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ΔF508 CFTR processing correction and activity in polarized airway and non-airway cell monolayers

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Abstract

We examined the activity of ΔF508 cystic fibrosis transmembrane conductance regulator (CFTR) stably expressed in polarized cystic fibrosis bronchial epithelial cells (CFBE41o[−]) human airway cells and Fisher Rat Thyroid (FRT) cells following treatment with low temperature and a panel of small molecule correctors of ΔF508 CFTR misprocessing. Corr-4a increased ΔF508 CFTR-dependent Cl[−] conductance in both cell types, whereas treatment with VRT-325 or VRT-640 increased activity only in FRT cells. Total currents stimulated by forskolin and genistein demonstrated similar dose/response effects to Corr-4a treatment in each cell type. When examining the relative contribution of forskolin and genistein to total stimulated current, CFBE41o[−] cells had smaller forskolin-stimulated I_{sc} following either low temperature or corr-4a treatment (10–30% of the total I_{sc} produced by the combination of both CFTR agonists). In contrast, forskolin consistently contributed greater than 40% of total I_{sc} in ΔF508 CFTR expressing FRT cells corrected with low temperature, and corr-4a treatment preferentially enhanced forskolin dependent currents only in FRT cells (60% of total I_{sc}). ΔF508 CFTR cDNA transcript levels, ΔF508 CFTR C band levels, or cAMP signaling did not account for the reduced forskolin response in CFBE41o[−] cells. Treatment with non-

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specific inhibitors of phosphodiesterases (papaverine) or phosphatases (endothall) did not restore $\Delta F508$ CFTR activation by forskolin in CFBE41o⁻ cells, indicating that the Cl⁻ transport defect in airway cells is distal to cAMP or its metabolism. The results identify important differences in $\Delta F508$ CFTR activation in polarizing epithelial models of CF, and have important implications regarding detection of rescued of $\Delta F508$ CFTR *in vivo*.

Keywords

cystic fibrosis; CFTR; ion transport

11. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the traffic ATPase protein family, and functions as a PKA-regulated Cl⁻ channel and regulator of other ion transport pathways [1–4]. $\Delta F508$ is the most common mutation identified in cystic fibrosis (CF), and is caused by the deletion of phenylalanine from position 508 of the protein. This mutation leads to a misfolded gene product, resulting in rapid degradation of $\Delta F508$ CFTR by ERAD in the proteosome [5,6]. The folding defect is believed to occur through altered domain-domain interactions within the nascent protein, and ER chaperones [7,8]. The end result is failure of $\Delta F508$ CFTR to mature from core glycosylated (B band) to a fully glycosylated (C band) protein, and extremely low levels of $\Delta F508$ CFTR at the apical surface of epithelial cells [9,10].

Several maneuvers allow $\Delta F508$ CFTR to escape from the ER and localize to the plasma membrane, including growth at low temperature [11,12], treatment with ‘chemical chaperones’ or inhibitors of the ERAD pathway [13–18], and exposure to small molecules identified by high throughput screening programs to correct aberrant CFTR processing through as yet undetermined mechanisms [19–22]. Once localized to the cell surface, $\Delta F508$ CFTR exhibits additional defects that limit ion transport activity, including reduced channel gating [23–25] and decreased half-life at the plasma membrane [26,27]. While these findings have been shown in a number of cell expression systems by both biochemical and electrophysiological means, studies of the isolated protein in cell free systems by Li and colleagues have indicated that mutant $\Delta F508$ CFTR retains normal PKA dependent regulation and activity relative to wild type CFTR [28]. This disparity suggests that defective Cl⁻ channel function seen in cells expressing $\Delta F508$ CFTR may reflect cell-specific influences on mutant protein regulation. Recent studies also indicate that CFTR processing and activity are strongly influenced by the cell model system of study [29,30], with enhanced maturation and surface stability demonstrated in polarizing models compared to a non-polarized environment [31]. Despite these observations, few studies have rigorously compared the activity of corrected $\Delta F508$ CFTR using low temperature and small molecules in polarizing systems, while also examining the influence of cell type on protein maturation and cAMP dependent regulation. This is of particular importance since the Fisher Rat Thyroid (FRT) cell type was recently used to identify a number of small molecules that rescue $\Delta F508$ CFTR activity [19,21], and the positive findings require confirmation in cells potentially more representative of the CF airway. Comparisons of this nature are highly relevant to drug discovery, particularly because results from commonly used preclinical model systems serve as the scientific foundation for selecting and prioritizing

¹BHK (baby hamster kidney cells), CF (cystic fibrosis), CFBE41o⁻ (cystic fibrosis bronchial epithelial cells), CFTR (cystic fibrosis transmembrane conductance regulator), ERAD (endoplasmic reticulum associated degradation), FRT (Fisher Rat Thyroid Cells), HTS (high-throughput screening), NBD (nucleotide binding domain), NPD (nasal potential difference), PKA (protein kinase A), PP (phosphatase), RT-PCR (reverse transcriptase polymerase chain reaction), TM (transmembrane domain)

therapeutic agents to restore $\Delta F508$ CFTR function that are advanced to the clinical testing phase.

In this manuscript, we examined the relative effectiveness of low temperature and small molecule correctors of $\Delta F508$ CFTR in two well-described cell types, FRT cells and cystic fibrosis bronchial epithelial cells derived from human airways (CFBE41o⁻), each stably transduced with $\Delta F508$ CFTR cDNA under regulatory control of the CMV promoter. Both cell types have been used by a number of independent laboratories to study $\Delta F508$ CFTR biogenesis and activity. Our results show that 1) $\Delta F508$ CFTR expressed in CFBE41o⁻ cells is less susceptible to rescue by either low temperature or the available small molecule correctors of CFTR misprocessing, 2) CFBE41o⁻ cells exhibit persistent defects in PKA-dependent regulation despite biochemical evidence of $\Delta F508$ CFTR restored to the plasma membrane, and 3) the small molecular corrector corr-4a preferentially restores cAMP mediated activation to $\Delta F508$ CFTR compared with low temperature in FRT but not CFBE41o⁻ cells. These findings have important implications for understanding and ultimately overcoming the cellular processes responsible for ER retention, degradation, and defective channel gating of misfolded $\Delta F508$ CFTR.

