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Mechanisms for Rescue of Correctable Folding Defects in CFTRΔF508

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Premature degradation of CFTRΔF508 causes cystic fibrosis (CF). CFTRΔF508 folding defects are conditional and folding correctors are being developed as CF therapeutics. How the cellular environment impacts CFTRΔF508 folding efficiency and the identity of CFTRΔF508's correctable folding defects is unclear. We report that inactivation of the RMA1 or CHIP ubiquitin ligase permits a pool of CFTRΔF508 to escape the endoplasmic reticulum. Combined RMA1 or CHIP inactivation and Corr-4a treatment enhanced CFTRΔF508 folding to 3–7-fold greater levels than those elicited by Corr-4a. Some, but not all, folding defects in CFTRΔF508 are correctable. CHIP and RMA1 recognize different regions of CFTR and a large pool of nascent CFTRΔF508 is ubiquitinated by RMA1 before Corr-4a action. RMA1 recognizes defects in CFTRΔF508 related to misassembly of a complex that contains MSD1, NBD1, and the R-domain. Corr-4a acts on CFTRΔF508 after MSD2 synthesis and was ineffective at rescue of ΔF508 dependent folding defects in amino-terminal regions. In contrast, misfolding caused by the rare CF-causing mutation V232D in MSD1 was highly correctable by Corr-4a. Overall, correction of folding defects recognized by RMA1 and/or global modulation of ER quality control has the potential to increase CFTRΔF508 folding and provide a therapeutic approach for CF.

INTRODUCTION

Cystic fibrosis (CF) is a lethal inherited disorder that is caused by mutations in the gene coding for the CF transmembrane conductance regulator (CFTR; Riordan *et al.*, 1989). The CFTR protein is a cAMP-regulated chloride channel that is localized to the apical surface of epithelial cells (Li *et al.*, 1988; Anderson *et al.*, 1991) where it functions to regulate water and salt homeostasis. Inheritance of the CFTRΔF508 gene, in which phenylalanine 508 is deleted from the nucleotide binding domain 1 (NBD1), is the major cause of CF. This prevalent mutation is found in ~90% of patients afflicted with CF (Bobadilla *et al.*, 2002). Loss of functional CFTR alters the hydration of airway epithelial and gives rise to microbial infections, which can ultimately lead to lung failure and death (Rowe *et al.*, 2005).

CFTR, an ATP-binding cassette (ABC) transporter superfamily member (Hyde *et al.*, 1990), is a 1480-residue polytopic membrane glycoprotein that is predicted to contain two membrane-spanning domains (MSD), two nucleotide-binding domains (NBD), and a regulatory domain (R; Riordan *et al.*, 1989). The folding of CFTR into a functional chloride channel is complex because it requires the coordinated folding and assembly of its membrane and cytoplasmic domains (Du *et al.*, 2005; Cui *et al.*, 2007). Consequently, CFTR folding is a relatively inefficient process with ~70% of the wild type and 99% of the ΔF508 mutant being partitioned from a folding to a degradation pathway (Ward and Kopito, 1994). However, although early stage biogenic intermediates of CFTR and CFTRΔF508 appear to have similar conformations (Zhang *et al.*, 1998), the folding of CFTRΔF508 becomes arrested at a poorly defined step. Interestingly, deletion of the F508 residue causes subtle structural changes in the NBD1 domain as well as defective interaction between NBD1 and other subdomains of CFTR (Du *et al.*, 2005; Thibodeau *et al.*, 2005).

The assembly of native CFTR into a conformation that can bypass the endoplasmic reticulum quality control (ERQC) system occurs in both co- and posttranslational steps (Kleizen *et al.*, 2005) that are proposed to involve critical interactions between the NBD and MSD interfaces (Dawson and Locher, 2006; Mendoza and Thomas, 2007; Mornon *et al.*, 2008; Serohijos *et al.*, 2008). Recent molecular modeling analysis and cross-linking studies suggest that NBD1 contacts the cytoplasmic loop (CL) 1 of MSD1 and CL3 and 4 of MSD2, whereas NBD2 interacts with CL1 and 2 of MSD1 and CL3 of MSD2 (He *et al.*, 2008; Mornon *et al.*, 2008; Serohijos *et al.*, 2008). The F508 residue, located in a surface exposed loop within the NBD1 domain (Lewis *et al.*, 2004; Thibodeau *et al.*, 2005), is proposed to help orient NBD1 and stabilize the overall CFTR structure by making contacts with solvent exposed surfaces of CL4 of MSD2 (Mornon *et al.*, 2008; Serohijos *et al.*, 2008). The F508 side chain also appears to be required for proper assembly of a folding intermediate that contains MSD1, NBD1, and the R-domain whose formation may be critical for completion of CFTR folding (Rosser *et al.*, 2008). This supposition is supported by the observation that loss of F508 leads MSD2 to accumulate in an altered conformation and hinders posttranslational folding of NBD2 (Du *et al.*, 2005; Du and Lukacs, 2009). Because deletion of F508

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from CFTR alters the assembly of more than one CFTR region, the folding defects that lead to its selection for premature degradation and the onset of CF remain unclear.

The folding progression of CFTR is monitored by at least two distinct ERQC complexes that are located in the ER membrane and cytosol. One ubiquitin ligase complex that recognizes CFTR is composed of the ERQC factor Derlin-1, the E2 Ubc6e, and the E3 ligase RMA1, which form an ER membrane–associated complex (Younger *et al.*, 2006). In addition, Hsc70, the E2 UbcH5a, and the E3 ligase CHIP also act in the cytosol to monitor the conformation of CFTR (Meacham *et al.*, 1999, 2001; Younger *et al.*, 2004). CFTR translation intermediates exhibit differential sensitivity to changes in RMA1 or CHIP activity, leading to the interpretation that these ubiquitin ligase complexes selectively recognize different regions of CFTR (Younger *et al.*, 2006). The membrane-associated RMA1 complex appears to recognize folding defects in CFTR that may involve misassembly of MSD2 into a complex with the amino-terminal regions of CFTR. In contrast, the cytosolic CHIP complex appears to sense folding defects that are related to misassembly of the NBD2. CFTRΔF508 is more sensitive to changes in RMA1 activity than CFTR, which suggests that RMA1 plays a critical role in CFTRΔF508's premature degradation (Younger *et al.*, 2004).

Misfolded forms of CFTR that are recognized by the ERQC machinery and are polyubiquitinated can subsequently be acted upon by gp78. Gp78, an E3 ligase that contains a polyubiquitin chain–binding CUE domain, interacts with RMA1 and acts to maintain ubiquitin chain length (Morito *et al.*, 2008) before CFTR's retrotranslocation by p97 (Dalal *et al.*, 2004) from the ER membrane and degradation by cytosolic proteasomes (Cheng *et al.*, 1990; Jensen *et al.*, 1995; Ward *et al.*, 1995; Meacham *et al.*, 2001). This pathway for quality control of CFTR is likely to contain additional components. In addition, how modulation of endogenous ERQC factors impacts the folding efficiency of CFTR and CFTRΔF508 is not clear. Furthermore, the nature of the correctable defects that limit folding of CFTRΔF508 is unknown.

What we know is that a portion of the folding defects caused by deletion of F508 are conditional and small pools of misfolded CFTRΔF508 intermediates can be rescued by low temperature incubations or by chemical chaperones in cell culture systems (Denning *et al.*, 1992; Brown *et al.*, 1996; Sato *et al.*, 1996). A number of high-throughput screens have been conducted and an array of small-molecule chemical correctors have been found to weakly enhance the folding of CFTRΔF508 (Pedemonte *et al.*, 2005; Van Goor *et al.*, 2006). Corr-4a, a bisaminomethylbithiazole derivative, is currently the most effective chemical corrector available to the research community (Figure 1A). Treatment with Corr-4a increases the cell surface expression of a small pool of CFTRΔF508 in human airway epithelia, making this compound a potentially attractive candidate for CF treatment (Pedemonte *et al.*, 2005). In addition, Corr-4a not only improves trafficking of CFTRΔF508 to the plasma membrane, but it also appears to enhance the cell surface stability of the rescued mutant protein (Varga *et al.*, 2008). The mechanism by which Corr-4a modulates the folding of CFTRΔF508 is unknown. Recent literature suggests that Corr-4a may bind directly to CFTR based on its ability to block specific cross-links between membrane segments of CFTR (Wang *et al.*, 2007); however, direct evidence of a drug-binding pocket on CFTR is lacking. Thus, it is currently unclear if Corr-4a has a direct or indirect affect on the folding of CFTRΔF508.

