Role of Phosphocholine Cytidylyltransferase α in Lung Development[∇][†]

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Lung development depends upon the differentiation and expansion of a variety of specialized epithelial cell types, including distal type I and type II pneumocytes in the late term. Previous studies have shown a strict dependence on the choline cytidylyltransferase α isoform (CCTα) to mediate membrane phospholipid formation in cultured cells and during preimplantation embryogenesis. CCTα expression is highest in lung, and there has long been speculation about its precise role, due to the dual requirement for phospholipid in proliferating cell membranes and for lung surfactant production from alveolar type II cells. We investigated the function of CCTα in lung development, using an inducible, epithelial cell-specific CCTα knockout mouse line. Deletion of CCTα beginning at embryonic day 7.5 did not restrict lung development but resulted in severe respiratory failure at birth. Alveolar lavage and lung lipid analyses showed significant decreases in the major surfactant phospholipid, dipalmitoyl-phosphatidylcholine. The fatty acids destined for the surfactant phospholipid were redirected to an expanded triglyceride pool. Transcripts encoding type II cell-specific markers were expressed in the knockout mice, indicating the expected progression of differentiation in lung epithelia. However, surfactant protein levels were reduced, with the exception of that for surfactant protein B, which was elevated. Ultrastructural analysis of the type II cells showed Golgi complex abnormalities and aberrant lamellar bodies, which deliver surfactant lipid and protein to the alveolar lumen. Thus, CCTα was not required for the proliferation or differentiation of lung epithelia but was essential for the secretory component of phospholipid synthesis and critical for the proper formation of lamellar bodies and surfactant protein homeostasis.

The CDP-choline pathway is the predominate route to phosphatidylcholine (PtdCho) in most tissues (18, 37). The choline cytidylyltransferase α (CCTα) protein is the major isoform that governs this pathway, while CCTβ2 and CCTβ3 mRNAs are usually expressed at 10- to 30-fold-lower levels (19). Loss of CCTα expression is lethal at day 3.5 of embryonic development (E3.5), and embryos fail to form blastocysts (40). These data illustrate a strict dependence on CCTα expression early in embryogenesis beyond the first few rounds of cell division following fertilization, which are otherwise supported by maternal CCTβ expression in the unfertilized ova (19). This early embryonic lethality reflects culture systems where cell death occurs when the CCT step is blocked (1, 11, 12, 29). On the other hand, selective deletion of the CCTα gene (*Pcyt1a*) does not inhibit the growth or differentiation of macrophages in vivo, although the viability of the CCTα-deficient macrophages is compromised when the cells are loaded with free cholesterol (45). The deletion of CCTα in hepatocytes also does not impair their development or differentiation, but they are larger, and secretion of high-density lipoprotein and very low density lipoprotein is impaired (21). The apparently normal development of these tissues is somewhat surprising. It may be due to compensation by the upregulation of CCTβ isoforms (21, 45); however, the inability of the hepatocytes with CCTα deleted to

proliferate in vitro suggests that there may be an alternate source of PtdCho that is not recapitulated in culture and that makes the CCTs dispensable in differentiated tissues.

In the lung, PtdCho biosynthesis via the CDP-choline pathway is a major contributor to formation of cell membranes and specialized intracellular lamellar bodies. The lamellar bodies found in type II epithelial cells are derived from lysosomes and contain dipalmitoyl-PtdCho, the major phospholipid component of lung surfactant (30, 31, 36). Expression of the CCTα isoform predominates in lung tissue, and the CCTβ mRNAs are expressed at about a 30-fold-lower level (19, 30, 40). There is a small, but significant, reduction in the total PtdCho content of adult lungs in *Pcyt1a*$^{+/-}$ heterozygous mice, whereas the PtdCho levels in other adult tissues are comparable to those of the wild-type littermates (19), suggesting that lung tissue may be less resilient to CCTα ablation than hepatocytes or macrophages. Also, the highest relative levels of CCTα mRNA expression occur in lung (19, 30), and in contrast to other cell types where CCTα is primarily nuclear (41), the CCTα protein is cytoplasmic (30) and localizes with developing lamellar bodies found in the glycogen stores of fetal lung type II cells (31). The lamellar bodies contain surfactant proteins (31) along with disaturated PtdCho (14) and are secreted into the alveoli to enable lung expansion and air exchange (14).

Our approach to probing the role of CCTα in lung biology was to derive mice with CCTα deficiency restricted to the lung epithelial cells. This was accomplished using a genetic system that controls Cre recombinase expression in lung epithelial progenitors using the doxycycline (Dox)-dependent human surfactant protein C (SP-C) promoter (27, 28, 43). These studies show that CCTα is not required for PtdCho synthesis sup-

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porting the development of lung epithelia but is essential for the production of secreted surfactant disaturated PtdCho.

MATERIALS AND METHODS

Mouse model. The floxed $Pcyt1a^{fl/fl}$ allele has been described previously (21, 40, 45). Briefly, the $Pcyt1a^{fl/fl}$ mice were generated on a C57BL6/J and 129/Sv mixed background. The strain was then backcrossed onto a C57BL6/J background for six generations and then crossed with the SP-C-rtTA and the $(tetO)_7$CMV-Cre strains. Mice with the SP-C-rtTA and $(tetO)_7$CMV-Cre transgenes (obtained from Jeffrey Whitsett, University of Cincinnati) had an FVB/N background (27, 28). $Pcyt1a^{fl/fl}$ mice were mated with SP-C-rtTA$^{tg/0}$/ $(tetO)_7$CMV-Cre$^{tg/tg}$ mice to produce SP-C-rtTA$^{tg/0}$/$(tetO)_7$CMV-Cre$^{tg/tg}$/ $Pcyt1a^{fl/fl}$ mice, which were maintained in a mixed C57BL6/FVB background for more than five generations. The tetO-Cre transgene and the $Pcyt1a^{fl/fl}$ allele were homozygous, and the SP-C-rtTA transgene was heterozygous, generating knock-out and wild-type progeny in the same litters. Pregnant dams received a chow diet containing 625 mg/kg Dox (Harlan Teklad) for the times indicated to produce SP-C-rtTA$^{tg/0}$/$(tetO)_7$CMV-Cre$^{tg/tg}$/$Pcyt1a^{\Delta/\Delta}$ mice. The SP-C-rtTA, $(tetO)_7$CMV-Cre, $Pcyt1a^{fl}$, $Pcyt1a^+$, and $Pcyt1a^\Delta$ alleles were identified by PCR of genomic DNA as described previously (27, 28, 40). All procedures concerning the care and use of animals were done according to St. Jude Children's Research Hospital ACUC-approved protocols.

