Novel Role for Pendrin in Orchestrating Bicarbonate Secretion in Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)-expressing Airway Serous Cells**

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In most HCO3\textsuperscript--secreting epithelial tissues, SLC26 Cl\textsuperscript--/HCO3\textsuperscript-- transporters work in concert with the cystic fibrosis transmembrane conductance regulator (CFTR) to regulate the magnitude and composition of the secreted fluid, a process that is vital for normal tissue function. By contrast, CFTR is regarded as the only exit pathway for HCO3\textsuperscript-- in the airways. Here we show that Cl\textsuperscript--/HCO3\textsuperscript-- anion exchange makes a major contribution to transcellular HCO3\textsuperscript-- transport in airway serous cells. Real-time measurement of intracellular pH from polarized cultures of human Calu-3 cells demonstrated cAMP/PKA-activated Cl\textsuperscript--/HCO3\textsuperscript-- exchange across the luminal membrane via CFTR-dependent coupled Cl\textsuperscript--/HCO3\textsuperscript-- anion exchange. The pharmacological and functional profile of the luminal anion exchanger was consistent with SLC26A4 (pendrin), which was shown to be expressed by quantitative RT-PCR, Western blot, and immunofluorescence. Pendrin-mediated anion exchange activity was confirmed by shRNA pendrin knockdown (KD), which markedly reduced cAMP-activated Cl\textsuperscript--/HCO3\textsuperscript-- exchange. To establish the relative roles of CFTR and pendrin in net HCO3\textsuperscript-- secretion, transepithelial liquid secretion rate and liquid pH were measured in wild type, pendrin KD, and CFTR KD cells. cAMP/PKA increased the rate and pH of the secreted fluid. Inhibiting CFTR reduced the rate of liquid secretion but not the pH, whereas decreasing pendrin activity lowered pH with little effect on volume. These results establish that CFTR predominately controls the rate of liquid secretion, whereas pendrin regulates the composition of the secreted fluid and identifies a critical role for this anion exchanger in transcellular HCO3\textsuperscript-- secretion in airway serous cells.

HCO3\textsuperscript-- is a vital component of epithelial secretions. Despite the growing awareness of its importance in epithelial function, the molecular mechanism of HCO3\textsuperscript-- secretion remains incompletely understood. Via its buffering role, HCO3\textsuperscript-- controls the pH of the luminal microenvironment, a function particularly important to the physiology of many epithelial tissues, including the airways. Consistent with a role for HCO3\textsuperscript-- secretion in airway function, a previous study found the airway surface liquid to be acidic in cystic fibrosis (CF) compared with normal cell cultures (1), and a similar finding was made in secretions from nasal submucosal glands (SMGs) from CF patients (2). Aberrant pH/HCO3\textsuperscript-- secretion probably contributes to CF lung pathogenesis in a number of fundamental ways. HCO3\textsuperscript-- is a chaotropic anion that facilitates efficient solubilization and transport of macromolecules, such as mucins (3). Also, the recent finding that HCO3\textsuperscript-- secretion is required for mucin secretion (4–6) and that mucin expansion and viscosity are regulated by HCO3\textsuperscript-- (7, 8) strongly suggests that adequate HCO3\textsuperscript-- is required for proper mucus homeostasis. In addition, acidic pH has been shown to reduce ciliary beat frequency (9) and impede bacterial killing by phagocytic cells (10, 11). Collectively, these consequences of inadequate HCO3\textsuperscript-- transport predispose the lungs to mucus blockage, bacterial infection, and disease, all hallmarks of the CF lung.

Current studies suggest that HCO3\textsuperscript-- exit (secretion) across the luminal plasma membrane of airway epithelial cells is mediated solely by the cystic fibrosis transmembrane conductance regulator (CFTR), the ion channel that is mutated in CF. Exactly how CFTR dysfunction leads to aberrant HCO3\textsuperscript-- secretion is unclear. In primary cultures of surface bronchial and tracheal epithelial cells from CF humans and pigs, a lack of CFTR is associated with reduced electrogenic HCO3\textsuperscript-- secretion (12, 13). Likewise, in ex vivo studies of liquid/mucus secretion from intact SMGs from a range of species, cAMP-stimulated fluid...
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secretion depends on both Cl⁻ and $\text{HCO}_3^-$ and was absent in CF glands (14–16). Furthermore, detailed studies of anion and fluid secretion from polarized cultures of human airway Calu-3 cells, a model of human tracheobronchial SMG serous cells, also concluded that CFTR was the sole mediator of apical Cl⁻ and $\text{HCO}_3^-$ secretion (17–19). However, in many $\text{HCO}_3^-$-secreting epithelia, including the pancreas (20), salivary glands (21), and gastrointestinal (22) and reproductive tracts (23), $\text{HCO}_3^-$ secretion is mediated by CFTR and one or more Cl⁻/$\text{HCO}_3^-$ exchangers belonging to the SLC26 gene family. This family comprises 10 members, and their functional characterization indicates distinct patterns of anion specificity and transport modes. Furthermore, structural analyses indicate that CFTR and SLC26 transporters can physically interact through their regulatory and STAS domains, respectively (24), a process that is enhanced by PKA phosphorylation of the regulatory domain (24). In most cases, these molecular interactions synergize the transport activity of CFTR and the SLC26 exchanger, resulting in enhanced $\text{HCO}_3^-$ and fluid secretion from epithelial tissues.

Therefore, SLC26 anion exchangers have a well documented role in $\text{HCO}_3^-$ secretion in non-airway tissues. Human tracheal airway epithelial cells express abundant SLC26A3, whereas delf508 cells do not (25). Furthermore, RNA analysis has shown that human lungs also express SLC26A9 (26) which appears to act as a constitutively active CFTR-regulated Cl⁻ channel in cultured human bronchial epithelial cells (27). However, to date, it is unknown whether SLC26 proteins are involved in $\text{HCO}_3^-$ secretion in the airways. Hence, the purpose of this study was to investigate the potential role of SLC26 Cl⁻/$\text{HCO}_3^-$ exchangers in transcellular $\text{HCO}_3^-$ secretion in airway epithelial cells. Our results show for the first time that human airway serous cells possess a luminal cAMP/PKA-activated Cl⁻/$\text{HCO}_3^-$ exchanger that exhibits functional properties consistent with those of SLC26A4 (pendrin). Short hairpin RNA (shRNA)-mediated knockdown of pendrin expression significantly reduced Cl⁻/$\text{HCO}_3^-$ exchange activity and markedly lowered the $\text{HCO}_3^-$ content of the secreted fluid. These studies therefore identify for the first time a critical role for pendrin in transcellular $\text{HCO}_3^-$ secretion by airway serous cells.

