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Functional Rescue of DeltaF508-CFTR by Peptides Designed to Mimic Sorting Motifs

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SUMMARY

The cystic fibrosis (CF)-causing mutant, deltaF508-CFTR, is misfolded and fails to traffic out of the endoplasmic reticulum (ER) to the cell surface. Introduction of second site mutations that disrupt a diarginine (RXR)-based ER retention motif in the first nucleotide binding domain rescues the trafficking defect of deltaF508-CFTR, supporting a role for these motifs in mediating ER retention of the major mutant. To determine if these RXR motifs mediate retention of the native deltaF508-CFTR protein in situ, we generated peptides that mimic these motifs and should antagonize mistrafficking mediated via their aberrant exposure. Here we show robust rescue of deltaF508-CFTR in cell lines and in respiratory epithelial tissues by transduction of RXR motif-mimetics, showing that abnormal accessibility of this motif is a key determinant of mistrafficking of the major CF-causing mutant.

INTRODUCTION

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) are associated with impaired chloride flux across epithelia lining the respiratory and gastrointestinal tracts, leading to altered epithelial fluid transport and production of mucus plugs, the hallmark pathology associated with the development of CF (Amaral, 2005; Cheung et al., 2008; Sheppard and Welsh, 1999). The most common CF mutation, deletion of F508 (deltaF508-CFTR) in the first nucleotide binding domain (NBD1), causes misfolding and misassembly of the mutant protein, resulting in its retention in the ER and failure to be functionally expressed at the apical surface of epithelia.

Misfolding of deltaF508-CFTR results in its altered interaction with proteins involved in ER quality control (Younger et al., 2004, 2006). Nontargeted maneuvers generally aimed at correcting the folding and misassembly defects in deltaF508-CFTR, including reduced cell culture temperatures (Denning et al., 1992) and chemical chaperones (Pedemonte et al., 2005; Van Goor et al., 2006), have been shown to "rescue" the functional expression of the mutant protein at the cell surface. However, because some of these chemical chaperones are known to exert off-target effects (Wang et al., 2007), there is a clear need to design more targeted therapeutic interventions.

Hypothetically, a targeted therapeutic intervention could modify the function of a protein known to contribute to the retention of deltaF508-CFTR in the ER. However, there are multiple barriers to the progression of deltaF508-CFTR to the cell surface, and the proteins that comprise these barriers have yet to be fully determined. As an alternative strategy, a specific intervention could be developed if we understood the structural defects inherent in deltaF508-CFTR that result in its mistrafficking. Recently, there has been remarkable progress in our understanding of the structural defects in the major mutant (Du et al., 2005; He et al., 2008; Serohijos et al., 2008). Intramolecular interactions within this multidomain protein appear to be perturbed by the deltaF508 mutation, possibly leading to the altered exposure of trafficking signals. For example, the anterograde trafficking of deltaF508-CFTR from the ER to the Golgi is thought to be impaired because the proper presentation of a diacidic motif in NBD1 that normally mediates interaction with Sec23/24 of the COPII vesicular pathway is lacking (Wang et al., 2004). To compound this defect in anterograde trafficking, Chang and colleagues identified RXR-based ER retention/retrieval motifs that are thought to be aberrantly exposed in the major mutant (Chang et al., 1999; Hegedus et al., 2006). Mutation of the four endogenous RXR-based retention motifs in the context of deltaF508-CFTR resulted in a significant increase in the functional expression of this mutant as a regulated chloride channel (albeit with abnormal channel activity) at the cell surface (Chang et al., 1999; Hegedus et al., 2006). A similar observation was described by Amaral and colleagues (Roxo-Rosa et al., 2006), who suggested that deltaF508-CFTR is targeted for retrograde trafficking back to the ER by virtue of aberrantly exposed RXR motifs. Previously, Teem and Welsh determined that second site revertant mutations in an endogenous RXR motif, proximal to the ABC signature sequence in NBD1 (including R553M and R555K in the motif: $R^{553}AR^{555}$), partially restored trafficking of deltaF508-CFTR, suggesting that the motif in this

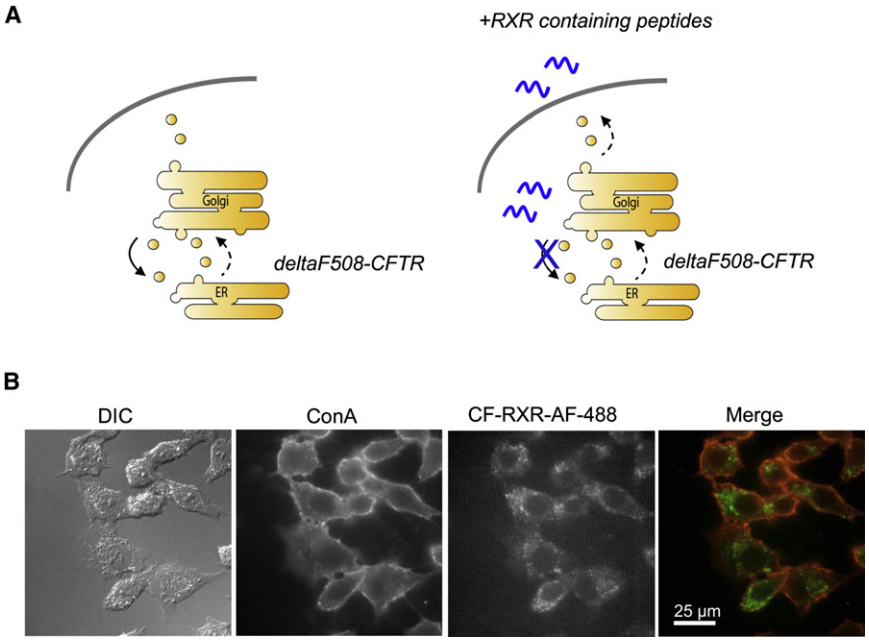

Figure 1. Synthetic Peptides Designed to Antagonize Retrograde Trafficking of DeltaF508-CFTR

(A) Schematic illustrating defective anterograde trafficking of deltaF508-CFTR to the Golgi from the ER (stippled arrow) and enhanced retrograde trafficking from Golgi to ER (solid arrow) (left). Right diagram shows the hypothesis wherein peptides containing strong ER retention motifs (blue line) will antagonize retrograde trafficking (blue cross) and increase cargo availability for delivery to the plasma membrane.

(B) Micrograph (63X magnification) of a 1 micron section through BHK cells exposed to fluorophore-conjugated CPP (1 μM) for 1 hr. AlexaFluor-488 (AF-488)-labeled CPPs are taken up into vesicular structures (green in merge, far right) from which diffusion into the cytosol occurs. The periphery of each cell is apparent in differential interference contrast (DIC) image (far left) and by concanavalin A-Rhd labeling (red in merge, far right).

region has particular functional significance (Teem et al., 1993, 1996).

However, interpretation of these and the previous studies is limited because mutation of RXR motifs in deltaF508-CFTR is known to incur secondary alterations in the conformation of NBD1 and in the full-length mutant protein (Hegedus et al., 2006; Lewis et al., 2005; Pissarra et al., 2008; Teem et al., 1996). Therefore, the role of RXR motifs in trafficking of the native mutant protein in situ has yet to be defined. In the current study, we tested the role of RXR motifs in deltaF508-CFTR trafficking by delivering exogenous peptides bearing RXR motifs. These peptides were conjugated to a cell-penetrating peptide module to facilitate their intracellular delivery (Figure 1A). The relative efficacy of these peptides in modifying the trafficking of the major mutant was determined in vivo in heterologous expression systems and in human primary respiratory epithelial cells obtained from CF patients homozygous for the deltaF508 mutation. Our observations indicate that the RXR motif proximal to the signature sequence in NBD1 is aberrantly exposed in the major mutant, and modification of its accessibility in situ will promote improved surface expression of the major CF-causing mutant.

