Up-regulation of AMP-activated Kinase by Dysfunctional Cystic Fibrosis Transmembrane Conductance Regulator in Cystic Fibrosis Airway Epithelial Cells Mitigates Excessive Inflammation*

AMP-activated kinase (AMPK) is a ubiquitous metabolic sensor that inhibits the cystic fibrosis (CF) transmembrane conductance regulator (CFTR). To determine whether CFTR reciprocally regulates AMPK function in airway epithelia and whether such regulation is involved in lung inflammation, AMPK localization, expression, and activity and cellular metabolic profiles were compared as a function of CFTR status in CF and non-CF primary human bronchial epithelial (HBE) cells. As compared with non-CF HBE cells, CF cells had greater and more diffuse AMPK staining and had greater AMPK activity than their morphologically matched non-CF counterparts. The cellular [AMP]/[ATP] ratio was higher in undifferentiated than in differentiated non-CF cells, which correlated with AMPK activity under these conditions. However, this nucleotide ratio did not predict AMPK activity in differentiating CF cells. Inhibiting channel activity in non-CF cells did not affect AMPK activity or metabolic status, but expressing functional CFTR in CF cells reduced AMPK activity without affecting cellular [AMP]/[ATP]. Therefore, lack of functional CFTR expression and not loss of channel activity in CF cells appears to up-regulate AMPK activity in CF HBE cells, presumably through non-metabolic effects on AMPK activation and AMPK expression and activity in non-CF cells under these conditions. This nucleotide ratio did not predict AMPK activity in differentiating CF cells. Inhibiting channel activity in non-CF cells did not affect AMPK activity or metabolic status, but expressing functional CFTR in CF cells reduced AMPK activity without affecting cellular [AMP]/[ATP]. Therefore, lack of functional CFTR expression and not loss of channel activity in CF cells appears to up-regulate AMPK activity in CF HBE cells, presumably through non-metabolic effects on upstream regulatory pathways. Compared with wild-type CFTR-expressing immortalized CF bronchial epithelial (CFBE) cells, ΔF508-CFTR-expressing CFBE cells had greater AMPK activity and greater secretion of tumor necrosis factor-α and the interleukins IL-6 and IL-8. Further pharmacologic AMPK activation inhibited inflammatory mediator secretion in both wild-type- and ΔF508-expressing cells, suggesting that AMPK activation in CF airway cells is an adaptive response that reduces inflammation. We propose that therapies to activate AMPK in the CF airway may be beneficial in reducing excessive airway inflammation, a major cause of CF morbidity.

Cystic fibrosis (CF),2 the most common lethal genetic disease among Caucasians, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP-activated, ATP-gated Cl− channel expressed in the apical membrane of a wide variety of epithelial tissues (1). However, many manifestations of CF that cause substantial morbidity and mortality cannot be easily explained by a simple loss of CFTR Cl− channel function, including abnormal regulation of other transport proteins, abnormal lipid metabolism, and excessive inflammation in the CF lung and pancreas (2–6). This diversity of CF-associated disorders suggests that CFTR may modulate other cellular pathways through as yet uncharacterized mechanisms. A clearer understanding of the cellular consequences of mislocalized or non-functional CFTR is, thus, critically important to a better understanding of the pathogenesis of CF. Defective CFTR function in the airway epithelium is responsible for CF lung disease, the most life-threatening complication of CF. The CF airway is characterized by colonization by bacteria such as Pseudomonas aeruginosa and chronic excessive inflammation, causing bronchiectasis and other chronic extensive damage to lung architecture and eventual respiratory failure (3). Whether there is an intrinsic hyperinflammatory state resulting directly from a lack of functional CFTR has been the subject of considerable debate in recent years (7–9). There is growing evidence for at least an exaggerated or prolonged inflammatory response to bacterial and other insults (10, 11). However, the underlying pathophysiological mechanisms and chain of events causing excessive inflammation are not well understood. Particular inflammatory mediators that have received considerable attention in recent years include pro-inflammatory cytokines such as interleukin (IL)-8, IL-6, and tumor necrosis factor (TNF)-α, which are up-regulated in the CF airway and anti-inflammatory mediators such as IL-10 and inducible nitric oxide synthase, which are down-regulated in the CF airway (9, 12–17).

We recently identified and characterized a novel functional interaction between CFTR and the metabolic sensor AMP-activated kinase (AMPK) through yeast two-hybrid and biochemical approaches (18). The catalytic α subunit of AMPK binds to the cytoplasmic COOH-terminal tail of CFTR, shares an overlapping predominantly apical distribution with CFTR in a variety of epithelial tissues, and phosphorylates and inhibits CFTR channel activity predominantly through effects on CFTR gating (19, 20). This AMPK-dependent inhibition of CFTR may provide a mechanism for cellular metabolic status to modulate CFTR...
AMPK Up-regulation in CF Airway Epithelial Cells

function. Because AMPK regulates CFTR function, it is of interest to determine whether CFTR reciprocally modulates AMPK function in epithelia and, if so, whether wild-type and mutant CFTR behave similarly.

