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The V510D Suppressor Mutation Stabilizes ΔF508-CFTR at the Cell Surface[†]

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ABSTRACT: Deletion of Phe508 (ΔF508) in the first nucleotide-binding domain (NBD1) of CFTR causes cystic fibrosis. The mutation severely reduces the stability and folding of the protein by disrupting interactions between NBD1 and the second transmembrane domain (TMD2). We found that replacement of Val510 with acidic residues (but not neutral or positive residues) promoted maturation of ΔF508-CFTR with V510D more efficiently than V510E. Promotion of ΔF508-CFTR maturation did not require NBD2 as introduction of V510D into a ΔNBD2/ΔF508-CFTR mutant restored maturation to levels similar to that of full-length protein. The V510D mutation increased the half-life of mature ΔF508-CFTR at the cell surface by about 5-fold to resemble the half-life of wild-type CFTR. It was also observed that introduction of the V510R/R1070D mutations into ΔF508-CFTR also promoted maturation whereas the V510D/R1070A mutations did not. We propose that the V510D mutation in NBD1 promotes maturation and stabilizes ΔF508-CFTR at the cell surface through formation of a salt bridge with Arg1070 in TMD2.

The cystic fibrosis transmembrane conductance regulator (CFTR) 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 maintain salt and water homeostasis in epithelial tissues (*2*).

Cystic fibrosis (CF) is caused by mutations that impair synthesis and trafficking of the protein (*3*). The most common lesion is deletion of the codon for Phe508 (ΔF508-CFTR). The ΔF508-CFTR protein is misfolded and is rapidly degraded in the endoplasmic reticulum (*4*). The ΔF508 mutation inhibits folding by disrupting interactions between NBD1[1] and intracellular loop 4 (ICL4) in the second transmembrane domain (TMD2) (*5*).

A treatment for CF would be to promote folding of ΔF508-CFTR to increase the amount delivered to the cell surface because ΔF508-CFTR retains some functional activity (*6*). Another goal would be to reduce the turnover of ΔF508-CFTR at the cell surface as its half-life is 5−10-fold shorter than that of wild-type protein (*7*).

Characterization of suppressor mutations could provide useful information needed to develop therapeutic strategies to repair ΔF508-CFTR. The V510D suppressor mutation is interesting because Val510 is predicted to reside at the domain−domain interface between NBD1 and TMD2 in close proximity to Phe508 (*8*). A negative charge appeared to be important because the V510D mutation promoted maturation of ΔF508-CFTR while mutation of Val510 to neutral amino acids (Cys, Gly, Ala, Ser, Asn, Pro, Thr, Tyr) did not (*9*). In this study, we tested whether the V510D mutation would reduce the turnover of ΔF508-CFTR at the cell surface.

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[1]Abbreviations: TM, transmembrane; NBD, nucleotide-binding domain; HEK, human embryonic kidney; BHK, baby hamster kidney.

MATERIALS AND METHODS

Construction and Expression of Mutants. Mutations were introduced into ΔF508-CFTR, ΔF508/ΔNBD2 (Δ1173−1480)-CFTR, or ΔF508/ΔNBD2 (Δ1197−1480)-CFTR cDNAs containing an A52 epitope tag at the C-terminal end (*10*) by site-directed mutagenesis as described by Kunkel (*11*). The mutant CFTRs were transiently expressed in HEK 293 cells as described previously (*5*). HEK 293 cells were transfected with the cDNAs, and the medium was changed 4 h later to fresh medium (Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum). Cells were harvested 18−36 h after the change in medium. Whole cell extracts of cells expressing untagged or A52-tagged CFTRs were subjected to immunoblot analysis using 6.5% (w/v) acrylamide gels and a CFTR polyclonal antibody or monoclonal antibody A52. Generation of stable BHK cell lines expressing wild-type or mutant ΔF508/V510D-CFTRs was performed as described previously (*9*).

