Activation of the Unfolded Protein Response by Δ F508 CFTR

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Environmental insults and misfolded proteins cause endoplasmic reticulum (ER) stress and activate the unfolded protein response (UPR). The UPR decreases endogenous cystic fibrosis transmembrane conductance regulator (CFTR) mRNA levels and protein maturation efficiency. Herein, we investigated the effects of the folding-deficient Δ F508 CFTR on ER stress induction and UPR activation. For these studies, we developed and characterized stable clones of Calu3 Δ F cells that express different levels of endogenous wild-type (WT) and recombinant Δ F508 CFTR. We also present a novel RT-PCR-based assay for differential quantification of wild-type CFTR mRNA in the presence of Δ F508 CFTR message. The assay is based on a TaqMan minor groove binding (MGB) probe that recognizes a specific TTT sequence (encoding phenylalanine at position 508 in human CFTR). The MGB probe is extremely specific and sensitive to changes in WT CFTR message levels. In RNA samples that contain both WT and Δ F508 CFTR mRNAs, measurement of WT CFTR mRNA levels (using the MGB probe) and total CFTR mRNA (using commercial primers) allowed us to calculate Δ F508 CFTR mRNA levels. The results indicate that overexpression of Δ F508 CFTR causes ER stress and activates the UPR. UPR activation precedes a marked decrease in endogenous WT CFTR mRNA expression. Furthermore, polarized airway epithelial cell lines are important tools in cystic fibrosis research, and herein we provide an airway epithelial model to study the biogenesis and function of WT and Δ F508 CFTR expressed within the same cell.

Keywords: endoplasmic reticulum stress; unfolded protein response; Δ F508 CFTR; quantitative PCR; Calu-3

Endoplasmic reticulum (ER) stress is often induced by misfolded mutant proteins (1, 2). Cystic fibrosis (CF) caused by the deletion of phenylalanine at the 508 position (Δ F508) of the human CF transmembrane conductance regulator (CFTR) is a protein folding disease. This common type of CF is representative of a class of protein folding diseases in which a misfolded, but partially functional, mutant protein is actively degraded by the proteasome (3, 4). Because Δ F508 CFTR is functional, large-scale efforts have been directed toward identification of compounds (chemical chaperones, correctors) that rescue this mutant CFTR from endoplasmic reticulum-associated degradation (ERAD). To further these efforts, it is essential to better understand not only how different levels of Δ F508 CFTR affect ER function, but also how ER stress responses might regulate CFTR expression.

Previous studies suggested that neither Δ F508 CFTR expressed in primary airway cells (5), or introduced by adeno-

CLINICAL RELEVANCE

Chronic inflammation may cause endoplasmic reticulum (ER) stress and activate the unfolded protein response. Studies regarding ER stress responses are important to understand the pathomechanism of these disorders.

virus gene transfer (6), activated the unfolded protein response (UPR). However, these studies did not examine different expression levels of Δ F508 CFTR. Moreover, a recent study revealed that the UPR may be triggered in CF by undefined insults exacerbating the disease (7). This finding also supported earlier observations that activation of the UPR inhibited endogenous CFTR expression (8). It is also conceivable that relatively high Δ F508 CFTR protein levels are necessary for its efficient rescue from ERAD. However, it is not known how the increased Δ F508 CFTR expression might affect ER functions and the UPR. Importantly, several previously described approaches for rescuing Δ F508 CFTR from ERAD (proteasome inhibition [9], ER Ca^{2+} transport blockers [10, 11], and depletion of ER Ca^{2+} [12]) also cause ER stress and activate the UPR (13–15).

Based on the idea that the UPR may be a critical component of CF and that emerging treatment approaches such as Δ F508 CFTR correction may in fact initiate the UPR, we investigated how increasing the expression of Δ F508 CFTR induced ER stress and activated the UPR. We also tested how the UPR affected endogenous and recombinant CFTR expression. To perform these studies, we developed a TaqMan minor groove binding (MGB) based assay aimed at the Δ F508 mutation site, for differential measurements of WT and Δ F508 CFTR mRNAs expressed in the same cell. Furthermore, we introduce novel epithelial cell line models expressing different levels of both WT and Δ F508 CFTR.

MATERIALS AND METHODS

In Vitro Transcription

CFTR transcripts were synthesized from XhoI-cut pcDNA-3.1-WT or pcDNA3.1- Δ F plasmids using MEGAscript T7 (Cat # AM1333; Ambion, Austin, TX). The reaction products were purified using MEGAclear (Ambion) according to the manufacturer's recommendations. The control transcript was obtained from the DNA supplied in the kit. RNA concentrations were established by absorbance at 260 nm ($A_{260\text{nm}}$).

Quantification of CFTR mRNA Using Real-Time RT-PCR

Isolation of cellular RNA. Total cellular RNA was isolated using RNeasy (Qiagen, Valencia, CA), according to the manufacturer's recommendations. RNA concentration was calculated based on the absorbance at 260 nm. RNA samples were stored at -20°C .

