Endoplasmic reticulum stress and the unfolded protein response regulate genomic cystic fibrosis transmembrane conductance regulator expression

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The unfolded protein response (UPR) is a cellular stress response that is required for cellular recovery following endoplasmic reticulum (ER) stress. UPR can be generated by a number of stress conditions referred to as conformational diseases. Although significant progress has been made toward understanding the cellular pathways associated with the UPR are understood.

Regulatory links between UPR and ERAD indicate that the two pathways work in concert. In addition to an important role in normal cellular physiology, ERAD of misfolded proteins is the basis of a growing number of pathological conditions referred to as conformational diseases. Furthermore, because ER stress activates both ERAD and UPR pathways, the biogenesis of numerous proteins with important cellular functions can be altered. Although significant progress has been made toward understanding the cellular pathways underlying the UPR (50) and ERAD (31, 48), the influence of these pathways on transcription, translation, and maturation of membrane glycoproteins following ER stress has not been elucidated in mammalian cells.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a multidomain membrane glycoprotein with diverse cellular functions (20, 28, 33). Mutations in the CFTR gene cause cystic fibrosis (CF) (35, 36). CF, caused most commonly by deletion of phenylalanine at the 508 position (F508), is a conformational disorder attributable to F508 CFTR misfolding and degradation by ERAD (34). In addition, whereas the folding and maturation of many newly synthesized wild-type (WT) glycoproteins is typically quite efficient regardless of expression system or cell type, CFTR has been shown to mature inefficiently in most model systems, particularly when it is overexpressed as recombinant protein (4, 25, 46). Although the significance and consequences of inefficient WT CFTR maturation are not understood, it is becoming evident that selection of F508 and WT CFTR for ERAD occur at different checkpoints (49) and that the pathways of degradation may differ significantly (10). Based on these findings, it is clear that understanding WT CFTR biogenesis will be necessary to reveal the defects associated with processing mutants such as F508.

We have previously shown that under physiological conditions, the maturation of WT CFTR is efficient in epithelial cells. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
endogenously expressing the protein (45). We also reported that cellular stress, caused by reactive oxygen nitrogen species (RONs), inhibits WT CFTR maturation and decreases steady-state CFTR levels and function (5). Recent studies have shown that cell-specific, cytokine-induced posttranscriptional regulation of CFTR expression occurs through the AU-rich elements of the 3'-untranslated region (UTR) of CFTR mRNA, and these elements have been shown to regulate mRNA stability (2). These studies suggest that the cellular milieu is extremely important in determining WT CFTR biogenesis. However, the role of ER stress and the UPR on WT CFTR expression regulation has not been studied in detail.

Although it is widely accepted that WT CFTR is inefficiently processed in recombinant models, studies regarding CFTR biogenesis have still been performed predominantly in recombinant overexpression systems for practical reasons. For example, in most cell lines, and in primary cells in particular, the synthesis rate of the endogenous WT CFTR is too slow to adequately monitor CFTR maturation in a metabolic pulse-chase experiment. It has also been noted that primary cells and endogenous CFTR-expressing cell lines often lose detectable CFTR expression during cell culture, but the reasons for this have not been determined.

The major objective of the present studies was to understand how the UPR, which is activated through pharmacologically induced ER stress, influences endogenous and recombinant WT CFTR biogenesis. Because the UPR regulates transcription and translation and increases the activity of ERAD, both CFTR mRNA levels and protein maturation efficiency were measured. We also tested the effects of recombinant WT CFTR overexpression on the activation of UPR and ERAD of CFTR in cells with efficient endogenous CFTR maturation. We found important differences in recombinant and endogenous WT CFTR expression regulation, and we suggest that perturbation of physiological ER functions that activate the UPR may significantly alter the expression of endogenous CFTR and other transport proteins. Moreover, because pathological conditions such as chronic inflammation and hypoxia may induce ER stress, it is critical to understand how WT CFTR expression is regulated under these conditions.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Calu-3, HT29, and T84 cells were obtained from the ATCC (www.atcc.org). HeLaWT (expressing recombinant WT CFTR) and Calu-3WT (overexpressing recombinant WT CFTR) were transduced with a TranzVector (Tranzyme, Birmingham, AL) and selected as previously described (3). Cells were cultured in DMEM (Life Technologies) with 10% FBS at 37°C in a humidified incubator in 5% CO2.

