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Pre- and Postnatal Lung Development, Maturation, and Plasticity

ClC-5: ontogeny of an alternative chloride channel in respiratory epithelia

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Edmonds, Rebecca D., Ian V. Silva, William B. Guggino, Robert B. Butler, Pamela L. Zeitlin, and Carol J. Blaisdell. ClC-5: ontogeny of an alternative chloride channel in respiratory epithelia. *Am J Physiol Lung Cell Mol Physiol* 282: L501–L507, 2002. First published October 26, 2001; 10.1152/ajplung.00207.2001.—Chloride transport is critical to many functions of the lung. Molecular defects in the best-known chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR), lead to impaired function of airway defensins, hydration of airway surface fluid, and mucociliary clearance leading to chronic lung disease, and premature death, but do not cause defects in lung development. We examined the expression of one member of the ClC family of volume- and voltage-regulated channels using the ribonuclease protection assay and Western blot analysis in rats. ClC-5 mRNA and protein are most strongly expressed in the fetal lung, and expression is maintained although downregulated postnatally. In addition, using immunocytochemistry, we find that ClC-5 is predominantly expressed along the luminal surface of the airway epithelium, suggesting that ClC-5 may participate in lung chloride secretion. Identifying candidate genes for critical ion transport functions is essential for understanding normal lung morphogenesis and the pathophysiology of several lung diseases. In addition, the manipulation of non-CFTR chloride channels may provide a viable approach for treating cystic fibrosis lung disease.

ion channels; developmental regulation; calcium channel family

CHLORIDE TRANSPORT BY AIRWAY epithelia is critically important to several functions of the lung including morphogenesis, airway defensins, and mucociliary transport. Distension of the developing lung by active fluid secretion is dependent on chloride transport across the respiratory epithelium (17, 20, 41), because lung liquid production can be interrupted by bumetanide (20), an inhibitor of Na^+-coupled Cl^- transport at the basolateral cell membrane. Interruption of lung fluid production or retention leads to alterations in lung architecture (1, 39, 40). The molecular identity of the chloride channel(s) (ClC) responsible for active chloride and fluid secretion across the developing airway lumen are unknown.

Molecular defects in the best-known chloride channel, cystic fibrosis (CF) transmembrane conductance regulator (CFTR), lead to impaired function of airway defensins (31), hydration of airway surface fluid, and mucociliary clearance (15, 24), leading to chronic pulmonary infections, obstructive lung disease, and death. Despite the interruption of normal lung defense postnatally, in utero abnormalities of cAMP-mediated CFTR chloride secretion do not lead to disturbances of lung morphogenesis (35). CF newborns have no impairment of lung fluid production or distension of the developing lung, although CF lung explants have abnormal cAMP-mediated chloride transport (21). In addition, the fetal lungs of CF knockout mice are well developed and have no abnormality of distal lung water nor transepithelial ion transport (32). Alternative Cl^- channels, such as those regulated by Ca^{2+}, may compensate for CFTR in these knockout animals.

The ClC family of volume- and voltage-regulated chloride channels plays essential roles in many epithelial tissues. Loss-of-function mutations in CLCN1 lead to myotonia (16, 42), and CLCNKB mutations lead to Bartter's syndrome, Type III (30). Although ClC-5 is

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expressed in several tissues, loss of function mutations lead to Dent's disease, an X-linked renal tubular disorder that results in low molecular weight proteinuria and hypercalciuric nephrolithiasis (19). Investigators have demonstrated ClC-5 expression in the plasma membrane and in endocytic vesicles of renal and intestinal epithelia (12, 27, 37). Mouse models suggest that ClC-5 is the key mediator of chloride conductance that is necessary for early endosomal acidification and is critical for receptor-mediated endocytosis (12, 38). No role for ClC-5 in the lung has yet been identified.

Our laboratory has previously shown that ClC-2 is expressed along the luminal surface of conducting airways in the fetal lung and downregulated at birth (22, 23). The developmental regulation of ClC-2 has recently been confirmed in the human lung and is similar to human CLCN3 expression (18). Acidic pH stimulates chloride secretion in fetal distal lung epithelial monolayers consistent with ClC-2 (2). Moreover, overexpression of ClC-2 in a human CF airway cell line (IB3–1) results in significantly increased chloride conduction, particularly at low pH (28). Taken together, these studies suggest that non-CFTR chloride channels may have the capacity to participate in important functions of airway epithelial ion transport.

