Upregulation of surfactant synthesis triggers ABCA1-mediated basolateral phospholipid efflux

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Abstract **Alveolar type II lung epithelia produce surfactant, an essential surface-active material highly enriched with disaturated phosphatidylcholine (DSPC), which requires a key regulatory enzyme, CTP:phosphocholine cytidylyltransferase α (CCTα), for its synthesis before its export apically into the alveolus. In this study, we examined whether surfactant phosphatidylcholine (PC) synthesis and export are physiologically linked. Stable overexpression of CCTα in lung epithelial cell lines increased rates of PC synthesis and cellular DSPC mass without altering total cellular PC content. Overexpression of CCTα was associated with *i*) increased basolateral, rather than apical, PC export catalyzed by ABCA1; *ii*) basolateral export of significant levels of unsaturated (nonsurfactant) PC; and *iii*) transcriptional activation of the ABCA1 gene via a liver X receptor/retinoic acid receptor-independent pathway. Cells exposed to PC vesicles exhibited a dose-dependent increase in ABCA1 transcriptional activity. These data provide the first evidence that surfactant PC synthesis is linked to its export via a basolateral lipid efflux pathway. This pathway is mediated, in part, by a phospholipid sensor, ABCA1, that appears to partake in the autoregulation of both cellular content and composition of PC, thereby providing a potentially novel exit route for a newly synthesized pool of PC distinct from surfactant.**—Zhou, J., Y. You, A. J. Ryan, and R. K. Mallampalli. **Upregulation of surfactant synthesis triggers ABCA1-mediated basolateral phospholipid efflux.** ***J. Lipid Res.*** **2004.** 45: **1758–1767.**

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Phosphatidylcholine (PC) is synthesized within alveolar type II epithelia and secreted into the alveolus as the major phospholipid component of surfactant, which is essential to establish normal breathing. Sufficient biosynthesis of the surface-active phospholipid, dipalmitoyl phosphatidylcholine (DPPC) [or disaturated phosphatidylcholine [DSPC]), coupled with its regulated secretion via a well-recognized apical secretory pathway, is critical for maintaining biophysical properties that optimize lung structure and function. Although not limited to the lung, the capacity to adequately synthesize and enrich phospholipids with DSPC is a fairly unique feature of type II cells compared with other polarized epithelia. Interference with surfactant DSPC synthesis or its apical secretion is recognized as a hallmark of the neonatal respiratory distress syndrome and to some degree a component of acute and chronic lung disease (1, 2).

PC synthesis in all eukaryotic cells occurs predominantly through the cytidine diphosphocholine (CDP-choline) pathway, controlled by the rate-limiting enzyme CTP: phosphocholine cytidylyltransferase (CCT; EC 2.7.7.15). CCT converts phosphocholine to CDP-choline with chemical energy provided by CTP (3, 4). To date, three CCT isoforms with identical residues in the catalytic domain have been reported, termed CCTα, CCTβ_1, and CCTβ_2 (5). Among them, CCTα is ubiquitously expressed and is the predominant species in alveolar epithelia. CCTα exists in both soluble and membrane-associated forms; in lung epithelia it is primarily soluble (6). CCTα contains four distinct functional domains: an amino-terminal nuclear localization signal, a catalytic core, a membrane binding region, and a carboxyl-terminal phosphorylation domain. Cellular CCTα activity is known to be regulated by several mechanisms, including *i*) interconversion between soluble and membrane-bound species; *ii*) reversible enzyme phosphorylation; *iii*) control at the level of protein stability; and *iv*) alterations in mRNA content (4). Until recently, limited information has been available on the con-

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Abbreviations: CCT, CTP:phosphocholine cytidylyltransferase; CDP-choline, cytidine diphosphocholine; DOPC, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine; DR-4, direct repeat separated by four nucleotides; DPPC, dipalmitoyl phosphatidylcholine; DSPC, disaturated phosphatidylcholine; ECL, enhanced chemiluminescence; GPC, glycerophosphocholine; 22HC, 22-hydroxycholesterol; LXR, liver X receptor; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; RA, 9-*cis* retinoic acid; RXR, retinoid X receptor.

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trol of CCTα gene transcription. In view of CCTα's regulatory role in PC metabolism, it has served as an attractive metabolic target for pharmacologic and genetic intervention in attempts to alter phospholipid synthesis.

Several studies demonstrate that maneuvers designed to stimulate CCTα activity increase PC synthesis concomitant with increased surfactant levels. For example, transgenic mice that overexpress CCTα have increased DSPC content (7). On the other hand, similar to cholesterol metabolism, eukaryotic cells harbor intricate feedback control mechanisms to tightly regulate cellular PC levels. For example, conditional upregulation of CCTα in nonepithelial cell lines increases PC synthesis but triggers phospholipase activation. The net result is that cellular PC levels are unchanged in these systems (3, 7, 8). Thus, the activation of specific phospholipid degradatory pathways is dependently regulated with PC synthesis, thereby allowing cells to exert tight homeostatic control of cellular phospholipids (5, 8–11). An alternative, although untested, issue is whether PC synthesis is directly coupled to its export from cells. Changes in rates of PC synthesis observed in prior studies may have been offset by increased rates of phospholipid secretion, resulting in the absence of a net increase in PC content.

ABCA1 has emerged as one of the most important genes related to cellular trafficking of cholesterol and phospholipids (12, 13). ABCA1 is involved in the export of cellular phospholipid and cholesterol from the plasma membrane (14, 15). Transactivation of the ABCA1 promoter by liver X receptor (LXR) and retinoic acid receptor ligands, such as 22-hydroxycholesterol (22HC) and 9-*cis* retinoic acid (RA), results in membrane expression of the transporter-triggering efflux of lipids to suitable acceptor proteins such as apolipoprotein A-I and HDL (12, 16). Thus, LXR/retinoid X receptor (RXR) ligand induction of ABCA1 appears to function as an important feedback control mechanism to eliminate excess cellular lipids in reverse cholesterol transport. Of interest is the fact that targeted disruption of the ABCA1 gene in mice results in respiratory death, reduced levels of serum phospholipids, and striking accumulation of lipid within alveolar type II cells (17). Moreover, ABCA3 gene mutations were recently identified in newborns with fatal surfactant deficiency (18). ABCA3 protein and regulated expression of ABCA1 are detected in alveolar epithelium (19). However, the precise role of these proteins with respect to either polarized alveolar secretion of surfactant phospholipid or their linkage with PC synthesis is unknown.

