CTP:Phosphocholine Cytidylyltransferase α Is a Cytosolic Protein in Pulmonary Epithelial Cells and Tissues*

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CTP:phosphocholine cytidylyltransferase (CCT) is a rate-determining enzyme in de novo synthesis of phosphatidylcholine (PC). The lung requires a steady synthesis of PC for lung surfactant of which disaturated PC is the essential active agent. Surfactant synthesis occurs in alveolar type II cells. Studies with non-pulmonary cells have suggested that CCT is both a nuclear and cytoplasmic protein. The unusual requirements of the lung for PC synthesis and, therefore, CCT activity suggest a unique mechanism of regulation and possibly localization of CCT. The localization of CCTα in lung epithelial cells and, of greater consequence, lung tissues are yet unknown. Three isoforms of CCT have been identified. Herein we investigated the localization of the ubiquitously expressed CCTα isoform. To ascertain CCTα localization in lungs and lung-related epithelial cells, we employed a number of localization methods. Immunogold electron microscopy using polyclonal antibodies raised to either the carboxyl terminus, catalytic domain, or amino terminus of CCTα localized CCTα mostly to the exterior plasma membrane or regions of the endoplasmic reticulum (ER) in both A549 and MLE-15 epithelial lung cell lines and primary cultures of fetal rat lung epithelial cells. In contrast to other studies, little or no nuclear labeling was observed. Indirect immunofluorescence of these cells with anti-CCTα antibodies resulted in a similar distribution. Indirect visualization of both hemagglutinin- and FLAG-tagged CCTα as well as direct visualization of enhanced green fluorescence protein-CCTα fusion protein corroborated a cytoplasmic localization of CCTα in pulmonary cells. Moreover, analysis of lung tissue from fetal and adult mouse by either immunogold electron microscopy or indirect immunofluorescence yielded a strong cytoplasmic CCTα signal with virtually no nuclear localization in epithelial cells lining the airways. The cytoplasmic localization of CCTα in type II cells was further substantiated with transgenic mice overexpressing FLAG-tagged CCTα using the lung-specific human surfactant protein C (SP-C) promoter. We conclude that CCTα does not localize to the nucleus in pulmonary tissues, and, therefore, nuclear localization of CCTα is not a universal event.

The major pathway for PC biosynthesis in animal cells is the CDP-choline or Kennedy pathway. CTP:phosphocholine cytidylyltransferase (CCT) catalyzes the transfer of choline from phosphocholine to CDP-choline. In many cells, CCT is the rate-limiting enzyme in PC production (1, 2). A steady, albeit low, production of PC is necessary to maintain suitable PC content for lipid homeostasis. However, elevated activity of CCT is required during cell division when increased PC production is needed for membrane biogenesis. The fetal lung has a further need for a high level of CCT activity to supply the large volumes of surfactant PC necessary to protect against respiratory distress (3). In late gestation, CCT activity increases in alveolar type II cells (3–6). There is a corresponding loss of intracellular glycogen stores, as the lung generates the necessary surfactant to establish respiration (3–6). High levels of PC production are a continued necessity postpartum and throughout life to maintain functional levels of pulmonary surfactant.

The activity of CCT is strongly affiliated with its association to phospholipid bilayers (for reviews see Refs. 7 and 8). Unbound CCT appears to have little or no activity whereas bound CCT is active. Many factors have been identified in controlling CCT lipid binding, including the phosphorylation state of its carboxyl terminus and the composition of the target membrane (9–12). The dependence of CCT activity on lipid binding makes the localization of CCT in a cell an important factor for CCT function.

To date, three CCT isoforms have been identified: CCTα, CCTβ1, and CCTβ2 (13, 14). The CCTβs are splice variants of the same gene, differing at their carboxyl termini (14). CCTα is encoded by a separate gene and differs from CCTβ at the amino and carboxyl termini (14). Based on sedimentation experiments, both CCT forms were thought to be cytosolic and endoplasmic reticulum (ER)-bound (15–17). Studies using indirect immunofluorescence demonstrated a predominant partitioning of CCTα to the nucleus in several cell lines and rat liver slices (14, 18, 19). Other indirect immunofluorescence and electron microscopic studies revealed that both CCT forms are cytosolic as well as nuclear in primary hepatocytes (18, 20). Recent direct immunofluorescence analysis of cell lines transfected with enhanced green fluorescence protein (EGFP)-CCTα supported the nuclear partitioning of CCTα (21). The CCTα form