Lung preparations. $Pcyt1a^{\Delta/\Delta}$ and control mice were exsanguinated, the lungs were lavaged three times with 0.4 ml phosphate-buffered saline (PBS), and the cell-free supernatant was separated into large and small aggregate fractions by centrifugation. The total protein (7) and phospholipid (9) contents were determined. Real-time quantitative reverse transcription-PCR (qRT-PCR) was done using total RNA isolated from lung with TRIzol reagent (Invitrogen Corp.) as previously described (40). For primer and probe sequences for the surfactant proteins, see Table S1 in the supplemental material. Lipids were extracted from lung by the method of Bligh and Dyer (5) and quantified using Iatroscan instrumentation as previously described (19). Mass spectrometry was performed by the St. Jude Hartwell Center for Biotechnology, using a Finnigan TSQ Quantum mass spectrometer operated in the positive ion mode, using parent ion scanning for PtdCho and neutral loss scanning for phosphatidylethanolamine and triglycerides, as outlined (15, 16).

Microscopy. Lung tissue was fixed in 4% paraformaldehyde, incubated in 30% sucrose overnight at 4°C, embedded in Tissue-Tek optimal-cutting-temperature medium, sectioned at 6 μm, and stained with hematoxylin and eosin, followed by dehydration and mounting. Unstained cryosections were washed, permeabilized with 0.2% Triton X-100 and 0.5% bovine serum albumin in PBS, blocked with 1.5% horse serum for 30 min, and incubated with primary antibodies overnight at 4°C. The primary antibodies were anti-CCTα antibody (1:500; goat polyclonal; Santa Cruz Biotechnology), ProSP-C antibody (1:1,000; rabbit polyclonal AB3428; Chemicon, Temecula, CA), and mature SP-B antibody (1:100; mouse monoclonal AB3282; ABCAM, Cambridge, MA). Sections were incubated with fluorescence-conjugated Alexa 594 or Alexa 488 secondary antibody (Molecular Probes), washed, and mounted. For electron microscopy at the University of Memphis Imaging Facility, lungs were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, postfixed in 0.8% osmium tetroxide–3% ferrocyanide–0.1 M PBS, washed, dehydrated through ethanol and propylene oxide, and embedded in Spurr's epoxy resin. Ultrathin sections (~75 nm) from selected blocks were mounted on 1- by 2-mm grids, stained with uranyl acetate and lead citrate, and examined in a Philips CM10 electron microscope (FEI; Hillsboro, OR) at 50 to 80 kV.

Immunoblotting. Lungs were lysed in a Dounce homogenizer in 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 μM Na_3VO_4, 5 mM NaF, 2% aprotinin, 10 μg of leupeptin/ml, and 1 mM phenylmethylsulfonyl fluoride. Lysate proteins (50 μg) were separated on 12% NuPAGE Bis-Tris gels, transferred to polyvinylidene difluoride membranes, washed in 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.4)–137 mM NaCl–0.1% (vol/vol) Tween 20, rinsed, and incubated with primary antibodies in 1% dry milk at 4°C overnight. The primary antibodies used were CCTα (rabbit polyclonal; 6 μg/ml), SP-A (1:500 dilution; AB3420; Chemicon), SP-B (1:100 dilution; AB3282; ABCAM; 1:5,000 dilution; WRAB-SpB; Seven Hills Bioreagents), SP-C (1:100 dilution; sc-13979; Santa Cruz Biotechnology), and GAPDH ([glyceraldehyde-3-phosphate dehydrogenase] 1:5,000 dilution; AB9485; ABCAM). The membranes were washed, and immunoreactive bands were detected, using the ECF Western blotting kit (Amersham Pharmacia Biotech), and quantified, using a Typhoon 9200 variable mode imager (Molecular Dynamics, Amersham Pharmacia Biotech) and ImageQuant software, version 5.2 (GE Healthcare).

Statistical analysis. The unpaired Student t test was used for statistical analysis, and all the data were calculated and plotted with Graphpad Prism 4 (Graphpad Software). The results are given as means ± standard errors or ± the standard deviation. The results are considered significant at P values of <0.05 or P values of <0.01 (see Fig. 1 to 4).