**EXPERIMENTAL PROCEDURES**

**Calu-3 Cell Culture**—The human adenocarcinoma-derived cell line, Calu-3 (passages 20–50 (28)), was grown in Eagle’s minimal essential medium plus 10% FCS, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% non-essential amino acids (Sigma) and incubated in humidified air containing 5% CO₂ at 37 °C. CFTR knockdown (KD) Calu-3 cells (29), SLC26A4, and cyclophilin B KD Calu-3 cells were cultured in the same media supplemented with Geneticin (CFTR KD, 400 μg/ml; G418; Sigma) or puromycin (10 μg/ml; Sigma), respectively. For experiments using polarized cells, Calu-3 cells were seeded onto clear Costar Transwell® or Snapwell® inserts (0.45-μm pore size, 1.12-cm² surface area) at 250,000 cells/cm², respectively. Calu-3 cells generally formed a confluent monolayer with a stable transepithelial resistance ($V_{te}$) of 700–900 ohms/cm² after 5 days of growth on Transwell inserts. Experiments were carried out 7–14 days postseeding.

**Fischer Rat Thyroid (FRT) Cell Culture**—Non-transfected FRT cells and FRT cells stably transfected with pendrin or CFTR were kindly provided by Drs. L. Galiotta and O. Zegarra-Moran (University of Genoa, Italy) and generated as described previously (30). The cells were cultured in Coon’s modified Ham’s F-12 medium supplemented with 10% FCS, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% non-essential amino acids. For CFTR-transfected FRT cells, medium was supplemented with 0.75 mg/ml Geneticin (G418; Sigma) and 0.6 mg/ml zeocin (Sigma). Pendrin-transfected FRT cells were grown in medium supplemented with 1 mg/ml Geneticin and 0.5 mg/ml hygromycin (Sigma).

**shRNA Knockdown of SLC26A4 in Calu-3 Cells**—Individual SLC26A4 and cyclophilin B (control)-deficient Calu-3 cell lines were produced using lentivirus-mediated delivery of shRNA (Sigma MISSION) knockdown with a set of four different shRNA sequences (supplemental Table S1). Lentiviral transduction particles were applied at a multiplicity of infection ratio of 1. Transduced cells were selected 48 h post-transduction using 10 μg/ml puromycin. KD cell lines used for this study had $V_{te}$ and growth patterns similar to that of WT Calu-3 cells.

**Measurement of Intracellular pH**—Cells were loaded with the pH-sensitive fluorescent dye 2’,7’-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (10 μM) for 45–60 min at 37 °C in a HEPES-buffered salt solution, which consisted of 130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM Na-HEPES, and 10 mM d-glucose set to pH 7.4. Transwells were placed in a perfusion chamber, mounted onto an inverted microscope stage (Nikon), and perfused with a HCO₃⁻-buffered Krebs solution (KRB), which consisted of 115 mM NaCl, 5 mM KCl, 25 mM NaHCO₃, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM d-glucose and adjusted to pH 7.4 by bubbling with a 95% O₂, 5% CO₂ mixture at 37 °C. Apical and basolateral bath volumes were 0.5 and 1 ml and were perfused at a rate of 3 and 6 ml/min, respectively. Intracellular pH (pHᵢ) was measured from 15–20 cells as described previously (31), using a Life Sciences Microfluorimeter System (Life Sciences Resources). Ratio values were calibrated to pHᵢ using the high K⁺-nigericin method (10 μM), using K⁺ solutions of various pH values from 5.6 to 8.6 (31). Mean changes in pHᵢ were estimated by calculating the average pHᵢ over 60 s (120 data points). The initial rate of pHᵢ change ($ΔpHᵢ/Δt$) was calculated by linear regression fitted to a minimum of 40 data points. Total buffering capacity was estimated by the ammonium pulse technique, using the Henderson-Hasselbalch equation as described previously (31). The $ΔpHᵢ/Δt$ values were converted to transmembrane efflux of $\text{HCO}_3^-$ ($J(B)$) using the equation, $J(B) = rate$ of $\text{HCO}_3^-$ change × total buffering capacity. For high K⁺ KRB, KCl was increased to 115 mM, and NaCl was reduced to 5 mM. For Cl⁻-free KRB, NaCl was substituted with sodium gluconate, with 6 mM calcium gluconate replacing 1 mM CaCl₂ to compensate for the Ca²⁺ buffering capacity of gluconate, and 5 mM KCl was replaced with 2.5 mM K₂SO₄. For Na⁺-free KRB, 115 mM NMDG-Cl replaced NaCl, and 25 mM choline-HCO₃ replaced NaHCO₃. Atropine (10 μM) was included to block muscarinic receptors. Cl⁻-free HEPES-buffered solution consisted of 130 mM sodium gluconate, 2.5 mM K₂SO₄, 1 mM magnesium gluconate, 6 mM calcium gluconate, 10 mM HEPES (free acid), and 10 mM d-glucose. All
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general chemicals were purchased from Sigma-Aldrich except for forskolin (Tocris), and GlyH-101 (Calbiochem).

Transepithelial Liquid Secretion Rates and pH Measurements—After washing confluent monolayers with PBS, to remove mucus, the rate of liquid secretion ($J_L$) was determined by applying 0.2 and 1 ml of Krebs solution to the apical and basolateral surfaces of the cells, respectively, with the desired agonist or inhibitors. Cells were placed in a humidified CO$_2$ incubator at 37 °C, and the volume of the apical fluid was measured using a calibrated micropipette after 24 h. For CO$_3^-$-free experiments, Transwells were bathed in HEPES-buffered solution and maintained in a humidified incubator at 37 °C without CO$_2$ gassing. Correction for evaporative apical volume loss (0.08 ± 0.02 μl/cm$^2$/h; $n = 12$) was determined empirically by measuring the reduction in apical volume from Transwells coated with silicone gel to stop fluid leakage across the membrane. The pH of 5% CO$_2$-saturated apical fluid was measured using a micro-pH electrode (Hamilton) within 60 s of removing individual Transwells from the incubator. Control experiments showed that pH drifted by 0.04 pH units/min using this approach.