Measurement of relative mRNA levels using real time RT-PCR. Real-time RT-PCR to measure WT and Δ F508 CFTR mRNA

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(total CFTR, standard assay) was performed using TaqMan One-Step RT-PCR Master Mix Reagents (Cat. No. 4309169; Applied Biosystems, Austin, TX) as described previously (8), using the manufacturer's handbook as a reference (Relative Quantification; Applied Biosystems 7300/7500 Real Time PCR System; 2004). *GAPDH* mRNA and 18S rRNA were amplified as internal controls and used as a reference, as specified for each experiment. WT *CFTR* mRNA levels were measured using a custom assay (Assay ID: 4331348, WT-CFTR-frag.txt) as described in RESULTS. Forward primer (WT-CFTR frag.txt-73F): GCCTG GCACCATTAAGAAAT. Reverse primer (WT CFTR frag.txt-145R): TTGATGACGCTCTGTATCTATATTCA. TaqMan minor groove binding (MGB) probe (WT CFTR frag.txt-96T): TCATCTT TGGTGTTTC. The MGB probe recognizes the presence of the TTT that encodes the phenylalanine at the 508 position in human WT *CFTR*.

Quantification of *In Vitro* Transcribed CFTR mRNA and the Assessment of Assay Sensitivity Using the MGB-Based Assay

Real-time RT-PCR was performed using TaqMan One-Step RT-PCR Master Mix Reagents and the custom probes containing the MGB probe, as described above. Standard mRNA concentrations were measured based on absorbance at 260 nm (A_{260nm}). Each point represents the average of six replicates.

Quantification of UPR Reporter and Control mRNA Levels

HSPA5/BiP (assay ID: Hs00607129_gH) and spliced *XBPI* (assay ID: Hs00231936_m1) mRNA levels were measured to test for UPR activity, as described previously (8). Transferrin receptor (TR, [assay ID: Hs99999911_m1]) was amplified and measured as an additional control.

Cell Lines and Culture Conditions

Calu-3 (16), CFPAC-1 (expressing endogenous Δ F508 CFTR) (17), and HeLa cells were obtained from ATCC (Manassas, VA). HeLaWT, HeLa Δ F (8, 18), and Calu-3 Δ F (expressing recombinant Δ F508 CFTR on the Calu-3 background) were transduced with a TranzVector (Tranzyme, Birmingham, AL) and selected as previously described (19). Briefly, Δ F508 CFTR cDNA was stably introduced into Calu-3 cells using TranzVector (Tranzyme). TranzVector system represents an HIV-based lentiviral vector with unique safety features as described (18). 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*) gene via the internal ribosomal entry site (IRES) of encephalomyocarditis virus, allowing for rapid selection of cells expressing CFTR in media containing puromycin. Calu-3 cells were transduced at multiplicity of infection of 1 followed by puromycin (4 μ g/ml) selection. Puromycin-resistant cells were expanded to form a pool of stable CFTR expressers and subjected to cloning.

Cloning of Calu-3 Δ F Cells Expressing Endogenous WT and Recombinant Δ F508 CFTR

A number of the individual Calu-3 Δ F clones described above were characterized and established based on the ratio of endogenous (WT) to recombinant (Δ F508) *CFTR* mRNA and protein levels. Clones were divided into three classifications based on whether they expressed (1) low levels of WT and Δ F508 CFTR, (2) similar amounts of both recombinant (Δ F508) and endogenous (WT) CFTR, and (3) significantly higher levels of recombinant (Δ F508) CFTR than endogenous WT. These clones were identified, expanded, and characterized according to *CFTR* mRNA distribution, protein levels, and morphology.

Antibodies

Anti-CFTR (24-1) C-terminal monoclonal antibody was purified from hybridoma supernatant at the UAB Hybridoma Core Facility (ATCC# HB-11947). Anti-ZO1 rabbit polyclonal antibody was purchased from Invitrogen (Carlsbad, CA). Anti-Sec61 β rabbit polyclonal antibody was purchased from Upstate (Temecula, CA).

Induction of ER Stress and Activation of the UPR

Pharmacologic induction of ER stress and activation of the UPR was performed according to previously described methods (8, 15, 20).

Briefly, cells were treated with 50 μ M ALLN (Sigma, St. Louis, MO) for the time intervals specified in each experiment (15). Tunicamycin (TM) (Sigma) was added to samples for the time periods specified at 5 μ g/ml final concentration (20).

Measurement of CFTR Protein Levels

CFTR was immunoprecipitated from 250 μ g total protein, *in vitro* phosphorylated with γ - 32 P ATP (NEN) and cAMP-dependent protein kinase-A (Promega, Madison, WI), separated on 6% gels, and analyzed using phosphorimaging, as described previously (8, 21).