2. Materials and Methods

2.1 Cell lines and cell culture

Wild type and $\Delta F508$ CFTR cDNA were stably transduced into CFBE41o⁻ cells using TranzVector™ (Tranzyme, Inc., Birmingham, AL). TranzVector™ system represents an HIV-based lentiviral vector with safety features as described [32]. To generate vector stock, CFTR cDNA was first cloned into the gene transfer component under the control of the human cytomegalovirus (hCMV) promoter. Expression of CFTR was also coupled to the puromycin-N-acetyltransferase gene (puro) via the internal ribosomal entry site (IRES) of encephalomyocarditis virus, allowing for rapid selection of cells expressing CFTR in media containing puromycin. CFBE41o⁻ cells were transduced at a multiplicity of infection of one followed by puromycin (4 μ g/ml) selection. Puromycin-resistant cells were expanded and clones were selected for high-level expression. FRT cells stably transduced with $\Delta F508$ CFTR cDNA (under regulatory control of the CMV promoter) were the generous gift of Michael Welsh and Joe Zabner (University of Iowa, Iowa City, IA).

Use of human cells and tissues was approved by the UAB Institutional Review Board. Primary human airway epithelial cells were derived from nasal polypectomies or lung explants of CF subjects who provided written informed consent and were confirmed to be $\Delta F508$ CFTR homozygotes by methods described previously [33]. Briefly, tissues were debrided immediately after surgical resection, washed twice in Minimum Essential Media with 0.5 mg/ml DTT (Sigma-Aldrich, St. Louis, MO) and 25 U/ml DNAase I (Roche, Basel, Switzerland), and then placed in dissociation media containing MEM, 2.5 U/mL DNAse I, 100 μ g/ml ceftazidime, 80 μ g/mL tobramycin, 1.25 μ g/mL amphotericin B, and 4.4 U/mL pronase (Sigma-Aldrich) for 24–36 hrs at 4 C. Loosened airway epithelial cells were then expanded in growth media containing BEGM (LONZA, Basel, Switzerland) supplemented with an additional 10 nM all trans-retinoic acid (Sigma) that was exchanged every 24 hrs. Following expansion, first or second passage cells were seeded on permeable supports for studies.

2.2 Voltage clamp studies in Ussing chambers

Inserts were mounted in Ussing chambers, and short-circuit current (I_{sc}) measured under voltage clamp conditions as previously described [34,35]. Briefly, cells expressing $\Delta F508$ or wild-type CFTR were seeded on Costar 0.4 μ m permeable supports (Bethesda, MD; 5×10^5 cells/filter, 6.5 mm diameter) after coating with fibronectin. Cells were grown to confluence

and then switched to at an air liquid interface (48 hours) prior to mounting in modified Ussing chambers (Jim's Instruments, Iowa City, IA), and initially bathed on both sides with identical Ringers solutions containing (in mM) 115 NaCl, 25 NaHCO₃, 2.4 KH₂PO₄, 1.24 K₂HPO₄, 1.2 CaCl₂, 1.2 MgCl₂, 10 D-glucose (pH 7.4). Bath solutions were vigorously stirred and gassed with 95% O₂:5% CO₂. Short-circuit current measurements were obtained using an epithelial voltage clamp (Warner Instruments, Hamden, CT). A three mV pulse of one second duration was imposed every 100 seconds to monitor resistance, which was calculated using Ohm's law. Since both CFBE41o⁻ and FRT monolayers require a serosal to mucosal Cl⁻ secretory gradient to detect maximal activity of CFTR, the mucosal bathing solution was changed to a low Cl⁻ solution containing (in mM) 1.2 NaCl, 115 Na gluconate, and all other components as above plus 100 μM amiloride to block residual ENaC current in CFBE41o⁻ cells. Agonists (2–20 μM forskolin and 50 μM genistein) were added to the bathing solutions as indicated (minimum five minutes observation at each concentration). 200 μM glybenclamide or CFTR_{Inh}-172 (10μM) was added to the mucosal bathing solution at the end of experiments to block CFTR-dependent I_{sc}. All chambers were maintained at 37°C, and agonist stimulation was initiated within 30 min of placement into the chambers. Permeabilization of the basolateral membrane was performed by application of nystatin (200 μg/mL) for 10 minutes to the basolateral compartment of the cells while mounted in Ussing chambers. Primary airway cells were handled the same, except seeded on Snapwell 1.13cm² permeable supports (Bayer, Pittsburgh, PN), and grown in differentiating media containing DMEM/F12 (Invitrogen, Carlsbad, California), 2% Ultrosor-G (Pall, New York, NY), 2 % Fetal Clone II (Hyclone, Logan, UT), 2.5 μg/ml Insulin (Sigma), 0.25 % Bovine Brain Extract (LONZA), 20nM Hydrocortisone (Sigma-Aldrich), 500 nM Triiodothyronine (Sigma), 2.5 μg/ml Transferrin (Invitrogen), 250 nM Ethanolamine (Sigma-Aldrich), 1.5 μM Epinephrine (Sigma-Aldrich), 250 nM Phosphoethanolamine (Sigma-Aldrich), and 10 nM Retinoic acid (Sigma-Aldrich) until terminally differentiated prior to studies, and were analyzed in MC8 voltage clamps and P2300 Ussing chambers (Physiologic Instruments, San Diego, CA)

2.3 CFTR Immunoprecipitation

For each sample, total protein concentration was measured by absorbance at 760 nm and quantified by the relative standard curve method (BSA standard) [36]. CFTR was immunoprecipitated from equal volume aliquots of cell lysate after normalizing for total protein concentration (final concentration = 1 mg/mL). CFTR was immunoprecipitated using 3 μg/mL 24-1, anti-C-terminal antibody (ATCC# HB-11947) coupled to 36 μl Protein A agarose beads (Roche Molecular Biochemicals, Nutley, NJ) as previously described, then analyzed by Western blot (see below). [18,34,37,38].

2.4 CFTR Biotinylation

All cell surface glycoproteins were biotinylated as previously described [35,38,39]. Biotinylated CFTR was immunoprecipitated using 24-1 antibody (ATCC# HB-11947), separated by SDS-PAGE and Western blotted. CFTR was detected with anti-CFTR NBD1 polyclonal antibody [40] and biotinylated CFTR detected with avidin–HRP followed by ECL (Pierce Biotechnology, Inc., Rockford, IL, USA).

