c-Cbl reduces stability of rescued ΔF508-CFTR in human airway epithelial cells
Implications for cystic fibrosis treatment

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CFTR is a PKA activated Cl⁻ channel expressed in the apical membrane of fluid transporting epithelia. We previously demonstrated that c-Cbl decreases CFTR stability in the plasma membrane by facilitating its endocytosis and lysosomal degradation in human airway epithelium. The most common mutation associated with cystic fibrosis, deletion of Phe508 (ΔF508), leads to a temperature sensitive biosynthetic processing defect in the CFTR protein. Mature ΔF508-CFTR that has been rescued by low temperature or chemical chaperones is partially functional as a Cl⁻ channel but has decreased plasma membrane stability due to altered post-maturational trafficking. Our present data demonstrate that c-Cbl controls the post-maturational trafficking of rescued ΔF508-CFTR. Partial depletion of c-Cbl increased stability of the plasma membrane associated mature ΔF508-CFTR and the ΔF508-CFTR mediated Cl⁻ secretion. These data indicate that correcting the post-maturational trafficking of ΔF508-CFTR may represent a therapeutic approach complementary to the biosynthetic rescue. Because c-Cbl functions as an adaptor and scaffolding protein during CFTR endocytosis, we propose that interfering with the c-Cbl mediated endocytic recruitment of ΔF508-CFTR may increase stability of Δ508-CFTR in the plasma membrane after its biosynthetic rescue.

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated Cl⁻ channel that mediates transepithelial Cl⁻ secretion in various fluid-transporting epithelia. Dysfunctions of the CFTR protein caused by CFTR gene mutations result in reduction or absence of Cl⁻ transport and lead to a common fatal genetic disease, cystic fibrosis (CF). In the airway, CFTR plays a critical role in regulating mucociliary clearance by maintaining the airway surface liquid. Control of CFTR mediated Cl⁻ secretion across polarized epithelial cells is achieved at the level of both CFTR Cl⁻ channel activity and protein stability in the plasma membrane. The plasma membrane stability of CFTR depends on its biosynthetic processing and post-maturational trafficking, which involves endocytic uptake followed either by recycling to the plasma membrane or degradation in the lysosome. The long plasma membrane stability of CFTR stands in contrast to its inefficient biosynthetic processing and depends primarily on efficient recycling to compensate for rapid endocytosis.

Both the biosynthetic processing and post-maturational trafficking of CFTR are critically affected by the common disease-associated mutation in the CFTR gene caused by loss of Phe508 (ΔF508). The ΔF508 mutation leads to a temperature sensitive processing defect that results in biosynthetic arrest of the ΔF508-CFTR protein in the immature, partially glycosylated form (band B) retained in the endoplasmic reticulum (ER). Low temperature and chemical chaperones rescue the biosynthetic processing defect of ΔF508-CFTR. The biosynthetic rescue allows the immature form of ΔF508-CFTR to exit the ER, complete glycosylation while passing through the Golgi system, and traffic to the cell membrane as a mature band C. Because rescued ΔF508-CFTR (rΔF508-CFTR) is partially functional as a Cl⁻ channel, therapies based on correction of the biosynthetic processing defect have been highly anticipated. Despite showing promising results in cultured cells, a small molecule corrector VX-809 had disappointing results in patients homozygous for the ΔF508 mutation. rΔF508-CFTR has decreased plasma membrane stability due to altered post-maturational trafficking. Correcting the post-maturational trafficking defect of rΔF508-CFTR may represent a therapeutic approach complementary to the biosynthetic rescue. Thus, CFTR interactions with protein adaptors involved in either endocytic uptake or lysosomal degradation or both are the logical targets for stabilizing...
ΔC. Partial silencing of c-Cbl increased the steady-state abundance of the partially glycosylated F508-CFTR band B (ΔCplasma membrane associated, fully glycosylated ΔCefficiency. Low temperature rescued the abundance of the mature, in WCL. Low temperature (27°C) did not affect the c-Cbl silencing on Figure 1. (ΔC).