Immunocytochemistry

Cells were grown on 12-mm polycarbonate cell culture inserts (Costar, St. Louis, MO) or on coverslips. Indirect immunocytochemistry and digital imaging were performed as described previously (19, 21).

Induction of Recombinant Δ F508 CFTR Expression

Sodium butyrate (NaBu, 1 or 2 mM) was added to cells for 12 hours to induce recombinant CFTR expression, based on previous reports that NaBu induces CFTR expression from a viral promoter (22) but decreases endogenous CFTR expression (23).

Statistical Analysis

Results were expressed as means $\pm$ SD. Statistical significance among means was determined using the Student's *t* test (two samples, paired and unpaired).

RESULTS

Validation of the TTT-Specific MGB Assay as a Method to Specifically Measure WT *CFTR* mRNA Levels

To confirm the specificity of the MGB-based assay (i.e., its ability to detect and quantitate WT but not Δ F508 *CFTR* mRNA), different cell lines expressing endogenous WT (Calu-3), recombinant WT (HeLaWT), endogenous Δ F508 (CFPAC-1), recombinant Δ F508 CFTR (HeLa Δ F), and cells without CFTR expression (HeLa parental) were tested. After isolation of RNA and reverse transcription, WT *CFTR* was amplified using custom primers and the TTT-specific MGB probe (*see MATERIALS AND METHODS*). A short 16-base probe with MGB at the 5' end was used to achieve high specificity in the assay. Average efficiency of this assay was 100% $\pm$ 10%. No PCR product was present in samples containing Δ F508 *CFTR* mRNA only (CFPAC-1 and HeLa Δ F), confirming the specificity of the assay (Figures 1A and 1B). We also designed a second MGB probe, intended to recognize Δ F508. However, assays using this probe did not produce a detectable fluorescent signal during RT-PCR experiments using RNA samples from HeLa Δ F or Calu3 Δ F cells. Therefore, instead of measuring Δ F508 *CFTR* mRNA levels directly, we performed parallel measurements on the same RNA templates, using a standard (commercial) *CFTR*-specific assay and the MGB-based assay. After determining the difference in the efficiencies of the two assays, we were able to calculate Δ F508 *CFTR* mRNA levels by subtracting WT *CFTR* mRNA levels (from the MGB-based assay) from total *CFTR* mRNA levels (from the standard assay).

MGB-based assays require either exogenous MGB control or additional calibration to standard TaqMan expression assay for relative quantification. This is necessary because the quality of fluorescent signal generated by the MGB-based product is different than the fluorescent signal generated by the standard assay (Figure 1C). Therefore, for each experimental measurement, we included RNA from parental Calu-3 cells (expressing WT *CFTR* mRNA only) as a calibration standard. Using this experimental system, we were able to establish the ratio of the fluorescent WT *CFTR* mRNA signal, generated using the MGB-based assay (WT CFTR), to the fluorescent signal

Figure 1. (A) Specific amplification of wild-type (WT) cystic fibrosis transmembrane conductance regulator (CFTR) using the TaqMan MGB-based probe. RNA templates from WT (Calu-3, HeLa WT) and $\Delta F508$ (CFPAC-1, CFBE $\Delta F508$, HeLa $\Delta F508$) CFTR-expressing cells were tested using the custom assay containing the TTT-specific MGB probe in real time RT-PCR experiments. Using the GTT-specific probe, fluorescent signal (PCR product) was only detected in samples obtained from cells expressing WT CFTR. NTC = no template control. (B) Real time RT-PCR amplification of WT CFTR using the MGB-based assay and a commercial probe that amplified both WT and $\Delta F508$ CFTR. RNA samples from Calu-3 cells were diluted (log), reverse transcribed, and amplified using either the commercial (A) or the MGB probe (B). NTC = no template control. Increases in reporter dye intensity are shown for the total CFTR probe (A) and the MGB-based probe (B) in the upper panel. Relative efficiency values (C_T) are plotted in the lower left (commercial probe) and lower right (MGB probe). (C) Raw fluorescence measurements using Standard TaqMan (A) and MGB (B) probe based assays. Different physical properties of the fluorescent signals generated by standard TaqMan expression (A), and MGB (B) based probes. (D) Formulas to calculate relative $\Delta F508$ CFTR levels. $CFTR_t$ = relative CFTR mRNA levels measured by the commercial assay; $CFTR_{WT}$ = relative WT CFTR mRNA levels measured by the MGB probe; $CFTR_{\Delta F508}$ = relative $\Delta F508$ CFTR mRNA levels calculated from $CFTR_t$ and $CFTR_{WT}$; a = ratio of fluorescence signal produced by the MGB probe to the signal produced by the commercial probe.