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1 The abbreviations used are: PC, phosphatidylcholine; BSA, bovine serum albumin; CCT, CTP:phosphocholine cytidylyltransferase; DIC, differential interference contrast; EM, electron microscopy; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; FRLE, primary fetal rat lung epithelial; HA, hemagglutinin; MEM, minimal essential medium; NGS, normal goat serum; NLS, nuclear localization signal; OCT, optimal cutting temperature; PBS, phosphate-buffered saline; PI, propidium iodide; SP-C, surfactant protein C; CHO, Chinese hamster ovary; kb, kilobase(s); EGFP, enhanced green fluorescent protein.
contains a nuclear localization signal (NLS), and removal of the NLS motif in CCTα has been shown to result in a cytoplasmic as well as nuclear localization (18, 22). Indirect immunofluorescence with CCTβ-specific antibodies has shown that the CCTβ isoforms, which lack the amino-terminal NLS, are confined to the ER (13, 14). The relevance of nuclear localized CCTα remains unclear. In IEC fibroblast cells, CCTα has been found to shuttle from the nucleus to the cytoplasm during G0 → G1 transition, suggesting that CCTα is sequestered to the nucleus until it is required (19).

Although the nuclear localization of CCTα is recently held as a universal phenomenon, no studies have been performed on pulmonary epithelial cell lines or on pulmonary tissues. Herein, we investigated the localization of CCTα in lung epithelial cell lines, primary cultured fetal rat lung epithelium, and whole lung tissue from fetal and adult mice. In all cases, CCTα was excluded from the nucleus indicating that nuclear localization of CCTα is not universal to all tissues.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chinese hamster ovary (CHO-K1) cells and human pulmonary epithelial cell lines (A549) were from American Type Culture Collection. Mouse lung epithelial MLE-15 cells were a generous gift from Dr. Jeff Whitsett (Cincinnati, OH). Rabbit polyclonal antibodies were raised against glutathione S-transferase fusion proteins encompassing either the first 31 amino acids of the amino terminus (anti-CCT-N) or the last 45 amino acids of the carboxyl terminus of rat lung CCT (anti-CCT-C). In addition, a rabbit polyclonal antibody was raised against a synthetic peptide corresponding to residues 164–176 of rat lung CCT (anti-CCT-M). We have previously shown that purified rat CCTα as well as baculovirus-expressed recombinant rat CCTα were recognized by all three antibodies (23, 24). Monoclonal antibodies to calnexin (endoplasmic marker), HA, and FLAG were from Affinity Bioreagents, Inc. (Golden, CO), BABC, (Richardson, CA) and Sigma (St. Louis, MO), respectively. Polyclonal antibodies against HA (BABC), rabbit (Richardson, CA) and FLAG (Affinity Bioreagents, Inc.) were used to confirm observations made with the monoclonal antibodies.

**Primary Fetal Rat Lung Epithelial (FRLE) Cell**—Pregnant rats were sacrificed by diethyl ether exposure on day 19 of gestation (term = 22 days). Fetuses and lungs were aseptically removed, and lung epithelial cells were isolated as previously described (23, 25, 26).

**Radioactive Choline Incorporation into PC**—CHO-K1, A549, MLE-15, and RFLE cells were plated into plastic 12-well culture plates. The next day, the cells were rinsed with serum-free media and 40 μg/ml [methyl-3H]choline (Amersham Pharmacia Biotech, Oakville). After 5 h of incubation, the medium was removed and the cells were washed with serum-free MEM. Following trypsinization to remove the cells, cellular lipids were extracted according to the method of Bligh and Dyer (27). Fifty milliliters of the cell suspension was used to determine the number of cells per well. PC was isolated from the lipid extract by thin-layer chromatography on Silica Gel H plates with CHCl3/MeOH/H2O (65:25:4, v/v) as developing solution. PC was visualized with a bromothymol blue solution. Bands were scraped from the surface of the plate, placed in scintillation vials with scintillation mixture, and counted on a Wallac 1219 Rackbeta liquid scintillation counter.

**Construction and Expression of EGFP, HA, and FLAG-tagged CCT**—For expression of EGFP-tagged CCTα protein in cells, the cDNA encoding for full-length CCTα was inserted into pEGFP expression vectors (CLONTECH, Palo Alto, CA) with EGFP being at either the amino or carboxyl termini of CCTα, named pEGFP-CCTα or pCCTα-EGFP, respectively. An HA epitope sequence (YPYDVPDYA) was affixed to the amino terminus of full-length CCTα and a flag epitope sequence (DYKDDDDK) was affixed to the carboxyl terminus of full-length CCTα.

