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Correctors Enhance Maturation of ΔF508 CFTR by Promoting Interactions between the Two Halves of the Molecule[†]

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ABSTRACT: Deletion of Phe508 in cystic fibrosis transmembrane conductance regulator (ΔF508 CFTR) causes cystic fibrosis. CFTR consists of two homologous halves with each containing a nucleotide-binding domain (NBD) and a transmembrane domain (TMD). ΔF508 CFTR appears to be trapped in an incompletely folded state. Small molecules (correctors) promote folding of ΔF508 CFTR with relatively low efficiency. Understanding the mechanism of repair may lead to the development of more effective correctors. Here we tested the effect of correctors and the ΔF508 mutation on interactions between the halves of CFTR when expressed as separate polypeptides. Glycosylation of C-half CFTR was defective when expressed alone as a mixture of core and unglycosylated proteins was detected. Coexpression of C-half CFTR with either wild-type N-half or ΔF508/N-half CFTR, however, increased the amount of core-glycosylated protein, but only coexpression with wild-type N-half promoted maturation of C-half CFTR (Endo H resistant). This suggested that the ΔF508 mutation inhibited some interactions between N-half and C-half CFTRs. Interaction of A52-tagged wild-type N-half or ΔF508/N-half CFTR with histidine-tagged C-half CFTR was then followed by nickel-chelate chromatography. Coexpression of A52-tagged wild-type N-half or ΔF508/N-half CFTR with histidine-tagged C-half CFTR resulted in the wild-type N-half CFTR but not ΔF508/N-half CFTR protein being retained on the column. Coexpression of ΔF508/N-half and C-half CFTR in the presence correctors VX-325 and corr-4a, however, restored interactions between the two halves. An interaction that was restored was that between NBD1 and TMD2 as the correctors restored cross-linking of mutant ΔF508/NBD1-(V510C)/TMD2(A1067C). Therefore, correctors promote proper interactions between the two halves of CFTR.

The cystic fibrosis transmembrane conductance regulator (CFTR)[1] is a cAMP-regulated chloride channel that is located on the apical surface of epithelial cells that line lung airways and ducts of various glands (reviewed in ref *1*). Its physiological role is to regulate salt secretion and reabsorption to maintain normal salt and water homeostasis in epithelial tissues (*2*).

Cystic fibrosis is a genetic disease caused by mutations in the CFTR gene that impair synthesis and trafficking of the protein or cause reduced chloride channel activity (*3*). The most common genetic lesion is deletion of the codon for Phe508 (ΔF508 CFTR). The ΔF508 CFTR protein undergoes rapid degradation because it is defective in folding (*4*). Defects in CFTR folding are recognized by ubiquitylation complexes (*5*−*7*) that direct the protein into the endoplasmic reticulum-associated degradation pathway (ERAD).

A potential treatment for cystic fibrosis would be to promote folding of ΔF508 CFTR to increase the amount of functional protein delivered to the cell surface as ΔF508 CFTR retains some functional activity (*8*). Small molecules called correctors have been identified that can increase the level of maturation of ΔF508 CFTR and trafficking to the cell surface (*9*−*11*). The compounds identified to date, however, are poor therapeutic candidates because they only promote delivery of a small fraction of the ΔF508 CFTR molecules to the cell surface. Knowledge of the mechanism of correctors would aid in the development of improved methods to promote folding and trafficking of ΔF508 CFTR.

CFTR belongs to the C subfamily of the ATP-binding cassette (ABCC7) family of transporters. Its 1480 amino acids are organized into two homologous halves that are joined by a regulatory (R) domain that contains phosphorylation sites that control the activation state of the channel (*3*). Each half begins with a transmembrane domain (TMD) containing six predicted transmembrane (TM) segments followed by a nucleotide-binding domain (NBD). Residue Phe508 is located in NBD1. Comparison of the crystal structures of wild-type and ΔF508 NBD1s revealed that the overall structures were quite similar (*12*). Therefore, it appeared that Phe508 might play a role in mediating interactions of NBD1 with other domains (*13*). There is no

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[1]Abbreviations: P-gp, P-glycoprotein; TM, transmembrane; NBD, nucleotide-binding domain; TMD1, NH_2-terminal transmembrane domain containing TM segments 1−6; TMD2, COOH-terminal transmembrane domain containing TM segments 7−12; HEK, human embryonic kidney; CFTR, cystic fibrosis transmembrane conductance regulator; corr-4a, *N*-[2-(5-chloro-2-methoxyphenylamino)-2′-yl]benzamide; VX-325, 4-(cyclohexyloxy)-2-{1-[4-(4-methoxybenzenesulfonyl)piperazin-1-yl]ethyl}quinazoline.

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atomic-resolution structure of full-length CFTR. A three-dimensional model of CFTR (*14*) has been constructed, however, on the basis of the atomic structures of the bacterial ABC transporters Sav1866 (*15*) and MsbA (*16*). In this model, Phe508 appeared to mediate interactions between NBD1 and TMD2 as the residue was positioned close to the fourth intracellular loop (ICL4) that connects TM segments 10 and 11. The proximity of Phe508 and ICL4 was confirmed by cross-linking analyses (*14*, *17*). It was observed in both studies that only mature forms of CFTR exhibited cross-linking between cysteines introduced into NBD1 and ICL4. Immature CFTR, including the ΔF508 mutant, exhibited little cross-linking. The cross-linking studies suggested that the ΔF508 mutation might inhibit CFTR folding by disrupting interactions between the two halves of CFTR. Establishment of interactions between the two halves of CFTR appears to be a late step in the folding process as the two halves of CFTR can be expressed as separate polypeptides but will associate when coexpressed in the same cell to yield functional channels at the cell surface (*18*, *19*). Here, we studied the effects of ΔF508 and correctors on the interactions between N-half and C-half CFTRs expressed as separate polypeptides.

MATERIALS AND METHODS

Construction of Mutants. Mutations were introduced into wild-type CFTR cDNA by site-directed mutagenesis as described by Kunkel (*20*). Plasmids that express N-half (residues 1−633) or C-half (residues 634−1480) molecules of CFTR were constructed as described previously (*21*). The cDNAs were further modified to encode an A52 epitope tag (*22*) at the C-terminal end or a 10-histidine tag (*23*) at the C-terminal end of C-half CFTR. The ΔF508 mutation was also introduced into N-half-A52. The construction of double-cysteine CFTR mutant ΔF508/V510C-(NBD1)/A1067C(TMD2) in a Cys-less background was described previously (*17*).