By contrast, partial silencing of c-Cbl did not increase the steady-state abundance of the partially glycosylated F508-CFTR band C, indicating that c-Cbl does not facilitate the biosynthetic processing of ΔF508-CFTR (ΔC).

Figure 1. Western blots demonstrating the effects of c-Cbl depletion on ΔF508-CFTR abundance in whole cell lysates (WCL) at steady-state in polarized CFBE410- cells. Cells were transfected with 50 nM siRNA against the human c-Cbl gene (si-c-Cbl) or the non-silencing negative siRNA control (si-CTRL). Low temperature (27°C) for 48h was used to rescue the biosynthetic processing defect of ΔF508-CFTR. Representative western blots (A, the bottom panel) and summary of experiments (B) demonstrating that si-c-Cbl decreased the c-Cbl protein abundance in WCL. Low temperature (27°C) did not affect the c-Cbl silencing efficiency. Low temperature rescued the abundance of the mature, plasma membrane associated, fully glycosylated ΔF508-CFTR band C. Partial silencing of c-Cbl increased the steady-state abundance of ΔF508-CFTR band C in polarized CFBE410- cells (A; the top panel and C). By contrast, partial silencing of c-Cbl did not increase the steady-state abundance of the partially glycosylated ΔF508-CFTR band B (A and C). Silencing c-Cbl in cells not subjected to low temperature (37°C) did not increase the abundance of the ΔF508-CFTR band C, indicating that c-Cbl does not facilitate the biosynthetic processing of ΔF508-CFTR (A). The ΔF508-CFTR abundance was normalized for actin. Actin expression was used as a loading control. *, p < 0.05 vs. siCTRL. Five experiments/group. Error bars, SE.

Silencing c-Cbl increases abundance of the plasma membrane associated ΔF508-CFTR in polarized human airway epithelial cells. Studies were conducted to determine whether c-Cbl regulates the post-maturational trafficking of ΔF508-CFTR. If c-Cbl facilitates ΔF508-CFTR endocytosis and lysosomal degradation, we predicted that c-Cbl depletion would attenuate the post-maturational degradation and thus, would increase the plasma membrane abundance of ΔF508-CFTR. To test this prediction, c-Cbl abundance was reduced in CFBE410- cells stably expressing ΔF508-CFTR by RNA mediated interference (siRNA). CFBE410- cells were transfected with siRNA specific for a non-conserved region of the human c-Cbl sequence (si-c-Cbl) or with a non-silencing, negative siRNA control (si-CTRL) as previously described. Low temperature (27°C) was used to facilitate the biosynthetic processing and rescue expression of the mature, fully glycosylated band C of ΔF508-CFTR. In addition, the effect of silencing c-Cbl on the biosynthetic processing of ΔF508-CFTR was examined in cells cultured exclusively at 37°C. Partial silencing of c-Cbl increased the steady-state abundance of ΔF508-CFTR band C (Fig. 1). By contrast, partial silencing of c-Cbl did not affect the abundance of the partially glycosylated band B of ΔF508-CFTR after temperature rescue, indicating that c-Cbl does not affect ΔF508-CFTR protein translation (Fig. 1). Moreover, in the absence of temperature rescue, partial depletion of c-Cbl did not increase the abundance of ΔF508-CFTR band C, indicating that c-Cbl does not facilitate the biosynthetic processing of ΔF508-CFTR (Fig. 1A).

