Increased Phosphatidylcholine Production but Disrupted Glycogen Metabolism in Fetal Type II Cells of Mice That Overexpress CTP:Phosphocholine Cytidyltransferase*

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CTP:phosphocholine cytidylyltransferase (CCT) is a rate-determining enzyme in the de novo synthesis of phosphatidylcholine (PtdCho). Alveolar type II cells synthesize large quantities of disaturated PtdCho, the surface-active agent of pulmonary surfactant, particularly at late gestation when the lung prepares itself for postnatal air breathing. To clarify the role of CCTα in lung surfactant maturation, we overexpressed CCTα1–367 using the surfactant protein-C promoter. Lungs of transgenic mice were analyzed at day 18 of gestation (term = 19 days). Overexpression of CCTα1–367 increased the synthesis and content of PtdCho in fetal type II cells isolated from the transgenic mice. Also, PtdCho content of fetal lung fluid was increased. No changes in surfactant protein content were detected. Interestingly, fetal type II cells of transgenic mice contained more glycogen than control cells. Incorporation studies with [U-14C]glucose demonstrated that overexpression of CCTα1–367 in fetal type II cells increased glycogen synthesis without affecting glycogen breakdown. To determine which domain contributes to this glycogen phenotype, two additional transgenes were created overexpressing either CCTα1–239 or CCTα239–367. Glycogen synthesis and content were increased in fetal type II cells expressing CCTα239–367 but not CCTα1–239. We conclude that overexpression of CCTα increases surfactant PtdCho synthesis without affecting surfactant protein levels but that it disrupts glycogen metabolism in differentiating type II cells via its regulatory domain.

In dividing cells, large quantities of phosphatidylcholine (PtdCho) are required for membrane synthesis. The lung also requires a steady synthesis of PtdCho for pulmonary surfactant, a lipoprotein that is synthesized and secreted by the alveolar type II epithelial cell into the thin liquid layer that lines the epithelium (1). One of the functions of pulmonary surfactant is to reduce surface tension at the air-liquid interface of the alveoli during expiration. This surface-active function requires sufficient amounts of dipalmitoyl-PtdCho (1). Deficiencies of surfactant have been associated with a variety of lung diseases, including respiratory distress syndrome in premature neonates, which is the most common respiratory disorder of premature infants. The production of surfactant is set into gear during the latter part of gestation (2). How maturing type II cells are able to increase and direct their PtdCho production toward pulmonary surfactant at late gestation without compromising PtdCho demand for membrane biogenesis and homeostasis remains unknown. The major pathway for de novo synthesis of PtdCho in most mammalian cells is the CDP-choline pathway. Output from this pathway is determined by the activity of the rate-limiting enzyme CTP:phosphocholine cytidylyltransferase (CCT), which catalyzes the formation of CDP-choline from phosphocholine (3). The mammalian genome contains two CCT genes that encode four isoforms (4–6). The α isoform (CCTα) is ubiquitously expressed, whereas the three β isoforms (CCTβ1, -2, and -3) are more restricted in distribution (4–6). Most tissues that express a CCTβ also express CCTα. Although the particular role of CCTβ is not understood, the overlapping expression suggests that CCTβ acts to augment PtdCho production in certain cell types. CCTα is the predominant isoform in the fetal and adult lung, and its expression and activity increases during the latter part of gestation (7–9). Structurally, CCTα can be divided into four domains (Fig. 1a). The N terminus contains a well characterized nuclear localization signal (10) and may play a role in dimerization (11). The highly conserved central domain is the catalytic domain (12, 13). This region shares high identity between isoforms and widely divergent species (12, 14). The catalytic domain is flanked on the C terminus by membrane (M) binding and phosphorylation (P) domains. The M region is an extended amphipathic α-helix that promotes reversible interaction with membranes (15). Membrane association of CCTα has been shown to increase catalysis (13, 14, 16). The P domain of the C terminus contains 16 serine and 2 tyrosine residues that are subject to multiple phosphorylations (17, 18). Increased phosphorylation is associated with decreased membrane binding and, therefore, decreased activity (17, 19, 20). Little is known about which kinases and phosphatases target CCTα and what role they play in modulating CCTα activity. The unusual requirements of the developing lung for PtdCho synthesis and, therefore, CCT activity suggest a unique mechanism of regulation of CCTα in differentiating type II cells.

In the present study we hypothesized that an increased CCTα activity in fetal type II cells would accelerate the maturation of the surfactant system in the developing lung. We overexpressed full-length and truncated CCTα isoforms in maturing type II cells using the surfactant protein (SP)-C promoter. There was an enhanced surfactant PtdCho production...
**FIG. 1.** Confirmation of CCTα<sup>1–367</sup> overexpression in mice. 