Quantitative RT-PCR Analysis of SLC26 Gene Expression—Total RNA was isolated using the Qiagen RNeasy miniprep kit and quantified using the Bioanalyzer 2100 and RNA total microchips and reagents (Agilent Technologies). cDNA synthesis was performed with 20 ng/ml total RNA/sample using the GeneAmp RT-PCR kit (Applied Biosystems). A mixed tissue standard was generated from total RNA (liver, spleen, kidney, lung, testis, pancreas, brain, and fetal brain; purchased individually from Clontech). Quantitative RT-PCR (Taqman) analysis was used to measure $SLC26$ gene expression, using previously optimized primers and probes (Applied Biosystems, Assays-on-Demand; supplemental Table S2). Amplicons were designed to span intron-exon boundaries to preclude possible amplification of genomic DNA sequences. Reactions were performed in 96-well optical plates and analyzed on the ABI PRISM 7700 (Applied Biosystems) using the following program: 50 °C for 2 min and 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were quantified relative to the standard curve for each gene. Expression was normalized to $GAPDH$ and expressed as percentage relative to the standard curve.

Immunocytochemistry—Calu-3 cells grown on Transwells were fixed and permeabilized with cold 100% methanol (−20 °C) for 15 min on ice. Samples were blocked with 3% horse serum for 1 h at room temperature and then incubated with a mouse polyclonal anti-pendrin (Abnova) at 1:200 overnight at 4 °C. After blocking with 3% goat serum for 1 h at room temperature, samples were incubated with a goat Alexa Fluor 488-coupled anti-mouse secondary antibody (Invitrogen) at 1:1000 in 3% goat serum. The samples were also stained for zona occludens 1 (ZO-1), using a rabbit anti-ZO-1 antibody (1:500; Invitrogen) and a goat anti-rabbit secondary antibody (1:1000; Red Alexa Fluor 568, Invitrogen). Negative controls omitted primary antibody. Images were collected by confocal laser-scanning microscopy, using a Leica TCS-NT system (Leica UK Ltd.) equipped with a ×100 oil immersion lens, using appropriate excitation and emission filter sets for dual fluorophore detection. Leica software was used to capture images, under identical conditions of imaging, illumination intensity, and photomultiplier settings.

Statistical Analysis—Results are presented as mean ± S.E., where $n$ indicates the number of experiments. Statistical analysis was performed with GraphPad Prism software (GraphPad Software Inc.), using either a paired Student’s $t$ test or one-way analysis of variance with Bonferroni’s $post hoc$ test. $p$ values of <0.05 were considered statistically significant.

RESULTS

Effects of Asymmetrical Cl$^-$ Removal in Unstimulated Cells—Under control conditions, removal of Cl$^-$ from the apical perfusate did not change pH$_i$, indicating little resting apical anion exchange (AE) activity (Fig. 1A), which would be expected to cause an alkalinization. In marked contrast, basolateral Cl$^-$ removal produced a large, monophasic alkalinization of 0.43 ± 0.01 pH units ($p < 0.001$; $n = 35$). Cl$^-$ readdition reversed this response at an initial rate of 0.52 ± 0.03 pH units/min (−$J_B$) = 49.1 ± 7.0 mm B min$^{-1}$). These results indicate functional AE activity in the basolateral, but not apical, membrane of Calu-3 cells under resting conditions, probably by anion exchanger 2 (AE2) (32, 33).

Effects of Cl$^-$ Removal Subsequent to cAMP Elevation—Exposure of cells to forskolin (fsk; 5 μM) under symmetrical high Cl$^-$ conditions significantly acidified pH$_i$ by 0.13 ± 0.01 units (Fig. 1A), at a rate of 0.08 ± 0.01 pH units/min. Subsequent apical Cl$^-$ removal markedly alkalinized fsk-treated cells by 0.64 ± 0.03 pH units ($p < 0.001$; $n = 35$; Fig. 1B). The pH response was typically biphasic, consisting of a fast initial alkalinization followed by a slower secondary rise in pH$_i$ that reached a steady state after 3–4 min (Fig. 1A). Upon Cl$^-$ readdition, pH$_i$ recovered to base-line values at an initial rate of 0.77 ± 0.08 pH units/min (−$J_B$) = 67.3 ± 13.3 mm B min$^{-1}$; Fig. 1B). Interestingly, under fsk stimulation, removal of basolateral Cl$^-$ produced no change in pH$_i$ (Fig. 1A), which indicates that fsk treatment both inhibits basolateral AE and stimulates apical AE activity. Dideoxyforskolin, an inactive analog of fsk, did not mimic these effects, thus confirming that a change in intracellular cAMP provided the fsk-induced “switch” in the Cl$^-$-dependent apical and basolateral pH$_i$ responses ($p > 0.05$ compared with non-stimulated apical and basolateral Cl$^-$-dependent changes in pH$_i$; $n = 3$; data not shown). Furthermore, both vasoactive intestinal peptide and adenosine, physiological, cAMP-mediated agonists in Calu-3 cells (34, 35), changed pH$_i$ in response to apical Cl$^-$ removal, similar to the fsk-stimulated response ($p > 0.05$; $n = 4$; Fig. 1C).

Consistent with a cAMP/PKA-dependent activation of an apical AE, the Cl$^-$-dependent pH$_i$ responses were reduced by the PKA inhibitor H-89 as well as by the general PK inhibitor staurosporine (Fig. 1D; Calu-3 cells were somewhat insensitive to H-89 and required preincubation at a relatively high concentration (50 μM), which may have PKA-independent effects). The changes in pH$_i$ caused by apical Cl$^-$ removal in fsk-treated cells depended entirely on HCO$_3^-$; replacement of NaHCO$_3$ with Na-HEPES markedly reduced the Cl$^-$-dependent alkalinization and pH$_i$ recovery (Fig. 1E). Finally, and in contrast to the results with cAMP agonists, raising cytosolic Ca$^{2+}$ had no effect.
on apical AE activity (supplemental Fig. S1). Taken together, these results suggest that the observed changes in pH\textsubscript{i} are due to cAMP/PKA activation of an apical Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−}-dependent HCO\textsubscript{3}\textsuperscript{−}/H\textsubscript{2}O transport that strongly selects for HCO\textsubscript{3}\textsuperscript{−}/H\textsubscript{2}O over OH\textsuperscript{−}.