Identifying candidate genes for critical ion transport functions is essential for understanding normal lung morphogenesis and the pathophysiology of several lung diseases. In addition, the manipulation of non-CFTR chloride channels may provide a viable approach for treating CF lung disease. The goal of this study is to examine the potential of ClC-5 as an alternative airway epithelial chloride channel in developing lung. Here, we are the first to report the ontogeny of ClC-5 expression in the lung using the ribonuclease protection assay (RPA) and Western blot analysis. In addition, we examine the localization of ClC-5 expression by immunocytochemistry. ClC-5 is predominantly expressed along the luminal surface of the airway epithelium, suggesting that ClC-5 may participate in lung chloride transport.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN) and euthanized by intraperitoneal injection of pentobarbital sodium. Lung tissue was harvested from fetal, newborn, and adult rats, flash frozen, and stored at −80°C until RNA and protein were isolated. For immunohistochemistry, lungs were perfused with 0.9% NaCl at 20 cm pressure distal to a tracheal ligation. Tissues were fixed in 4% paraformaldehyde in PBS.

Quantification of ClC-5 transcripts by ribonucelase protection assay. Total RNA was isolated with TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Optical density 260 readings were used to quantify total RNA, and quality was assessed by ethidium bromide staining on 1% agarose gels. A linearized PCRscript (Stratagene, La Jolla, CA) plasmid containing a 264-bp cDNA fragment of ClC-5 corresponding to nucleotides 1236–1461 of rat ClC-5 (European Molecular Biology Laboratories/GenBank accession No. embZ56277) was transcribed off the T3 promotor and labeled with α-[^{32}P]UTP using a MAXIscript in vitro transcription kit (Ambion, Austin, TX) (29). Ribosomal 18S internal control templates were purchased from Ambion, and riboprobes were prepared according to the manufacturer's instructions. Ten micrograms of rat lung total RNA was hybridized with 1–3× 10^5 counts/min (cpm) of the labeled antisense cRNA probe at 37°C overnight using the RPAIII RNAse protection assay kit (Ambion). RNAse digestion at 1:100 removed unhybridized single-stranded RNA. Protected fragments were electrophoresed on a 6% urea gel and exposed to autoradiography film. For controls, 5 μg of yeast tRNA were used to confirm complete RNAse digestion and specificity of the probes, and 18S ribosomal RNA was used to confirm consistency of total RNA quantitation in samples.

Image files were saved using a Kodak image-grabbing system. The intensity of each band was quantitated using two-dimensional Kodak software. Peak absorbances for ClC-5 and 18S bands were determined for independent experiments. Data were expressed as mean ratio ± SE of the fetal 18-day mRNA in each gel.

Statistical analysis. Data were analyzed by one-way ANOVA using the GLM procedure in SAS software (SAS/STAT User's Guide, Version 8, by SAS Institute, Cary, NC). Statistically significant differences were reported at $P < 0.05$.

Western blot analysis. Lung homogenates were prepared from rat lung in 250 mM sucrose/5 mM Tris/1 mM EDTA using a protease inhibitor cocktail as previously described (23). The DC assay (Bio-Rad Laboratories, Hercules, CA) was used to quantify total protein. Twenty micrograms of total protein were separated by 8% SDS-PAGE and transferred to nitrocellulose (Bio-Rad). The membrane was blocked in 5% nonfat milk/1% BSA in buffer containing 50 mM $NaPO_4$/150 mM NaCl/0.05% Tween 20. A polyclonal chicken anti-rat antibody generated against the putative extracellular loop between transmembrane *domains 8* and *9* was used at 1:5,000 dilution for immunoblotting. Specificity of this antibody has been previously described by Silva et al. (29). The signal was amplified by sequential incubation with a 1:5,000 dilution of biotin labeled goat anti-chicken IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and a 1:5,000 dilution of streptavidin-horseradish peroxidase (Amersham Pharmacia, Piscataway, NJ). Enhanced chemiluminescence (Amersham Pharmacia) was used to detect the signal. A commercially available antipolyclonal antibody raised in rabbit, against a fusion protein with residues 592–661 of rat ClC-3 (Alomone Labs, Jerusalem, Israel) was used to examine potential cross-reactivity of ClC-5 antisera with other ClC family members at a dilution of 1:300. A mouse β-actin monoclonal antibody (Sigma, St. Louis, MO) was used as an internal control at a 1:5,000 dilution.