RESULTS

Deletion of $Pcyt1a$ in the respiratory epithelium. SP-C-rtTA$^{tg/0}$/$(tetO)_7$CMV-Cre$^{tg/tg}$/$Pcyt1a^{fl/fl}$ mice were derived and mated to produce $Pcyt1a^{\Delta/\Delta}$ mice in which $Pcyt1a$ was deleted by activation of Cre recombinase in respiratory epithelial cells by administration of Dox to the pregnant dams. The deletion removed exons 5 and 6, thus inactivating the catalytic function of CCTα, and was documented by PCR analysis of lung genomic DNA with specific primers which yielded a 255-bp product (Fig. 1A). Complete ablation of expression in whole lung was never observed because the gene remained intact in other lineages. SP-C-rtTA$^{0/0}$/$(tetO)_7$CMV-Cre$^{tg/tg}$/$Pcyt1a^{fl/fl}$ littermate embryos were generated in each cross, along with the SP-C-rtTA$^{tg/0}$/$(tetO)_7$CMV-Cre$^{tg/tg}$/$Pcyt1a^{\Delta/\Delta}$ mice, and were used as the experimental controls. There are two genes in the CCT family, $CCT\alpha$ and $CCT\beta$, and a second pathway for PtdCho synthesis via the N methylation of phosphatidylethanolamine (18). Quantification of these four mRNAs in $Pcyt1a^{\Delta/\Delta}$-knockout lungs showed that $Pcyt1a$ mRNA was significantly decreased, but there was no change in expression of the genes that produced CCTβ1, CCTβ2, or phosphatidylethanolamine N-methyltransferase (Fig. 1B).

Lung morphology and epithelial differentiation. The type II pneumocytes are cuboidal cells clustered at the junction between the alveolar ducts and the alveoli that express very high levels of surfactant protein and have large cytoplasmic lamellar bodies. Type I pneumocytes are flattened, do not express surfactant proteins, and lie in close apposition to the capillaries of the alveoli. Histological examination of control mice revealed normal lung morphology with dilated terminal lung, thin interstitial septa, and normal growth of pulmonary capillaries in close proximity to squamous type I respiratory epithelial cells (Fig. 1C). The gas exchange surface was fully formed. Lung pathology in $Pcyt1a^{\Delta/\Delta}$ mice was severe, with focal or extensive atelectasis (Fig. 1C), hyaline membrane formation, and pulmonary congestions. Neonatal $Pcyt1a^{\Delta/\Delta}$ mice were in respiratory distress and moribund. Dilation of the peripheral saccules was reduced, and the septa remained thickened, but the septations appeared normal, and the peripheral saccules were lined with cuboidal type II cells and squamous type I cells.

Immunohistochemistry of control and $Pcyt1a^{\Delta/\Delta}$ mouse lungs at birth (E19.5) confirmed a reduction of CCTα protein in lung epithelium, particularly in the type II epithelial cells which were heavily stained in the control lungs (Fig. 1D). For the control mice, CCTα antibody primarily labeled the cytoplasms of type II cells rather than the nuclei. Lung weights in $Pcyt1a^{\Delta/\Delta}$ mice were similar to those of the controls, and there was no evidence of cell injury or inflammation at birth. The number of brightly stained type II cells was markedly reduced in the terminal lung saccules of all $Pcyt1a^{\Delta/\Delta}$ mice (Fig. 1D). Western blot analyses of the lung homogenates of control and $Pcyt1a^{\Delta/\Delta}$ mice verified that a reduced level of CCTα protein correlated with the $Pcyt1a^{\Delta/\Delta}$ phenotype (Fig. 1D). Quantification of the Western blots indicated a 75% decrease in the

total amount of CCTα in the $Pcyt1a^{\Delta/\Delta}$ mice (Fig. 1E), which was consistent with the immunohistochemistry (Fig. 1D).

Respiratory distress phenotype. Deletion of $Pcyt1a$ in endodermal progenitor cells with Dox exposure beginning at E6.5 to E7.5 resulted in 100% neonatal mortality for the $Pcyt1a^{\Delta/\Delta}$ mice, while the control $Pcyt1a^{fl/fl}$ littermates had an almost 100% survival rate ($n = 12$ litters). $Pcyt1a^{\Delta/\Delta}$ mice developed severe respiratory distress immediately after birth, became cyanotic, and died within 10 min of delivery. Dox administration beginning at either E10.5 or E12.5 and continuing until birth (E18.5 to E19.5) recapitulated the results at E6.5 with 100% neonatal death ($n = 19$ and 11 pups, respectively) for the $Pcyt1a^{\Delta/\Delta}$ animals. Neonatal lungs on the day of birth were excised intact and floated in saline to determine whether the lungs were inflated. The lungs from $Pcyt1a^{\Delta/\Delta}$ animals sank (100%, $n = 18$ pups), whereas the lungs from the $Pcyt1a^{fl/fl}$ littermates floated (100%, $n = 19$ pups), indicating that the knockout lungs did not inflate. However, Dox administration from E16.5 to term resulted in only 10% mortality in the $Pcyt1a^{\Delta/\Delta}$ neonates ($n = 11$ pups), and the excised lungs from the viable $Pcyt1a^{\Delta/\Delta}$ mice were inflated and had normal lung morphology, with type II pneumocytes clustered at the junction between the alveolar ducts and the alveoli (data not shown). Although toxicity from expression of the Cre-recombinase and rtTA proteins has been reported (44), we did not observe significant neonatal death related to the SP-C-rtTA heterozygous and tetO-Cre homozygous genotypes, where the $Pcyt1a$ alleles were homozygous wild-type.