AMPK is a ubiquitous Ser/Thr kinase that exists as a heterotrimer composed of a catalytic α subunit and regulatory β and γ subunits (21). The activity of AMPK increases during conditions of metabolic stress, in response to elevated intracellular [AMP]/[ATP] ratios. A parallel AMPK activation pathway involves phosphorylation of the α subunit at Thr-172 in its "activation loop" by upstream AMPK kinases (22), which include the LKB1 complex (23, 24) and the Ca²⁺/calmodulin-dependent protein kinase kinase (25–27). Other as yet undefined upstream kinases likely exist (28), and further upstream signaling mediators and pathways may be important for nucleotide-independent regulation of AMPK, although the specific mechanisms involved remain obscure (21). As a cellular metabolic sensor, AMPK acts on a wide variety of metabolic pathways including the regulation of cellular energy and metabolic processes (21, 29, 30).

Therefore, a disruption in its normal functioning could have broad effects on overall cellular functioning. Specifically, it is plausible that changes in AMPK function associated with CFTR mutations could affect some of the pleiotropic manifestations of CF.

Accordingly, we have undertaken studies to examine the role of CFTR in affecting AMPK function using model CF and non-CF human bronchial epithelial (HBE) cells. We find that the presence or absence of functional CFTR expression in polarized airway epithelial cells significantly modulates AMPK cellular localization, expression, activity, and function. We further demonstrate that AMPK activation may play a beneficial role in reducing the expression of pro-inflammatory mediators that contribute to excessive CF airway inflammation.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals—All chemicals were obtained from Acros Organics (Fisher) unless otherwise stated. NH₄-terminal green fluorescent protein (GFP)-tagged full-length CFTR and GFP alone adenoviral vectors were synthesized, amplified, and purified by the Vector Program of the Gene Therapy Program at the University of Pennsylvania. Mouse monoclonal CFTR antibodies (M3A7 and L12B4) were obtained from Upstate Biotech (Lake Placid, NY). AMPK-α1 rabbit polyclonal antibody to the catalytic subunit of AMPK was generated as described previously (31). Cy3-conjugated goat anti-rabbit IgG, Alexa-Fluor 488-conjugated goat anti-mouse IgG, and Cy5-conjugated phalloidin were obtained from Molecular Probes (Eugene, OR). Polyclonal antibodies against AMPK-α and AMPK-α-Thr(P)-172 were from Cell Signaling Technology. Monoclonal antibodies against β-actin were obtained from Sigma. The SAMS peptide (HMRSAMSGGLHVLKRR) used for in vitro kinase assays was synthesized by the University of Pittsburgh Peptide Synthesis Facility. CFTR-Inh₁₇₂ was obtained from Calbiochem. 5-Aminoisimidazole-4-carboxamide-1-β-D-ribose (AICAR) was obtained from Toronto Research Chemicals (North York, ON, Canada).

Cell Growth, Culture, Generation of Stable Cell Lines, and Adenoviral Transduction—HBE cells were isolated from fresh lung transplant specimens as approved by the University of Pittsburgh Institutional Review Board, propagated in flasks, and then transferred to 0.33-cm² Costar Transwell filters and grown at an air-liquid interface, as described previously (32). Six of the 10 CF HBE cell lines were from patients who were homozygous for the ΔF508 CF mutation, and the remaining lines were from compound heterozygotes who had one ΔF508 allele along with a Class I or II mutation in the other allele, including G542X, W1282X, and G551D (one each); one mutation was unknown. At least three independent tissue samples were used for all analyses with primary airway cells. Cells were used for study while undifferentiated (within 2 weeks of seeding at air-liquid interface) or when fully differentiated. Differentiation status was assessed by phase microscopy to assure reproducible differentiation states. Undifferentiated cells were morphologically squamous and had no discernible cilia by phase microscopy or tubulin staining. Cells were considered differentiated only when the cells were uniformly columnar in shape and ≥25% of the cells had appreciable cilia by phase microscopy. Only morphologically matched non-CF and CF cells were used for comparative analyses between wild-type and mutant CFTR-expressing cells. A previous study of cells grown and studied under these conditions demonstrated insignificant differences in transepithelial resistance between non-CF and CF cells (32).

Parental CFBE41o– cells (33) were cultured as described previously (34). Wild-type and ΔF508 CFTR CDNA was stably introduced into CFBE41o– cells, as recently described (35). The biochemical, morphological, and electrophysiological properties of these cell lines have been well characterized (35).

For transduction of polarized CF HBE cells with adenoviral vectors expressing either GFP-CFTR or GFP alone, well differentiated cells grown for at least 4 weeks on filters were first washed apically in warm PBS and then exposed to PBS containing 15 mM sodium caprate for 5–10 min to temporarily disrupt tight junctions, as described previously (36). Adenovirus at a multiplicity of infection of 500 diluted in PBS was then added to the apical side and incubated for 2 h at a 37 °C 5% CO₂ incubator. The recombinant adenovirus was removed, and fresh growth medium was added to the basolateral chamber. Filters were observed by fluorescence microscopy for GFP expression and used for experimentation 4–5 days after infection.