*a*, schematic diagram of rat CCTα protein sequence and three constructs that were generated based on the rat sequence. **NLS**, nuclear localization signal; **MBD**, membrane binding. 

**b**, *upper panel*, PCR genotyping for SPC-CCTα<sup>1–367</sup> using genomic DNA from mouse tails. The negative control sample was wild type C57BL/6 genomic DNA. The positive control was a C57BL/6 genomic sample spiked with 20 pt SPC-CCTα<sup>1–367</sup> plasmid. 

*Middle panel*, immunoblot of E18 murine lungs using the anti-FLAG antibody. 

*Lower panel*, immunofluorescence image of E18 lung with anti-FLAG antibody and a corresponding differential interference contrast (DIC) image from the same region. Immunolabeling is restricted to alveolar type II cells. **kb**, kilobases.
without a change in surfactant protein levels. However, the most striking feature of these mice was that CCT\textsubscript{o} overexpression led to increased glycogen content in the maturing type II cells. This contrasts the normal decline in glycogen content with increased surfactant synthesis at late gestation (21, 22). The increased glycogen content was dependent on the presence of the regulatory (M and P) domain of CCT\textsubscript{o} and occurred due to an increase in glycogen synthesis. No change in surfactant proteins was noted. Although no overall acceleration in the maturation of the surfactant system was noted, the results corroborate recent findings (29) linking glycogen and surfactant \textit{PtdCho} metabolism in maturing type II cells via CCT\textsubscript{o}.

**Experimental Procedures**

**Materials**—C57BL/6 and SJL mice were obtained from Charles River (St. Constant, Quebec, Canada). Anti-SPA and SPB antibodies were obtained from Chemicon (Temecula, CA). Anti-pro-N-SPC antisemur was generously provided by Dr. M. Beers (University of Pennsylvania, Philadelphia, PA). Epon was purchased from Marivac (Montreal, Quebec, Canada). All culture medium was obtained from Invitrogen. Osmium tetroxide and anti-FLAG M2 antibodies were obtained from Sigma (St. Louis, MO). 100-mesh carbon-coated nickel-plated grids were obtained from Ted Pella (Salinas, CA). Methyl-[\textsuperscript{\textit{U}}-\textsuperscript{14}C]glucose and [\textsuperscript{\textit{U}}-\textsuperscript{14}C]glucose were purchased from Amersham Biosciences.

**Transgene Construction**—Rat CCT\textsubscript{o} (9) was used as the basis to generate three constructs, each augmented with a FLAG sequence (DYKDDDK) at the C terminus. The first construct encompassed the full translated sequence for CCT\textsubscript{o} (CCT\textsubscript{o}-1–367), the second truncated construct contained the N terminus and the catalytic domain (CCT\textsubscript{o}-1–239), and the third construct was composed of the membrane (M) and phosphorylation (P) domains (CCT\textsubscript{o}203–367) (Fig. 1a). The resultant 1.2-, 0.8-, and 0.5-kilobase FLAG-tagged CCT\textsubscript{o} DNA subclones were isolated from the 3'-7.3-kilobase human SPC promoter (25) and 5' of the SV40 small T intron and polyadenylation signal contained within the RSV LTR. The SPC promoter was previously used to express the human \textit{CYP2C11} enzyme in COS-7 cells (25). The FLAG sequence was inserted between the SV40 small T intron and the RSV LTR to block enhancer activity. The primers used were 5'-TCACCTCTGTCCCCTCTCC-