Immunohistochemistry. Lung and trachea were fixed in 4% paraformaldehyde, embedded with paraffin, and 4-μm tissue sections were mounted on glass slides for immunolocalization. Sections were deparaffinized in xylene and rehydrated in ethanol/water. Antigen retrieval was performed by boiling for 20 min in 10 mM sodium citrate (pH 6.0). Nonspecific sites were blocked with 5% donkey serum in 0.1% Triton X in PBS, and sections were incubated with a 1:200 dilution of our ClC-5 anti-serum overnight in a humidity chamber at room temperature. Controls consisted of no primary antibody, competition with ClC-5 fusion protein (29), and 1:200 dilution of preimmune serum. After overnight incubation, sections were thoroughly washed with PBS and incubated with Texas red conjugated donkey anti-chicken IgG (Jackson Immunoresearch, West Grove, PA). Sections were washed with PBS, and nuclei were counterstained with a 1:3,000 dilution of Hoechst 33342 (Molecular Probes, Eugene, OR). After a quick PBS wash, sections were mounted with Flouromount-G (Southern Biotechnology

Associates, Birmingham, AL) and stored at 4°C until examination on a Zeiss confocal microscope.

RESULTS

Gestational regulation of ClC-5 mRNA. RPA was used to examine the developmental regulation of ClC-5 mRNA in rat lung. A 225-bp protected fragment, corresponding to ClC-5 mRNA, was most easily detectable in fetal and early postnatal rat lung. In the mature lung, the quantity of ClC-5 protected fragments decreased by 14–21 days postnatal and even more significantly by 28 days and in the adult. Figure 1 was one of five representative RPAs, which demonstrates the cohybridization of ClC-5 and 18S ribosomal probes with total RNA from 18- and 20-day fetal; 7-, 14-, 21-, and 28-day postnatal; and adult lung. The 18S protected fragments cohybridized in all samples demonstrate that RNA loaded in each lane were comparable.

To quantify ClC-5 mRNA expression, mean densitometry measurements from five experiments are presented in Fig. 2. Values of the 225-bp protected ClC-5 bands were normalized to the internal control 18S protected RNA fragment and expressed relative to the value of the 18-day fetal sample (n = 4 for 7-day fetal and adult samples, n = 5 for all others). Significant differences in mean ClC5/18S values for 14- and 28-day and adult samples compared with 18-day fetal samples