Properties of cAMP-stimulated Responses to Apical Cl\textsuperscript{−} Removal—The ability of different anions to support reacidification following Cl\textsuperscript{−} withdrawal was next measured. Recovery of pH\textsubscript{i} was supported by a range of monovalent but not divalent anions. A clear selectivity exists among the monovalent anions, with I\textsuperscript{−} and Br\textsuperscript{−} exhibiting the highest rate of reacidification compared with the other anions (p < 0.01; n = 5). The anion/Cl\textsuperscript{−} selectivity ratio sequence was as follows: HCOO\textsuperscript{−}, 0.6; NO\textsubscript{3}\textsuperscript{−}, 0.8; SCN\textsuperscript{−}, 0.8; Cl\textsuperscript{−}, 1; Br\textsuperscript{−}, 1; I\textsuperscript{−}, 1.3 (Fig. 2A). Removal of Na\textsuperscript{+} had no significant effect on the rate of reacidification (ΔpH\textsubscript{RA}) (p > 0.05; n = 4; Fig. 2B). Similarly, hyperpolarization of the resting membrane potential (V\textsubscript{m}) using the K\textsuperscript{+} channel...
Bilateral H2-DIDS-sensitive "base" transporter strongly influenced both due to inhibition of HCO3
exposure to GlyH-101 caused an alkalinization that is probably

**FIGURE 2. Ionic dependence of apical Cl−-dependent HCO3− transport in forskolin-stimulated Calu-3 cell monolayers.** A, anion selectivity of the apical Cl−-dependent pHi changes measured by the ability of the replacement anion (for Cl−) to support reacidification under fsk-stimulated conditions. Anions were added iso-osmotically to replace Cl−. (n = 5; paired observations; *, p < 0.01 compared with chloride; †, p < 0.01 compared with formate, nitrate, and thiocyanate). B, the effect of bilateral Na+ removal on ΔpHRA upon apical Cl− readministration in fsk-treated cells. Data are expressed as a percentage of the rate obtained with fsk-treated monolayers in the presence of bilateral Na+ (n = 4; Na+ containing and Na+ free Krebs solutions applied in separate experiments, performed in parallel). C, the effect of bilateral high K+ (115 mM) and K+ channel opener EBIO (1 mM) on the percentage ΔpHRA upon apical Cl− readministration in fsk-stimulated cells. Shown are pHi responses in high K+ and EBIO compared with control apical Cl− plus fskolin (FSK) responses. Each condition was measured in separate experiments performed in parallel (n = 4; *, p < 0.05 compared with apical Cl− plus fskolin). D, the effect of apical H2-DIDS (500 μM) on the ΔpHRA upon apical Cl− readministration in fsk-stimulated cells (n = 4; paired observations). Error bars, S.E.

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open 1-ethyl-2-benzimidazolinone (EBIO; 1 mM) likewise was without effect (Fig. 2C). However, clamping Vm to 0 mV through a combination of high extracellular K+ plus EBIO did reduce the rate of reacidification by 23.7 ± 2.3% (p < 0.05; n = 4; Fig. 2C), indicating some dependence on Vm. The ΔpHRA was also insensitive to the general anion transport inhibitor 4,4'-disothiocyanato-1,2-diphenylethane-2,2'-disulfonate (H2-DIDS) (p > 0.05; n = 4; Fig. 2D). The observed changes in pHi depicted in Figs. 1 and 2 are therefore due to the activity of a Na+-independent, H2-DIDS-insensitive, electrogenic, monovalent anion transporter, consistent with the known properties of CFTR.

**Contribution of CFTR and Basolateral Cl−/HCO3− Exchange to pHi Responses—** To determine whether CFTR contributes to the alkalization caused by apical Cl− removal, the effect of CFTR inhibition was tested. GlyH-101 (10 μM) abolished the pHi response to apical Cl− removal in fsk-stimulated Calu-3 monolayers (lack of alkalization in the presence of GlyH-101, shown by the black trace in Fig. 3A). CFTRinh-172 produced similar results (10 μM) (data not shown). Note that the initial exposure to GlyH-101 caused an alkalization that is probably due to inhibition of HCO3− efflux from the cells.

In the absence of a CFTR blocker, the activity of a basolateral H2-DIDS-sensitive "base" transporter strongly influenced both the magnitude of the pHi response to apical Cl− removal and the ΔpHRA (supplemental Fig. S2). To ascertain whether a basolateral H2-DIDS-sensitive transporter also affects the Cl−-dependent pHi response in the presence of GlyH-101, Calu-3 monolayers were first exposed to basolateral H2-DIDS before GlyH-101. This maneuver restored apical Cl−-induced pHi changes to GlyH-101-treated cells (Fig. 3A, blue trace) and indicated the presence of an apical Cl−/HCO3− exchanger. In contrast to the rates of alkalization with Cl− removal, the ΔpHRA upon apical Cl− readministration was significantly reduced under these conditions (p < 0.001; n = 4; Fig. 3C). These data suggest that apical Cl−/HCO3− exchange is functionally coupled to Cl− transport by CFTR or that CFTR itself directly contributes to pH changes, in addition to the exchanger.

