Oxysterols Inhibit Phosphatidylcholine Synthesis via ERK Docking and Phosphorylation of CTP:Phosphocholine Cytidylyltransferase

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The abbreviations used are: PtdCho, phosphatidylcholine; LXR, liver X receptor; CCTα, CTP:phosphocholine cytidylyltransferase; 22HC, 22-hydroxycholesterol; RA, retinoic acid; RXR, RA receptor; FBS, fetal bovine serum; CK, choline kinase; CPT, cholinephosphotransferase; CDP-choline, cytidine diphosphocholine; DSPtdCho, disaturated phosphatidylcholine; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; cdc2 kinase, cell division control 2 kinase; Elk, Ets-like transcription factor; ATF-2, activating transcription factor 2; JNK, c-Jun N-terminal kinase; MLE-12, murine lung epithelia.

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Running title: Oxysterols regulate phosphatidylcholine synthesis.
SUMMARY

Surfactant deficiency contributes to acute lung injury, and may result from the elaboration of bioactive lipids such as oxysterols. We observed that the oxysterol, 22-hydroxycholesterol (22HC), in combination with its obligate partner, 9 cis-retinoic acid (RA), decreased surfactant phosphatidylcholine (PtdCho) synthesis by increasing phosphorylation of the regulatory enzyme, CTP: phosphocholine cytidylyltransferase (CCTα). CCTα phosphorylation decreases its activity. 22HC/RA inhibition of PtdCho synthesis was blocked using PD98059 or a dominant negative ERK (p42 kinase). Overexpression of constitutive active MEK1, the kinase upstream of p42 kinase, increased CCTα phosphorylation. Expression of CCTα truncated mutants lacking proline-directed sites within the carboxyl-terminal phosphorylation domain partially blocked oxysterol-mediated inhibition of PtdCho synthesis. Mutagenesis of Ser315 within CCTα was both required and sufficient to confer significant resistance to 22HC/RA inhibition of PtdCho synthesis. A novel putative ERK docking domain NH2-terminal to this phosphoacceptor site was mapped within the CCTα membrane-binding domain (residues 287-300). The results are the first demonstration of a physiologically relevant phosphorylation site and docking domain within CCTα that serves as a target for ERK kinases resulting in inhibition of surfactant synthesis.

Key Words: phosphatidylcholine, oxysterol, cytidylyltransferase, ERK
INTRODUCTION

Pulmonary surfactant is an essential mixture containing primarily disaturated phosphatidylcholine (DSPtdCho) and key proteins that provide stability to alveoli by lowering surface-tension. Surfactant is synthesized within lung type II alveolar epithelia and packaged within lamellar bodies, an intracellular storage form of surfactant, prior to secretion into the alveolar lumen. Deficiency of surfactant DSPtdCho contributes to the pathogenesis of acute lung injury, a disorder characterized by leakage of serum proteins into the alveolus resulting in severe respiratory compromise (1). Recent studies in our laboratory and by others suggest that cholesterol-enriched low-density lipoproteins are important components of serum that accumulate in the alveolus (2, 3). Once becoming oxidized, these modified lipoproteins have the ability to impair DSPtdCho synthesis within alveolar type II cells (2, 3).

Oxysterols are oxygenated derivatives of cholesterol and important constituents of oxidized low-density lipoproteins that are detected in association with surfactant during acute lung injury (4). Oxysterols are present in human lung and exert potent biological effects by controlling expression of diverse genes via binding to liver X receptors (LXR), members of the nuclear receptor superfamily (5, 6). As a prerequisite for nuclear transduction of oxysterol signaling, LXR receptors form heterodimers with the obligate partner, 9-cis retinoic acid receptor (RXR), that binds to LXR/RXR response elements within target genes thereby regulating transcription (5). However, not all regulation by oxysterols and 9-cis retinoic acid are transcriptional, as rapid activation of target proteins (e.g. kinases) has been demonstrated within minutes by these agonists via nongenomic
mechanisms (7, 8). One recognized target for oxysterols is the ATP-binding cassette transporter (ABC transporter) family of transmembrane proteins. The ATP-binding cassette transporter 1 (ABCA1) gene is involved in the efflux of cellular phospholipid and cholesterol from the plasma membrane; our recent studies show that oxysterols deplete surfactant PtdCho in lung epithelia, in part, by ABCA1-driven basolateral phospholipid export (9). Effects of oxysterols on PtdCho synthesis, however, have not been investigated.

The synthesis of surfactant PtdCho is tightly regulated by the rate-limiting enzyme CTP:phosphocholine cytidylyltransferase ([CCT] EC 2.7.7.15), which catalyzes conversion of cholinephosphate to CDP-choline utilizing CTP (10). CCT has been purified to homogeneity and cDNAs from several species have been identified and cloned (10). The primary structure of CCT (termed CCTα) in mammals consists of four functional domains, including an amino terminal nuclear localization signal (NLS), a catalytic core, an α-helical lipid binding domain, and a carboxyl-terminal phosphorylation domain (10). CCTα is localized to the nuclear membrane, but in alveolar epithelia it is cytoplasmic, largely associated with plasma and endoplasmic reticulum membranes (11). In addition to CCTα, two additional splice variants encoded by a different gene (CCTβ1 and CCTβ2) have been identified in the human (12). These isoforms differ in extent of their carboxyl-terminal phosphorylation but share some similar regulatory features.

CCT activity in cells is regulated extensively by exogenous lipids as both activating and inhibitory lipids have been identified (10). CCTα is also a phosphoenzyme although the precise physiological role of CCTα phosphorylation in vivo is unknown. The degree by which lipids alter CCT activity is influenced by its phosphorylation status and CCTα
phosphorylation reduces its activity in vitro (13, 14). CCTα phosphorylation is restricted to sixteen serines located within the carboxyl-terminus. Seven of these Ser are followed by Pro, suggestive of a role for proline-directed kinases, such as p34\textsuperscript{cdc2} or mitogen-activated protein (MAP) kinase, in CCTα regulation (15). Accordingly, CCTα is an in vitro substrate for MAP kinase and ras-transfected cells exhibit increased CCTα phosphorylation in vivo (16-18). However, mutagenesis of consensus proline-directed phosphorylation sites or expression of a truncated CCTα mutant lacking the phosphorylation domain results in an enzyme that is phenotypically similar to wild-type CCTα, thus calling into question the physiologic role of proline-directed kinases and their link with PtdCho synthesis (19, 20).

There is mounting data suggesting that proline-directed kinases target not only specific motifs (Ser/Thr-Pro) within substrates but efficiency and specificity of phosphorylation of the acceptor site is regulated by binding of MAP kinases to scaffolding complexes and distinct docking sites (21). Docking domains are present within all members of the MAP kinase cascade including c-Jun N-terminal kinase (JNK), p38 kinase, and extracellular signal-regulated kinase (ERK), such as p42/p44 kinases. Many MAP kinases use a negatively charged conserved docking domain that interacts with motifs within target substrates that contain basic or hydrophobic residues; these sequences are usually located upstream of the kinase phosphorylation site (22).

In this study we investigated the pathophysiologic role of CCTα phosphorylation within the context of oxysterol exposure in alveolar epithelia. We observed that LXR/RXR agonists greatly diminish cellular PtdCho levels by inhibiting its biosynthesis via site-specific phosphorylation of CCTα catalyzed by ERK. In the process of investigating MAP kinase
phosphorylation of CCTα, we identified a distinct docking domain for ERK residing within the membrane-binding domain of the enzyme.
EXPERIMENTAL PROCEDURES