**Transgene Construction**—The SPC-CCTα vector was constructed using the full-length rat CCTα (4). A FLAG sequence (DYKDDDDK) was connected to the carboxyl terminus of CCTα. The 1.2-kb FLAG-tagged CCTα CDNA was subcloned 3’ of the 3.7-kb human SP-C promoter (28) and 5’ of the SV40 small T intron and polyadenylation sequences. The expression cassette was excised with NdeI and NotI, purified using Glass Milk (Geneen Kit Bio101, Biocan, Canada) and Etulip-D columns (Schleicher and Schuell, NY), and ethanol-precipitated. Following Packaging of Transgene Constructs, the transgene constructs were generated according to Hogan et al. (4). DNA injections into the pronuclei of (C57BL/6 × SJL) F2 embryos were carried out at a concentration of 3 ng/μl. Of the total of nine embryos at 18 days post coitus, three were transgenic; of the total of seven newborns, two were transgenic. The genotype was established by polymerase chain reaction analysis and confirmed by Southern blot analysis. The primers used were 5’-TACCTCTTGCCTCCCTCCTGTTAC-3’ (SP-C primer for 5’) and 5’-TGCTGCTGTCCCTTGTTAGTGTGCTTTAT-3’ (CCTα primer for 3’).

**Direct Immunofluorescence Microscopy**—Cells transfected with pEGFP-CCTα or pCCTα-EGFP were observed after 48 h under a Zeiss LSM500e confocal microscope. Digital images of cells were recorded.

**Immunoblot Analysis**—SF9 cells transfected with rat CCTα (24) or untransfected MLE-15 cells were washed with PBS, lysed in homogenization buffer using a Dounce homogenizer. Homogenates were diluted in Laemmli loading buffer to a final concentration of 25 μg/μl for SF9 cells and 40 μg/μl for MLE-15 cells and then boiled for 5 min. Samples (250 μg for SF9 cells and 40 μg for MLE-15 cells) were subjected to SDS-polyacrylamide gel electrophoresis on a 10% (w/v) polyacrylamide gel and then electrophoretically transferred to PVDF membranes. Non-specific binding was blocked by incubating the nitrocellulose membrane with 3% (w/v) powdered skim milk in PBS at 4 °C for 60 min, then 1:100 diluted rabbit anti-CCT-anti-antiserum was added to detect CCT in the homogenates. After overnight incubation at 4 °C, the nitrocellulose membrane was washed three times with PBS, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20,000) for 1 h at room temperature. The membranes were then thoroughly washed with cold PBS (5 × 5 min) and detected with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

**Indirect Immunofluorescence Microscopy on Cells**—Cells seeded on glass coverslips were fixed in 4% (w/v) paraformaldehyde in PBS and permeabilized with 0.2% (w/v) Triton X-100 in PBS containing 1% (w/v) bovine serum albumin (BSA). Coverslips were then washed with PBS and treated successively with 5% (w/v) normal goat serum (NGS) and 1% (w/v) BSA in PBS (NGS/BSA) for 1 h, first antibody solution in NGS/BSA overnight at 4 °C and fluorescein (FITC)- or rhodamine-conjugated secondary antibody solution in NGS/BSA for an additional hour, with liberal washing with PBS in each interval. Rabbit anti-CCT-N, rabbit anti-CCT-M, or rabbit anti-CCT-C antibodies; mouse monoclonal anti-HA or anti-FLAG were diluted 1:50 and used as primary antibodies. The FITC- and rhodamine-labeled secondary antibodies to rabbit (goat anti-rabbit) and mouse (goat anti-mouse) IgG were diluted 1:300 and 1:100, respectively. Coverslips were mounted on microscope slides with mounting medium alone or mounting medium with propidium iodide (PI)-stained cells were then observed under a Leitz Laborlux microscope equipped with an HBO xenon arc light source and a fluorescence filter. Digital images of cells were recorded using a Leica DC200 camera.