2.5 Western Blot

Total and biotinylated CFTR were detected as described previously [38]. Briefly, immunoprecipitated CFTR was separated by SDS-PAGE 8% gels (Invitrogen). Membranes were blocked overnight in 3% (w/v) BSA and 0.5% Tween 20 in PBS. All antibodies were incubated for 1 h at room temperature. Total CFTR was detected with anti-CFTR NBD1 polyclonal antibody (1:2500 dilution) [40]. Biotinylated CFTR was detected with HRP (horseradish peroxidase)-conjugated avidin (1:5000 dilution; Sigma-Aldrich, St. Louis, MO).

Chemiluminescence was induced with high-sensitivity Immobilon Western substrate (Millipore, Billerica, MA). The membranes were exposed using CemiDoc XRS HQ (Bio-Rad, Hercules, CA, USA) for different time periods (up to 2 min) and calibrated in the linear range for a standard set of diluted samples.

2.6 cAMP levels

Cellular cAMP was measured using an ELISA-based detection kit (Cayman Chemicals, Ann Arbor, MI) as previously described [35,41]. Briefly, cells grown on 35 mm dishes ($\sim 2.5 \times 10^6$ cells/dish) were stimulated with agonist for 10 minutes, and cellular cAMP was extracted with ice cold ethanol. The supernatants were vacuum dried, resuspended in phosphate buffer, and cAMP levels quantified per manufacturer's directions. For all experiments, papaverine (nonspecific nonxanthine phosphodiesterase inhibitor, 100 μ M) was included to improve cAMP detection.

2.7 Real time RT-PCR

A TaqMan One Step RT-PCR protocol (Applied Biosystems) was used to quantify CFTR mRNA transcripts using "Assays on Demand" Gene Expression Products, coupled with the ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA) as previously described [35,42]. Briefly, total RNA was isolated using the Qiagen RNeasy mini kit according to manufacturer's instructions. To prevent possible DNA contamination, the samples were pretreated with RNase-free DNase (Qiagen, Valencia, CA). Sequence specific primers and probes for human *CFTR* and 18S rRNA were purchased from *Assays on Demand* (ABI); Assay ID for *CFTR*: Hs00357011_m1; the probe extends across the exon 21/22 boundary of the human *CFTR* sequence. TaqMan One Step PCR Master Mix Reagents Kit (ABI, Foster City, CA) was used for reverse transcription and PCR. The reaction volume was 25 μ l and contained 12.5 μ l of 2 $\times$ Master Mix without UNG, 0.625 μ l of 40 $\times$ MultiScribe and RNase Inhibitor Mix, 1.25 μ l of 20 $\times$ target primer & probe, 5.625 μ l of Nuclease-free water (Ambion, Austin, TX), and 5 μ l of RNA sample. Reaction plates were covered with an optical cap and centrifuged briefly to remove bubbles. Thermocycler conditions were as follows: Stage 1: 48°C for 30 min; Stage 2: 95°C for 10 min; Stage 3: 95°C for 15 sec, repeat 40 cycles, 60°C for 1 min. All experiments were run in triplicate for verification. The absolute value of the slope of log input amount vs. Δ Ct was > 0.1 , implying that the efficiencies of CFTR and 18S rRNA amplification were not equal. Therefore, the relative quantification of transcript levels (CFTR compared with endogenous 18S rRNA) was performed using the standard curve method.

2.8 Statistics

For I_{sc} , cAMP, and RT-PCR measurements, descriptive statistics (mean, SD, and SEM) and paired and unpaired t-tests were performed using SPSS (Chicago, IL) and Microsoft Excel (Seattle, WA). ANOVA were performed for multiple comparisons using SPSS software (Chicago, IL). All statistical tests were two-sided and were performed at a 5% significance level (i.e., $\alpha = 0.05$).

2.9 Reagents

Small molecule Δ F508 correctors were obtained from the CFFT Chemical Compound Distribution Program and generously provided by Robert Bridges, Ph.D. at Rosalind-Franklin University of Medicine and Science. All agonists were purchased from commercially available sources: endothall and forskolin were purchased from Calbiochem (San Diego, CA) and papaverine and genistein from Sigma-Aldrich.

3. Results

3.1 Functional correction of $\Delta F508$ CFTR by low temperature and chemical agents in FRT and CFBE41o⁻ cells

We first examined the activity of $\Delta F508$ CFTR in CFBE41o⁻ and FRT cells following treatment (16 hrs) with a panel of small molecule correctors available through the CFMT Modulator Compound Resource. Each was tested at the published EC₅₀ concentration [19, 21,22], and compared to activity of $\Delta F508$ CFTR rescued by growth at low temperature (27°C for 48 hrs) (Figure 1). Incubation at low temperature increased the short-circuit currents in both cell lines following stimulation with the combination of forskolin (20 μ M) and genistein (50 μ M). In contrast, only corr-4a treatment (2 μ M) increased $\Delta F508$ CFTR currents in both cell types above vehicle treated controls (maintained at 37°C). A relative hierarchy of corr-4a (C4) > low temperature > VRT-325 (C3) = VRT-640 (C2) > corr-3a (C1) was demonstrated in FRT cells, compared with low temperature >> corr-4a > VRT-325 = VRT-640 = Corr-3a in CFBE41o⁻ cells. Corr-4a demonstrated similar dose/response relationships in FRT and CFBE41o⁻ cells stably transduced with $\Delta F508$ CFTR, with higher total currents (absolute and normalized for baseline currents, as shown in Figures 1B – 1D) seen in FRT cells.

