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A loop abnormality in ΔF508-CFTR

A defective flexible loop contributes to the processing and gating defects of the predominant cystic fibrosis-causing mutation

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Footnote
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ABBREVIATIONS
CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; IBI, interburst interval; ICL, intracellular loop; MBD, mean burst duration; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; P₀, open probability; RI, regulatory insertion; RE, regulatory extension; REMD, replica-exchange molecular dynamics; RMSF, root mean square fluctuation.
ABSTRACT

People with the genetic disease cystic fibrosis (CF) often carry a deletion mutation ΔF508 on the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel. This mutation greatly reduces the CFTR maturation process and slows the channel opening rate. Here, we investigate whether residues near F508 contribute to these defects in ΔF508-CFTR. Most deletion mutations, but not alanine substitutions, of individual residues from position 503 to 513 impaired CFTR maturation. Interestingly, only protein processing of ΔY512-CFTR, like that of ΔF508-CFTR, was greatly improved by low-temperature culture at 27 °C or small molecule corrector C18. The two mutant Cl⁻ channels were equally slow to open, suggesting that they may share common structural flaws. Studies on the H3-H4 loop that links residues F508 and Y512 demonstrate that G509A/V510G mutations, moving G509 one position backward in the loop, markedly enhanced ΔF508-CFTR maturation and opening rate, while promoting protein stability and persistence of the H3 helix in ΔF508 NBD1. Moreover, V510A/S511A mutations noticeably increased ΔY512-CFTR maturation at 27 °C and its opening rate. Thus, loop abnormalities may contribute to ΔF508- and ΔY512-CFTR defects. Importantly, correcting defects from G509 displacement in ΔF508-CFTR may offer a new avenue for drug discovery and CF treatments.

Keywords: cystic fibrosis transmembrane conductance regulator (CFTR), ΔF508 mutation, chloride channel, low temperature and corrector C18.
INTRODUCTION
The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl⁻ channel on the apical membrane of epithelial cells (1, 2). CFTR is comprised of two membrane-spanning domains (MSDs) that form a transmembrane ion pathway, two nucleotide-binding domains (NBDs) that chelate and hydrolyze ATP to drive channel opening and closing, termed channel gating, and a regulatory domain that serves as a platform for PKA-dependent phosphorylation to activate the channel (3). Mutations in CFTR disrupt channel function, causing the multi-organ disease cystic fibrosis (CF) (1, 2). Currently, about 90% of people with CF carry the ΔF508 mutation, which deletes a phenylalanine residue at position 508 in NBD1 of CFTR (Fig. 1A and S1A). This mutation markedly diminishes CFTR function in epithelial cells by two major mechanisms. First, the ΔF508 mutation greatly reduces CFTR expression in the cell membrane due to impaired protein delivery from the endoplasmic reticulum (ER) to Golgi apparatus, known as the processing defect (4), and also due to accelerated ubiquitin-dependent lysosomal degradation that quickly removes any mutant proteins that reach the cell membrane (5). Second, the ΔF508 mutation slows the channel opening rate of CFTR to decrease channel activity to about only 1/4 of that in wild-type CFTR, known as the gating defect (6-9). The mutation also delays and desensitizes CFTR activation by PKA-dependent phosphorylation (10-12). These findings reveal multiple functional deficits in ΔF508-CFTR. Yet how a single residue deletion elicits such an array of abnormalities in CFTR function is not fully understood.