Expression of Mutants. The mutant CFTRs were transiently expressed in HEK 293 cells as described previously (*17*). HEK 293 cells were transfected with the cDNAs, and the medium was changed 7 h later to fresh medium [Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum] containing various concentrations of VX-325 (4-(cyclohexyloxy)-2-{1-[4-(4-methoxybenzenesulfonyl)piperazin-1-yl]ethyl}quinazoline) and corr-4a {*N*-[2-(5-chloro-2-methoxyphenylamino)-2′-yl]benzamide}, combinations of VX-325 and corr-4a, or no correctors. Cells were harvested 24 h after the change in medium. Whole cell extracts of A52-tagged CFTRs were subjected to immunoblot analysis using 6.5% (w/v) acrylamide gels and monoclonal antibody A52. In some cases, samples of the whole cell extracts were treated with endoglycosidase H or PNGase F as described previously (*24*) prior to immunoblot analysis. Whole cell SDS extracts of cells expressing untagged CFTRs were subjected to immunoblot analysis using a polyclonal antibody against NBD2 of CFTR (*25*).

Nickel-Chelate Chromatography. N-Half-A52 or ΔF508 N-half-A52 was expressed alone or coexpressed with C-half-His in the presence or absence of 50 μM VX-325 with 15 μM corr-4a for 24 h after transfection of HEK 293 cells. The cells were harvested and solubilized with 1% (w/v) *n*-dodecyl β-D-maltoside, and the supernatants were subjected to nickel-chelate chromatography as described previously (*26*). Samples of the supernatant obtained after centrifugation at 16000*g* for 15 min and material eluted with 300 mM imidazole were subjected to immunoblot analysis with monoclonal antibody A52.

Disulfide Cross-Linking Analysis. CFTR double-cysteine mutant ΔF508/V510C(NBD1)/A1067C(TMD2) was transiently expressed in HEK 293 cells in the presence or absence of 50 μM VX-325 and 15 μM corr-4a for 24 h. Membranes were prepared as described previously (*17*) and suspended in TBS (pH 7.4). Membrane samples were then treated with oxidant [1 mM Cu^{2+}-(1,10-phenanthroline)$_3$] for 15 min at 0 °C. The reactions were stopped by addition of 2× SDS sample buffer [125 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, and 4% (w/v) SDS] containing 50 mM EDTA and no reducing agent. The reaction mixtures were then subjected to SDS−PAGE [7% (w/v) polyacrylamide gels] and immunoblot analysis with a rabbit polyclonal antibody against CFTR (*27*). Intramolecular disulfide cross-linking between domains of CFTR can be detected because the cross-linked product migrates with a slower mobility on SDS−PAGE gels (*27*).

RESULTS

Effect of Correctors on Interactions between CFTR Half-Molecules. The mechanism that correctors use to promote maturation of CFTR processing mutants is unknown. One hypothesis is that correctors affect the cellular quality control mechanism. Another possibility is that correctors directly interact with the protein to promote proper interactions between the two halves of CFTR. A characteristic feature of ABC transporters such as CFTR is that they are composed of two homologous halves (Figure 1A). Each half contains an N-terminal TMD followed by an NBD. Establishment of domain−domain interactions between the two halves of an ABC transporter appears to be a key folding step in generation of a functional transporter as it has been demonstrated that expression of half-molecules of CFTR (*18*, *21*) or the P-glycoprotein (P-gp) drug pump (*28*) will yield a functional complex only if they are coexpressed in the same cell. The predicted structures (*14*) of the TMDs and NBDs of the N-half (Figure 1B) and C-half (Figure 1C) CFTR proteins are shown in Figure 1. The R domain is not shown for the sake of clarity and because it has been shown that CFTR deletion mutants lacking the R domain can mature and undergo trafficking to the cell surface (*29*). It is predicted from the models that the main contact point between the TMD and NBD within each half-molecule is the first intracellular loop. The second intracellular loop in each TMD (between TM segments 4 and 5 in TMD1 and between TM segments 10 and 11 in TMD2) is predicted to mediate interactions between the two halves (Figure 1D). The model depicting the interaction between the two halves (Figure 1D) shows that residue Phe508 lies at a contact point between the two halves. Phe508 lies at the interface between NBD1 and the second intracellular loop connecting TM segments 10 and 11 of TMD2. Previous studies suggested that the ΔF508 mutation traps CFTR as a partially folded immature protein with incomplete domain−domain contacts between the two halves. For example, mature CFTR shows cross-linking between cysteines in TM segment 6 (N-half) and TM segment 12 (C-half) or between cysteines in NBD1 [N-half (V510C)] and ICL4 [C-half (A1067C)], but the immature form of ΔF508 does not (*17*, *25*).

We tested for interaction between the two halves of CFTR by constructing the cDNAs encoding A52-tagged N-half (residues 1−633) or C-half (residues 634−1480) CFTRs followed by

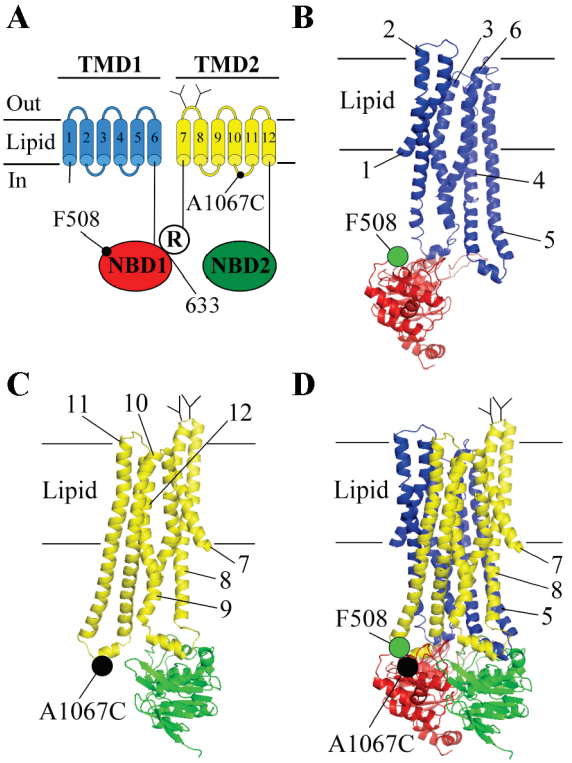

FIGURE 1: Schematic models of CFTR. (A) The cartoon depicts the various domains of CFTR and the location where CFTR was split into half-molecules (residue 633). The cylinders represent TM segments, and the branched lines represent the locations of the glycosylated sites. The predicted structures (*14*) of the TMD and NBD domains of N-half (B), C-half (C), or both (N-half and C-half CFTR) (D) are shown using the same color scheme as in panel A but with the R domain omitted for the sake of clarity. The helices that contain the predicted TM segments are numbered. The location of Phe508 is indicated by a green dot. The black dot indicates the predicted location of an introduced cysteine (A1067C) in TMD2 that will directly cross-link to a cysteine introduced into NBD1 (V510C). The pair of branched lines between TM segments 7 and 8 represents the glycosylated sites.