Representative tracings of FRT and CFBE41o⁻ cell monolayers grown at 37°C (with vehicle), 27°C, and 2 μ M corr-4a (37°C) are shown in Figures 1C and 1D. Neither forskolin nor genistein produced significant Cl⁻ conductance in FRT or CFBE41o⁻ cells without $\Delta F508$ CFTR transduction (37°C, 27°C, or corr-4a treatment, data not shown) confirming specificity of effects for $\Delta F508$ CFTR. Figure 2 compares the $\Delta F508$ CFTR conductance produced by treatment with corr-4a relative to low temperature in each cell type (optimized for correction by treatment with 20 μ M corr-4a x 8 hrs in CFBE41o⁻ cells, and 16 hrs in FRT cells; higher concentrations or longer exposure were toxic). Using these optimized conditions, corr-4a treatment led to higher stimulated $\Delta F508$ CFTR Cl⁻ currents relative to low temperature in FRT cells, while low temperature remained more potent in CFBE41o⁻ cell monolayers. While $\Delta F508$ CFTR activity could be detected following treatment with both low temperature and chemical agents in either cell type, the results of Figure 1 and Figure 2 demonstrate significantly greater $\Delta F508$ CFTR activity in FRT monolayers compared to CFBE41o⁻ cells, and point to the importance of cell-specific influences on $\Delta F508$ CFTR rescue *in vitro*. These results were then compared to the degree of I_{sc} rescue seen in primary human airway epithelial monolayers derived from $\Delta F508$ homozygous donors and incubated with corr-4a (10 μ M x 24 hrs) compared to 27°C growth (48 hrs). As shown in Figure 3, correction was seen in some but not all donors, although the relative effects of corr-4a compared to 27°C reflected the relative efficacy observed in CFBE41o⁻ compared to FRT cells.

3.2 Defects in cAMP stimulation of $\Delta F508$ CFTR in corrected CFBE41o⁻ cells

Based on the results of the studies above, we carefully examined the activity of corrected $\Delta F508$ CFTR in the two heterologous cell types, comparing the relative contribution of cAMP stimulation (produced by forskolin treatment) and potentiation of $\Delta F508$ CFTR (with genistein) to total $\Delta F508$ CFTR conductance. As $\Delta F508$ CFBE41o⁻ cells are highly sensitive to temperature correction, we used this model for examining the nature of forskolin stimulated I_{sc} in these cells. Following temperature correction of $\Delta F508$ CFTR processing, increasing doses of forskolin had small effects on Cl⁻ conductance in CFBE41o⁻ cells, while the same treatment was a robust stimulus of Cl⁻ conductance in FRT cells (Figures 4A and 4B). The order of stimulus did not influence the Cl⁻ secretory response produced by forskolin compared with genistein in CFBE41o⁻ cells (Figure 4C), indicating that the response of $\Delta F508$ CFTR to cAMP remained refractory regardless of the agonist sequence in this cell type. To more closely mimic *in vivo* signaling through cAMP [43], we examined the relative contribution of a submaximal forskolin (2 μ M) concentration, followed by genistein (50 μ M) to determine total CFTR activity. CFBE41o⁻ and FRT monolayers were studied after growth at 37°C (control),

37°C with corr-4a (2 μ M, 8–16 hr exposure to maximize the effect for each cell type), and 27°C growth for 48 hrs (Figure 5). In CFBE41o⁻ cells, forskolin again provided small contributions to the total Δ F508 CFTR Cl⁻ conductance (i.e. cumulative I_{sc} response to forskolin and genistein) under all three conditions, despite increased total stimulated Cl⁻ currents following either low temperature or corr-4a treatment (see Figure 2). In contrast, Δ F508 FRT cells had higher forskolin-stimulated currents (absolute, and as a percentage of total I_{sc} stimulated by forskolin and genistein) when grown under each of the three study conditions. Δ F508 CFTR rescue with corr-4a specifically increased forskolin stimulated I_{sc} compared to both 37°C control (vehicle) and low-temperature conditions in FRT monolayers. Permeabilization of the basolateral membrane with nystatin did not meaningfully alter the I_{sc} response in either CFBE41o⁻ or FRT cells expressing Δ F508 CFTR following low temp or pharmacologic rescue with corr-4a, and the relative proportion of activation due to cAMP activation remained significantly different between the two cell types (Figure 5B–C).

3.3 Defects in Δ F508 CFTR activity in CFBE41o⁻ cells do not reflect differences in transgene expression levels, cAMP production, total cellular phosphodiesterase or phosphatase activity

To explore the nature of the defect in cAMP regulation of Δ F508 CFTR in CFBE41o⁻ cells, we examined Δ F508 CFTR transcript levels in CFBE41o⁻ and FRT monolayers relative to Calu-3 cells, an established, polarizing human airway cell line that expresses chromosomal wild type CFTR [44]. We have previously reported that the CFBE41o⁻ cells (in the absence of Δ F508 CFTR transduction) exhibit extremely low levels of endogenous Δ F508 CFTR expression [45]. Figure 6A shows that the transcript levels of the Δ F508 CFTR transgene in CFBE41o⁻ and FRT cells were only modestly increased relative to endogenous wtCFTR expression in Calu-3 cells (as assessed by real time RT-PCR), suggesting that mRNA expression provides a reasonable model system in these cells. We next compared mature Δ F508 CFTR levels in FRT and CFBE41o⁻ cells by immunoprecipitation. The mature (Band C) form of Δ F508 CFTR was detectable at low levels in polarized FRT cells under control (37°C) conditions, and was not seen in the transduced CFBE41o⁻ cells, consistent with the functional studies described above (Figure 6B). Moreover, rescue of Band C Δ F508 CFTR was seen following corr-4a (10 μ M) or low temperature (27°C x 48 hours) exposure in both cell types, but was particularly robust in FRT cells. More sensitive biotinylation studies to specifically detect Δ F508 CFTR localized to the plasma membrane demonstrated strong rescue of Δ F508 misprocessing in FRT cells treated with corr-4a or low temperature incubation. Although correction of Δ F508 CFTR processing with corr-4a was also seen in CFBE41o⁻ cells, the effects were much less potent compared to low temperature, or to that seen with corr-4a in FRT cells.

Increasing concentrations of forskolin had more potent effects on cellular cAMP levels in both the Calu-3 and CFBE41o⁻ human airway cell lines relative to FRT cells, indicating that inadequate or blunted forskolin-stimulated cAMP production was not likely to be responsible for diminished forskolin-stimulated Cl⁻ currents in corrected Δ F508 CFBE41o⁻ cells (Figure 7A). Neither PDE inhibition by the nonspecific PDE inhibitor papaverine (100 μ M, Figure 7B) nor phosphatase (PP) inhibition with endothall (400 μ M, Figure 7C) restored forskolin activation to Δ F508 CFTR, despite potent activation of wtCFTR in these cells (results summarized in Figure 7 legend). Together, the results confirm that corrected Δ F508 CFTR is refractory to activation by forskolin in CFBE41o⁻ cells (but not FRT cells), and that transgene expression, protein levels, stimulated cAMP production, and excessive PDE or PP activity are unlikely to be independently responsible for this defect.