**Production of Transgenic Mice**—Transgenic embryos were generated according to Hogan et al. (34). DNA fragments into the pronuclei of (C57BL/6 × SJL) F2 embryos were carried out at a concentration of 3 ng/µl. The genotype was established by PCR analysis of genomic DNA extracted from the embryonic tail (Fig. 1b) and confirmed by Southern blot analysis. The primers used were 5'-TGGCCGTCTTCTCTCCCTTCCCTCCTAG-3' (SPC primer for 5') and either 5'-TGGCCGTCTTCTCTGGTGTA-TCATTAT-3' (CCT\textsubscript{o}-1–367 primer for 3'), 5'-GGGGCCGGTTCTCTCCTCCT-CCAT-3' (CCT\textsubscript{o}-1–239 primer for 3'), or 5'-TGAACAGACTGTAGTGTA- GGA-3' (CCT\textsubscript{o}203–367 primer for 3'). The PCR conditions were 90 °C for 10 min, 58 °C for CCT\textsubscript{o}-1–367, 55 °C for CCT\textsubscript{o}203–367, and 53 °C for CCT\textsubscript{o}-1–239. A double wash distilled water to remove the excess stain. Samples were then rinsed 3 times in PBS and exposed to 1% (w/v) osmium tetroxide for 1 h followed by another three rinses with PBS. The samples were then dehydrated through an ascending alcohol series ending in propylene oxide. Propylene oxide was then exchanged with an increasing concentration of Epon (Marivac, St. Laurent, Quebec, Canada) until the samples were fully infiltrated with 100% Epon. Samples were placed in molds, and the Epon was polymerized at 70 °C overnight. For immunogold electron microscopy the tissue was processed as described previously (29). Ultrathin sections of the resulting blocks were cut using a diamond knife on a Reichert Ultracut microtome to gold thickness and stained with uranyl acetate and lead citrate. Immunogold labeling was performed as previously (29) using a 1:200 diluted anti-FLAG antibody (Sigma) followed by a 1:300 diluted 10-nm gold-conjugated goat anti-mouse IgG (Nanoprobes, Yaphank, NY). All samples were analyzed by transmission electron microscopy (TEM).

**Immunoblot Analysis**—Three CCT\textsubscript{o} positive and three negative E18 lungs were rinsed with PBS and lysed in homogenization buffer using a Dounce homogenizer. Homogenates were diluted in Laemmli loading buffer to a final concentration of 25 µg/ml protein and then boiled for 5 min. Samples (60 µg) were subjected to SDS-PAGE on a 10% (w/v) polyacrylamide gel and then electrophoretically transferred to a nitrocellulose membrane. Nonspecific binding was blocked by incubating the nitrocellulose membrane with 3% (w/v) dry skim milk in Tris-buffered saline at 4 °C for 60 min, and the membrane was then treated with the specified primary antibody (see “Results”). After overnight incubation at 4 °C, the nitrocellulose membrane was washed 3 times with Tris-buffered saline plus % Tween 20 followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000 diluted) at room temperature. The membranes were then washed thoroughly with cold Tris-buffered saline plus Tween 20 (5 × 5 min), and bands were visualized using an enhanced chemiluminescence detection kit (Amer sham Biosciences).

**Cytidyltransferase Assay**—Lungs were collected and homogenized in homogenization buffer of 145 mM NaCl, 50 mM Tris-HCl (pH 7.4), 50 mM NaF, and 2.5 mM EDTA. Postmitochondrial supernatant and membrane-rich and cytosolic fractions were obtained as previously described (31, 32).

**Measurement of Choline-containing Metabolites**—The aqueous phase remaining after lipid extraction from fetal lung tissue was used to determine the choline metabolites (33). Briefly, a 200-µl aliquot of the aqueous layer was spiked with 5 nm deuterated choline and phosphocholine and subjected to liquid chromatography on an Altrec Absorbosphere silica column fitted with a Altrec Solvent Miser silica guard column (Deerfield, IL), and peaks were analyzed by an API4000 triple-quadrupole mass spectrometer (MDS SCIEX, Concord, ON, Canada) using multiple reaction monitoring and quantified by Analyst 1.2 software (MDS SCIEX).

**Liquid Chromatography—**Liquid Chromatography—E18 pregnant mice were euthanized with diethyl ether, and fetuses were extracted with caesarian section. With the aid of a dissecting microscope, a thoracotomy was performed to expose the lungs, and a 30-gauge needle (with a blunted tip) was inserted through a tracheostomy. The fluid-filled lungs were lavaged with 50 µl of PBS containing 0.05 mg/ml 70-kDa dextran-FITC (PBS/dextran-FITC) (Molecular Probes, Eugene, OR). The recovered lung liquid was diluted with PBS/dextran-FITC to a final volume of 200 µl.