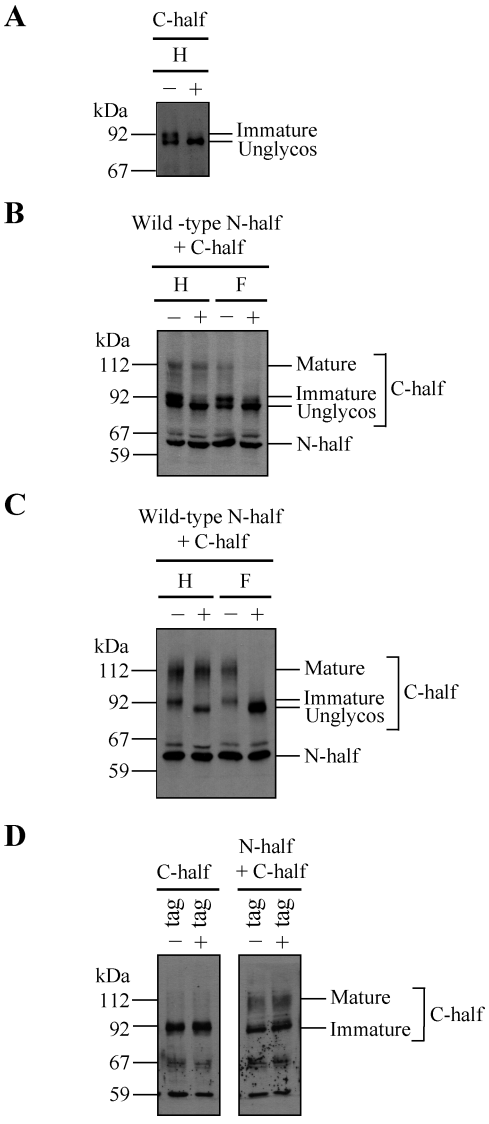

FIGURE 2: Expression of wild-type CFTR half-molecules. A52-tagged C-half CFTR was expressed alone (A) or coexpressed with A52-tagged N-half CFTR at a 1:1 (B) or 1:3 (C) cDNA ratio in HEK 293 cells. Whole cell SDS extracts were treated with (+) or without (−) endoglycosidase H (H) or PNGase F (F) and samples subjected to immunoblot analysis with monoclonal antibody A52. (D) Untagged (−tag) or A52-tagged (+tag) C-half CFTR was expressed alone (C-half) or coexpressed with untagged (−tag) or A52-tagged (+tag) N-half (N-half + C-half). Whole cell SDS extracts were subjected to immunoblot analysis with a rabbit polyclonal antibody against CFTR-NBD2. The positions of N-half CFTR and mature, immature, and unglycosylated (Unglycos) forms of C-half CFTR are indicated.

expression in HEK 293 cells. CFTR was split after residue 633 because previous studies showed that coexpression of these severed molecules yielded functional chloride channels at the cell surface (*19*, *21*, *30*). The N-half molecule (residues 1−633) contains TMD1 and NBD1, while the C-half molecule (residues 634−1480) contains the R domain, NBD2, and TMD2 (Figure 1A). Both glycosylation sites are located in the extracellular loop connecting TM segments 7 and 8 in the C-half molecule (Figure 1A). The glycosylation state of CFTR serves as a tool to monitor folding of the protein during synthesis. Core glycosylation is a cotranslational (reviewed in ref *31*) event that can be used to monitor the efficiency of topogenesis of the extracellular loop connecting TM segments 7 and 8. Incomplete folding of CFTR causes the protein to accumulate as a core-glycosylated intermediate that is sensitive to endoglycosidase H. Folding into a native structure allows the protein to proceed to the Golgi for processing of the carbohydrate into an endoglycosidase H-resistant state. Accordingly, the half-molecules were expressed in HEK 293 cells and whole cell SDS extracts subjected to immunoblot analysis. Expression of only the C-half molecule yielded both unglycosylated (86 kDa) and core-glycosylated (92 kDa, endoglycosidase H sensitive) proteins (Figure 2A). The presence of both core-glycosylated and unglycosylated C-half protein in the cell suggests that insertion of the first two TM segments (7 and 8) of C-half CFTR into the lipid bilayer was inefficient.

Coexpression of the A52 epitope-tagged N-half and C-half CFTRs at a 1:1 cDNA ratio yielded additional A52-reactive protein bands after immunoblot analysis of whole cell SDS extracts. Figure 2B shows that the 65 kDa protein corresponds to the predicted size of N-half CFTR. The N-half protein was not glycosylated as it was resistant to both endoglycosidase H and PNGase F. The 110 kDa protein represents mature C-half protein, as it was resistant to endoglycosidase H but sensitive to PNGase F (Figure 2B). A mixture of core-glycosylated (92 kDa) and unglycosylated (86 kDa) C-half protein was also detected. The ratio of core-glycosylated to unglycosylated C-half