 Immunofluorescent Staining and Confocal Microscopy—Polarized CF and non-CF HBE cells grown on 0.33-cm² filters were immunofluorescently stained as follows with all steps and solutions at room temperature. Cell monolayers were washed once in PBS and then fixed in 2% paraformaldehyde for 30 min. They were washed again in PBS and then permeabilized by addition of PBS plus 1% bovine serum albumin plus 0.1% Triton X-100 for 10 min. After an additional wash with PBS, primary antibodies diluted in PBS plus 1% bovine serum albumin (M3A7 and L12B4 each at 1:1000 (cf. Ref. 37) mixed with AMPK-α antiseraum at 1:100) were added for 2 h. After another wash with PBS, secondary antibodies (Cy3-conjugated goat anti-rabbit IgG and Alexa-Fluor 488-conjugated goat anti-mouse IgG each at 1:500) along with Cy5-conjugated phalloidin (at 1:250) mixed in PBS plus 1% bovine serum albumin were added and incubated in the dark. After a final wash in PBS, the filters were cut from the support and mounted on a slide with a thin coverslip. Slides were stored at 4 °C in the dark before confocal microscopy. Confocal imaging was performed with an Olympus FluvioView 500 confocal laser scanning microscope with the following settings: 60× oil immersion objective lens, 3 channels at excitation/emission settings optimized for fluorescein isothiocyanate, Cy3, and Cy5, 1024 × 1024 pixels per image, 124-μm pinhole diameter, and 0.45-μm z-step interval. Identical photomultiplier voltage and gain settings were used for all images shown of both cell types. MetaMorph software (Molecular Devices Corp., Downingtown, PA) was employed for xz reconstruction of xy-plane images.

TIFF xz-plane images were analyzed using Adobe Photoshop 7.0 to quantitate and compare subcellular AMPK-α1 localization in CF and non-CF cells. Specifically, the outlines of individual cells were defined as...
regions of interest within each slice, and the slices encompassing the full thickness of each analyzed cell were determined. The total integrated signal for each cell slice, determined from the product of the mean red pixel intensity and the number of pixels within the cell slice, was summed up for all slices, yielding a total integrated signal for the cell. Cell slices were then sorted into four equal quartiles in the z-dimension from the apical to basolateral side of the cell, and the fraction of total cellular staining found in each quartile was calculated. This analysis was performed on 20 cells total from three separate cell lines derived from F508 homozygous CF and non-CF patients, and the relative staining by quartile was compared across the two conditions.

Real-time PCR—Real-time RT-PCR was employed using Taqman technology for analysis of relative AMPK-α1 or -α2 gene expression levels. Total RNA was purified from CF and non-CF HBE cell lysates (two 0.33-cm² Transwell filters per condition) using RNeasy columns (Qiagen, Valencia, CA). First strand cDNA (RT reaction) was synthesized from 500 ng of RNA using Superscript II (Invitrogen) according to the manufacturer’s directions. A negative control was also performed without enzyme (NRT reaction). RT and NRT reactions were also performed on 500 ng of commercially purchased liver RNA (Ambion, Austin, TX) to serve as the calibrator for the Taqman reactions. Duplicate aliquots of the RT reaction and duplicate aliquots of the NRT reaction served as templates for the AMPK-α1 and -α2 subunit genes (PRKAA1 and PRKAA2) and the β-glucuronidase control gene (GUSB). Reaction components, including the ROC internal control, were obtained from Applied Biosystems, Inc. (ABI). The probes and primers for PRKAA1, PRKAA2, and GUSB were also obtained from Applied Biosystems (Assays on Demand numbers Hs00178993_m1, Hs00178993_m1, and Hs99999908_m1, respectively). The real-time PCR reactions were run on an ABI7000 sequence detection system with the following cycle conditions: 95 °C for 12 min, then (95 °C for 15 s, 60 °C for 1 min) × 40 cycles. Differential gene expression was calculated by the ΔΔCt calculation (38). Unlike calculations based on standard curves, the ΔΔCt method controls for potential differences in efficiency of the RT reactions as well as the PCR.

Immunoblotting and Quantification of AMPK Expression—Cells grown on at least two 0.33-cm² filters per condition were washed twice in ice-cold PBS and then lysed as described previously for AMPK in vitro kinase assays (20). After pooling lysates from duplicate filters, pelleting the lysates, and measuring protein concentrations of the supernatants, equal amounts of cellular protein per lane were loaded on a 4–12% gradient gel (NuPage, Invitrogen) and subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane. Membranes were blocked in 5% milk or 1% bovine serum albumin, and immunoblotting was performed using AMPK-α (1:1000), AMPK-α-Thr(172) (1:1500), or β-actin (1:1000) primary antibodies. Secondary donkey anti-rabbit or sheep anti-mouse antibodies (Amersham Biosciences) were used at 1:5000. Intensities of all relevant bands corrected for local background intensity on the blot were quantitated using a VersaDoc Imager with Quantity One software (Bio-Rad).

In Vitro AMPK Activity Measurements—Polarized HBE cells grown on 0.33-cm² filters were lysed in AMPK lysis buffer (20), and the lysates from 5–7 identical filters were pooled for each kinase assay. After immunoprecipitation of AMPK-α from cell lysates, AMPK activity in the cellular lysate samples was measured using the SAMS peptide in vitro kinase assay, as described previously (20, 31).