In this study, we hypothesized that surfactant PC biosynthesis and secretion are physiologically coupled. Stable CCTα expression in lung epithelia resulted in a significant increase in the rate of PC synthesis and DSPC mass, but this did not impact total cellular PC content. Interestingly, CCTα overexpression was linked to transcriptional activation of ABCA1 via a phospholipid-sensitive LXR/RXR-independent mechanism, resulting in the efflux of high levels of unsaturated PC by a basolateral exit route. These results demonstrate the dependency of PC export with its biosynthesis and suggest that alveolar epithelia use an ABCA1-driven export pathway as a novel mechanism to enrich themselves with surfactant phospholipid.

METHODS

Materials

The human lung adenocarcinoma cell line A549 and murine lung epithelial cell line MLE-12 were purchased from the American Type Culture Collection (Manassas, VA). Media were obtained from the University of Iowa Tissue Culture and Hybridoma Facility (Iowa City, IA). The pGL3-basic and pSV-β-galactosidase plasmids were purchased from Promega (Madison, WI). The polyclonal antibodies to human ABCA1, scavenger receptor class B (CD36), and β-actin were purchased from R&D Systems (Minneapolis, MN). The polyclonal CCTα antibody against synthetic peptide was generated as described (20). The enhanced chemiluminescence (ECL) kit was obtained from Pierce (Rockford, IL). All restriction enzymes were purchased from New England Biolabs (Beverly, MA). The pCR-TOPO4 plasmid and *Escherichia coli* Top10 competent cells were obtained from Invitrogen (Carlsbad, CA). QIAprep Miniprep and Maxiprep kits were obtained from Qiagen (Valencia, CA). The FuGENE6 transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN). The Geneclean2 Kit was obtained from Bio101 (Carlsbad, CA). The luciferase assay system was obtained from Promega (Madison WI), and the Galacto-Light Plus™ kit was obtained from Tropix (St. Louis, MO). Individual lipids and Tri-Reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Specifically, egg yolk PC from Sigma was 99% pure by TLC and contained varying amounts of palmitate (16:0), linoleate (18:2), oleate (18:1), and stearate (18:0) as acyl groups using gas-liquid chromatography. 22HC and RA were purchased from Steraloids (Newport, RI). Glyburide was from EMD Biosciences (San Diego, CA). The pTRE2 hygromycin expression vector and Advantage cDNA polymerase were obtained from Clontech (Palo Alto, CA). All DNA sequencing was performed by the University of Iowa DNA core facility. Luciferase and β-galactosidase activities were determined using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The Taqman reverse transcription reagents, SYBR Green PCR master mix, rodent GAPDH control reagent, and nitrocellulose membrane were obtained from Applied Biosystems (Foster City, CA). Transwell (24-mm) plates were purchased from Corning Costar Corp. (Cambridge, MA).

Cell culture

A549 cells were maintained in MEM supplemented with 10% FBS. The MLE-12 cells were grown in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12) with 2% FBS. All cell cultures were incubated at 37°C with 5% CO_2.

Construction of pTRE2 hyg-CCTα

The cDNA that encodes the open reading frame of the rat liver CCTα gene was cloned using the same procedure used in our previous study, a two-step PCR amplification using the sense primer 5′-GGATCCATGGATGCACAGAGTTCAGC-3′ and the antisense primer 5′-CGCTAGCATTAGTCCTCTTCATCCTCGC-3′ (3). It was inserted into the pTRE2 hyg vector using the *Bgl*II and *Nhe* restriction sites. pTRE2 hyg-CCTα was verified by DNA sequencing.

Construction of a stable CCTα-overexpressing A549 cell line

A549 cells were grown to ~80% confluence in MEM plus 10% FBS medium in 100 mm dishes and transfected with 1 μg of

pTRE2 hyg-CCTα plasmids, as described previously (3). After a 48 h recovery, cells were selected using 400 μg/ml hygromycin for ~8 weeks. Antibiotic-resistant colonies were isolated, cloned, and analyzed by CCTα activity and immunoblot analysis. The stable cell line exhibiting the highest level of CCTα expression was selected for experiments (termed the CCTα-overexpressing cell line), and untransfected A549 cells were used as a control.

Immunoblot analysis

Equal amounts of total protein from cell lysates were solubilized in 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromphenol blue, and 5% β-mercaptoethanol and heated at 95°C for 5 min. Samples were then electrophoresed through a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoreactive CCTα, ABCA1, CD36, and β-actin were detected using the ECL Western blotting detection system. The dilution factors for all polyclonal antibodies were 1:1,000 (3).

Real-time PCR analysis

Total cellular RNA from cells was obtained using Tri-Reagent. Taqman reverse transcription reagents were used to generate cDNA from cellular RNA. Real-time PCR was then performed on cDNA using the Applied Biosystems 7700 real-time PCR instrument and the SYBR Green PCR master mix. Primers used in PCR for the detection of genes involved in lipid metabolism are listed in **Table 1**. Taqman rodent GAPDH control regents were used as the internal control. Standard curves were generated for target genes and compared with GAPDH using serial dilutions of mRNA, and they were found to be linear from 0.08 to 50 ng RNA in the reaction mixture. This range included effective concentrations used in the experiments (3).

CCTα activity

CCTα activity was determined by measuring the rate of incorporation of [methyl-^{14}C]phosphocholine into CDP-choline using a charcoal extraction method (3).

Construction of human ABCA1 promoter plasmids

A 644 bp human ABCA1 core promoter fragment [AF258623, −618/+26(21)] was generated using the same PCR procedure as previously described (3) using the sense primer 5′-GACTCGAGCAGTAAGATGTTCCTCTCG-3′ and the antisense primer 5′-AGATCTTACTATCGGTCAAAGCCTG-3′. This fragment was then directionally cloned into pGL3-basic at *Xho*I/*Bgl*II sites, generating pGL3-ABCA1$_{644}$. It included the proximal 5′ flanking region, the transcription start site, and up to 26 nt of the first exon corresponding to the 5′ untranslated region of the human ABCA1 transcript (21).

A 1694 bp human ABCA1 promoter fragment [AF275948, −1453/+241(16)] was also generated using PCR using the sense primer 5′-GGTACCCCTGGAGATCCTGTTGACTG-3′ and the antisense primer 5′-AGATCTGTCACTGGAGAGCCTCTTACC-3′. The cloned fragment included the proximal 5′ flanking region, the transcription start site, and 241 nt of the first exon (16). It was cloned into pGL3-basic at *Bgl*II/*Kpn*I sites, generating a larger fragment, termed pGL3-ABCA1$_{1694}$. Several additional ABCA1 deletion constructs were generated using PCR with an identical antisense primer for ABCA1$_{1694}$ and the following sense primers: ABCA1$_{-706/+241}$, 5′-GGTACCCAGTAAGATGTTCCTCTCG-3′; ABCA1$_{-411/+241}$, 5′-GGTACCAGCAGGATTTAGAGGAAGC-3′; ABCA1$_{-145/+241}$, 5′-GGTACCACGTGCTTTCTGCTGAGTG-3′. These fragments were also cloned into pGL3-basic at *Bgl*II/*Kpn*I sites. All promoter sequences were confirmed by DNA sequencing.