The ΔF508 mutation may cause dysfunction of CFTR due to loss of the backbone function (13, 14) and side-chain interactions of the F508 residue (13-16). Consistent with this scenario, protein misfolding (14, 15, 17, 18) and aberrant domain-domain interactions between NBD1 and other regions, such as intracellular loop 4 (ICL4) (14, 16, 19), ICL3 (19) and NBD2 (15), have been documented in ΔF508-CFTR. Recently solved cryo-EM structures of wild-type CFTR also reveal intensive interactions of ICL4 with F508 and nearby residues in NBD1 (20-22). In addition, impaired domain-domain interactions might accelerate ΔF508-CFTR degradation by the peripheral protein quality control mechanism (5), decrease channel thermostability (23, 24) and reduce open probability (Pₒ) (14, 16). Correcting individual structural defects (23, 25) seems to be a promising strategy to restore ΔF508-CFTR function. However, it is unclear whether loss of function of the F508 residue could account for all abnormalities in ΔF508-CFTR.

NBD1 crystal structures of human wild-type and ΔF508-CFTR are similar, except for the loop that connects H3 and H4 helices (H3-H4 loop) (Fig. 1A) (18, 26). Flexibility of the H3-H4 loop in the NBD1 crystal structure (26) or synthetic peptides (27) may be enhanced in ΔF508-CFTR. Moreover, the orientation of loop residues 509-511 is also changed in ΔF508 NBD1 (18, 26). Alterations around this loop, such as the ΔI507
A loop abnormality in ΔF508-CFTR mutation (28-30) or mutations of the Y512 residue (31), all impair CFTR processing. Moreover, structural differences between crystal structures of wild-type and ΔF508 NBD1 (18) might be underestimated because solubilizing mutations in these constructs partially correct ΔF508-CFTR processing and gating defects (32). The structure of ΔF508 NBD1 in the previous study (18) is found different from the recent one (33) that contains no solubilizing mutations and removes protein segments of the regulatory insertion (RI) and extension (RE). Therefore, we hypothesized that the ΔF508 mutation might cause structural abnormalities of the H3-H4 loop, as well as neighboring residues. Such alterations might contribute to functional defects of ΔF508-CFTR.

We tested this hypothesis by deleting residues near F508 and then examining their effects on CFTR processing. After discovering that the ΔY512 mutation behaved like ΔF508, we further tested whether abnormalities of the H3-H4 loop might be a mechanism that initiates both CFTR processing and gating defects.

MATERIALS AND METHODS

CFTR plasmid construction

Wild-type human CFTR in the pcDNA3.1 expression vector was a generous gift from MJ Welsh, University of Iowa. Various CFTR mutations were created using Stratagene QuickChange II site-directed mutagenesis kit (Agilent Technologies, CA) and verified by DNA sequencing.

CFTR expression and small molecule treatments

Wild-type or mutant CFTRs were transiently expressed in HeLa cells by plasmid transfection. 4 µg plasmid and 8 µl Lipofectamine 2000 (Invitrogen) were mixed in Opti-MEM I reduced serum medium (Invitrogen) and incubated with cells for 8 h at 37 ºC. Transfected cells were further cultured at 37 or 27 ºC for 40 h before harvest of proteins. CFTR correctors C4 and C18 (Cystic Fibrosis Foundation Therapeutics), each at 10 µM, were added to the culture medium 24 h before protein was harvested. All small molecules were dissolved and frozen in DMSO (x1000, 10 mM) as stocks prior to use.

Immunoblotting

The Western hybridization method was adopted from a previous study (9). After washing with ice-cold Ca²⁺/Mg²⁺-free PBS solution 3 times, transfected HeLa cells were digested with the lysis buffer containing 150 mM NaCl, 1% NP40 (Thermo Fisher Scientific, Waltham, MA), 1 mM EDTA, 50 mM Tris (pH 7.4), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and mixed protease inhibitors (cOmplete, Roche) for 1 h at 4 ºC. Then,
A loop abnormality in ∆F508-CFTR cell lysates were centrifuged at ~50,000 g (20,000 RPM) by using a Type 50.4 Ti rotor (Beckman Coulter, CA) for 20 mins at 4 ºC. The supernatant was collected and analyzed by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) to determine the protein concentration. Electrophoresis of 60 or 120 µg total protein in each well was run at 150 V for 4 h in 6% SDS-PAGE gels using the Mini-Protean Tetra Cell or Protean II Xi Cell (Bio-Rad Laboratories, Hercules, CA). The running buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS.