Cellular Nucleotide Analysis—Samples for analysis were obtained from HBE cells grown on 5–7 0.33-cm² Transwell filters per condition. Before lysis, the medium was aspirated, and the cell monolayers were washed three times with ice-cold PBS. Extraction was performed by scraping the cells in each filter with 5 µl of ice-cold acetonitrile followed by 15 µl of cold water (39). The soluble and precipitated fractions were centrifuged at 16,000 × g for 10 min at −20 °C. The supernatant fraction was stored at −80 °C. The pellet was solubilized with 1 N NaOH, and the protein content was analyzed using the Bradford assay (Bio-Rad).

The HPLC column used was a 4-µm Nova-Pack C18 cartridge (100 mm × 8 mm inner diameter) equipped with a radial compression chamber (Waters, Milford, MA). The buffer consisted of 20% acetonitrile, 10 mM ammonium phosphate and 2 mM PIC-A ion pairing reagent (Waters) and was run isocratically at 2 ml/min (40, 41). Samples were gassed for 10 min with N2 to evaporate acetonitrile and then diluted with 10–15 µl of ice-cold water depending on the retrieved volume from each dish. The injection volume varied between 75 and 90 µl, again depending on the retrieved volume. An HP LC-MS and Chemstation model 1100 were used (Hewlett-Packard, Wilmington, DE) with the UV detector set at 254 nm. HPLC grade nucleotide standards were used to calibrate the signals and identify peaks, which were integrated by the software. Standards were run daily because the retention of the column varied with time. Internal standards were occasionally added to the samples to test recovery. It exceeded 90% for all nucleotides.

Short Circuit Current Measurements—Short circuit current measurements were performed as described previously (20) with the following modifications. Modified Ussing chambers designed to accept 0.33-cm² Transwells were used (42). Ringer’s solution was used in both the apical and basolateral chambers and contained 120 mM NaCl, 25 mM NaHCO3, 3.3 mM KH2PO4, 0.8 mM K2HPO4, 1.2 mM MgCl2, 1.2 mM CaCl2, and 10 mM d-glucose. As measured by periodic ±5 mV pulses away from the continuous clamp at 0 mV, polarized HBE cell monolayers had base-line transepithelial resistance values ranging from 800 to 2000 ohm-cm².

Inflammatory Mediator Expression Analysis—Apical supernatant samples (50-µl aliquots) were taken from each filter growing polarized CFBE cells and frozen for subsequent analysis using the human cytokine Lincoplex kit (Linco Research, St. Charles, MO) run on a Bio-Plex suspension array system (Bio-Rad), as previously described (43). Data were analyzed using the Bio-Plex manager software and exported to Microsoft Excel for statistical analysis.

RESULTS

We have previously found that AMPK binds to and phosphorylates CFTR and inhibits CFTR currents in polarized colonic and lung epithelial cells (18–20). This AMPK-CFTR functional interaction may afford the coupling of ion transport to cellular metabolic status (30) and raises the question of whether changes in the normal function or expression of CFTR, as occur in the CF airway, may modulate AMPK function. AMPK is involved in regulating a wide variety of cellular processes, including metabolic, inflammatory, growth, apoptotic, and gene transcriptional (21, 29). Thus, CFTR-associated effects on AMPK function could greatly impact on the pleiotropic pathogenic mechanisms of CF airway disease. To determine whether the presence of functional CFTR in the airway epithelium is involved in the regulation of AMPK function, we initially examined the effects of mutant versus normal CFTR on the localization, amount, and activity of AMPK and on cellular metabolic profiles in polarized primary HBE cells derived from CF patients versus patients with other non-CF lung diseases or normal donors.

AMPK and CFTR Localization in Polarized Primary HBE Cells—Confocal microscopic images of immunofluorescently stained polarized non-CF HBE cell monolayers demonstrate that the catalytic α1 subunit of AMPK localized predominantly to apical and sub-apical
regions (Fig. 1A), a finding consistent with earlier immunolocalization studies in various rat epithelial tissues (18, 19). However, this AMPK distribution appeared substantially disrupted in morphologically matched polarized HBE cells from CF patients, which exhibited staining in a more generally diffuse cytoplasmic/perinuclear distribution (Fig. 1B). This apparent difference in the subcellular distribution of AMPK staining between the two cell types was verified by comparing the fraction of total AMPK staining falling within each of four evenly spaced layers (quartiles) defined within the cell from the apical to basolateral membrane (Fig. 1C). In the most apical quartile (Q1) there was much less fractional AMPK staining in the CF cells compared with non-CF controls (0.12 ± 0.01 versus 0.36 ± 0.02, respectively). Conversely, in the more basolateral quartiles (Q3 and Q4) there was significantly greater fractional AMPK staining in the CF cells compared with non-CF cells (0.33 ± 0.02 versus 0.16 ± 0.01 for Q3 and 0.11 ± 0.01 versus 0.03 ± 0.01 for Q4, respectively). Moreover, the overall amount of AMPK immunofluorescent staining was generally greater in the CF than in the non-CF polarized monolayers. Finally, as expected from earlier studies (3, 44, 45), CFTR staining was substantially decreased and less apically localized in the CF than in the non-CF HBE cells (Fig. 1). These findings suggest that CFTR affects AMPK expression and localization in airway epithelial cells.