Stage-specific human SP-C promoter activation and CCTα deletion. Activation of the $(tetO)_7CMV$ promoter with 4-day Dox pulses was performed to determine when ablation of CCTα expression occurred during lung development. Promoter activation was expected to be maximal 48 h after starting Dox treatment and to diminish to zero 48 h after Dox removal (28). Dox administration between E0.5 to E4.5 did not result in a significant change in the PtdCho content of late-term lungs (Fig. 2A), as expected, because the embryo develops independently without maternal influence until implantation at E5.5. Providing Dox between E4.5 and E8.5 would delete $Pcyt1a$ in lung progenitor cells (28) and in the ventral foregut of the E9.5 embryo. However, activation of the SP-C promoter in endodermal progenitors prior to lung bud formation (E9.5) resulted in no significant alteration in PtdCho production (Fig. 2A). Between E10.5 and E14.5, the proximal-distal differenti-
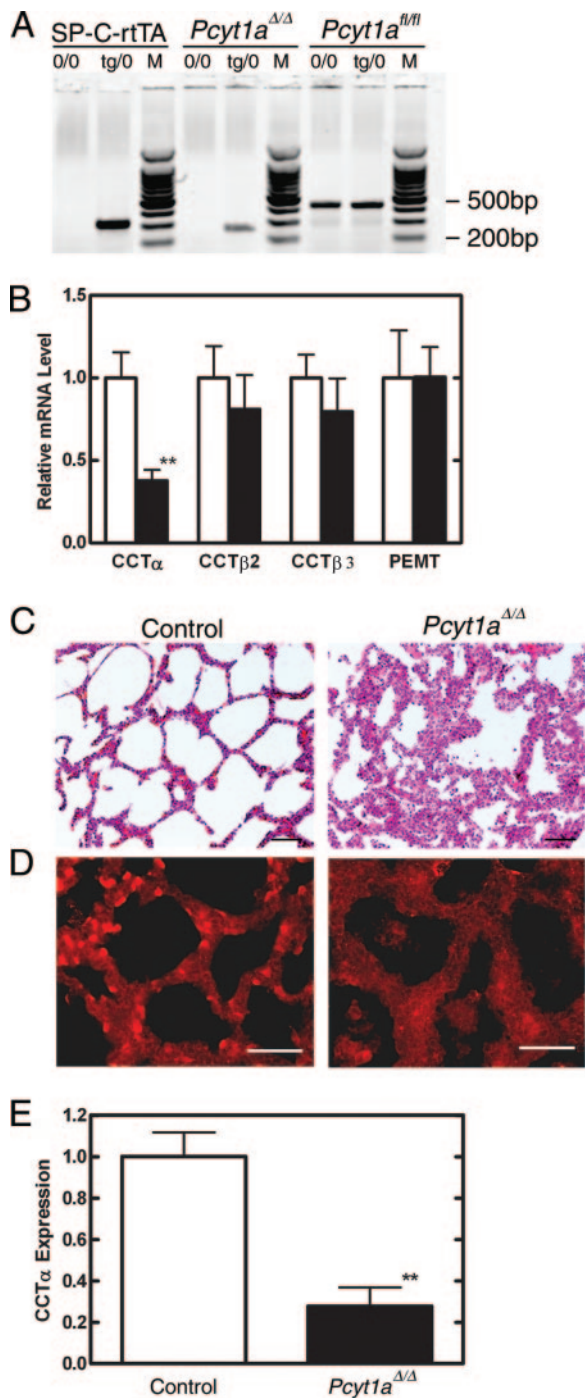
FIG. 1. Lung epithelial-specific ablation of CCTα. (A) PCR analysis on lungs obtained from Dox-treated perinatal mice. The deleted $Pcyt1a$ allele ($Pcyt1a^{\Delta/\Delta}$) was detected as a 255-bp PCR product, in contrast to the 301-bp product from the $Pcyt1a^{fl/fl}$ allele. (B) Real time qRT-PCR of phospholipid biosynthetic pathway transcripts in lungs from $Pcyt1a^{\Delta/\Delta}$ (black bars) and control (open bars) mice at E18.5. The primers and probes used for detection of CCTα, CCTβ2, CCTβ3, and phosphatidyl ethanolamine N-methyltransferase are listed by Wang et al. (40). The results are given as mean mRNA levels ± the standard error of the mean (SEM) (∗∗, $P < 0.01$; $n = 4$) normalized to the level of GAPDH, using the ΔC_T method. Relative expression was calculated based on the cycle number at which fluorescence exceeded the threshold of detection (C_T). Specifically, the C_T for GAPDH was subtracted from that of the target gene for each well (ΔC_T). The percentage of change in gene expression, relative to the reference control group, was defined as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ equals the group ΔC_T minus the ΔC_T of the control group. The value for the control lung set at 1. (C) Perinatal lung sections from littermate controls and $Pcyt1a^{\Delta/\Delta}$ mice were stained with hematoxylin and eosin. Scale bars, 50 μm. (D) Perinatal lung sections of $Pcyt1a^{\Delta/\Delta}$ and control littermate mice were stained with a goat polyclonal antibody against an internal region of the $Pcyt1a$ gene product (CCTα). The photomicrographs are representative of eight mice per group. Scale bars, 50 μm. (E) Western blots of perinatal mouse lungs from $Pcyt1a^{\Delta/\Delta}$ mice and control littermates, using a rabbit polyclonal C-terminal anti-CCTα (45). Quantification of the Western blots ($n \geq 4$ per genotype) was performed using ImageQuant software, and the results are given as means ± SEM (∗∗, $P < 0.01$) standardized to the GAPDH loading control and normalized to results for the littermate controls.
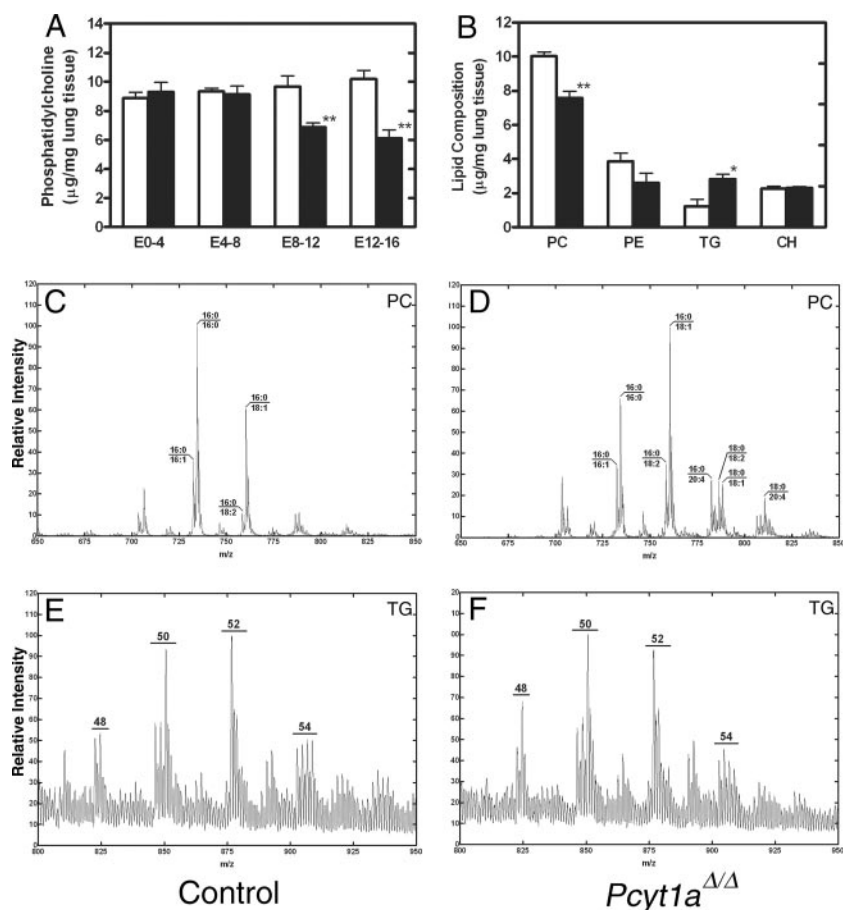
FIG. 2. Lipid composition and molecular distribution in $Pcyt1a^{\Delta/\Delta}$ newborn lungs. Lung lipids were analyzed as described in Materials and Methods. The open bars represent results for the littermate controls and the black bars represent results for the $Pcyt1a^{\Delta/\Delta}$-knockout mice. (A) Total lung PtdCho in embryos pulsed with Dox between the indicated days of gestation. (B) Ptdcho (PC), phosphatidylethanolamine (PE), cholesterol (CH), and triglyceride (TG) contents of lungs from embryos treated with Dox from E0 to E17. Comparisons were made between means ± SEMs ($n = 3$). $*$, $P < 0.05$; $**$, $P < 0.01$. (C to F) Molecular species profile of PtdCho (C and D) and triglyceride (E and F) levels in $Pcyt1a^{\Delta/\Delta}$ (D and F) and control (C and E) neonatal lungs from embryos treated with Dox from E0 to E17. Fatty acid chains are indicated as number of carbon atoms to number of double bonds (C and D) or the total carbon numbers of the peak clusters (E and F). The spectra shown are representative of 4 to 10 spectra obtained on individual control and $Pcyt1a^{\Delta/\Delta}$ mouse lungs.