In this study we sought to define the extent to which activity of the ERQC machinery limits CFTRΔF508 folding and to identify which folding defects in CFTRΔF508 are correctable. Toward this goal, we observed an increase in both the folding of CFTRΔF508 and the activity of Corr-4a when endogenous levels of the E3 ligases RMA1 or CHIP were depleted by small interfering RNA (siRNA). We also demonstrated that deletion of F508 causes folding defects that are both correctable and noncorrectable by Corr-4a. The noncorrectable defect in CFTRΔF508 occurs early in its biogenesis and involves misfolding of N-terminal regions that contain MSD1, NBD1, and the R-domain. The correctable folding defect occurs after synthesis of MSD2. Corr-4a acts in part via stabilization of the MSD2, which may enhance critical contacts between MSD2 and other subdomains of CFTR, such as MSD1 or NBD1. Surprisingly, we also discovered that Corr-4a can strongly correct the folding defects of less common CF disease-causing point mutations. Collectively, our data indicate that modulation of ERQC activity can increase the accumulation of foldable pools of CFTRΔF508 and enhance Corr-4a action. Some of the misfolded CFTR intermediate species can be brought back on pathway, whereas others appear more difficult to fix.

MATERIALS AND METHODS

Plasmids, Antibodies, and Reagents

CFTR expression plasmids pcDNA3.1(+)-CFTR and pcDNA3.1(+)-CFTRΔF508 have been described elsewhere (Meacham *et al.*, 2001). CFTR constructs representing CF disease-causing point mutants or biogenic intermediates were made using the QuikChange protocol (Stratagene, La Jolla, CA). Antibodies used in this study were as follows: α-CFTR MM13–4 (N-terminal tail epitope) and α-CFTR M3A7 (NBD2 epitope) were from Millipore (Billerica, MA); α-RMA1 (sc81716) was from Santa Cruz Biotechnology (Santa Cruz, CA); and α-tubulin was purchased from Sigma (St. Louis, MO). The polyclonal α-CHIP antibody used in our studies was generated against full-length recombinant CHIP protein. Polyclonal α-CFTR, generated against a glutathione *S*-transferase (GST) fusion protein that contained CFTR residues 1–79, was a generous gift from Dr. Kevin Kirk (University of Alabama Birmingham). Corr-4a was provided by the Cystic Fibrosis Foundation and Dr. Robert Bridges (Rosalind Franklin University). An initial titration of Corr-4a, prepared in DMSO, indicated that a final concentration of 5 μM was sufficient to observe maximal maturation of CFTRΔF508.

Cell Culture and Transfection

Human embryonic kidney (HEK) 293 cells from Stratagene were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Invitrogen) at 37°C in an atmosphere of 5% CO_2. Cell transfections were performed using Effectene reagent (Qiagen, Valencia, CA) or Lipofectamine 2000 (Invitrogen) where indicated. The empty pcDNA3.1(+) vector was used to ensure equal microgram quantities of DNA were used in all transfection reactions.

Analysis of CFTR Biogenesis

CFTR Steady-State Levels. Steady-state levels of CFTR and its mutants were determined by Western blot analysis. HEK293 cells were transiently transfected with the indicated plasmids. The transfected cells were allowed to recover for ~18 h before addition of DMEM (10% FBS and antibiotics) supplemented with Corr-4a (5 μM final concentration) or DMSO. The cells were incubated with the chemicals for 24 h before isolating the cells for Western blot analysis. The harvested cells were diluted with 2× SDS sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.05% bromophenol blue, and 20% glycerol), sonicated, and heated at 37°C before resolving the proteins on SDS-PAGE gels. The proteins were transferred to nitrocellulose membranes, and the membranes were probed with the designated antibodies. α-Tubulin was used to indicate loading controls.

CFTR Processing Efficiency. CFTR processing efficiency was measured by pulse-chase analysis. Transiently transfected HEK293 cells were allowed to recover for 18 h. The cells were then incubated with DMEM (10% FBS and antibiotics) supplemented with Corr-4a or DMSO for 2 h. Next, cells were starved in methionine-free MEM (Sigma) for 20 min, pulse-labeled for 20 min with [^{35}S]methionine (100 μCi/6 well; 1200 Ci/mmol; MP Biomedicals, Ir-

vine, CA), and then chased for the indicated amount of time. Corr-4a or DMSO was also included in the media during these steps of the pulse-chase reaction. Cells were then lysed in PBS buffer supplemented with 1% Triton (PBS-T, 1%), 1 mM PMSF, and Complete protease inhibitor cocktail (Roche, Indianapolis, IN). Soluble lysates were obtained by centrifugation at 20,000 rpm for 10 min in a Beckman Allegra 64R centrifuge (Fullerton, CA). Equal microgram quantities of cell lysate were subjected to immunoprecipitation by incubation with a polyclonal α-CFTR antibody directed against the N-terminus followed by addition of a 50% protein G bead slurry. The beads were washed with PBS-T (1%) supplemented with 0.2% SDS, the bound CFTR was eluted with 2× SDS sample buffer, and the samples were heated at 55°C for 10 min. The samples were analyzed by SDS-PAGE and visualized by autoradiography.

RNA Interference Analysis

HEK293 cells were transfected at a final total concentration of 100 nM with oligonucleotides (oligos) directed at either RMA1 (sequence 1, GCGCGACCUUCGAAUGUAA; sequence 2, CGGCAAGAGUGUCCAGUAU), CHIP (sequence 1, GGAGCAGGGCAAUCGUCUG; sequence 2, CCAAGCACGACAAGUACAU), or a nonspecific control (Dharmacon, Lafayette, CO) by using the transfection reagent Lipofectamine 2000 (Invitrogen). Forty-eight hours later 1 μg of CFTR or CFTRΔF508 was introduced into the cells using Effectene (Qiagen) as the transfection reagent. For steady-state analysis Corr-4a or DMSO was added to the cells 4 h after the second transfection, and the cells were harvested 24 h later. Then 2× SDS sample buffer was added to cell pellets, and after sonication the samples were normalized to contain the same total amount of protein. The reactions were resolved on SDS-PAGE gels, transferred to nitrocellulose membranes, and the membranes were probed with the indicated antibodies. α-Tubulin was used to indicate loading controls. For pulse-chase analysis the cells were allowed to recover for 18 h after the second transfection. The cells were then incubated with DMEM (10% FBS and antibiotics) supplemented with Corr-4a or DMSO for 2 h. The pulse-chase procedure utilized is similar to that described above within the CFTR processing efficiency subsection.