Materials - 22-(R) hydroxycholesterol (22HC) was purchased from Steraloids, (Newport, RI). 9-cis-retinoic acid (RA) were obtained from Sigma Chemicals (St. Louis, MO). The murine lung epithelial (MLE-12) cell line was obtained from American Type Culture Collection (Manassas, VA). Hite’s medium was from the University of Iowa Tissue Culture and Hybridoma Facility (Iowa City, IA). Radiochemicals were purchased from DuPont New England Nuclear Chemicals (Boston, MA). Immunoblotting membranes were obtained from Millipore (Bedford, MA). Anti-CCTα rabbit polyclonal antiserum to synthetic peptide was generated by Covance Research Products Inc. (Richmond, CA), and the phosphoserine antibody was from Zymed Laboratories (San Francisco, CA). The recombinant p42 kinase and the p42/44 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to PKC and the Rabbit IgG TrueBlot Set were from e-Bioscience (San Diego, CA) and the cdc2 kinase was purchased from Cell Signaling Technology (Beverly, MA). The p44 kinase, GST-agarose, and p38 NH2-terminal GST tagged protein were from Upstate (Lake Placid, N.Y.). The ECL Plus Western blotting detection system was purchased from Amersham Biosciences, (Piscatawy, NJ). The dominant negative p42 kinase and constitutive active MEK1 plasmids were a kind gift from Dr. Roger Davis (U of Mass Medical Center, (23)) and the phosphatase inhibitor cocktail and PD98059 was obtained from Calbiochem (La Jolla, CA). The CCTα314 phosphoterminal deletion mutant (pCMV5-CCTα314) was a kind gift from Dr. Claudia Kent, (University of Michigan, (19)). The TnT reticulocyte assay system were from Promega (Madison, WI). The ERK-GST agarose was obtained from StressGen (Victoria, BC,
Canada). The Advantage cDNA polymerase, and *In Vivo* Transactivator Kinase Assay (MAPK) kits containing the pTRE-luc and pTET-Elk plasmids were obtained from Clontech, (Palo Alto, CA). The Geneclean2 Kit was obtained from Bio101 (Carlsbad, CA). The Quikchange Site-Directed Mutagenesis Kit was from Stratagene (La Jolla, CA). The pCR-TOPO4 plasmids and *E. coli* Top10 competent cells were obtained from Invitrogen (Carlsbad, CA), and FuGENE6 transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN). All DNA sequencing was performed by the University of Iowa DNA core facility.

**Cell Culture**- MLE cells were maintained in Hite’s medium with 2% fetal bovine serum (FBS) at 37°C in atmosphere containing 5% CO₂. After reaching 70% confluence, the cells were harvested using 0.25% trypsin with 0.1% EDTA and plated onto either 12-well or 60 mm tissue culture dishes. After incubation overnight, the medium was replaced with serum-free Hite’s medium alone (control medium) or in combination with various amounts of 22HC (5-30 µM) with or without 9-cis RA (1 µM) for up to 48 hrs. In some studies, cells were exposed to PD98059 (10 µM) for 1 hr prior to addition of 22HC and 9-cis RA. Cells lysates were prepared by brief sonication in Buffer A (150 mM NaCl, 50 mM Tris, 1.0 mM EDTA, 2 mM DTT, 0.025% sodium azide, 1 mM PMSF, pH 7.4) at 4°C.

*Phosphatidylcholine (PtdCho) and Disaturated PtdCho (DSPtdCho) Analysis*- Cells cultured in Hite’s medium alone, or medium containing agonists for up to 48 hrs were pulsed with 2 µCi [*methyl³H]-choline chloride during the final 2-4 hrs of incubation. Total
cellular lipids were extracted from equal amounts of cellular protein using the method of Bligh-Dyer (24). Lipids were resolved using thin layer chromatography and PtdCho or DSPtdCho quantitated using scintillation counting as described (25). PtdCho mass was assayed by measuring lipid phosphorus content (26).

*Enzyme Activities-* CCT activity was performed as described without inclusion of lipid activator in the reaction mixture (26).

*Immunoblot Analysis* - Immunoblotting was performed as described (2). In some studies, CCTα was immunoprecipitated from equal amounts of cell lysate prior to SDS-PAGE (26). Immunoreactive proteins were probed for using the ECL Western blotting detection system. The dilution factor for anti-CCTα, phosphorylated p42/44 MAP kinase, and cdc2 kinase antibodies was 1:1000 and for PKC 1:100. To control for loading, blots were also probed with rabbit polyclonal antibody to β-actin or total p42/44 kinase at 1:1000 dilution. In separate studies, lysates from cells transiently transfected with pCMV5-CCTα-histidine tag plasmids were harvested using the M-PER mammalian protein extraction reagent. CCTα- histidine tag proteins were purified using the B-PER 6Xhis spin purification kit following the manufacturer’s instructions (Pierce, Rockford, IL). Levels of phosphoserine, phosphorylated p42/44 MAP kinase, PKC, cdc2 kinase, or CCTα were then determined by probing membranes with antibody at 1:1000 dilution and for PKC at 1:100.
**ERK Kinase Assay** - To determine assess ERK kinase activity *in vivo*, cells were co-transfected with pTET-Elk and pTRE-luc plasmids per the manufacturer’s instructions in the presence or absence of a constitutive active MEK1 plasmid as previously shown (27, 28). The pTET-Elk plasmid expresses Elk-1, an ERK-dependent transactivator, that if phosphorylated, activates the luciferase reporter gene contained in pTRE-luc. After 24 hrs, cells were stimulated with or without 22HC (25 µM) and 9-cis RA(1 µM) for up to 24 hrs and luciferase activity, which was normalized to protein, was measured (27).

**Immunoprecipitation of CCTα and p42/44 kinase** - Immunoprecipitation was performed with the Rabbit IgG TrueBlot Set according to the manufacturer’s instructions. Cellular lysates (250 µl) were first precleared with 25 µl of anti-rabbit IgG beads for 1 hour on ice. Subsequently, 2.5 µg of primary antibody (to CCTα, p42/44 kinase, normal rabbit IgG, or preimmune serum) was added to precleared lysates and incubated on ice for an additional hour. Samples were then incubated with 25 µl of anti-rabbit IgG beads overnight at 4 °C. The next day, beads were washed 3 times with TrueBlot lysis buffer and immunoprecipitates were released from beads with 5 min boiling in Laemmli buffer prior to separation by SDS-PAGE. Immunoblotting was performed with a CCTα or p42/44 primary antibody as described above. A rabbit IgG TrueBlot at a 1:1,000 dilution was used as a secondary antibody.
Construction of CCTα Putative Docking-Domain Mutants – The cDNA encoding the open reading frame (ORF) (~1100 bp) for rat CCTα was generated using PCR as described previously and ligated into a pCMV5 expression vector (pCMV5-CCTα) (2). A C-terminal histidine-tagged full-length CCTα (pCMV5-CCTα–his) was generated by PCR using pCMV5-CCTα as a template as we described (2). A CCTα NH₂-terminal truncated deletion mutant (termed CCTN₄₀), lacking the first 40 amino acid residues was generated as follows: pCMV5-CCTα was used as a template for PCR using the sense primer: 5'-aga tct atg tta cgg cag cca gct cc –3' and anti-sense primer: 5'-tct aga tta gtc ctc ttc atc ctc gct g-3' in a two-step PCR amplification using Advantage cDNA polymerase under the following reaction conditions: 94°C 2 min; 94°C 30 sec, 68°C 3 min, 18 cycles. A ~1000 bp fragment was purified using the Geneclean2 kit, cloned into pCR4-TOPO, and plasmid minipreps verified by DNA sequencing.

A CCTα variant, termed CCT₁₅₈, where three residues L¹⁵⁸AEHR were mutated to A¹⁵⁸SEHA was generated using the QuikChange Site-Directed Mutagenesis kit. The oligonucleotides used were: 5'-cgc ccc cgg cgt cga gct cc ttg att tct gcg –3'(sense) and 5'-cga aat caa tcg cgt gct cgc tcg cga act cgg gcg –3'(antisense), with pCMV5-CCTα plasmid DNA used as a template (2). PCR conditions were as follows: 95°C for 30 sec, 18 cycles at 95°C for 30 sec, 55°C for 60 sec, and 68°C for 6 minutes.

A CCTα internal deletion mutant (CCT₂₈₉) lacking the membrane binding domain (residues 240-290) has been described previously (2). A related CCTα variant, termed CCTm₂₈₉, was generated as following: two residues K¹³RRK were mutated to K¹³WWK by site-directed mutagenesis with oligonucleotides: 5'-gtc aaa gtc aat tca agg aag tgg tgg
aaa gag gta cct ggc cct –3’ (sense) and 5’-agg gcc agg tac ctc ttt cca cca ctt cct tga att
gac ttt agc –3’ (antisense), using pCMV5-CCT\textsubscript{289} as a template. PCR conditions were as follows: 95°C for 30 sec, 18 cycles at 95°C for 30 sec, 55°C for 60 sec, and 68°C for 5 minutes.