The thymidine kinase promoter reporter construct was generated as described (22).

Cloning of murine ABCA1

The cDNA encoding the open reading frame for the mouse ABCA1 gene (National Center for Biotechnology Information accession number NM013454) was generated using mouse liver cDNA as a template and three sets of primers: for cloning of fragment 1, sense primer 5′-GGTACCGCCATGCCGTCTGCAGGAAC-3′ and antisense primer 5′-GGATCCACCCACGAAGGCCAAG-3′; for fragment 2, sense primer 5′-GGGATGCAGAGAAAGCTGTCTG-3′ and antisense primer 5′-GACACGAGGACGTCGACAGAGG-3′; for fragment 3, sense primer 5′-CCTCTGTCGACGTCCTCGTGTC-3′ and antisense primer 5′-TCTAGACCTTTCATTCACCCTGTGTG-3′, in a two-step PCR amplification using Advantage 2 cDNA polymerase under the following reaction conditions: 94°C for 2 min, 94°C for 30 s, 68°C for 3 min, 25 cycles. Three PCR fragments, ~3, ~1.8, and ~2 kb, were purified using the Geneclean2 kit and cloned into pCR4-TOPO, and plasmid minipreps were prepared. After verification by DNA sequencing, a clone of fragment 1 was digested by *Kpn*I/*Bam*HI; fragment 2 was digested by *Bam*HI/*Aat*II; and fragment 3 was digested by *Aat*II/*Xba*I; digestion products were purified and ligated into a pcDNA3.1-V5-His-B expression vector previously digested with *Kpn*I/*Xba*I, termed pcDNA-ABCA1. The construct was verified by partial DNA sequencing.

Transient transfection and reporter gene assays

Because the stable CCTα-overexpressing clonal cell line was technically difficult to generate, we performed comparative analysis using transient transfectional studies. Cells maintained in growth medium were harvested using 0.25% trypsin plus 0.1% EDTA, plated onto 12-well tissue culture dishes, and allowed to reach ~80% confluence before transient transfection. Transfections were performed for 120 min in 0% FBS medium using FuGENE6 reagent, 0.75 μg/well of test plasmid, and 0.25 μg/well of pSV-β-galactosidase, which was used as a transfection efficiency control. Immediately after transfection, cells were trans-

TABLE 1. Real-time PCR primer sets for this research

Target Gene	Sense Primer	Antisense Primer
CCTα	CCTGGAAATGTTTGGTCCAGA	CTCTGCTTGGGACTGATGG
ABCA1	GAGAAGGAGGCACGGCTG	AGCTAAACCAGAGGATGCTGTTG
CD36	TGTAACCCAGGACGCTGAGG	GGAGGTTCGAAGATGGCACC
PPARγ	GGATGTCTCATAATGCCATCAGG	CGCCAACAGGTTCTCCTTCT
LPL	CAGGAGCATTACCCAGTGTCC	GATAAACCGGGCCACATCC
SPHK1	TCCTGACCAACTGCACGCT	CAGCAGGTTCATGGGTGACA
SPHK2	ATCACCCCTGACCTGCTACCT	CGACCCCCAAAGGGATTG
SPT1	CCGGCCAGGGATACTGCT	AGGGCCTCSATTGCTGCA
SPT2	CCTGCTCTTGTTGGCAAAGG	GCTCCCAGAACCAGTGATGC

CCTα, CTP:phosphocholine cytidylyltransferase α; PPARγ, peroxisome proliferator-activated receptor γ.

ferred to medium containing FBS and allowed to recover for 24 h before cell lysates were harvested in reporter lysis buffer for analysis of luciferase and β-galactosidase activities (22). In some studies, cells were allowed a 4 h recovery after transfection and were exposed to 1–50 μM CDP-choline, cholinephosphate, PC, lyso-PC, or glycerophosphocholine (GPC) for 24 h before analysis of reporter activity.

PC and DSPC analysis

Cells were pulsed with 1 μCi of [methyl-^{3}H]choline chloride during the final 2 h of incubation. Total cellular lipids were extracted and spotted onto LK5D plates, and PC was resolved using thin layer chromatography (20). DSPC determination was measured as described (23).

Apical and basolateral PC efflux

Cells were plated in the upper chamber of 24-mm Transwell plates at a density of 120,000 cells per well with 1.5 ml (upper chamber) and 2.6 ml (lower chamber) of medium in cluster plates. After cells were 70% confluent on the membrane, medium was changed, and 2 μCi of [^{3}H]choline was added to the upper chamber of each well for 18 h. After rinsing the medium five times to remove unincorporated [^{3}H]choline, cells were incubated in medium containing 25 μg/ml HDL (the PC acceptor). HDL was added to both chambers of plates. After another 24 h of incubation, medium from both chambers was collected by a 5-min spin at 1,500 rpm. Lipids were extracted and subjected to PC analysis as described (3). In some cases, 1 μM glyburide was added in the medium of both chambers.

To assess total cellular PC efflux on cells cultured on plastic, cells were plated on 60 mm plates at a density of 600,000 cells per plate and labeled with 2 μCi of [^{3}H]choline, and medium was harvested for PC analysis as described above.

Statistical analysis

All data were analyzed using statistical software SPSS 11.5. Data are presented as means ± SEM. Statistical significance was accepted at the $P < 0.05$ level by unpaired samples *t*-test or one-way ANOVA for multiple group analysis.

RESULTS

Overexpression of CCTα

We first investigated whether manipulation of CCTα by stable expression increases PC synthesis in lung epithelia. We focused on CCTα because this enzyme is rate regulatory in the PC biosynthetic pathway (3). Indeed, the stable CCTα cell line exhibited a 450% increase in CCTα activity compared with control A549 cells (**Fig. 1A**). CCTα-overexpressing cells expressed 2-fold higher levels of immunoreactive CCTα content as determined by densitometric analysis of immunoblots (Fig. 1B), and levels of enzyme transcripts were ~7-fold greater than control levels using real-time PCR at 24 and 48 h of analysis (Fig. 1C).