RESULTS

Inactivation of ERQC Checkpoints Permits CFTRΔF508 Folding and Enhances Corr-4a Activity

The extent that activity of the ERQC machinery determines the fate of nascent forms of CFTR and CFTRΔF508 is unclear. In addition, whether activity of the ERQC machinery impacts the effectiveness of drugs developed to correct misfolding of CFTRΔF508 is unknown. Thus, we sought to gain insight into the mechanism for premature degradation of CFTR and CFTRΔF508 through inactivating the known ERQC checkpoints and determining the effect this had on CFTR and CFTRΔF508 folding in the presence or absence of Corr-4a. This was accomplished by evaluating the accumulation of the folded C-form of CFTR and CFTRΔF508 when endogenous levels of either the E3 ligase RMA1 or the E3 ligase CHIP was depleted by siRNA in the absence and presence of Corr-4a (Figure 1). Knockdown of endogenous RMA1 or CHIP levels resulted in a respective 3.4- and 2.3-fold increase in the accumulation of the C-form of CFTR, which was also accompanied by an increase in B-form levels (Figure 1B). As shown in Figure 1C, the endogenous levels of either RMA1 or CHIP are reduced to greater than 90% using RNA interference (RNAi). Corr-4a treatment alone is able to elevate the levels of the folded, maturely glycosylated, C-form of CFTR 3.9-fold, whereas the levels of nonnative, ER localized and immaturely glycosylated B-form were increased by 1.7-fold (Figure 1B). The combined knockdown of either RMA1 or CHIP and treatment with Corr-4a further enhanced accumulation of CFTR to levels around fivefold greater than the DMSO-treated control siRNA reaction. Thus, inactivation of either the RMA1 or CHIP E3 quality control checkpoints permits a larger pool of folded CFTR to accumulate and greatly influences the potency of Corr-4a action.

The positive effects of the combined knockdown of ERQC factors and Corr-4a treatment on CFTRΔF508 folding were more dramatic than those observed with CFTR. Knockdown

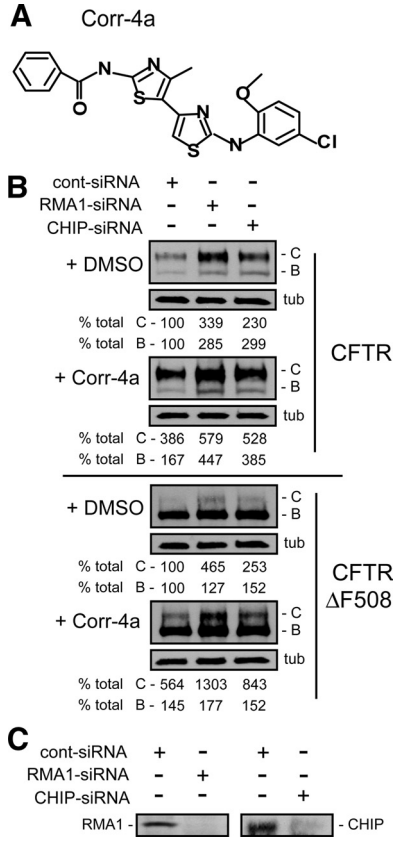

Figure 1. Knockdown of ER quality control checkpoints enhance CFTRΔF508 folding and Corr-4a activity. (A) Chemical structure of small molecule corrector Corr-4a. (B) Transfections with siRNA oligos and CFTR or CFTRΔF508 were performed as described in *Materials and Methods.* The siRNA samples were treated with DMSO or Corr-4a as indicated. Twenty-four hours later the samples were lysed in 2× SDS sample buffer and normalized to total amount of protein, and Western blot analysis was used to determine the steady-state levels of either CFTR or CFTRΔF508. Bands B and C represent the immature and maturely glycosylated forms of CFTR, respectively. Tubulin blots were used as loading controls. The B- and C-band levels for each reaction were quantified by densitometry and were normalized to the DMSO-treated reaction which contained the siRNA control duplex (cont-siRNA). Results shown are representative of one experiment, but trends were identical when the experiment was repeated three individual times. (C) A monoclonal RMA1 antibody and a polyclonal CHIP antibody were used to monitor the impact of RNAi addition on endogenous RMA1 and CHIP levels, respectively.

of RMA1 was nearly as effective as Corr-4a treatment at permitting CFTRΔF508 to accumulate in its folded C-form, whereas knockdown of CHIP was about half as effective (Figure 1B). Amazingly, the knockdown of endogenous RMA1, in addition to Corr-4a treatment, led to a 13-fold increase in the accumulation of the C-form of CFTRΔF508 and only a 1.8-fold increase in levels of its B-form. Furthermore, the combination of CHIP knockdown and Corr-4a treatment increased the accumulation of the C-form of CFTRΔF508 eightfold while its B-form levels were increased by 1.5-fold. Thus, inactivation of either RMA1 or CHIP permits a foldable pool of CFTRΔF508 to accumulate and the folding of this pool of CFTRΔF508 is greatly augmented by Corr-4a.

To determine whether modification of the cellular folding environment through inactivation of ERQC factors increased the folding efficiency of CFTR, we conducted a series

of pulse-chase experiments under the same experimental conditions as described in Figure 1. In the presence of control siRNA, 20% of the immature B-form of CFTR was converted to its folded C-form with DMSO treatment, whereas the addition of Corr-4a increased the efficiency of this process around 2.5-fold (Figure 2A). Interestingly, knockdown of RMA1, but not CHIP, led to a twofold increase in CFTR folding efficiency. When RMA1 siRNA cells were treated with Corr-4a, 83% of the total quantity of the B-form of CFTR could be converted to its C-form. Although knockdown of CHIP did not detectably increase CFTR folding efficiency, the combination of CHIP siRNA and Corr-4a treatment increased CFTR maturation from 20 to 65%. Thus, inactivation of ERQC factors has the potential to increase the folding efficiency of nascent CFTR and appears to permit the accumulation of larger pools of foldable molecules that can be acted upon by chemical corrector Corr-4a in the ER.

Increased accumulation of the C-form of CFTRΔF508 was observed when RMA1 or CHIP levels were knocked down by siRNA and also upon Corr-4a treatment (Figure 1B). Yet, increased folding of CFTRΔF508 was not readily observed in pulse-chase experiments (Figure 2B). Nevertheless, as compared with the appropriate control (Figure 2B, top panel) 2–4-fold more of the B-form of CFTRΔF508 was observed at the end of pulse-chase time courses when either RMA1 or CHIP levels were decreased and/or Corr-4a was present. Thus, the half-life of CFTRΔF508 is increased upon inactivation of ERQC complexes and/or Corr-4a treatment, but the quantity that can fold to the native state is too small to detect after a 20-min pulse-labeling period and subsequent chase. Nevertheless, inactivation of ERQC factors can spare a pool of nascent CFTRΔF508 from degradation and permit its proper folding and this pool is detected via western blot as a maturely glycosylated species after a 24-h time period.

These data indicate that large portions of foldable nascent CFTR and CFTRΔF508 are selected by the ERQC machinery for degradation before reaching the native state. Therefore, inactivation of the ERQC machinery can spare both nascent CFTR and CFTRΔF508 from degradation and permits accumulation of a larger pool of protein that can be brought onto the folding pathway in the ER by Corr-4a.

Corr-4a Appears to Act on CFTR and CFTRΔF508 Biogenic Intermediates in the ER after Synthesis of MSD2

Data presented thus far indicate that the folding pathway of CFTR can be altered by Corr-4a. Yet, large pools of CFTRΔF508 in Corr-4a treated cells are still recognized by ERQC machinery and irreversibly leave the folding pathway. To understand why Corr-4a action can be enhanced by inactivation of ERQC machinery, we sought to define the correctable defective folding steps in CFTR and CFTRΔF508. To accomplish this we determined the effect of Corr-4a on the steady-state levels of CFTR and CFTRΔF508 fragments that resemble different length biogenic intermediates (Younger *et al.*, 2006; Cui *et al*., 2007; Rosser *et al.*, 2008; Figure 3).