ation of the lung endoderm becomes evident and gives rise to the bronchi and bronchioles lined with distinct epithelial cell types, including ciliated cells, mucus-secreting cells, and Clara cells. Activation of the SP-C promoter between E8.5 and E12.5 effectively deleted *Pcyt1a* in cells destined for expansion to form the gas-exchange region in the lung (28) and reduce PtdCho content (Fig. 2A) and viability (data not shown). Dox administration between E12.5 and E16.5 deleted *Pcyt1a* in the distal epithelial cells destined to form type I and type II pneumocytes, and PtdCho content was reduced significantly (Fig. 2A). Dox treatment of adult SP-C-rtTA$^{tg/0}$/(tetO)$_7$CMV-Cre$^{tg/tg}$/ *Pcyt1a*$^{fl/fl}$ mice did not give rise to a phenotype, due to a lack of Cre-recombinase expression (28). These data showed that CCTα deletion via Dox activation of the SP-C promoter yielded a phenotype between days 8 and 16 of embryonic development.

Lipid and protein composition of *Pcyt1a*$^{\Delta/\Delta}$ lungs. The lung phospholipid composition was examined in lungs from embryos treated with Dox between E0 and E17. There was significantly reduced PtdCho content in the late-term lungs

(E17.5) from SP-C*Pcyt1a*$^{\Delta/\Delta}$ mice compared to that for the *Pcyt1a*$^{fl/fl}$ littermate controls (Fig. 2B). Neither phosphatidylethanolamine nor cholesterol was significantly decreased, but there was a significant increase in triglycerides (Fig. 2B). The molecular species of PtdCho in *Pcyt1a*$^{\Delta/\Delta}$ lungs showed a pronounced decrease in the proportion of dipalmitoyl-PtdCho (16:0/16:0; $m/z = 734$), suggesting a selective depletion of the $C_{16:0}$ fatty acid that was used in the formation of the surfactant (compare Fig. 2C and D). The triglyceride fraction exhibited a corresponding increase in the proportion of species containing $C_{16:0}$, notably in the species containing 48 and 60 carbons (compare Fig. 2G and F). These analytic data indicated that the production of dipalmitoyl-PtdCho was selectively affected by loss of CCTα activity. The enrichment of the lungs with triglycerides containing palmitic acid ($C_{16:0}$) suggested that surfactant fatty acid synthesis continued in *Pcyt1a*$^{\Delta/\Delta}$ lungs and shunted into the triglyceride pool when PtdCho formation was blocked. These data reflect findings for cultured cells where the diversion of fatty acids to the triglyceride pool was observed when CCTα was inhibited (20).
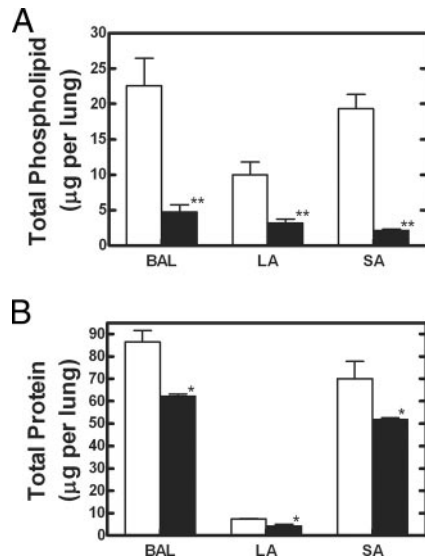
FIG. 3. Total phospholipid and protein levels in the bronchial-alveolar lavage fluid (BAL) from neonatal $Pcyt1a^{\Delta/\Delta}$ mice. The total BAL was fractionated into large aggregates (LA) and small aggregates (SA). Total phospholipid (A) or total protein (B) levels in BAL, LA, and SA lungs from $Pcyt1a^{\Delta/\Delta}$ mice (black bars) and littermate control animals (white bars) are shown. The results are given as means ± the standard deviation ($n = 4$). *, $P < 0.05$; **, $P < 0.01$.