D

Formula #1
(All CFTR mRNA is WT)

$$CFTR_t = CFTR_{WT} \times a$$

$$a = CFTR_t / CFTR_{WT}$$

Formula #2
(WT and $\Delta F508$ CFTR mRNA are present)

$$CFTR_t = (CFTR_{WT} \times a) + CFTR_{\Delta F508}$$

$$CFTR_{\Delta F508} = CFTR_t - (CFTR_{WT} \times a)$$

produced by the standard assay (total CFTR). To reduce calculation errors resulting from the different efficiencies of the primers in each sample, four replicates of the calibration samples were conducted for all experiments.

An “a” value (the ratio of the fluorescent signals generated by total [standard] to the WT-CFTR specific [MGB] probes) was calculated for each experiment using samples containing WT CFTR mRNA only (Figure 1D, *Formula #1*). Subsequently, to calculate amounts of $\Delta F508$ CFTR mRNA, we used this “a” value to normalize our experimental measurements from samples containing both WT and $\Delta F508$ CFTR mRNA. We could then subtract the amount of WT CFTR mRNA (MGB probe) from the amount of “total” CFTR mRNA (standard assay). The result of this calculation represented the amount of $\Delta F508$ CFTR mRNA in the sample (Figure 1D, *Formula #2*).

Quantification of CFTR mRNA Copy Number Using the MGB-Based Assay

Next, we tested the sensitivity and range of the MGB-based assay using *in vitro* transcribed WT CFTR mRNA as template. Samples containing known copy numbers of CFTR mRNA were studied. The results indicated that the MGB-based assay was reliable for absolute quantification of WT CFTR mRNA within a wide range, from 10^6 to as low as 2 to 3 copies (Figure 2), meaning that the assay is suitable for CFTR mRNA measurements in tissue samples or cells with low CFTR expression levels.

CFTR Expression Levels in the Calu-3 ΔF Clones

Cloning of Calu-3 ΔF cells allowed us to select individual clones with different ratios of recombinant $\Delta F508$ to endogenous WT CFTR. After expansion of 12 individual clones, we measured their CFTR mRNA and protein levels and calculated their ratios of endogenous WT to recombinant $\Delta F508$ CFTR mRNA, as follows: We measured relative total (endogenous WT + recombinant $\Delta F508$) CFTR mRNA levels in the clones using the commercial CFTR probe (Figure 3A). We then determined relative WT CFTR mRNA levels using the MGB probe on the same RNA templates (Figure 3B). We used these two measurements to calculate levels of $\Delta F508$ CFTR mRNA levels using the method described above. Finally, we calculated the ratios of WT to $\Delta F508$ CFTR mRNA using the formula presented in Figure 1C.

Clones with similar WT and $\Delta F508$ expression levels showed comparable morphologic and physiologic characteristics during the screening process. Therefore, we chose three representative clones (Calu-3 $\Delta FC1$, Calu-3 $\Delta FC3$, and Calu-3 $\Delta FC5$) expressing different ratios of WT to $\Delta F508$ CFTR mRNA as models for the present studies (Figure 3C). In Calu-3 $\Delta FC1$, the ratio of endogenous WT to recombinant $\Delta F508$ CFTR mRNA was 1:1, but both mRNA levels were approximately 30% of levels in Calu-3 parental cells. In Calu-3 $\Delta FC3$, the ratio of endogenous WT to recombinant $\Delta F508$ CFTR was also approximately 1:1, but these cells expressed CFTR mRNA levels that were approximately 50% higher than in Calu-3 parental cells. In Calu-3 $\Delta FC5$, recombinant $\Delta F508$ CFTR expression was 8-fold higher than endogenous WT, and endogenous WT CFTR mRNA levels were significantly lower than in Calu-3 $\Delta FC3$ or Calu-3 parental cells.

Based on their varying mRNA levels, we predicted that CFTR protein levels would also vary between the clones. To confirm this prediction, we used immunoprecipitation and *in vitro* phosphorylation to quantitatively compare CFTR protein levels in Calu-3, Calu-3 $\Delta FC1$, Calu-3 $\Delta FC3$, and Calu-3 $\Delta FC5$ (Figure 3D). Our results demonstrated that in Calu-3 cells,

Figure 2. Sensitivity of the MGB-based TaqMan probe for WT CFTR. To determine the sensitivity of MGB-based assay to measure WT CFTR mRNA, we performed absolute quantification RT-PCR using known copy numbers of WT CFTR mRNA generated by *in vitro* transcription reactions. Measurements of CFTR mRNA copies in the range of 10^4 to 10^1 are plotted in the upper panel. Low copy number measurements (2–250) are plotted in the lower panel. Each point represents the average of six replicates. R^2 values are similar for both high and low copy number measurements.