Disulfide Cross-Linking Analysis. HEK 293 cells were transfected with W356C/W1145C-, ΔF508/W356C/W1145C-, or ΔF508/V510D/W356C/W1145C-CFTR cDNAs, and the cells were incubated for 4 h at 37 °C. The transfection medium was removed, and the cells were incubated in fresh medium overnight at 30 °C. The cells were then treated for 15 min at 20 °C with 3 μM BMH. The medium was removed, and fresh medium containing 0.1 mM cysteine was added. At various time points the cells were cooled on ice, harvested, and washed with PBS, and whole cell SDS extracts were prepared. The reaction mixtures were then subjected to SDS−PAGE (6.5% (w/v) polyacrylamide gels) and immunoblot analysis with a rabbit polyclonal antibody against CFTR (*12*). 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 (*12*). The gel lanes were scanned and quantitated using the NIH Image Program and an Apple computer.

Pulse−Chase and Cell Surface Labeling. Pulse−chase experiments (*13*) or cell surface labeling with biotin-LC-hydrazide

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after oxidation of carbohydrate with sodium periodate (*14*) were performed using HEK 293 cells transfected with A52-tagged wild-type, ΔF508-, or ΔF508/V510D-CFTR cDNAs as described previously. Pulse−chase assays were performed using transfected HEK 293 cells maintained at 37 °C. For cell surface labeling, the medium was replaced 4 h after transfection, and the cells were grown at 30 °C for 18 h. The cells were biotinylated and then grown in fresh media at 37 °C for various intervals.

Iodide Efflux. Measurement of cAMP-stimulated iodide efflux was performed on baby hamster kidney (BHK) cells or BHK cells expressing wild-type or mutant ΔF508/V510D-CFTRs as described previously (*9*, *15*).

RESULTS

Effect of Charged Amino Acids at Position Val510 on Maturation of ΔF508-CFTR. Models of CFTR structure predict that the segment in NBD1 containing Phe508 to Val510 would lie close to the fourth intracellular loop (ICL4) in TMD2 that connects transmembrane segments 10 and 11 (Figure 1, panels A and B). Recent crystal structures of wild-type NBD1 and ΔF508 NBD1 showed that deletion of Phe508 caused only local structural changes in residues 509−511 such as increased exposure of Val510 to solvent (*16*) (Figure 1, panels C and D). In a modeling study, it was predicted that the negative charge of the V510D mutation promoted maturation of ΔF508-CFTR because it restores defective NBD1−TMD2 interactions by forming a salt bridge with Arg1070 (TMD2) (*17*). We tested this prediction by introducing the charged amino acids Glu or Arg into ΔF508-CFTR. HEK 293 cells were transfected with the cDNA of wild-type CFTR (Figure 1E) or mutants ΔF508 (Figure 1, panels E−H), V510D/ΔF508 (Figure 1F), V510E/ΔF508 (Figure 1G), and V510R/ΔF508 (Figure 1H), and whole cell SDS extracts were subjected to immunoblot analysis 18 h after transfection. Mature and immature forms of CFTR can be distinguished in SDS−PAGE gels because of differences in glycosylation. The V510E mutation also promoted maturation, but it was less efficient than the V510D mutation (12% and 35%, respectively) (Figure 1, panels F and G). Little mature CFTR was observed in mutant ΔF508 (Figure 1, panels E-H) or V510R/ΔF508 (Figure 1H).

It was possible that the high level of expression of V510D/ΔF508 in the transiently transfected HEK 293 cells was also a factor in enhancing maturation of the mutant. To examine the extent of maturation at lower levels of expression, HEK 293 cells were transfected with lower concentrations of plasmid containing V510D/ΔF508 cDNA and whole cell extracts subjected to immunoblot analysis 42 h after transfection. The time of incubation was increased so CFTR could be detected after transfection with low cDNA concentrations. It was observed that expression of CFTR was reduced when cells were transfected with lower concentrations of plasmid (Figure 1I) but the relative steady-state level of mature V510D/ΔF508-CFTR remained at about 50% of total CFTR (Figure 1J).