**Indirect Immunofluorescence Microscopy on Tissue**—Pregnant female mice were sacrificed by exposure to diethyl ether on either day 17 or day 18 of a timed pregnancy (term = 22 days). Fetuses were aseptically removed, and lungs rinsed with 1 unit/ml heparin in PBS to remove blood. Fetal lungs were then incubated for 20 min in 12% (w/v) sucrose in 2 volumes of PBS to 1 volume Tissue-Tek OCT Compound (OCT) (Sakura, Torrance CA). Adult mouse lungs were perfused with 1:1 PBS/OCT through the trachea. All lungs were then coated with OCT and frozen on dry ice. Six-micron frozen sections were used for indirect immunofluorescence. Frozen sections were fixed with 3% (v/v) paraformaldehyde for 10 min, washed three times in PBS, and permeabilized with 2% (v/v) Triton X-100 in PBS for 10 min. Sections were then blocked with 3% (v/v) normal goat serum and 1% (v/v) BSA in PBS for 1 h at room temperature. Sections were incubated with diluted anti-CCT-N (1:100), anti-CCT-M (1:100), anti-CCT-C (1:100), pre-immune serum (1:100), or anti-FLAG M2 (1:150) in PBS overnight at 4 °C. Following repeated washes in PBS, the sections were exposed to goat anti-rabbit or goat anti-mouse FITC- or rhodamine-conjugated secondary antibodies (1:500). Sections were then counterstained with 1:75 dilution of DAPI. Samples were imaged with a Zeiss LSM500e confocal microscope.

**Immunogold Electron Microscopy**—Cells were cultured in 15 cm² tissue culture flasks until nearly confluent. The cells were then washed three times in PBS and scraped from the surface of culture plates. Lung tissue was removed from mouse or rat and rinsed in 1 unit/ml heparin in PBS to remove blood and then minced in 1-mm pieces. Cells were
washed three times in PBS and fixed in 4% (w/v) paraformaldehyde and
0.1% (w/v) glutaraldehyde for 1 h. Following three washes in PBS,
samples were transferred to beam capsules, dehydrated through an
alcohol series, and incubated in 1:1 lowicryl KM4:100% ethanol. The
samples were placed in 100% lowicryl KM4 overnight with repeated
changes. The lowicryl was polymerized by exposure to UV light over-
night at −20 °C. For freeze substitution, minced tissues were fixed in
4% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde for 1 h. They
were incubated overnight in 1% (w/v) sucrose in PBS overnight for
cryoprotection and frozen with liquid nitrogen. The tissues were per-
mitted to dehydrate slowly in 3% (w/v) uranyl acetate in 100% methanol at
−20 °C for 4 days. The methanol was switched for 1:1 lowicryl
HM20:100% methanol for 2 days at −20 °C followed by several changes
of 100% lowicryl HM20 (8 h per change). The samples were then
exposed to UV light overnight at −20 °C to polymerize the lowicryl.
Ultrathin sections of these samples were cut using a diamond knife on
a Reichert Ultracut microtome to light gold thickness and floated onto
Formvar-coated 300-mesh nickel grids. Grids were treated with a series of
blocking solutions: 0.5% (w/v) glycine, 1% (w/v) BSA in PBS for 10 min,
and three changes of 1% (w/v) BSA in PBS, 10 min each. Primary
antibodies were added to the samples diluted in 1% (w/v) BSA in PBS.
The final concentrations of the primary antibodies were 1:150 of anti-
FLAG M2 (Sigma-Aldrich) and 1:100 for anti-CCT-N, anti-CCT-M, and
anti-CCT-C. Following 1 h of incubation, samples were washed four
times, 10 min each in 1% (w/v) BSA in PBS. Ten-nanometer gold-
conjugated antibodies (goat anti-mouse secondaries for samples incu-
bated with anti-FLAG primaries or goat anti-rabbit secondaries for
samples incubated with anti-CT primaries) were diluted 1:20 in 1%
(w/v) BSA in PBS and spun 10 min at 10,000 rpm in a 1.5-ml Eppendorf
tube at 4 °C. The grid samples were incubated with the supernatant
for 1 h at room temperature in a humid chamber. Grids were washed three
times in PBS and then three times in double-distilled water, 10 min
per wash. Finally the samples were stained 10 min in 3% (w/v) uranyl
acetate in double-distilled water, 5 min in 1% (w/v) lead citrate followed
by double-wash distilled water to remove excess stain. Samples were
examined on a Philips 430 electron microscope. Controls included:
omission of primary antibodies and samples treated with pre-immune
serum (at 1:100 dilution).

RESULTS

Choline Incorporation into PC—First, we evaluated the rate of
PC synthesis in the different cell types (Fig. 1a). Incorporation
of radioactive choline into PC was similar for CHO-K1 and
FRLE cells. The rate of PC synthesis of A549 and MLE-15 cells
was approximately half of that of CHO-K1 and FRLE cells. To
determine if CCTβ was expressed, we performed RT-polymer-
ase chain reaction analysis using CCTβ-specific primers (14).
Both CHO-K1 cells and fetal rat lung expressed small amounts
of CCTβ mRNA whereas no message was detected in any of the
other samples (data not shown). Similar findings have been pre-
viously reported (15, 14, 29). Thus, PC synthesis of pulmo-
ary epithelial cells can be attributed to the activity of CCTα
whereas PC synthesis of CHO-K1 cells may be the synergistic
activity of both CCTα and CCTβ.