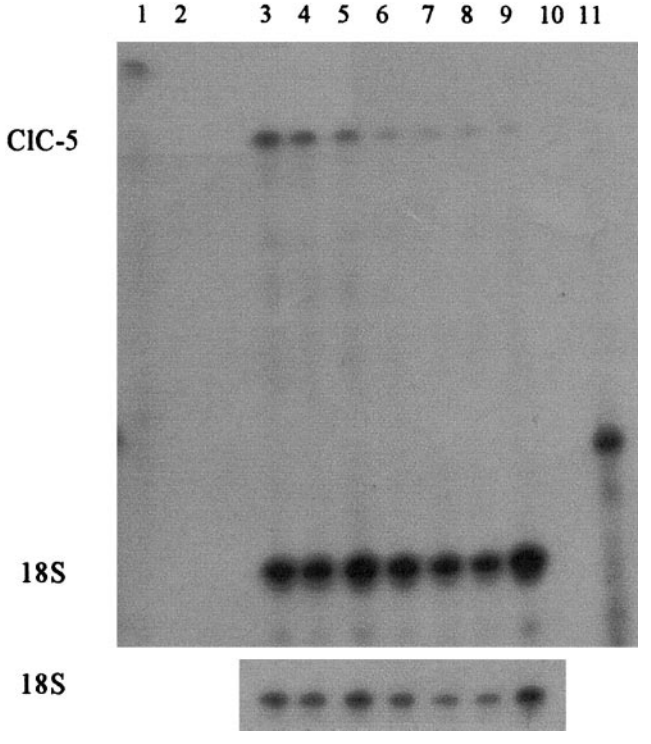

Fig. 1. Ribonuclease protection assay (RPA) demonstrating ClC-5 and 18S protected fragments cohybridized with total RNA from fetal and postnatal rat lung exposed for 60 h (*above*) with 20-h exposure of the same blot (*below*) (n = 5). ClC-5 mRNA is abundantly expressed in 18- and 20-day fetal, and 7-day postnatal (*lanes 3*, *4*, *5*), but is downregulated in 14-, 21-, 28-day postnatal and adult lung (*lanes 6*, *7*, *8*, *9*). Control *lanes 1* and *2* show full-length ClC-5 probe uncut and cut; *lanes 10* and *11* show 18S probe uncut and cut.

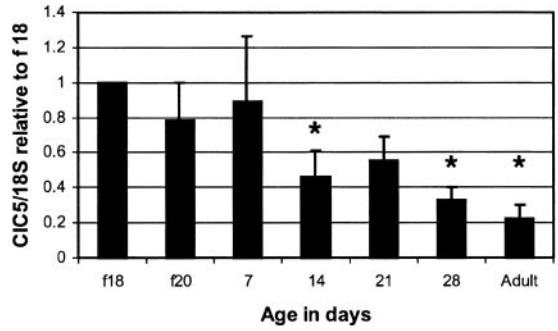

Fig. 2. Gestation-dependent differences in ClC-5 mRNA expression by RPA (n = 5). Quantitation of ClC-5 mRNA transcripts by densitometry measurements of protected ClC-5 and 18S fragments. Bars indicate mean ± SE as a ratio of ClC-5 18-day levels, corrected for 18S. Significant differences in mean ClC-5/18S values for 14- and 28-day fetal, and adult samples compared with 18-day fetal are represented with asterisk (P = 0.024, 0.02, 0.0043 respectively). The above reported P values are not adjusted for multiple comparisons.

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Gestational regulation of ClC-5 protein. To verify the gestational dependence of ClC-5 expression, the ontogeny of ClC-5 protein expression was examined using a polyclonal antibody raised in chicken against rat ClC-5, as previously described (29). The expected 83-kDa signal (33) was detected in fetal (18- and 20-day gestation) and newborn lung (0- to 14-day postnatal) homogenates, but decreased in 21-day lungs, and was nearly undetectable in adult lung homogenates. Figure 3 shows a representative experiment (n = 4). Incuba-

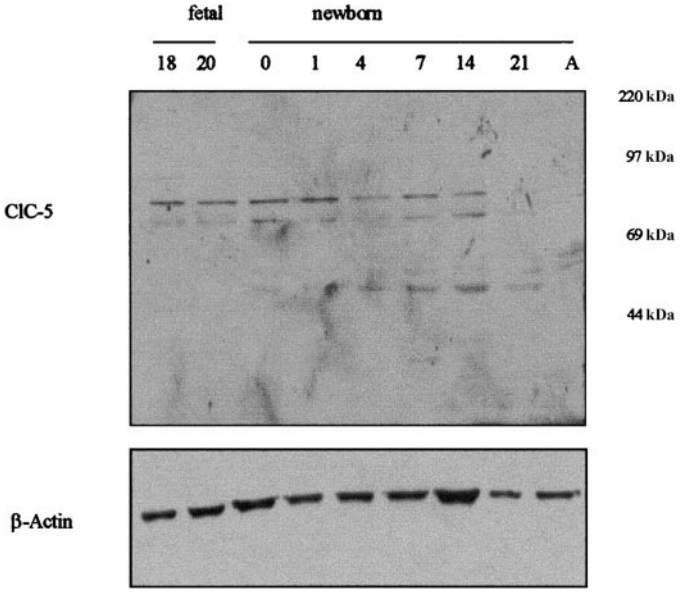

Fig. 3. Detection of ClC-5 protein in rat lung by Western blotting. Fetal (18- and 20-day gestation), postnatal (0–21 day), and adult (*A*) lung homogenates were separated on an SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with anti-ClC-5 antibody. Antisera detected an 83-kDa protein in fetal and early postnatal samples, but weakly reacted with 21-day and adult lung. β-Actin immunoblotting of same membrane shows that amounts of lung homogenate protein examined were similar.