To test if the mutant was active, BHK cell lines were generated that stably expressed wild-type or V510D/ΔF508-CFTRs for use in iodide efflux assays. The relative expression level of mature to total CFTR for mutant V510D/ΔF508 in BHK cells was also found to be about 50% (data not shown). Iodide efflux assays were performed rather than chloride channel measurements because very few channels other than CFTR can conduct iodide ions (*18*). Cells expressing wild-type or V510D/ΔF508-CFTRs exhibited iodide efflux upon addition of forskolin (Figure 2).

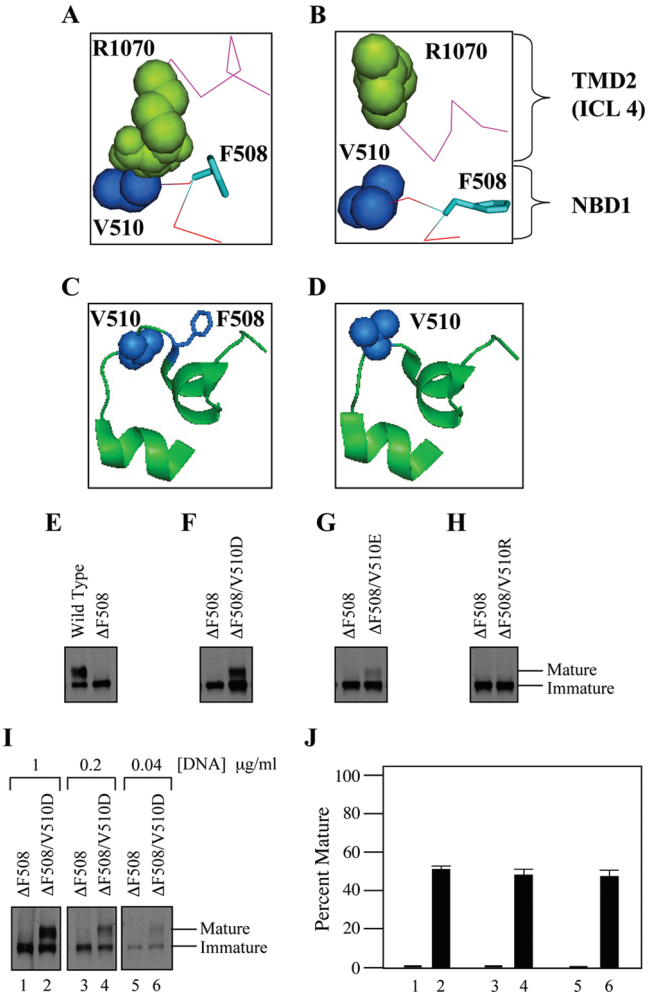

FIGURE 1: Effect of charged amino acids at position Val510 at the NBD1−TMD2 interface on maturation of ΔF508-CFTR. Amino acids located at the NBD1−TMD2 (ICL4) interface in models of CFTR (A (*17*); B (*8*)) that were mutated in this study are shown. The crystal structure of NBD1 of wild-type (C) and ΔF508-CFTR (D) (segment between Ile497 and Val520 is shown) shows that deletion of Phe508 exposes Val510 to solvent (*16*). Whole cell extracts of HEK 293 cells expressing full-length wild-type (E) or ΔF508-CFTRs (E−H) or ΔF508-CFTR containing the V510D (F), V510E (G), or V510R mutation (H) were subjected to immunoblot analysis 18 h after transfection. To test the maturation efficiency of ΔF508- and ΔF508/V510D-CFTRs at various levels of expression, HEK 293 cells were transfected with various levels of plasmid followed by immunoblot analysis and enhanced chemiluminescence 42 h after transfection (I). The level of mature CFTR relative to total CFTR in panel I was determined (J). The positions of mature and immature CFTRs are indicated.