Antibody Specificity—To test the specificity of the CCT ant-
obodies, we performed immunoblot analysis using homoge-
lates of S9 insect cell transfected with rat CCTα (30) and untransfected MLE-15 cells. All three CCT antibodies recognized proteins with molecular masses of ~42 kDa (Fig. 1b), similar to the molecular mass of rat CCTα (31).

Localization of CCTα in CHO-K1 Cells—We first monitored the
CCTα localization in CHO-K1 cells. The main purpose was to
confirm the reported CCTα nuclear localization in these cells
(14, 18, 21). A similar localization would validate our tech-
niques. When CHO-K1 cells were either transfected with
pCCTα-EGFP or pEGFP-CCTα for direct visualization, an
extremely intense nuclear signal was observed (Fig. 2a, inset).
When we increased the detector gain of the confocal microscope
on these samples to levels similar to those used for the EGFP
controls, a clear cytoplasmic signal was seen, although the
resultant nuclear signal was saturated (Fig. 2a). Thus, al-
though we observe a high nuclear signal in CHO-K1 cells, the
localization of CCTα-EGFP and EGFP-CCTα appears not to be
exclusively nuclear. Although some nuclear signal was noted
with the pEGFPs without a CCTα insert, they displayed a
more generalized signal throughout the CHO-K1 cells when
compared with pEGFPs with inserts (Fig. 2b). Indirect immu-
nofluorescence was performed with both HA-CCTα and CCTα-
HA-tagged fusion proteins, co-localized with endogenous CCTα (Fig. 3b).
In contrast to the intense nuclear signal seen with EGFP-CCTα fusion proteins,
FLAG- and HA-tagged CCTα distributed across the nucleus and the cytoplasm (Fig. 3b). This finding was corroborated by the indirect immunogold EM experiments. Immunogold label
for CCTα in CHO-K1 cells was detected in both cytoplasm and
nucleus (Fig. 3a). The nuclear immunogold signal was weaker
than that expected based on the CCTα-EGFP transfection ex-
periments and previously published data (21). CCTα localized
clearly to the nuclear envelope in proximity of the outer
circumference (Fig. 3a, arrowheads). There was no obvious
preference for gold localization to heterochromatin, euchroma-
tin, or nuclear lamina. No gold particles were observed within
the nucleolus region. Controls for all localization experiments
included the omission of primary antibody and pre-immune
serum treatment. When the primary anti-sera were omitted
the occurrence of gold particles on sectioned material was very
sparse (data not shown), suggesting that the gold-conjugated

FIG. 1. Choline incorporation into PC and CCT antibody spec-
cificity. a, CHO-K1, A549, MLE-15, and FRLE cells were pulse-labeled
for 5 h with [methyl-3H]choline and incorporation into PC was assessed.
Experiments, carried out in quadruplicate, were repeated twice with
similar results. b, immunoblot of transfected S9 insect cell homogenate
using either anti-CCT-N, anti-CCT-M, or anti-CCT-C antibodies. A single
band of approximately 42 kDa was observed. No other bands were
visible. c, immunoblot of MLE-15 homogenate using either anti-
CCT-N, anti-CCT-M, or anti-CCT-C antibodies. A strong single band
of approximately 42 kDa was observed with minimal signal from other
bands. Protein standard sizes are in kilodaltons.
Localization of Lung CCTα

EGFP control plasmids (no CCT b-fected cells. The CHO-K1 cell panel shows the same cells at a lower intensity. The inset for A549 shows the same cells under DIC. Arrows indicate transfected cells. CHO-K1, A549, and MLE-15 cells were transfected with EGFP control plasmids (no CCTα insert). The EGFP-C plasmid was used to generate the EGFP-CCTα construct, and the EGFP-N plasmid was used to generate the CCTα-EGFP construct.

Secondary antibody contributed little to the signal. The substitution of anti-serum for pre-immune serum resulted in the observation of only occasional gold particles (data not shown). There was not specific localization of these gold particles to any cellular compartment. Rather, the gold was close to evenly distributed over the cells and surrounding embedding material.