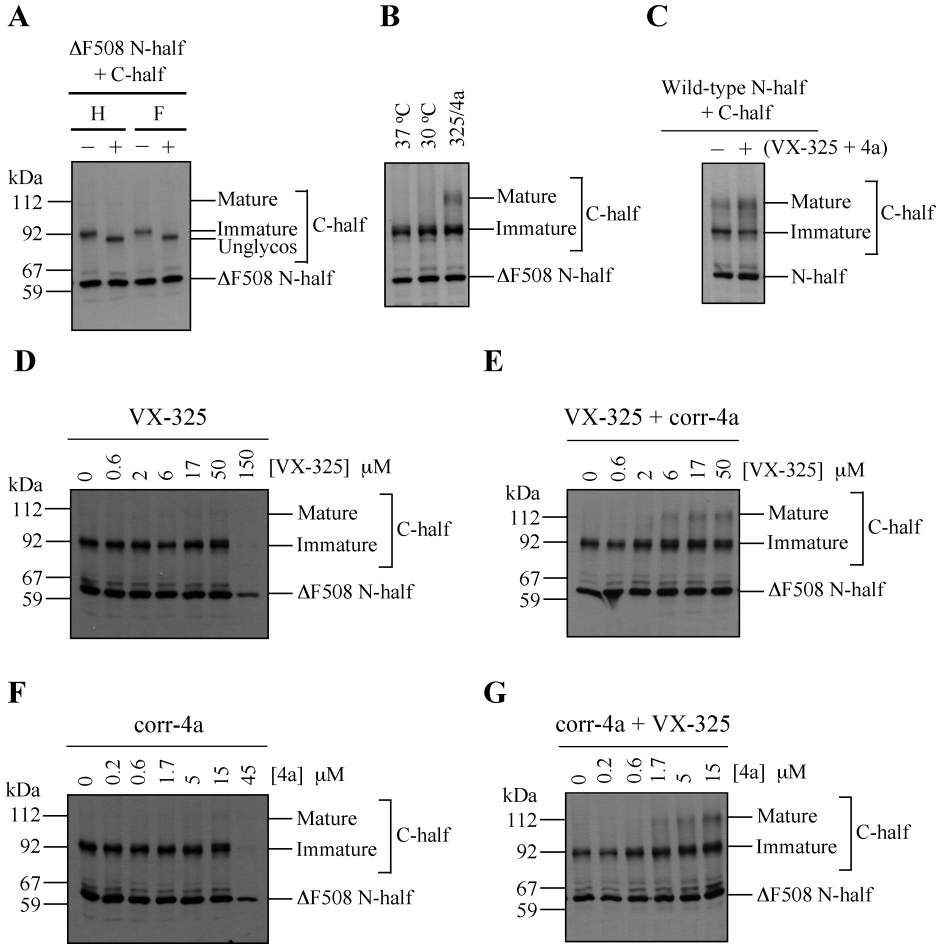

FIGURE 3: Coexpression of ΔF508 N-half and wild-type C-half CFTRs. (A) HEK 293 cells were transfected with A52-tagged ΔF508/N-half and A52-tagged wild-type C-half CFTR cDNAs at a 3:1 ratio. Whole cell SDS extracts were treated with (+) or without (−) endoglycosidase H (H) or PNGase F (F) and samples subjected to immunoblot analysis with monoclonal antibody A52. (C) Cells were transfected as described above. After 24 h, the medium was changed to fresh medium and incubated at 37 or 30 °C or replaced with fresh medium containing 50 μM VX-325 and 15 μM corr-4a (325/4a). After an additional 24 h, whole cell extracts were subjected to immunoblot analysis as described above. (C) A52-tagged wild-type N- and C-half (transfected at a 1.5:1 ratio, respectively) were expressed in the absence (−) or presence (+) of 50 μM VX-325 and 15 μM corr-4a (VX-325+4a) for 24 h followed by immunoblot analysis of whole cell SDS extracts. A52-tagged ΔF508/N-half and C-half CFTR were expressed for 24 h in the presence of various concentrations of VX-325 (D), various concentrations of VX-325 and 15 μM corr-4a (E), various concentrations of corr-4a (F), or various concentrations of corr-4a and 50 μM VX-325 (G) followed by immunoblot analysis. The positions of ΔF508 N-half, mature, immature, and unglycosylated (Unglycos) forms of C-half CFTR are indicated.

protein was increased, however, when cells were transfected with a 3:1 ratio of N-half cDNA to C-half cDNA (Figure 2C). This result suggests that interactions of the C-half protein with the N-half protein during synthesis may promote insertion of TM segments 7 and 8 into a native topology that undergoes glycosylation. Increasing the ratio of N-half protein to C-half protein also increased the efficiency of maturation of C-half CFTR. When the cDNAs were transfected at an equivalent ratio (Figure 2B), the amount of mature C-half protein (110 kDa) was ~12% of total C-half protein. An excess of N-half protein (Figure 2C) enhanced maturation of C-half CFTR such that the amount of mature C-half protein increased to ~65% of the total amount of C-half protein.

To test if the C-terminal tags affected maturation of C-half CFTR, we constructed untagged N-half (residues 1−633; insertion of a stop codon at residue 634) and C-half (residues 634−1480) molecules of CFTR. The cDNAs were expressed in HEK 293 cells and whole cell SDS extracts subjected to immunoblot analysis with a rabbit polyclonal antibody to NBD2 (*25*). Figure 2D shows that the C-terminal tags did not affect maturation of C-half CFTR because similar levels of mature C-half CFTR were detected in the presence or absence of the tags.

To test if the ΔF508 mutation affected the ability of N-half CFTR to promote maturation of C-half CFTR, cells were cotransfected with A52-tagged ΔF508/N-half and C-half CFTRs at a 3:1 cDNA ratio. After 24 h, whole cell SDS extracts were treated with or without endoglycosidase and samples subjected to immunoblot analysis. Figure 3A shows that the major products were the 65 kDa N-half and the 92 kDa C-half proteins. The 92 kDa protein represented core-glycosylated C-half CFTR as it was sensitive to both endoglycosidase H and F (Figure 3A). No unglycosylated (86 kDa) or mature (112 kDa) forms of the C-half protein were observed. The results suggest that the ΔF508 mutation did not impair the ability of N-half CFTR to promote core glycosylation of C-half CFTR. In contrast to wild-type N-half CFTR, however (Figure 2C), the ΔF508 mutation appeared to inhibit other interactions between the half-molecules because no maturation of C-half CFTR was observed (Figure 3B). This observation supports the recent findings of Rosser et al. (*32*). The authors studied interactions between split molecules of CFTR (severed at a different site, residue 837) and

reported that the ΔF508 mutation affected a late-stage folding event between the severed molecules.

Full-length ΔF508 CFTR will mature when expressed at reduced temperatures (*8*). Recently, it was reported that promotion of maturation of ΔF508 CFTR at reduced temperature could be due to changes in the folding environment as temperature could affect the levels of molecular chaperones in the cell (*33*). Accordingly, we tested whether C-half CFTR would undergo maturation if coexpressed with ΔF508/N-half CFTR at 30 °C. Cells were cotransfected with cDNAs of A52-tagged ΔF508/N-half and C-half CFTR and incubated at 37 or 30 °C for 18 h and whole cell SDS extracts subjected to immunoblot analysis. Figure 3B shows that mature C-half (110 kDa) CFTR was not detected at either temperature. Maturation of C-half CFTR in the presence of ΔF508/N-half CFTR could be induced, however, when the cells were incubated with correctors VX-325 (50 μM) and corr-4a (15 μM). When C-half CFTR and ΔF508/N-half CFTR were coexpressed in the presence of both correctors, the mature (110 kDa) protein made up 21% of the total C-half CFTR (Figure 3B). The level of maturation of wild-type C-half CFTR when coexpressed with wild-type N-half CFTR also increased from 24% in the absence of correctors to 51% in the presence of correctors (50 μM VX-325 and 15 μM corr-4a) (Figure 3C). Both correctors VX-325 and corr-4a were used because only low levels of mature C-half CFTR were detected when it was coexpressed with ΔF508/N-half CFTR in the presence of only one corrector (Figure 3D,F), and correctors have been shown to have an additive effect on maturation of CFTR processing mutants (*34*). Panels E and G of Figure 3 show that VX-325 and corr-4a had an additive effect on C-half CFTR maturation when coexpressed with ΔF508/N-half CFTR. Higher concentrations of correctors appeared to be toxic to the cells [150 μM VX-325 (Figure 3D) or 45 μM corr-4a (Figure 3F)] and resulted in detachment of the cells from the plates. Another modification was that we used 50 μM VX-325 in 10% (v/v) serum rather than in 2% (v/v) (*9*) serum because the higher concentration of serum and VX-325 increased the level of expression and maturation of CFTR processing mutants (unpublished observations).