Wild-type CFTR lacking NBD2 shows efficient maturation (*19*). It was recently reported that rescue of ΔF508-CFTR by other suppressor mutations in NBD1 (I539T, G550E, R553M, R555K) was drastically reduced in wild-type CFTR lacking NBD2 (ΔNBD2) (*20*). It was concluded that these suppressor mutations likely promoted maturation through interactions with NBD2 as well as other domains. We introduced the V510D mutation into ΔF508-CFTR lacking NBD2 (truncated after residue 1196 and containing the epitope for monoclonal antibody A52 (Δ1197−1480)-A52) to test if it still promoted maturation. Immunoblot analysis of whole cell SDS extracts (Figure 3, left panel) showed that ΔF508/V510D/ΔNBD2-A52 (Δ1197−1480) had a maturation efficiency (about 50%) that was similar to that of the full-length ΔF508/V510D mutant (about 50%; Figure 1, panel F).

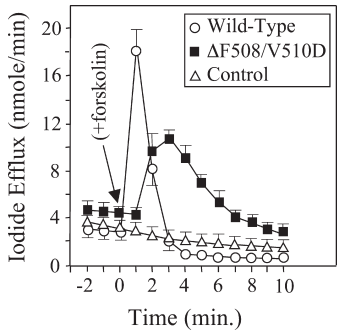

FIGURE 2: Iodide efflux activity of wild-type and ΔF508/V510D-CFTR. Iodide efflux assays were performed on BHK cells stably expressing wild-type CFTR, mutant ΔF508/V510D, or no CFTR (control). At time 0, forskolin was added to start stimulation of the iodide-loaded cells. Each value is the mean ± SD (n = 9).

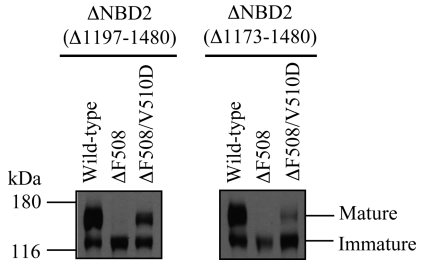

FIGURE 3: Effect of V510D on maturation of ΔF508/ΔNBD2 mutants. Immunoblots of HEK 293 cells expressing A52-tagged CFTR mutants lacking NBD2 that were truncated after residue 1196 (Δ1197−1480) or 1172 (Δ1173−1480). The positions of mature and immature ΔNBD2-CFTRs are indicated.

These results suggest that the V510D suppressor mutation differs from other NBD1 suppressor mutations because the absence of NBD2 did not reduce its effect. A potential problem with this interpretation, however, is that the two studies used different ΔF508/ΔNBD2 constructs. This study used a construct truncated after residue 1196 (Δ1197−1480) whereas the other study used a construct truncated after residue 1172 (Δ1173−1480) (*20*). To test if the position of the truncation affected maturation, mutant ΔF508/V510D/ΔNBD2-A52 (Δ1173−1480) was constructed and expressed in HEK293 cells. Immunoblot analysis of whole cell extracts showed that the V510D mutation promoted maturation of the truncation mutant but the relative level of mature was only about 20% of total CFTR protein.

It was predicted in a modeling study (*17*) that the V510D mutation may promote NBD1−TMD2 interactions in ΔF508-CFTR by forming a salt bridge with positively charged residue Arg1070 (TMD2). We tested the contribution of Arg1070 by introducing the R1070A change into ΔF508/V510D. As shown in Figure 4A, mature protein was not detectable in mutant ΔF508/V510D/R1070A. To test if the R1070A mutation alone affected maturation of ΔF508, mutants ΔF508/R1070A and ΔF508 were expressed at 30 °C. Maturation of both mutants was similar (about 20%) (Figure 4B). Therefore, it was unlikely that the R1070A mutant caused further misfolding of ΔF508-CFTR. This is consistent with the observation that introduction of the R1070A mutation into wild-type CFTR also did not affect its maturation (data not shown).