**Electron Microscopy**—Lung tissue removed from fetuses was rinsed in 1 unit heparin in PBS to remove blood and minced in 1-mm pieces. For routine electron microscopy the tissue was fixed for 1 h in 4% (w/v) paraformaldehyde and 1% (w/v) glutaraldehyde in PBS. Tissues were then rinsed 3 times in PBS and exposed to 1% (w/v) osmium tetroxide for 1 h followed by another three rinses with PBS. The samples were then dehydrated through an ascending alcohol series ending in propylene oxide. Propylene oxide was then exchanged with an increasing concentration of Epon (Marivac, St. Laurent, Quebec, Canada) until the samples were fully infiltrated with 100% Epon. Samples were placed in molds, and the Epon was polymerized at 70 °C overnight. For immunogold electron microscopy the tissue was processed as described previously (29). Ultrathin sections of the resulting blocks were cut using a diamond knife on a Reichert Ultracut microtome to gold thickness and stained with uranyl acetate and lead citrate. Immunogold labeling was performed as previously (29) using a 1:200 diluted anti-FLAG antibody (Sigma) followed by a 1:300 diluted 10-nm gold-conjugated goat anti-mouse IgG (Nanoprobes, Yaphank, NY). All samples were analyzed by transmission electron microscopy (TEM).
A standard curve was generated using PBS/dextran-FITC and PBS alone and compared with lung liquid samples to correlate loss of FITC signal with a volume of diluted PBS/dextran-FITC. Samples were measured fluorometrically using a Molecular Devices SpectraMax Gemini electron microscope (Sunnyvale, CA). This provided an accurate measurement of the volume of fetal lung liquid obtained by the lavage method.

**Laser Capture Microscopy (LCM)**—Optimal cutting temperature compound frozen lung sections from control and transgenic mice were fixed with 75% (v/v) ethanol, rehydrated, stained with rabbit pro-SPC antibody diluted in normal goat serum followed by FITC-anti rabbit IgG, and then rehydrated. Alveolar II cells identified by pro-SPC immunofluorescence were dissected using a PixCell II System (Arcturus Engineering, Mountain View, CA), and the RNA was extracted. For mass spectral analysis of PtdCho, sections were fixed with 3% (w/v) paraformaldehyde in PBS for 5 min. These sections were then rinsed in distilled water for 5 min. Excess water was removed, and the tissue was rapidly frozen on dry ice. Tissues were thawed in distilled water and stained with rabbit pro-SPC antibody diluted in 5% (w/v) bovine serum albumin followed by FITC-anti rabbit IgG. The tissue was washed, rapidly frozen on dry ice, and immediately freeze-dried for 1 h. Alveolar type II cells were captured as described above.

**Real-time Reverse Transcription-PCR**—Total RNA was extracted from the LCM-captured type II cells using the PicoPure RNA isolation kit (Arcturus Engineering). After DNase I treatment, total RNA was reverse-transcribed using random hexamers (Applied Biosystems, Foster City, CA). The resulting templates (20 ng of cDNA for our target genes and 2 ng for 18 S) were quantified by real-time PCR (ABI Prism 7700, Foster City, CA). Primers and TaqMan probes for CCT genes and 2 ng for 18 S) were quantified by real-time PCR (ABI Prism 7700, Foster City, CA). A dilution series determined the efficiency of amplification, allowing the relative quantification method to be employed (34). For relative quantitation, PCR signals were compared between groups after normalization using 18 S as an internal reference. Fold change was calculated according to Livak and Schmittgen (34).

**Mass Spectral Analysis of PtdCho**—Homogenized tissues, fetal lung liquid material, or microdissected type II cells were spiked with 2.5 nm deuterated dipalmitoyl-PtdCho as an internal standard and then extracted by a two-step Bligh and Dyer (26). For lipid analysis the chloroform layers were removed and dried under nitrogen gas. Samples were then resuspended in 200 μl of 3:1 chloroform/methanol, and 30 μl was injected by auto-sampler into an API4000 triple-quadrupole mass spectrometer (MDS SCIEX). Individual PtdCho species were detected at M/z 55949/H9251 and M/z 55969/H9251 and identified by tandem mass spectrometry of PtdCho, sections were fixed with 3% (w/v) paraformaldehyde in PBS for 5 min. These sections were then rinsed in distilled water for 5 min. Excess water was removed, and the tissue was rapidly frozen on dry ice. Tissues were thawed in distilled water and stained with rabbit pro-SPC antibody diluted in 5% (w/v) bovine serum albumin followed by FITC-anti rabbit IgG. The tissue was washed, rapidly frozen on dry ice, and immediately freeze-dried for 1 h. Alveolar type II cells were captured as described above.