**Knockdown of CFTR Provides Additional Evidence for a Role of both CFTR and a Coupled Anion Exchanger—** The role of CFTR in regulating apical Cl−/HCO3− exchange was next investigated using CFTR KD Calu-3 cells (29). CFTR content of KD cells was determined to be ~28 ± 5% that of wild-type (WT) cells (supplemental Fig. S3, A and B). Compared with WT cells, cAMP-stimulated secretory capacity in these CFTR KD cells was also reduced by ~25% (29). Mean pHi values did not differ significantly between WT (7.43 ± 0.03; n = 50) and CFTR KD (7.39 ± 0.05; n = 32; p > 0.05) Calu-3 cells. The switch from basolateral to apical AE activity in response to cAMP is preserved in CFTR KD cells (Fig. 4A). However, in CFTR KD cells,
the basolateral anion exchanger was not fully inhibited under fsk-stimulated conditions (compare Figs. 1A and 4A). The reduced expression of CFTR in the CFTR KD Calu-3 cells was reflected in a reduced ΔpHRA (~48%), which decreased from 0.75 ± 0.09 pH units/min (−J(B) = 97.6 ± 13.3 mm B min⁻¹) in WT cells to 0.39 ± 0.02 pH units/min (−J(B) = 65.9 ± 9.8 mm B min⁻¹) in KD cells (p < 0.01; n = 4; Fig. 4B). These values are similar to those obtained in GlyH-101/H₂-DIDS-treated WT Calu-3 cells (Fig. 3B). Although ΔpHRA was slower, the profile of the apical Cl⁻-dependent change in pHᵢ in CFTR KD Calu-3 monolayers broadly resembled that of WT cells, being H₂-DIDS-insensitive, abolished by GlyH-101 addition in the absence of basolateral H₂-DIDS (data not shown), and supported only by monovalent anions. However, the I⁻/Cl⁻ selectivity ratio significantly increased from 1.3 in WT cells to 2.1 in CFTR KD cells (p < 0.001; n = 4; Fig. 4C; see “Discussion”). Taken together, these results suggest that both CFTR and an apical Cl⁻/HCO₃⁻ exchanger contribute to Cl⁻-induced pHᵢ changes in Calu-3 cells.

**Pendrin Is Expressed in Calu-3 and Human Airway Cells—**
Apical SLC26A family Cl⁻/HCO₃⁻ exchangers contribute to transepithelial HCO₃⁻ secretion in other HCO₃⁻-secreting epithelia. To identify the apical AE, quantitative RT-PCR was performed for all 10 members of the SLC26A family (36) using RNA extracted from polarized Calu-3 cells. Fig. 5A shows that a number of SLC26A transporters are expressed in Calu-3 cells, several of which are known to function as Cl⁻/HCO₃⁻ exchangers (SLC26A4, -A6, -A7, and -A9). Of these four candidates, only pendrin (SLC26A4) has properties that are consistent with our results: a monovalent anion transporter with high affinity for iodide and insensitivity to H₂-DIDS (37–40). A mouse polyclonal anti-SLC26A4 antibody detected a band migrating at ~100 kDa in immunoblot analysis of Calu-3 whole cell lysates (supplemental Fig. S4). The size of the detected species is consistent with that of the band detected using heterologously expressed pendrin fusion proteins as well as with previous reports (41). Confocal immunofluorescence images revealed a punctate localization of pendrin near the apical membrane of Calu-3 monolayers (Fig. 5C, top, green), when compared with ZO-1 expression (Fig. 5C, bottom, red). Immunohistochemical studies on native human tissue showed that pendrin is highly expressed in surface ciliated cells, is not present in mucous-secreting cells of submucosal glands, and is present, albeit at a lower level than in ciliated cells, in serous-like cells from SMGs (supplemental Fig. S5). This work provides additional support for pendrin expression in human airway serous cells and also confirms that pendrin is expressed in surface bronchial epithelial cells (42).
Pendrin Knockdown Provides Insights into Its Function in Calu-3 Cells—To establish if pendrin functions in HCO$_3^-$ transport in Calu-3 cells, pendrin (SLC26A4) and cyclophilin B (control) KD Calu-3 cells were produced by shRNA inhibition. Knockdown was verified using both quantitative RT-PCR and immunodetection methods. Pendrin mRNA expression in these KD cells was reduced to 8.5 ± 0.7% compared with cyclophilin B (control) KD Calu-3 cells (p < 0.05; n = 3; Fig. 5B). Confocal immunofluorescence imaging verified pendrin expression in both control KD Calu-3 monolayers and CFTR KD Calu-3 cells, whereas no signal was detected in the pendrin KD cells (Fig. 5C).

Although pendrin KD cells showed a fsk-stimulated apical Cl$^-$-dependent change in pH$_i$, the δpH$_{RA}$ was reduced by 47.6 ± 2.4%, compared with control KD cells (p < 0.001; n = 5; Fig. 5D). Like CFTR KD cells, the profile of the apical Cl$^-$-dependent change in pH$_i$ in pendrin KD cells was reminiscent of WT Calu-3 cells. It was only supported by monovalent anions (Fig. 5E), was H$_2$-DIDS-insensitive (Fig. 5F), and was abolished by GlyH-101 addition (data not shown). Interestingly, unlike WT as well as CFTR KD cells, the pendrin KD cells showed no significant difference in the δpH$_{RA}$ when iodide replaced chloride (p > 0.05; n = 4; Fig. 5E). This observation is consistent with the KD of an AE which has a high affinity for iodide. Neither pendrin nor control KD Calu-3 cells showed significant differences in CFTR protein expression (p > 0.05; n = 3; supplemental Fig. S3, A and B). Thus, these differences in apical Cl$^-$-induced changes in pH$_i$ in pendrin KD cells do not reflect a change in CFTR expression. In addition, short circuit current (I$_{SC}$) measurements demonstrated a similar GlyH-101-sensitive fraction of fsk-stimulated I$_{SC}$ (indicative of CFTR-dependent anion transport) in pendrin and control KD Calu-3 monolayers (p > 0.05; n = 9; supplemental Fig. S3C). The H$_2$-DIDS insensitivity of the apical Cl$^-$-dependent changes in pH$_i$ in WT and pendrin KD Calu-3 cells suggests that other SLC26 members highly expressed in these cells, such as SLC26A2 or SLC26A6 (both are sensitive to H$_2$-DIDS (43, 44)), are unlikely to be involved in these responses.