A third CCT\textalpha{} internal deletion mutant, CCT\textsubscript{d21}, (devoid of residues 231-251) was constructed using the same PCR protocol for generation of full-length CCT\textalpha{}, using pCMV5-CCT\textalpha{} as a template, and the sense primer: 5’-gga gct caa agt gaa aga tgt gga gg
3’; anti-sense primer: 5’- tcc ccg ggt cta gat tag tcc tct tca tcc tgc c-3’ resulting in a 351 bp PCR product that was cloned into pCR4-TOPO for transformation into Escherichia coli TOP10 competent cells. Plasmids were double digested using Sac I and Xba I, while pCMV5-CCT\textalpha{} was digested using Bgl II and Sac I. The 351 bp and 687 bp fragments were purified and then ligated into pCMV5 (previously digested by Bgl II and Xba I) using T4 ligase at 15 °C overnight, and then transferred into TOP10 competent cells. The 1038 bp insert was directionally cloned into pCMV5 as confirmed by partial DNA sequencing and restriction enzyme digestion.

A series of carboxyl-terminal truncated CCT\textalpha{} mutants were generated as follows: pCMV5-CCT\textalpha{} was used as a template for PCR using the sense primer, 5’-gga tcc ata tgg atg cac aga gtt cag c -3’ and different anti-sense primers: 5’-acg cgt tta tga ctt ttc ctc cac atc -3’ for CCT\textsubscript{260}, 5’-acg cgt tta ctc ctc cca ctt ctg gat gag-3’ for CCT\textsubscript{280}, 5’-acg cgt tta aat gaa ctc tgc gga ctt c -3’ for CCT\textsubscript{286}, and 5’-acg cgt tta ctt cag cgc tcc ttc tgg acc-3’ for CCT\textsubscript{300}, respectively. The PCR products were purified using the Geneclean2 kit, cloned into pCR4-TOPO, and plasmids minipreps verified by DNA sequencing.
Construction of CCTα Phosphorylation Mutants – A truncated deletion mutant (termed CCTα328), lacking the distal portion of the phosphorylation domain of CCTα (residues 329-367) was generated as follows: pCMV5-CCTα was used as a template for PCR using the sense primer: 5’-aga tct atg gat gca cag agt tca g -3’ and anti-sense primer, 5’-tct aga tta gcg ctc atg agt agg gct gc-3’ to generate a ~990 bp fragment. The PCR product was purified using the Geneclean2 kit, cloned into pCR4-TOPO, and plasmids minipreps verified by DNA sequencing. This clone was then digested by Bgl II and Xba I, purified by Geneclean2, and ligated into a pCMV5 expression vector previously digested with the same restriction enzymes.

A phosphorylation variant (termed CCTα328SA), similar to CCTα328 but where Ser321-Ser322-Ser323 were mutated to Ala321-Ala322-Ala323 was constructed using site-directed mutagenesis. The oligonucleotides used were: 5’-ccc aag cag agt ccc gca gca gca cct act cat gag cgc-3’ and 5’-gcg ctc atg agt agg tgc tgc tgc ggg act ctg ctt ggg-3’, with CCTα328 plasmid DNA used as a template. PCR conditions were as follows: 95°C for 30 sec, 18 cycles at 95°C for 30 sec, 55°C for 60 sec, and 68°C for 6 minutes.

Another truncated phosphorylation variant, termed CCTα328SAquad, where Ser315-Ser321-Ser322-Ser323 were mutated to Ala was generated similarily using the oligonucleotides: 5’-gat gct gca ggc cat cgc tcc cca gca gag tcc-3’ and 5’-ggg tgc tgc ttg gga gcg atg gcc tgc agc atc-3’, with CCTα328SA plasmid as a DNA template. PCR conditions were identical to conditions used for construction of CCTα328SA.

A full length mutant, termed CCTα315, was constructed where an Ala was substituted only at Ser315 using oligonucleotides 5’-ccc aag cag agt ccc gca gca gca cct act cat gag
cgc -3' and 5'-gcg ctc atg agt agg tgc tgc ggg act ctg ctt ggg -3', with pCMV5-CCTα plasmid DNA used as a template (2). PCR conditions were identical to those used for construction of CCTα_{328SAquad}. A second full-length CCTα phosphorylation mutant, termed CCTα_{quad}, was generated where Ser^{315}-Ser^{321}-Ser^{322}-Ser^{323} were changed to Ala as follows: pCMV5-CCTα_{328SAquad} and pCMV5-CCTα were digested by Bgl II/Xba I, generating ~1 kb and ~1.1 kb inserts, respectfully. These fragments were purified and digested using BspHI. A ~970 bp fragment from CCTα_{328SAquad} and a ~200 bp fragment from CCTα were purified and ligated into pCMV5 that was predigested with Bgl II/Xba I by T4 ligase, and transferred into Top10 cells. All CCTα constructs above were verified by DNA sequencing.

**Preparation of p38-GST Agarose** - p38–GST agarose was prepared from NH₂-terminal GST tagged full length p38 recombinant protein and GST protein–agarose. Proteins (5 µg of p38-GST and 25 µl of GST-agarose beads) were incubated in binding buffer containing 20 mM Tris-HCl, 150 mM NaCl and 5 mM Imidazole, pH 7.5 at 4 °C for 2 hours according to the manufacturer’s recommendations. The beads were subsequently washed 3 times with washing buffer (20 mM Tris-HCl, 150 mM NaCl and 50 mM Imidazole, pH 7.5) and suspended in PBS containing 50% glycerol and 0.05% sodium azide. Immunoblotting of preparations using p38 antibody confirmed that the kinase was effectively conjugated to GST-agarose.
In Vitro CCTα Transcription and Translation and ERK Pull-Down Assays- For in vitro synthesis of CCTα mutants, cDNA constructs cloned into pCR4-TOPO4 (2µg plasmid/reaction) were added directly to the rabbit reticulocyte lysate (TNT Coupled Reticulocyte Lysate System) and incubated with T7 RNA polymerase in a 50 µl reaction containing [35S]-methionine (20 µCi/reaction) for 90 minutes at 30 °C per the manufacturer’s instructions. Aliquots (10µl) of in vitro translation products were boiled for 5 min in loading buffer and stored at –80°C; alternatively, aliquots (40µl) were directly rotated with ERK-GST agarose, p38–GST agarose, or GST-agarose alone at 4 °C overnight. After 18 hrs, agarose beads were washed three times with ice-cold PBS and proteins were eluted from beads after resuspension in 4X Laemmli buffer and boiling for 5 min. Proteins eluted from GST and products of in vitro translation (10 µl aliquots) were resolved using SDS-PAGE, gels were dried, and autoradiography performed.

Transfectional Analysis- For expression of CCTα plasmids, MEK1, or the p42 kinase dominant negative plasmid, cells were transfected for 4 hrs (4 µg/60 mm dish) with test plasmids. Immediately after transfections, cells were transferred to serum-free medium containing agonists for various times before pulsing cells with [methyl 3H]-choline chloride as above for PtdCho determination.

CCTα Phosphorylation In Vivo and In Vitro- Purified his-tagged CCTα, CCTα315 and CCTαquad were dephosphorylated using alkaline phosphatase (5U) in 50 mM Tris-HCl, 100 µM EDTA (pH 8.5)(16). After 1 hr incubation at 30 °C, CCTα proteins were purified again
using the B-PER 6Xhis spin kit. *In vitro* phosphorylation was catalyzed using 10 µg p42 (or p44) kinase in MAP kinase buffer (25 mM MOPS, 6 mM MgCl₂, 25 mM β-glycerol phosphate, 100 µM ATP, 2 mM DTT) in the presence of phosphatase inhibitor cocktail (1:100) with 10 µCi of (γ-³²P) ATP (29). After 1 hr incubation at 30 °C, reactions were terminated with 4X protein loading buffer, and products heated at 5 min at 100 °C. Samples were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by CCTα immunoblotting and autoradiography.

For *in vivo* ³²P labeling, MLE cells were exposed to medium with or without agonists as described above. After 20 hrs, cells were washed twice with phosphate-free medium, and incubated in the same media with 750 µCi ³²P orthophosphate for 24 hr (13). Cells were then harvested in RIPA buffer (10 mM Na₂HPO₄, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 mM NaF, 10 mM Na₃VO₄, 1 mM PMSF, 20 µM leupeptin, 1% (v/v) Triton X-100) and precleared for 2 hr at 4°C using Sepharose CL-4B, protein A and preimmune rabbit serum. Cleared supernatants (500 µl) were incubated overnight at 4°C with 1 µg of rabbit anti-CCT antibody, which was previously bound to protein A. The following morning, the immunoprecipitates were washed with lysis buffer (50 mM HEPES, 150 mM NaCl, 0.5 mM EGTA, 50 mM NaF, 10 mM Na₃VO₄, 1 mM PMSF, 20 µM leupeptin, 1% (v/v) Triton X-100), and the pellets were placed in SDS protein sample buffer and heated to 95°C for 5 min. Soluble proteins were separated using 10% SDS-PAGE, the gels dried and subjected to autoradiography as above (2). Alternatively, to determine CCTα phosphorylation, cells were transfected with his-tagged CCTα constructs with or without
constitutive active MEK-1 plasmid as described above. After 24 hrs, cells were harvested, purified, and processed for phosphoserine immunoblotting.