Separated proteins in the gel were transferred to the PVDF membrane (IPVH00010, Merck Millipore, Billerica, MA) at 30 V and 4 ºC overnight, using the Tetra blotting module (Bio-Rad Laboratories, Hercules, CA) with the transfer solution containing 25 mM Tris, 192 mM glycine and 20% methanol. To detect CFTR, PVDF membranes in the 0.1% Casein blocking solution were treated with a mixture of anti-CFTR antibodies: M3A7 (Millipore), MM13-4 (Millipore, Billerica, MA) and 13-1 (R&D Systems, Minneapolis, MN) (each diluted 1:3000) for 2 h at room temperature. Then, PVDF membranes were sequentially washed 3 times with the TBST solution containing 140 mM NaCl, 0.1% Tween 20 and 20 mM Trizma (pH 7.6 with HCl), incubated with the goat anti-mouse IgG-HRP antibody (AP308P, Millipore, Billerica, MA) (1:3000 dilution) in the 0.1% Casein blocking solution for 1 h at room temperature and then washed again 3 times with the TBST solution. WesternBright ECL HRP substrate (Advansta, Menlo Park, CA) was used to illuminate CFTR. Images were captured using the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA) and analyzed with the software Image J (NIH). To calculate the Band C %, the background intensity was subtracted from the measured values.

**Electrophysiology**

CFTR currents in excised inside-out membrane patches were recorded using Axopatch 200B patch-clamp amplifiers and analyzed with pCLAMP software (both from Molecular Devices, Union City, CA), as described previously (34). The pipette (extracellular) solution contained (mM): 140 N-methyl-D-glucamine (NMDG), 140 aspartic acid, 5 CaCl₂, 2 MgSO₄ and 10 TES, pH 7.3 with Tris ([Cl⁻], 10 mM). The control bath (intracellular) solution contained (mM): 140 NMDG, 3 MgCl₂, 1 CsEGTA, 5 Trizma base and 5 Bis-Tris, pH 7.3 with HCl, ([Cl⁻], 147 mM; free [Ca²⁺], <10⁻⁸ M) at room temperature.

The channel activity of CFTR in excised inside-out membrane patches was activated and maintained by adding PKA (75 nM) and ATP (1 mM) to the bath solution in the recording chamber (Brook Industries, IL, USA). Membrane voltage was clamped at ~50 mV. For data analysis and storage, CFTR currents were filtered with an 8-pole Bessel filter (model 900; Frequency Devices, Inc., Ottawa, IL) at a corner frequency (fₒ) of 500
A loop abnormality in ΔF508-CFTR Hz and acquired using a Digidata 1440 interface (Molecular Devices) and pCLAMP software at the sampling rate of 10 kHz.

For analyzing the patch-clamp data, the number of active channels (N) in a membrane patch was determined by the maximum number of recorded channels that opened simultaneously at any one time during the entire experiment. For open probability (P_o) and burst analysis, event lists of open- and closed-times were created using pCLAMP software with a half-amplitude crossing criterion. Transitions ≤ 1 ms were excluded from event lists (eight-pole Bessel filter rise time (T_{10-90}) ~0.73 ms at f_c = 500 Hz).