4. Discussion

In this report we examined CFTR expression, activity, and regulation in CFBE41o⁻ human airway cells and FRT cells grown in polarizing conditions following correction of the Δ F508 processing defect with low temperature or a panel of putative small molecule Δ F508 CFTR processing correctors available through the CFFT Chemical Compound Distribution Program. Both cell types are frequently used to examine Δ F508 CFTR maturation, cell membrane stability, and ion transport activity in preclinical testing of novel Δ F508 CFTR corrector agents [19,22,46]. Our results indicate that Δ F508 CFTR exhibits distinctly different processing and activation efficiency in each cell line. These findings are therefore relevant to the secondary evaluation of compounds identified in Δ F508 CFTR high-throughput screening programs [19,47], and provide a rationale to evaluate lead compounds across redundant model systems during drug development. They are also relevant to the interpretation of clinical trials examining Δ F508 CFTR correctors, as rescued Δ F508 CFTR may exhibit persistent regulatory defects despite localization to the plasma membrane.

In our studies of FRT cells, Δ F508 CFTR exhibited low level protein maturation even when grown at 37°C (Figure 6, Panel B - without exposure to a corrector) that was readily enhanced by several Δ F508 corrector agents (VRT-640, VRT-325, and corr-4a) (Figure 1). Moreover, Δ F508 CFTR in FRT cells was strongly activated by both the cAMP agonist forskolin, and a well described potentiator of surface CFTR activity, genistein. In contrast, Δ F508 CFTR expressed in CFBE41o⁻ cells was less sensitive to correction by these reagents, exhibiting limited rescue only following corr-4a treatment. Confirmation of corr-4a effects in primary airway epithelial cells derived from a Δ F508 homozygous donor suggested low level functional correction in some but not all subjects (Figure 3). Similar levels of Δ F508 CFTR mRNA were demonstrated in the two cell types by real time RT-PCR, while biochemical analysis showed increased levels of C Band Δ F508 CFTR in FRT cells relative to CFBE41o⁻ cells at 37°C that became even more pronounced following corr-4a treatment (Figure 6B). In addition, Δ F508 CFTR expressed in CFBE41o⁻ cells and rescued by corr-4a or low temperature was poorly activated by forskolin (Figure 5). Production of cAMP by forskolin was not reduced in CFBE41o⁻ cells relative to the other cell types, and promotion of R domain phosphorylation via inhibition of PDEs or PP_s was insufficient to restore forskolin regulation to surface Δ F508 CFTR in CFBE41o⁻ cells (Figures 7A–7C). Papaverine alone was a modest stimulus of Δ F508 CFTR-dependent I_{sc} (Figure 7C), and also reduced subsequent activation via genistein. The nature of this mixed effect is unclear from our studies, but supports previous work that has implicated PDE inhibition as a means to activate Δ F508 CFTR [25, 48,49]. Despite these positive effects on Δ F508 CFTR activity, papaverine failed to restore Δ F508 CFTR activation by subsequent stimulation with a potent cAMP-elevating agonist, suggesting compartmental PDE expression (such as that reported for PDE3 [50]) does not account for the distinctive activation pattern in these cell lines. The observed differences in activated Cl⁻ conductance between the cell lines were unlikely to reflect limitations of Cl⁻ entry and/or K⁺ channel activation across the basolateral cell membrane, since genistein remained an effective stimulus in either polarized cell model before or after forskolin pre-stimulation (Figures 4A–4C). This conclusion is supported by the findings in Figure 5, as basolagteral permeabilization failed to restore forskolin activated I_{sc} in CFBE41o⁻ cells. Furthermore, the biotinylation studies (Figure 6) demonstrated that the levels of Δ F508 CFTR at the plasma membrane following temperature correction were similar to those of wtCFTR grown at 37°C in CFBE41o⁻ cells. Thus, the total amount of corrected Δ F508 CFTR available at the cell membrane was not solely responsible for the observed defects in Δ F508 CFTR activation by cAMP in these cells. In aggregate, our results suggest that differences in cellular processing efficiency, in addition to cell specific effects at the plasma membrane, contribute to the failure of cAMP agonists to activate Δ F508 CFTR in CFBE41o⁻ cells. Coupled with our previously published reports using this cell type [35,42], the present studies provide a better

understanding of $\Delta F508$ CFTR activity in commonly used preclinical model systems. Our results are also consistent with a recent report by Pedemonte et al., who showed a similar phenotype when $\Delta F508$ CFTR was expressed in a pulmonary alveolar cell line (A549), including the observation that these cells exhibited a higher threshold for rescue of $\Delta F508$ CFTR activity relative to FRT cells, and failed to respond to cAMP stimulation [51].

The results presented here are relevant to the design of future clinical trials of molecules intended to correct $\Delta F508$ CFTR maturation. Standard assays to detect CFTR activity in early phase clinical studies (such as the nasal potential difference (NPD)) rely on stimulation via G-protein coupled receptors that signal through cAMP (*e.g.*, stimulation by catecholamines through β_2 adrenergic receptors or by adenosine through A_{2B} adenosine receptors) [43,52–54]. We have previously reported that agonists that stimulated these receptor pathways fail to activate temperature corrected $\Delta F508$ CFTR in CFBE41o⁻ cells (5). Our findings suggest that cAMP-dependent stimuli used in current NPD protocols may be insufficient to fully activate $\Delta F508$ CFTR at the cell membrane of human airway epithelia [35,43,52,54]; we speculate that this defect may have contributed to reduced measurable rescue of CFTR activity reported in previous clinical trials of CFTR processing correctors [55,56], and could be improved by co-administration of an agent that can overcome cAMP-dependent gating defects, such as reported for the flavonoid quercetin [57].