Statistical Analysis- Statistical analysis was performed using the one-way ANOVA or a student’s t-test (30). Data are presented as means ± SEM.
RESULTS

Oxysterols/RA Inhibit Phosphatidylcholine (PtdCho) Synthesis - Incubation of MLE cells with 22HC and 9-cis RA significantly decreased incorporation of [methyl $^3$H]-choline into PtdCho and DSPtdCho by nearly 60% and 76%, respectively after 24 hrs of exposure (Fig. 1A and Fig. 1A [inset]). However, significant inhibitory effects on [methyl $^3$H]-choline incorporation into PtdCho were also achieved with 22HC and 9-cis RA as early as 6 hrs after exposure with maximal effects seen by 48 hrs (Fig. 1A). Increasing concentrations of oxysterol in combination with fixed amounts of 9-cis RA (1 µM) produced a dose-dependent decrease in choline incorporation into PtdCho, (Fig. 1B). Comparative analysis with other oxysterols revealed that 25OH also produced similar inhibitory effects on PtdCho synthesis ([Fig. 1C]). LXR/RXR agonists decreased PtdCho synthesis in cells by significantly altering activities of enzymes within the CDP-choline pathway. There was no significant effect of 22HC and 9-cis RA on activity of choline kinase, the first committed enzyme of the pathway or of cholinephosphotransferase activity, the terminal enzyme involved in PtdCho synthesis (data not shown). However, after 6, 24, or 48 hrs of exposure, 22HC/9-cis RA inhibited CCT activity by approximately 30%-50% relative to control (Fig.1D). Only modest inhibitory effects of 22HC and 9-cis RA on CCT activity were observed when these agents were used individually (Fig. 1E-F). These effects of 22HC and 9-cis RA on PtdCho synthesis resulted in a net decrease in the mass of PtdCho by 30% within MLE cells (139 ± 16 nmol lipid phosphorus [control] vs 96 ± 5 nmol lipid phosphorus [22HC/9-cis RA], n=12, p<0.05).
Oxysterols/RA Increase CCTα Phosphorylation- To investigate mechanisms whereby 22HC and 9-cis RA reduced CCT activity, we assayed CCTα protein mass. Overall, 22HC and 9-cis RA did not alter enzymes levels relative to control (Fig. 2A). We next examined whether these agents might increase levels of CCTα phosphorylation thereby reducing enzyme activity and PtdCho synthesis. First, to examine phosphorylation status of endogenous CCTα, cells were stimulated with agonists, and labeled with 32P orthophosphate. Cells were then harvested, CCTα immunoprecipitated, and processed for autoradiography (Fig. 2B). 22HC and 9-cis RA clearly increased intensity of a major product at ~42 kDa compared to control (Fig. 2B). Next, cells were transfected with plasmids encoding his-tagged full-length CCTα and exposed to agonists, the transfectants then purified to remove endogenous CCTα, and lysates processed for immunoblotting using an anti-phosphoserine antibody (Fig. 2C). As a control, we also probed for total levels of overexpressed CCTα protein (Fig 2C, [lower panel]). After correction for quantity of total enzyme, 22HC and 9-cis RA increased levels of CCTα phosphorylation nearly 2-fold and 3-fold at 6 and 24 hrs of analysis, respectively (Fig. 2D). In separate experiments, endogenous CCTα was immunoprecipitated and probed with an anti-phosphoserine antibody after stimulation of cells with 22HC and RA (Fig. 2D, [inset]). Three bands exhibiting varying mobilities (~42 kDa) on autoradiograms were detected; these CCTα phosphorylation bands increased in intensity after 22HC and 9-cis RA treatment. Thus, 22HC and 9-cis RA increased phosphorylation of both endogenous and overexpressed CCTα.
Oxysterols/RA Increase CCTα Phosphorylation via a MAP kinase- We used two complementary approaches to assess p42/44 activation by 22HC and 9-cis RA. As shown by immunoblotting, 22HC and 9-cis RA exerted a biphasic temporal pattern of activation of p42/44 kinase in MLE cells as described elsewhere (31-34). First, the agonists increased levels of phosphorylated p42/44 MAP kinase in cells within 15 min and kinase activation was detected for up to 6 hrs (Fig. 3A). In addition, a late-phase pattern of p42/44 MAP kinase activation was seen from 18-21 hrs after the stimulus with activity waning by 24 hrs (Fig. 3A). As a second method, we measured p42/44 kinase activity in vivo using a luciferase reporter plasmid, driven by Elk (Fig. 3B). In this assay, when Elk is phosphorylated by p42/44 kinase via constitutive active MEK1, phosphorylated Elk binds to a tetracycline-response element within the luciferase promoter to induce reporter expression. Cells were transiently co-transfected with the luciferase reporter plasmid, the Elk plasmid, and either an empty vector or the constitutive active MEK1. Cells exposed to 22HC and 9-cis RA exhibited a ~2-fold increase in reporter gene expression compared to control. In addition, expression of constitutively active MEK1 also produced a ~30% increase in p42/44 kinase activation in response to agonist stimulation (p<0.001, n=4, Fig. 3B). Additional studies examining the kinetics of p42/44 kinase activation using this system demonstrated that luciferase activity was increased by 22HC and 9-cis RA uniformly from 6 to 24 hrs (Fig. 3C). These latter studies strongly link 22HC and 9-cis RA inhibition of CCT activity with p42/44 kinase activation (Fig. 3A) as the luciferase reporter method is a functional readout that assesses activation of the transcription factor Elk, an important down-stream physiologic target for p42/44 kinase.
To determine if effects of 22HC and 9-cis RA on PtdCho synthesis are ERK-dependent, cells were pretreated with an inhibitor of MEK1, the upstream kinase of p42/44 kinase, or transfected with a dominant negative plasmid encoding p42 kinase. Both pretreatment with the MEK1 kinase inhibitor, PD98059, and expression of a dominant-negative p42 kinase significantly blocked 22HC and RA-induced inhibition of \([\text{methyl}^3\text{H}]\)-choline incorporation into PtdCho (Fig. 3D-E). In these studies, 22HC and 9-cis RA decreased CCT activity from 1.57±.04 nmol/min/mg protein [control] to 1.12 ±.01 nmol/min/mg protein [22HC and 9-cis RA]; PD98059 alone did not alter CCT activity (1.50±0.02 nmol/min/mg protein) but in combination with 22HC and 9-cis RA significantly blocked agonist-induced inhibition of enzyme activity (1.38±0.02 nmol/min/mg protein, p<0.05 22HC and 9-cis RA versus all other groups). Finally, to further investigate p42/44 kinase and CCT\(\alpha\) interaction, cells were transfected with pCMV5-CCT\(\alpha\)-his in the presence or absence of 22HC and 9-cis RA, CCT\(\alpha\) was purified using a his-column, and proteins resolved using SDS-PAGE prior to immunoblotting for phosphorylated or total p42/44 kinase (Fig. 3F). Results show that phosphorylated (active) p42/44 kinase was variably associated with CCT\(\alpha\) protein and that this association increased at 3, 6, 21, and 24 hrs after 22HC and 9-cis RA exposure (Fig. 3F). In contrast, nonspecific binding to the his-column was not observed either from endogenous CCT\(\alpha\) protein or p42/44 kinase in cell lysates (Fig. 3G, [lanes 2-3]) or after recombinant p44 kinase was applied to the column (data not shown). To further assess specificity of kinase binding to our his-columns, we first detected immunoreactive CCT\(\alpha\) protein, p42/p44 kinase, cdc2 kinase, and PKC in MLE cells following cellular transfection with pCMV5-CCT\(\alpha\)-his (Fig. 3H, [lysates]). Next, CCT\(\alpha\) was purified from cells
transfected with pCMV5-CCTα-his as above. Unlike p42/p44 kinase, we did not detect cdc2 kinase or PKC in association with CCTα protein in these studies (Fig. 3H, [fraction]). To determine if endogenous CCTα was associated with total p42/44, we performed immunoprecipitation studies after cells were cultured in the presence or absence of agonists (Fig. 3I). We first performed immunoprecipitation using anti-p42/44 kinase or normal rabbit IgG followed by immunoblotting with anti-CCTα antibodies. As shown in Fig. 3I (left panel), CCTα was detected with immunoprecipitated p42/44 kinase in the presence or absence of oxysterol and 9-cis RA exposure whereas this association was not detectable using control (rabbit IgG) antibody. Conversely, immunoprecipitation using CCTα antibodies followed by immunoblotting with an antibody to total p42/44 kinase also revealed that the kinase was detected in association with CCTα (Fig. 3I, right panel). As a negative control, this association was not observed using pre-immune serum. Thus, endogenous CCTα and total p42/p44 kinase are detected in close association as are overexpressed CCTα and the activated kinase.