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**Statistics**—All values are shown as the means ± S.E. Statistical analysis was done by Student’s t test or, for comparison of more than two groups, by one-way analysis of variance followed by Duncan’s multiple-range comparison test, with significance defined as *p < 0.05.**

### RESULTS

**CCTa Expression in Transgenic Mice Lungs**—To establish a role for CCTa in regulating pulmonary surfactant formation during late fetal development, we expressed FLAG-tagged full-length CCTa1–367 in lung epithelial type II cells using the SPC enhancer/promoter. Western blot analysis using monoclonal FLAG M2 antibodies demonstrated exogenous CCTa-FLAG expression in the E18 transgenic lungs, whereas no expression was noted in the lungs of littermate wild type controls (Fig. 1b). As anticipated, CCTa-FLAG protein expression at E18 was restricted to the distal lung epithelial cells of the transgene (Fig. 1b). Overexpression of full-length CCTa in distal lung epithelial cells caused no embryonic or postnatal lethality. The transgenic mice were in no obvious distress. No differences were observed in body or lung weight between wild type and transgenic mice (results not shown).

**CCT Activity in CCTa1–367 Transgenic Lungs**—E18 fetal lungs were fractionated by centrifugation, and CCT activity was measured. The postmitochondrial (3,000 × g) and membrane-enriched (300,000 × g) pellet of transgenic lungs displayed a significantly greater CCT activity than those of littermate control lungs (Fig. 2a). Examination of the soluble choline metabolites from the fetal lung tissue indicated that there was a significant decrease in phosphocholine content of E18 transgenic lungs compared with littermate controls with no significant changes in the amount of choline and CDP-choline (Fig. 2b). This increased consumption of phosphocholine supports the idea of an increased flux through the reaction catalyzed by CCTa. The amount of glycerophosphocholine, a breakdown product of PtdCho, was not different between transgenic and control lungs (Fig. 2b).

**Choline Incorporation and PtdCho Content in Type II Cells of CCTa1–367 Transgenic Lungs**—Because the SPC promoter restricts CCTa protein expression to the distal epithelial cells of the lung, we measured radioactive choline incorporation into PtdCho and PtdCho content in E18 epithelial type II cells (Fig. 3). First, we found using laser capture microscopy (Fig. 3a) and real-time PCR (Fig. 3b) that E18 type II cells of transgenic mice had a 6-fold greater CCT mRNA expression than E18 type II cells from control mice. For [methyl-3H]-choline incorporation studies, multiple litters had to be pooled to provide sufficient cell numbers. Progeny of CCTa negative parents produced wild type (control) cell cultures, whereas progeny of homozygous transgenic males and negative females were used to obtain enriched fetal epithelial type II cell cultures in which 100% of the epithelial cells overexpressed CCTa. In agreement with the aforementioned increased mRNA expression and CCT activity, choline incorporation into total lipids of E18 type II cells isolated from the transgenic mice was significantly (>6-fold) greater than that of E18 control type II culture cells (Fig. 3c). In previous studies we have found that >90% of the radioactive choline incorporated in total lipids is incorporated into PtdCho (36). To determine whether this increase in PtdCho synthesis led to increased PtdCho content, we quantitatively assessed PtdCho in LCM-dissected E18 type II cells using tandem mass spectrometry. In line with the increased PtdCho synthesis, E18 type II cells of transgenic mice had an ~2-fold greater dipalmitoyl-PtdCho content compared with cells of control mice (Fig. 3d).

**PtdCho Content in CCTa1–367 Transgenic Lungs**—To determine whether the increase in CCTa expression and activity of E18 type II cells also resulted in an increase in surfactant PtdCho content, we isolated fetal lung liquid fluid from E18 mice and with the aid of tandem mass spectrometry quantitatively analyzed the PtdCho molecular species of both fetal lung liquid and residual lung tissue (Fig. 4). Although a moderate increase in dipalmitoyl-PtdCho content was observed in whole fetal lung tissue of mice overexpressing CCTa (Fig. 4b), no significant difference in total PtdCho content was noted between the CCTa transgenics and their wild type siblings (Fig. 4a). Conversely, lung liquid fluid of E18 transgenes displayed a significant increase in the amount of all major PtdCho species when compared with wild type (control) siblings (Fig. 4, a and b). However, the PtdCho species composition in the fetal lung fluid of CCTa transgenics was not different from that of control siblings (Fig. 4c). Fetal lung fluid of control and transgenic mice alike contained larger amounts of palmitoylmyristoyl (16:0/14:0)-PtdCho and palmitoylpalmitoyl (16:0/16:0)-PtdCho than expected, based on published adult rat surfactant PtdCho values (37). Fetal lung tissue and lung liquid were also examined for phosphatidylglycerol and phosphatidylinositol content and composition, but no significant differences were observed between the transgenic and control mice (not shown).

**Surfactant Proteins in CCTa Transgenic lungs**—To examine if CCTa overexpression in lung epithelial type II cells had any impact on SP production, we measured SPA, SPB, and pro-SPC content by immunoblotting (Fig. 5). Because the surfactant proteins are mainly expressed by distal lung epithelial cells (38), surfactant proteins were only assessed in E18 whole lung
No significant difference in protein content was observed for any of the three surfactant proteins between the transgenic mice and littermate controls.