Overexpression of CCTα regulates PC metabolism

Overexpression of CCTα was associated with increased PC synthesis, as reflected by rates of incorporation of [^{3}H]choline into PC that were 210% greater in the stable CCTα cell line compared with control cells (**Fig. 2A**). These changes, interestingly, were not associated with significant alterations in total PC mass between 24 and 72 h

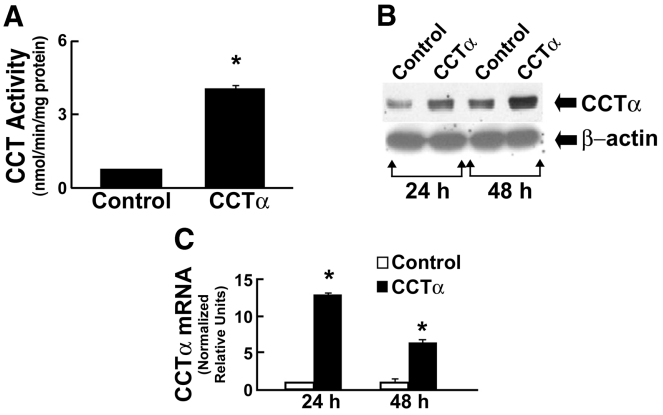

Fig. 1. CTP:phosphocholine cytidylyltransferase α (CCTα) overexpression in A549 cells. Control cells and cells exhibiting stable CCTα overexpression were analyzed for CCT activity (A), CCTα protein by immunoblotting (B), and steady-state mRNA by real-time PCR (C). The times shown represent the duration of cells in culture after passaging. In B, β-actin was used as a protein-loading control, and in C, transcripts were normalized for loading using GAPDH as a housekeeping control. Results are presented as means ± SEM (n = 3). Statistical analyses were performed using Student's *t*-test; * $P < 0.05$ versus control.

after plating of cells (Fig. 2B). There were some day-to-day variations in the mass of PC and DSPC in control cultures after seeding, but these changes were not statistically significant (Fig. 2B, C). The cellular mass of DSPC, a marker

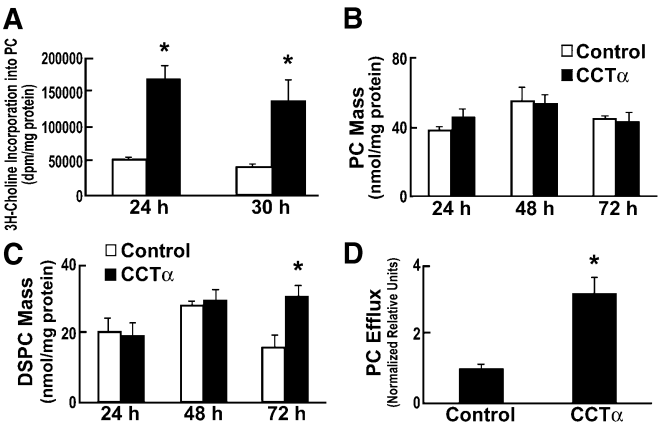

Fig. 2. CCTα overexpression regulates cellular phosphatidylcholine (PC) and dipalmitoyl phosphatidylcholine (DSPC) metabolism. A: Control cells and cells exhibiting stable CCTα overexpression were analyzed for rates of PC synthesis by pulsing cells with 1 μCi of [^{3}H]choline for the final 2 h of incubation, extracting cell lipids, and measuring radioactivity in cellular [^{3}H]PC using TLC. B and C: The cellular masses of PC (B) and DSPC (C) were assayed in control and CCTα-overexpressing cells. The times shown represent the duration of cells in culture after passaging. D: Cells were pulsed in medium containing 2 μCi of [^{3}H]choline overnight, the medium was removed and cells were rinsed, and the secretion of [^{3}H]PC was assayed from control and CCTα-overexpressing cells. Data were normalized by correcting the dpm of [^{3}H]PC recovered in medium for total cellular protein in each dish and then expressed as the ratio of average dpm/mg protein value from all experiments in each group. Data are presented as means ± SEM (n = 3). * $P < 0.05$ versus control.

of surfactant lipid, however, was observed to be 2-fold greater in CCTα-overexpressing cells compared with control cells after long-term (72 h) culture (Fig. 2C). To further investigate the physiologic basis whereby the stable expression of CCTα increased rates of PC synthesis but did not translate into increased PC mass, we examined phospholipid export. CCTα overexpression resulted in a 3-fold increase in PC efflux (Fig. 2D). Collectively, these results indicate that upregulation of PC biosynthesis triggers its export, resulting in minimal changes in total cellular PC content; such coordinated activity in this system, however, appears to enrich alveolar epithelia long term with surfactant-associated phospholipids.

CCTα overexpression is coupled to ABCA1-mediated basolateral PC efflux

To examine PC efflux further, we used a Transwell system to separate apical from basolateral secretion. Cells constitutively released phospholipid (**Fig. 3A**). Compared with control cells, rates of PC secretion in CCTα-overexpressing cells was 3-fold greater via a basolateral efflux pathway, with no statistical differences between cells with regard to apical secretion (Fig. 3A). Moreover, unlike apical release, basolateral secretion in CCTα-overexpressing cells was significantly blocked using glyburide, an ABCA1 inhibitor, such that PC efflux was reduced to levels comparable to those of control cells. These data suggest that, unlike apical secretion, CCTα-driven basolateral PC efflux is at least partly mediated by ABCA1. In agreement with these functional data, CCTα-overexpressing cells also exhibited high-level expression of ABCA1 protein (Fig. 3B). The levels of CD36 protein and transcripts of several lipogenic genes were also increased after CCTα overexpression (Fig. 3B, C). There were no significant differences in the expression of other genes involved in lipid metabolism, including sphingosine kinase and serine palmitoyltransferase (data not shown).