HEK293 cells, transiently transfected with the indicated CFTR biogenic fragments (Figure 3A), were incubated with Corr-4a or DMSO and the steady-state levels of CFTR were assessed (Figure 3B). CFTR 370X, which represents MSD1 of CFTR, was unaffected by Corr-4a treatment. The MSD1-NBD1 fragment CFTR 653X and CFTR 837X, which is truncated after the R-domain, were also unaffected by Corr-4a. Deletion of F508 causes a modest decrease in accumulation of CFTR 653X and has a more dramatic effect on CFTR 837X (Figure 3B; Rosser *et al.*, 2008). However, Corr-4a did not enhance the accumulation of the F508 deleted forms of either CFTR 653X or CFTR 837X. Yet, we know that accumulation

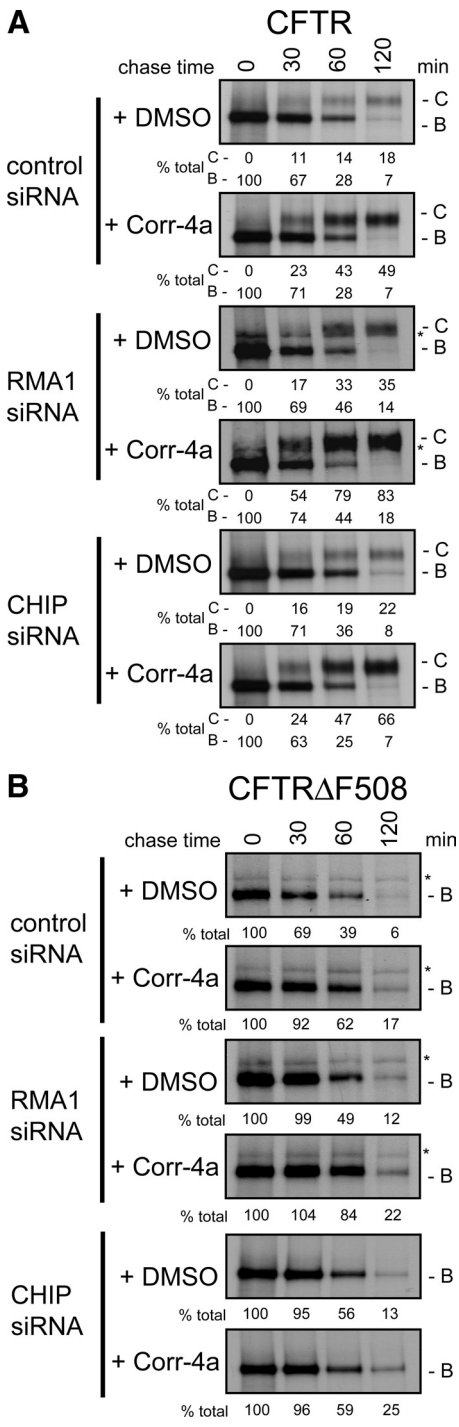

Figure 2. Modulation of the cellular folding environment by siRNA and Corr-4a affects the folding efficiency of CFTR and stability of CFTRΔF508. Transfections with siRNA oligos and CFTR (A) or CFTRΔF508 (B) were performed as described in *Materials and Methods.* The siRNA reactions were incubated with DMSO or Corr-4a for 2 h before being pulse-labeled with [^{35}S]methionine and chased for the indicated time periods in the presence of chemical treatment. Cell lysates were normalized to total amount of protein, immunoprecipitated with a polyclonal CFTR N-terminal antibody, and visualized by SDS-PAGE analysis and autoradiography. Asterisk denotes a background band. Results were quantified by densitometry and normalized relative to the amount of B-band at t = 0 min for each condition. Similar results were observed upon three repeats of the pulse-chase experiment.

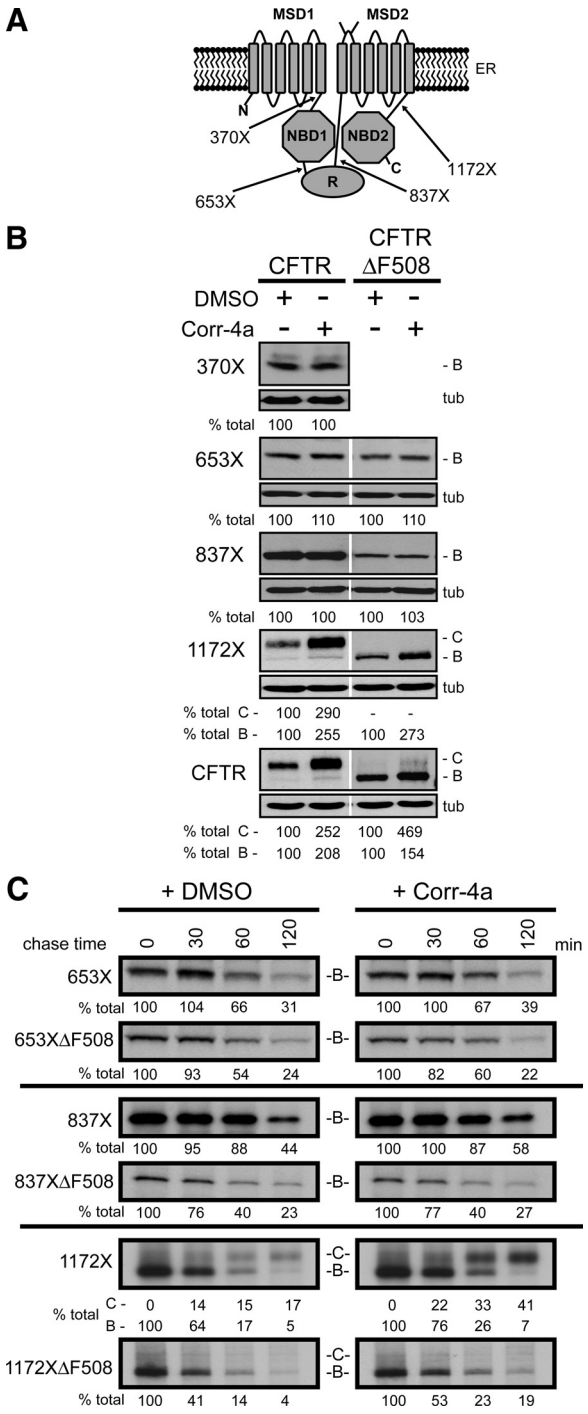

Figure 3. Corr-4a appears to act on CFTR and CFTRΔF508 biogenic intermediates after synthesis of MSD2. (A) A schematic depicting the amino acid position in CFTR where stop codons were introduced to generate the CFTR biogenic intermediates utilized in B and C. HEK293 cells were transiently transfected with 1 μg of the indicated CFTR constructs. (B) Corr-4a or DMSO was added to the cells 18 h after transfection, and the cells were incubated with the chemicals for 24 h. The samples were subjected to Western blot analysis with an antibody that specifically recognizes the N-terminus of CFTR to determine steady-state levels of the CFTR fragments. Bands B and C represent the immature and maturely glycosylated forms of CFTR, respectively. Tubulin blots were performed to indicate loading controls. The B- and C-band levels for each reaction were quantified by densitometry and were normalized to the DMSO-treated control. (C) For the pulse-chase reactions, 18 h after transfection the cells were pretreated for 2 h with the indicated chemicals, labeled with [^{35}S]methionine, and chased for the indicated amounts of time in the presence of chemical treatment. The isolated cells were lysed, soluble cell lysates were normalized to contain the same total amount of protein, and CFTR was immunoprecipitated with a polyclonal N-terminal CFTR antibody. The immunoprecipitates were visualized by SDS-PAGE analysis and autoradiography. Results were quantified by densitometry and normalized to the amount of B-band at t = 0 for each CFTR construct and chemical treatment. For each CFTR fragment, the steady-state (B) and pulse-chase (C) results are consistent and the observed trends reproducible.

of CFTR 837XΔF508 is dramatically enhanced by siRNA knockdown of RMA1 (Rosser *et al.*, 2008).

It is not until after synthesis of MSD2, shown here with the CFTR 1172X biogenic intermediate, that we observed a significant affect of Corr-4a. The CFTR 1172X fragment is able to fold into a conformation that can escape the ERQC system, become maturely glycosylated and traffic to the plasma membrane (Cui *et al.*, 2007). We observed an increase in the accumulation of both the B- and C-forms of CFTR 1172X in the presence of Corr-4a (Figure 3B). Similar to the full-length CFTRΔF508 mutant, deletion of F508 inhibits formation of the maturely glycosylated C-form of CFTR 1172X (Cui *et al.*, 2007). Yet, in contrast to what was observed with shorter fragments, Corr-4a increased the accumulation of the B-form of CFTR 1172XΔF508 (Figure 3B). However, Corr-4a was ineffective at promoting the folding of CFTR 1172XΔF508.