Type II Cell Development in CCTα Transgenic Lungs—
The most striking morphological feature of differentiating type II cells at late fetal gestation is the decrease in glycogen content.
and a concomitant increase in the number and size of lamellar bodies, the intracellular storage organelles of surfactant (2, 27). To investigate whether overexpression of CCTα/H92511–367 had any ultrastructural effects on differentiating type II cells, E18 mouse lungs were fixed, processed, and examined by transmission electron microscopy. Contrary to the normal decline in glycogen content at late gestation, CCTα/H92511–367 overexpression led to increased glycogen content in differentiating type II cells (Fig. 6b). However, secreted surfactant material (lamellar bodies and tubular myelin) was clearly visible in the fluid-filled alveolar spaces of the CCTα/H9251 transgene (Fig. 6b), suggesting that surfactant maturation was not delayed. Biochemical assessment of glycogen content confirmed that E18 CCTα/H92511–367 transgenic lungs contained significantly more glycogen than E18 wild type control lungs (Fig. 6c). Additionally, labeling studies showed that [U-14C]glucose incorporation into glycogen of E18 epithelial type II cells isolated from SPC-CCTα/H92511–367 transgenic lungs was significantly greater than that of wild type control cells (Fig. 7a).

PtCho and Glycogen Metabolism in CCTα/H92511–239 and CCTα/H9251203–367 Mutant Lungs—The glycogen accumulation in differentiating type II cells of SPC-CCTα/H92511–367 transgenes could be a direct result of increased CCTα activity. Alternatively, it could be the result of an excess of CCTα protein and, therefore, CCTα phosphorylation sites, which interfere with the phosphorylation/dephosphorylation regulation of glycogen metabolism. Interestingly, exogenously expressed CCTα localized predominantly to the glycogen stores in E18 fetal type II cells (Fig. 7b). The glycogen stores of fetal type II cells are also important cellular sites for endogenous CCTα (29), implicating a direct linkage of CCTα and glycogen. To test the aforementioned possibilities, two additional transgenes were created overexpressing truncated CCTα proteins in the differentiating type II cells. One protein (CCTα/H92511–239) consisted of the N terminus and catalytic domain but lacked the regulatory M and P domains. This truncated CCTα protein is thought to be constitutively active (39). The second construct (CCTα/H9251203–367) contained the regulatory M and P domains but lacked the catalytic domain.

**FIG. 3.** CCTα mRNA and PtCho analysis of type II cells of SPC-CCTα/H92511–367 mice. a, an example of laser captured E18 type II cells identified with pro-N-SPC antibody. Top panels from right to left, a rhodamine-positive E18 type II cell before capture and after capture. Bottom panels from right to left, tissue remaining on the slide after removal of the LCM cap and captured E18 type II cell on the cap. b, relative abundance of CCTα transcript in LCM captured type II cells from E18 transgenic and control mice as determined by real-time PCR. c, incorporation of [methyl-3H]choline into PtCho by type II cells isolated from E18 transgenic and control mouse lungs. d, dipalmitoyl-PtCho content in LCM captured type II cells from E18 CCTα overexpressors and negative littermates. Open bars are control mice, and closed bars are CCTα/H92511–367 transgenic mice. n = 3; **, p < 0.01.
Both constructs included a FLAG epitope, and the expression of the truncated proteins in the E18 transgenic lungs was confirmed by immunoblotting using an anti-FLAG antibody (Fig. 6a). Both CCTα mutants were analyzed at 18 days of gestation for CCT activity and PtdCho content in whole lung tissue and bronchiolar alveolar fluid. No increase in CCT activity compared with control siblings was noted (Fig. 6a). Also, no significant difference in PtdCho content of fetal lung liquid was
observed between CCT<sup>1–239</sup> and CCT<sup>203–367</sup> mutant mice and their littermate controls (Fig. 6d). E18 lungs of CCT<sup>1–239</sup> transgenes had a similar ultrastructural appearance as their control siblings. In contrast, CCT<sup>203–367</sup> transgenes showed an increased glycogen deposition in E18 type II cells (Fig. 6b), and biochemically, the lungs of these mice showed a modest but significant increase in glycogen content (Fig. 6c). In addition, E18 type II cells isolated from CCT<sup>203–367</sup> transgenes incorporated significantly greater amounts of [U-<sup>14</sup>C]glucose into glycogen relative to epithelial type II cells of CCT<sup>1–239</sup> transgenic lungs or nontransgenic lungs (Fig. 7a).