pH$_i$ Response to Cl$^-$ Removal by Fisher Rat Thyroid Cells Expressing CFTR or Pendrin—The significant changes in I$^-$/Cl$^-$ ratio for the δpH$_{RA}$ across the different cell types (Figs. 2A, 4C, and 5E), together with expression data and pharmacological responses to H$_2$-DIDS and GlyH-101, indicate that both CFTR and pendrin contribute to the Cl$^-$-induced pH$_i$ responses. As a further test, the pH$_i$ responses to Cl$^-$ removal and readdition were evaluated in polarized FRT monolayers stably expressing either CFTR or pendrin alone (30). Fig. 6A shows that FRT cells expressing CFTR alkalized in response to apical Cl$^-$ removal in the presence of fsk, supporting the notion that CFTR can conduct HCO$_3^-$ into the cell under an imposed outwardly directed Cl$^-$ gradient. The pH$_i$ response to apical Cl$^-$ was significantly augmented after fsk treatment.
FIGURE 5. Pendrin knockdown Calu-3 cells show reduced apical Cl\(^{-}\)-dependent HCO\(_3\)^{-} transport. A, quantitative RT-PCR (Taqman) analysis of SLC26 mRNA expression in WT Calu-3 cells grown on Transwell supports, relative to standard curve and normalized to GAPDH (%) (n = 3 separate cell cultures). B, quantitative RT-PCR analysis of the percentage of pendrin expression in pendrin KD Calu-3 cells normalized to GAPDH compared with that of cyclophilin B KD (Control KD) Calu-3 cells (n = 3). C, confocal micrographs showing pendrin (green; upper panels) and ZO-1 (red; lower panels) staining in wild type (WT), CFTR knockdown (CFTR KD), pendrin KD (A4 KD), and cyclophilin B KD (control KD) Calu-3 cell monolayers. WT no 1\(^{\circ}\), immunofluorescence from WT Calu-3 cells with only the secondary antibodies applied. Note that cells treated with either antibody are separate xy sections taken from the same confluent monolayer of each cell type, and images were acquired under identical conditions of illumination intensity and photomultiplier settings. Scale bars, 10 μm (n = 3). D, comparison of the mean ΔpH\(_{\text{IA}}\) upon readdition of Cl\(^{-}\) in cyclophilin B (control KD) and pendrin KD Calu-3 cells. Control KD and pendrin KD cell experiments were carried out in parallel (n = 5; * p < 0.001 compared with control KD). E, the mean ΔpH\(_{\text{IA}}\) in pendrin KD Calu-3 cells upon introduction of monovalent (chloride, formate, and iodide) and divalent (oxalate and sulfate) anions following apical Cl\(^{-}\) removal under fsk-stimulated conditions (n = 4; paired observations). F, the effect of apical H\(_2\)-DIDS (500 μM) on the ΔpH\(_{\text{IA}}\) upon apical Cl\(^{-}\) readdition in fsk-stimulated pendrin KD cells. Data expressed as a percentage of the rate obtained with control fsk-treated monolayers (n = 4; paired observations). Error bars, S.E.
FIGURE 6. Properties of Cl⁻-dependent changes in pH in CFTR- and pendrin-transfected FRT cells. A and D, experimental traces showing the effect of forskolin (5 μM) on changes in pH following the removal of apical Cl⁻ in CFTR-transfected (A) and pendrin-transfected (D) FRT cell monolayers. Note that untransfected FRT cells produced no response to this maneuver (data not shown). B and E, the effect of fsk and fsk plus GlyH-101 (10 μM) on ΔpH latina on apical Cl⁻ readdition in CFTR-transfected (B) and pendrin-transfected (E) FRT cells (n = 4; paired observations; *, p < 0.05 compared with apical 0Cl⁻. †, p < 0.01 compared with forskolin and GlyH-101). C and F, the mean ΔpH latina produced by the introduction of monovalent (iodide, formate, and chloride) and divalent (oxalate and sulfate) anions following the removal of apical Cl⁻ in CFTR-transfected (C) and pendrin-transfected (F) FRT cells (n = 4; paired observations; *, p < 0.05 compared with Cl⁻ and formate). Error bars, S.E.
This increase could be completely inhibited by GlyH-101; thus, CFTR mediated the changes in pH
\(i\) (Fig. 6, A and B). Anion substitution studies revealed that only monovalent anions sup-
ported reacidification, and I\(^-\) and Cl\(^-\) selectivity did not differ (Fig. 6C). Pendrin-expressing FRT cells responded to apical Cl\(^-\) removal very differently. Significant Cl\(^-\)/HCO\(_3\) AE activity was not enhanced by fsk
or inhibited by GlyH-101 (Fig. 6, D and E). Importantly, the \(\Delta p\text{H}_{i}\) was markedly higher for I\(^-\) compared with Cl\(^-\) (Fig. 6F; I\(^-\)/Cl\(^-\) = 3.2), with no ability to transport divalent anions, results that are entirely consistent for pendrin (39, 40).

**Anion Transport Is Critical for Fluid Secretion**—We next measured and compared both the rate of transepithelial liquid secretion (\(J_v\)) and the pH of secreted fluid samples from WT, CFTR KD, and pendrin KD monolayers. WT Calu-3 monolay-
ers, submerged in HCO₃⁻-KRB, increased the apical fluid volume from 200 μl to 210 ± 1 μl over 24 h (p < 0.05; n = 6; Fig. 7A), corresponding to a Jᵥ of 0.42 ± 0.02 μl/cm²/h. Forskolin increased Jᵥ ~5-fold to 2.23 ± 0.11 μl/cm²/h (p < 0.001; n = 6). Bilateral Cl⁻ removal abolished basal liquid secretion and reduced fsk-stimulated secretion by 88% (p < 0.001; n = 6). No significant fluid secretion was observed from Calu-3 monolayers bathed in HEPES-buffered KRB under either non-stimulated or forskolin-stimulated conditions (p > 0.05; n = 6). These results show that both basal and fsk-stimulated Calu-3 liquid secretion are entirely Cl⁻ and HCO₃⁻-dependent.