Localization of CCTα in A549 and MLE-15 Cells—Human lung A549 cells and mouse lung MLE-15 cells were studied for CCTα localization. When A549 cells were transfected with pEGFP-CCTα, a high intensity perinuclear fluorescence signal was observed with no observable signal from either the nucleus or cytoplasm (Fig. 2a). However, when the CCTα-EGFP construct was used the EGFP fusion protein was distributed throughout the cytoplasm (Fig. 2a). The pEGFP controls (no CCTα insert) showed an even EGFP distribution throughout both the nucleus and cytoplasm (Fig. 2b). A perinuclear localization similar to that of A549 cells transfected with pEGFP-CCTα was observed in some A549 cells transfected with the HA-CCTα construct (Fig. 3d). Anti-CCT-N antibodies localized CCTα to both the perinuclear space and the cytoplasm in HA-CCTα transfected cells (Fig. 3d). CCTα-FLAG protein in A549 cells was predominantly cytoplasmic (Fig. 3d). MLE-15 cells were also transfected with EGFP-, HA-, and FLAG-tagged full-length CCTα constructs. The transfected cells were then examined for direct and indirect fluorescence. As can be seen in Fig. 2a, MLE-15 cells transfected with either pEGFP-CCTα or pCCTα-EGFP displayed primarily cytoplasmic fluorescence. None of the fusion constructs tested targeted CCTα to the perinuclear region. The majority of cytoplasmic CCTα protein in MLE-15 cells co-localized with the ER as demonstrated by double-indirect labeling with anti-CCT-N and anti-calnexin, an integral membrane protein of the ER (Fig. 3f).

The three antibodies for CCTα were tested on thin sections from both human pulmonary A549 cells and mouse lung MLE-15 cells using immunogold-labeled secondary antibodies. Anti-CCT-N and anti-CCT-C were raised against the amino-terminal and carboxyl-terminal domains of CCTα, respectively, which both differ significantly from CCTβ1 and -β2, and, therefore, anti-CCT-N and anti-CCT-C recognize selectively CCTα. In contrast, anti-CCT-M, will recognize all three CCT isoforms, CCTα, CCTβ1, and CCTβ2. As mentioned earlier, we were unable to detect CCTβ1 message in A549 and MLE-15 cells, implying that the antibody signals are attributable to CCTα. Because we obtained similar results with all three CCT anti-
bodies, we only show the results obtained with anti-CCT-N antibody. In both cell lines, nuclear labeling was rare and approached the nuclear labeling of the pre-immune controls (Fig. 3, c and e). Gold particles were almost exclusively observed in the cytoplasm. Frequently, gold was found in association with the ER or the nuclear envelope. Furthermore, gold particles were occasionally found associated with the plasma membrane. No mitochondrial-associated gold particles were observed. Indirect immunofluorescence microscopy using the anti-CCT-N antibody revealed a similar localization pattern of CCT in A549 and MLE-15 cells (Fig. 3, d and f). No nuclear signal was detected. Indirect immunofluorescence controls (omission of primary antibody or pre-immune serum treatment) produced no appreciable signal (not shown).

Localization of CCTα in Primary Cultures of FRLE Cells—Fetal rat lung epithelial cells (FRLE) were examined using all three CCTα antibodies by indirect immunogold EM (Fig. 4a, c, f, and i) and immunofluorescence microscopy (Fig. 4a: a and b; d and e, g, and h). Furthermore, FRLE cells were transfected with CCTα-EGFP to confirm CCTα localization by direct fluorescence (Fig. 4a, j and k). Using immunogold EM, no nuclear localization was observed in any of the samples. Gold particles were primarily localized to the cytoplasm and repeatedly observed bound to the nuclear envelope and plasma membrane when anti-CCT-N (Fig. 4c), anti-CCT-M (Fig. 3f), or anti-CCT-C (Fig. 4i) was used as primary antibody. The mitochondria were never labeled. Both direct and indirect fluorescence microscopy indicate a cytoplasmic localization of CCTα in FRLE cells. Anti-CCT-N (Fig. 4a), anti-CCT-M (Fig. 4d), and anti-CCT-C (Fig. 4g) antibodies showed a strong cytoplasmic signal that localized to the perinuclear region. A similar distribution was observed with both CCTα-EGFP fusion proteins (Fig. 4a, j and k).