**Effects of CFTR and Differentiation Status on AMPK Gene and Protein Expression in Primary HBE Cells**—Measurements from polarized HBE cell lysates of AMPK-α mRNA expression, AMPK-α protein expression, cellular AMPK activity, and cellular metabolic profiles were performed in parallel. These parameters were analyzed as a function of CFTR and differentiation status, comparing cells lysed within 1–2 weeks of seeding onto permeable supports (exhibiting an undifferentiated, squamous morphology) with cells lysed after 4–6 weeks of growth on permeable supports (exhibiting a well differentiated, columnar, and ciliated morphology). As measured by real-time PCR, both AMPK-α1 (Fig. 2A) and AMPK-α2 (Fig. 2B) relative mRNA expression increased with increasing differentiation in both non-CF and CF cells. There was, however, no significant difference in AMPK-α mRNA levels between CF and non-CF cells matched for differentiation. In contrast, AMPK-α protein expression tended to decrease in both cell types with differentiation (Fig. 3). These AMPK-α mRNA and protein expression data taken together suggest that there is an increased rate of AMPK protein turnover or degradation in well differentiated HBE cells independent of CFTR status. Consistent with the immunofluorescent staining results (Fig. 1), there was also a trend toward greater overall AMPK-α protein expression in CF than in non-CF differentiated HBE cells (Fig. 3B).
Effects of CFTR and Differentiation Status on AMPK Activity and Cellular Metabolic Profiles in Primary HBE Cells—In vitro kinase activity measurements revealed that AMPK activity in both CF and non-CF HBE cells decreased by 65–70% as the cells transitioned from an undifferentiated to a well differentiated state (Fig. 4A). However, both undifferentiated and differentiated CF cells had greater AMPK activity (by 76 and 66%, respectively) than their non-CF counterparts (Fig. 4A). Parallel immunoblot performed on cell lysates to detect the activated form of AMPK-α phosphorylated at Thr-172 confirmed these changes with qualitatively similar results (Fig. 4B). Because AMPK activity responds to changes in underlying cellular metabolic status, we examined whether differences in AMPK activity under these conditions correlated with changes in the cellular [AMP]/[ATP] ratio, a critical parameter for the regulation of AMPK activity (46). Cellular nucleotide profiles of acetonitrile-extracted cell samples were obtained by reverse-phase HPLC. A representative chromatogram with the various nucleotide peaks identified is shown in Fig. 5A. The cellular [AMP]/[ATP] ratio was higher in undifferentiated than in differentiated non-CF cells (0.15 ± 0.02 versus 0.05 ± 0.01, respectively; Fig. 5B, right), a finding that correlated with the measured AMPK activities under these conditions (Fig. 4A, right). In contrast, [AMP]/[ATP] ratios in undifferentiated CF cells were lower than in undifferentiated non-CF cells (0.03 ± 0.01 versus 0.15 ± 0.02, p = 0.02; Fig. 5B) despite the higher AMPK activity in CF cells (Fig. 4A). Moreover, the [AMP]/[ATP] ratio increased to 0.10 ± 0.04 with differentiation in the CF cells (Fig. 5B, left) despite the measured fall in AMPK activity (Fig. 4A, left). Together, these data indicate that, unlike in non-CF HBE cells, changes in cellular metabolic status do not predict AMPK activity in CF HBE cells. Of note, recent studies suggest that upstream kinases and other signaling mediators may also regulate AMPK activity independently of changes in cellular metabolic status (21, 47, 48). It is, thus, plausible that the increased AMPK activity in CF cells reflects effects of nonfunctional or mislocalized CFTR on upstream kinase or signaling pathways (i.e. non-metabolic factors), which in turn modulate AMPK activity.

CFTR Inhibition and Complementation Studies—To determine whether the presence or absence of functional CFTR is sufficient to modulate AMPK function in polarized airway cells, we tested the effects on AMPK activity and cellular metabolic status of both inhibiting functional CFTR activity in non-CF HBE cells and rescuing mutant CFTR expression in CF HBE cells. Treatment of polarized, well differentiated non-CF cells for 48 h with 30 μM CFTR-Inh172, a specific CFTR channel blocker (49), did not affect either AMPK activity (Fig. 6A) or the cellular metabolic profile ([AMP]/[ATP] ratio; Fig. 6B), as compared with vehicle-treated controls. As expected, unlike the controls, the CFTR-Inh172-treated monolayers exhibited no significant cAMP-stimulated short cir-
Recent biochemical, morphological, and functional studies suggest that a CFTR interaction-dependent effect on AMPK activity may also exist.