To determine the role played by CFTR in transepithelial HCO₃⁻ and liquid secretion, experiments were performed with the CFTR blocker, GlyH-101. In the presence of apical GlyH-101, non-stimulated WT Calu-3 cells absorbed rather than secreted fluid (~0.18 ± 0.01 μl/cm²/h; p < 0.001; n = 3; Fig. 7B), suggesting that all basal liquid secretion involves CFTR. Forskolin-stimulated fluid secretion was substantially inhibited by GlyH-101 (60 ± 7% inhibition; p < 0.001; n = 3) but nonetheless achieved levels significantly greater than control (p < 0.001; n = 3). Assuming complete block of CFTR in these experiments, these results suggest that although the majority of the cAMP-stimulated liquid secretion depends on CFTR, a significant CFTR-independent, cAMP-stimulated, component of liquid secretion does exist. The pH of the apical fluid from non-stimulated Calu-3 monolayers did not differ significantly from that of the bathing Krebs solution (compared with pH 7.4; p > 0.05; n = 3; Fig. 7C). However, apical fluid pH significantly increased to 7.8 ± 0.1 (~60 mM HCO₃⁻) following fsk stimulation (p < 0.001; n = 3). Surprisingly, despite reducing the rate of liquid secretion, GlyH-101 addition had no effect on the pH of the secreted fluid under either non-stimulated or forskolin-stimulated conditions (p > 0.05; n = 3).

The CFTR dependence of Calu-3 liquid secretion was further explored using CFTR KD Calu-3 cells. Under non-stimulated conditions, CFTR KD Calu-3 cells failed to increase the volume of apical fluid over 24 h (p > 0.05; n = 4; Fig. 7D) and secreted significantly less liquid compared with WT cells under fsk-stimulated conditions (34 ± 3% compared with WT; p < 0.001; n = 4). Like GlyH-101 addition in WT Calu-3 cells, CFTR KD had no significant effect on the pH of secreted apical fluid under either non-stimulated or fsk-stimulated conditions (p > 0.05; n = 3; Fig. 7E). These results establish a critical role for CFTR in regulating the rate of transepithelial liquid secretion but not in controlling the pH ([(HCO₃⁻)]) of the secreted fluid.

In contrast, pendrin KD did not significantly affect either the rate of transepithelial liquid secretion or the pH of secreted fluid under basal conditions, compared with control KD Calu-3 cells (p > 0.05; n = 3; Fig. 7, D and E). However, although pendrin KD Calu-3 cells produced an increase in fluid volume (p < 0.05; n = 3) and pH (p < 0.01; n = 3) in response to fsk addition, comparatively less fluid was secreted (p < 0.05; n = 3; Fig. 7D). Importantly, the secreted fluid was markedly less alkaline (pH 7.54 ± 0.01; p < 0.001; n = 3; Fig. 7E). Taken together, these results are consistent with pendrin mediating the majority of HCO₃⁻ secretion (exit) across the luminal membrane of Calu-3 monolayers via coupled Cl⁻/HCO₃⁻ exchange. Furthermore, because the osmolarity of the secreted fluid from all treatment groups did not significantly differ from the Krebs buffer (data not shown), any increase in [HCO₃⁻] indicates that the [Cl⁻] of the secreted fluid must decrease proportionally to maintain constant osmolarity. From the [Cl⁻] and volume of fluid secreted, it is possible to estimate total Cl⁻ secretion capacity for the different cell types (supplemental Table S3).

The results show that CFTR KD substantially reduced whereas pendrin KD increased total Cl⁻ secretion, as would be predicted from their proposed roles in transepithelial salt and fluid secretion in Calu-3 cells (see Fig. 8).
Role of Pendrin in $HCO_3^-$ Secretion in the Airways

DISCUSSION

Our findings demonstrate for the first time the presence of an apical $Cl^-/HCO_3^-$ exchange activity in cAMP-stimulated Calu-3 cells. This cAMP-activated apical AE activity is Na$^+$-independent, H$_2$-DIDS-insensitive, and capable of transporting a broad range of monovalent, but not divalent, anions in exchange for $HCO_3^-$. The selectivity profile is $I = Br > Cl = HCOO = NO_2 = SCN > OH$, consistent with pendrin-mediated transport (38, 45). Our results agree with data from *Xenopus* oocytes expressing human SLC26A4, which show that pendrin can function as a $Cl^-/HCO_3^-$ exchanger as well as a $Cl^-/I^-$ exchanger (39). Furthermore, mouse parotid duct cells show forskolin-stimulated $I^-$ secretion, which was absent in *Sclc26a4*−/− mice (40).

We measured active apical AE activity in cAMP-stimulated cells exposed to no $Cl^-$ (0$Cl^-$) even in the presence of the CFTR inhibitor GlyH-101 (Fig. 3). This strongly suggests that apical $HCO_3^-$ transport can occur independently of CFTR, contrary to many previous Calu-3/SMG studies (17–19, 46, 47). However, in CFTR-transfected FRT cells, apical $Cl^-$ removal also increased pH$_i$. This was completely GlyH-101-sensitive, indicating that $HCO_3^-$ influx through CFTR does occur under 0$Cl^-$ conditions (Fig. 6, A and B). Therefore, the changes in pH$_i$ following removal of luminal $Cl^-$ in cAMP-stimulated Calu-3 cells could also be interpreted as being due to $HCO_3^-$ influx through CFTR alone or in conjunction with a $Cl^-/HCO_3^-$ exchanger. We favor the latter possibility based on the monovalent anion selectivity, particularly to $I^-$, of the pH$_i$ response in the different cell types studied. In WT Calu-3 cells, the rate of $HCO_3^-$-dependent reacidification displayed an anion selectivity of $I > Cl = HCOO$, consistent with the high selectivity of pendrin for $I^-$ (validated in pendrin-transfected FRT cells; Fig. 6F). In contrast, CFTR-transfected cells transported each of these monovalent anions at equal rates (Fig. 6C). Furthermore, CFTR knockdown in Calu-3 cells (Fig. 4C) enhanced the relative $I^-$ selectivity ($I/Cl = 2.1$), consistent with pendrin mediating a larger component of the $HCO_3^-$-dependent reacidification. Under physiological situations, luminal $Cl^-$ levels probably remain much higher (30–50 mM). Thus, it is unlikely that CFTR contributes significantly to $HCO_3^-$ flux across the apical membrane, a prediction supported by our fluid pH measurements (Fig. 7). The hypothesis that Calu-3 cells secrete $HCO_3^-$ solely via CFTR clearly opposes our present findings. Our data do support a critical role for CFTR in “switching” Calu-3 cell $HCO_3^-$ transport capability from pH$_i$ regulatory (via basolateral AE) to $HCO_3^-$ secretory modes (via activation of an apical AE) in response to cAMP elevation and activation of PKA (for discussion, see the legend to Fig. 8). This dual role of CFTR is particularly evident in the CFTR KD Calu-3 cells, where the reduced CFTR expression in these cells not only lowered the rate of cAMP-stimulated apical AE activity but also led to partial relief of basolateral AE inhibition normally seen in stimulated cells. Taken together, these observations suggest that CFTR participates in both processes. Furthermore, FRT cells expressing pendrin alone (Fig. 6) show a basal level of AE activity, which could not be further enhanced by cAMP elevation (Fig. 6, D and E). These results may indicate that in Calu-3 cells, CFTR tonically inhibits pendrin activity until a cAMP stimulus is received. Exactly how this occurs requires further clarification. Previous studies on CFTR and SLC26A3 and A6 transporters postulate physical interaction between the phosphorylated regulatory domain of CFTR with the STAS domain of the SLC26 transporters, which promote enhanced channel and AE activity (24). Co-expression studies in HEK cells showed that CFTR expression led to a marked increase in $Cl^-/OH^-$ and $Cl^-/HCO_3^-$ exchange mediated by SLC26A3, -A4, and -A6 (48). It is also possible that, under resting conditions, association between CFTR and pendrin blocks AE activity, a phenomenon recently described for SLC26A9 and the regulatory domain of CFTR in *Xenopus* oocyte co-expression studies (49).