To examine the mechanisms for CCTα-driven ABCA1 induction, we cloned two fragments of the human ABCA1 promoter ($ABCA1_{644}$ and $ABCA1_{1694}$), coupled them to a reporter gene, and performed transient transfections to assess transcriptional regulatory activity. The ABCA1 gene is regulated by an LXR/RXR-dependent pathway via binding of ligands, such as 22HC and RA to LXR and RXR nuclear heterodimeric receptors, respectively, resulting in transactivation of its promoter within a specific response motif [direct repeat separated by four nucleotides (DR-4 locus)]. The $ABCA1_{644}$ construct lacks a DR-4 element, whereas the $ABCA1_{1694}$ fragment contains this response element. The $ABCA1_{1694}$ construct exhibited a 7-fold higher level of baseline activity in lung epithelia compared with the $ABCA1_{644}$ construct (Fig. 3D). Furthermore, transfection of the $ABCA1_{644}$ or $ABCA1_{1694}$ promoter-reporter constructs resulted in a 2- or 3-fold higher level of transcriptional activity, respectively, in CCTα-overexpressing cells compared with control cells (Fig. 3D). As a control, we exposed cells to 22HC and RA after cellular transfection with either the $ABCA1_{1694}$ or $ABCA1_{644}$ construct and assayed promoter activity. As expected, the LXR/RXR ligands, 22HC and RA, selectively activated $ABCA1_{1694}$ promoter activity but did not stimulate $ABCA1_{644}$ activity (data not shown). Thus, the observation that CCTα overexpression resulted in higher ABCA1 promoter activities compared with control cells irrespective of the promoter fragment used suggests that increased PC synthesis activates the ABCA1 gene via an LXR/RXR-independent pathway. Finally, to determine if these observations linking stable CCTα overexpression with ABCA1 expression were unique to the clonal cell line or were a more widespread phenomenon, we transiently transfected two transformed cell lines, A549 and MLE cells, with the $ABCA1_{1694}$ promoter-reporter plasmid with or without cotransfection of a full-length CCTα plasmid. Transient overexpression of CCTα stimulated ABCA1 promoter activity in A549 cells but did not alter thymidine kinase promoter activity (Fig. 3E); similar effects were observed in MLE cells (see below).

PC, generated in response to CCTα overexpression, activates the ABCA1 gene

We hypothesized that lipid products resulting from PC synthesis might activate the ABCA1 gene. We tested this hypothesis by exposing cells to various metabolic intermediates of PC metabolism after performing transient transfections in cells with ABCA1 promoter-reporter constructs to assess gene transactivation. For these studies, we selected MLE cells because at early passages these cells display higher levels of DSPC, have intact responses to surfactant secretagogues, and have higher transfection efficiencies for promoter-reporter constructs compared with A549 epithelia (22). As with A549 cells, overexpression of CCTα in MLE cells increased ABCA1 steady-state mRNA (**Fig. 4A**). Similarly, cells cotransfected with a CCTα plasmid and either the $ABCA1_{644}$ or $ABCA1_{1694}$ promoter-reporter construct showed induction of reporter activity (Fig. 4B). Cells were next transfected with the $ABCA1_{1694}$ fragment and exposed to various metabolites involved in PC synthesis. In contrast to choline, cholinephosphate, CDP-choline, lyso-PC, or GPC, only PC, when present in the context of lipid vesicles, activated ABCA1 promoter activity in a dose-dependent manner (Fig. 4C). Dose-response analysis revealed that PC produced maximal effects on the stimulation of ABCA1 promoter activity at 50 and 30 μM (Fig. 4D, E) using the $ABCA1_{1694}$ and $ABCA1_{644}$ constructs, respectively. Exposure of cells to PC also increased steady-state levels of immunoreactive ABCA1 content and ABCA1 mRNA (Fig. 4F, G). Because the phospholipid used in these experiments (egg yolk) is a mixture of several species, we tested the effects of different PC species varying in fatty acyl composition. Interestingly, unlike DSPC, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (DOPC), or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), only egg yolk PC was observed to produce a 2- to 3-fold increase in $ABCA1_{1694}$ promoter activity (Fig. 4H). Individual fatty acids alone did not activate ABCA1 promoter activity in lung epithelia (Fig. 4I). Deletion analysis using ABCA1 promoter-reporter fragments varying in 5′ extent were tested