To gain additional evidence that the increased abundance of ΔF508-CFTR band C after c-Cbl depletion resulted from changes in the post-maturational trafficking rather than increased...
Taken together, the above data indicate that in human airway epithelial cells, endogenous c-Cbl decreases stability of the plasma membrane associated rΔF508-CFTR by controlling its post-maturational trafficking. Silencing c-Cbl increases rΔF508-CFTR-mediated Cl- secretion across polarized human airway epithelial cells. Control of CFTR-mediated Cl- secretion across polarized epithelial cells is achieved at the level of both CFTR Cl- channel activity and protein stability in the plasma membrane. Because silencing c-Cbl increased the abundance and stability of rΔF508-CFTR band C and partial silencing of c-Cbl had a synergistic effect and completely prevented the disappearance of the temperature rescued rΔF508-CFTR band C at 37°C without affecting the stability of the Epidermal Growth Factor Receptor (EGFR) or Transferrin Receptor (TR). Low temperature (27°C) alone decreased the disappearance of the rescued rΔF508-CFTR band C and partial silencing of c-Cbl had a synergistic effect and completely prevented the disappearance of the temperature rescued rΔF508-CFTR band C (B and C). Partial silencing of c-Cbl did not attenuate the disappearance of the rΔF508-CFTR band B at 37°C or 27°C (A, B and D). The rΔF508-CFTR abundance was normalized for ezrin. Ezrin expression was used as a loading control. *, p < 0.05 vs. time zero in siCTRL. Five experiments/group. Error bars, SE.

Figure 2. Western blots demonstrating the effects of c-Cbl depletion on rΔF508-CFTR abundance in whole cell lysates (WCL) in polarized CFBE41o-cells as a function of time. Cells were transfected with 50 nM siRNA against the human c-Cbl gene (si-c-Cbl) or the non-silencing negative siRNA control (si-CTRL). Low temperature (27°C) for 48h was used to facilitate the biosynthetic processing of rΔF508-CFTR band C. Disappearance of rΔF508-CFTR band C (A and C) or band B (A and D) from WCL was monitored over time in the presence of 20 μg/ml cycloheximide (CHX) at 37°C (A, C and D) or 27°C (B–D). In si-CTRL-transfected cells, at least 50% of rΔF508-CFTR band C disappeared in 4h. Thus, data are reported at the 4h time point (C and D). Representative western blots (A) and summary of experiments (C) demonstrating that partial silencing of c-Cbl attenuated the disappearance of the temperature rescued rΔF508-CFTR band C at 37°C without affecting the stability of the Epidermal Growth Factor Receptor (EGFR) or Transferrin Receptor (TR). Low temperature (27°C) alone decreased the disappearance of the rescued rΔF508-CFTR band C and partial silencing of c-Cbl had a synergistic effect and completely prevented the disappearance of the temperature rescued rΔF508-CFTR band C (B and C). Partial silencing of c-Cbl did not attenuate the disappearance of the temperature rescued rΔF508-CFTR band B at 37°C or 27°C (A, B and D). The rΔF508-CFTR abundance was normalized for ezrin. Ezrin expression was used as a loading control. *, p < 0.05 vs. time zero in siCTRL. Five experiments/group. Error bars, SE.
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Low temperature (27 °C) A

Low temperature is non-specific for correcting the biosynthetic defect of ΔF508-CFTR and is not suitable for use in patients. Several small molecules called CFTR correctors have been shown to rescue the functional defect of ΔF508-CFTR by correcting its biosynthetic processing defect in cultured cells.13,25 The present generation of CFTR correctors does not improve the decreased plasma membrane stability of rΔF508-CFTR and their clinical efficacy has been very limited in patients homozygous for the ΔF508 mutation.14,25 Thus, we examined whether increasing stability of rΔF508-CFTR by silencing c-Cbl would complement the corrector effect on the functional defect of ΔF508-CFTR. Despite an overall lower lsc at 37°C compared with 27°C, partial silencing of c-Cbl increased the forskolin/IBMX-stimulated lsc across CFBE41o-monolayers after rescue with the CFTR corrector CF-106951 (Fig. 3C and D). These results are consistent with the above data that si-c-Cbl increased rΔF508-CFTR abundance and stability and demonstrate that endogenous c-Cbl inhibits the rΔF508-CFTR-mediated Cl− secretion by limiting the rΔF508-CFTR Cl− channel abundance in the plasma membrane in human airway epithelial cells. These data indicate that targeting the interaction between rΔF508-CFTR and c-Cbl could serve as a complementary approach to the biosynthetic rescue in order to correct the functional defect of ΔF508-CFTR.