**Glycogen Metabolism in CCT<sup>1–367</sup> Mutant Lungs**—Initially, we assessed the incorporation of [U-<sup>14</sup>C]glucose into glycogen over a 24-h period (Fig. 7a). Such a long labeling period does not differentiate between glycogen synthesis and degradation. To determine whether CCT<sup>1–367</sup> overexpression increased the rate of glycogen synthesis, we pulse-labeled type II cells isolated from E18 control and SPC-CCT<sup>1–367</sup> mice for 2–4 h with radioactive glucose. As shown in Fig. 7d, the rate of [U-<sup>14</sup>C]glucose incorporation into glycogen was significantly increased in the cells overexpressing CCT<sup>1–367</sup> when compared with wild type control cells. In contrast, no significant differences in glycogen degradation were observed between wild type control and CCTeta-overexpressing cells (Fig. 7d). Glycogen synthase activity was determined in whole lung homogenates and type II cells isolated from E18 control and SPC-CCT<sup>1–367</sup> mice. Both whole lung homogenate and isolated type II cells of the CCT<sup>1–367</sup> transgene had significantly greater GS activity relative to littermate controls (Fig. 7e). Together, the data suggest that overexpression of CCTeta affects the glycogenic pathway, thereby increasing the glycogen content of the fetal type II cells.

**DISCUSSION**

Herein, we demonstrate that overexpression of CCTeta in fetal distal epithelial cells results in an increased surfactant PtdCho formation without affecting surfactant protein levels. Most interestingly, CCTeta overexpression led to increased glycogen content in the maturing type II cells, which contrasts with the normal decline in glycogen content and increased surfactant PtdCho synthesis at late gestation. The increased glycogen deposition appeared to be dependent on the presence of the regulatory domain of CCTeta. The increased glycogen content was due to an augmented glycogen synthesis. Earlier studies have suggested a precursor-product relationship between glycogen and surfactant production during development (27, 40, 41), and recently we have shown that endogenous CCTeta localizes within the glycogen pools of maturing type II cells (29). The data herein strengthen the idea that CCTeta provides a link between glycogen and surfactant PtdCho metabolism in differentiating type II cells.

Although transgenic mice overexpressing CCTeta in epithelial type II cells using the SPC promoter have been created, those mice were only investigated for their effect on the adult surfactant system (35). In agreement with our findings, the lungs of the transgenic mice exhibited a 6–7-fold greater expression of CCTeta relative to wild type controls (35). The authors reported that the rate of disaturated PtdCho synthesis was significantly increased in adult type II cells isolated from the transgenic mice but that the disaturated PtdCho content of alveolar lavage and lung tissue did not differ between transgenic and control mice. This suggests that the increase in surfactant PtdCho synthesis may be counterbalanced by an increase in surfactant PtdCho degradation. In contrast to adult lung, we found that the increase in the rate of PtdCho synthesis in fetal type II cells from transgenic mice was accompanied by an increase in (disaturated) PtdCho content in whole lung tissue, dissected type II cells, and lung liquid fluid. Given that macrophages are involved in surfactant clearance (42), it is plausible that the excess PtdCho content generated in the postpartum lungs was consumed by resident macrophages that are not present in the fetal lung. In the present study, we observed that the content of glycerophosphocholine, a PtdCho degradation product, did not significantly differ between transgenic and control mice, suggesting that overexpression of CCTeta in fetal type II cells does not increase PtdCho degradation.

Mass spectral analysis showed a significant increase in PtdCho content in the fetal lung liquid of the transgenic mice; however, no significant change in species distribution was observed. Therefore, although PtdCho synthesis was increased, the mechanisms involved in generating the PtdCho species profile remained unaffected. Independent of CCTeta overexpression, the concentrations of palmitoylmyristoyl-PtdCho and palmitoylpalmitoleoyl-PtdCho were much higher in the fetal mouse lung liquid than the reported values for adult rat lung lavage fluid (10% 16:0/14:0-PtdCho and 30% 16:0/16:1-PtdCho versus 4% 16:0/14:0-PtdCho and 10% 16:0/16:1-PtdCho) (37). A postpartum decrease of both PtdCho species has been reported for rodent and pig surfactants (43). The content of both PtdCho species was also significantly higher in the fetal murine lung liquid than that of newborn rat (43), suggesting that they may play an important role at term when the air/fluid interface is first established.
FIG. 6. Glycogen and PtdCho content of SPC-CCTα<sup>1–367</sup>, SPC-CCTα<sup>1–239</sup> and SPC-CCTα<sup>203–367</sup> mice. 