Band B (core glycosylated, ER form) CFTR was undetectable at steady state, an observation that is consistent with previous results (18) (Figure 3D, *lane 1*). In contrast, we readily detected Band B CFTR in all three clones (Figure 3D, *lanes 2–4*). Because no Band B was detected in parental Calu-3 cells, we concluded that the observed Band B in the clones corresponded to recombinant $\Delta F508$ CFTR (Figure 3D, *Band B*). Band C CFTR levels in Calu-3 $\Delta FC1$ were higher than we expected based on the mRNA analysis. Band C CFTR levels were highest in Calu-3 $\Delta FC3$. In Calu-3 $\Delta FC5$ cells, Band C CFTR levels were lower than those in parental Calu-3 cells or in the other two clones (Figure 3D, *Band C*).

We performed immunocytochemistry to investigate potential morphologic differences between clones, compare cell surface CFTR staining, and visualize the ER (Figure 3E). As an ER marker, we probed for Sec-61 β , a component of the ER translocon (24). All clones retained the ability to form polarized monolayers, as indicated by the formation of tight junctions (ZO-1 staining, *red*) (Figure 3E, *upper panels*). Cell surface CFTR staining is dominant in Calu-3, Calu-3 $\Delta FC1$, and Calu-3 $\Delta FC3$ cells grown on coverslips (*green*). In contrast, in Calu-3 $\Delta FC5$ cells, most of the CFTR fluorescence co-localized with Sec-61 β in the ER, and only minimal CFTR staining is present at the cell surface (Figure 3E, *lower panels*). Since the parental Calu-3 cell line is not clonal, endogenous WT CFTR expression varied between individual cells. This variability may be responsible for the varying levels of endogenous CFTR mRNA in the clones. Another possibility is that the introduction of $\Delta F508$ CFTR to Calu-3 cells induced ER stress, activated the UPR, and modulated endogenous CFTR levels (8).

Figure 3. Characterization of Calu-3 Δ F cells. (A) Relative total CFTR mRNA levels. Total CFTR mRNA levels in Calu-3, Calu-3 Δ FC1, Calu-3 Δ F, and Calu-3 Δ FC5 are plotted relative to GAPDH mRNA levels ($n = 6$). (B) Endogenous CFTR mRNA levels. Endogenous CFTR mRNA levels were measured and plotted relative to GAPDH mRNA ($n = 6$). (C) Relative distribution of WT (endogenous) and Δ F508 (recombinant) CFTR. Recombinant CFTR mRNA levels were calculated from total (recombinant Δ F508 and endogenous WT) and endogenous CFTR mRNA. Results are plotted as relative distribution of WT and Δ F508 CFTR compared with total CFTR mRNA in parental Calu-3 cells ($n = 6$). (D) CFTR protein levels. CFTR was immunoprecipitated from 250 μ g total cellular protein using anti-CFTR C-terminal monoclonal antibody (24-1), *in vitro* phosphorylated using 32 P-ATP and PKA, separated on 6% PAGE, and detected by phosphorimaging. Arrows show ER (Band B) and post-ER forms (Band C) of CFTR. (E) Immunocytochemical detection of tight junctions, CFTR, and Sec-61 (ER marker) in Calu-3 and Calu-3 Δ F cells. Cells were grown on permeable supports, and immunocytochemistry was performed on methanol-fixed monolayers. Tight junctions were detected with anti-ZO1 polyclonal antibody (red). CFTR is stained green (24-1 antibody). Images show the apical cell surface at the level of tight junctions (upper panel). CFTR (green) and the ER (Sec-61 β , red) were stained to demonstrate CFTR in the ER and post-ER compartments in each clone (lower panel).

The UPR Is Activated in Calu3 Δ FC5

To analyze how different levels of Δ F508 CFTR affected ER stress and consequent UPR activation, we tested the Calu-3 Δ F clones, Calu-3 cells, and CFPAC-1 cells for UPR activity. Using two classic reporters of the UPR (relative *sXBP1* and *BiP* mRNA levels) as readouts, we did not detect UPR activity in

CFPAC-1, Calu-3 Δ FC1, or Calu-3 Δ FC3 cells. In contrast, Calu3 Δ FC5 cells, which expressed the highest level of recombinant Δ F508 CFTR, contained 2-fold higher levels of relative *sXBP1* mRNA ($P < 0.05$; Figure 4A) and 6-fold higher levels of *BiP* mRNA ($P < 0.001$, Figure 4B) than the controls. Relative TR mRNA levels, measured as a control, were similar in all of

Figure 4. The UPR is constitutively activated in Calu-3 $\Delta FC5$. To monitor UPR activity under basal conditions, *sXBP1* and *BiP* mRNA levels were measured in Calu-3, Calu-3 $\Delta FC1$, Calu-3 $\Delta FC3$, Calu-3 $\Delta FC5$, and CFPAC-1 cells. Transferrin receptor (*TR*) mRNA was measured as a control and did not change significantly. Constitutive UPR activity was observed only in Calu-3 $\Delta FC5$. Results are plotted relative to *GAPDH* mRNA. $n = 6$, $*P < 0.001$.

the Calu-3 clones and in CFPAC-1 cells (Figure 4C). These results revealed constitutive UPR activity in Calu-3 $\Delta FC5$, the clone expressing the highest level of $\Delta F508$ CFTR.