Correctors may induce maturation of C-half CFTR by promoting domain−domain interactions with ΔF508/N-half CFTR. We then utilized nickel-chelate chromatography to test the effect of correctors on interactions between ΔF508/N-half CFTR and C-half CFTR. The rationale was that ΔF508/N-half CFTR and C-half CFTR might loosely associate when they accumulate in the endoplasmic reticulum in immature conformations because interactions between the two halves are incomplete. These relatively weak interactions may be sensitive to the high-salt (0.3 M) and detergent [1% (w/v) *n*-dodecyl β-D-maltoside] conditions used during nickel-chelate chromatography and could be used to monitor interaction between A52-tagged N-half CFTR and histidine-tagged C-half CFTR. Nickel-chelate chromatography has previously been demonstrated to be a useful technique for distinguishing between immature and mature forms of P-gp half-molecules (*35*). It was shown that an epitope-tagged wild-type P-gp half-molecule would copurify with a wild-type histidine-tagged half-molecule using nickel-chelate chromatography. The interaction between the half-molecules was disrupted by the presence of a processing mutation in either half-molecule. Expression in the presence of a drug substrate could reverse the effects of the processing mutation (*35*).

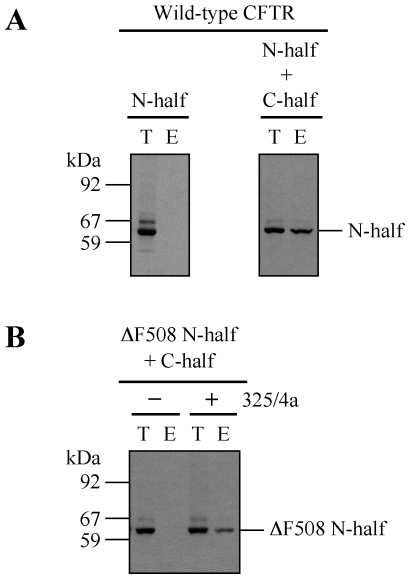

FIGURE 4: Nickel-chelate chromatography of CFTR half-molecules. (A) Wild-type A52-tagged N-half CFTR was expressed alone (left) or together with histidine-tagged C-half CFTR (right). Whole cell detergent extracts were applied to nickel columns. The columns were washed and histidine-tagged protein eluted with 300 mM imidazole. Samples corresponding to 1% of the total solubilized material (T) or 5% of the eluted material (E) were subjected to immunoblot analysis with monoclonal antibody A52. (B) A52-tagged ΔF508/N-half CFTR and histidine-tagged wild-type C-half CFTRs were coexpressed in HEK 293 cells in the absence (−) or presence (+) of correctors VX-325 and corr-4a (325/4a). Cell extracts were subjected to nickel-chelate chromatography as described above. Samples corresponding to 1% of the total solubilized material (T) or 10% of the eluted material (E) were subjected to immunoblot analysis with monoclonal antibody A52. The position of ΔF508 N-half CFTR is indicated.

The first step was to test if both half-molecules of CFTR would copurify using nickel-chelate chromatography if one half-molecule contained a histidine tag. Accordingly, the C-half CFTR cDNA was modified to encode a 10-histidine tag at the C-terminal end of the protein. To test if A52-tagged wild-type N-half CFTR would interact with histidine-tagged C-half protein, it was expressed alone or with histidine-tagged C-half CFTR. The cells were solubilized with 1% (w/v) *n*-dodecyl β-D-maltoside and then subjected to nickel-chelate chromatography. Samples of the total detergent extract and eluted material were subjected to SDS−PAGE, and the presence of A52-tagged N-half protein was detected by immunoblot analysis. Figure 4A shows that A52-tagged wild-type N-half CFTR was not retained on the column unless it was coexpressed with the histidine-tagged C-half protein. We then coexpressed A52-tagged ΔF508/N-half CFTR and histidine-tagged C-half CFTR in the absence or presence of correctors (VX-325 and 4a) followed by nickel-chelate chromatography. The cells were solubilized with 1% (w/v) *n*-dodecyl β-D-maltoside and subjected to nickel-chelate chromatography. Samples solubilized with 1% (w/v) *n*-dodecyl β-D-maltoside and eluted from the column were subjected to immunoblot analysis. Figure 4B shows that the ΔF508/N-half protein was not eluted from the column in the absence of correctors. Expression of A52-tagged ΔF508/N-half CFTR and histidine-tagged C-half CFTR in the presence of correctors, however, promoted interactions between the two halves as the N-half protein was retained on the column and eluted with imidazole (Figure 4B). Although the half-molecules

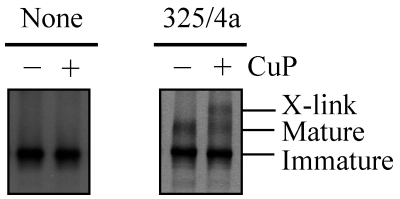

FIGURE 5: Effect of correctors on cross-linking of mutant ΔF508/V510C(NBD1)/A1067C(TMD2). Membranes prepared from cells expressing mutant ΔF508/V510C(NBD1)/A1067C(TMD2) in the absence (None) or presence (325/4a) of VX-325 and corr-4a were treated without (−) or with (+) 1 mM copper phenanthroline (CuP) for 15 min at 0 °C. Samples were subjected to immunoblot analysis. The positions of immature, mature, and cross-linked (X-link) forms of CFTR are indicated.

likely associate early in synthesis to increase the efficiency of insertion of TM8 into the membrane (Figure 2B), it appears that expression in the presence of correctors enhanced interactions between the half-molecules such that they remained associated even after being exposed to relatively high concentrations of detergent [1% (w/v)] and NaCl (0.3 M). These results suggest that the presence of the ΔF508 mutation disrupted interactions between the two halves and that in the absence of correctors, the ΔF508/N-half CFTR and C-half CFTR molecules appear to be trapped in loosely folded conformations. Expression of the half-molecules in the presence of VX-325 and corr-4a may have promoted further domain−domain interactions to increase the number of contacts between the two halves.

An interaction that may be sensitive to the ΔF508 mutation and correctors occurs between NBD1 and TMD2. It has been demonstrated in cysteine mutagenesis and cross-linking studies that residue 508 in NBD1 lies close to ICL4 in TMD2 (*14*) and that the ΔF508 mutation disrupts the NBD1−TMD2 interaction (*17*). For example, the cysteines in mutant V510C(NBD1)/A1067C(TMD2) were directly cross-linked in a wild-type background but not in a ΔF508 background (*17*). Although an equivalent interaction occurs between NBD2 and TMD1, only the NBD1−TMD2 interaction appears to be critical for maturation as a deletion mutant lacking NBD2 can mature (*24*, *36*). Therefore, it was possible that a crucial step induced by correctors in ΔF508 CFTR was to promote interactions between the segment encompassing position 508 and ICL4. To test if correctors VX-325 and corr-4a promoted NBD1−TMD2 interactions in ΔF508 CFTR, mutant ΔF508/V510C(NBD1)/A1067C-(TMD2) was expressed in the presence or absence of the correctors. The full-length molecule was used rather than the Cys-less half-molecules because we found that ΔF508/Cys-less N-half CFTR was unstable. The unstable nature of Cys-less N-half CFTR has been reported previously (*19*). Therefore, membranes were prepared from cells expressing the full-length mutant CFTR, and samples were treated with or without copper phenanthroline [Cu^{2+}-(1,10-phenanthroline)$_3$] for 15 min on ice. Copper phenanthroline catalyzes the air oxidation of sulfhydryl groups (*37*) to catalyze the formation of a disulfide bond between adjacent cysteines. The reactions were stopped by the addition of EDTA, and samples were subjected to immunoblot analysis (Figure 5). Cross-linking can be readily detected because the cross-linked protein migrates with a lower mobility on SDS−PAGE gels (*25*). In the absence of correctors, mutant ΔF508/V510C(NBD1)/A1067C(TMD2) yielded immature CFTR (160 kDa protein) that showed no detectable cross-linking (Figure 5, left). Expression of ΔF508/V510C(NBD1)/A1067C-(TMD2) in the presence of correctors, however, yielded mature CFTR (180 kDa protein) (Figure 5, right). Treatment of the mature CFTR with copper phenanthroline yielded cross-linked CFTR that migrated with a lower mobility on the gel. The results suggest that correctors promote NBD1−TMD2 interactions.