Antibodies

Anti-CFTR COOH-terminal monoclonal antibody (24-1), which was originally produced and characterized by Genzyme (Cambridge, MA), was purified from hybridoma supernatant (ATCC no. HB-11947) and used as previously described (3, 45).

Induction of ER and Cytosolic Stress

Cells were treated with 100 μM N-acetyl-l-leucyl-l-leucyl-l-norleucinal (ALLN; Sigma) or 20 μM epoxymycin (Biomol) for time intervals specified in each experiment (15). Brefeldin A (eBioscience) was added to the tissue culture medium at 3 μg/ml (final concentration) for 4 h (16). Tunicamycin (Sigma) was added to samples for 12-14 h at 5 μg/ml (final concentration) (16). To induce cytotoxic stress, we treated cells with 100 μM arsenite for 6 h (29, 47).

Immunoprecipitation of CFTR

CFTR was immunoprecipitated using anti-COOH-terminal 24-1 monoclonal antibody followed by in vitro phosphorylation with [γ-32P]ATP (NEN) and cAMP-dependent protein kinase (Promega). Labeled CFTR was analyzed using SDS-PAGE and autoradiography as described previously (5). Densitometry was performed using IPLab software (Scanalytics). All lysis buffers were supplemented with a protease inhibitor cocktail (Complete Mini; Roche).

Metabolic Pulse Chase

After a 30-min methionine starvation in methionine/cysteine-free MEM at 37°C, 300 μCi/ml of EasyTag protein labeling mixture ([35S]methionine/cysteine; NEN) was added to pulse label proteins for 30 min. Following the pulse, the medium was changed to nonradioactive methionine-rich solution for the time intervals specified. Cells were lysed in RIPA buffer, and CFTR was immunoprecipitated using 1 μg of 24-1 monoclonal antibody and 20 μl of protein A+G-agarose. Immunoprecipitated samples were analyzed using SDS-PAGE (6% gels) and detected using autoradiography (PhosphorImager; Molecular Dynamics). CFTR maturation efficiency was measured using IPLab software as described previously (45).

Real-Time RT-PCR

Total cellular RNA was isolated using the RNeasy mini kit (catalog no. 74106; Qiagen) according to the manufacturer’s protocol. RNA concentration was calculated based on the absorbance of samples at 260 nm. RNA samples were stored at −20°C. RT-PCR was performed using TaqMan One-Step RT-PCR master mix reagents (catalog no. 4309169; Applied Biosystems) containing carboxy-X-Rhodamine (ROX) as a passive reference to normalize non-PCR-related signal in each reaction. Probes were conjugated to carboxyfluorescein (5-end) and a nonfluorescent quencher (3-end). TaqMan assays utilize the 5’-exonuclease activity of the DNA polymerase to free the fluorescent dye from the quencher during the PCR reaction. The proportionally rising dye concentration results in a crescent absolute fluorescence. Total CFTR was evaluated using Assay-On-Demand primer mix (assay ID: Hs00357011_m1). Endogenous CFTR was amplified using assays CFTR5UTR-TS7, CFTR5UTR-TS7F, and CFTR5UTR-TS7/R (GACATCAGACAGGTCAAGAAAA and GCTTCCTAATGGCAGAACCTACTA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; assay ID: Hs99999905_m1) and 18S RNA (assay ID: Hs99999901_s1) were studied in parallel as internal controls. HSPAS/BiP (assay ID: Hs00607219_gH) and XBP1 (assay ID: Hs00231936_m1) were amplified to test for UPR activity. Transferrin receptor (TR; assay ID: Hs00999991_m1) was amplified as an additional control. TaqMan RT-PCR reactions were performed in 25-μl final volumes containing 5 μl (10×) dilution of stock) of RNA sample, AmpErase UNG (2×, 12.5 μl), MultiScribe reverse transcriptase and RNase inhibitor (40×, 0.625 μl), primers and probe (20×, 1.25 μl), and RNase-free water (5.625 μl). Quantitative real-time PCR was performed using the ABI PRISM 7500 sequence detection system. Five 1-log serial dilution reactions were conducted in duplicate. Data were exported from ABI PRISM 7500 software into Microsoft Excel and analyzed using the relative standard curve method. Cycle threshold values for each serial dilution were plotted, and the values were calculated from the y-intercept and slope of the standard curve using the Excel Trendline option. These values were then used to calculate the input amount of mRNA samples. The input amount of target mRNA was normalized to GAPDH mRNA as an endogenous control. Results are plotted as CFTR mRNA levels relative to 18S RNA (means ± SD).