Mutant ΔF508/V510R/R1070D was constructed to reverse the positions of the charged residues. Figure 4C shows that the V510R/R1070D mutations promoted maturation of ΔF508-CFTR. Both mutations were required because the presence of

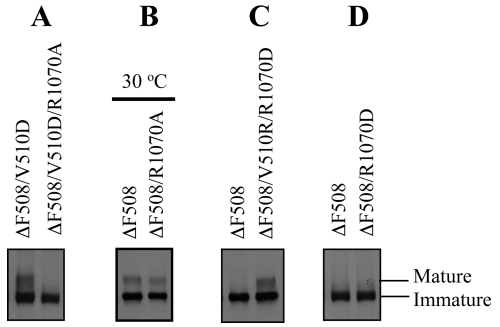

FIGURE 4: Effect of Arg1070 and Val510 mutations on maturation of ΔF508-CFTR. Whole cell immunoblots were performed on HEK 293 cells that were transfected with the indicated ΔF508-CFTR mutants and incubated for 18 h at 37 °C (A, C, D) or 30 °C (B). The positions of mature and immature CFTRs are indicated.

V510R (Figure 1H) or R1070D (Figure 4D) alone did not promote maturation of ΔF508-CFTR. These results suggest that V510D may form a salt bridge with Arg1070 in ΔF508-CFTR.

The ΔF508 mutation reduces the stability of CFTR at the cell surface (*7*). To test if the V510D mutation increased the stability of ΔF508-CFTR, a cross-linking assay was used to measure the half-life of mature CFTR at the cell surface. We previously showed that cysteines introduced into TM segments 6 (W356C) and 12 (W1145C) could be cross-linked in mature CFTR when whole cells were treated with bifunctional thiol cross-linkers (*14*, *15*). Cross-linked mature CFTR was readily detected because it migrates with a slower mobility on SDS−PAGE gels. Cross-linking of immature CFTR was not detected. Therefore, the cross-linking can be used as a reporter to monitor turnover of mature CFTR at the cell surface. Accordingly, the W356C and W1145C mutations were introduced into wild-type, ΔF508-, and ΔF508/V510D-CFTRs. Transfected cells were incubated for 24 h at 30 °C and then treated with 3 μM 1,6-bismaleimidohexane (BMH) for 15 min at 21 °C. BMH was used instead of the bifunctional methanethiosulfonate cross-linkers (*14*) because maleimide-type cross-links are not reduced by thiol-reducing compounds such as dithiothreitol. BMH was then removed, and fresh medium containing 0.1 mM cysteine was added. Cells were harvested at various intervals and whole cell extracts subjected to immunoblot analysis. Panels A and B of Figure 5 show that the half-lives of cross-linked wild-type/W356C/W1145C-, ΔF508/W356C/W1145C-, and ΔF508/V510D/W356C/W1145C-CFTRs were about 12, 3, and 14 h, respectively. These results indicate that the stability of mutant V510D/ΔF508-CFTR resembled that of wild-type protein.

A potential problem with the cross-linking assay is that it required introduction of a pair of cysteines into the TM segments of CFTR. It was possible that cysteines W356C and W1145C may influence the stability of the protein. To examine the effect of the V510D mutation on turnover of ΔF508-CFTR without the introduced cysteines, a pulse−chase assay was performed. It was observed that the rate of conversion of immature wild-type CFTR to the mature form was nearly complete by 2−4 h (Figure 6A, top panel). By contrast, the conversion of immature to mature form in mutant ΔF508/V510D was slower since there was considerable amount of immature CFTR still present at 2−4 h (Figure 6A, bottom panel). No mature protein was detected in mutant ΔF508-CFTR because there is little maturation when expression is performed at 37 °C (Figure 6A, middle panel).