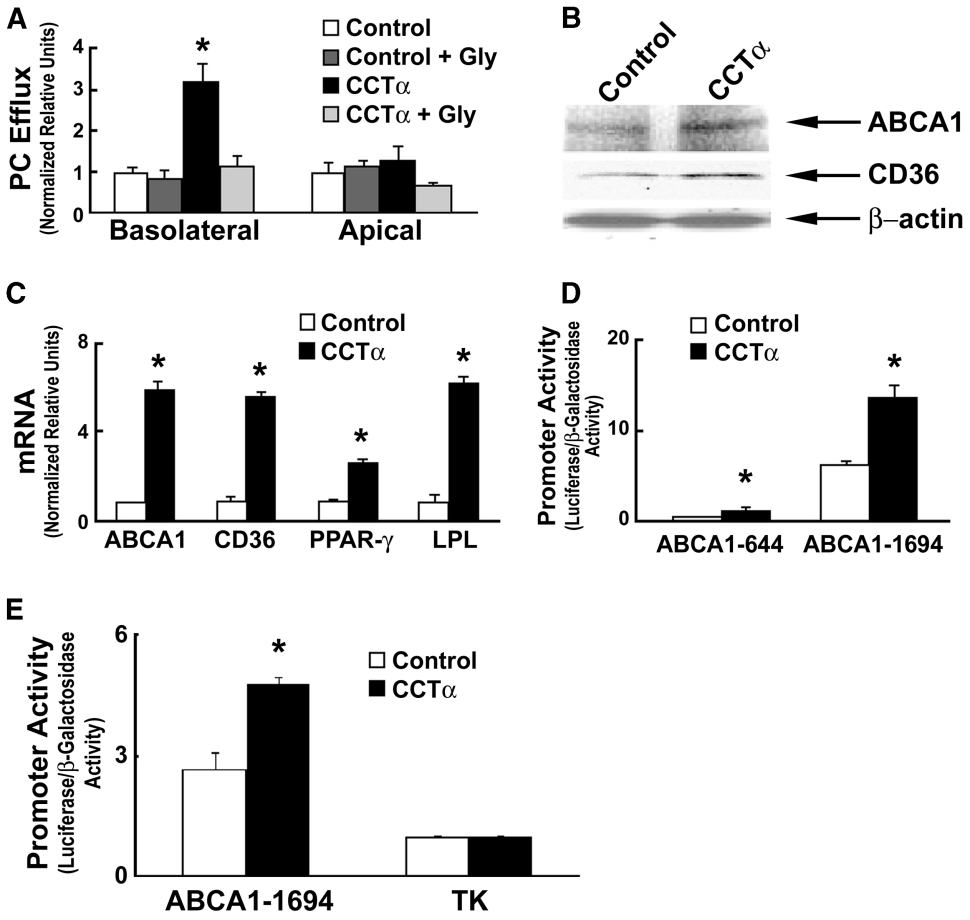

Fig. 3. CCTα overexpression is coupled to ABCA1-driven PC efflux. A: PC efflux within the basolateral and apical routes was assayed in control and CCTα-overexpressing cells (stably transfected) by plating cells on Transwell dishes containing 20 μg/ml HDL and labeling with [^{3}H]choline overnight. Cells were also treated with or without glyburide (Gly; 1 μM), an ABCA1 inhibitor, for 1 h. Data were normalized by correcting the dpm of [^{3}H]PC recovered in medium for total cellular protein in each dish and then expressed as a ratio of average dpm/mg protein value from all experiments in each group. B: Immunoblotting for ABCA1, CD36, and β-actin protein in control and CCTα-overexpressing cells. C: Real-time PCR was performed to determine the levels of ABCA1, CD36, peroxisome proliferator-activated receptor γ (PPAR-γ), and LPL transcripts in cells. Transcripts were normalized for loading using GAPDH as a housekeeping control. D: ABCA1 promoter activity was measured in control and stably transfected CCTα-overexpressing cells after cellular transfection with $ABCA1_{644}$ and $ABCA1_{1694}$ promoter-reporter plasmids, constructs that lack or harbor a liver X receptor/retinoid X receptor (RXR) response element [direct repeat separated by four nucleotides (DR-4)], respectively. ABCA1 promoter-reporter activities were assayed after cotransfection with pSV-β-galactosidase as a control for transfection efficiency. E: A549 cells (nonclonal cells) were transiently transfected with an $ABCA1_{1694}$ or a thymidine kinase (TK) promoter-reporter plasmid with or without cotransfection using a full-length CCTα plasmid. ABCA1 promoter activity was then assayed after controlling for transfection efficiency using pSV-β-galactosidase. Data are presented as means ± SEM (n = 3). * $P < 0.05$ versus control.

for PC sensitivity (**Fig. 5A**). All constructs ranging in size from −1453 to −145/+241 bp exhibited a response to the phospholipid (Fig. 5B). These results demonstrate the existence of a PC-sensitive *cis*-acting positive regulatory element within the core ABCA1 promoter.

Overexpression of ABCA1 increases efflux of unsaturated PC

To examine directly if ABCA1 selectively increases the export of different PC species within alveolar epithelia, we transiently expressed full-length ABCA1 in MLE cells and assayed the relative proportion of DSPC and unsaturated PC within the basolateral medium. Transfection of cells resulted in increased immunoreactive ABCA1 content (**Fig. 6A**). Under baseline conditions, cells constitutively effluxed ∼70% unsaturated PC basolaterally (Fig. 6B). ABCA1 overexpression increased the efflux of total PC ∼2-fold compared with untransfected cells, and stimulatory effects of ABCA1 on DSPC and unsaturated PC efflux were significantly greater than control values (Fig. 6C). Moreover ABCA1 expression increased the percentage of unsaturated PC effluxed into the medium by 94%, whereas DSPC efflux increased 60% versus control, although these differences did not achieve statistical signifi-