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**RESULTS**

**Induction of ER Stress and Activation of the UPR Reduce CFTR Maturation Efficiency in Calu-3 Cells**

The effects of ER stress and the UPR on endogenous WT CFTR maturation were tested in Calu-3 cells. Previous studies showed that inhibition of the proteasome causes ER stress and activates the UPR (15, 19, 38). Based on these results, Calu-3 cells were treated with 100 μM ALLN for 6 h. Activation of the UPR was tested by measuring spliced XBP1 and HSPA5/GRP78/BiP (BiP) mRNA levels using real-time RT-PCR. XBP1 is a transcription factor that is activated upon frameshift splicing by IRE1 RNase (1, 30). The spliced XBP-1 mRNA encodes a basic leucine zipper transcription factor that enters the nucleus and acts during ER stress to activate UPR target genes via direct binding to the UPR consensus element (UPRE) (1, 30). BiP is a central regulator of ER stress that controls the activity of IRE1, PERK, and ATF6 through binding and release as a major ER chaperone (22). Therefore, we used these reporters to monitor UPR activity. After 6 h of ER stress, significant increases in spliced XBP1 and BiP mRNA levels were detected, indicating UPR activation (Fig. 1A).

It has been suggested that the functions UPR and ERAD are coordinated and that activation of the UPR leads to enhanced ERAD (44). We therefore tested whether activation of the UPR results in enhanced ERAD of CFTR with subsequent decreased WT CFTR maturation efficiency. Following activation of the UPR, we performed metabolic pulse-chase experiments to follow CFTR maturation efficiency in Calu-3 cells. Untreated Calu-3 cells were tested as controls (Fig. 1B). These exper-
ments were performed to test the hypothesis that CFTR becomes a substrate for ERAD if the activity of ERAD is enhanced. In agreement with our previous results (45), CFTR maturation is efficient in control Calu-3 cells (>90% maturation). However, following induction of ER stress and activation of the UPR, which enhances the activity of ERAD, endogenous WT CFTR maturation was severely compromised (~40% maturation) as shown in Fig. 1B. These results suggest that increased ERAD activity may cause inefficient WT CFTR maturation measured in most models.

**Induction of ER Stress and Activation of the UPR Decrease Endogenous CFTR mRNA Levels and Protein Synthesis**

The extent and specificity of protein expression regulation following ER stress and UPR activation are not understood in mammalian cells (40). To test whether CFTR mRNA levels are affected, we measured genomic CFTR mRNA levels in three different cell lines, Calu-3, T-84, and HT-29, before and after activation of the UPR. An increase in spliced XBP1 mRNA was used as the criterion for UPR activity (Fig. 2A, left). When