RESULTS

Transduction of a Synthetic Peptide Predicted to Disrupt RXR-Mediated Trafficking Modifies the Functional Expression of DeltaF508 at the Surface of Stably Transfected Cells

A potential role for diarginine-based ER retention motifs in mistrafficking of deltaF508-CFTR was revealed using a mutagenesis approach in which the consequences of disrupting each motif were determined (Chang et al., 1999). The goal of the current study was to determine the role for these motifs in the context of the deltaF508-CFTR protein with its native sequence, by delivering peptides that should interfere with their function in a specific and targeted manner. Previous studies by Zerangue and colleagues demonstrated that the molecular context of RXR motifs dictates their efficiency as ER retention signals (Zerangue et al., 2001). Using high-throughput screening of a peptide combinatorial library, the sequence TLRKRIR was scored as a strong ER retention sequence whereas ENRLRLQ was scored as a relatively weak ER retention sequence (Zerangue et al., 2001). One would therefore predict that intracellular delivery of the TLRKRIR peptide should be more effective than the delivery of the ENRLRLQ peptide in antagonizing the retention of deltaF508-CFTR mediated by exposed RXR motifs.

To enable intracellular delivery, the two synthetic peptides encoding the RXR motifs described above were conjugated to cell-penetrating peptides (CPPs) based on the Tat transduction module (Kaplan et al., 2005). CPPs have been used extensively to deliver peptides and larger cargo (Kaplan et al., 2005; Snyder and Dowdy, 2004) and we confirmed by flow cytometry that fluorescently (AlexaFluor-488) labeled CPP-linked peptides were effectively taken up by approximately 95% of BHK-deltaF508-3HA cells (see Figure S1 available online). The intracellular localization of the peptides was also verified by fluorescence microscopy (Figure 1B).

The effect of CPP-conjugated TLRKRIR (CPP-TLRKRIR) or ENRLRLQ (CPP-ENRLRLQ) on the functional expression of deltaF508-CFTR was monitored using HEK293MSR-GT cells stably expressing deltaF508-CFTR and transfected with the halide-sensitive mutant version of the yellow fluorescent protein (YFP-H148Q/I152L) initially described by Pedemonte and colleagues (Galietta et al., 2001; Pedemonte et al., 2005). An increase in YFP quenching upon addition of NaI (52 mM) is indicative of an increase in deltaF508-CFTR function at the cell surface. In this previously published work, pretreatment of cells stably expressing deltaF508-CFTR with Corrector 4a for 24 hr led to a significant increase in the functional expression of deltaF508-CFTR at the cell surface. We reproduced this "correction," which is shown in Figure 2A. Addition of the strong ER retention sequence CPP-TLRKRIR (1 μM) for 24 hr to HEK293MSR-GT-deltaF508

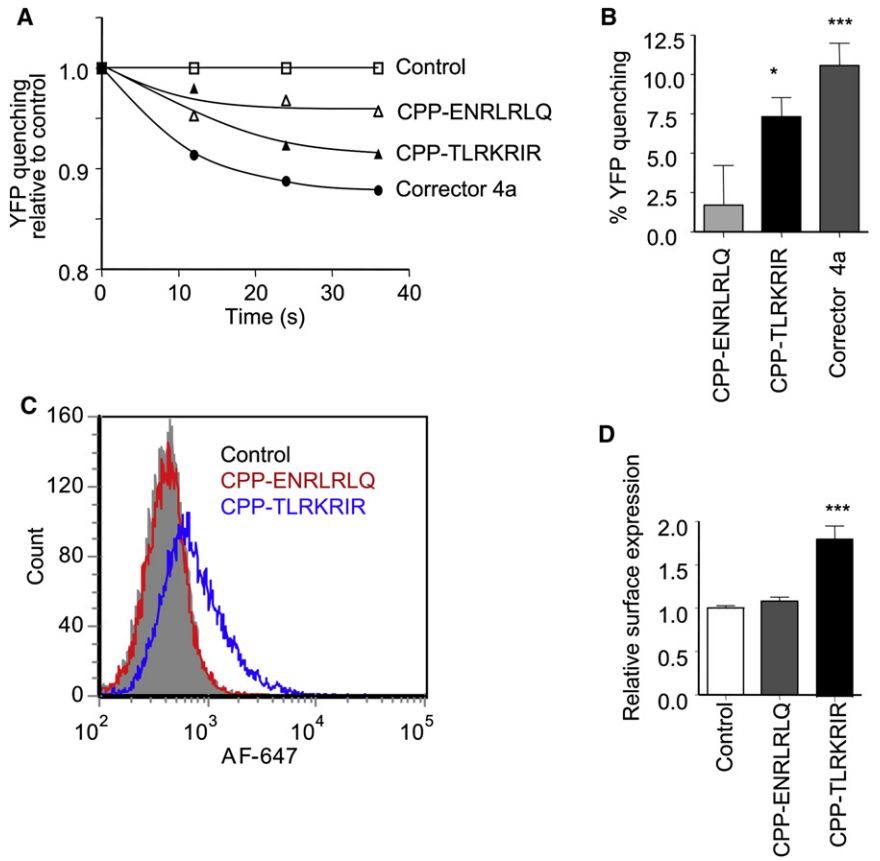

Figure 2. Synthetic Peptides Encoding RXR Motifs Modulate DeltaF508-CFTR Trafficking

(A) Curves show quenching of yellow fluorescent protein (YFP) after NaI addition to HEK-fibroblasts stably expressing deltaF508-CFTR and transiently expressing enhanced YFP. Prior to NaI addition, mutant CFTR had been activated using a cocktail of cAMP agonists plus genistein. Cells were pretreated with CPP-conjugated RXR peptides, the corrector 4a compound, or vehicle alone (control) for 24 hr.

(B) Bars show mean YFP quenching in wells exposed to peptides or Corrector 4a relative to control (vehicle treated) (n = 7). CPP-TLRKRIR (1 μM) and 1 μM corrector 4a significantly increased YFP quenching at 36 s by 7.3 ± 1.2% (*$p < 0.05$) and 10.6 ± 1.4% (***$p < 0.001$), respectively. Mean ± SEM are shown, and statistical significance was assessed using ANOVA and a secondary Bonferroni test of certain data set pairs.

(C) Analysis of surface expression of deltaF508-CFTR bearing exofacial 3HA tag by flow cytometry. A representative histogram of cells treated with CPP-TLRKRIR (blue), CPP-ENRLRLQ (red) versus untreated BHK-deltaF508-3HA cells (shaded gray).

(D) Bars show mean change in surface expression in multiple studies (n = 5). CPP-TLRKRIR [1 μM] significantly increases relative cell surface expression 1.8 ± 0.1-fold (***$p < 0.001$). Mean ± SEM are shown, statistical significance assessed using ANOVA and a secondary Bonferroni test of certain data set pairs.

cells transfected with the mutant YFP also led to a significant (7.3 ± 1.2%) increase in the rate of YFP quenching (*$p < 0.05$), whereas the weak ER retention sequence CPP-ENRLRLQ failed to elicit a significant response (Figures 2A and 2B). The differential effect of CPP-TLRKRIR and CPP-ENRLRLQ peptides supports the utility of this transduction system to specifically target known protein interaction motifs and suggests that antagonism of the interaction between deltaF508-CFTR and the ER retention machinery modulates the trafficking of the major mutant. Interestingly, the success of the CPP-TLRKRIR in promoting functional rescue of deltaF508-CFTR was comparable to that of Corrector 4a (1 μM), a compound previously shown to be an effective corrector in this assay (Pedemonte et al., 2005) (Figure 2B).