Similar effects of Corr-4a on the different length CFTR fragments were also observed in pulse-chase studies (Figure 3C). First, we found that the addition of Corr-4a does not increase the biosynthesis of the various length ^{35}S-labeled CFTR fragments (compare t = 0 for DMSO controls vs. Corr-4a reactions for each CFTR fragment). Second, it was apparent that deletion of F508 impacts the amount of ^{35}S-labeled starting product (t = 0) and the stability of CFTR 837X and CFTR 1172X, and to a lesser extent CFTR 653X. However, Corr-4a did not increase the shortened half-life of CFTR 653XΔF508 or CFTR 837XΔF508. Yet, Corr-4a did increase the folding efficiency of CFTR 1172X around 2.5-fold and also increased the half-life of CFTR 1172XΔF508.

The extent that deletion of F508 can differentially affect the levels of N-terminal fragments of CFTR are intriguing and have previously been documented (Rosser *et al.*, 2008). Deletion of F508 has only a modest affect on the stability of the MSD1-NBD1 fragment CFTR 653X, but the presence of the R-domain results in a much more pronounced defect. It appears that deletion of F508 in CFTR 837X dramatically disrupts the assembly of MSD1, NBD1, and the R-domain into a stable complex. Consequently, a large population of 837XΔF508 is selected for proteasomal degradation by the E3 ligase RMA1 (Rosser *et al.*, 2008). Thus, it is not surprising that the accumulation and ^{35}S-labeled starting product (t = 0) of CFTR 837XΔF508 is significantly lower than its wild-type counterpart CFTR 837X.

Major conclusions that can be drawn from analyzing how chemical corrector Corr-4a affects the biogenesis of CFTR fragments that resemble biogenic intermediates are as follows: first, corrector-induced accumulation of CFTR fragments was not observed until after synthesis of MSD2; second, folding defects caused by deletion of F508 that occur before synthesis of MSD2 do not appear to be corrected by Corr-4a addition alone; and third, the corrector-induced maturation of CFTRΔF508, but not CFTR 1172XΔF508, suggests that NBD2 has an important role in the conformational maturation of CFTRΔF508.

Overall, these data suggest that the mechanism of action of Corr-4a involves the stabilization of an interaction interface between MSD2 and a more N-terminal domain of CFTR, which is disrupted by deletion of F508. In fact cross-linking data suggests that deletion of F508 disrupts interactions between MSD2 and MSD1 (Chen *et al.*, 2004) as well as MSD2 and NBD1 (Serohijos *et al.*, 2008). Furthermore, the fact that we observe correction of CFTRΔF508, but not CFTR 1172XΔF508, suggests that NBD2 may play a role in further stabilizing the interface formed after Corr-4a addition.

Folding Defects Caused by CF Disease-related Mutations in Different CFTR Subdomains Exhibit Varied Degrees of Correction

Data presented herein, and by others (Chen *et al.*, 2004; Du *et al.*, 2005; Mornon *et al.*, 2008; Rosser *et al.*, 2008; Serohijos *et al.*, 2008) indicate that there are multiple folding defects in the pathway for CFTRΔF508 folding, yet the present data implies that not all defects are correctable. Thus, we asked to what extent can disease related folding defects caused by mutations in MSD1 (G85E, G91R, and V232D), MSD2 (M1137R), and NBD2 (N1303K) be corrected relative to those caused by deletion of F508 in NBD1 (Figure 4A). The G85E, G91R, M1137R, and N1303K mutations all hinder folding of the nascent B-form of CFTR via a mechanism that involves insertion of a charged amino acid into an inappropriate region (Gregory *et al.*, 1991; Xiong *et al.*, 1997; Vankeerberghen *et al.*, 1998). The V232D mutation has been proposed to introduce an abnormal hydrogen bond within the transmembrane (TM) region of MSD1, which impedes normal TM assembly (Therien *et al.*, 2001), but the affect of this mutation on CFTR biogenesis is unknown.

The effect of Corr-4a on the NBD2 mutant N1303K was similar to that observed with CFTRΔF508 in that a small degree of correction was observed (Figure 4A). The levels of the immature form of CFTR N1303K were slightly elevated while accumulation of the mature form was enhanced upon addition of Corr-4a. Surprisingly, we observed dramatic differences in the ability of Corr-4a to increase the quantity of the folded forms of four different MSD mutants (Figure 4A). The CFTR G85E and G91R point mutations are contained within TM1, whereas the V232D mutation lies within TM4 of CFTR's MSD1 domain. The CFTR M1137R mutation exists within TM12 of CFTR's MSD2 domain. The addition of Corr-4a slightly increased the steady-state levels of CFTR G85E B-form, but Corr-4a–dependent folding of this mutant was not detected. Furthermore, chemical treatment of CFTR G91R or CFTR M1137R did not significantly affect the accumulation of the immature B-form; however, the mature C-forms of both CFTR G91R and CFTR M1137R were apparent. Thus, Corr-4a is not specific for the F508 deletion and can increase the folding of a small pool of some, but not all, CFTR mutants.

Intriguingly, introduction of the V232D mutation generates an unstable CFTR biogenic mutant with decreased levels of the immature B-form and no apparent maturation product (Figure 4A). Yet, treatment with Corr-4a resulted in a dramatic increase in accumulation of both the immature and mature forms of CFTR V232D. However, folding defects in CFTR V232D were not significantly rescued by low temperature incubations (data not shown). Pulse-chase analysis indicated that Corr-4a strongly increases the folding efficiency of CFTR V232D, and does not simply increase the synthesis of this point mutant (Figure 4B). The ability of Corr-4a to dramatically enhance CFTR V232D folding suggests that this mutant may have a single folding defect in MSD assembly that is correctable by Corr-4a. In agreement with this idea, Corr-4a is able to enhance the accumulation of the V232D-containing MSD1 fragment, CFTR 370X V232D (data not shown). In contrast, the multiple folding defects caused by deletion of F508 do not appear to be as readily repaired by Corr-4a.