We analyzed the bronchial-alveolar lavage (BAL) fluid from four $Pcyt1a^{\Delta/\Delta}$ and control mice to verify the anticipated deficiency in surfactant production (Fig. 3). The total BAL fluid contained significantly less phospholipid per lung than that of the controls, and the fractionation of BAL into the large (LA) and small (SA) aggregate fractions showed that both were severely depleted in total phospholipid (Fig. 3A). The protein content of BAL and its subfractions was also reduced in $Pcyt1a^{\Delta/\Delta}$ mice compared to that for the controls (Fig. 3B); however, the reduction in surfactant protein was less than the reduction in total phospholipid. This translated into a 4.7-, 2.8-, and 7.4-fold increase in the protein:phospholipid ratio in BAL, LA, and SA, respectively. These data showed that the $Pcyt1a^{\Delta/\Delta}$ pups had a deficiency in surfactant that was most pronounced in the phospholipid fraction.

Surfactant protein expression. By E14.5, the distal epithelial cells begin to express surfactant proteins. Between E16.5 and E17.5, the distal epithelial cells become flattened as the distal endoderm begins to form terminal sacs and the epithelia differentiate into two distinct cell types, type I and type II pneumocytes. Expression of mouse surfactant protein becomes restricted to the type II alveolar pneumocytes, which have large cytoplasmic secretory vacuoles, known as lamellar bodies, containing whorls of surfactant protein and lipid (see Fig. 5B). Deletion of CCTα expression from the type II cell population did not reduce SP-B or SP-C transcript levels, and differences in the levels of SP-A or SP-D mRNAs were not significant (Fig. 4A). In contrast, the $Pcyt1a^{\Delta/\Delta}$-knockout lungs had significantly reduced amounts of SP-A, SP-C, and SP-D proteins and elevated SP-B protein content (Fig. 4B). These data show that CCTα depletion did not blunt surfactant protein gene expression but altered their translation or turnover.
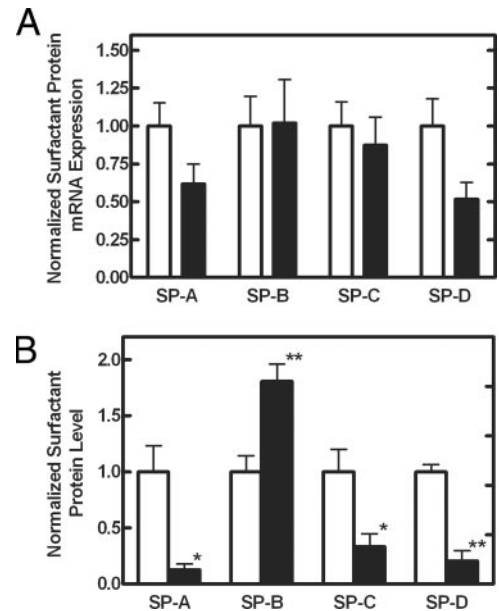

FIG. 4. Expression of surfactant proteins in $Pcyt1a^{\Delta/\Delta}$ mice. (A) Real time qRT-PCR of SP-A, SP-B, SP-C, and SP-D transcripts in lungs from $Pcyt1a^{\Delta/\Delta}$ (black bars) and control (open bars) mice at E17.5. The results are given as mean mRNA levels ± SEM ($n = 4$) normalized to the level of GAPDH, using the ΔC_T method, with the value for the control lung set at 1. (B) Results of immunoblotting of total lung homogenates (50 μg protein) from $Pcyt1a^{\Delta/\Delta}$ mice (black bars) and control littermates (white bars) at postnatal day 0. The results are given as mean SP-A, SP-B, SP-C, and SP-D protein levels ± SEM ($n = 4$ to 8 animals per group). *, $P < 0.05$; **, $P < 0.01$.