DISCUSSION

In this study, we present evidence for early- and late-stage interactions between the two half-molecules of CFTR. It was found that ΔF508 and correctors appeared to affect the late-stage interactions. An early interaction that appeared to take place between the two half-molecules was an interaction of a region containing TM segments 7 and 8 (C-half) with N-half CFTR because it influenced the efficiency of core glycosylation of the C-half protein. When C-half CFTR was synthesized alone, glycosylation of the extracellular loop (between TM segments 7 and 8) appeared to be inefficient as approximately half of the protein detected in whole cell extracts was unglycosylated (Figure 2A). Defects in this region have also been observed by Carveth et al. (*38*), who reported that TM segment 8 exhibited poor stop transfer activity when both TM segments (7 and 8) were attached to a reporter molecule. When the TM 7−8 CFTR fusion protein was expressed in *Xenopus laevis* oocytes, it was found that ~75% of the protein was core-glycosylated. The efficiency of topological maturation of the CFTR TM 7−8 segments may be lower in mammalian cells than in *X. laevis* oocytes. For example, aquaporin 1 remains in its immature four-spanning topology when synthesized in canine rough microsomes but acquires its mature six-spanning topology when incorporated into *Xenopus* microsomes (*39*).

The efficiency of glycosylation of C-half CFTR was markedly improved upon coexpression with an excess of N-half CFTR (Figure 2C). It is predicted from the model of CFTR (*14*) that TM segments 7 and 8 interact with the last two TM segments (5 and 6) of N-half CFTR (Figure 1D). Interaction of TM segments 7 and 8 (C-half) with TM segments 5 and 6 (N-half) may assist in positioning both glycosylation sites in the extracellular loop connecting TM segments 7 and 8 in the membrane so they can be modified by oligosaccharyl transferases. The glycosylation sites must be spaced a minimum distance from the membrane surface to be glycosylated cotranslationally (*40*). The acceptor site must be located at least 12 amino acids from the preceding TM segment and 14 amino acids from the beginning of the following TM segment (reviewed in ref *31*). An increased level of core glycosylation of C-half CFTR was also observed upon coexpression with the ΔF508/N-half protein, suggesting that the ΔF508 mutation did not block the early interactions between the two proteins (Figure 3A). It has been reported that CFTR folds mostly cotranslationally (*41*).

The ΔF508 mutation, however, must have inhibited another folding step because coexpression of C-half CFTR with the ΔF508/N-half protein did not promote maturation of the C-half protein (Figure 3A). This observation is in agreement with a recent study on interactions of calnexin with CFTR split molecules that showed that the ΔF508 mutation seemed to affect a late stage of folding (*32*). In contrast to our study, Rosser et al. (*32*) severed CFTR at a different location such that the R domain formed part of the N-half protein. The N-half molecule consisted of residues 1−837, whereas C-half CFTR consisted of residues 837−1480. When C-half CFTR (residues 837−1480) was expressed alone, it was observed that the protein accumulated

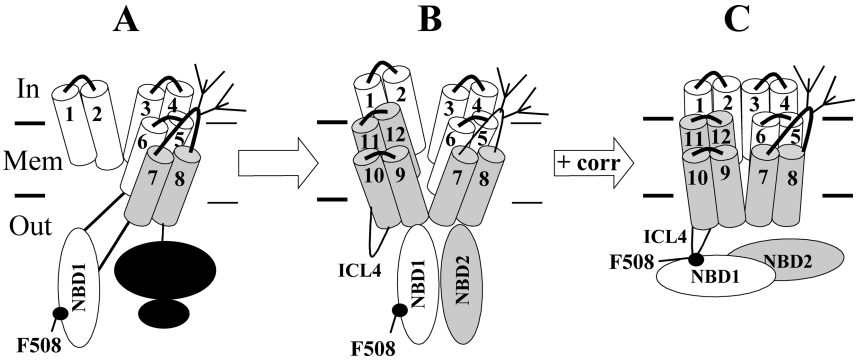

FIGURE 6: Effects of ΔF508 and correctors on interactions between CFTR half-molecules. In the models, the numbered cylinders represent the TM segments within the membrane (Mem) and the branched lines in the loop connecting TM segments 7 and 8 represent the glycosylation sites. The R domain is not shown for the sake of clarity. (A) An initial interaction that occurs between the halves of CFTR may be association of TM segments 7 and/or 8 (C-half) with N-half TM segments since the efficiency of cotranslational glycosylation of C-half CFTR was enhanced by the presence of excess N-half protein. The model depicts TM segments 7 and 8 of the N-half protein before the rest of the molecule has emerged from the ribosome (colored black). (B) CFTR folding then proceeds to yield an intermediate that has incomplete domain−domain interactions. Processing mutations such as ΔF508 introduce a thermodynamic hurdle to trap the protein at this stage. Specific folding defects between the halves are incomplete packing between TM segments (*25*) and incomplete interactions between the segment containing Phe508 in NBD1 and ICL4 of TMD2. (C) Correctors (+corr) can interact with the folding intermediates of processing mutants to lower the thermodynamic hurdle and allow the protein to fold into a native structure. Interactions that would enhance association of the two halves are packing of the TM segments and interaction of the Phe508 segment in NBD1 to ICL4 of TMD2.

as an unstable core-glycosylated protein that was rapidly degraded. The C-half (residues 837−1480) protein was stabilized, however, upon coexpression with wild-type or ΔF508 N-half (residues 1−837) protein. Maturation of C-half CFTR (conversion to endoglycosidase H-resistant protein) was only observed, however, when it was coexpressed with the wild-type (residues 1−837) protein. Therefore, the authors concluded that the ΔF508 mutation affected only a late-stage folding event between the two halves of CFTR.