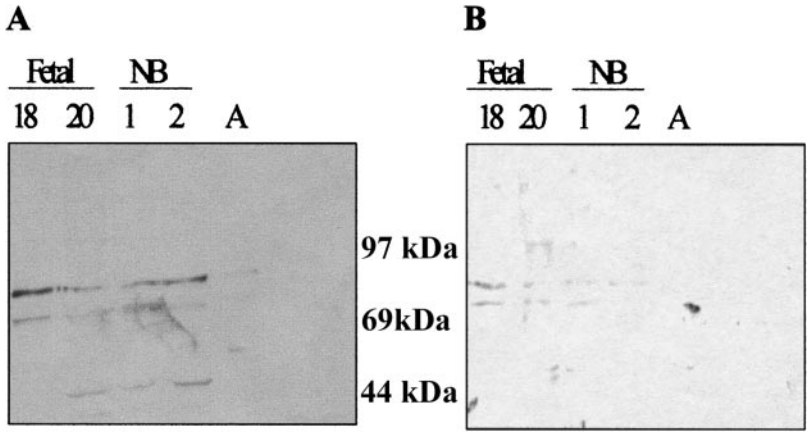

Fig. 4. Inhibition of ClC-5 antibody binding by incubation of ClC-5 antisera with 1 mg/ml ClC-5 fusion protein before Western blotting (*B*) compared with immunoblotting with ClC-5 antisera alone (*A*). The larger 83-kDa band and 50-kDa band are competed more effectively than the 78-kDa band. Fetal (18 and 20 day), newborn (NB; *days 1* and *2*), and adult lung homogenates were separated on an SDS-PAGE gel.

tion of antibody with 1 mg/ml fusion protein decreased, but did not abolish the 83-kDa signal, suggesting that the predominant immunoreactivity is due to ClC-5 (Fig. 4). In addition, this band was not recognized by preimmune serum (not shown). This does not exclude the possibility that the ClC-5 antisera might cross-react with other proteins.

Because ClC-3 is also expressed in lung (18) and may be a potential calcium-activated conductance (14), we also examined the ontogeny of ClC-3 protein expression in rat lung. The commercially available anti-ClC-3 rabbit polyclonal antibody was raised against residues 592–661 (Alomone Labs). This region has 66 and 70% homology with ClC-4 and ClC-5 respectively (33). Expression of ClC-3 protein was nearly absent in the prenatal lung, apparent on the day rat pups were born and abundant in the postnatal lung by *day 7* (Fig. 5). The immunoreactivity of the ClC-3 antibody was abolished by competition with threefold microgram excess of affinity purified ClC-3 fusion protein ("cont," Fig. 5; Alomone Labs). Use of β-actin as an internal control confirms that similar quantities of total lung homogenate were used.

Localization of ClC-5 in rat lung and trachea. RPA and Western blotting quantitate mRNA transcripts and protein levels in whole lung but may not predict regional protein localization. We used immunohistochemical techniques to detect the compartment where ClC-5 is expressed in the lung (Fig. 6). ClC-5 was most abundantly expressed in the airways of the lung (red reaction product along bronchiolar and tracheal airway). Expression was not prominent in the air exchange units (alveoli), interstitium, or vascular spaces as has been seen with its homolog, ClC-3 (18). There was a nonspecific signal in red blood cells in the vascular compartment (small arrow), as seen with positive reaction with second antibody alone. The trachea was a prominent site of expression in both early neonatal lung and in adult lung. The large airways and small airways also had significant ClC-5 expressed. Specific cell types expressing ClC-5 were not determined in this study, but the signal intensity was fairly homogeneous along the airway epithelium. More signal was evident on the luminal surface of the epithelium compared with the basolateral surface or the cytosolic compartment.

DISCUSSION

These studies using RPA, Western blotting, and immunofluorescence demonstrate that ClC-5 expression is gestationally regulated and that it is localized in the respiratory epithlelium. This suggests that ClC-5 could participate in chloride ion transport across the developing airway epithelium.