Induction of Recombinant $\Delta F508$ CFTR Expression in Calu-3 $\Delta FC3$ Cells Activated the UPR and Decreased Endogenous *CFTR* mRNA Levels

To clarify the role of $\Delta F508$ CFTR on UPR activation, we conducted follow-up experiments to test whether increased levels of $\Delta F508$ CFTR in the ER activated the UPR. We also wanted to confirm that the high $\Delta F508$ CFTR levels in Calu-3 $\Delta FC5$ were responsible for the increases in *sXBP1* and *BiP* mRNA levels. For these experiments, we boosted the recombinant CFTR expression in the Calu-3 $\Delta FC3$ cells (1:1 recombinant to endogenous CFTR expression under unstimulated conditions) and tested for UPR activity. To enhance recombinant CFTR expression, we treated Calu-3 $\Delta FC3$ cells with NaBu, which induces the expression of CFTR from retroviral vectors (22). In contrast, treatment of these cells with 1 mM NaBu for 12 hours decreased WT *CFTR* mRNA levels to 20 to 30% (Figure 5A). NaBu treatment of the Calu-3 $\Delta FC3$ cells also decreased levels of endogenous WT *CFTR* mRNA, but it increased levels of total *CFTR* mRNA (Figure 5B). *TR* mRNA levels, measured as a control, decreased slightly but not significantly in both Calu-3 and Calu-3 $\Delta FC3$ cells after NaBu

treatment (Figure 5C). In both Calu-3 and Calu-3 $\Delta FC3$ cells, NaBu decreased endogenous *CFTR* mRNA levels. Therefore, the increased levels of total *CFTR* mRNA in Calu-3 $\Delta FC3$ cells must have corresponded to increased levels of recombinant $\Delta F508$ CFTR. The significant decrease in endogenous WT and increase in recombinant $\Delta F508$ *CFTR* mRNA levels were accompanied by decreased Band C CFTR levels in Calu-3 and decreased Band C and increased Band B CFTR protein levels in Calu-3 ΔF cells (Figure 5E). Taken together, these results demonstrated that in Calu-3 $\Delta FC3$ cells, NaBu treatment enhanced the expression of recombinant $\Delta F508$ CFTR at the mRNA and protein levels.

We investigated whether we had activated the UPR in these cells by increasing $\Delta F508$ CFTR expression. To explore this possibility, we compared the relative *sXBP1* and *BiP* mRNA levels in untreated and NaBu-treated Calu-3 $\Delta FC3$ cells. We also examined parental Calu-3 cells as control. NaBu-treated Calu-3 $\Delta FC3$ cells exhibited significantly higher levels of both *sXBP1* and *BiP* mRNA than untreated cells (Figure 5D). We observed no increase in either of these UPR markers after NaBu treatment of parental Calu-3, HeLaWT (18), or Calu-3WT cells (8) transduced with TranzVector encoding WT CFTR (data not shown). Based on these results, we concluded that the NaBu-mediated increase in recombinant $\Delta F508$ CFTR expression resulted in ER stress and UPR activation. These results support the idea that, in contrast to what was shown for WT CFTR (8), $\Delta F508$ CFTR caused ER stress and activated the UPR when expressed at high levels.

Pharmacologic Induction of ER Stress and Activation of the UPR in Calu-3 ΔF Cells Decreased Endogenous WT *CFTR* Expression

To confirm that ER stress and activation of the UPR mediated endogenous, but not recombinant, *CFTR* mRNA repression, we induced ER stress in Calu-3, Calu-3 $\Delta FC1$, Calu-3 $\Delta FC3$, Calu-3 $\Delta FC5$, and CFPAC-1 cells by two classic pharmacologic methods (tunicamycin or proteasome inhibition) (8, 15). Throughout our studies herein, we obtained similar results using either of these ER stressors. We monitored for UPR activity by following *sXBP1* mRNA levels. As expected, induction of ER stress increased *sXBP1* mRNA levels in parental Calu-3 cells, all Calu-3 ΔF clones, and CFPAC-1 cells (Figure 6A). Interestingly, although *sXBP1* mRNA levels were constitutively higher in Calu-3 $\Delta FC5$ cells, pharmacologic ER stress mediated an additional increase.