**Fig. 2. Induction of ER stress and activation of the UPR decrease endogenous CFTR mRNA and protein levels.**

A: sXBP1 and CFTR mRNA levels. Real-time RT-PCR was used to measure sXBP1 (left) and CFTR mRNA levels (right) in Calu-3, T84, and HT29 cells expressing endogenous CFTR following induction of ER stress (100 μM ALLN, 6 h). Spliced XBP1 increased significantly in all 3 cell lines tested, whereas CFTR mRNA levels decreased to 50–80% of controls following UPR activation (n = 8). *P < 0.01. No change in GAPDH mRNA levels was detected, and GAPDH was used as reference to quantify sXBP1 and CFTR mRNA levels. B: transferrin receptor (TR) mRNA levels. Real-time RT-PCR was used to measure TR mRNA levels from the same samples as sXBP1 and CFTR as a control. No significant changes in TR mRNA levels were detected. C: CFTR band B protein levels. Control and ER-stressed Calu-3, T84, and HT29 cells were lysed, and CFTR was immunoprecipitated from 500 μg of total cellular proteins in triplicate. Samples were in vitro phosphorylated (MATERIALS AND METHODS) and separated by SDS-PAGE on 6% gels. Representative gels of 3–5 individual experiments are shown from each cell line (left). CFTR band B levels were compared by densitometry (right) using IPLab software (n = 3) *P < 0.05.
the UPR was activated, CFTR mRNA levels decreased significantly to 25–45% of controls in all three cell lines tested (Fig. 2). Similar results were obtained using either GAPDH mRNA or 18S RNA as reference. Because GAPDH mRNA levels are closer to the mRNA levels of CFTR and XBP1 than 18S RNA, results are plotted as the function of GAPDH mRNA (Fig. 2A, right). Transferrin receptor (TR) mRNA levels were measured as an additional control. TR was chosen because it is a membrane protein expressed in most cells at high levels, and its expression is regulated both transcriptionally and posttranscriptionally (8, 81). Transcriptional regulation of TR is necessary to maintain cellular homeostasis, and iron levels regulate TR mRNA stability (8, 81). Both TR and CFTR mRNAs contain AU-rich elements at the 3′-UTR that are important in exosome-mediated mRNA decay, and these elements regulate protein expression posttranscriptionally through mRNA stability (2, 9, 43). Therefore, decreases in both CFTR and TR mRNA levels would suggest an enhanced exosome-mediated mRNA decay. The results shown in Fig. 2B indicate that TR mRNA levels were not changed after induction of ER stress, suggesting that under these conditions, there was not a global enhancement of cytosolic RNase activity.

We also measured steady-state CFTR protein levels in these cell lines following UPR activation and observed a marked decrease in newly synthesized band B CFTR (Fig. 2C). Only a very small amount of band B CFTR is present in endogenous CFTR-expressing cells at steady state, even under optimal conditions, since the synthesis rate is low (45). Because the half-life of the fully processed band C CFTR is long (≥24 h) in these cells (45), no significant changes in total band C CFTR were detected following 6 h of ER stress.

The previous experiments were performed following a 6-h ER stress. We next asked whether decreased CFTR mRNA correlates with the activation of the UPR and production of XBP1 transcription factor in a time-dependent fashion. Spliced XBP1, CFTR, and TR mRNA levels were measured hourly for 8 h following induction of ER stress. The results indicate that spliced XBP1 mRNA levels reach a maximum after 3 h and remain significantly higher up to 8 h (Fig. 3). In contrast, CFTR mRNA begins to decrease after 3 h (XBP1 maximum) and reaches a minimum at ~20% by 8 h (Fig. 3). The specificity of CFTR mRNA downregulation by the UPR was assessed by measuring TR mRNA levels from the same samples. TR mRNA remained constant during the 8-h ER stress. As an additional control, cellular ATP/ADP ratios were measured at each time point and extended to 12 h to test for general cellular toxicity (see MATERIALS AND METHODS). No significant changes in ATP levels were detected (data not shown).

**ER Stress Caused by Brefeldin A or Tunicamycin Decreases Endogenous CFTR mRNA Levels**

To test whether the observed changes in CFTR biogenesis were specific to ER stress and UPR activation caused by proteasome inhibition, we conducted experiments using two alternative ER stress agents, brefeldin A (16, 24) and tunicamycin (32). Real-time RT-PCR was performed in Calu-3 cells treated with brefeldin A or tunicamycin to measure endogenous CFTR mRNA levels. The spliced variant of XBP1 was measured to monitor UPR activation. ER stress caused by brefeldin A or tunicamycin resulted in an increase in spliced XBP1 levels. Calu-3 cells were treated with 100 μM ALLN for the time periods specified (1–8 h), and total RNA was isolated. Spliced XBP1, CFTR, GAPDH, and TR mRNA levels were measured from each sample. No changes in TR or GAPDH mRNA levels were measured following arsenite treatment (data not shown). These results further support the idea that the changes seen in CFTR mRNA levels following ER stress induction using three different methods were specifically caused by ER stress responses.
Overexpression of Recombinant WT CFTR in Calu-3 Cells Results in Inefficient Maturation Without Increasing UPR Activity