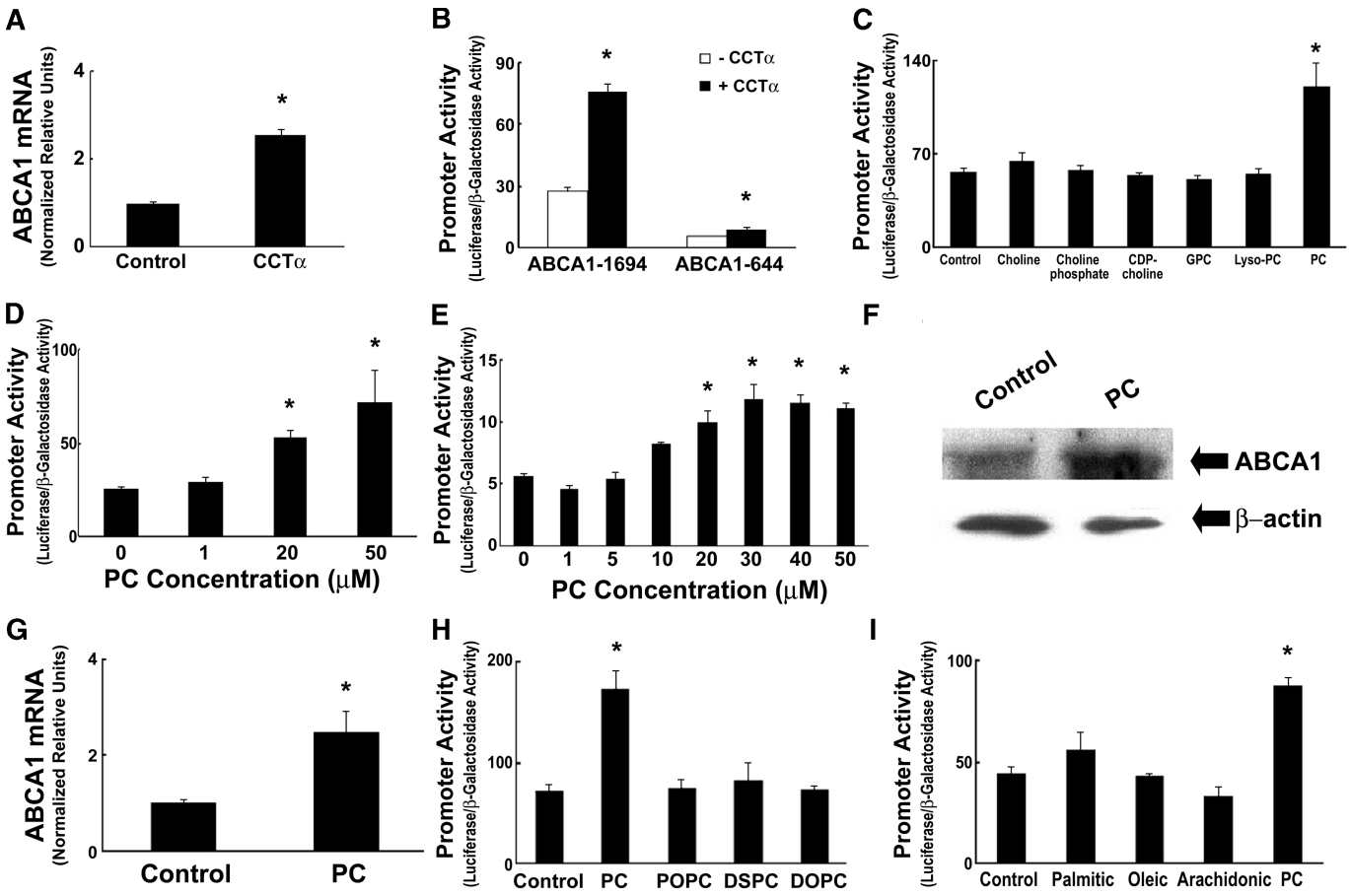

Fig. 4. PC, generated in response to CCTα overexpression, transactivates the ABCA1 promoter. A: MLE cells were transiently transfected with a full-length CCTα plasmid (2 μg), and levels of ABCA1 mRNA were assayed using real-time PCR. Transcripts were normalized for loading using GAPDH as a housekeeping control. B: Cells were transfected with either the $ABCA1_{1694}$ or the $ABCA1_{644}$ promoter-reporter plasmid with or without cotransfection using the CCTα plasmid. ABCA1 promoter activity was then assayed after controlling for transfection efficiency using pSV-β-galactosidase. C: Cells were transfected with the $ABCA1_{1694}$ plasmid and after a 4 h recovery exposed to various lipid and water-soluble intermediates (each at 20 μM) for 24 h before harvesting for analysis of ABCA1 promoter activity. CDP-choline, cytidine diphosphocholine; GPC, glycerophosphocholine. D and E: Cells were transfected with either the $ABCA1_{1694}$ (D) or the $ABCA1_{644}$ (E) promoter-reporter plasmid and after a 4 h recovery period subsequently exposed to various amounts of egg yolk PC vesicles for 24 h, and ABCA1 promoter activity was then assayed. F and G: Cells were exposed to egg yolk PC vesicles (20 μM) for 24 h, and ABCA1 protein (F) and mRNA (G) were determined using immunoblotting and real-time PCR, respectively. H and I: Cells exposed to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), DSPC, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (DOPC), and egg yolk PC (H) or the individual fatty acids palmitic, oleic, or arachidonic acid or egg yolk PC (I) were tested for their ability to activate the ABCA1 promoter as in C. Cells were exposed to 20 μM of each individual lipid in the presence of fatty acid-free BSA for 24 h. Data are presented as means ± SEM (n = 3). Statistical analyses were performed using ANOVA. * $P < 0.05$ versus control.

cance (Fig. 6C). These data indicate that in the native state, lung epithelia secrete predominantly nonsurfactant-associated phospholipids, and ABCA1 significantly augments this effect.

DISCUSSION

These data demonstrate that surfactant phospholipid synthesis is linked to its export by a viable basolateral lipid efflux pathway in lung epithelia. This pathway is mediated, in part, by an exquisite phospholipid-sensing mechanism provided by ABCA1 that appears to serve in an adaptive capacity to eliminate excess cellular PC. Specifically, PC appears to be a critical mediator generated in response to CCTα overexpression, triggering ABCA1 gene activation via *cis*-acting positive regulatory element(s) contained within its promoter. Unlike other ABC-expressing polarized epithelia, our data suggest that this basolateral alveolar efflux pump might have a functionally distinct role by facilitating the export of high levels of unsaturated (nonsurfactant) PC, thereby enriching cells with DSPC, a lipid destined for apical secretion into the surfactant film.

The existence of an ABCA1-driven basolateral PC export pathway in alveolar epithelia is noteworthy for two reasons. First, it expands on the current model whereby surfactant DSPC is initially synthesized in the endoplasmic reticulum, packaged within storage organelles (lamellar bodies), and then extruded into the alveolar lumen by a well-established apical secretory route. A basolateral phos-

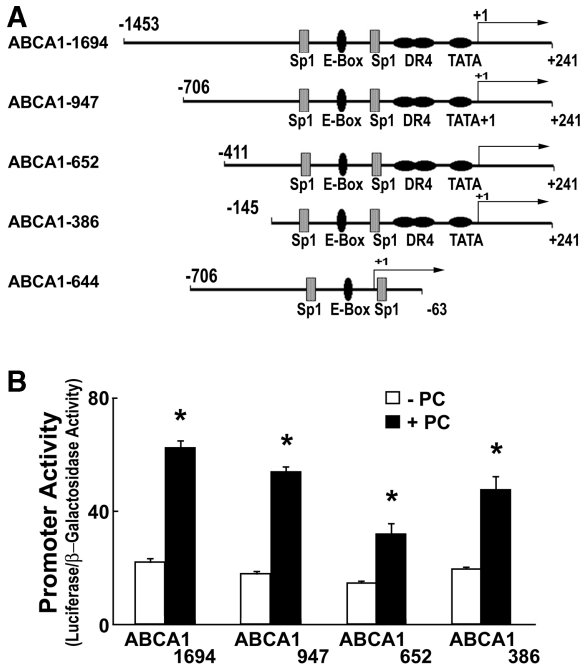

Fig. 5. PC transactivates the ABCA1 core promoter. A: Maps of the ABCA1 promoter deletion constructs. B: Deletion analysis was performed by transfecting various ABCA1 promoter reporter constructs in MLE cells in the presence or absence of 20 μM PC. Data are presented as means ± SEM (n = 3). Statistical analyses were performed using ANOVA. * $P < 0.05$ versus control.

pholipid pathway mediated by ABCA1 could be coordinated with PC synthesis or modulated during pathophysiologic stress, thereby affecting the mass of surfactant lipid destined for apical secretion into the alveolus. The fact that targeted deletion of the ABCA1 gene results in a phenotype of severe respiratory impairment and histological abnormalities of type II cells is suggestive of deleterious effects on surfactant trafficking (17). Second, ABCA1 activity might directly influence the phospholipid composition of alveolar cells. Type II cells are somewhat unique as they are highly enriched with DSPC (24). Presumably, this lipid phenotype results from either increased de novo synthesis of DSPC or remodeling of unsaturated PC molecules to DSPC to achieve a lipid composition characteristic of surfactant (25). Consistent with this, sustained CCTα overexpression increased PC synthesis and cellular DSPC levels (Figs. 2, 3); the increase in DSPC may have been achieved from utilization of diacylglycerol substrates harboring disaturated (palmitic) acyl groups within the de novo pathway or may have resulted from export of unsaturated PC species. Our results show that CCTα overexpression stimulates ABCA1 activity, that lung epithelia normally secrete high levels of unsaturated PC basolaterally, and that this process was facilitated by forced expression of ABCA1. This pathway contrasts with the apical secretion of DSPC-enriched surfactant that is highly conserved across species (26). Taken together, these data suggest that a basolateral export pathway plays a role in modulating lung epithelial PC composition by eliminating a significant proportion of nonsurfactant PC. However, because ABCA1 expression effluxed both DSPC and unsaturated PC in our studies, we speculate that this basolateral route probably serves in an auxiliary capacity to the de novo synthesis and remodeling mechanisms that may provide more rapid modification of PC molecular species. Further studies, however, will be required to elucidate the role of this transporter in regulating lung phospholipid composition within primary isolates of alveolar type II cells and in vivo.