After an 8 h chase, the majority of labeled CFTR in wild-type and mutant ΔF508/V510D was present as the mature protein

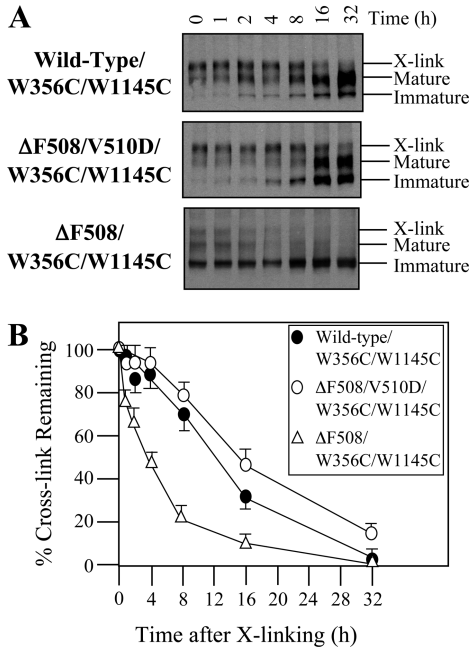

FIGURE 5: Measurement of cell surface stability by cross-linking assay. HEK 293 cells expressing wild-type, ΔF508-, or ΔF508/V510D-CFTRs containing the W356C and W1145C cysteines were treated with the thiol cross-linker BMH. The cross-linker was then removed at time 0, and whole cell extracts were subjected to immunoblot analysis at the indicated times (A), and the level of cross-linked CFTR remaining at each time point was quantitated (B). The positions of immature, mature, and cross-linked (X-link) forms of CFTR are indicated.

(Figure 6A). Therefore, we used this time point to compare the lifetimes of the mature protein in wild-type and mutant ΔF508/V510D. Figure 6 (panels B and C) shows that the half-lives of both proteins were similar (12−14 h). The results indicate that V510D stabilizes ΔF508-CFTR.

To test if V510D stabilizes ΔF508-CFTR at the cell surface, cells expressing wild-type, ΔF508-, or ΔF508/V510D-CFTRs were first incubated at 30 °C for 18 h so that ΔF508-CFTR would be present at the cell surface. CFTR at the cell surface was then biotinylated. The cells were washed to remove biotin-LC-hydrazide, and cells were harvested at various time points after labeling. Labeled CFTR was immunoprecipitated, samples were subjected to SDS−PAGE, and CFTR was detected with streptavidin conjugated to horseradish peroxidase. It was found that mature forms of wild-type or ΔF508/V510D-CFTRs had similar half-lives of about 12 h while ΔF508-CFTR had a short half-life of about 2 h (Figure 7).

DISCUSSION

The folding and trafficking defects of ΔF508-CFTR can be partially overcome by expressing the mutant at lower temperatures to yield a partially functional molecule at the cell surface (*6*). The mutant is unstable at the cell surface, however, because it shows a 5−10-fold shorter half-life than wild-type protein when cells are returned to 37 °C (*21*). This study showed that the V510D mutation increased the cell surface stability of mature ΔF508-CFTR to levels that were similar to the wild-type protein (Figures 5−7).

A similar effect was observed when the combination of four NBD1 suppressor mutations (I539T, G550E, R553M, R555K) was introduced into ΔF508-CFTR (*20*). Mature wild-type CFTR and mature ΔF508-CFTR containing the four suppressor mutations