To test the effect of recombinant CFTR overexpression on CFTR ERAD and activation of the UPR, we developed a stable cell line, Calu-3WT, that expresses recombinant CFTR on a background of endogenous CFTR expression. It has been suggested that overexpression of recombinant proteins causes ER stress and activates both ERAD and the UPR (38). To characterize the recombinant CFTR-overexpressing Calu-3 cell line (Calu-3WT), we measured steady-state CFTR levels by immunoprecipitation from 250 μg of total cell lysates and directly compared them with that of parental Calu-3 cells expressing only endogenous CFTR. Surprisingly, overexpression of recombinant WT CFTR under the control of the CMV promoter did not increase steady-state CFTR levels in Calu-3WT cells (Fig. 5A). In contrast to protein levels, total CFTR mRNA levels were higher in the transduced Calu-3WT cells than in the parental cell line (Fig. 5B). This suggested either decreased CFTR synthetic rates or inefficient CFTR maturation in Calu-3WT cells. To discriminate between these possibilities, we performed metabolic pulse-chase studies. The results indicate a significant decrease in CFTR maturation efficiency in Calu-3WT cells (~60% maturation) compared with the parental cells (~100% maturation) (Fig. 5C). CFTR synthesis rates were slightly elevated in Calu-3WT cells as shown by the increased density of band B immediately after the pulse (Fig. 5C). This result supports the hypothesis that recombinant CFTR overexpression results in inefficient CFTR maturation. As a consequence, steady-state CFTR protein levels in Calu-3 and Calu-3WT cells remain similar.

To test whether overexpression of recombinant WT CFTR activates the UPR in Calu-3WT cells, we measured spliced XBP1 and BiP mRNA levels and compared them with those of parental Calu-3 cells. A mixed population of Calu-3WT and two clones, one with low (Calu-3WT1) and another with high (Calu-3WT2) recombinant CFTR expression levels, were examined. sXBP1 and BiP mRNA levels were normalized to either GAPDH mRNA or 18S RNA. The results indicate that overexpression of recombinant WT CFTR does not activate the UPR as monitored by an increase in spliced XBP1 or BiP levels (Fig. 6A). Next, we tested whether induction of recombinant WT CFTR synthesis activates the UPR. To increase recombinant CFTR synthesis, we treated Calu-3 and Calu-3WT cells with 1 mM Na-butyrate for 12 h. Na-butyrate treatment increased recombinant CFTR mRNA and protein levels without a corresponding positive effect on endogenous CFTR levels (Fig. 6B). The decrease in total CFTR mRNA and protein levels in Calu-3 parental cells may be attributed to a toxic effect by Na-butyrate. Considering that steady-state CFTR levels in Calu-3 and Calu-3WT cells are similar in the absence of induction (Fig. 5), the increase in CFTR levels following induction with Na-butyrate is likely to be a temporary effect. To test whether increased recombinant CFTR synthesis causes ER stress and activates the UPR, we measured spliced XBP1 mRNA levels. Again, no significant changes in XBP1 levels were detected, indicating that an acute increase in WT CFTR synthesis rates does not activate the UPR (Fig. 6C).

ER Stress and the UPR Have No Effect on Recombinant CFTR mRNA Levels

Because ER stress and activation of the UPR resulted in decreased endogenous CFTR mRNA levels, we next tested whether ER stress regulates recombinant CFTR transcript levels. In these studies, a HeLaWT cell line (3, 45) expressing recombinant WT CFTR only and Calu-3WT expressing both recombinant and endogenous CFTR were examined. Following induction of ER stress by proteasome inhibition, the levels of sXBP1 and BiP mRNAs were measured to monitor UPR activation. CFTR mRNA levels were measured using two different primer sets, a set designed to amplify endogenous and recombinant CFTR, and a set constructed to amplify only endogenous CFTR (see MATERIALS AND METHODS). In contrast to endogenous WT CFTR-expressing cells (Fig. 2), no changes in CFTR mRNA levels were detected in HeLaWT cells expressing recombinant CFTR only (Fig. 7A). To further differentiate the effects of ER stress and the UPR on recombinant and endogenous CFTR mRNA levels, we tested Calu-3WT cells. In these cells expressing considerably more (~5×) recombinant than endogenous CFTR mRNA (Fig. 5B), there was no significant change in total CFTR mRNA (endogenous + recombinant) levels following ER stress (Fig. 7B, top). However, endogenous CFTR mRNA levels decreased significantly (Fig. 7B, bottom). Under the same conditions, TR mRNA levels remained unchanged (data not shown). These results further demonstrate that the decreased CFTR mRNA following ER stress is specific to endogenous CFTR, even when recombinant and endogenous CFTR are expressed in the same cell.