In order to determine directly if the trend for increased functional expression following treatment with peptides bearing the strong retention motif reflected an absolute increase in deltaF508-CFTR trafficking to the surface, we studied cells stably expressing deltaF508-CFTR bearing an exofacial triple-HA tag (Du et al., 2005) by flow cytometry. The total deltaF508-CFTR protein trafficking to the cell surface was assessed after treatment with vehicle alone (control), or with CPP bearing the weak ER retention motif (1 μM) or the strong ER retention motif (1 μM), as detected using anti-HA antibody and secondary antibody conjugated with AlexaFluor-647 (AF-647). Treatment of cells with CPP-TLRKRIR (1 μM, blue line) increased the relative cell surface expression of deltaF508-CFTR by 1.8 ± 0.1-fold (***$p < 0.001$), whereas CPP-ENRLRLQ (1 μM, red line) caused no significant increase in surface expression of the mutant protein relative to control samples (shaded gray) (Figures 2C and 2D). The results of this direct assay of surface expression support the findings of the YFP quenching assay, and show that there is a differential effect of the "weak" and "strong" motif-mimetic peptides on trafficking of deltaF508-CFTR, consistent with their predicted efficacies in competing ER retention mechanisms.

Transduction of a Peptide Containing Endogenous RXR Motif Proximal to the ABC Signature Motif of NBD1 (CF-RXR) Increases DeltaF508-CFTR Function by Promoting Cell Surface Trafficking

We then asked if delivery of a synthetic peptide bearing an RXR motif endogenous in the CFTR protein and previously implicated in ER retrieval of deltaF508-CFTR (Chang et al., 1999; Hegedus et al., 2006; Teem et al., 1993, 1996) was also effective in modulating trafficking of the mutant protein. The current studies focus on the RXR motif residing in NBD1, proximal to the ABC signature motif and starting at the arginine at position 553, as it has been implicated in ER retrieval of deltaF508-CFTR with mutations R553M/Q or R555K leading to the enhanced surface expression of the major mutant (Teem et al., 1993, 1996). We therefore predict that transduction of a peptide containing this CF-specific RXR motif (GGQRARISLARAVYK) should effectively antagonize the retention of deltaF508-CFTR if this region is aberrantly exposed in the mutant protein.

To assess deltaF508-CFTR trafficking and function in the presence of CPP-conjugated CF-RXR (CPP-CF-RXR), we

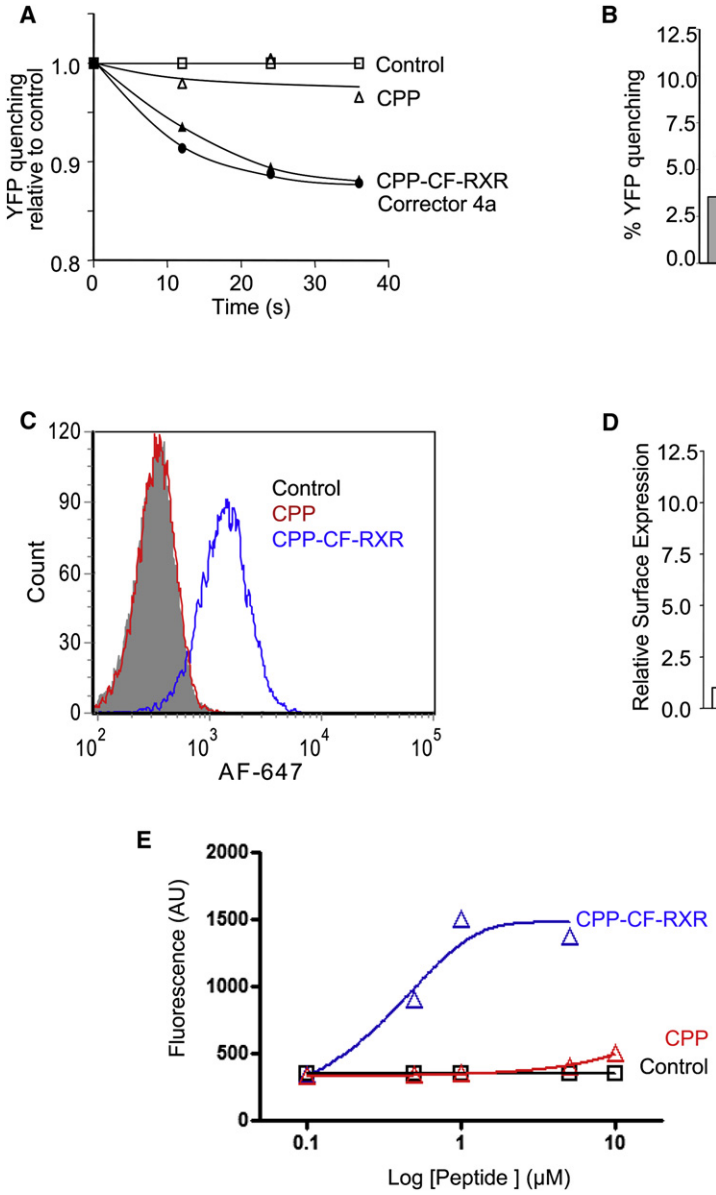

Figure 3. CPP-CF-RXR Enhances DeltaF508-CFTR Trafficking

(A) Representative trace of YFP quenching detected in cells treated with CPP, CPP-CF-RXR, and corrector 4a relative to control (vehicle-treated) cells.

(B) Bars show mean YFP quenching (±SEM, n = 8). CPP-CF-RXR (1 μM) and 1 μM corrector 4a significantly increased YFP quenching at 36 s by 10.7 ± 1.7% (***$p < 0.001$) and 10.6 ± 1.4% (***$p < 0.001$), respectively. Statistics were processed using ANOVA and a secondary Bonferroni test of certain data set pairs.

(C) Representative histograms of cells expressing deltaF508-CFTR-3HA at the cell surface, as detected using anti-HA monoclonal and Alexa-647-labeled anti-mouse secondary antibody following pretreatment with CPP-CF-RXR (blue), CPP (red), or untreated BHK-deltaF508-3HA cells (shaded gray) as analyzed by flow cytometry.

(D) Bars show mean ± SEM (change in surface expression following different peptide treatments in multiple experiments [n = 8] relative to control). CPP-CF-RXR (1 μM) significantly increased relative cell surface expression 10.0 ± 2.4-fold (**$p < 0.01$). Statistics were processed using ANOVA and a secondary Bonferroni test of certain data set pairs.

(E) Concentration-dependent effects of CPP-CF-RXR exhibit saturation (blue, single site binding algorithm, EC_{50} = 0.48 μM, r^2 = 0.94). The carrier (CPP, red) alone does not increase surface expression of deltaF508-CTFR relative to control (no treatment).

performed both the YFP quenching assay and flow cytometry assays as described earlier for assessment of CPP-TLRKRIR and CPP-ENRLRLQ. In Figures 3A and 3B, we show that delivery of the CPP-CF-RXR (1 μM) induced a significant 10.7 ± 1.7% (C***$p < 0.001$) increase in the functional expression of deltaF508-CFTR (detected as YFP quenching), an increase comparable to that evoked by the Corrector 4a (1 μM), (10.6 ± 1.4%). In contrast, cells treated with CPP alone showed no significant increase in YFP quenching relative to untreated samples. Although the magnitude of quenching cannot be directly correlated with halide flux because there is a nonlinear relationship between these two parameters (Galietta et al., 2001), the CPP-CF-RXR peptide is clearly effective in promoting the functional expression of deltaF508-CFTR.

The change in functional expression of deltaF508-CFTR evoked by CPP-CF-RXR treatment was associated with its enhanced localization at the surface because there was increased presentation of its exofacial 3HA tag in deltaF508-CFTR as detected by an anti-HA antibody and a fluorescent secondary antibody by flow cytometry. Addition of CPP-CF-RXR (1 μM, blue) for 3 hr induced a significant increase in deltaF508-CFTR cell surface expression by 10.0 ± 2.4-fold (*$p < 0.01$) relative to CPP-treated (red) or untreated (shaded gray) cells (Figures 3C and 3D). The ability of the CPP-CF-RXR peptide to increase the cell surface expression of deltaF508-CFTR-3HA (as determined by flow cytometry) exhibits a saturable concentration dependence with an estimated EC_{50} of 0.48 μM peptide (Figure 3E).