Mapping of ERK Docking Sites within CCTα -MAP kinase phosphorylation of substrates involve docking interactions at motifs often very distant to phosphoacceptor sites that are essential for efficient phosphorylation. These motifs often harbor clusters of basic residues, FXFP motifs, or LXL motifs (35). Data base analysis of the CCTα sequence uncovered similar regions suggestive of potential p42/44 kinase docking sites. Thus, we initially used a candidate approach by deleting or mutating these core regions within CCTα (Fig. 4A). Four mutants, CCTN40, CCT158, CCTm289, and CCTd21 were constructed where candidate
sites were removed or mutated and tested for kinase binding after in vitro translation (Fig. 4, left panels) and analyzed using an ERK pull-down assay (Fig. 4, right panels). As shown in Figure 4, each of these mutants were sufficiently translated (Figs 4B, D, F). Deletion of 40 residues from the CCTα NH2-terminus (CCTN40), 21 residues (231-251) internally (CCTd21), or mutations within potential docking sites at CCTαm158 failed to alter CCTα-ERK binding (Fig. 4C, E). Moreover, a double mutant (CCTm289) lacking the entire membrane binding domain (residues 240-290) and harboring an NH2-terminal site mutation at CCTα R14R15 resulted in total loss of kinase-CCTα binding (Fig. 4C). Results using a similar mutant (CCT289) devoid of this membrane binding region (but lacking the NH2-terminal mutation) confirmed that an ERK docking domain likely resides between residues 252-314 of CCTα (Fig. 4E). CCT289 is an internal deletion mutant that contains the entire phosphorylation domain (residues 315-367 of CCTα) and absence of kinase binding with this construct indicates that this domain is not sufficient for ERK docking. Conversely, ERK pull-down analysis using a carboxyl-terminal truncated mutant lacking only the phosphorylation domain (CCT314) demonstrated ERK-CCTα binding similar to full-length CCTα indicating that the phosphorylation domain was not required for kinase docking (data not shown).

We next tested several CCTα mutants that were progressively truncated within the membrane binding domain (at the carboxyl-terminus) to map an ERK docking site (Fig. 4F-G). Although full-length CCTα and a CCT mutant harboring the first 300 residues effectively bound ERK, this binding was not observed in mutants that contained less than 287 amino acids. Thus, the results indicate that a putative ERK docking domain likely
resides with a span of 14 residues localized within the membrane binding domain (CCTα 287-300) (Fig. 4E,G). Finally, to assess binding specificity, newly synthesized full-length CCTα was incubated with either ERK GST agarose, p38 GST agarose, or GST-agarose beads alone. As shown in Fig. 4H, only ERK exhibited robust binding to CCTα.

Because the above data suggested that an ERK-CCTα docking domain is present within the membrane binding region, we further investigated if these in vitro results could be recapitulated in vivo by transfecting lung epithelia with histidine-tagged full-length CCTα or CCT289. CCT was then purified on a nickel column and samples processed for immunoblotting for phosphorylated p42/44 kinase (Fig. 4 I, [middle row]) and blots probed again with anti-CCTα antibody (Fig. 4 I, [lower row]). Further, to determine if p42/44 kinase docking was necessary for efficient CCTα phosphorylation, similar transfectants were purified from cells and reacted in vitro with recombinant p44 kinase in the presence of γ-32P ATP after dephosphorylation using alkaline phosphatase (Fig. 4 I, [upper row]). Compared to full-length CCTα, minimal γ-32P labeling, if any, was detected with the internal deletion mutant suggesting that CCT289 was less efficiently phosphorylated compared with wild-type CCTα (Fig. 4 I, upper row). As anticipated, significantly greater amounts of active kinase were bound to CCT in transfectants where full-length CCTα was expressed compared to cells transfected with the mutant lacking the membrane binding domain (Fig. 4 I, middle row). Thus, ERK docking with CCTα in the membrane binding domain is observed in vivo and required for optimal phosphorylation of the enzyme.
Altered Oxysterol/RA and MAP kinase Sensitivity by CCTα Phosphodomain Mutants- To investigate physiologically relevant ERK phosphorylation sites within CCTα in response to 22HC and 9-cis, we expressed other truncated enzyme mutants that lacked various portions of the carboxyl-terminal phosphorylation domain. In addition, we generated CCTα full-length or truncated mutants that harbored point mutations at specific proline-directed sites (Fig. 5A). Sensitivity of cells to inhibitory effects of 22HC and 9-cis RA on PtdCho synthesis varied after transfection with CCTα, CCTα314, CCTα328, CCTα328SA, and CCTα328SAquad plasmids. Cells transfected with full-length CCTα exhibited a 53% decrease in rates of [methyl 3H]-choline incorporation into PtdCho after 24 hrs of 22HC and 9-cis RA exposure whereas similar treatment did not significantly alter synthetic rates in cells transfected with CCTα314 (Fig. 5B). In these studies, 22HC and 9-cis RA treatment decreased CCT activity from 3.11±0.1 to 1.76±0.2 nmol/min/mg protein after transfection of full-length CCTα whereas the agonists did not significantly alter enzyme activity after expression of CCTα314 (1.92±0.3 [control] to 1.71±0.1 nmol/min/mg protein [22HC and 9-cis RA]). Thus, deletion of the carboxyl-terminal phosphorylation CCTα domain totally abolished inhibitory effects of 22HC and 9-cis RA on PtdCho synthesis. When cells were transfected with CCTα328, treatment with 22HC and 9-cis RA led to a 41% decrease in [methyl 3H]-choline incorporation into PtdCho compared to matched controls (CCTα328 transfected, but untreated cells). Thus, expression of a construct lacking eleven of sixteen consensus serine phosphorylation sites within CCTα still led to significant agonist-induced inhibition of PtdCho synthesis. These results suggest that additional sites contained within the proximal carboxyl-terminal domain were targets for p42/44 kinase phosphorylation of
CCTα in response to 22HC and 9-cis RA. To investigate this, cells were transfected with a CCTα328SA, where one of three proline-directed sites within CCTα328 (Ser323-Pro324) was modified to alanine and subsequently tested for 22HC and 9-cis RA sensitivity. As shown in Fig. 5B, cells transfected with CCTα328SA still displayed a 42% decrease in rates of 3H-choline incorporation into PtdCho after agonist stimulation compared to untreated control transfectants. We next expressed a CCTα mutant similar to CCTα328SA but also contained an alanine substitution at Ser315 (CCTα328SAquad). Cells transfected with CCTα328SAquad were resistant to treatment with 22HC and 9-cis RA as rates of PtdCho synthesis were comparable between treatment and control groups.

When [3H] choline incorporation into PtdCho was expressed as dpm/mg of protein for these constructs, 22HC and 9-cis RA decreased labeling from 39,828±5,780 dpm/mg protein (control) to 19,063±2258 dpm/mg protein (22HC/RA) in wild-type CCTα, whereas expression of the CCTα328SAquad construct resulted values that were comparable between the groups (34,102±4,665 dpm/mg protein (control) to 36,117±5455 dpm/mg protein (22HC/RA)). Thus, Ser315 within CCTα was a required phosphorylation site for 22HC and 9-cis RA to maximally inhibit PtdCho synthesis. To determine if mutagenesis of Ser315 was sufficient to confer oxysterol resistance, cells were transfected with a full-length construct where alanine was substituted only at the Ser315 position (CCTα315) (Fig. 5C). We also tested a full-length CCTα construct with identical mutations as in CCTα328SAquad. Indeed, rates of PtdCho synthesis were not significantly different between cells treated with 22HC and 9-cis RA versus control after transfection with either CCTα315 or CCTαquad. Further analysis revealed that 22HC and 9-cis RA treatment decreased CCT activity from 4.05±0.4
to 2.58±0.3 nmol/min/mg protein (p<0.01) after transfection of full-length CCT\(\alpha\) whereas the agonists did not significantly alter enzyme activity after expression of CCT\(\alpha_{315}\) (4.67±0.3 [control] to 4.47±0.3 nmol/min/mg protein [22HC and 9-cis RA]). Collectively, these results suggest that mutagenesis of Ser\(^{315}\) is both necessary and sufficient to significantly overcome inhibition of PtdCho synthesis after LXR/RXR agonist treatment (Fig. 5C).