Decreased Endogenous CFTR mRNA Levels are Not the Result of Enhanced Cytosolic mRNA Decay

To determine whether decreased endogenous CFTR mRNA levels were the result of transcriptional repression or enhanced
mRNA degradation in the cytosol, we performed experiments to test for increased cytosolic RNase activity following induction of ER stress. Cytosolic fractions from ER stressed and control Calu-3 cells were incubated with RNA extracted from Calu-3 cells containing CFTR mRNA. Nuclease activity was stopped by the addition of TRIzol reagent, followed by RNA extraction and real-time RT-PCR to amplify CFTR mRNA. RNA incubated with buffer only was tested as control. The results indicate no differences in RNase activity between control and ER-stressed Calu-3 cell cytosolic fractions (Fig. 8). The presence of active RNase in the cytosolic fractions is demonstrated by the ~20% decrease in CFTR mRNA levels in both control and ER-stressed Calu-3 cell cytosolic fractions compared with the control incubated with buffer (Fig. 8). The CFTR mRNA decay was similar to previously reported results (2). These results indicate that decreased genomic CFTR mRNA levels following ER stress and activation of the UPR are not the result of enhanced cytosolic mRNA decay.

DISCUSSION

The studies presented concentrate on the differences in genomic and recombinant WT CFTR biogenesis regulation following induction of ER stress. ER stress activates cellular recovery mechanisms such as the UPR and ERAD. The UPR increases the folding capacity of the ER by increasing the synthesis of chaperones and ER membrane components (38) and decreases the protein load both transcriptionally (18) and posttranslationally by decreasing protein synthesis (13, 17). The UPR also increases the capacity of ERAD, the main posttranslational mechanism that regulates protein biogenesis (11). These pathways are equally relevant for both normal cellular homeostasis as well as disease pathogenesis (for review, see Refs. 38, 39). Because our studies concentrate on WT CFTR expression regulation, the results presented are not directly relevant to the pathogenesis of cystic fibrosis caused by mutations in the cftr gene. In contrast, our results highlight the potentially important consequences of ER stress responses regarding membrane protein expression regulation. In agreement with our previous studies (45), we have shown that endogenous WT CFTR is not normally a substrate for ERAD but that under ER stress conditions, endogenous WT CFTR biogenesis becomes regulated by ERAD. We also have shown that following induction of ER stress and activation of the UPR, but not under cytosolic stress conditions, endogenous...

Fig. 5. A: CFTR protein levels are the same in Calu-3 and Calu-3 wild-type (Calu-3WT) cells. Total cellular lysates (250 µg) were immunoprecipitated (1 µg/sample 24-1 anti-CFTR antibody, 20 µl of protein A-agarose), in vitro phosphorylated using [γ-32P]ATP and cAMP-dependent protein kinase, and analyzed using SDS-PAGE and autoradiography. A gel representative of 3 experiments is shown (left). Densitometry results are plotted to show relative amounts of CFTR expressed in the cell lines (right). n = 3, N.S., no significant difference. B: increased CFTR mRNA levels in Calu-3 WT cells. CFTR mRNA levels were measured using real-time RT PCR. GAPDH was amplified as internal control, and results are plotted relative to GAPDH mRNA levels (n = 6; *P < 0.05). C: CFTR maturation efficiency is decreased in Calu-3 WT cells compared with Calu-3. Calu-3 cells were pulse labeled with 300 µCi/ml 35S-labeled amino acids. After the pulse, the [35S]methionine/cysteine-containing medium was replaced with complete medium. Cells were lysed at the time points specified, and CFTR was immunoprecipitated with anti-CFTR 24-1 antibody. Samples were separated using SDS PAGE on 6% gels and analyzed using a PhosphorImager. CFTR maturation efficiency was measured by comparing the density of labeled band B (100%) after a 30-min pulse to the density of band C after 4 h of chase, using IPLab software. Results are plotted as percentages of newly synthesized band B converted to band C by the end of a 4-h chase (average ± SD, n = 4; *P < 0.05).
WT CFTR mRNA levels decrease significantly. The decrease in endogenous WT CFTR mRNA levels is likely the result of transcriptional repression through the UPR, since incubation of RNA samples containing CFTR mRNA with cytosolic fractions (containing nuclease complexes such as the exosome) did not reveal an increase in nuclease activity in the ER stressed cytosolic samples. In contrast to endogenous CFTR, ER stress had no effect on recombinant CFTR mRNA levels, suggesting that upstream elements or intronic sequences present only in endogenous CFTR are responsible for decreased CFTR mRNA levels. Furthermore, we have shown that overexpression of recombinant CFTR does not activate ER stress responses, and this would help to explain the inefficient recombinant WT CFTR maturation seen in most models.