The interdependence of PC export with its biosynthesis as shown here adds a new level of regulation by which type II cells maintain steady-state levels of phospholipid. Upregulation of CCTα activity stimulates PC synthesis but leads to variable levels of PC content in different systems (3, 5, 7–11). Some of these discrepancies in PC metabolism may be attributed to secondary effects after genetic manipulation of CCTα. Alternatively, increased rates of PC synthesis could have simultaneously activated a phospholipid export pathway, providing a plausible explanation for some of the disparities between various studies (5, 7, 9, 10, 27). Upregulation of PC export in response to increased PC biosynthesis might represent a compensatory mechanism to either maintain steady-state levels of phospholipid mass or modify existing lipid composition or a means by which cells escape the lipotoxicity typically seen in other tissues (28).

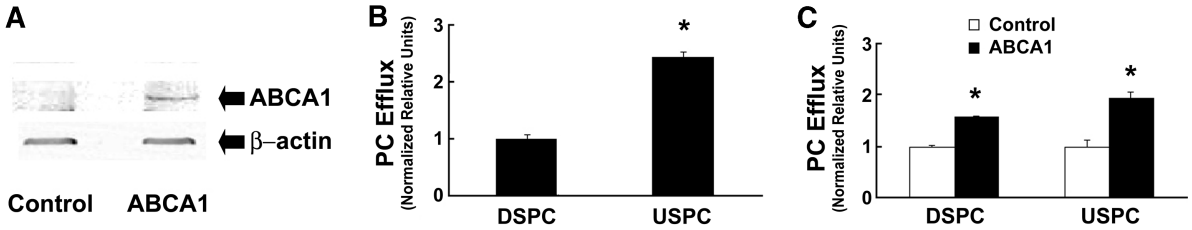

Fig. 6. ABCA1 overexpression effluxes PC. A: Cells were incubated in medium containing 20 μg/ml HDL on Transwell dishes, transfected with or without full-length ABCA1 plasmid (2 μg) for 90 min, cultured for an additional 24 h, and subsequently pulsed with 1 μCi of [^{3}H]choline for the final 2 h of incubation. Cells were harvested from Transwells, and lysates were processed for ABCA1 (above) or β-actin (below) immunoblotting. B and C: Basolateral medium lipids from the experiments shown above were extracted, and radioactivity within effluxed [^{3}H]unsaturated PC (USPC) and [^{3}H]DSPC was determined using TLC under baseline conditions (B; untransfected) or after ABCA1 plasmid transfection (C). Data were normalized by correcting the dpm of [^{3}H]PC recovered in medium for total cellular protein in each dish and then expressed as the ratio of the average dpm/mg protein value from all experiments in each group. Data are presented as means ± SEM (n = 3). * $P < 0.05$ versus control.

We used two complementary cell models to investigate the linkage between PC synthesis and secretion. We specifically exploited A549 cells as they are of human origin, exhibiting lower endogenous CCTα expression and DSPC mass compared with other alveolar-like cell models (data not shown). These cells typically lack lamellar bodies; thus, apical surfactant phospholipid secretion would less likely confound our data analysis of ABCA1 transport activity. We then expanded our studies to a more surfactant-producing type II cell line (MLE) to investigate molecular linkages. Using these models, we observed that CCTα overexpression increased ABCA1 levels but also the expression of other lipogenic genes. Some of these genes, such as CD36 and LPL, are involved in the uptake and internalization of substrates used in PC synthesis. In contrast, ABCA1 is a well-characterized sterol and phospholipid exporter, and some studies suggest that phospholipids may be primary substrates for ABCA1 (12, 29). Although cells constitutively released PC both apically and basolaterally, only the basolateral pathway in CCTα-overexpressing cells was blocked with pharmacologic ABCA1 inhibition or small interfering RNA (30). Thus, ABCA1 mediates CCTα-driven basolateral PC efflux, whereas apical and constitutive basolateral phospholipid export are likely independent of ABCA1.

To examine the molecular basis for CCTα-driven ABCA1 induction, we first attempted to identify biomolecules involved in PC metabolism that might trigger ABCA1 gene activation. Although several water-soluble and lipid species were tested, only PC was observed to activate the ABCA1 promoter. We specifically determined that egg yolk PC containing a mixture of fatty acyl groups effectively activated the ABCA1 promoter, whereas other PCs (DOPC, DPPC, and POPC) did not. This suggests that the molecular presence of PCs harboring a combination of saturated and unsaturated species or yet unidentified structural properties of the lipid is important for optimal ABCA1 gene transactivation. To date, the characterization and regulation of two distinct proximal promoters for ABCA1 have been reported (29, 31). Our $ABCA1_{644}$ construct harbors a weak TATA box (21), lacks a DR-4 element, and shows weak activity. A larger promoter fragment ($ABCA1_{1694}$) was also cloned, in view of studies suggesting different transcription initiation sites (16). This construct harbors a TATA box and a DR-4 element that appears to confer more robust core activity (16, 32). CCTα overexpression activated both ABCA1 promoter-reporter constructs, indicating that increased PC synthesis activates the ABCA1 gene via an LXR/RXR-independent pathway.

We were able to localize PC activation of the ABCA1 gene to its core promoter. Database analysis reveals that aside from the basal transcriptional complex and DR-4 locus, the proximal 5′ flanking region harbors several candidate PC response elements, including two Sp1 or GnC motifs (GGGGCGGGGA, binding to Sp1) (32) and a repressor E-box (33). One of the two Sp1 elements, located at −157 bp within $ABCA1_{1694}$, appears to be a negative regulatory element. The other Sp1 site (+12 bp) is involved in RA-induced ABCA1 gene activation (32, 33). Transcriptional activation of the ABCA1 gene by either PC loading or CCTα overexpression is likely indirect and might involve peroxisome proliferator-activated receptor γ, which modulates ABCA1 expression (34, 35). Whether these or other regulatory elements are involved in the molecular regulation of ABCA1 transcription in response to PC will require further analysis.

In summary, these data provide the first evidence that surfactant phospholipid synthesis is linked to its export via a basolateral lipid efflux route in lung epithelia. This pathway is mediated, in part, via phospholipid transactivation of the ABCA1 gene that appears to autoregulate cellular PC content in response to lipid excess. This pathway might optimize phospholipid composition of type II epithelia by eliminating unsaturated fatty acids, resulting in high-level saturated PC typical of surfactant films; the existence of this pathway also raises the possibility that this homeostatic mechanism might be disrupted during alveolar inflammation, resulting in altered alveolar surfactant composition.

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