Chloride channels in respiratory epithelia. Several chloride conductances have been characterized in the mammalian lung in addition to the cAMP-dependent, CFTR. These include the Ca^{2+}-dependent chloride channel (10, 11, 14), the outwardly rectifying chloride

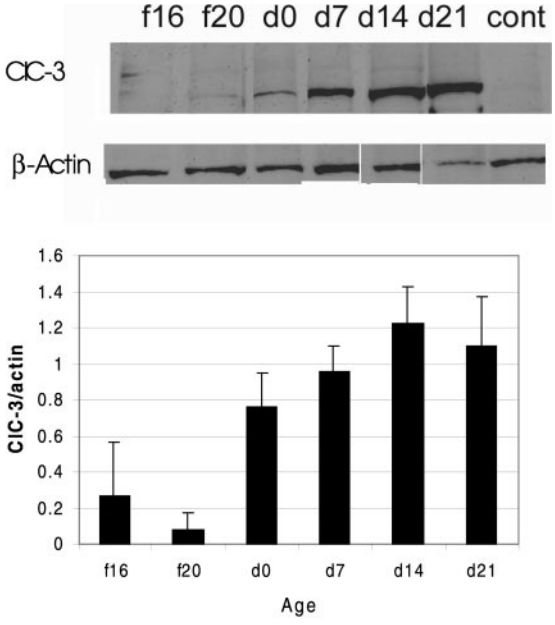

Fig. 5. Immunoblot demonstrating that ClC-3 expression in rat lung begins after birth and increases postnatally. F16 and 20 are fetal lung homogenates of 16- and 20-day gestation. D0–21 are postnatal lung homogenates from rats ages 0–21 days. Cont is *day 21* lung homogenate immunoblotted with ClC-3 polyclonal antibody preincubated with threefold microgram excess of control fusion protein. β-Actin control lanes are from same immunoblot incubated with β-Actin to demonstrate that similar amounts of protein are examined. Graph demonstrates mean ClC-3/actin expression ± SE for 4 experiments.