What is the significance of these results? Early studies indicated that only 20–55% of newly synthesized 140-kDa, core glycosylated WT CFTR molecules acquire complex oligosaccharide chains, and this represents the percentage of CFTR molecules that traffic through post-ER compartments (25, 46). Although the inefficiency in WT CFTR maturation was first suggested to be the consequence of overexpression (7), it later was also shown in cell lines expressing low levels of the protein (46). On the basis of these results, it has been proposed that the absence of assembly factors or other changes caused by transformation of cells may account for inefficient CFTR maturation (20). However, the significance of this post-translational CFTR expression regulation is not understood. Furthermore, although transcriptional (for review see Ref. 27) and posttranscriptional (through mRNA stability; Ref. 2) regulation of CFTR expression have been investigated in endogenous CFTR-expressing cells and during development, the role of ER stress on CFTR expression regulation has not been examined. The studies presented are the first to show endogenous CFTR expression regulation at multiple levels by ER stress responses and will contribute to our understanding of how membrane protein expression is regulated under stress conditions.
Our careful investigation of recombinant and endogenous WT CFTR biogenesis in epithelial cell lines shows significant differences in WT CFTR maturation efficiency between different model cell lines (45). When sufficient levels of the protein are synthesized that allow for metabolic pulse-chase analysis, the results indicate that the maturation of endogenous WT CFTR is efficient (45). To be able to label sufficient endogenous CFTR, cell culture conditions have to be carefully optimized, because either starvation or overgrowth of the culture results in significantly decreased endogenous CFTR synthesis and low labeling efficiency (Bebo ˝k Z, unpublished observation). Interestingly, starvation is one of the original methods for inducing ER stress that results in the activation of both ERAD and the UPR (50), suggesting that the biogenesis of endogenous WT CFTR is downregulated under these conditions.

Oxidative cytosolic stress following a 12- to 24-h exposure to butylhydroquinone has been shown to decrease CFTR function by decreasing mRNA stability (6). Cigarette smoke extract also has been shown to decrease chloride secretion in human bronchial epithelial cells, suggesting that this too may be the result of decreased CFTR expression (21). Furthermore, CFTR mRNA stability by cytokines is regulated through the AU-rich element of the 3′-UTR, which plays role in exosome-mediated mRNA decay (2). These studies suggest that oxidative stress and inflammation decrease CFTR levels posttranscriptionally by decreasing the half-life of CFTR mRNA. However, it is not clear whether these methods induce ER stress. In contrast, previous reports (29) and our results demonstrate that treatment with arsenate for the same time period as ALLN or tunicamycin does not cause ER stress and has no effect on CFTR or GAPDH message levels. This suggests that the time frame for posttranscriptional (mRNA stability) regulation is longer than the likely transcriptional regulation presented. Furthermore, the specific downregulation of genomic CFTR mRNA levels argues against decreased mRNA stability, since endogenous human TR mRNA levels with similar AU-rich regions in the 3′-UTR that would mediate exosomal decay (2) remained unchanged following ER stress and UPR activation. Decreases in both TR and CFTR mRNA would indicate an increased exosome-mediated mRNA turnover in our experiments. However, based on the results and the known pathways regulated by the UPR (transcription, translation, and ERAD), it is most likely that the decrease in CFTR mRNA seen in our studies results from transcriptional downregulation.