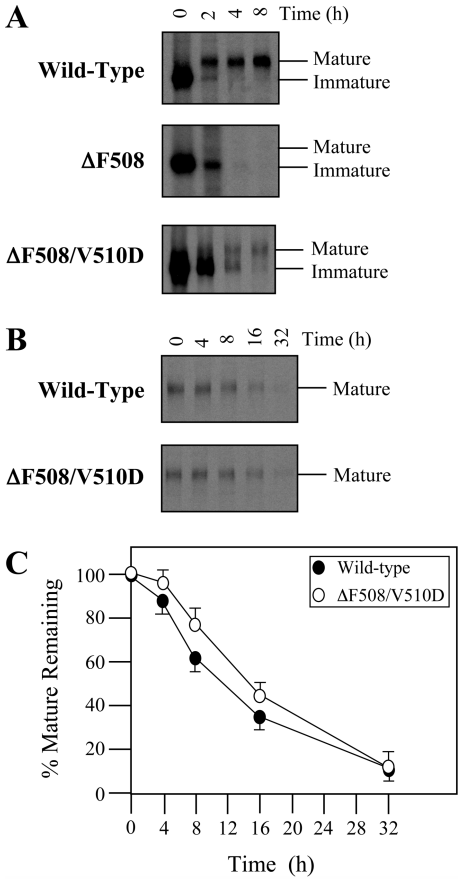

FIGURE 6: Metabolic labeling of CFTR. HEK 293 cells were transfected with A52-tagged wild-type, ΔF508, or ΔF508/V510D cDNAs. After 24 h, the cells were pulse-labeled with [^{35}S]-L-methionine and [^{35}S]-L-cystine for 30 min at 37 °C. Cells were chased and samples collected immediately (time 0 h) and at various intervals (A). Time 0 h in (B) is 8 h after chase. At each time point cells were solubilized and labeled CFTR immunoprecipitated with monoclonal antibody A52. The immunoprecipitates were subjected to SDS−PAGE and fluorography. The amount of mature labeled CFTR at each time point (B) was quantitated and expressed relative to time 0 (C). The positions of mature and immature CFTRs are indicated.

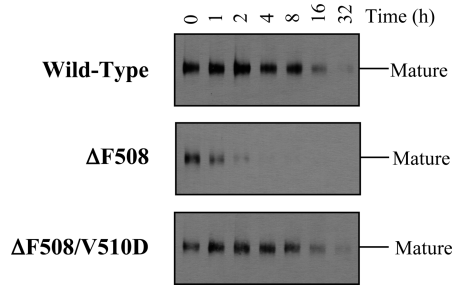

FIGURE 7: Cell surface labeling of CFTR. HEK 293 cells were transfected with the cDNAs of A52-tagged wild-type, ΔF508-, or ΔF508/V510D-CFTR. To compare the half-life of ΔF508-CFTR to wild-type or mutant ΔF508/V510D-CFTR, the cells were first incubated at 30 °C to promote maturation and increase the level of ΔF508-CFTR at the cell surface. After 18 h at 30 °C, cells were biotinylated. The biotin-LC-hydrazide was removed and replaced with fresh media. The cells were then harvested immediately (time 0) and at the indicated time points. The cells were solubilized and labeled CFTR collected by immunoprecipitation with monoclonal antibody A52. The samples were subjected to SDS−PAGE and CFTR detected with streptavidin conjugated to horseradish peroxidase followed by enhanced chemiluminescence. The position of mature CFTR is indicated.

had half-lives of about 12 h. Although the effects of these suppressor mutations on full-length CFTR were similar to that of V510D, there were different effects on NBD2 deletion mutants. This suggested that the mutations might be affecting different domain−domain interactions. The V510D mutation (present study) appeared to restore stability by promoting NBD1−TMD2 interactions in ΔF508-CFTR since deletion of NBD2 had little effect (Figure 3; Δ1197−1480). By contrast, He et al. (*20*) concluded that the NBD1 suppressor mutations enhanced maturation by promoting NBD1−NBD2 interactions because the effects were greatly diminished when NBD2 was absent. These differences in the two studies could be due to different NBD2 mutants used. Maturation of CFTR in C-terminal deletion mutants is sensitive to the location of the truncation (*22*). Therefore, mutant ΔF508/ΔNBD2 (Δ1173−1480) might have been more difficult to rescue compared to full-length ΔF508-CFTR as it was less sensitive to reduced temperature (*20*). In contrast, maturation of the ΔF508/ΔNBD2 used in this study (Δ1197−1480) was similar to that of full-length ΔF508-CFTR at 30 °C (data not shown). Other evidence suggesting that ΔF508/ΔNBD2 (Δ1173−1480) was more difficult to rescue was the observation that introduction of V510D into the mutant yielded lower levels of mature protein compared to the ΔF508/ΔNBD2/V510D (Δ1197−1480) construct (Figure 3).