In order to compare the level of correction in the functional expression of deltaF508-CFTR mediated by the synthetic CPP-CF-RXR peptide to the functional expression of the wild-type CFTR protein, we compared two BHK cell lines: one stably expressing the major mutant and the other, wild-type CFTR. Treatment of BHK cells expressing the mutant protein with CPP-CF-RXR peptide (1 μM) for 1.5 hr led to a significant 2.9 ± 0.5-fold (**$p < 0.01$) increase in the rate of normalized halide transport relative to untreated samples (Figures 4A and 4B), and represents approximately 30% of the cyclic-AMP activated iodide efflux measured for wild-type CFTR expressed in the

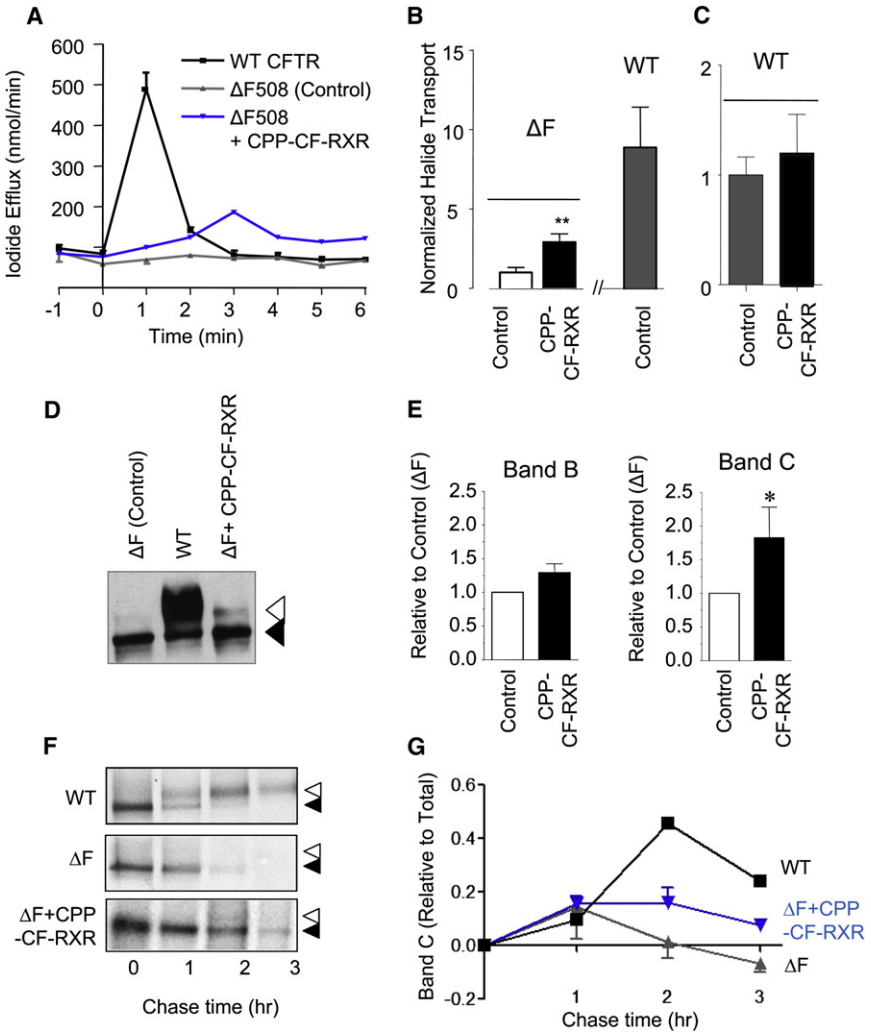

Figure 4. CPP-CF-RXR Enhances Functional Expression of DeltaF508-CFTR to 30% Levels of Normal CFTR and Enhances N-Glycan Processing

(A) Representative iodide efflux response to cAMP stimulation cocktail of BHK cells expressing wild-type CFTR or deltaF508-CFTR (ΔF508) (mean ± SEM of triplicate experiments). Agonist cocktail induces peak in the rate of iodide efflux from cells expressing wild-type CFTR but not deltaF508-CFTR unless pretreated with 1 μM CPP-CF-RXR for 1.5 hr (or 3 hr: 224 ± 79 nmol/min).

(B) Bars show mean ± SEM peak iodide efflux rates in multiple comparative experiments of deltaF508-CFTR (n = 4), or wild-type CFTR (n = 4). Efflux rates normalized relative to control rates determined for cells expressing deltaF508-CFTR. CPP-CF-RXR (1 μM) significantly enhances cAMP-dependent deltaF508-CFTR function by 2.9 ± 0.5-fold (**$p < 0.01$, paired t test) over untreated (control) cells, to achieve approximately 30% of the cAMP response exhibited by wild-type CFTR in a distinct set of studies (9 ± 2.5-fold over control).

(C) Bars show lack of effect of 1 μM CPP-CF-RXR peptide on functional expression of wild-type CFTR where efflux rates are normalized relative to control values in untreated cultures expressing wild-type CFTR (n = 7, paired t test).

(D) Western analysis of deltaF508-CFTR maturation. DeltaF508-CFTR protein migrates primarily as core glycosylated 150 kDa protein (band B, solid triangle), and wild-type CFTR migrates primarily as complex glycosylated 170-180 kDa protein (band C, open triangle). CPP-CF-RXR (10 μM) for 3 hr at 37°C stabilizes band B (closed arrow) and increases maturation of band C (open arrow).

(E) Quantification of band B and band C in multiple studies (n = 4). Mean ± SEM are shown. Statistical significance was assessed using an unpaired t test (*$p < 0.05$).

(F) Top panel shows conversion of ^{35}S-methionine pulse-labeled precursor form of wild-type CFTR (band B, solid triangle, time 0 hr) to mature form (band C, empty triangle) after chase times shown. Cells were starved of methionine, pulsed for 15 min with ^{35}S-methionine + ^{35}S-cysteine labeling mix (150 μCi/ml), and chased for 0, 1, 2, or 3 hr in methionine-containing medium. Middle panel shows failure of conversion for deltaF508-CFTR. Lower panel shows partial conversion for deltaF508-CFTR following a 2 hr pretreatment with CPP-CF-RXR (10 μM, as final concentration) at 37°C followed by subsequent treatment with 1 μM CPP-CF-RXR in the chase media. This protocol was developed to maintain total peptide exposure during the entire pulse-chase experiment at levels comparable to biochemical experiments shown in (d). Control samples (pretreatment with a similar volume of the peptide vehicle, PBS) were not significantly different than deltaF508-CFTR untreated (data not shown).

(G) Biosynthetic maturation efficiency for different chase times was determined as the intensity of ^{35}S-methionine incorporated band C relative to the total amount of ^{35}S-methionine band B precursor at time 0 hr. Symbols show mean data (±SEM) from deltaF508-CFTR (gray triangles, three independent trials) and CPP-CF-RXR-pretreated deltaF508-CFTR (inverted blue triangles, three independent trials). At 3 hr of chase, there is a significant difference in maturation efficiency resulting from peptide pretreatment (unpaired t test, $p < 0.01$). Black squares show mean maturation efficiency of wild-type CFTR over time for two independent trials.

same cell type. We also investigated the effect of treating deltaF508-CFTR with CPP-CF-RXR for 3 hr (so as to be consistent with flow cytometry assays) and found that the efflux measured was similar to that produced from a 1.5 hr peptide treatment (data not shown). The delay in the peak iodide efflux of the CPP-CF-RXR-treated deltaF508-CFTR-expressing cells relative to the peak observed in cells expressing wild-type CFTR has been observed for pharmacological correctors and likely reflects the defect in gating kinetics exhibited by the mutant protein (Clarke et al., 2004; Sheppard et al., 1993). In Figure 4C, we show that treatment of cells expressing wild-type CFTR with CPP-CF-RXR (n = 7) fails to promote a significant increase in the relative functional expression of CFTR at the cell surface relative to control (n = 7). These findings suggest that this peptide is targeting a molecular recognition site that is aberrantly exposed in the mutant, deltaF508-CFTR.