CFTR is not an abundant protein, even in cells with relatively high endogenous CFTR levels such as the Calu-3 cells used in this study. If transcription inhibition is a normal component of the cellular processes used to decrease ER load under stress conditions, then why is CFTR a target? Although it has been shown in yeast that cellular stress may downregulate the expression of certain membrane proteins transcriptionally by decreasing mRNA stability (6), it is not clear whether these methods induce ER stress. In contrast, previous reports (29) and our results demonstrate that treatment with arsenate for the same time period as ALLN or tunicamycin does not cause ER stress and has no effect on CFTR or GAPDH message levels. This suggests that the time frame for posttranscriptional (mRNA stability) regulation is longer than the likely transcriptional regulation presented. Furthermore, the specific downregulation of genomic CFTR mRNA levels argues against decreased mRNA stability, since endogenous human TR mRNA levels with similar AU-rich regions in the 3′-UTR that would mediate exosomal decay (2) remained unchanged following ER stress and UPR activation. Decreases in both TR and CFTR mRNA would indicate an increased exosome-mediated mRNA turnover in our experiments. However, based on the results and the known pathways regulated by the UPR (transcription, translation, and ERAD), it is most likely that the decrease in CFTR mRNA seen in our studies results from transcriptional downregulation.

Fig. 7. ER stress and UPR have no effect on recombinant CFTR mRNA levels. The effects of ER stress and activation of the UPR on recombinant CFTR expression levels were tested in HeLaWT (A; expressing recombinant WT CFTR only) and in Calu-3WT (B; expressing endogenous and recombinant CFTR). UPR was induced with ALLN (100 μM, 6 h). Total and recombinant CFTR mRNA levels were measured using real-time RT-PCR. No changes in recombinant CFTR mRNA were detected following activation of the UPR. Endogenous CFTR mRNA levels decreased to ~50% in Calu-3 WT cells (n = 6, *P < 0.001).

Fig. 8. Cytosolic RNase activity is not increased following induction of ER stress. Cytosolic fractions were isolated from control and ER-stressed Calu-3 cells and incubated with equal amounts of total RNA isolates from Calu-3 cells containing CFTR mRNA (20 μg of cytosolic proteins, 20 μg of RNA, 25°C, 30 min) in RNA digestion buffer, followed by extraction of RNA and real-time RT-PCR of CFTR mRNA levels. RNA samples incubated with buffer alone (no nucleases) were used as control. No differences in nuclease activities on CFTR mRNA and control and ER-stressed cytosolic samples were measured. The ~20% decrease in CFTR mRNA levels following incubation with cytosolic proteins compared with nonincubated mRNA samples indicates the presence of active nucleases in the cytosolic fraction. These experiments were performed on 4 individual samples under each condition and repeated twice. *P < 0.05.
ally (18), the mechanism of this process and the nature of the inhibiting transcription factors are not known and require further investigation.

Our finding that overexpression of recombinant WT CFTR does not cause ER stress or activate the UPR also is significant. Previous studies have indicated that WT CFTR carries structural features that may contribute to inefficient folding (42). This suggests that overexpression of CFTR could increase the likelihood of misfolding, resulting in activation of the UPR. However, in agreement with our results, recent studies investigating ER stress responses following the overexpression of LDL receptor showed that only the ER retention mutant, not WT LDL receptor overexpression, activated the UPR (41). We can speculate that overexpression of the ER retention mutant ΔF508 CFTR may cause ER stress responses similar to those of the mutant LDL receptor, considering that ΔF508 CFTR is selected for degradation at different checkpoints than the WT protein.

During recent years, significant progress has been made in identifying the pathways of the mammalian ERQC, ERAD, and UPR. However, it remains unclear how these processes regulate the protein load in different cell types. If recombinant protein overexpression following gene transfer activates the UPR, this could lead to cell damage and apoptosis (37), resulting in inefficient transgene expression levels and tissue damage. Furthermore, because cellular stress responses are often activated under inflammatory conditions, the biogenesis of proteins may be compromised.

In summary, the studies presented showed a relationship among ER stress, UPR activation, and CFTR expression regulation. Protein overexpression following gene transfer activates the UPR, this could lead to cell damage and apoptosis. To promote efficient translation and folding, ER stress conditions. In contrast, ERAD appears to be the main pathway for limiting recombinant CFTR protein levels. These experiments also illustrate new features concerning the role of ER stress and the UPR in the biogenesis of CFTR and other integral membrane proteins. Further studies, however, are required to reveal the precise mechanism by which the UPR regulates CFTR and other protein expression.

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