AMPK Activity in ΔF508- and WT-CFTR-corrected Polarized CFBE Cells and Its Role in the Modulation of Inflammatory Mediators—An immortalized HBE cell line originally derived from a ΔF508 homozygote (CFBE410− cells) was stably transfected to express either WT- or ΔF508-CFTR on the ΔF508 genetic background, as described (35). Recent biochemical, morphological, and functional studies suggest that these immortalized cells are a useful model system to study CFTR biogenesis, trafficking, and regulation (35). These two cell lines sharing the same genetic background were, thus, used to more specifically examine the role of functional CFTR in the modulation of both AMPK activity and inflammation. Cell lysates immunoblotted for CFTR and AMPK-α revealed comparable CFTR and AMPK-α protein expression in both cell lines (not shown). Consistent with our findings in primary HBE cells (Fig. 4), polarized CFBE cells expressing ΔF508-CFTR had greater AMPK activity than CFBE cells expressing WT-CFTR under base-line conditions (Fig. 8A). Previous studies have demonstrated that there is increased expression of pro-inflammatory cytokines and chemokines in CF airway epithelial cells relative to that in non-CF airway cells (9, 50).

Interestingly, as measured by an automated microsphere-based multiplex protein assay, secretion levels of the pro-inflammatory hormone TNF-α and the cytokines IL-6 and IL-8 were also significantly enhanced in the ΔF508-CFTR-expressing CFBE cells relative to those expressing WT-CFTR (by 7-, 1-, and 1.5-fold, respectively; Fig. 8, C–E). Thus, these CFBE cells corrected with either WT- or ΔF508-CFTR recapitulated both the effects on AMPK activity observed in the primary HBE cells and the CFTR-dependent differences in the expression of inflammatory mediators. Because this increase in inflammatory mediators was associated with increased AMPK activation in the ΔF508-expressing cells, we reasoned that the increased AMPK activity could either contribute to the excessive inflammation in the ΔF508-expressing cells or help to counteract it as a secondary (adaptive) response. To distinguish between these possibilities, we treated CFBE cells with the AMPK activator AICAR (51), which further enhanced cellular AMPK activity in both WT- and ΔF508-expressing cells, as assessed by immunoblotting for the activated form of AMPK (Fig. 8B). AICAR treatment significantly decreased TNF-α, IL-6, and IL-8 expression levels in both WT- and ΔF508-expressing CFBE cells by ~60, ~50, and ~70%, respectively (Fig. 8, C–E, dark bars). Moreover, a preliminary experiment also demonstrated that AICAR treatment prevented the further rise in IL-6 and IL-8 expression in ΔF508-CFBE cells after a 16-h stimulation with TNF-α (data not shown). These results strongly suggest that AMPK activation in CF lung epithelial cells plays an adaptive role in helping to counteract the increased expression of pro-inflammatory mediators.
DISCUSSION

Because we have previously shown that CFTR is regulated by AMPK in lung epithelial cells (20), this study was undertaken to further examine the potential interregulation of CFTR and AMPK in the airway and the potential role of AMPK in CF lung disease. We found that the predominantly apical and subapical localization of AMPK observed in non-CF HBE cells was disrupted in polarized CF HBE cells (Fig. 1). It is reasonable to speculate that a loss of normal AMPK-CFTR interaction due to a loss of CFTR expression at or near the apical membrane contributes to the disrupted AMPK localization in the CF HBE cells. However, this mechanism is unlikely to fully explain the effect for at least two reasons. First, it is clear that although there is an overlapping distribution of CFTR and AMPK in non-CF HBE cells, most of the cellular pools of both proteins do not directly interact with one another (Fig. 1A). Thus, even if all of the AMPK that was in the co-localizing pool in the non-CF cells were to redistribute diffusely in the cytoplasm, it would probably not account for a substantial change in subcellular AMPK localization. Second, if as seems likely, there is much greater cellular AMPK expression than CFTR expression on a molar basis, direct binding of AMPK to CFTR could play only a minor role in AMPK localization. Rather, the loss of normal functional CFTR expression in the CF cells most likely leads to increased AMPK protein expression (Figs. 1 and 3) and a more diffuse subcellular localization pattern (Fig. 1) largely through indirect cellular effects.

FIGURE 5. Cellular nucleotide profiles in HBE cells as a function of differentiation and CFTR status. A, representative HPLC chromatogram showing various nucleotide peaks from CF cell lysates. The small peak flanking AMP represents IMP plus GMP and was not included in the AMP area calculation. B, mean cellular [AMP]/[ATP] ratios (± S.E.) derived from lysates of 3–5 different primary cell lines for each condition (*p < 0.02, #p < 0.02, unpaired t tests, relative to undifferentiated non-CF cells).

AMPK Up-regulation in CF Airway Epithelial Cells

FEBRUARY 17, 2006• VOLUME 281 • NUMBER 7

JOURNAL OF BIOLOGICAL CHEMISTRY 4237
AMPK Up-regulation in CF Airway Epithelial Cells

