatment with phorbol ester. No change in intracellular localization, specific activity or phosphorylation state as measured by autoradiography of ^{32}P-labelled CT was detected. However, a small but significant decrease in the level of

[^{14}C]phosphocholine was observed in phorbol-ester-treated cells relative to controls, leading the authors to conclude that CT was activated to some extent in phorbol-ester-treated cells. The study of Watkins and Kent [28] is consistent with our observations with FFCM-treated MLE-12 cells in that phorbol ester enhanced choline incorporation into PC and depressed phosphocholine levels without producing a detectable increase in CT activity. More recently, Utal et al. [36] reported a twofold stimulation in [^{14}C]choline incorporation into PC and a threefold enhancement of membrane-associated CT activity. Despite this apparent translocation, greater than 90% of the total CT remained soluble after phorbol ester treatment [36]. In contrast, oleate stimulation of HeLa cells results in a tenfold stimulation in choline incorporation with a 98% translocation of soluble CT to the particulate fraction [12].

Throughout the course of the present study we were able to assay significant amounts of CT activity in membrane fractions, but only in oleate-stimulated cells was this membrane-associated CT immunologically detectable. Studies by Fraslon and Batenburg [20] and Hogan et al. [48] have shown that both the amount of CT mRNA and the amount of membrane-associated CT increases in fetal-rat type-II cells during late gestation. It is noteworthy that a recent study by Mallampalli and Hunninghake [16] reported a decrease in immunodetectable CT during the perinatal period up to adulthood in whole rat lungs . The basis for the observations in [16] and the apparent discrepancies with the study of Hogan et al. [48] indicate the complexity of the system. The difference may be related to the fact that different antibodies were used in the two studies and that the former [16] study used whole lung, while that of Hogan et al. [48] used isolated type-II pneumocytes.

The suggestion that fetal type-II cells contain a large immunodetectable pool of mostly inactive CT while adult cells possess a small pool of highly active enzyme is intriguing. The manner in which adult cells maintain a small pool of active enzyme is unknown. In the present study we observed that FFCM enhances choline incorporation into PC in an adult type-II cell line. While some of our results suggest that CT is activated in response to FFCM in MLE-12 cells, we were unable to demonstrate this activation in cell extracts. The study suggests that adult type-II cells may remain responsive to factors produced by surrounding fibroblasts (and perhaps other cells such as macrophages) and that 'cross-talk' between different cells in the lung could play a role in the production of surfactant lipid.

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