Discussion

Data presented in this manuscript demonstrate that c-Cbl regulates the rΔF508-CFTR mediated Cl− secretion by controlling the post-maturational trafficking of rΔF508-CFTR in polarized human airway epithelial cells. Several lines of evidence in the present study support our conclusion. Compared with controls, partial silencing of c-Cbl increased the steady-state abundance and stability of the mature, plasma membrane associated ΔF508-CFTR after its biosynthetic rescue (Figs. 1 and 2). Moreover, partial silencing of c-Cbl increased the rΔF508-CFTR mediated Cl− secretion (Fig. 3). We previously reported a similar effect of c-Cbl on the WT-CFTR and demonstrated that c-Cbl controls the post-maturational trafficking of WT-CFTR by facilitating WT-CFTR endocytosis and lysosomal degradation in human airway epithelial cells.23 Data in this manuscript indicate that c-Cbl does not decrease the ΔF508-CFTR protein translation because c-Cbl depletion did not increase the abundance of the partially glycosylated ΔF508-CFTR (Fig. 1). Moreover, c-Cbl has no effect on the biosynthetic processing of the partially glycosylated immature ΔF508-CFTR because c-Cbl depletion did not increase the abundance of fully glycosylated mature ΔF508-CFTR in the absence of biosynthetic rescue (Fig. 1). Similar abundance of c-Cbl at 27°C and 37°C confirm that differences observed in experiments performed at these temperatures did not result from differences in siRNA silencing efficiency.

Correcting ΔF508-CFTR to resemble WT-CFTR functionally will require rescue of the biosynthetic processing defect, increasing the Cl− channel activity, and stabilizing rΔF508-CFTR in the plasma membrane. Recent data demonstrate promising effects of combining a corrector with a potentiatort—an agent that increases the rΔF508-CFTR Cl− channel function—although the level of functional correction by such complementary therapy in CF patients is still far from that of WT-CFTR.26 Partial depletion of c-Cbl increased stability of rΔF508-CFTR and increased the rΔF508-CFTR mediated Cl− secretion (Figs. 2 and 3). Our data demonstrate that controlling the post-maturational trafficking of rΔF508-CFTR should be considered as a complementary approach to improve the efficacy of correction of the functional defect of ΔF508-CFTR in CF patients.

Figure 3. Ussing chamber experiments performed to determine the effects of c-Cbl depletion on the ΔF508-CFTR mediated Cl− secretion across CFBE41o-monolayers. Cells were transfected with 50 nM siRNA against the human c-Cbl gene (si-c-Cbl) or the non-silencing negative siRNA control (si-CTRL). Low temperature (27°C; A and B) or a corrector CF-106951 (C and D) for 48h was used to facilitate the biosynthetic processing and rescue abundance of ΔF508-CFTR band C. CFBE41o-cells were bathed in solutions with apical-to-basolateral Cl− gradient in the presence of amiloride (50 μM) in the apical bath solution to inhibit Na+ absorption through ENaC. lsc was stimulated with forskolin (20 μM) and IBMX (50 μM) and si-c-Cbl transfected cells, respectively). Representative experiment (A) and summary of data (B) demonstrating that partial silencing of c-Cbl increased the forskolin/IBMX-stimulated lsc across CFBE41o-cells compared with the non-transfected cells (data not shown). Si-c-Cbl did not change the transepithelial resistance across cell monolayers (398.8 ± 72 Ω cm² vs. 404.7 ± 140.8 Ω cm² for the si-CTRL and si-c-Cbl transfected cells, respectively). Representative experiment (A) and summary of data (B) demonstrating that partial silencing of c-Cbl increased the forskolin/IBMX-stimulated lsc across CFBE41o-cells after the low temperature rescue. Moreover partial silencing of c-Cbl increased the forskolin/IBMX-stimulated lsc across CFBE41o-cells after the CF-106951 rescue (C and D). *, p < 0.05 vs. si-CTRL. Six monolayers/group. Error bars, SE.
c-Cbl is a multifunctional protein critical for many cellular functions. In our study depletion of c-Cbl by 60–70% did not change the stability of membrane receptors such as EGFR and TR or the cytoskeletal proteins actin and ezrin and did not change the transepithelial resistance while increasing the stability and function of rΔF508-CFTR (Figs. 1–3). We previously demonstrated that off-target effects can be prevented by partial depletion of target proteins at levels similar to those used in our study. Depletion of endogenous c-Cbl is a useful approach to elucidate its function in the post-maturational trafficking of rΔF508-CFTR in cultured cells. Such approach would be impractical in vivo where control of the post-maturational trafficking of rΔF508-CFTR requires high specificity. Targeting directly the interaction between c-Cbl and rΔF508-CFTR will allow c-Cbl to control other cellular functions and thus, will be highly specific. We propose that targeting the c-Cbl-rΔF508-CFTR interaction may serve as a useful biochemical tool to uncovering disease modifying, complementary approaches for CF. Future studies will focus on elucidating the complex formation between c-Cbl and rΔF508-CFTR in human airway epithelial cells.