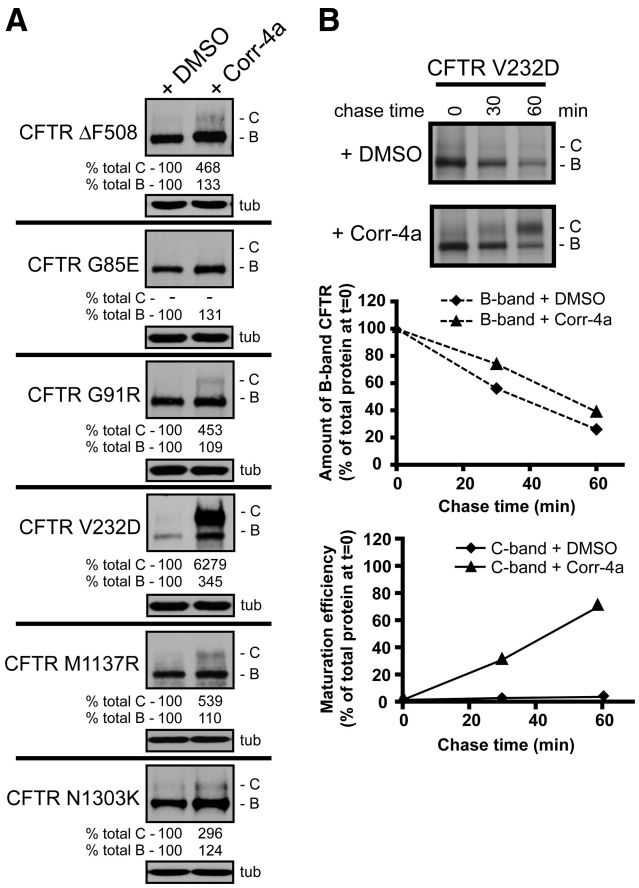

Figure 4. Folding defects caused by CF disease-related mutations in different CFTR subdomains exhibit varied degrees of correction. (A) HEK293 cells were transiently transfected with 1 μg of the indicated CF disease-causing mutants. The transfected cells were allowed to recover for 18 h before addition of Corr-4a or DMSO. The cells were incubated with chemicals for 24 h before using Western blot analysis to determine the steady-state levels of the mutant CFTR proteins. The immature and maturely glycosylated forms of CFTR are designated as B- and C-bands, respectively. Tubulin is used as a loading control. The B- and C-band levels of each CFTR point mutant were quantified by densitometry and were normalized to the DMSO-treated samples for each mutant. The affect of Corr-4a on the studied CF disease causing mutants was reproducible (n = 3). (B) Corr-4a increases the biosynthetic maturation of CFTR V232D. HEK293 cells transfected with CFTR V232D (1 μg) were preincubated with Corr-4a or DMSO for 2 h, labeled with [^{35}S]methionine, and chased for the indicated amounts of time in the continuous presence of chemical treatment. The cells were lysed, soluble cell lysates were normalized to contain the same total amount of protein, and CFTR was immunoprecipitated with a polyclonal N-terminal CFTR antibody. The reactions were visualized by SDS-PAGE analysis and autoradiography. Results were quantified by densitometry and graphed.

Corr-4a Stabilizes the MSDs of CFTR

To define in greater detail the defective step(s) in the CFTRΔF508 folding pathway that can be corrected by Corr-4a, we studied assembly of wild-type and mutant forms of

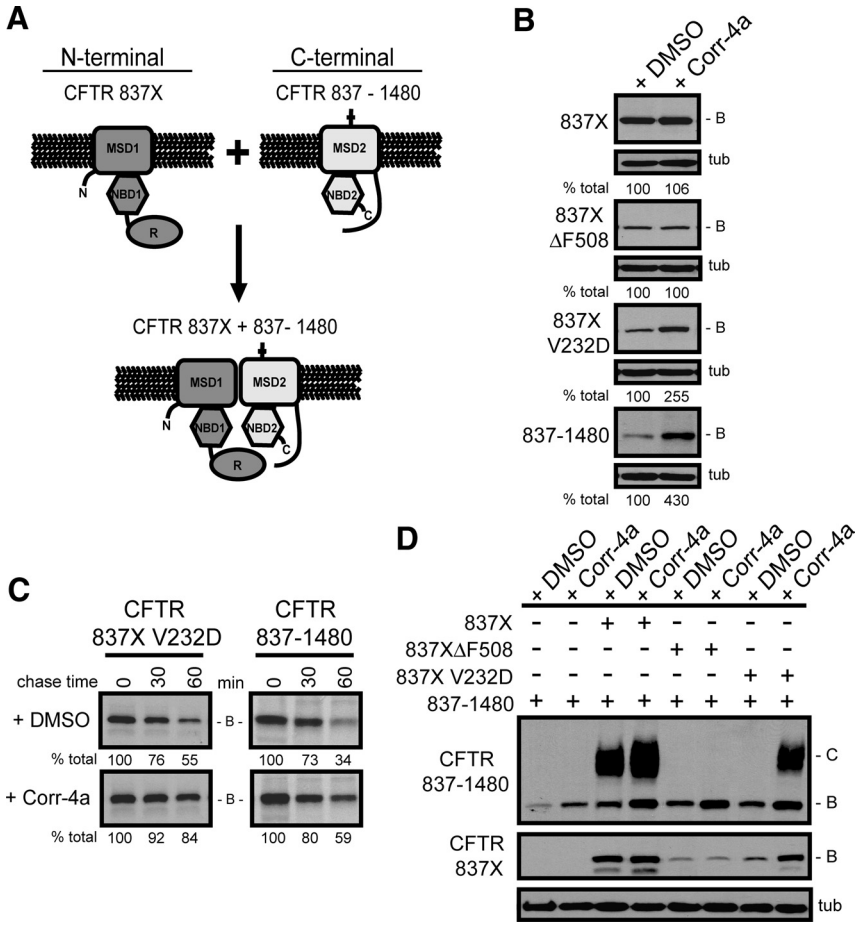

Figure 5. Corr-4a stabilizes the MSDs of CFTR. (A) Domain schematic of CFTR halves: CFTR 837X and CFTR 837-1480. When CFTR 837-1480 is expressed alone it accumulates as an immaturely glycosylated species. Coexpression of the two halves of wild-type CFTR promotes the formation of a maturely glycosylated form of CFTR 837-1480. (B) HEK293 cells transfected individually with CFTR 837X (1 μg), CFTR 837XΔF508 (1 μg), CFTR 837X V232D (1 μg), or CFTR 837-1480 (1 μg) were cultured for 18 h before addition of Corr-4a or DMSO. Cells were incubated with chemicals for 24 h. Steady-state levels were then determined by Western blot analysis using CFTR N-terminal or NBD2-specific antibodies. Loading consistency was indicated with tubulin blots. The B-band levels for each reaction were quantified by densitometry and were normalized to the DMSO-treated control. (C) Corr-4a increases the stability of CFTR 837X V232D and CFTR 837-1480. HEK293 cells transfected with either CFTR 837X V232D or CFTR 837-1480 were preincubated with Corr-4a or DMSO for 2 h, labeled with [^{35}S]methionine, and chased for the indicated amounts of time in the continuous presence of chemical treatment. Soluble cell lysates were normalized to contain the same total amount of protein, and CFTR was immunoprecipitated with a CFTR N-terminal– or NBD2–specific antibody. The reactions were visualized by SDS-PAGE analysis and autoradiography. Results were quantified by densitometry and normalized to the amount of B-band at t = 0 for each CFTR construct and chemical treatment. (D) HEK293 cells were transfected with either CFTR 837-1480, CFTR 837X and CFTR 837-1480, CFTR 837XΔF508 and CFTR 837-1480, or CFTR 837X V232D and CFTR 837-1480. Transfected cells were cultured and incubated with chemicals as in B. Steady-state levels of CFTR 837-1480 were determined by Western blot using a NBD2 specific antibody, whereas the levels of the N-terminal fragments were analyzed with a CFTR N-terminal antibody. Maturely glycosylated CFTR 837-1480 is designated as C-band, whereas the immaturely glycosylated form is indicated as B-band. Tubulin is used as a loading control. Results shown are representative of one experiment; however, the reported observations were repeatedly observed.

split CFTR fragments that individually contain the N- and C-terminal subdomains (Figure 5A). As shown earlier in Figure 3, Corr-4a treatment does not affect accumulation of the stable N-terminal fragment CFTR 837X (Figure 5B). However, the C-terminal fragment CFTR 837-1480 is unstable (Rosser *et al.,* 2008), and steady-state levels of it are increased by Corr-4a (Figure 5B). In addition, pulse-chase analysis indicates that Corr-4a increases the half-life of CFTR 837-1480 (Figure 5C).

Coexpression of the two halves of CFTR increases the accumulation of CFTR 837-1480, as well as, promotes the formation of a maturely glycosylated form of CFTR 837-1480 (Rosser *et al.,* 2008). Addition of Corr-4a to the coexpressed CFTR halves significantly increased the levels of CFTR 837-1480 and also enhanced interactions of the two halves of CFTR such that more folded maturely glycosylated CFTR 837-1480 accumulates (Figure 5D). Thus, Corr-4a can stabilize membrane inserted domains of CFTR and may thereby enhance the association and folding of N- and C-terminal regions.