The inhibition of the basolateral $Cl^-/HCO_3^-$ exchanger under cAMP-elevated conditions fits with a primary role of this AE in maintaining pH$_i$. It is likely that the basolateral AE is also important for $Cl^-$ accumulation under resting conditions, as has already been suggested for Calu-3 cells (33) as well as for $HCO_3^-$-secreting pancreatic duct cells (50). Our results also suggest that GlyH-101 inhibition of CFTR in stimulated cells leads to the reactivation of the basolateral AE, possibly via the resulting alkalinization. This may help prevent an intracellular alkali load by shunting $HCO_3^-$ accumulation by the basolateral Na$^+^-HCO_3^-$ cotransporter (NBC) (Fig. 8). The exact mechanisms involved in the cAMP/CFTR-dependent inhibition of the basolateral AE presently are not clear. AE2 is known to be very pH-sensitive (51). Thus, intracellular acidification produced by the efflux of $HCO_3^-$ across the apical membrane upon fsk stimulation could provide the basis of one potential mechanism.

We obtained an insight into the role of the apical $Cl^-/HCO_3^-$ exchanger in Calu-3 cells by examining the composition of the secreted fluid (Fig. 7). Our pH$_i$ measurements predict fsk-stimulated, apical $HCO_3^-$ secretion facilitated by pendrin-mediated $Cl^-/HCO_3^-$ exchange. Forskolin-stimulated pendrin KD Calu-3 cells showed reduced apical AE activity and produced a fluid of lower pH$_i$ consistent with these predictions. Although CFTR knockdown or inhibition by GlyH-101 had no effect on the pH of fsk-stimulated secreted fluid, both maneuvers reduced the volume of secreted fluid. These findings are consistent with electrogenic $Cl^-$ efflux via CFTR-driven transcellular fluid secretion and oppose the notion of $HCO_3^-$ efflux through CFTR-driven fluid secretion, which is consistent with recent work on native serous cells isolated from pig and human SMGs (52). Cl$^-$ secretion through CFTR is predicted to support apical $Cl^-/HCO_3^-$ exchange by maintaining an inward $Cl^-$ gradient for efficient anion exchange, which has also been observed for SLC26A3 (Dra) activity in mouse duodenum (53).

Our transcellular liquid secretion measurements showed that $J_i$, was dependent on the presence of both $Cl^-$ and $HCO_3^-$ and was increased, dose-dependently, up to ~5-fold by fsk, in a time-dependent fashion (supplemental Fig. S6, A and B). We also found that much of the fluid secretion could be inhibited by basolateral anion transport inhibitors, H$_2$-DIDS and bumetanide (supplemental Fig. S6C), suggesting that Na$^+$, K$^+$, and $Cl^-$ uptake via the basolateral Na$^+$-$K^+$-$2Cl^-$ cotransporter and $HCO_3^-$ uptake via the basolateral NBC (and/or $Cl^-/HCO_3^-$ exchange by basolateral AE2) support overall luminal fluid secretion from Calu-3 cells under cAMP-stimulated conditions.
Role of Pendrin in $\text{HCO}_3^-$ Secretion in the Airways

Recent findings that pendrin expression is coupled to mucin gene expression in response to inflammatory cytokines is important in that it suggests that mucus production and mucus expansion may be tightly co-regulated (42).

Mutations in the gene encoding pendrin lead to Pendred syndrome, a condition characterized by deafness and in some cases goiter, reflecting its expression in the inner ear and thyroid (67, 68). To our knowledge, Pendred syndrome patients have not been reported to develop disturbances in lung physiology. This may be because the lungs use other transporters to regulate $\text{HCO}_3^-$ secretion, such as CFTR, in the absence of pendrin or because compensatory up-regulation of other SLC26A CI$^-$/HCO$_3^-$ exchangers occurs when pendrin is not present. A similar conclusion was reached in studies on pendrin in the kidney, where it functions in acid-base status (69, 70), yet no obvious renal abnormalities are observed in either Pendred patients or in SLC26A4 knockout mice (69, 70). Interestingly, the inflammatory cytokine, IL-4, up-regulates the functional expression of pendrin in primary cultures of human bronchial epithelial cells, whereby it mediates secretion of $\text{SCN}^-\$ and promotes the production of $\text{OSCN}^-\$, a potent antimicrobial agent (30). Thus, in the context of airway serous SMGs, pendrin may also participate in CI$^-$/coupled SCN$^-$ secretion in addition to $\text{HCO}_3^-$ secretion.

In conclusion, our results are consistent with pendrin mediating the majority of $\text{HCO}_3^-$ secretion across the apical membrane of Calu-3 monolayers via coupled exchange of CI$^-$ for $\text{HCO}_3^-$. This work establishes a critical and novel role for this anion exchanger in transporting $\text{HCO}_3^-$ across human airway serous epithelial cells.

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