Expression of the half-molecules in the presence of VX-325 and corr-4a appeared to promote a later interaction between ΔF508/N-half CFTR and C-half CFTR. Evidence for an increased level of interaction was that ΔF508/N-half CFTR was retained on a nickel column only upon coexpression with histidine-tagged C-half CFTR in the presence of VX-325 and corr-4a (Figure 4B). A late-stage ΔF508 CFTR folding event that appeared to be promoted by conducting expression in the presence of correctors was domain−domain interaction between NBD1 and TMD2. Cross-linking of cysteines in mutant ΔF508/V510C(NBD1)/A1067C(TMD2) was observed only if maturation of the mutant was induced by conducting expression in the presence of VX-325 and corr-4a. In a previous study, we were unable to detect maturation of mutant ΔF508/V510C(NBD1)/A1067C(TMD2) using only VX-325 or corr-4a (*17*). Interaction between TMD1 and TMD2 may be another late-stage folding event that is inhibited by processing mutants (*25*) and restored by expression in the presence of correctors (*42*).

A model of the effect of the ΔF508 mutation and correctors on CFTR folding is shown in Figure 6. An initial interaction between the two halves appears to be a cotranslational step as TM segments 7 and 8 are synthesized and results in efficient core glycosylation that is not inhibited by the presence of the ΔF508 mutation (Figure 6A). The protein then becomes trapped as a folding intermediate after synthesis due to incomplete TMD1−TMD2 and NBD1−TMD2 contacts (Figure 6B). The trapped intermediate is likely recognized by the RMA1 E3 ubiquitin complex to target the protein for degradation. Since folding of the protein is incomplete, treatment of cells with proteasome inhibitors does not promote maturation and export out of the endoplasmic reticulum (*43*). The protein completes the folding process to establish native TMD1−TMD2 and NBD1−TMD2 contacts, however, when ΔF508 CFTR is expressed in the presence of VX-325 and corr-4a (Figure 6C).

The mechanism of corrector rescue of CFTR appears to resemble drug rescue of P-gp. Processing mutations can disrupt interactions between P-gp half-molecules. These effects could also be reversed by expression in the presence of drug substrates (*35*). The presence of drug substrates appeared to enhance a late-stage folding process that was termed "superfolding". Drug substrates promoted folding of the P-gp processing mutants through interactions with the TM domains (*44*). In wild-type P-gp, NBD−TMD interactions appear to be important for topological maturation of the TM segments since a truncation mutant lacking the NBDs accumulates as an immature protein (*44*). The NBDs of CFTR and P-gp likely contribute to topological maturation through interactions with the intracellular loops (*14*, *17*). These observations suggest that several regions of ΔF508 CFTR could be potential targets for correctors. One region would be the TM domains as the mutation traps the protein in a conformation with incomplete packing of the TM segments. Studies on P-gp suggest that drug substrates promote maturation of processing mutants through hydrogen bond interactions with multiple TM segments (*45*). Therefore, improved correctors with improved ability to form hydrogen bond interactions (increased fit) with residues in CFTR TM segments may improve their efficiency. Another target would be to design correctors capable of promoting interactions of NBD1 with ICL4 of TMD2 as NBD−TMD interactions also appear to contribute to packing of the TM segments in yielding a native structure. In both cases, correctors must be designed to be sufficiently hydrophobic that they are able to diffuse into the cell to directly interact with CFTR as it is being synthesized in the endoplasmic reticulum.