DeltaF508-CFTR migrates primarily as a 140–150 kDa protein (band B) when analyzed by SDS-PAGE, whereas the normal protein migrates primarily as a 170–180 kDa protein (band C). The differential migration of the mutant protein reflects its relative retention in the ER and failure to traffic to the Golgi where complex glycosylation is conferred to generate the mature

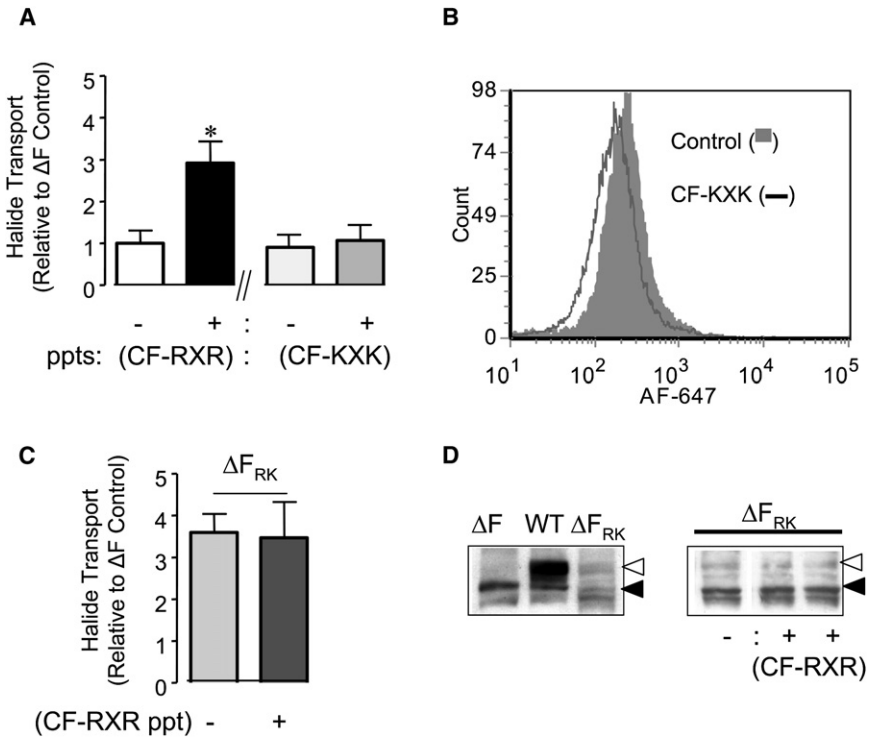

Figure 5. Functional Rescue of DeltaF508-CFTR by CF-RXR Is Sequence Specific and Mediated by Interfering with Endogenous RXR

(A) Bars show mean cAMP-dependent activation in BHK cells expressing deltaF508-CFTR with (+, n = 4) or without (−, n = 4) peptide (ppt) pretreatment for 1.5 hr. Data obtained using the CF-derived peptide bearing the endogenous motif and coupled to the carrier (CPP): CF-RXR is shown for comparison [2.9 ± 2.5-fold over control (*$p < 0.01$)] with the mutated CF-derived peptide wherein arginines are replaced with lysines: CF-KXK. No statistically significant change was stimulated by the mutant peptide. Mean ± SEM are shown. A paired t test was used to perform statistical analysis.

(B) BHK-deltaF508-3HA cells failed to show enhanced surface expression after treatment with CPP-coupled CF-KXK peptides (dark gray) relative to untreated cells (shaded gray), as determined using flow cytometry.

(C) Bars show cAMP-activated iodide efflux by deltaF508-CFTR protein bearing a mutant $R^{553}XR^{555}$ wherein arginine residues are mutated to KXK (ΔF_{RK}). Treatment of cells expressing ΔF_{RK} with CF-RXR peptide (ppt, n = 3) caused no additional cAMP response (paired t test).

(D) Western blotting of deltaF-CFTR (ΔF), wild-type CFTR, and deltaF-CFTR with mutation of $R^{553}XR^{555}$ to KXK (ΔF_{RK}) shows partial correction of processing defect of deltaF508-CFTR caused by genetic modification of RXR with increase in band C generation (open triangle). As expected for effect mediated by $R^{553}XR^{555}$, the CF-RXR peptide (+, 10 μM) fails to cause an additive effect on band C (open triangle) generation in cells expressing deltaF508-CFTR (KXK) (right panel).

form of the protein. We reasoned that if CPP-CF-RXR is acting to modulate trafficking of deltaF508-CFTR by antagonizing its ER retention, we would detect enhanced Golgi-dependent glycosylation. As shown in Figures 4D and 4E, treatment of BHK-deltaF508-3HA with CPP-CF-RXR (10 μM) for 3 hr at 37°C led to a significant 1.8 ± 0.4-fold (*$p < 0.05$) enhancement in the appearance of complex glycosylated band C (open arrow). Interestingly, treatment of cells with CPP-CF-RXR peptide at the lower concentration of 1 μM, the concentration that is effective in promoting functional cell surface expression as detected by flow cytometry and halide flux assays, does not consistently lead to the enhanced appearance of band C. Rather, the requirement for the higher concentration of 10 μM peptide suggests that this biochemical assay is less sensitive than functional analyses or flow cytometry in detecting surface expression of the major mutant. The relative insensitivity of western blotting and detection of band C production relative to direct measures of surface expression (similar to the flow cytometry assay) has been observed previously (Pedemonte et al., 2005).

We performed kinetic pulse-chase experiments to directly test our hypothesis that the CPP-CF-RXR peptide promotes forward trafficking of newly synthesized deltaF508-CFTR to the Golgi. First, we confirmed previous studies showing that the trafficking of newly synthesized deltaF508-CFTR protein from the ER to the Golgi (conversion from the band B to band C form of the protein) is impaired relative to the wild-type CFTR protein (Figures 4F and 4G). As in previous publications by Riordan and colleagues (Chang et al., 1999), we found no conversion to band C for deltaF508-CFTR over the 3 hr chase period of ^{35}S-methionine labeled protein. However, 30%–40% of wild-type CFTR protein undergoes conversion during this time. These values are consistent with several publications (Chang et al., 1999; Pedemonte et al., 2005; Van Goor et al., 2006). Treatment of deltaF508-CFTR-expressing cells with CPP-CF-RXR (10 μM) at 37°C led to a modest (15%) but significant increase in conversion of band B to band C in three independent trials. This result is consistent with studies by Riordan and colleagues (Chang et al., 1999) wherein mutagenesis of intrinsic RXR-based retention motifs led to a ~10% increase in maturation efficacy of deltaF508-CFTR. Therefore, these kinetic data support the hypothesis that the CPP-CF-RXR peptide modulates the trafficking of deltaF508-CFTR through the biosynthetic pathway.