Type II cell ultrastructure. The selective deletion of CCTα expression in lung epithelia resulted in marked differences in the ultrastructure of type II cells at birth (Fig. 5). In the control lungs, the type II alveolar epithelial cells contained numerous forming and mature lamellar bodies and an extensive elaboration of apical microvilli (Fig. 5A and B). The peripheral saccules were well dilated, with a thin alveolar wall with squamous-like type I cells and cuboidal type II cells lining the vascularized saccules. In $Pcyt1a^{\Delta/\Delta}$ lungs, the numbers and lengths of apical microvilli in type II cells were reduced and few secretary vesicles or mature lamellar bodies were observed (Fig. 5C). The lungs were collapsed, with the epithelial cells clustered tightly around nondilated alveolar saccules. Alveolar walls were thickened but were still lined with organized type I and type II cells. The integrity of the pulmonary vascular system was damaged somewhat, with capillary leakage and macrophage accumulation inside the alveolar space. Higher magnification revealed large and clearly abnormal membrane-enclosed structures that appeared to arise from expansion of the Golgi complex adjacent to the nucleus (Fig. 5C). In many type II cells, accumulation of what appeared to be lipid droplets was frequently observed, a finding that correlated with the increased triglyceride levels in $Pcyt1a^{\Delta/\Delta}$ lungs (Fig. 3B). In contrast, the ultrastructure of type I alveolar cells appeared normal (data not shown). We also examined the lung ultrastructure of unborn pups just prior to birth, and similar changes were detected in type II alveolar cells from the $Pcyt1a^{\Delta/\Delta}$ animals (data not shown).
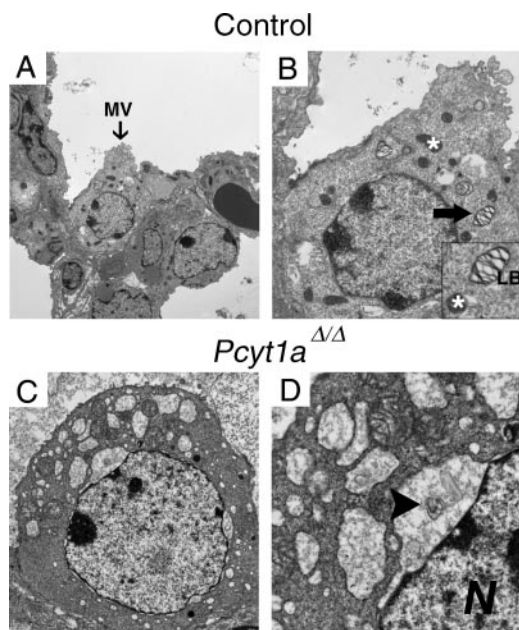
FIG. 5. Ultrastructural analysis of type II alveolar epithelia in $Pcyt1a^{\Delta/\Delta}$ mice. Electron microscopy was performed with perinatal lung samples from control (A and B) and $Pcyt1a^{\Delta/\Delta}$ (C and D) mice. (A) In the control mice, normal flattened type I and cuboidal type II cells with apical microvilli (MV) containing a normal number and structure of lamellar bodies and glycogen stores were observed. (B and inset) Higher magnifications of a type II cell shown in panel A. The arrow indicates a normal lamellar body (LB) near a mitochondrion (∗). (C) In the $Pcyt1a^{\Delta/\Delta}$ mice, lamellar body formation was aberrant, and a reduction in apical microvilli was observed. (D) A higher magnification of a type II cell shown in panel C. The arrow indicates an immature/malformed lamellar body from a $Pcyt1a^{\Delta/\Delta}$ mouse. N indicates the nucleus. The micrographs are representative of results for three $Pcyt1a^{\Delta/\Delta}$ mice and their littermate controls.

FIG. 6. Distribution of surfactant proteins in $Pcyt1a^{\Delta/\Delta}$ mice. Immunohistochemistry was performed on serial sections from control and $Pcyt1a^{\Delta/\Delta}$ lungs, using antibodies against proSP-C (A) and mature SP-B (B). A higher magnification of type II alveolar cells is shown in the insets for each genotype in panel A. The location of SP-B protein in the alveolar lumen is indicated by an arrow. The images are representative of results for eight individual control and $Pcyt1a^{\Delta/\Delta}$ mice, and the lungs were obtained on E19.5 within minutes of birth or just prior to birth. Scale bars, 50 μm.

Surfactant protein distribution. SP-B and SP-C are secreted proteins which play crucial roles in surfactant function and homeostasis. In light of the aberrant Golgi structures in the $Pcyt1a^{\Delta/\Delta}$ type II cells, we investigated whether the level of the unprocessed proSP-C protein, which is the cellular precursor of the mature SP-C, was altered. It was possible that the reduced level of mature SP-C that we detected in the immunoblots from the knockout lungs (Fig. 4B) was due to inefficient processing and accumulation of the proSP-C form. In the control mice, proSP-C antibody stained perinuclear structures in the cytoplasms of all type II peripheral cells (32). The $Pcyt1a^{\Delta/\Delta}$ mice showed similar subcellular localization in the type II epithelial cells (Fig. 4b, inset), although, overall, weaker staining was observed, indicating a reduced amount of proSP-C protein (Fig. 6A). These data suggested that the steady-state level of the precursor protein, rather than processing to the mature form during secretion from the Golgi structures, accounted for the lower amount of mature SP-C in the $Pcyt1a^{\Delta/\Delta}$ lungs (Fig. 4B). Immunostaining for mature SP-B was evident in the control lungs in the cytoplasms of type II cells and lining the luminal surfaces of the peripheral saccules (Fig. 6B). In $Pcyt1a^{\Delta/\Delta}$ lungs, apparent constriction of the alveolar spaces was accompanied by accumulation of aggregated SP-B-positive material inside the alveolar spaces, while the
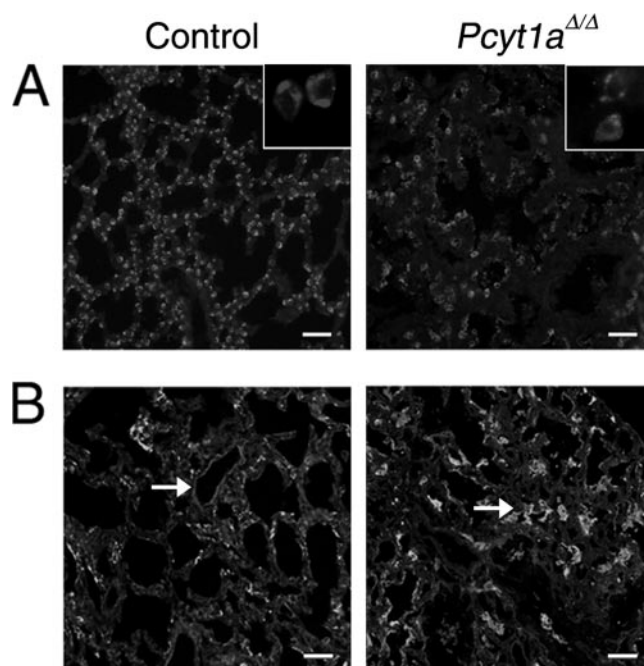
type II alveolar cells retained cytoplasmic staining. The aggregates corresponded to the elevated level of SP-B observed in the immunoblots from the knockout animals (Fig. 4B), and these data suggested that SP-B protein synthesis and secretion into the alveolar space might be intact but that aggregation might reduce the uptake and recycling of the protein by the type II cells. The aberrant Golgi structures in the $Pcyt1a^{\Delta/\Delta}$ lung type II cells were apparently related to impairment of PtdCho synthesis and affected surfactant PtdCho secretion more than secretion of SP-B or the maturation of SP-C.