a, left panel, immunoblot of lungs from E18 CCTα<sup>1–239</sup> (28 kDa) and CCTα<sup>203–367</sup> (15 kDa) transgenic mice and littermate controls using the anti-FLAG antibody. n = 3 separate mice. Right panel, CCTα activity in lung tissue of E18 CCTα<sup>1–367</sup>, CCTα<sup>1–239</sup>, and CCTα<sup>203–367</sup> transgenic mice and littermate controls. 

b, representative ultrastructural electron micrographs of E18 lung tissue. Glycogen stores are marked by an asterisk. Scale bar = 200 nm. 

c, total PtdCho content of fetal lung fluid. Open bars are control mice, and closed bars are CCTα transgenic mice. n = 4; **, p < 0.01.
FIG. 7. Glycogen metabolism of type II cells isolated from SPC-CCTα1–367 mice, SPC-CCTα1–239, and SPC-CCTα203–367 mice. 

(a) Incorporation of [U-14C]glucose into glycogen over a 24-h period in isolated type II cells from E18 transgenic and control mice. Open bars are control mice, and closed bars are either SPC-CCTα1–367, SPC-CCTα1–239, or SPC-CCTα203–367 transgenic mice. 

(b) Immunogold localization of the exogenously expressed FLAG-tagged CCTα in a type II cell from an E18 SPC-CCTα1–367 transgenic mouse lung using a monoclonal FLAG antibody. Circles indicate gold particles. The scale bar equals 100 nm. 

(c) Time course of 14C glucose incorporation into glycogen (dpm/cell) over 2–4 h in isolated type II cells from E18 CCTα1–367 (black circles) and control mice (black squares). Cells were labeled for 2 h with 5 μCi/ml of [U-14C]glucose, washed, and incubated with fresh medium. At the indicated time intervals, incubations were terminated, and the disappearance of 14C-labeled glycogen was measured. 

(d) Pulse-chase study monitoring loss of 14C-labeled glycogen in isolated type II cells from E18 CCTα1–367 (black circles) and control mice (black squares). Cells were labeled for 2 h with 5 μCi/ml of [U-14C]glucose, washed, and incubated with fresh medium. 

(e) Glycogen synthase activity in whole lung tissue and isolated type II cells from E18 CCTα1–367 (black bar) and control C57Bl/6 mice (white bar). 

n = 4–8; *, p < 0.05.
The increase in PtdCho content in the lung liquid fluid was significantly greater than that of type II cells or lung tissue, suggesting an activation of the apical secretory pathway. How the type II cell senses the excess of PtdCho and stimulates its apical efflux remains to be investigated.

No significant change in content and composition of fetal lung liquid was observed for phosphatidylglycerol or phosphatidylserine, indicating that other lipid synthetic pathways were unaffected by the increase in surfactant PtdCho production. Interestingly, the large increase in pulmonary surfactant PtdCho content within and outside (fetal lung liquid) type II cells also did not affect the expression levels of pulmonary surfactant proteins A, B, or C. Similar observations have been made in transgenic mice, where surfactant protein levels were altered without affecting surfactant PtdCho levels (44–48). Thus, although both surfactant lipid and protein components are closely regulated during development (49), there appears to be no cross-regulation between the surfactant protein and lipid biosynthetic pathways.

Our findings of high glycogen content occurring concurrently with high PtdCho production were unexpected since previous morphological studies had suggested an opposite correlation, i.e. glycogen depletion with increased surfactant production (21, 22). We suspected that the increase in glycogen content was not the direct result of increases in CDP-choline production and speculated that the excess of phosphorylation targets in the CCTα transgenes (14-fold increase in CCTα expression relative to littermate controls) interfered with the coordinated phosphorylation-dephosphorylation control of glycogen metabolism in the fetal lung. To test this possibility we generated two transgenic mice, where surfactant PtdCho levels were unaffected by the increase in surfactant PtdCho production. The localization of exogenously expressed CCTα to the glycogen stores in the fetal type II cells agrees with an interaction of CCTα with glycogen-metabolizing enzymes. Recent immunogold data have revealed a similar cellular localization of endogenous CCTα to the glycogen deposits in fetal type II cells (29), supporting the idea of a physiologic linkage between glycogen and surfactant PtdCho via CCTα during gestation. The current data indicate that this relationship is more than glycogen utilization. It would appear that CCTα may also play a role in regulating glycogen production in the type II cells.

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