Next, we were prompted to determine if this peptide is acting to correct mistrafficking of deltaF508-CFTR by interfering with ER retention mediated by the endogenous $R^{553}XR^{555}$ motif in deltaF508-CFTR. First, we synthesized a mutant peptide, wherein the arginines were replaced by lysines (CF-KXK). Substitution of one of the two arginines in the motif was previously shown to inactivate this retention motif (Chang et al., 1999; Hegedus et al., 2006; Teem et al., 1996). In Figure 5A, we show that 1 μM CF-KXK (GGQKAKISLARAVYK conjugated to the same CPP as the CF-RXR peptide) was ineffective in causing rescue of the functional expression of deltaF508-CFTR, in contrast to the previous results obtained for CF-RXR. Furthermore, we found that CF-KXK (1 μM) also failed to cause an increase in the surface expression of deltaF508-CFTR bearing an exofacial triple-HA tag (Figure 5B). To further probe the specific activity of the exogenous CF-RXR peptide on function

of the endogenous $R^{553}XR^{555}$ motif in deltaF508-CFTR, we constructed a deltaF508(RK)-CFTR mutant, whereby the arginines at position R553 and R555 were mutated to lysines. We predict that if our CPP-CF-RXR peptide is specifically mediating rescue of deltaF508-CFTR by interfering with interactions occurring between aberrantly exposed RXR motifs and the ER retention machinery, then the CPP-CF-RXR peptide should exert no effect on deltaF508(RK)-CFTR. As expected on the basis of previous studies (Teem et al., 1993, 1996), we found that deltaF508(RK)-CFTR exhibits partial maturation to the complex glycosylated form of the protein (Figure 5D). Furthermore, fraction of deltaF508(RK) at the surface is at least partially functional because it mediates greater cAMP-dependent halide flux than deltaF508-CFTR (as determined using the iodide efflux assay, and analyzed by unpaired t test, $^{**}p < 0.01$). Interestingly, the rate of iodide efflux mediated by the deltaF508(RK)-CFTR mutant was similar to that observed for deltaF508-CFTR treated with CPP-CF-RXR peptide (Figures 5A and 5C, unpaired t test). Importantly, treatment of the deltaF508(RK)-CFTR mutant construct with CPP-CF-RXR peptide (1 μM) for 1.5 hr failed to increase the rate of cAMP-dependent iodide efflux over the untreated sample (Figure 5C). Furthermore, even relatively high concentrations of the CPP-CF-RXR peptide (10 μM) did not cause an increase in deltaF508(RK)-CFTR processing to the band C form of the protein (Figure 5D, right panel), in contrast to its effect on the native deltaF508-CFTR sequence (Figure 4D). Together, these studies support our claim that the cellular delivery of CPP-CF-RXR causes partial correction of the trafficking defect of deltaF508-CFTR by antagonizing the effects of this motif.

Because certain pharmacological small-molecule modulators exhibit dual activity and rescue the trafficking defect of deltaF508-CFTR as well as directly enhance or potentiate channel activity (Loo et al., 2006), we were prompted to assess the activity of the CPP-CF-RXR peptide as a potentiator. For these studies, BHK cells stably expressing deltaF508-CFTR were first subjected to biosynthetic rescue by incubation at 27°C for 24 hr, and the effect of the addition of CPP-CF-RXR (1 μM) on surface-expressed and cAMP-activated deltaF508-CFTR protein was assessed using the iodide efflux assay. We found that the rate of halide (iodide) efflux was not significantly altered by 1 μM CPP-CF-RXR treatment: efflux rate 180 ± 42 (standard error of the mean [SEM]) nmol/min (n = 3) versus 120 ± 18 (SEM) nmol/min (n = 3) (p = 0.28, unpaired t test) for control samples. Therefore, these findings suggest that CPP-CF-RXR is not acting as a potentiator, but rather it rescues functional expression through its effect on trafficking.

CPP Transduction Mediates Epithelial Uptake of Peptide Cargo and the CF-RXR Peptide Partially Rescues the Functional Expression of DeltaF508-CFTR Expressed in Human Bronchial Epithelial Cell Monolayers

We proceeded to investigate the consequences of peptide treatment in primary cultures of human bronchial epithelia obtained from patients homozygous for the deltaF508 mutation. These cells were grown and maintained in the presence of an air-liquid interface on collagen coated inserts to form polarized and fully differentiated monolayers (Farmen et al., 2005). Because multiple monolayers were generated from a single patient, the consequences of peptide treatment could be determined

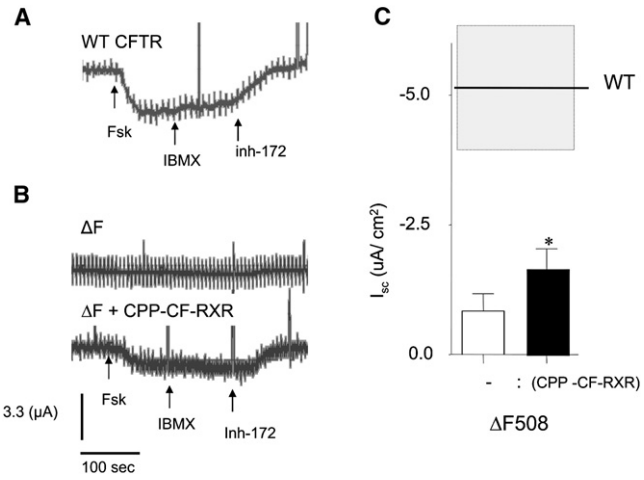

Figure 6. CPP-CF-RXR Partially Rescues Functional Expression of DeltaF508-CFTR in Airway Epithelia

(A) Representative short circuit current traces on bronchial epithelial monolayers obtained from non-CF control patients. Both apical and basolateral epithelial surfaces exposed to physiological salt solutions (containing 137 mM NaCl) with endogenous ENaC currents inhibited by amiloride (100 μM) in the apical compartment. CFTR currents were activated by 20 μM forskolin and 500 μM IBMX. Activated currents are inhibited by the CFTR-specific inhibitor CFTRinh-172 (5 μM).

(B) Representative traces of currents mediated by monolayer of primary bronchial epithelial cells generated from tissue obtained from a patient homozygous for the deltaF508-CFTR mutation, treated with vehicle alone or CPP-CF-RXR (1 μM) for 3 hr. DeltaF508-CFTR currents in peptide treated epithelium are activated by agonists of cAMP and inhibited by inhibitor CFTRinh-172.

(C) Bars show mean ± SEM short circuit current responses to cAMP agonists in paired studies of vehicle or peptide (CPP-CF-RXR) treated bronchial monolayers derived from CF patients bearing the deltaF508-CFTR mutation (n = 9 pairs). CPP-CF-RXR (1 μM) significantly increased forskolin and IBMX response 2-fold ($^{*}p < 0.05$) relative to untreated monolayers. Statistical analysis was performed using a paired t test. Solid line above bars shows the mean cAMP-dependent response by non-CF monolayers, with the gray rectangle representing the SEM (n = 8).

relative to control (vehicle-alone) treatments for each patient in a pairwise fashion, thereby eliminating the influence of patient-to-patient variability. These inserts were treated with 1 μM CPP-CF-RXR on both apical and basolateral sides for 3 hr (control samples were treated with a similar volume of the vehicle for peptide). The inserts were mounted into Ussing chambers and studied in the presence of physiological chloride solutions under voltage clamp conditions. ENaC currents were inhibited using 100 μM amiloride and non-CFTR chloride channels were inhibited with 250 μM 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS). CFTR-associated chloride currents were stimulated with 20 μM forskolin and 500 μM 3-isobutyl-1-methylxanthine (IBMX) and inhibited using 5 μM CFTR-inh172, a specific CFTR inhibitor (Ostedgaard et al., 2002; Pedemonte et al., 2005; Van Goor et al., 2006) (Figure 6B). We examined a total of nine monolayers from three different patients. As previously mentioned, the studies were performed in a paired fashion so that the effect of peptide treatment was compared with vehicle alone on monolayers obtained from the same patient. As shown in Figure 6C, monolayers treated with 1 μM CPP-CF-RXR for 3 hr displayed an almost 2-fold ($^{*}p < 0.05$) increase in the forskolin and IBMX

response (−1.6 ± 0.4 μA/cm^2) relative to non-peptide-treated controls (−0.8 ± 0.3 μA/cm^2). The results from comparative studies using monolayers obtained from subjects with normal CFTR are also shown in Figures 6A and 6C. On the basis of these comparative studies, we estimate that the CPP-CF-RXR-treated deltaF508-CFTR monolayers have approximately 30%–40% of normal CFTR chloride channel activity. These findings suggest that the CPP is taken up by epithelial tissues, and further that the cell surface expression of deltaF508-CFTR can be modulated specifically in human respiratory epithelial cells by delivering a peptide designed to antagonize a specific recognition motif for ER retention.