Localization of CCTα in Mouse Tissue—CCTα localization has been primarily studied in cultured cells. Little is known about CCTα distribution in native tissue. For this reason we analyzed whole lung tissue from fetal and adult mouse using the anti-CCT-N antibodies (Fig. 5). In adult mouse lung, all cells displayed some positive signal by indirect immunofluorescence (Fig. 5, d and f). Strong fluorescence signals were observed in Type II cells (arrowheads) and in the epithelium of the upper airways (arrow). These epithelial cells demonstrated a clear absence of nuclear signal. Even in other cells that displayed relatively weak signal strength, no clear nuclear signal was observed. In the immunogold EM experiments we primarily focused our attention on Type II cells (Fig. 5, a–c). However, a survey of all the differing cell types in the lung yielded occurrences of nuclear localization as infrequent as that observed in negative controls. In adult Type II cells, CCTα localized to the same subcellular sites as described above for FRLE cells, namely, cytoplasm, ER, nuclear envelope, and plasma membrane (Fig. 5a). Unique to adult Type II cells was the association of CCTα to lamellar bodies. In the fetal mouse lung, indirect immunofluorescence microscopy yielded a strong signal that was evenly distributed among the maturing cell types (Fig. 5, g–j). The nucleus displayed no distinguishable signal. Immunogold labeling gave a similar result as that seen with the adult, including the association with lamellar bodies (Fig. 5c). Both adult and fetal tissue had no mitochondrial labeling. Unlike adult lung cells, immature type II cells contain glycogen deposits (Fig. 5, b and c). Surprisingly, there was a strong labeling of CCTα in these glycogen pools.

CCTα-FLAG Localization in Transgenic Mice—Transgenic mice were generated that express CCTα-FLAG under the lung-specific SP-C promoter. To confirm the previous observations made with the anti-CCT antibodies, lung tissues from these mice were tested using a monoclonal anti-FLAG antibody (Fig. 6). Because SP-C expression is confined to the Type II cells, indirect immunofluorescence yielded a strong signal in the Type II cells with minimal background in other cells (Fig. 6, a and b). In the Type II cells, CCTα-FLAG labeled exclusively the cytoplasm. No nuclear fluorescence signal was noted. Immunogold localization of FLAG-tagged CCTα in fetal tissue resulted in labeling of the cytoplasmic glycogen along with the lipid
containing surfaces of the plasma membrane and the nuclear envelope (Fig. 6c). Although CCTα-FLAG could be localized to the surfaces of many organelles, labeling of the mitochondria was not observed.

**DISCUSSION**

In the present study we have used various imaging techniques on multiple lung cell and tissue samples to reduce the risk of artifact. Multiple antibodies (anti-CCT-N, anti-CCT-M, and anti-CCT-C) against CCT and epitope-tagged CCTα (anti-FLAG and anti-HA) were employed in the indirect localization with consistent agreement in results. Western blotting of Sf9 and MLE15 cells (Fig. 1, b and c) was performed to test the specificity of the anti-CCT antibodies. The blotting results showed one strong band that was consistent in size to CCTα suggesting the specificity of these antibodies was high. The strong consensus between the divergent methods and samples is that CCTα in lung cells is localized nearly exclusively to the plasma membrane.

**CCTα Localization in CHO-K1 Cells**—The nuclear localization of CCTα in CHO-K1 cells confirmed previously reported studies (14, 18, 21). CCTα could be detected in the cytoplasm of CHO-K1 cells under all conditions tested. However, the strong nuclear signal seen with the EGFP-tagged CCTα constructs suggested a higher degree of nuclear compartmentalization than that observed with either HA- and FLAG-tagged CCTα or immunogold labeling using anti-CCTα antibodies. One possible explanation is that the EGFP fused to either terminus of CCTα contributed to nuclear retention in these cells. The tendency for EGFP alone to compartmentalize to the nucleus supports this possibility. High resolution immunogold EM did not define any specific ultrastructural location for CCTα in the nucleus of CHO-K1 cells. Although CCTα was close to the outer end of the nucleus, it did not bind specifically to the nuclear lamina nor was it found in specific association with heterochromatin or euchromatin. Instead, CCTα appeared to be randomly distributed within the nucleus, although excluded from the nucleolus. This randomness of nuclear distribution gives no clues regarding the functionality of nuclear CCTα in CHO-K1 cells.

**CCTα Localization in A549 and MLE-15 Cells**—Using immunogold EM all three CCT antibodies revealed a consistent localization pattern in A549 and MLE-15 cells. Both cell lines displayed a strong cytoplasmic localization with some gold particles in close proximity to the ER. Although the occurrence was relatively infrequent, a second population of CCTα molecules appeared to be bound to the outer nuclear envelope and the plasma membrane. The function of nuclear envelope-bound
Furthermore, it can be surmised that nuclear CCT/H9251 plasm in HA-CCT/H9251 fused to the amino terminus of CCT/HA-CCT/H9251 produced a higher level of nuclear fluorescence than that of EGFP fused to the carboxyl terminus of CCT/HA-CCT/H9251. The fluorescence signal was also stronger than that observed with indirect immunofluorescence. This intense nuclear signal in EGFP-CCT/HA-CCT/H9251 transfected CHO-K1 cells is also likely the result of trapping. Taken together, these data suggest that amino-terminal tagging of CCT results in artifactual (peri-)nuclear localization. This could be the result of the fusion tag being in proximity to the nuclear localization domain, which is near the amino terminus (22). No trapping effects were apparent in MLE-15 cells with any of the constructs tested, suggesting that trapping of amino-terminal tagged CCT is cell type-specific. We believe that CCTα localization studies in the future need to confirm fusion-tagged constructs with antibodies directed to CCTα.