We next investigated if CCT\(\alpha\) mutants exhibited different sensitivities to in vitro phosphorylation by ERK (Fig. 5D). Following cellular expression, CCT\(\alpha\) mutants were purified, dephosphorylated in vitro with alkaline phosphatase, and phosphorylated with p42 kinase in the presence of \(\gamma\)\(^{-32}\)P ATP. Wild-type CCT\(\alpha\) was substantially phosphorylated under these conditions whereas CCT\(\alpha_{315}\) or CCT\(\alpha_{quad}\) exhibited relatively low levels of \(\gamma\)\(^{-32}\)P labeling (Fig. 5D). To determine if p44/42 kinase differentially increased phosphorylation of CCT\(\alpha\) mutants in vivo, cells were co-transfected with his-tagged mutants and MEK1 plasmid, and CCT\(\alpha\) was purified and processed for phosphoserine analysis. Compared to control cells (untransfected with MEK1), cells co-transfected with MEK1 and full-length CCT\(\alpha\) plasmid displayed increased levels of enzyme phosphorylation (Fig. 5E, [lanes 1-2]). In contrast, levels of phosphorylation were unchanged in cells co-transfected with MEK1 and either CCT\(\alpha_{315}\) or CCT\(\alpha_{quad}\) compared to matched controls untransfected with the kinase plasmid. These results demonstrate that CCT\(\alpha_{315}\) is significantly less sensitive to MAP kinase mediated phosphorylation both in vivo and in vitro. Finally, cells co-transfected in this manner were also analyzed for [\(^3\)H] choline incorporation into PtdCho. While cells co-transfected with MEK1 and full-length CCT\(\alpha\)
plasmid exhibited a 38% reduction in PtdCho synthesis, MEK1 did not alter radiolabeling into the phospholipid in cells transfected with either CCT\(_{\alpha_{315}}\) or CCT\(_{\alpha_{quad}}\) (Fig. 5F).
DISCUSSION

These results show that oxysterols in combination with their obligate partner, 9-cis RA, significantly inhibit cellular PtdCho biosynthesis by ERK-dependent phosphorylation of the regulatory enzyme, CCTα. The ERK kinase binds CCTα via a unique docking region that was mapped to an amphipathic helical region previously shown to interact with membranes (36, 37). This docking region is upstream to a CCTα phosphoacceptor site that was functionally characterized in vivo; indeed, by expressing CCTα variants harboring truncated phosphodomain or mutations at proline-directed sites, we observed marked variations in sensitivity of cells to LXR/RXR agonists. Specifically, we show that Ser\textsuperscript{315} was a critical site that was targeted by p42/44 kinase as mutagenesis of this site was not only required but sufficient to substantially block inhibitory effects of 22HC/9-cis RA on PtdCho synthesis. The significance of these studies is that oxysterol activation of stress kinases could significantly reduce de novo synthesis of PtdCho and alveolar secretion of surfactant phospholipid thereby accelerating pulmonary atelectasis.

Reversible phosphorylation of CCTα was the most likely mechanism whereby LXR/RXR agonists inhibited CCT activity and PtdCho synthesis. CCTα phosphorylation is linked to membrane phospholipid synthesis with cell division, but to our knowledge these studies are the first showing physiologic relevance of specific phosphoacceptor sites (18, 38, 39). Our results mechanistically differ from inhibitory effects of 25HC on PtdCho synthesis that are dependently regulated with cholesterol biosynthesis (40, 41). In our studies, we used 22HC, a more potent LXR ligand that in combination with 9-cis-RA did not reduce CCTα protein expression. Rather, the magnitude by which 22HC and 9-cis RA
inhibited CCT\(\alpha\) activity inversely correlated with the degree of enzyme phosphorylation (Figs. 1-2). Our data also indicate that p42/44 MAP kinase pathway was the primary mediator for 22HC and 9-cis RA signaling as i) the kinetics of p42/44 kinase activation by 22HC and 9-cis RA coincided with CCT\(\alpha\) docking, phosphorylation, and inhibition of enzyme activity, ii) CCT\(\alpha\) and p42/44 kinase were physically associated in vivo, iii) p42/p44 MAP kinase phosphorylated CCT\(\alpha\), and iv) effects of these agents on PtdCho synthesis were reversed using PD98059 and a dominant-negative p42 kinase (Fig. 3). In particular, we observed a fairly tight correlation between p42/44 kinase activation and inhibition of CCT activity by 22HC and 9-cis RA. The biphasic activation of MAP kinases (up to 6 hrs and at 18-21 hrs) in response to 22HC and 9-cis RA was temporally linked to inhibition of PtdCho synthesis at 6 and 24 hrs by these agents (Figs. 1, 3A). Even though total cellular phosphorylated p42/44 kinase activity dissipated by 24 hrs, a distinct pool of active kinase was still bound to CCT\(\alpha\) after 22HC and 9-cis RA exposure (Fig. 3F); this together with the extended half-life of CCT\(\alpha\) protein (26) provides a plausible explanation for reduced levels of CCT activity and PtdCho synthesis observed at 24 hrs following agonist treatment (Fig. 1). Further, this pattern is highly consistent with the kinetics of ERK kinases as they regulate a variety of physiologic readouts such as platelet derived growth factor-induced mitogenesis (31), cellular propagation of influenza (32), leukotactin-1 control of cell cycle progression (33), and effects of phorbol-esters and nerve growth factors on cell survival (34, 42). Although the data favor 22HC and 9-cis RA activation of p42/44 kinase, concurrent activation of p38 kinase, JNK, or p34\(^{cdc2}\) kinase is also possible as these enzymes utilize similar minimal recognition motifs ((Ser/Thr) Pro) for substrate
phosphorylation (43, 44). On the other hand, because (Ser/Thr) Pro sequences are fairly ubiquitous within substrates, specificity and prevention of inappropriate cross-talk between related kinases is provided by assembly of scaffolding proteins and interaction with docking regions on target proteins (45).

We localized an ERK docking region to the distal membrane binding region of CCTα. This motif (GSFLEMGPEGALK) is rich in nonpolar residues that helps form a putative amphipathic α-helix, helix-1, for membrane insertion and lipid activation (36). This interaction resembles that of p44 kinase docking with PDE4 cAMP phosphodiesterase where a docking site is also located on an exposed α-helix (46). Results of deletional analysis using truncated mutants of helix-1 showed that residues 287-300 were required for p42/44 kinase-CCTα binding, and that this domain was also required for optimal phosphorylation of the enzyme. Our studies do not rule out the possibility that the putative docking region within CCTα might involve a broader motif encompassing the entire α-helix or that other residues (e.g. 260 to 286) might optimize ERK-CCTα binding. This dock sequence is enriched with hydrophobic residues typical of many dock sites, but it has few basic residues and lacks LXL or FXF motifs commonly seen in some substrates (35, 47-49). However, many MAP kinase substrates either lack or display little similarity in their core sequences for docking suggestive of yet unidentified binding motifs. Indeed, sequence alignment for the ERK docking region within CCTα shows little similarity with other ERK targets but resembles that of ATF-2, a p38/JNK substrate, suggestive of the existence of a novel MAP kinase docking motif (47-49). It is possible that this variation in docking sequence for CCTα provides greater promiscuity for kinase binding or simply
provides a recognition site whereby p42/44 kinase can influence CCT\(\alpha\) membrane association. This scenario might be analogous to mutations within hydrophobic docking residues for mitogen-activated protein kinase kinase (MEK1) that alter cellular distribution of p44 kinase (50), thioredoxin-1 docking with PTEN phosphatase inhibiting its catalytic activity and membrane association (51), or protein kinase C binding and phosphorylation of its adaptor molecule triggering translocation of the complex to the cytoplasm (52). It is also possible that ERKs utilize other motifs within the CCT\(\alpha\) sequence for binding. For example, the sequence K\(^{13}\)RRK within the NLS, L\(^{156}\)AEHR within the catalytic domain, and L\(^{242}\)QER in the membrane binding region of CCT\(\alpha\) share high similarity with known dock sites in ribosomal S6 kinase (53), p90\(\text{rsk}\) (54), and MAP kinase phosphatase (55), respectively. However, candidate mutagenesis or deletion of these regions failed to interrupt p42/44 binding with CCT\(\alpha\).