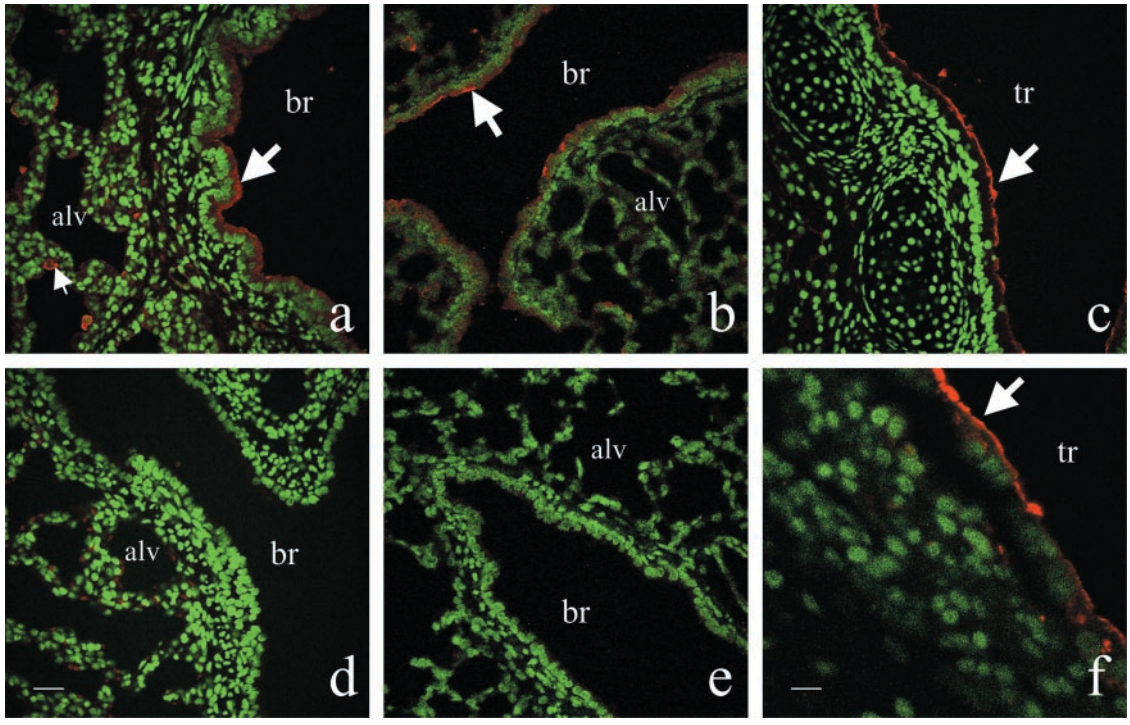

Fig. 6. ClC-5 is expressed predominantly along airway luminal surfaces by immunohistochemistry. Texas Red immunoreaction demonstrates ClC-5 expression in 4-day-old (*A*) rat lung, 18-day-old lung (*B*), trachea (*C*), and adult trachea (*F*) (large arrows). Bronchiole = br; alveoli = alv; trachea = tr. Small arrow points to nonspecific staining of red blood cells. Negative control (lacking primary antibody) for 4-day-old (*D*) and 18-day-old (*E*) lungs are also shown for comparison. Bar in 6*D* = 50 μm for *A*–*E*. Bar = 20 μm for *F*.

channel (ORCC) (8, 13), and at least one member of the voltage- and volume-regulated, ClC family of chloride channels (ClC-2) (2). Although mutations in 2 CFTR alleles are associated with the most common life-shortening autosomal recessive disease, CF does not alter lung morphogenesis (35), can be associated with preserved lung function, and is not uniformly lethal (25). Creation of CF knockout mice, which have no lung disease, has demonstrated that alternative chloride channels in respiratory epithelia exist, and that they can compensate for defects in CFTR (32).

Although the molecular identity of compensatory chloride channels in the lung is unknown, the large ClC family offers some candidates. Of the eight members of the mammalian ClC family, several are associated with human disease due to loss-of-function mutations, suggesting important roles in chloride transport (16, 19, 30, 42). In the developing lung, ClC-2 was the first to be described. We have previously demonstrated that mRNA and protein of the volume- and pH-regulated ClC channel, ClC-2, are abundantly expressed in the fetal lung and expression is markedly diminished at birth (22, 23). ClC-2 may participate in fetal lung chloride secretion as acidic pH-activated chloride currents exist in the polarized fetal respiratory epithelium that can be inhibited by CdCl but not 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, which blocks ClC-3 and ClC-5 or glibenclamide, which blocks CFTR, consistent with properties of ClC-2 (2). Although the knockout model for ClC-2 suggests that it is not essential for fetal lung fluid secretion and morphogenesis (4), its role in the fetal lung has yet to be determined. Upregulation of ClC-2 expression postnatally would likely be necessary as a strategy to overcome defects in CFTR chloride secretion. The identification of an endogenous airway epithelial chloride channel that is expressed throughout postnatal life would be advantageous for developing therapies that could compensate for defects in CFTR chloride channel functions.

With homology cloning and the characterization of single-channel properties of ClC family members, candidate genes for the airway epithelial ORCC have been identified ClC-3, ClC-4, and ClC-5 (7, 26, 33, 36). These three form a distinct branch of the ClC gene family with ~80% amino acid identity among them. Loss of function mutations of ClC-5 have been linked to Dent's disease, an X-linked hereditary form of nephrolithiasis (19). Knockout mice have confirmed the crucial role that ClC-5 plays in the kidney (38). ClC-5 encodes a 746 amino acid protein with a predicted molecular mass of 83 kDa and has consensus sites for phosphorylation by protein kinase A (33). The entire ClC-5 mRNA transcript is 9.5 kb. The region encoding the chloride channel is only 2.2 kb, suggesting there is a large untranslated 5′ region. ClC-5 produces strong outward rectification in *Xenopus* oocytes, which can be reproduced in the plasma membrane of HEK293 cells (9). These studies demonstrate that ClC-5 has the capacity to function in the plasma membrane and may not have functions restricted to the endosomes. In

contrast to ClC-2, ClC-5 currents are reduced by acidic extracellular pH. Although chloride transport via ClC-5 may not be favorable at biological potentials, activation by other influences should be sought.