Our findings are also in agreement with recent reports by Ostedgaard [29] and Liu et al. [30], who showed that $\Delta F508$ CFTR exhibited different maturation patterns when expressed in murine, porcine, and human airway cells. In those studies, $\Delta F508$ CFTR maturation and activity were relatively preserved in murine and porcine cells compared to isolated human airway cells, demonstrating detectable $\Delta F508$ CFTR at the cell membrane under normal (37°C) growth conditions. Previous experience from our laboratory indicates that aberrant $\Delta F508$ CFTR maturation and function are much more pronounced in human airway cells compared with other cell types of human origin. For example, forskolin remains an effective stimulus of low temperature corrected $\Delta F508$ CFTR in HeLa cells [35], a human, non-airway, non-polarizing cell type. Further studies of $\Delta F508$ CFTR behavior between different human cell lines, human and non-human cells, or cells grown under polarizing and non-polarizing conditions might therefore be used to identify novel regulatory pathways that influence $\Delta F508$ CFTR, either through effects on $\Delta F508$ CFTR processing in the ER, or regulation of CFTR activity at the cell surface [58].

To date, the most extensive analyses of $\Delta F508$ CFTR activity have been performed in single cells or cell free expression systems, without direct comparison using intact epithelial models or between human and non-human cell types. Several laboratories have shown that $\Delta F508$ CFTR is refractory to activation by cAMP and PKA *in vitro*, with defects in channel gating and open channel probability by patch clamp analysis of $\Delta F508$ CFTR in BHK and NIH 3T3 cells [23,59,60]. In these studies, $\Delta F508$ CFTR activity was not fully restored without co-stimulation with molecules that potentiate CFTR independent of cAMP and PKA (such as genistein, NPPB-AM, and curcumin). The present studies therefore extend earlier observations, and provide new evidence of cell-specific effects that may reflect species differences in the processing and regulation of $\Delta F508$ CFTR. Other investigators have demonstrated that $\Delta F508$ CFTR rescued by small molecules retains cAMP/PKA dependent activation [22,61]. Whether this difference compared to CFBE41o⁻ or alveolar cells represents effects of stable transgene expression (versus endogenous CFTR expression), or variation across a heterogeneous population of human donors of primary cells is unknown. For example, Bronsveld and colleagues reported that $\Delta F508$ CFTR maturation and function can be detected in epithelial cells isolated from a subset of human CF subjects homozygous for the $\Delta F508$ CFTR mutation [62]. In other reports [63], $\Delta F508$ CFTR activity correlated with clinical phenotype, and the investigators provided evidence that maturation and function of $\Delta F508$

CFTR likely varies among individuals with the disease. Whether heterogeneity among individuals reflects modifier genes that contribute to $\Delta F508$ CFTR maturation and/or the membrane activity observed in our experiments will require further study, but are certainly suggested by heterogeneous correction with *corr-4a* in primary airway epithelial cells described here.

It is not clear from our studies whether the differences in cAMP responsiveness exhibited by $\Delta F508$ CFTR in CFBE41o⁻ cells compared with FRT cells represent differences in $\Delta F508$ CFTR structure (e.g. subtle folding differences of $\Delta F508$ CFTR in the two cell lines) producing differences in channel gating and/or kinetics), or altered $\Delta F508$ CFTR regulation between the two cell lines (e.g. different binding partners at the plasma membrane that affect $\Delta F508$ CFTR regulation by cAMP). Single channel analysis of $\Delta F508$ CFTR in the two cell lines may help resolve this question, as differences in channel behavior could be discriminatory between direct (e.g., ion channel) or indirect (e.g., binding partners not isolated within the same membrane patch) effects. We speculate that the differences in cAMP responsiveness could represent unique protein-protein interactions in human airway cells relative to FRT cells, potentially explaining these results. A number of CFTR binding partners that have been described at or near the plasma membrane in human airway cells (NHERF-1, syntaxin-1A, ENaC, Cal) [64–66], including CK2, a protein implicated to alter wild-type CFTR Cl⁻ transport, while not affecting $\Delta F508$ CFTR [67]. Adenosine monophosphate-stimulated kinase (AMPK) is known to co-localize with CFTR, and as a constitutively active inhibitor of CFTR, represents another potential modifier of CFTR activation pattern between cell types. [68]. An improved understanding of other CFTR binding partners relevant to CFTR activation could lead to new therapeutic targets to restore cAMP activation to the mutant channel. Regardless, compounds that restore cAMP dependent regulation and also correct aberrant $\Delta F508$ CFTR processing would seem to be ideal candidates for further clinical development [22,69].

In summary, the present studies provide evidence for cell and species specific properties of $\Delta F508$ CFTR that are magnified by small molecule correctors of protein misprocessing. Refractory cAMP-dependent regulation of rescued $\Delta F508$ CFTR in human airway cells may be a potential barrier to future therapies that correct $\Delta F508$ CFTR misprocessing in human subjects, and will benefit from the evaluation of epithelial monolayers derived from a number of CF subjects to understand its effects.

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Figure 1A

Figure 1B

Figure 1C

Figure 1C

Figure 1D

Figure 1. Comparison of chemical and temperature correction of $\Delta F508$ CFTR dependent I_{sc} in polarized CFBE41o⁻ and FRT cells

Panel A. Summary of stimulated I_{sc} produced by $\Delta F508$ CFTR small molecule correctors. $\Delta F508$ CFBE41o⁻ (black bars) and FRT (white bars) cells were incubated with CFTR correctors (corr-3a, VRT-640, VRT-325, and corr-4a) at the published EC₅₀ (3 μ M, 10 μ M, 2 μ M, and 2 μ M, respectively) overnight (16 hours) and compared with vehicle control. Cells were studied in modified Ussing chambers and total I_{sc} was determined after sequential treatment with forskolin (20 μ M) and genistein (50 μ M). Values reported are I_{sc} (μ A/cm²) normalized for vehicle treated controls within each cell type. In all conditions, FRT cells were more sensitive to rescue of $\Delta F508$ CFTR mediated I_{sc} . * $p < 0.05$, ** $p < 0.01$, $n = 3-6$ per condition, $\pm$ SEM. Normalized to vehicle treatment shown here, WT CFTR transduced I_{sc} are 8.5 μ A/cm² in CFBE41o⁻ and 1.6 μ A/cm² in FRT cells (absolute currents 59.9 ± 19.0 and 60.2 ± 9.8 μ A/cm², respectively; $n=4$). **Panel B. Corr-4a dose response effects on I_{sc} .** Corr-4a had similar dose response effects in $\Delta F508$ CFTR-expressing FRT and CFBE41o⁻ cells. CFBE41o⁻ and FRT $\Delta F508$ cells were incubated at increasing concentrations of corr-4a for 18–24 hours and compared with vehicle treated controls. Short-circuit current was measured after CFTR stimulation with forskolin (20 μ M) and genistein (50 μ M). Results are normalized for vehicle treated conditions within cell type. Corrector concentrations higher than 20 μ M were toxic in both cell types. † $p < 0.01$ vs. CFBE41o⁻ at same concentration of corr-4a, * $p <$