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REFERENCES

1. Riordan, J. R. (2008) CFTR function and prospects for therapy. *Annu. Rev. Biochem. 77*, 701–726.
2. Welsh, M. J., and Smith, A. E. (1993) Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell 73*, 1251–1254.
3. Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., and Tsui, L.-C. (1989) Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. *Science 245*, 1066–1073.
4. Cheng, S. H., Gregory, R. J., Marshall, J., Paul, S., Souza, D. W., White, G. A., O'Riordan, C. R., and Smith, A. E. (1990) Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell 63*, 827–834.
5. Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M., and Cyr, D. M. (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat. Cell Biol. 3*, 100–105.
6. Meacham, G. C., Lu, Z., King, S., Sorscher, E., Tousson, A., and Cyr, D. M. (1999) The Hdj-2/Hsc70 chaperone pair facilitates early steps in CFTR biogenesis. *EMBO J. 18*, 1492–1505.
7. Younger, J. M., Chen, L., Ren, H. Y., Rosser, M. F., Turnbull, E. L., Fan, C. Y., Patterson, C., and Cyr, D. M. (2006) Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator. *Cell 126*, 571–582.
8. Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E., and Welsh, M. J. (1992) Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature 358*, 761–764.
9. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2005) Rescue of ΔF508 and other misprocessed CFTR mutants by a novel quinazoline compound. *Mol. Pharmaceutics 2*, 407–413.
10. Pedemonte, N., Lukacs, G. L., Du, K., Caci, E., Zegarra-Moran, O., Galietta, L. J., and Verkman, A. S. (2005) Small-molecule correctors of defective ΔF508-CFTR cellular processing identified by high-throughput screening. *J. Clin. Invest. 115*, 2564–2571.
11. Van Goor, F., Straley, K. S., Cao, D., Gonzalez, J., Hadida, S., Hazlewood, A., Joubran, J., Knapp, T., Makings, L. R., Miller, M., Neuberger, T., Olson, E., Panchenko, V., Rader, J., Singh, A., Stack, J. H., Tung, R., Grootenhuis, P. D., and Negulescu, P. (2006) Rescue of ΔF508 CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. *Am. J. Physiol. 290*, L1117–L1130.
12. Lewis, H. A., Zhao, X., Wang, C., Sauder, J. M., Rooney, I., Noland, B. W., Lorimer, D., Kearins, M. C., Conners, K., Condon, B., Maloney, P. C., Guggino, W. B., Hunt, J. F., and Emtage, S. (2005) Impact of the ΔF508 mutation in first nucleotide-binding domain of human cystic fibrosis transmembrane conductance regulator on domain folding and structure. *J. Biol. Chem. 280*, 1346–1353.
13. Du, K., Sharma, M., and Lukacs, G. L. (2005) The ΔF508 cystic fibrosis mutation impairs domain-domain interactions and arrests post-translational folding of CFTR. *Nat. Struct. Mol. Biol. 12*, 17–25.
14. Serohijos, A. W., Hegedus, T., Aleksandrov, A. A., He, L., Cui, L., Dokholyan, N. V., and Riordan, J. R. (2008) Phenylalanine-508 mediates a cytoplasmic-membrane domain contact in the CFTR 3D structure crucial to assembly and channel function. *Proc. Natl. Acad. Sci. U.S.A. 105*, 3256–3261.
15. Dawson, R. J., and Locher, K. P. (2006) Structure of a bacterial multidrug ABC transporter. *Nature 443*, 180–185.
16. Ward, A., Reyes, C. L., Yu, J., Roth, C. B., and Chang, G. (2007) Flexibility in the ABC transporter MsbA: Alternating access with a twist. *Proc. Natl. Acad. Sci. U.S.A. 104*, 19005–19010.
17. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2008) Processing mutations disrupt interactions between the nucleotide binding and transmembrane domains of P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (CFTR). *J. Biol. Chem. 283*, 28190–28197.
18. Ostedgaard, L. S., Rich, D. P., DeBerg, L. G., and Welsh, M. J. (1997) Association of domains within the cystic fibrosis transmembrane conductance regulator. *Biochemistry 36*, 1287–1294.
19. Mense, M., Vergani, P., White, D. M., Altberg, G., Nairn, A. C., and Gadsby, D. C. (2006) In vivo phosphorylation of CFTR promotes formation of a nucleotide-binding domain heterodimer. *EMBO J. 25*, 4728–4739.
20. Kunkel, T. A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.
21. Chan, K. W., Csanady, L., Seto-Young, D., Nairn, A. C., and Gadsby, D. C. (2000) Severed molecules functionally define the boundaries of the cystic fibrosis transmembrane conductance regulator's NH_2-terminal nucleotide binding domain. *J. Gen. Physiol. 116*, 163–180.
22. Loo, T. W., and Clarke, D. M. (1994) Functional consequences of glycine mutations in the predicted cytoplasmic loops of P-glycoprotein. *J. Biol. Chem. 269*, 7243–7248.
23. Loo, T. W., and Clarke, D. M. (1995) Rapid purification of human P-glycoprotein mutants expressed transiently in HEK 293 cells by nickel-chelate chromatography and characterization of their drug-stimulated ATPase activities. *J. Biol. Chem. 270*, 21449–21452.
24. Wang, Y., Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2007) Modulating the folding of P-glycoprotein and cystic fibrosis transmembrane conductance regulator truncation mutants with pharmacological chaperones. *Mol. Pharmacol. 71*, 751–758.
25. Chen, E. Y., Bartlett, M. C., Loo, T. W., and Clarke, D. M. (2004) The ΔF508 mutation disrupts packing of the transmembrane segments of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem. 279*, 39620–39627.
26. Loo, T. W., and Clarke, D. M. (1996) The minimum functional unit of human P-glycoprotein appears to be a monomer. *J. Biol. Chem. 271*, 27488–27492.
27. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2008) Correctors promote folding of the CFTR in the endoplasmic reticulum. *Biochem. J. 413*, 29–36.
28. Loo, T. W., and Clarke, D. M. (1994) Reconstitution of drug-stimulated ATPase activity following co- expression of each half of human P-glycoprotein as separate polypeptides. *J. Biol. Chem. 269*, 7750–7755.
29. Ostedgaard, L. S., Zabner, J., Vermeer, D. W., Rokhlina, T., Karp, P. H., Stecenko, A. A., Randak, C., and Welsh, M. J. (2002) CFTR with a partially deleted R domain corrects the cystic fibrosis chloride transport defect in human airway epithelia in vitro and in mouse nasal mucosa in vivo. *Proc. Natl. Acad. Sci. U.S.A. 99*, 3093–3098.
30. Csanady, L., Seto-Young, D., Chan, K. W., Cenciarelli, C., Angel, B. B., Qin, J., McLachlin, D. T., Krutchinsky, A. N., Chait, B. T., Nairn, A. C., and Gadsby, D. C. (2005) Preferential phosphorylation of R-domain Serine 768 dampens activation of CFTR channels by PKA. *J. Gen. Physiol. 125*, 171–186.
31. Cheung, J. C., and Reithmeier, R. A. (2007) Scanning N-glycosylation mutagenesis of membrane proteins. *Methods 41*, 451–259.
32. Rosser, M. F., Grove, D. E., Chen, L., and Cyr, D. M. (2008) Assembly and misassembly of cystic fibrosis transmembrane conductance regulator: Folding defects caused by deletion of F508 occur before and after the calnexin-dependent association of membrane spanning domain (MSD) 1 and MSD2. *Mol. Biol. Cell 19*, 4570–4579.
33. Wang, X., Koulov, A. V., Kellner, W. A., Riordan, J. R., and Balch, W. E. (2008) Chemical and biological folding contribute to temperature-sensitive ΔF508 CFTR trafficking. *Traffic 9*, 1878–1793.
34. Wang, Y., Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2007) Additive effect of multiple pharmacological chaperones on maturation of CFTR processing mutants. *Biochem. J. 406*, 257–263.
35. Loo, T. W., and Clarke, D. M. (1998) Superfolding of the Partially Unfolded Core-glycosylated Intermediate of Human P-glycoprotein into the Mature Enzyme Is Promoted by Substrate-induced Transmembrane Domain Interactions. *J. Biol. Chem. 273*, 14671–14674.
36. Cui, L., Aleksandrov, L., Chang, X. B., Hou, Y. X., He, L., Hegedus, T., Gentzsch, M., Aleksandrov, A., Balch, W. E., and Riordan, J. R. (2007) Domain Interdependence in the Biosynthetic Assembly of CFTR. *J. Mol. Biol. 365*, 981–994.
37. Kobashi, K. (1968) Catalytic oxidation of sulfhydryl groups by o-phenanthroline copper complex. *Biochim. Biophys. Acta 158*, 239–245.
38. Carveth, K., Buck, T., Anthony, V., and Skach, W. R. (2002) Cooperativity and flexibility of cystic fibrosis transmembrane conductance regulator transmembrane segments participate in membrane localization of a charged residue. *J. Biol. Chem. 277*, 39507–39514.
39. Lu, Y., Turnbull, I. R., Bragin, A., Carveth, K., Verkman, A. S., and Skach, W. R. (2000) Reorientation of aquaporin-1 topology during maturation in the endoplasmic reticulum. *Mol. Biol. Cell 11*, 2973–2985.
40. Nilsson, I. M., and von Heijne, G. (1993) Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. *J. Biol. Chem. 268*, 5798–5801.
41. Kleizen, B., van Vlijmen, T., de Jonge, H. R., and Braakman, I. (2005) Folding of CFTR is predominantly cotranslational. *Mol. Cell 20*, 277–287.
42. Loo, T. W., Bartlett, M. C., Wang, Y., and Clarke, D. M. (2006) The chemical chaperone CFcor-325 repairs folding defects in the

transmembrane domains of CFTR processing mutants. *Biochem. J. 395*, 537–542.
43. Ward, C. L., Omura, S., and Kopito, R. R. (1995) Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell 83*, 121–127.
44. Loo, T. W., and Clarke, D. M. (1999) The transmembrane domains of the human multidrug resistance P-glycoprotein are sufficient to mediate drug binding and trafficking to the cell surface. *J. Biol. Chem. 274*, 24759–24765.
45. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2008) Arginines in the first transmembrane segment promote maturation of a P-glycoprotein processing mutant by hydrogen bond interactions with tyrosines in transmembrane segment 11. *J. Biol. Chem. 283*, 24860–24870.