Materials and Methods

Cell lines and cell culture. CFBE410- cells stably expressing ΔF508-CFTR were a generous gift from Dr. J.P. Clancy, Cincinnati Children’s Hospital Medical Center Cincinnati, OH. CFBE410- cells were seeded on Transwell (4.67 cm² or 44 cm²) (Corning Corporation) permeable supports coated with plating medium containing Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) and 10% purified collagen (PureColTM; Advanced Biomatrix, San Diego, CA) and maintained as polarized monolayers in air-liquid interface culture as described previously. To rescue the biosynthetic processing of ΔF508-CFTR cells were cultured in fresh medium at 27°C for 48 h before experiments. Alternatively, to rescue the biosynthetic processing of ΔF508-CFTR the corrector CF-106951 (1-[benzo[d][1,3]dioxol-5-yl]-N-(5-((S)-(2-chlorophenyl)((R)-3-hydroxypropyrrolidin-1-yl)methyl)thiazol-2-yl)cyclopropanecarboxamide; 6 μM) was added daily in fresh medium and cells were cultured at 37°C for 48 h before experiments. Cultures were plated on tissue culture plates and incubated with the optimized transfection mixture containing 50 nM of siRNA at 37°C. After 24 h, cells were trypsinized and plated on collagen-coated Transwell permeable supports and cultured for an additional 4 d to establish polarized monolayers (total 5 d in culture). Silencing the target genes under these conditions resulted in the corresponding protein depletion by at least 60% (Fig. 1B).

Biochemical determination of the complex (band C) and partially glycosylated (band B) ΔF508-CFTR. The biosynthetic defect caused by the ΔF508 mutation results in processing arrest of the partially glycosylated, immature ΔF508-CFTR band B. Temperature rescue (27°C for 48 h) was used to correct the biosynthetic defect and facilitate processing to the mature, fully glycosylated form of ΔF508-CFTR band C. Because CFTR band C is expressed at the cell surface, unlike band B, the abundance of band C in whole cell lysates (WCL) was used as a measure of the cell surface associated rΔF508-CFTR. To inhibit protein translation cells were incubated with cycloheximide (Sigma-Aldrich; 20 μg/ml), at 37°C or 27°C and the disappearance of the fully and partially glycosylated form of ΔF508-CFTR was monitored over time. CFBE410- cells expressing ΔF508-CFTR were lysed in buffer containing 25 mM HEPES, pH 8.0, 1% Triton, 10% glycerol, and Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN), as described previously. Proteins were visualized by western blotting with specific primary antibodies and horseradish peroxidase secondary antibodies using the Western Lightning™ Plus-ECL detection system (Perkin Elmer Inc.; USA) followed by chemiluminescence. Quantification of the immunoreactive bands was performed by densitometry using exposures within the linear dynamic range of the film.