To identify the phosphorylation targets for ERK in response to 22HC and 9-cis RA, we expressed other enzyme mutants containing phosphodomain truncations or point mutations at proline-directed sites within the CCT\(\alpha\) carboxyl-terminus. Indeed, the CCT\(\alpha\) carboxyl-terminal region was a necessary signal to direct inhibition of PtdCho synthesis by LXR/RXR agonists as expression of a mutant devoid of the entire phosphoterminal domain (CCT\(\alpha_{314}\)) significantly blocked inhibitory effects of 22HC and 9-cis RA. In contrast, cells transfected with CCT\(\alpha_{328}\), a mutant that lacked eleven of sixteen carboxyl-terminal serines, largely retained sensitivity to these agonists. Thus, five carboxyl-terminal phosphorylation sites (Ser\(^{315}\), Ser\(^{319}\), Ser\(^{321}\), Ser\(^{322}\), Ser\(^{323}\)) within CCT\(\alpha_{328}\) were potential kinase targets. Sequence analysis of CCT\(\alpha_{328}\) reveals three Ser/Pro motifs recognized by p42/44 kinases.
at Ser\textsuperscript{315}, Ser\textsuperscript{319}, and Ser\textsuperscript{323} indicating that an oxysterol sensitive region might be localized to residues within the aminoterminal end of the phosphorylation domain. To examine this, two additional CCT\textalpha\textsubscript{3} mutants were tested. By using CCT\textalpha\textsubscript{328SA}, we observed that mutagenesis of Ser at positions 321, 322, and 323 was insufficient to overcome inhibitory effects of 22HC and 9-\textit{cis} RA on PtdCho synthesis. Mutagenesis of Ser\textsuperscript{315} to alanine within the context of serial mutations at Ser 321, 322, and 323, (CCT\textalpha\textsubscript{328SA}quad) dramatically abolished agonist-induced inhibition of phospholipid synthesis (Fig. 5). Moreover, expression of CCT\textalpha\textsubscript{315}, a construct with a point mutation at Ser\textsuperscript{315}, restored ability of cells to synthesize PtdCho to near control levels after oxysterol treatment. Limited $\gamma$-$^{32}$P labeling was also detected by CCT\textalpha\textsubscript{315} or CCT\textalpha\textsubscript{quad} using p42 kinase \textit{in vitro}. Taken together, these data suggest that Ser\textsuperscript{315} is a mechanistically relevant site mediating p42/44 kinase directed CCT\textalpha phosphorylation following 22HC and 9-\textit{cis} RA exposure. Single site modification of such serine targets for p42/44 kinase leading to altered biological activity is well-described for several proteins including hormone sensitive lipase, sterol regulatory element-binding protein, GTPase-activating protein, and the transcription factor GATA4 (56-59). Our demonstration that expression of CCT\textalpha mutants with modified ERK phosphorylation sites restores surfactant lipid synthesis to high levels raises intriguing possibilities for use of gene transfer approaches using “designer” surfactant biosynthetic enzymes in lung injury.
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**FIGURE LEGENDS**

**Figure 1. Oxysterols/RA Inhibit Phosphatidylcholine (PtdCho) Synthesis and CCT Activity in Lung Epithelia.** (A) Murine lung epithelial (MLE) cells were incubated in serum-free medium supplemented with or without 22HC (25 µM) and 9-cis RA (1 µM) for various times. Cells were pulsed with 2 µCi [³H]-choline the final 2 hrs of incubation, and radioactivity in cellular [³H] PtdCho was determined following cellular lipid extraction, separation using TLC, and quantification by scintillation counting. [Inset]: [³H]-choline incorporation into DSPtdCho at 24 hrs was also determined as above. Values are mean±SEM from 3 independent experiments. (B-C) Cells were exposed to various amounts of 22HC (B) or (C) different oxysterols: 24-hydroxycholesterol (24-OH), 25-hydroxycholesterol (25-OH), or 7-β-hydroxycholesterol (7-β diol) (25 µM) for 24 hrs and [³H]-choline incorporation into PtdCho was determined. Both panels (B) and (C) were performed with inclusion of 9-cis RA (1 µM) in the medium. (D-F) MLE cells were cultured with 22HC (25 µM) and 9-cis RA (1 µM) in combination (D) or individually (E-F) for 6 hrs, 24 hrs or 48 hrs. CCT activity was assayed in the absence of exogenous lipid activator in the assay mixture. For panels A-F, * p<0.05 or **p<0.005 for 22HC and 9 cis-RA vs. control; †p<0.001 22HC/RA vs control or 25-OH group vs. all other groups. Values are mean ± SEM from 3 independent experiments.
Figure 2. Oxysterols/RA Increase CCTα Phosphorylation. (A) MLE cells were exposed to medium with (+) or without (-) 22HC (25 µM) in combination with 9-cis RA (1 µM) for various times and cells harvested for CCTα immunoblotting. (B) Cells were exposed to medium with (+) or without (-) 22HC (25 µM) with 9-cis RA (1 µM) as above for 24 hrs, cells labeled with [32-P] orthophosphate (750 µCi/dish), and CCTα was immunoprecipitated. The immunoprecipitates were run on SDS-PAGE, gels dried, and autoradiography was performed to detect [32-P] - CCTα. (C) MLE cells were transiently transfected with a his-tagged full-length pCMV5-CCTα plasmid and cultured with (+) or without (-) 22HC (25 µM) in combination with 9-cis RA (1 µM) for various times. Lysates were harvested and fusion proteins purified on a his-tagged column. Purified proteins were separated by SDS-PAGE as above and levels of phosphoserine CCTα (upper panel) were determined by probing immunoblot membranes with rabbit phosphoserine polyclonal antibody or probed for levels of total CCTα (lower panel). (D) Densitometric analysis of autoradiograms showing amounts of immunoreactive phosphorylated CCTα versus control using arbitrary densitometric values after correction for levels of CCTα loading. [Inset]: Cells were cultured with (+) or without (-) 22HC (25 µM) in combination with 9-cis RA (1 µM) for 24 hrs. CCTα was immunoprecipitated using a polyclonal antibody to the enzyme and resolved using SDS-PAGE prior to immunoblotting with the phosphoserine antibody. Arrowheads show multiple CCTα phosphorylated forms. Results from panel (A) are from 4 independent experiments, and panel B is a representative experiment. Panels C is from n=4 experiments and panel D [inset] is from n=2. * p<0.05 for 22HC and 9 cis-RA vs. control.
**Figure 3. Oxysterol/RA Inhibition of PtdCho Synthesis is Mediated by p42/44 Kinase.** (A). MLE cells were cultured with (+) or without (-) 22HC (25 µM) with 9-cis RA (1 µM) for various times and levels of phosphorylated (above) and total levels (below) of p42/44 kinase was assayed by immunoblotting. (B). Cells were transiently transfected with pTRE-luc and pTET-Elk and either an empty vector or constitutively active pCMV-MEK-1 expression vector. After 24 hours, the cells were stimulated with ethanol or 22HC with 9-cis RA for 6 hours. Luciferase activity, which is normalized to protein, is expressed as fold increase from control. (C) Using the same assay system as in (B), cells were cultured with 22HC and 9-cis RA for up to 24 hrs, and luciferase activity measured. (D) Cells were cultured in 12 well plates with or without 22HC and 9-cis RA for 24 hrs, with PD98059 (10 µM) alone for 1 hr and then exposed to medium for 24 hrs, or pretreated with PD98059 (10 µM) for 1 hr prior to agonist stimulation for 24 hrs. [3H]-choline incorporation into PtdCho was then determined. (E) Cells were cultured with or without 22HC and 9-cis RA in 60 mm dishes as in (D), transfected with a p42 kinase dominant negative (DN) plasmid (4 µg) alone for 4 hrs and then exposed to medium for 24 hrs, or transfected with the p42 kinase DN plasmid prior to agonist stimulation. (F) Cells were transfected with his-tagged full-length pCMV5-CCTα plasmid and cultured with (+) or without (-) 22HC (25 µM) in combination with 9-cis RA (1 µM) for 3, 6, 21, or 24 hrs. Lysates were harvested and purified on a his-tagged column, and then either probed with a polyclonal antibody to phosphorylated p42/44 kinase (above) or total p42/44 kinase (below). (G) To control for nonspecific kinase binding to the his-tagged column, untransfected MLE cell lysates ((200 µg) alone were applied to the column and protein was eluted in two fractions (Fractions 1
and 2). We then used equal amounts of protein from crude cell lysate (L), and eluted fractions (Lanes 1, 2) for SDS-PAGE and immunoblotting for CCTα (left) and 42/p44 kinase (right). (H) In separate studies, cells were transfected with his-tagged full-length pCMV5-CCTα plasmid and lysate harvested and used for immunoblotting [left] for: CCTα (Lane1), p42/44 kinase (Lane 2), cdc2 kinase (Lane 3), and PKC (Lane 4). Transfected lysate was also applied to the nickel column, the protein was eluted, and samples used for immunoblotting [right] for analysis of protein binding: CCTα (Lane1), p42/44 kinase (Lane 2), cdc2 kinase (Lane 3), and PKC (Lane 4). (I) (Left Panel): Immunoprecipitation of ERK and immunoblotting for CCTα. Cells were cultured in the presence (+) or absence (-) of agonists for 24 hrs as above. After treatment, cells were harvested in lysis buffer, and samples processed using the Rabbit IgG TrueBlot Set. Thus, lysates were precleared and incubated with either p42/44 kinase antibody (far left two lanes) or normal rabbit IgG (Ig) followed by immunoblotting with anti-CCTα antibodies. The far right lane represents CCTα detected in cell lysates. (Right Panel): Immunoprecipitation of CCTα and immunoblotting for ERK. Lysates from cells cultured as above were also immunoprecipitated using CCTα antibodies followed by immunoblotting with an antibody to total p42/44 kinase (right panel, left two lanes). As a negative control, lysates were first incubated with pre-immune serum (P) followed by immunoblotting with antibody to total p42/44 kinase. The far right lane represents levels of total p42/44 in cell lysates. In studies above, *p<0.05 vs. control. **p<0.01 and +p<0.001 vs. control. Values are mean ± SEM from 3 independent experiments for all panels except panels D and E (at least n=6) and panel F (n=4), and panels G-I (n=1).
Figure 4. Mapping of a CCTα Docking Domain For ERK. (A) Map illustrating sequences of individual CCTα mutants using candidate or deletional mutagenesis of putative dock sites. Dashed lines represent deleted residues. Specific mutations within motifs are underlined. Constructs tested include wild-type CCT (CCTα), a variant harboring mutations at a candidate dock site (CCTm158), and an aminoterminal deletion mutant (CCTN40) devoid of the nuclear localization signal (NLS). A double mutant lacking the membrane binding region and containing mutations within a putative NH₂-terminal dock site (CCTm289), an internal deletion mutant devoid of only the membrane binding region (CCT289), and another internal deletion mutant lacking 21 residues within the catalytic-membrane binding hinge region (CCTd21) were also tested. Finally, a series of deletion mutants progressively truncated within the distal membrane-binding region (CCT260, CCT280, CCT286, CCT300) were generated. (B, D, F) Individual mutants were synthesized in vitro using rabbit reticulocyte lysate in a reaction containing [³⁵S]-methionine (20 µCi/reaction). (C, E, G) Newly translated reactions products were reacted with ERK-GST agarose and processed subsequently for SDS-PAGE and autoradiography as described in METHODS. Each panel is representative of at least n=4 separate experiments. (H). CCTα was synthesized in vitro using rabbit reticulocyte lysate as above and then reacted with ERK-GST agarose, p38-GST agarose, or GST agarose alone and processed subsequently for SDS-PAGE and autoradiography. (I). Cells were transfected with plasmids encoding his-tagged full-length CCTα or the membrane binding deletion mutant, CCT289. After 18 hrs, lysates were harvested and purified on a his-tagged column. In the upper row, lysates from CCTα and CCT289 transfectants were dephosphorylated using alkaline phosphatase, and
then phosphorylated using recombinant p44 kinase (10 µg) in the presence of \( \gamma \)-32P ATP. Reaction products were purified, resolved using SDS-PAGE and processed for autoradiography to detect levels of \( \gamma \)-32P–labeled CCT\( \alpha \). In the middle and lower rows, lysates were processed for immunoblotting and probed with either a polyclonal antibody to phosphorylated p42/44 kinase (middle) or to CCT\( \alpha \) (lower).