We also investigated relative total and endogenous *CFTR* mRNA levels after pharmacologic ER stress (Figure 6B), and our measurements indicated that pharmacologic activation of the UPR decreased endogenous *CFTR* mRNA levels in parental Calu-3 cells to less than 20% of controls. Subjecting Calu-3 $\Delta FC5$ cells to the same conditions increased total *CFTR* mRNA levels, whereas endogenous *CFTR* mRNA dropped to almost undetectable levels. These observations suggested that despite the existing signs of ER stress in Calu-3 $\Delta FC5$ under basal conditions, the additional acute increase in UPR activity completely abolished endogenous *CFTR* expression. We observed no significant changes in relative *TR* mRNA levels under identical experimental conditions (data not shown).

To elucidate whether ER stress mediated similar effects on endogenous $\Delta F508$ *CFTR* mRNA levels, we included CFPAC-1 cells in these experiments. Although $\Delta F508$ *CFTR* mRNA levels are very low in CFPAC1 cells under basal conditions, activation of the UPR using proteasome inhibition or TM further decreased their endogenous $\Delta F508$ *CFTR* mRNA levels. We measured no significant changes in *TR* mRNA levels

A Calu-3

B Calu-3ΔFC3

C Calu-3 Calu-3ΔFC3

Figure 5. Induction of recombinant $\Delta F508$ CFTR expression and activation of the UPR in Calu-3 $\Delta FC3$. Calu-3 $\Delta FC3$ cells were treated with NaBu (1 or 2 mM for 12 h) to induce recombinant $\Delta F508$ CFTR expression. (A) Relative CFTR mRNA levels in Calu-3 cells. Total and endogenous CFTR mRNA levels were measured in control and NaBu-treated Calu-3 cells. A significant decrease in CFTR mRNA was detected upon NaBu treatment. Results are plotted as relative CFTR mRNA compared with control (untreated) cells. (B) Relative CFTR mRNA levels in Calu-3 $\Delta FC3$ cells. Calu-3 $\Delta FC3$ cells express both endogenous and recombinant CFTR. A significant increase in total and a decrease in endogenous CFTR mRNA were observed in NaBu-treated cells compared with control cells. Results are plotted relative to CFTR mRNA levels in control (untreated) Calu-3 $\Delta FC3$ cells. (C) Relative TR mRNA levels in Calu-3 $\Delta FC3$ cells. TR mRNA levels were monitored as a control and were slightly decreased after NaBu treatment. TR mRNA levels are plotted relative to 18S rRNA. (D) Relative sXBP1 and BiP mRNA levels. Relative sXBP1 and BiP mRNA levels are plotted as a fold increase after NaBu treatment. Calu-3 parental cells were tested as controls; $n = 6$, $*P < 0.01$ (sXBP1); $P < 0.005$ (BiP). (E) Changes in CFTR protein levels in Calu-3 and Calu-3 $\Delta FC3$ following NaBu treatment. CFTR was immunoprecipitated from 500 μ g total cellular protein using anti-CFTR C-terminal monoclonal antibody (24-1), *in vitro* phosphorylated using 32 P-ATP and PKA, separated on 6% PAGE, and detected by phosphorimaging.

under the same conditions (data not shown). These results from CFPAC-1 cells indicated that the UPR has a pronounced negative effect on endogenous CFTR mRNA, an effect that is detectable even in cells with very low CFTR mRNA expression.

DISCUSSION

Our studies concentrate on membrane protein expression regulation during ER stress. Earlier studies indicated that

Figure 6. Activation of the UPR in Calu-3, Calu-3 $\Delta FC1$, Calu-3 $\Delta FC3$, Calu-3 $\Delta FC5$, and CFPAC-1 cells with tunicamycin (TM). (A) *sXBP1* mRNA levels were measured to monitor UPR activity after TM treatment. Results are plotted relative to *sXBP1* mRNA in Calu-3 parental cells. (B) Relative total (left panels) and endogenous (right panels) CFTR mRNA levels in Calu-3 $\Delta FC3$ and Calu-3 $\Delta FC5$ after induction of ER stress and activation of the UPR. ALLN or TM were used to induce ER stress and activate the UPR. Results are plotted relative to CFTR mRNA levels in Calu-3 cells. $n = 6$, $P < 0.005$. (C) Induction of ER stress and activation of the UPR decrease endogenous CFTR mRNA levels in CFPAC-1 cells. CFPAC-1 cells were treated with TM to induce ER stress. The UPR was monitored by measuring *sXBP1* mRNA (left panel). CFTR mRNA levels were measured using a primer set that amplifies both endogenous and recombinant CFTR (right panel). Results are plotted relative to control (untreated) cells.

pharmacologically induced ER stress and the UPR decreased endogenous, but not recombinant, CFTR expression at the mRNA and protein maturational levels, and that overexpression of WT CFTR did not by itself cause ER stress (8). Herein, we showed that $\Delta F508$ CFTR is fundamentally different from WT, in that $\Delta F508$ CFTR can induce ER stress when expressed at high levels.