0.001 and $**p < 0.05$ vs. vehicle, $n=4-20$ /condition, $\pm$ SEM. **Panels C and D. Examples of corr-4a and temperature correction in FRT (C) and CFBE41o⁻ (D) monolayers.**

Pretreatment with corr-4a augments CFTR dependent I_{sc} in both $\Delta F508$ transduced FRT cells and CFBE41o⁻. Representative short-circuit current tracings show enhanced I_{sc} response in $\Delta F508$ CFBE41o⁻ and FRT monolayers pretreated with corr-4a (2 μ M). Following establishment of a Cl^- secretory gradient (LoCl), amiloride (Am, 100 μ M) was added to the apical compartment followed by forskolin (Forsk, 20 μ M – apical and basolateral), and genistein (Gen, 50 μ M – apical and basolateral); glybenclamide (Glyb, 200 μ M - apical) was then added to block CFTR-dependent conductance.

Figure 2. Summary comparison of total stimulated Cl^- currents following corr-4a and low temperature treatment in ΔF508 CFTR-transduced CFBE41o $^-$ and FRT monolayers

The data show the total I_{sc} after forskolin (2 μM) and genistein (50 μM) stimulation. Results are presented relative to ΔF508 CFTR cells corrected with low temperature (27°C x 48 hours) within the same cell type. Corr-4a was a more potent corrector than low temperature in FRT cells, while low temperature was more potent than corr-4a in CFBE41o $^-$ cells. * $p < 0.005$ and ** $p < 0.001$ vs. vehicle, $n = 10\text{--}17$ filters per condition, $\pm\text{SEM}$. Corr-4a treatment induced increased total stimulated I_{sc} in 13 of 16 paired CFBE41o $^-$ monolayers compared to 13 of 13 FRT monolayers

Figure 3. The effects of CFTR processing modulation on CFTR-dependent chloride short-circuit current in fully-differentiated primary epithelial cells derived from homozygous $\Delta F508$ donors $\Delta F508$ homozygous primary human airway epithelial cells were grown in polarizing conditions until terminally differentiated, and the treated with corr-4a (10 μM) or DMSO (vehicle) to the apical and basolateral surface for 24hr prior to assay. Low temperature growth was for 48 hours. Following treatment, monolayers were mounted in modified Ussing chambers under voltage clamp conditions, receiving sequential exposure to amiloride (100 μM), chloride-free gluconate, forskolin (20 μM), and CFTR_{Inh}-172 (10 μM). Each marker designates forskolin stimulated Isc in monolayers derived from a single CF donor. Mean changes are designated by the black bar. $n = 9$. Mean stimulated Isc in WT cells studied during the same time period was $24.0 \pm 3.1 \mu A/cm^2$, $n=8$.

Figure 4A

Figure 4B

Figure 4C

Figure 4. Forskolin stimulates dose-dependent increases in I_{sc} in $\Delta F508$ CFTR transduced FRT monolayers but not in $\Delta F508$ CFTR transduced CFBE41o $^-$ monolayers

Panel A. Representative I_{sc} tracings are shown for CFBE41o $^-$ cells (top panel) and FRT cells (bottom panel). Following establishment of a basolateral to mucosal Cl^- secretory gradient ($LoCl^-$) and addition of amiloride to the apical compartment (Am, 100 μM), increasing concentrations of forskolin (Forsk) were added to the apical and basolateral membranes, followed by the addition of genistein (Gen 50 μM – apical and basolateral) and glybenclamide (Glyb, 200 μM - apical). **Panel B: Summary data of studies represented in Figure 3A.**

$\Delta F508$ CFTR transduced FRT cells exhibit dose dependent activation of I_{sc} with forskolin, while $\Delta F508$ transduced CFBE41o cells are refractory to similar maneuvers. Genistein (50 μM) potently stimulates I_{sc} in both cell types. * $p < 0.005$, $n = 4$. **Panel C: The sequence of agonist addition has no effect on forskolin stimulation I_{sc} in CFBE41o $^-$ monolayers.**

Temperature corrected $\Delta F508$ CFBE41o $^-$ monolayers were stimulated with either forskolin (Forsk, 20 μM) followed by genistein (Gen, 50 μM) (top panel – agonists added to both the apical and basolateral compartments), or genistein followed by forskolin (bottom panel). Glybenclamide (Glyb, 200 μM - apical) was added to confirm the CFTR dependence of stimulated I_{sc} . Forskolin accounted for $7.2 \pm 0.1\%$ of the total stimulated current when forskolin preceded genistein, while forskolin did not stimulate additional I_{sc} when added after genistein ($-4.4 \pm 0.3\%$). In contrast, in temperature corrected $\Delta F508$ CFTR-transduced FRT cells,

forskolin accounted for $43.9 \pm 0.2\%$ of the total I_{sc} when preceding genistein (data not shown). Data are representative of 6 filters completed in each condition.