To further probe the nature of the correctable and noncorrectable folding defects in CFTR, we deleted F508 from the CFTR N-terminal fragment 837X and examined its ability to facilitate the formation of the maturely glycosylated form of CFTR 837-1480 in the presence of Corr-4a. As reported above, deletion of the F508 residue destabilizes CFTR 837X in a manner that is noncorrectable by Corr-4a (Figures 3B and 5B). Coexpression of CFTR 837XΔF508, and the wild-type C-terminal fragment resulted in increased levels of the B-form of CFTR 837-1480, but no observable mature CFTR product (Rosser *et al.,* 2008; Figure 5D). Interestingly, the addition of Corr-4a did not result in the appearance of the mature, highly glycosylated form of CFTR 837-1480 when coexpressed with CFTR 837XΔF508. Yet, Corr-4a treatment still promotes the accumulation of CFTR 837-1480 even when it is coexpressed with the N-terminal CFTR 837XΔF508 fragment. Thus, factors in addition to stabilization of the C-terminal domains of CFTR by Corr-4a appear to be involved in the correction of ΔF508 folding defects.

Next, we probed the ability of Corr-4a to enhance the proper association of CFTR 837X V232D with CFTR 837-1480. We observed that, when compared with the wild-type 837X fragment, the steady-state levels of CFTR 837X V232D were decreased (Figure 5B). Yet, the levels of CFTR 837X V232D were increased upon Corr-4a treatment. Furthermore, pulse-chase analysis demonstrated that Corr-4a stabilizes CFTR 837X V232D (Figure 5C). In addition, the levels of the C-terminal fragment, 837-1480, were increased when coexpressed with the CFTR 837X V232D mutant fragment (Figure 5D). However, coexpression of this mutant N-terminal fragment with the wild-type C-terminal fragment did not produce a mature product. Yet, when Corr-4a was present, a large subpopulation of the 837X V232D and 837-

1480 fragments could assemble to form a mature CFTR product (Figure 5D). Thus, Corr-4a is able to correct defects in MSD1 folding to the degree that it facilitates the proper assembly of CFTR 837X V232D and CFTR 837-1480. Collectively, it appears that Corr-4a can impact the stability of CFTR fragments that contain MSD2 and mutant forms of MDS1, which in turn helps to augment CFTR folding.

Enhancement of CFTRΔF508 Folding by Combined Use of Corr-4a and Suppressor Mutations

Corr-4a is capable of stabilizing fragments of CFTR that contain MSD1 as well as MSD2 and may consequently increase the folding efficiency of CFTR and different CFTR mutants (Figure 5). Yet, it appears that multiple folding defects limit the rescue of CFTRΔF508, whereas a less complex mutant, such as CFTR V232D, is readily rescued. The loss of contact between NBD1 and surfaces in CL4 of MSD2 was recently implicated as a critical step in CFTRΔF508 misfolding (Mornon *et al.*, 2008; Serohijos *et al.*, 2008). Thus, we sought to restore such contacts and determine if this augmented the potency of Corr-4a action in promoting the folding of full-length CFTRΔF508.

Molecular modeling suggests that conversion of V510 to an Asp in NBD1 generates contacts with R1070 in CL4, thereby reinforcing the NBD1-MSD2 interface that is destabilized by loss of F508 (Mornon *et al.*, 2008). Indeed, introduction of the V510D suppressor point mutation into CFTRΔF508 partially restored folding of some CFTRΔF508 V510D (Wang *et al.*, 2007), but the majority of CFTRΔF508 V510D accumulated in its B-form (Figure 6A), and the level of repair remained below the observed levels of folded wild-type CFTR (compare to CFTR panel in Figure 3B). Corr-4a was able to enhance the accumulation of the folded C-form of CFTRΔF508 V510D (Figure 6A). Pulse-chase analysis indicated that Corr-4a stabilizes the B-form and enhances the folding efficiency of CFTRΔF508 V510D (Figure 6B). These data are impressive since Corr-4a-dependent enhancement of CFTRΔF508 folding efficiency is weak and difficult to detect in pulse-chase experiments (Figure 2B). Overall, the V510D suppressor mutation and Corr-4a appear to act in an additive manner to restore the folding of CFTRΔF508 toward wild-type levels.

Corr-4a appears to be able to stabilize CFTRΔF508 after MSD2 synthesis, yet this compound can only stabilize the B-form of CFTR 1172XΔF508 and does not enhance folding of this CFTR fragment to a level that can be detected in biochemical assays (Figure 3). We reasoned that this result reflects a role for the proposed NBD2-MSD1 interactions (Mornon *et al.*, 2008; Serohijos *et al.*, 2008) in stabilizing the conformation of CFTR when the F508 residue is missing. To test this model, we asked if the V510D suppressor mutation permits Corr-4a to correct the misfolding of CFTR 1172XΔF508. Suppression of CFTR 1172XΔF508 misfolding upon introduction of the V510D mutation was similar to the folding correction observed with CFTRΔF508 V510D (Figure 6A). Corr-4a was now able to promote the proper folding of a small pool of CFTR 1172XΔF508 V510D and enhance the accumulation of its maturely glycosylated C-form. However, again the level of correction was low and an increase in the folding efficiency of CFTR 1172XΔF508 V510D was not detected in pulse-chase experiments (Figure 6B).

These data suggest that proper folding of CFTR becomes dependent upon NBD2 when the F508 residue is deleted, and the global conformation of CFTR is destabilized by the loss of interactions between NBD1 and CL4 of MSD2. Our data indicates that Corr-4a modulates the conformation of CFTR MSDs, which in turn promotes proper folding of CFTR by permitting the formation of stable contacts between its N- and C-terminal regions.

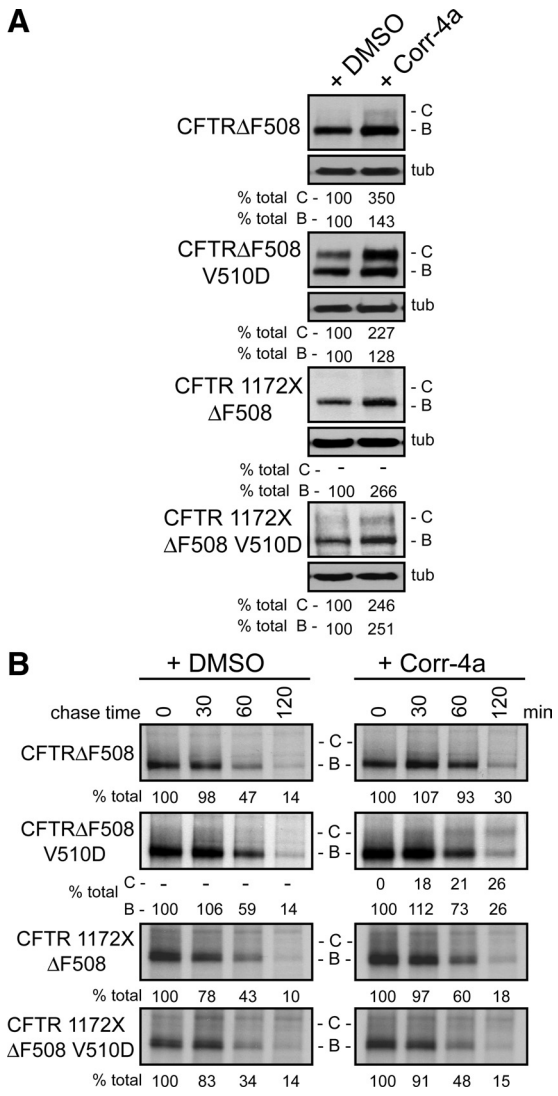

Figure 6. Enhancement of CFTRΔF508 folding by combined use of Corr-4a and suppressor mutations. (A) HEK293 cells were transfected with 1 μg of either CFTRΔF508, CFTRΔF508 V510D, CFTR 1172XΔF508, or CFTR 1172XΔF508 V510D. Chemicals Corr-4a or DMSO were added to the cells 18 h after transfection, and the cells were incubated with the chemicals for 24 h. Steady-state levels were then determined by Western blot analysis. Tubulin serves as a loading control. The B- and C-band levels for each reaction were quantified by densitometry and were normalized to the DMSO-treated control. (B) HEK293 cells transfected with the indicated CFTR plasmids were incubated with Corr-4a or DMSO for 2 h. Next, the cells were pulse-labeled with [^{35}S]methionine and chased for the indicated time periods in the presence of chemical treatment. Soluble cell lysates were normalized to total amount of protein, immunoprecipitated with a CFTR N-terminal antibody, and visualized by SDS-PAGE analysis and autoradiography. Results were quantified by densitometry and normalized to the amount of B-band at t = 0 min for each condition. Similar trends were observed upon repetition of the experiments shown in A and B.