CCTα Localization in FRLE cells—It has been previously reported that primary cultures of hepatocytes displayed strong cytoplasmic localization with significantly less nuclear localization of CCTα than CHO-K1 cells (20). Considering that immortalized cell lines may have divergent phenotypes relative to the original cells from which they are derived, we examined primary cultures of fetal rat lung epithelial (FRLE) cells to ascertain CCTα localization in these untransformed cells. Similar to hepatocytes, CCTα displayed a cytoplasmic localization in FRLE cells, but in contrast to the previous study (20) no nuclear CCTα was observed.

CCTα localization was similar as A549 and MLE-15 cells, whereas its rate of PC synthesis was similar to that of CHO-K1 cells. The presence of nuclear CCTα in CHO-K1 cells and its absence in pulmonary epithelial cells suggests that CCTα distribution varies with cell type and is not dependent on the rate of PC synthesis. However, CCTα within the pulmonary epithelium may alternatively regulate or act in a functionally distinct manner relative to other cell types that compartmentalize CCTα to the nucleus.

CCTα Localization in Adult Mouse Lung—Nuclear exclusion of CCTα was clearly observed in adult mouse lung tissue. This was particularly evident in the strongly labeled epithelium of the upper airway and alveolar Type II cells. Many cells within the lung labeled too weak to resolve nuclear signal. However, no strong nuclear signal, like that seen with the CHO-K1 cells, was ever observed in any of the cells of the adult lung tissue. CCTα distributions throughout the tissue, as determined by immunogold localization, displayed a strong association with lipid structures. As with pulmonary cells, gold was observed bound to the interior leaflet of the plasma membrane, the exterior leaflet of the nuclear envelope, and to the ER. Furthermore, CCTα was localized to lamellar bodies, the storage organelle of pulmonary surfactant. The promiscuous binding of CCTα to so many lipid structures was unexpected. Because lipid binding is a determinant of CCTα activity (for reviews see Refs. 32 and 33), it is tempting to speculate that the promiscuously bound CCTα is the active form.

CCTα Localization in Fetal Mouse Lung—Localized CCTα was also absent in fetal lung. Unlike adult tissue, the immunofluorescence was not concentrated to any particular cell type. This could be attributed to a need for PC production for the processes of cell growth and differentiation. It is expected that some cells will be at differing stages of the cell cycle, however, no difference in CCTα distribution was observed. Like the adult mouse lung tissue, gold particles were localized to many of the lipid surfaces, including lamellar bodies, implying a promiscuous lipid binding ability of CCTα. Although CCTα was associated with many organelles, the absence of mitochondrial binding suggests there is some specificity to its association. The large stores of glycogen, not found in adult tissue, proved another common site for CCTα.

FLAG Localization of CCTα-FLAG Overexpression Mouse Lungs—When anti-FLAG antibodies were applied to CCTα-FLAG-positive transgenic mice, a positive signal was exclu-
sively observed in Type II cells. This signal was again non-nuclear and in strong agreement with the anti-CCT antibodies tested. Given the distinct nature of the FLAG and CCT antibodies, it seems unlikely that the observed labeling could be artifactual. Gold particles were positioned in the expected locations, but they were also observed within the glycogen pools artifactual. Gold particles were positioned in the expected locations, but they were also observed within the glycogen pools artifactual. Gold particles were positioned in the expected locations, but they were also observed within the glycogen pools artifactual. Gold particles were positioned in the expected locations, but they were also observed within the glycogen pools artifactual. Gold particles were positioned in the expected locations, but they were also observed within the glycogen pools artifactual. Gold particles were positioned in the expected locations, but they were also observed within the glycogen pools artifactual. Gold particles were positioned in the expected locations, but they were also observed within the glycogen pools.

Taken together these results indicate that CCTα is a cytoplasmic protein in pulmonary cells and that nuclear localization of CCTα is not a universal phenomenon.

REFERENCES
CTP:Phosphocholine Cytidylyltransferase α Is a Cytosolic Protein in Pulmonary Epithelial Cells and Tissues
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