DISCUSSION

Much of our current understanding of the molecular mechanisms that underlie mistrafficking of the major CF-causing mutant deltaF508-CFTR has been developed through genetic manipulation of protein components in the biosynthetic compartment and/or mutagenesis of the deltaF508-CFTR itself. However, mutational analyses of ER trafficking motifs might introduce changes in the deltaF508-CFTR protein in addition to the intended disruption of a trafficking event. Because second-site mutations introduced in the context of deltaF508-CFTR protein might induce conformational changes (Pissarra et al., 2008), it is important to verify that the mechanisms identified in the previous studies are functional for the native deltaF508-CFTR protein (without second site mutations) in their appropriate cell and tissue context. Such studies require the intracellular delivery of targeted probes. We show here that peptides designed to mimic RXR ER retention motifs in a suitable sequence specific context, and delivered intracellularly via a cell penetrating transduction module, can function as such targeted probes. These peptides modified trafficking of deltaF508-CFTR in a sequence-specific manner. Furthermore, our observations allow us to conclude that RXR-based ER retention motifs are aberrantly exposed in the disease-causing mutant, bearing the single deletion mutation of phenylalanine 508. Interestingly, the RXR motif proximal to the signature motif of NBD1 of the major mutant plays a key role as a recognition element leading to its ER retention.

We conclude that the motif mimetic peptide described in this study, CF-RXR, caused an increase in the functional expression of deltaF508-CFTR by interfering with its retention in the ER. This conclusion is based on several observations. First, the treatment of cell lines stably expressing the mutant protein with the CF-RXR peptide enhanced the maturation efficiency of deltaF508-CFTR as assessed in kinetic pulse chase experiments and in studies of steady-state band C expression. An increase in its expression as a regulated chloride channel at the cell surface was also observed within this time frame in primary airway epithelial monolayers from patients homozygous for this mutation. Furthermore, removal of the endogenous ER retention motif ($R^{553}XR^{555}$) in *cis* with deltaF508-CFTR abolished the effect of the motif mimetic peptide on Golgi modification, surface expression, and function at the cell surface in the heterologous expression system mentioned above. Our halide efflux studies using temperature-corrected deltaF508-CFTR showed that these peptides did not act as potentiators of the channel activity of deltaF508-CFTR protein. Together, these findings suggest that the CF-RXR peptide partially rescued the mistrafficking of deltaF508-CFTR.

The molecular basis for the exceptional effect of the CFTR-derived peptide (CF-RXR) relative to the strong hypothetical ER retention signal peptide identified from a combinatorial library by high-throughput screen (TLRKRIR) (Zerangue et al., 2001) remains to be fully elucidated. Our findings indicate that this motif might be aberrantly exposed in the mutant protein and plays a key role in ER retention. Thus, in the context of the full-length wild-type CFTR protein, the $R^{553}AR^{555}$ motif on NBD1 likely contributes to intramolecular interactions between NBD1 and NBD2 or MSD2 (He et al., 2008; Mense et al., 2006; Serohijos et al., 2008; Vergani et al., 2005). However, with full-length deltaF508-CFTR protein, our findings suggest that this particular motif is exposed, consistent with recent biochemical data showing that normal intramolecular interactions are perturbed in the major mutant (Du et al., 2005; Loo et al., 2008; Serohijos et al., 2008). Interestingly, although protein fragments corresponding to NBD1 of CFTR alone are known to be susceptible to aggregation, the introduction of so-called solubilizing mutations in this domain helps to prevent this process. Both arginines at positions 553 and 555 are mutated to enhance solubility of the human NBD1 protein fragment (Lewis et al., 2005). These observations suggest that, if exposed, the arginine residues in these positions might be predisposed to promote non-native protein-protein interactions.

With respect to the molecular basis for ER retention via retrograde trafficking of deltaF508-CFTR, the COPI coatomer protein complex has been implicated in retrograde trafficking of the limited number of previously studied cargo proteins, including the misassembled ATP-sensitive K^+ channel (Zerangue et al., 1999), $GABA_B$ receptor (Margeta-Mitrovic et al., 2000), NMDA receptor (Scott et al., 2001; Standley et al., 2000), ROMK channel (Yoo et al., 2005), and the Kainate receptor (Jaskolski et al., 2004). An interaction between the β-COP subunit and deltaF508-CFTR was determined in a heterologous expression system following chemical crosslinking and analysis by mass spectrometry (Wang et al., 2006). Small interfering RNA-mediated knockdown of certain components of the COPI pathway was shown to affect anterograde trafficking of CFTR (Rennolds et al., 2008; Szul et al., 2007). These overall findings suggest that the COPI complex might not have a primary role in mediating retrograde transport of deltaF508-CFTR or that particular coatomer subunits, not as yet studied, contribute to this function. Therefore, identification of the interaction partner (or partners) for the aberrantly exposed RXR motif in NBD1 that mediates ER retention remains a priority for future study.

A further dimension to the present work is our demonstration of the feasibility of delivering specific cargo into CF-affected epithelia using a cell-penetrating-based peptide transduction domain (CPP). The efficacy of this delivery module has been documented extensively, not only for a wide range of cargo types (DNA, RNA, and protein), but also in various tissues in in vitro and in vivo experiments (Duchardt et al., 2007; Henriques et al., 2006; Kaplan et al., 2005; Potocky et al., 2003; Snyder and Dowdy, 2004; Wadia et al., 2004). A wide variety of diseases are effectively treated in mouse models using CPP-protein conjugates. For example, TAT-survivin, which competes or antagonizes the interaction between Hsp90 and the survivin

protein and causes apoptosis when taken up by tumor cells, shows promise in the treatment of prostate, breast, leukemia, melanoma, and glioma in mice (Ma et al., 2006). The present studies, together with a recent publication by Frizzell and colleagues (Sun et al., 2008), suggest that peptide or protein transduction might provide a useful strategy for targeting aberrant intermolecular interactions with deltaF508-CFTR. Furthermore, although the peptide employed in the current study requires optimization for efficacy and bioavailability, it might provide the template for designing correctors of defective deltaF508 trafficking. Interestingly, a CPP-conjugated δPKC inhibitor peptide completed phase 2 clinical studies for the treatment of a distinct clinical condition, acute myocardial infarction, with promising results (Bates et al., 2008). There is, accordingly, significant promise for the potential application of this approach in therapy development.

SIGNIFICANCE

The major CF-causing mutation, deletion of phenylalanine in position 508, leads to misfolding, misassembly and mistrafficking of the CFTR protein with its retention in the ER. Despite these defects, mistrafficking of the protein can be partially corrected by various manipulations, including treatment with chemical chaperones, and its function detected on the cell surface. These findings suggest that effective pharmacological treatment of patients bearing this mutation might be feasible. Ideally, interventions that promote proper trafficking of deltaF508-CFTR could be developed if the structural features that confer ER retention are defined. Prior to this current study, such features have typically been probed by mutagenesis that might cause secondary conformational changes not normally existing in the mutant protein in situ. In this paper, we introduce a novel peptide-based approach that targets an ER retention motif that ostensibly becomes aberrantly exposed in the deltaF508-CFTR protein. The results of the current study support the role for diarginine motifs—in particular a diarginine motif proximal to the signature motif in NBD1—in conferring a molecular recognition element that contributes to mistrafficking of the major mutant in situ. This structural feature might thus comprise a molecular target for effective CF therapies.