AMPK Expression, Turnover, and Non-metabolic Regulation—The factors that govern AMPK gene expression, protein turnover rates, and non-metabolic regulation by upstream pathways are currently ill-defined. Of note, the opposite trends in mRNA and protein expression with differentiation in both CF and non-CF cells (Figs. 2 and 3) suggest that there is a greater rate of AMPK protein turnover or degradation in the differentiated HBE cells. In addition, AMPK activity in both CF and non-CF undifferentiated HBE cells was greater than that in differentiated cells (Fig. 4), suggesting that differentiation status and/or the development of epithelial cell polarity may play an important role in modulating AMPK function. With non-CF cells, this difference in AMPK activity correlated well with the underlying metabolic status ([AMP] / [ATP]) present in the cells (Fig. 5), suggesting that the undifferentiated non-CF cells may be under greater metabolic stress (e.g. from greater rates of cell growth and division) than the differentiated cells. However, the mechanism for the greater AMPK activity in undifferentiated CF cells is unclear. In addition, CF HBE cells had greater AMPK activity than non-CF cells matched for differentiation status (Fig. 4). In the case of CF cells, changes in the cellular metabolic status did not correlate with differentiation-associated changes in AMPK activity (Fig. 5). Together, these findings suggest that the increased AMPK activity in CF HBE cells results from non-metabolic factors and, more generally, that a lack of functional CFTR in these cells may modulate upstream AMPK signaling pathway(s), resulting in changes in AMPK localization, expression, and activity.

Potential Mechanisms of AMPK Up-regulation in CF Airway Cells—The upstream pathways involved in AMPK activation in CF airway cells are currently unclear. However, it has become apparent that upstream kinases, including the immediate upstream AMPK kinases (i.e. the LKB1 complex, Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase, and possibly others) may activate AMPK in the absence of an increased [AMP]/[ATP] ratio (25–27, 52, 53). Interestingly, it was recently proposed that chronic infection or inflammation in CF airway epithelia induces an expansion of cellular Ca\(^{2+}\) stores and increased cytoplasmic [Ca\(^{2+}\)], with a secondary Ca\(^{2+}\)-dependent hyperinflammatory response (11, 54). Such an inflammation-induced rise in cellular Ca\(^{2+}\) signaling could also potentially up-regulate Ca\(^{2+}\)/calmodulin-dependent protein kinase-associated AMPK activation chronically. The pathway involved in AMPK activation with exposure to either the anti-diabetic drug metformin or oxidative stress (peroxynitrite) suggests an additional potential mechanism for AMPK up-regulation in the CF airway. Specifically, Zou et al. (47, 48) have reported that metformin and peroxynitrite activate AMPK through activation of the tyrosine kinase c-Src and phosphoinositide 3-kinase, which appear to be upstream activators of the AMPK kinases. Relevant to this mechanism, Gonzalez-Guerrero et al. (55) found that both the expression and activity of c-Src were significantly increased in a CF airway epithelial cell line relative to CFTR-corrected control cells (55). Based on this study, it is conceivable that enhanced c-Src activation is involved in the up-regulation of AMPK activity in the CF airway. However, a more recent study reported defective activation of c-Src in CF airway epithelial cells (56). Elucidating the upstream cellular and molecular events involved in AMPK activation in CF airway epithelial cells is clearly an important goal for further study.

CFBE Cells as a Model System for AMPK Function and Airway Inflammation—As suggested in the c-Src example given above, an important potential source of inconsistency in the literature regarding the role of various signaling pathways in the manifestations of CF lung disease may stem from the use of model cell lines with differing genetic backgrounds to compare CF and non-CF phenotypes or by comparing phenotypes of stably transfected, CFTR-expressing CF cells with the untransfected parental CF cell line. In these cases, in addition to the difference in CFTR status, there is the potential for other confounding variables to be present in the two comparison cell types. To avoid this issue, we used clonal CFBE cells that were then stably transfected to express either WT- or ΔF508-CFTR on an endogenous ΔF508-CFTR genetic background (Fig. 8). These CFTR-corrected CFBE cells appear to be a good model cell system to study CFTR-dependent effects, as they recapitulated both the effects on AMPK activity observed in the primary HBE cells (cf. Figs. 4A and 8A) and the CFTR-dependent differences in the expression of pro-inflammatory mediators (Fig. 8, C–E) that have been well documented previously and play an important role in the pathogenesis of CF lung disease (9). Indeed, although the difference between the inflammatory profiles of CF and non-CF primary HBE cells has been attributed to the greater recent inflammation and infection in vivo in the CF cells (11), our present data and those from others (57) suggest an intrinsic up-regulation of pro-inflammatory mediators associated with the expression of defective CFTR in airway epithelial cells.