DISCUSSION

This work identifies CCTα expression as essential for lung epithelial cells to synthesize and secrete PtdCho lung surfactant, but it is not required to produce the PtdCho needed for the proliferation or differentiation program of lung epithelia. The loss of CCTα leads to failure of the lungs to inflate at birth, a dramatic reduction in disaturated and total PtdCho, accumulation of triglycerides, and the absence of mature lamellar bodies coupled with a significant disruption of the Golgi structures in alveolar type II cells. A large number of lung epithelial cells express type II cell differentiation marker proteins in $Pcyt1a^{\Delta/\Delta}$ mice. These findings are counterintuitive in light of the strict requirement for CCTα in cultured cells (11, 12) and the early stage lethality of $Pcyt1a^{-/-}$ embryos (40). However, the normal development of the lung epithelia is

reminiscent of the normal development of hepatocytes and macrophages when CCTα expression is selectively deleted (45, 47). Cells must derive their PtdCho for proliferation from either the presence of the CCTβ isoform or the serum lipoproteins. In the lung, as in the liver and macrophage systems, CCTβ expression was detected, albeit at a much lower level than for CCTα, but the potential contribution of dietary PtdCho provided via the serum lipoproteins should not be overlooked.

There is little cross talk between the formation of palmitate, disaturated PtdCho, and surfactant protein expression in type II cells. In the $Pcyt1a^{\Delta/\Delta}$ mice, palmitate destined for surfactant formation continues to be synthesized in the absence of CCTα and, instead of being incorporated into phospholipid, accumulates in the triglyceride pool. When CCTα is overexpressed in mouse lung epithelia, the animals do not produce excess surfactant PtdCho (23), illustrating that the supply of palmitate via fatty acid synthase, rather than CCTα, sets the upper limit for surfactant PtdCho production. The high level of CCTα expression in lung (17) is explained by the need to prevent other pathways from effectively competing for the pool of intracellular fatty acids produced by fatty acid synthase. CEBP/α is essential for lung maturation, and disaturated PtdCho and surfactant proteins are severely reduced in C/EBPα$^{\Delta/\Delta}$ knockout mice (24). The reduction in PtdCho can be explained by the decreased expression of fatty acid synthase, since $Pcyt1a$ was not identified as under C/EBPα control in the lung. Similarly, deletion of the forkhead transcription factors $Foxa1$ and $Foxa2$ results in failure of the lung epithelia to produce surfactant proteins or phospholipids (4, 38, 39), but $Pcyt1a$ was not identified as a target gene for these factors (39). Keratinocyte growth factor upregulates lipogenesis in alveolar type II cells through an SREBP-1c-dependent pathway by stimulating acetyl coenzyme A carboxylase and fatty acid synthase transcription; however, $Pcyt1a$ mRNA levels are not affected (8, 26). Nonetheless, lung has the highest abundance of CCTα mRNA (40), and the CCTα promoter drives high levels of transgene expression in type II lung epithelial cells (46), suggesting that robust transcription drives CCTα protein accumulation. Whether transcription factors identified in cell culture systems that upregulate $Pcyt1a$, such as Ets-1 and Sp-1 (2, 3, 33, 34), are involved is unknown.

Surfactant contains four major proteins, SP-A, SP-B, SP-C, and SP-D. SP-A and SP-D are relatively hydrophilic proteins that contribute to antimicrobial defense in the lung (25). SP-C and SP-B are hydrophobic proteins that enhance the surface-active properties of the surfactant phospholipids (42). SP-B is the only one of the four required for lung function and postnatal survival (6, 10, 13, 22). Like the $Pcyt1a^{\Delta/\Delta}$ mice, SP-B$^{-/-}$ mice lack mature lamellar bodies, although PtdCho accumulation continues (10). The SP-B$^{-/-}$ mice have numerous small electron-dense vesicles that are thought to harbor disaturated PtdCho (10), in contrast to the distorted Golgi structures observed in our $Pcyt1a^{\Delta/\Delta}$ mice. The transcription and content of the other surfactant proteins persist in SP-B$^{-/-}$ mice (10, 35), and although the steady-state mRNA levels of the four surfactant proteins is maintained in $Pcyt1a^{\Delta/\Delta}$ mice, the accumulation of all of these proteins, except for SP-B, was reduced in $Pcyt1a^{\Delta/\Delta}$ mice. These data indicate that the stability of SP-A, SP-C, and SP-D proteins may be coupled to the formation of

lamellar bodies and progression through the secretory pathway.

In summary, $Pcyt1a$ plays an essential role in phospholipid synthesis, which is required for postnatal adaptation to air breathing. Deletion of $Pcyt1a$ in epithelial cells of the developing mouse lung caused neonatal respiratory failure with characteristics of respiratory distress syndrome in preterm human infants. Thus, we provide a useful mouse model for future studies of surfactant lipid deficiency in newborns, a common developmental defect in humans.

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