Ussing chamber measurements. Ussing chamber measurements were performed as previously described. Monolayers of airway cells grown on Snapwell permeable supports were mounted in an Ussing-type chamber (Physiologic Instruments; USA) and bathed in solutions (see below) maintained at 37°C and stirred by bubbling with 5% CO₂ / 95% air. Short circuit current (Isc) was measured by voltage-clamping the transepithelial voltage across the monolayers to 0 mV with a voltage/current clamp (model VCC MC8, Physiologic Instruments) as described previously. Data collection and analysis were done with Acquire and Analyze Data Acquisition System (Physiologic Instruments). CFBE410- cells were bathed in solutions with an apical-to-basolateral Cl gradient in the presence of amiloride (50 μM) in the apical bath solution to inhibit Na⁺ absorption. The apical bath solution contained (in mM): 115 Na-glucosinate, 5 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, 10 mannitol (pH 7.4). The basolateral bath solution contained (in mM): 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, 10.0 glucose (pH 7.4). A low Cl⁻, high-Na⁺, high-glucosinate, apical bath solution was used to

RNA mediated interference. Transfection of CFBE410- cells with siRNA targeting human c-Cbl gene (si-c-Cbl; Hs_Cbl_7 siRNA) or the non-silencing siRNA control (AllStars, si-CTRL; Qiagen, USA) was conducted using HiPerfect Transfection Reagent (Qiagen) according to the manufacturer’s instructions as described in our recent publication. Briefly, CFBE410- cells were plated on tissue culture plates and incubated with the optimized transfection mixture containing 50 nM of siRNA at 37°C. After 24 h, cells were trypsinized and plated on collagen-coated Transwell permeable supports and cultured for an additional 4 d to establish polarized monolayers (total 5 d in culture). Silencing the target genes under these conditions resulted in the corresponding protein depletion by at least 60% (Fig. 1B).

Antibodies and reagents. The following antibodies were used: mouse anti-human CFTR (clone 596; Cystic Fibrosis Foundation Therapeutics Inc.; www.cftrfolding.org), anti-actin (mouse monoclonal or rabbit polyclonal; Sigma-Aldrich; St.Louis, MO), rabbit anti-c-Cbl (C-15; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Epidermal Growth Factor Receptor (EGFR) D38B1 (Cell Signaling Technology, Danvers, MA) and mouse anti-human Transferrin Receptor (TR; CD71; eBioscience, Inc., San Diego, CA). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit (BioRad Laboratories; Hercules, CA) secondary antibodies were used. All antibodies were used at the concentrations recommended by the manufacturer or as indicated in the figure legends.

Materials and Methods

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RNA mediated interference. Transfection of CFBE410- cells with siRNA targeting human c-Cbl gene (si-c-Cbl; Hs_Cbl_7
prevent cell swelling due to the increased apical Cl− permeability under these conditions, as previously described.23 Isc was stimulated with 20 μM forskolin and 50 μM IBMX added to the apical and basolateral bath solution. Thiazolidinone CFTRinh-172 (5 μM; Tocris Bioscience; Ellisville, MO) was added to the apical bath solution to inhibit CFTR-mediated Isc. Data are expressed as forskolin/IBMX stimulated Isc, calculated by subtracting the baseline Isc from the peak stimulated Isc.

Data analysis and statistics. Statistical analysis of the data was performed using GraphPad Prism version 4.0 for Macintosh (GraphPad Software Inc., USA). The means were compared by a two-tailed t-test. A P value < 0.05 was considered significant. Data are expressed as mean ± SE.

Disclosure of Potential Conflicts of Interest

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Acknowledgments

Data are apical bath solution to inhibit CFTR-mediated Isc, calculated by subtracting the baseline Isc from the peak stimulated Isc.

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