The importance of using endogenous CFTR-expressing cell lines (such as Calu-3) as model systems is clear from previous studies (8, 18). However, endogenous $\Delta F508$ CFTR-expressing models are limited and the expression level of the mutant CFTR is usually too low to study trafficking. Therefore, we developed and characterized clonal cell lines that express $\Delta F508$ CFTR on the WT Calu-3 background. These Calu-3 ΔF clones stably express different levels of WT and $\Delta F508$ CFTR, as defined herein. Together, these cell lines provide a novel model system in which the effects of different WT and $\Delta F508$ CFTR expression levels can be explored. In addition, these cells also represent a model system in which endogenous and recombinant species of CFTR exist within the same cells, facilitating a wide range of studies such as the NaBu induction experiments presented herein.

In previous studies, NaBu has been used to facilitate CFTR expression and to enhance the rescue of recombinant $\Delta F508$ CFTR mediated by low temperature culture (22, 25, 26). In contrast, other studies showed that in parental Calu-3 cells, NaBu treatment decreased endogenous CFTR function (27) and mRNA levels (23). Our results are in agreement with the latter two studies, in that NaBu increased recombinant CFTR expression but significantly decreased endogenous CFTR mRNA levels.

Because we found that the decrease in endogenous CFTR mRNA levels was not accompanied by UPR activation, and because recombinant CFTR levels increased by more than 5-fold, we used NaBu treatment to test the effect of increased $\Delta F508$ CFTR expression on ER stress induction and UPR activation. These experiments confirmed our previous hypothesis that $\Delta F508$ CFTR differs from WT in its ability to cause ER stress (8). These results also support previous reports that ER traffic and degradation of WT and $\Delta F508$ CFTR are different (28, 29).

Although our studies concentrate on ER stress, and the decrease in CFTR mRNA levels under ER stress results from transcriptional repression (8), it is likely that ER stress occurs in combination with cytosolic stress *in vivo*. In support of this idea, oxidative stress caused by butylhydroquinone exposure decreased CFTR mRNA stability in a previous report (30). In addition, exposure to cigarette smoke extract, most likely a source of ER and cytosolic stress, decreased chloride secretion in human bronchial epithelial cells (31). Furthermore, inflammatory cytokines decreased CFTR mRNA stability by activating exosomes through the AU-rich element of the 3'UTR (32). Although none of these reports included a study of ER stress and UPR activity under the described conditions, other investigators have reported that infection and inflammation in CF airway epithelia triggered the UPR (33). Therefore, it is feasible that the cumulative negative effects of cellular stress on CFTR expression might down-regulate functional CFTR levels to pathologically low levels, especially considering that epithelial cells often bear the brunt of these stress conditions. These observations are especially relevant with regard to CFTR function in patients with compound heterozygote CF. If our hypotheses are correct, any strain on the cells might disturb the partially balanced CFTR function in these patients, resulting in a suppression of CFTR expression with potential detrimental effects.

Understanding the role of $\Delta F508$ CFTR in ER stress and UPR activation is critical to develop therapies for CF caused by the $\Delta F508$ mutation. Significant efforts toward identifying $\Delta F508$ CFTR rescue compounds have produced some promising candidates (34–39), but the mechanisms by which any of these rescue agents work are not fully understood, and many of them are likely to increase $\Delta F508$ CFTR levels in the ER. It is therefore vital to understand how increased levels of the mutant protein may initiate other cellular responses. If any of these

compounds causes ER stress, the inhibitory effect on CFTR expression would only be apparent in endogenous CFTR-expressing models. For all of these rescue agents, the initial characterization studies were conducted in stable recombinant Δ F508 CFTR-expressing cell lines, and only a few endogenous Δ F508-CFTR expressing models have been used (36, 38).

In addition, it is necessary to accurately differentiate between WT and Δ 508 CFTR mRNA levels when analyzing therapeutic approaches and pathologic influences on CFTR transcript levels or in heterozygote settings. Moreover, differential quantification of WT and Δ F508 mutant CFTR transcripts has furthered our understanding of how epigenetic factors influence CFTR expression and function (8, 40). Various studies have introduced a number of PCR- and non-PCR-based methods to investigate CFTR transcript levels (41, 42), but future studies will require novel quantitative, specific, and sensitive assays that are able to discriminate between WT and Δ F508 CFTR mRNA. As such, our WT CFTR-specific, MGB-based RT-PCR protocol fits these requirements and will serve as an important tool to study the molecular pathomechanism of CF.

In summary, to clarify how Δ F508 CFTR mRNA levels relate to activation of the UPR, we developed novel cell lines and a novel RT-PCR based quantitative assay to differentially investigate WT and Δ F508 CFTR mRNA levels. Our findings showed that overexpression of recombinant Δ F508 CFTR induced ER stress and activated the UPR. Endogenous CFTR (WT and Δ F508) expression decreased under ER stress conditions, independent of the pathways of ER stress induction. Therefore, these studies provide novel insight into how cellular stress responses regulate CFTR expression.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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