Mutational analysis of Arg1070 in TMD2 yielded evidence that was consistent with the prediction that an aspartic acid introduced into position Val510 may stabilize ΔF508-CFTR through interactions with Arg1070. Mutation of Arg1070 to a neutral amino acid abolished V510D rescue of ΔF508-CFTR while V510R only rescued the mutant when the R1070D change was introduced (Figure 4C). The pattern of effects of the Val510 and Arg1070 mutations was similar to that reported for residues that were predicted to form a salt bridge between TM segments 6 (Arg347) and 8 (Asp924) in wild-type CFTR (*23*). It was found that mutation of Arg347 to neutral amino acids or Asp destabilized channel function but the D924R mutation complemented R347D to yield a channel that behaved like wild-type CFTR. The authors concluded that a salt bridge between Arg347 and Asp924 plays an important structural role in CFTR.

Comparison of the initial crystal structures of wild-type and ΔF508 NBD1s from CFTR suggested that the deletion only caused local structural changes restricted to residues 509−511 (*24*, *25*). A potential problem with these initial structures, however, was that additional mutations had to be introduced to enhance the solubility of the proteins. The solubility-enhancing mutations may have altered the structure of ΔF508-NBD1. A recent study addressed the concerns about the impact of the solubility-enhancing mutations by examining NBD1 crystal structures that had fewer and different solubilizing mutations (*16*). It was found that the only consistent conformational changes occurred at residues 509−511. Residue Val510 was buried in all non-ΔF508 structures but completely solvent exposed in all ΔF508 structures. Mass spectroscopy measurements also showed that the ΔF508 mutation increased backbone dynamics at residues 509−511. It was suggested that increased exposure and dynamics of the 509−511 loop could promote aberrant intermolecular interactions that impede trafficking *in vivo*. Residue Val510 was predicted to be particularly important because increased exposure of its hydrophobic side chain to solvent could promote local unfolding and chaperone interactions leading to retention in the endoplasmic reticulum. Second-site suppressor mutations at Val510 to less hydrophobic residues such as aspartic acid may reverse the alteration in the local structure of the protein caused by the ΔF508 mutation or prevent intermolecular interactions that lead to retention in the endoplasmic reticulum or increased turnover at the cell surface. It is also possible that changes to Val510 may have multiple effects because we observed that various mutations had different effects in promoting maturation of Cys-less or Δ508-CFTR (see below).

In this study we found that the ΔF508/V510D mutant was active while we failed to detect activity when V510D was introduced into Cys-less CFTR (*9*). Although Cys-less/V510D-CFTR matured, its chloride channel activity was below the limits of detection in the iodide efflux assay. An explanation may be due to structural differences between Cys-less and ΔF508-CFTRs. Cys-less CFTR was constructed by mutating the 18 endogenous cysteines to alanine or leucine. Mutation of all the cysteines yielded a mutant that also failed to mature. While introduction of V510D into Cys-less or ΔF508-CFTRs promoted maturation, the Cys-less mutant was different because its maturation could also be promoted by changing Val510 to Thr, Cys, Gly, Ala, or Ser. Therefore, the structural differences in the 508−511 region of Cys-less CFTR may explain why the V501D/Cys-less mutant did not show iodide efflux activity (*9*).

In summary, the V510D mutation increases the stability of ΔF508-CFTR by promoting NBD1−TMD2 interactions likely through formation of a salt bridge with Arg1070 in ICL4. A potential therapeutic strategy would be to mimic these effects using a compound that directly binds to the protein (pharmacological chaperone).

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