EXPERIMENTAL PROCEDURES

Cell Lines and Synthetic Peptides

Baby hamster kidney (BHK) cells stably expressing deltaF508-CFTR bearing an exofacial triple-HA tag (BHK-deltaF508-3HA) or wild-type CFTR bearing the exofacial triple HA tag (BHK-CFTR-3HA) obtained from Dr. G.L. Lukacs were maintained as described (Pedemonte et al., 2005). Griptite 293 MSR cells stably expressing deltaF508-CFTR (293MSR-GT-deltaF508) obtained from Dr. D. Rotin were maintained in Dulbecco's modified Eagle's medium (DMEM) media (MultiCell) with 10% fetal bovine serum (FBS) (Wisent), 0.1 mM NEAA (Invitrogen), 600 μg/ml Geneticin (Invitrogen), and 5 μg/ml Blasticidin (Invitrogen). Human bronchial epithelia cells from transplant patients homozygous for the deltaF508-CFTR mutation (provided by Drs. P. Karp and J. Zabner, manager and director, respectively, of the Iowa Cell Culture Facility) were maintained as described (Zabner et al., 1996; Zhang et al., 2004). DeltaF508(R553/555K) construct was synthesized via site-directed mutagenesis.

Peptides were synthesized by Dr. N.C. Wang (Advance Protein Technology Centre, Hospital for Sick Children, Toronto). Peptides were conjugated to the CPP TAT derived from HIV1 (Kaplan et al., 2005) to facilitate intracellular delivery (Kaplan et al., 2005; Snyder and Dowdy, 2004).

Halide Flux Assays

YFP Quenching Assay

Briefly, HEK293MSR-GT-deltaF508 cells were seeded (~65,000 cells per well) and transfected with 250 ng YFP-H148Q/I152L (Galietta et al., 2001; Pedemonte et al., 2005) 48 hr before measurement. Cells were treated with 1 μM peptides at 24 hr, and surface channels were stimulated with 25 μM forskolin, 10 μg/ml IBMX, and 50 μM genistein (all chemicals purchased from Sigma). The rate of YFP quenching in the presence of NaI at a final concentration of 52 mM was acquired using Cellomics KineticScan® HCS reader (SIDNET Facility, Research Institute, Hospital for Sick Children). The development of this assay is described elsewhere by Verkman and colleagues (Galietta et al., 2001; Pedemonte et al., 2005) and by Trzcinska-Daneluti et al. (2009). Kinetic analysis was performed using Python analysis software. Analysis of variance (ANOVA) statistical analysis of cumulative quenching results was performed using GraphPad Prism software.

Iodide Efflux Studies

Experiments performed as previously described (Du et al., 2005) using BHK-CFTR-3HA or BHK-deltaF508-3HA cells in the presence or absence of peptide [1 μM] for 1.5 or 3.0 hr. Intracellular cAMP levels were raised using 10 μM forskolin, 1000 μM IBMX, and 100 μM cpt cAMP (all compounds purchased from Sigma) in order to stimulate CFTR activity. An iodide sensitive electrode was used to measure efflux and delta response was calculated as the maximum iodide response after stimulation minus the response before stimulation. Statistics were processed using Prism 4 (GraphPad Software), and an unpaired t test was used to determine significance of treated samples.

Ussing Chamber Studies

Primary human bronchial epithelial cells homozygous for deltaF508-CFTR were provided by the Iowa Cell Culture Facility. Epithelia were cultured on collagen coated membrane permeable inserts as previously described (Zabner et al., 1996). Monolayers were treated (apical and basolateral) with 1 μM peptide for 3 hr at 37°C. Inserts were mounted in an Ussing chamber apparatus (PlexiCraft, Iowa City) and studied under voltage clamp (Physiologic Instruments) conditions as previously described (Ostedgaard et al., 2002; Zabner et al., 1996). The bathing solution, which was added to both apical and basolateral sides, contained 5.4 mM KCl, 0.3 mM $Na_2HPO_4 \cdot 7H_2O$, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 137 mM NaCl, 5.6 mM D-glucose, 1.8 g/l sodium bicarbonate, 1.2 mM $MgCl_2$, and 1.2 mM $CaCl_2$ (pH 7.4). ENaC sodium channels were inhibited with 100 μM amiloride (MacVinish et al., 1997); non-CFTR chloride channels were inhibited with 250 μM DNDS; CFTR currents were stimulated using 20 μM forskolin and 500 μM IBMX (Sigma) and inhibited using 5 μM CFTRinh-172 (provided by CFFT courtesy of Dr. R. Bridges) (Pedemonte et al., 2005). Data were recorded and analyzed using Labview (HSC). A paired t test was performed to compare vehicle-treated and peptide-treated samples using GraphPad Prism software.

Flow Cytometry

BHK-deltaF508-3HA were grown to a density of 10^6 cells/ml and treated with 1 μM peptides for 3 hr at 37°C. To detect the total amount of CFTR reaching the cell surface within the 3 hr incubation period, anti-HA antibody (1/1000) and secondary goat-anti-mouse AlexaFluor647 (1/1000) were incubated with the cells during peptide treatment (Haggie et al., 2006; Jin et al., 2007; Watson et al., 2004). AlexaFluor647 signal was detected using the BD Aria Cell Sorter or LSR II analyzer (λex = 650 nm; λem = 668 nm). Data were analyzed using FlowJo 8.5 software. ANOVA statistical analysis was performed using GraphPad Prism software.

Monitoring N-glycan Status

BHK-deltaF508-3HA were grown to ~70%, confluency and treated with 10 μM peptide. Cells were lysed in a modified RIPA lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, Compete Mini EDTA-free protease inhibitor tablet [Roche]). Protein concentration was determined by Lowry protein assay method and ~40 μg total protein was loaded per sample and separated on SDS-PAGE (6% SDS-polyacrylamide gel). M3A7 (1/1000), goat-anti-mouse (1/1000) antibodies, and an ECL detection kit (Amersham) were used to detect CFTR. An unpaired t test was used to determine significance of glycan processing in treated samples.

Metabolic Labeling and Immunoprecipitation

BHK cells stably expressing deltaF508-CFTR were preincubated with 10 μM CPP-CF-RXR or an equal volume of the vehicle control for 2 hr at 37°C. Metabolic labeling assays were performed as previously described (Lukacs et al., 1994) with the following modifications. Cells were serum starved in DMEM media containing 1 μM peptide or an equal volume of the vehicle (phosphate-buffered saline [PBS]) but lacking methionine and cysteine. Cells were pulsed for 15 min with 150 μCi/ml ^{35}S-methionine + ^{35}S-cysteine labeling mix (Perkin-Elmer). Cells were subsequently washed and chased in media supplemented with 1 mM methionine, 1 mM cysteine, 2 mM glutamine, and 10% FBS in the presence of 1 μM CPP-CF-RXR or an equal volume of vehicle (PBS) for 0–3 hr. This protocol was developed to maintain total peptide exposure during the entire pulse-chase experiment at levels comparable to other biochemical experiments. At each time point, cells were harvested in RIPA lysis buffer and CFTR was immunoprecipitated using L12B4 antibody (Chemicon). Samples were analyzed by SDS-PAGE and amount of radioactively labeled CFTR quantitated using Phosphorimager software (GE Healthcare, Piscataway, NJ).

Confocal Microscopy

BHK-deltaF508-3HA were treated with AlexaFluor488 tagged CPP (1 μM) for 60 min at 37°C to evaluate peptide uptake. Plasma membrane was labeled with 10 μg/ml concanavalin A-Rhd (Molecular Probes). Images were acquired under live cell conditions using the Zeiss Axiovert 200 inverted fluorescence microscope equipped with a META emission scan head (63X magnification) and analyzed on Volocity software (AlexaFluor488: λex = 495 nm, λem 519 nm; Rhd: λex = 530 nm, λem = 555 nm).

Statistical Analyses

In all comparative studies of multiple groups, one-way ANOVA was performed with secondary analyses of all pairs using the Bonferroni test for significant differences (unless otherwise stated). A single asterisk (*) denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes ($p < 0.001$). In comparison of two groups, the unpaired t test or paired t test was employed as appropriate. Prism software (Version 4) was employed to conduct these tests (GraphPad software, San Diego, CA).

SUPPLEMENTAL DATA

Supplemental Data include one figure and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00138-0.

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