DISCUSSION

Decreasing the endogenous pools of the E3 ubiquitin ligases RMA1 or CHIP permits a subpopulation of CFTRΔF508 to

fold, escape the ER, and accumulate as a maturely glycosylated species. In the absence of these ERQC factors, Corr-4a is dramatically more effective at rescuing folding defects in CFTRΔF508. The increases in folding efficiency observed via these manipulations are not complete, so it is clear that folding kinetics as well as the activity of ERQC machinery control the fate of nascent CFTR and CFTRΔF508. Previous approaches to inactivate ERQC factors and rescue CFTRΔF508 misfolding relied on overexpression of dominant negative E2 proteins (Younger *et al.,* 2004). Such an approach stabilized CFTRΔF508 in a foldable state, but did not permit its folding to a form that could escape the ER (Younger *et al.,* 2004). The observation that knockdown of endogenous RMA1 or CHIP can rescue folding defects of CFTRΔF508 is consistent with observations that knockdown of Hsp70/Hsp90 cochaperones permits proper folding of CFTRΔF508 (Wang *et al.,* 2006a). Cells often have a difficult time compensating for the loss of cochaperones because they have a broad array of general and essential cellular housekeeping functions (Cyr *et al.,* 2002). However, cells often tolerate the loss of ER quality control factors well (Wilhovsky *et al.,* 2000; Dai *et al.,* 2003). Thus, the development of approaches to inactivate specific E3 ubiquitin ligases (Burger and Seth, 2004; Lee *et al.,* 2008) have the potential to permit foldable pools of CFTRΔF508 or other disease-causing mutants to accumulate and may serve as a means to treat CF.

Deletion of F508 from CFTR causes multiple folding defects that occur during and after completion of biosynthesis (Chen *et al.,* 2004; Du *et al.,* 2005; Thibodeau *et al.,* 2005; Mornon *et al.,* 2008; Rosser *et al.,* 2008; Serohijos *et al.,* 2008; Du and Lukacs, 2009). Our studies on the mechanism for Corr-4a action indicate that deletion of F508 causes both correctable and noncorrectable defects in CFTR folding. Formation of a stable folding intermediate by MSD1, NBD1, and the R-domain requires F508 and assembly defects caused by loss of F508 in CFTR 837X appear to be recognized by the RMA1 E3 ligase (Rosser *et al.,* 2008). Interestingly, Corr-4a is not able to correct the folding defects of CFTR 837XΔF508. Thus, studies with this CFTR fragment suggests that Corr-4a acts after large pools of nascent CFTRΔF508 have been targeted for degradation by the RMA1 ligase. This observation explains why Corr-4a is ineffective at rescuing larger populations of misfolded CFTRΔF508. However, wild-type CFTR becomes resistant to RMA1 action once MSD2 is present (Younger *et al.,* 2006), and Corr-4a is now able to increase the folding efficiency of CFTR 1172X and stabilize CFTR 1172XΔF508. The E3 ligase CHIP does not appear to monitor the folding status of CFTR until the last subdomain, NBD2, is synthesized (Younger *et al.,* 2006). Thus, the population of CFTR that is able to make it past the RMA1 E3 ligase can be acted upon by Corr-4a, and its folding status can be monitored by the CHIP E3 ligase.

CFTR 1172X can fold and escape the ER (Cui *et al.,* 2007), but we were unable to biochemically detect folding of chemical-treated CFTR 1172XΔF508. Thus, although NBD2 is not required for maturation of CFTR 1172X, the contribution of NBD2 to the folding of ΔF508-containing mutants is required for maximal Corr-4a action. It appears that the predicted contacts between side chains in NBD2 and MSD1 (Mornon *et al.,* 2008; Serohijos *et al.,* 2008) are required for the maximal activity of Corr-4a in correction of folding defects caused by loss of the F508 residue. The need for such contacts can be overcome when contacts between NBD1 and MSD2, which are disrupted when F508 is deleted, are restored upon introduction of suppressor mutations in NBD1. Thus, interactions between solvent-exposed residues on NBD2 and MSD1, as well as NBD1 and MSD2, participate in CFTR and CFTRΔF508 assembly (Mornon *et al.,* 2008; Serohijos *et al.,* 2008).

Our data, and those of others (Wang *et al.,* 2006b; Loo *et al.,* 2008), suggest that the mechanism of Corr-4a action involves the alteration of the conformation of CFTR's MSDs. The conformation of MSD1 is altered after deletion of F508 (Cui *et al.,* 2007; Rosser *et al.,* 2008). As well, MSD2 is an unstable domain in wild-type CFTR that is chaperoned by calnexin (Rosser *et al.,* 2008) and becomes misfolded and more sensitive to proteolytic digestion in CFTRΔF508 (Du and Lukacs, 2009). Corr-4a promotes the accumulation of the MSD2 containing fragment CFTR 837-1480 and enhances assembly of CFTR 837-1480 with CFTR 837X. Corr-4a is also able to act on CFTR 837X V232D, and enhance its accumulation. In full-length CFTR, Corr-4a induced correction is not observed until after synthesis of MSD2. Thus, Corr-4a appears able to alter the conformation of MSDs of CFTR to enhance formation of proper interdomain contacts between MSD2 and other CFTR subdomains. Yet, Corr-4a is significantly more effective at promoting the folding of CFTRΔF508 V510D than CFTRΔF508. The V510D mutant is designed to facilitate interactions between NBD1 and MSD2 that are defective when F508 is deleted (Mornon *et al.,* 2008). Thus, it appears that restoration of these contacts combined with stabilization of MSD2 by Corr-4a is important for correction of CFTRΔF508 misfolding.

Interestingly, the affect of Corr-4a on the accumulation of CF disease-causing mutations contained within MSDs of CFTR was highly variable. Corr-4a–dependent rescue of MSD biogenic mutants G91R and M1137R was no greater than that observed with corrector-treated CFTRΔF508. Furthermore, the folding defect of the MSD1 mutant G85E appeared to be largely noncorrectable by Corr-4a. Surprisingly, there was a dramatic enhancement of CFTR V232D folding by Corr-4a. In addition, Corr-4a was able to enhance the accumulation of CFTR 837X V232D and to increase levels of the mature, highly glycosylated form of CFTR 837-1480 with CFTR 837X V232D. The V232D mutant presents a mild form of CF (Alonso *et al.,* 2007); however, the molecular basis for CF associated with this mutation has not been well-defined. The V232D mutation is proposed to form an aberrant hydrogen bond in MSD1 (Therien *et al.,* 2001; Choi *et al.,* 2004) that may hinder formation of interdomain contacts between MSD1 and MSD2 that are proposed to occur in the folded CFTR channel (Dawson and Locher, 2006; Serohijos *et al.,* 2008). Corr-4a is a hydrophobic molecule that may act by forming contacts within the hydrophobic transmembrane helices of CFTR or it may influence the lipid environment that surrounds CFTR. Nevertheless, Corr-4a can act on N- and C-terminal regions of CFTR and its mechanism of action is not specific to correction of folding defects caused by the F508 deletion.

Complex folding defects that involve multiple domains caused by deletion of F508 have thus far been difficult to correct with small molecules. Yet, rare mutations, such as V232D, which cause more specific folding defects (Therien *et al.,* 2001; Choi *et al.,* 2004) are easier to correct. It therefore seems prudent to define the mechanism for misfolding of additional rare CF-causing mutations and to screen for compounds that correct the folding defects caused by mutations other than deletion of F508. Rare mutations impact a small population of CF patients, but helping at least some of this patient population appears to be on the immediate horizon.

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