Figure 5. $\Delta F508$ CFTR-transduced FRT cells exhibit a significantly greater proportion of forskolin stimulated I_{sc} compared with $\Delta F508$ CFTR-transduced CFBE41o⁻ monolayers following temperature or corr-4a correction

Panel A: After either temperature (27° C x 48 hours) or chemical (corr-4a = 20 μ M, 18 hrs) corrector treatment, I_{sc} was measured in modified Ussing chambers following sub-maximal forskolin (2 μ M) and genistein (50 μ M) stimulation. FRT cells exhibited a significantly greater proportion of total stimulated I_{sc} following forskolin stimulation ($\dagger p < 0.005$ vs. CFBE41o⁻ cells under paired conditions). In addition, corr-4a preferentially rescued forskolin-stimulated Cl⁻ conductance in $\Delta F508$ CFTR-transduced FRT cells compared to low temperature and vehicle treated conditions. Concentrations of forskolin were chosen to more closely reflect cAMP production anticipated by G-protein coupled receptors [43]. * $p < 0.001$ vs. vehicle control, $n = 14-17$ per condition, $\pm$ SEM. **Panel B & C:** CFBE41o⁻ (B) and FRT (C) cells transduced with $\Delta F508$ CFTR were treated with low temperature growth (27° C) or Corr-4a (10 μ M) for 24 hrs then mounted in Ussing chambers. The basolateral membrane was premeabilized with nystatin (50 μ g/mL x 10 min) or control in Ringer's solution prior to I_{sc} assay. Stimulated I_{sc} following addition of forskolin (2 μ M, black) and genistein (50 μ M, white) are shown. ($n = 4$ per condition, $\pm$ SEM). No significant differences in total I_{sc} between control and nystatin treated cells were present. The proportion of total I_{sc} stimulated by forskolin in CFBE41o⁻ cells remained low relative to FRT cells under all conditions tested. This proportion was 6.8% in CFBE41o⁻ vs. 28.4% in FRT cells ($p < 0.01$) and 11.5% in CFBE41o⁻ vs. 32% in FRT cells ($p < 0.01$) following 27° C growth with and without nystatin permeabilization, respectively; following treatment with corr-4a, the proportion of total I_{sc} stimulated by forskolin was 34.4% in CFBE41o⁻ vs. 48.5% in FRT cells ($p < 0.05$) and 26.1% in CFBE41o⁻ vs. 57.0% in FRT cells ($p = 0.06$) with and without nystatin permeabilization, respectively.

Figure 6A

Figure 6B

Figure 6. Comparison of $\Delta F508$ CFTR expression**Panel A. $\Delta F508$ CFTR transgene mRNA transcript levels in FRT and CFBE41o⁻ cells.**

CFTR mRNA levels were measured by real time RT-PCR and compared with 18S rRNA endogenous control. Results are presented relative to wtCFTR levels in Calu-3 cells (which express endogenous CFTR). $\Delta F508$ CFTR-transduced FRT and CFBE41o⁻ cells demonstrated 2.0 – 2.6-fold higher $\Delta F508$ CFTR transgene mRNA levels vs. wtCFTR transcript levels in Calu-3 cells (n=4–5 per condition, $\pm$ SEM).

Panel B. Biochemical comparison of temperature and chemical correction by corr-4a in $\Delta F508$ -transduced CFBE41o⁻ and FRT cells.

Immunoprecipitation of $\Delta F508$ -transduced FRT (left) and CFBE41o⁻ (right) cells grown in polarizing conditions following incubation in control (37°C), low temperature (27°C x 48 hours), or corr-4a treated (10 μ M x 16 hours) conditions with anti-CFTR antibody (top). Band B and C CFTR are illustrated by white and black arrows, respectively. All IPs and biotinylation studies were performed with the same amount of cell lysate (500 μ gm), and all lanes had matched protein content.. Cells transduced with wild-type CFTR are included as controls. Detection of CFTR localized to the plasma membrane by biotinylation of surface glycoproteins and blotting with anti-avidin antibody following immunoprecipitation of CFTR are shown from the same conditions (bottom panel).

Figure 7A

Figure 7B

Figure 7C

Figure 7. cAMP production and regulation of CFTR in the different model cell types

Panel A. Comparison of cAMP levels in Calu-3, CFBE41o⁻, and FRT cells. Total cAMP levels (normalized for total protein) were measured as described in the Methods. Forskolin raised cAMP in all cell types in a dose dependent fashion. CFBE41o⁻ cells demonstrated the strongest response compared with FRT and Calu-3 cells (*p<0.01 versus control-treated cells of the same cell type; † p<0.001 versus FRT cells at the same concentration of forskolin; n = 4 per condition, ± SEM). **Panel B. Phosphodiesterase inhibition does not rescue forskolin-stimulated I_{sc} in temperature corrected ΔF508 CFBE41o⁻ monolayers.** ΔF508

CFBE41o⁻ cells were grown in polarizing conditions and studied in modified Ussing chambers following temperature correction (27°C x 48 hours). After placing cells in chambers with a basolateral to apical low Cl⁻ gradient and blocking apical Na⁺ transport with amiloride (100 μM), monolayers were stimulated with papaverine 100μM (or vehicle) followed by forskolin (20 μM) and then genistein (50 μM – all agonists added to the apical and basolateral compartments). Papaverine alone (100 μM) activated I_{sc} [(6.8 ± 0.4 μA/cm² versus 0.1 ± 0.1 μA/cm² for vehicle treated cells], **p<0.001), but had no effect on subsequent forskolin-mediated I_{sc} (p=NS). Genistein (50 μM) further stimulated I_{sc}, although this was blunted in cells pretreated with papaverine (*p<0.05; n=5 ± SEM). CFBE41o⁻ monolayers expressing wtCFTR had a robust Cl⁻ secretory response to 100 μM papaverine that was sensitive to glybenclamide blockade (59.8 ± 14.3 vs. 1.3 ± 0.7 μA/cm² with vehicle, p<0.005, n = 8, data not shown). **Panel C. Phosphatase inhibition does not rescue forskolin-stimulated I_{sc} in temperature corrected ΔF508 CFBE41o monolayers.** ΔF508 CFTR-transduced CFBE41o⁻ cells were treated as described above in Panel 6B, except that the phosphatase inhibitor endothall (400 μM, apical and basolateral addition) was used in place of papaverine.

Endothall alone did not activate Cl^- conductance (1.6 ± 0.4 vs. $1.1 \pm 1.3 \mu\text{A}/\text{cm}^2$ in vehicle treated cells, $p=\text{NS}$). Endothall pretreatment had no effect on I_{sc} following addition of forskolin ($20 \mu\text{M}$) or genistein ($50 \mu\text{M}$), $n=5$, $\pm\text{SEM}$. CFBE41o $^-$ monolayers expressing wtCFTR had a robust Cl^- secretory response to $400 \mu\text{M}$ endothall that was sensitive to glybenclamide blockade (40.1 ± 14.4 vs. $8.8 \pm 4.8 \mu\text{A}/\text{cm}^2$ with vehicle, $p=0.05$, $n=8$, data not shown).