**Figure 5.** Altered Oxysterol/RA Sensitivity by CCT\( \alpha \) Phosphorylation Domain Mutants. (A) Cartoon illustrating sequences of individual CCT\( \alpha \) mutants. All constructs contain a nuclear localization signal (NLS), a catalytic core, and membrane-binding domain but vary in the carboxyl-terminal domain. Wild-type CCT (above, CCT\( \alpha \)) and a mutant lacking the entire phosphorylation domain (bottom, CCT\( \alpha_{314} \)) were used as controls. Three truncated constructs each containing 328 residues (CCT\( \alpha_{328} \), CCT\( \alpha_{328SA} \), and CCT\( \alpha_{328SAquad} \)) only differ in numbers of carboxyl-terminial proline-directed sites. Two full-length CCTs were tested: CCT\( \alpha_{quad} \) contains the entire carboxyl-terminus but with modified residues identical to CCT\( \alpha_{328SAquad} \) whereas CCT\( \alpha_{315} \) harbors a point mutation at Ser\( ^{315} \). (B) Cells were transfected with plasmids encoding full-length CCT\( \alpha \), or one of four truncated mutants (CCT\( \alpha_{quad} \), CCT\( \alpha_{328SA} \), CCT\( \alpha_{328SAquad} \), and CCT\( \alpha_{314} \)). Cells were subsequently exposed to medium alone (control) or medium in combination with 22HC (25 µM) with 9-cis RA (1 µM) for 24 hrs and pulsed with 2 µCi [\(^3\)H]-choline to determine radioactivity in cellular [\(^3\)H] PtdCho as described in Fig.1. (C) Cells were transfected with plasmids encoding wild-type CCT\( \alpha \), or one of two full-length mutants (CCT\( \alpha_{quad} \), and CCT\( \alpha_{315} \)) harboring mutations within the carboxyl-terminus. Cells were subsequently exposed to medium with or without 22HC with 9-cis RA and processed for...
PtdCho synthesis as in (B). Data in (B-C) is from at least n=3 independent experiments. (D). In vitro $^{32}$P labeling of CCT$\alpha$ mutants. Cells were transfected with his-tagged CCT$\alpha$ constructs, CCT$\alpha$ purified, dephosphorylated using alkaline phosphatase, and then phosphorylated using recombinant p42 kinase (10 $\mu$g) in the presence of $\gamma^{32}$P ATP. Reaction products were purified, resolved using SDS-PAGE and processed for autoradiography (above) or CCT$\alpha$ immunoblotting (below). The bar graph shows the densitometric ratio of CCT$\alpha$ phosphorylated mutants to total CCT$\alpha$ protein. (E). In vivo phosphorylation of CCT$\alpha$ mutants. Cells were transfected with his-tagged CCT$\alpha$ constructs with (+) or without (-) constitutive active MEK-1 plasmid. After 24 hr, cells were rinsed and harvested, his-tagged CCT$\alpha$ purified, and samples processed for phosphoserine immunoblotting. (F). Cells transfected as in (E) were also pulsed with 2 $\mu$Ci $[^3]$H-choline as above and radioactivity in cellular $[^3]$H PtdCho was then determined. *p<0.05 vs. control plasmids as determined using an ANOVA.
Figure 3

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

I.
Figure 5

A. CCT\(\alpha\)        
NLS  | Catalytic | Membrane Binding
CCT\(\alpha\)        
NLS  | Catalytic | Membrane Binding
CCT\(\alpha328\)     
NLS  | Catalytic | Membrane Binding
CCT\(\alpha328SA\)   
NLS  | Catalytic | Membrane Binding
CCT\(\alpha328SAquad\) 
NLS  | Catalytic | Membrane Binding
CCT\(\alphaquad\)    
NLS  | Catalytic | Membrane Binding
CCT\(\alpha315\)     
NLS  | Catalytic | Membrane Binding
CCT\(\alpha314\)     
NLS  | Catalytic | Membrane Binding

B. Effect of 22HC/RA on 3H-Choline Incorporation into PutCho (dpm/mg protein)

C. Effect of 32P incorporation into CCT\(\alpha\) ( Arbitrary Optical Density)

D. Total CCT\(\alpha\)

E. MEK1

F. PhosphoCCT\(\alpha\)
Oxysterols inhibit phosphatidylcholine synthesis via ERK docking and phosphorylation of CTP: Phosphocholine cytidylyltransferase
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