To determine whether ClC-5 could be an airway epithelial chloride channel with the potential for important functions in the developing lung, we used three independent techniques: RPA, Western blotting, and immunofluorescence. We have demonstrated that ClC-5 is expressed not only in the fetal airway but continues to be expressed postnatally but less abundantly in young and mature adult lung. This is consistent with Northern and Western blots comparing ClC-5 expression in many tissues (12, 33, 37). ClC-5 has significant homology with two family members, ClC-3 and ClC-4 (33). Although cross-reactivity of ClC-5 cRNA probe and antibody with these homologs is possible and cannot be entirely excluded, the RNAse protected fragment was of the expected size, and specificity of the antibody was demonstrated by competition with the ClC-5 fusion protein (reduction in 83-kDa signal, Fig. 4). The D8-D9 domains of ClC-5 are less conserved among these three homologs, and specificity of this region as an epitope for ClC-5 has been previously reported (6, 29). To examine the potential cross-reactivity of ClC-5, we also tested a ClC-3 antibody with 70% homology to ClC-5. ClC-3 had a later onset of expression in the rat lung (Fig. 5), suggesting that the dominant band detected at 83 kDa in Fig. 3, was most likely ClC-5 and not ClC-3. Protein degradation leading to faster migration of proteins by SDS-PAGE is possible; however, samples were kept frozen until use and were homogenized with protease inhibitors. The 78-kDa band in Figs. 3 and 4 is likely nonspecific as it did not compete away as effectively as the 83- and 50-kDa bands in Fig. 4 (22). Alternatively, the 78- and 50-kDa bands could be homologs of the ClC family, which have yet to be identified. Interestingly, the timing of expression of the 50-kDa band is similar to that of ClC-3 (Fig. 5) and could be a short form of the 84-kDa ClC-3 protein.

Although examination of whole lung homogenates suggests less abundant expression of ClC-5 in the adult lung, immunolocalization demonstrates that ClC-5 is expressed in adult trachea and airways. This is compatible with CFTR, ClC-2, and ClC-3 localization (5, 18, 22, 23). CLCN3 expression in developing human lung localizes not only to airway epithelium but also to vascular endothelium (18). Nonspecific immunoreactivity with our ClC-5 polyclonal antibody was seen in occasional red blood cells but not in vascular endothelium, again suggesting that immunodetection in this study was most likely ClC-5. Within the kidney and intestine, ClC-5 is localized in vesicles and likely plays a role in acidification of early endosomes (38). This study was not designed to examine ClC-5 expression in subcellular compartments of the airway epithelium, so whether or not ClC-5 is involved in vesicular trafficking in the lung has yet to be determined.

Although no pulmonary manifestations of Dent's disease have been described in humans and the ClC-5 knockout mouse has no significant lung disease, loss of ClC-5 function in the lung may not be as critical as CFTR dysfunction. ClC-2 and ClC-3 are also expressed in the developing lung and may contribute to the chloride gradient necessary for lung fluid production and morphogenesis. Disruption of ClC-2 (4), ClC-3 (34), and ClC-5 (38) have not resulted in severe lung defects, suggesting that they may have overlapping functions and that if there is a critical chloride channel for lung morphogenesis, it has not yet been identified. Alternative chloride channels in the airways may explain the heterogeneity in CF lung disease, by compensating when CFTR is defective (43). The expression of ClC-5 in the lung postnatally along the luminal surface of the airway compartment makes it an attractive candidate for further study. Gene regulation and activation of chloride secretion in CF lung disease may be a useful alternative to gene therapy. The potential for manipulation of an alternative chloride channel before CF lung disease manifests (as early as infancy) is likely to be important.

Chloride channel functions differ by tissue and site of expression. Although CFTR is expressed in many epithelial tissues, disease manifestations are most prominent in the respiratory tract and the kidney is relatively preserved. In contrast, ClC-5 disease manifestations are most evident in the kidney, although the intestine may also be affected (19, 29, 37). Given our findings that ClC-5 is more strongly expressed along the lumen of the airway epithelium, endocytic recycling of proteins may not be the primary function of ClC-5 in the lung. Developmental regulation of ClC-5 expression in lung and predominant expression in airway epithelium suggests that further study is warranted to determine its potential chloride transport functions in the lung.

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