**FIGURE 6. Effect of chronic CFTR channel inhibition on AMPK activity and nucleotide profile in polarized non-CF HBE cells.** Well differentiated, polarized non-CF HBE cell monolayers were treated for 2 days with 30 μM CFTR-Inh-172 or vehicle (Me2SO) control (applied apically). Medium was replaced with fresh inhibitor or vehicle after the first day. A, mean (±S.E.) AMPK activity of vehicle control or CFTR-Inh-172-treated cells normalized to the mean AMPK activity of vehicle control-treated samples from all experiments (p = 0.55, paired t test, samples obtained from 7 different cell lines). B, mean (±S.E.) cellular AMPK activity as measured by HPLC for the two treatment conditions (p = 0.44, paired t test, samples obtained from 6 different cell lines).
Potential Mechanisms of AMPK-dependent Modulation of Inflammation—AMPK activation in polarized CFBE cells through AICAR treatment dramatically reduced the expression of the pro-inflammatory cytokines TNF-α, IL-6, and IL-8 (Fig. 8). Although the mechanism of AICAR-dependent down-regulation of inflammation has not been tested in airway epithelial cells, it has been reported recently that treatment of neuronal cells with AICAR attenuates TNF-α- or lipopolysaccharide-induced expression of pro-inflammatory cytokines through down-regulation of IκB-kinase/α/β activity and inhibition of NF-κB (58). The protein factor NF-κB is required for maximal transcription and cellular production of a variety of pro-inflammatory cytokines, including IL-6 and IL-8 (9). Of note, relative to non-CF cells, it has been reported that CF cells have increased (exaggerated) NF-κB activation and IL-8 secretion in response to TNF-α (10, 12). It is, thus, plausible that AMPK activation in airway epithelial cells down-regulates expression of pro-inflammatory cytokines through inhibition of the IκB kinase/IκB/NF-κB axis that controls their transcription. An additional mechanism by which AMPK may dampen inflammation is through its phosphorylation and inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase (59), the rate-limiting enzyme in sterol biosynthesis and the production of isoprenoids. Isoprenoids are increased in the CF airway and may contribute to excessive airway inflammation (17, 60, 61). Indeed, treatment with the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor mevastatin was recently found to confer anti-inflammatory effects by inducing nitric-oxide synthase 2 expression in models of CF epithelia, an effect that may be mediated through modulation of the RhoA and STAT1 (signal transducer and activator of transcription 1) signaling pathways (17). Further studies are, thus, warranted to define the mechanisms of AMPK-dependent modulation of inflammation in lung epithelial cells.

Other Effects of AMPK Up-regulation in CF Lung Disease—Based on our previous studies (18–20), it might be predicted that increased
AMPK Up-regulation in CF Airway Epithelial Cells

FIGURE 8. AMPK activity, AICAR-dependent activation, and secretion of inflammatory mediators in WT- versus ΔF508-CFTR-expressing polarized CFBE cells. Polarized WT- and ΔF508-CFTR-expressing CFBE cells grown on filters were lysed for AMPK SAMS peptide in vitro kinase assays (A) and/or AMPK α-Thr-172 (AMPK-α-pThr-172) immunoblotting (B). For the results shown in panels B–E, cell monolayers were treated with 1 mM AICAR (or medium alone (Control)) in both the apical and basolateral baths for 16 h before aspiration of the apical supernatant for Bio-Plex analysis followed by cell lysis. A, mean AMPK activity levels (±S.E.) relative to WT-CFTR-expressing cells. ΔF508-CFTR cells had 59 ± 14% greater AMPK activity than WT-CFTR cells (*, p = 0.01, paired t test, n = 4). B, immunoblot of WT- and ΔF508-CFTR-expressing cell lysates with or without prior AICAR treatment probed for activated AMPK (upper section) and β-actin (lower section). Apical supernatants aspirated before cell lysis (n = 3–7 filters for each condition) underwent multiplex analysis for TNF-α (C), IL-6 (D), and IL-8 (E) expression. Mean levels for each inflammatory mediator (±S.E.) are reported relative to the mean level measured for WT-CFTR-expressing cells under control conditions (dashed line at 1); *, p < 0.01 for comparison of ΔF508 control sample to WT control sample for each mediator; $, p = 0.04, p < 0.01, and p < 0.01 for comparison of AICAR-treated ΔF508 sample to control ΔF508 sample for TNF-α, IL-6, and IL-8, respectively, *, $ = 0.01 for comparison of AICAR-treated WT sample to control WT sample for TNF-α and IL-8; #, p = 0.07 for comparison of AICAR-treated WT sample to control WT sample for IL-6.

AMPK activity in the CF airway would further inhibit CFTR channel activity, a potentially detrimental effect. However, functional CFTR activity in the CF airway is already very low at base line (cf. Fig. 7B), and the importance of CFTR Cl− channel activity alone in the pathogenesis of CF lung disease is unclear (62). Importantly, AMPK activation also inhibits ENaC activity in both renal and lung polarized epithelial cell systems (63, 64). AMPK-dependent down-regulation of ENaC in the CF airway could be viewed as an adaptive response, especially given recent evidence suggesting that up-regulation of ENaC by itself may cause many of the features of CF lung disease in a mutant ENaC transgenic mouse model (62). Interestingly, ENaC down-regulation has also been reported to occur in response to the exposure of lung epithelial cells to inflammatory stimulators (65). Whether AMPK activation is at least partly responsible for the inflammation-induced down-regulation of ENaC in lung epithelial cells is an interesting question that awaits further study. In summary, it thus appears that AMPK up-regulation in the CF airway may act as an adaptive response in both dampening the production of pro-inflammatory cytokines and down-regulating ENaC activity and, thus, excessive salt and water reabsorption from the airway surface. Accordingly, as has been proposed for inflammatory diseases of the central nervous system such as multiple sclerosis (66), AICAR and other AMPK-activating drugs might also be beneficial as novel anti-inflammatory therapies for patients with CF lung disease. Further in vivo studies to examine the role of AMPK and AMPK modulators in this setting may prove fruitful.

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