To measure mean burst duration (MBD), interburst interval (IBI) and P_o within a burst (P_o-burst), burst analysis was performed using recordings from membrane patches that contained 1-4 active channels. The delimiter time (t_c) that separates interburst closures from intraburst closures was determined from the point of intersection between the two exponential curves fitting fast and slow populations of channel closures in the closed-time histogram, as described previously (35). Event lists and t_c values were used to derive MBD and P_o-burst with pCLAMP software. To obtain burst durations, opening bursts formed by only one active channel were measured. Finally, IBI was calculated using the equation:

\[
P_o = \frac{\text{MBD} \times P_{o-burst}}{\text{MBD} + \text{IBI}}
\]

To obtain N accurately, the time required to observe an event where all active channels open simultaneously was estimated by the equation: \((3 \times \tau_o/N)/(P_o)^N\) (36), in which the open time \(\tau_o\) could be estimated by MBD. For wild-type CFTR with \(P_o = 0.39\) and MBD = 401 ms (Fig. 4C, D), it requires ~13 s for observing all 4 channels opening at the same time and ~27 s for 5 channels. Moreover, for ΔF508/AG (G509A/V510G)-CFTR with \(P_o = 0.21\) and MBD = 351 ms (Fig. 6C, D), it requires ~2.3 mins for \(N = 4\) and ~8.6 mins for \(N = 5\). Thus, we recorded CFTR currents in the presence of ATP (1 mM) and PKA (75 nM) for 6-30 mins for most experiments. To maintain channel activity, we replaced the bath solution with fresh ATP and PKA after recording for 15 mins. However, N would be underestimated for mutant CFTR with large gating anomalies such as ΔF508- and ΔY512-CFTR. Therefore, reported P_o of these two CFTR mutants could be considered as maximum P_o and their IBI as minimal IBI.
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For the purpose of illustration, recordings were further digitized at 1 kHz, in which current tracings of 10 s could clearly demonstrate the gating kinetics of CFTR with reduced data points, noise and some very brief current transitions (e.g., those < 1 ms).

Molecular dynamics simulation

Simulations were performed for ΔF508-G509A/V510G human ΔRI, ΔRE-NBD1, comprising residues 387-646 (excluding a deletion of residues 405-436) with no additional solubilizing mutations (PDB entries 2PZF, 2.0 Å resolution). This structure was solved by introducing G509A and V510G mutations into the crystal structure of ΔF508 NBD1 (PDB: 2PZF) with conformational optimization of neighboring residues within a distance of 5 Å using software Discovery Studio 2018 (37). To incorporate ATP and Mg$^{2+}$ into the structure, the parameters for ATP were obtained from the OPLS/AA force field as implemented in Schrodinger (Schrodinger Maestro, 9.0; Schrodinger: NY, 2009) while those for Mg$^{2+}$ are included in the GROMACS implementation of this force field. Similar trajectories obtained for wild-type and ΔF508-NBD1 were adopted from the previous study (38).

Replica-exchange molecular dynamics (REMD) simulation was performed using the GROMACS package as described previously (38). In brief, the construct was simulated three times each time starting from a different random seed. The simulation consisted of 32 replicas covering a temperature range of 300 °K (26.85 °C) to 349.38 °K (76.23 °C) with each replica running for 10 ns. Thus, the construct was simulated for a total of 960 ns (32 replicas x 10 ns x 3 times).

All analyses were performed on the lowest temperature (ground state) replicas. The dynamic behavior of the protein was evaluated by calculating the root mean square fluctuation (RMSF) profile for each trajectory. Prior to RMSF calculation, the entire trajectory was superposed on the starting structure using all backbone atoms. The dynamics of a residue was defined to be significantly different between two constructs (e.g., between ΔF508 and ΔF508/AG NBD1) when RMSF values of the residue were consistently higher or lower in one construct than those in the other construct across all three simulation repeats. To predict and compare the secondary structure propensity of a polypeptide segment across the different constructs, a comparative analysis of the trajectories was performed using the dictionary of secondary structure of proteins (DSSP) algorithm.

Reagents, chemicals and resources
CFTR correctors are generously provided by the Chemical Compound Distribution Program from Professor Robert J. Bridges, Rosalind Franklin University of Medicine and Science, and Cystic Fibrosis Foundation Therapeutics. PKA purified from bovine heart was purchased from Calbiochem/Merck Millipore (Darmstadt, Germany). Stock solutions of ATP were prepared fresh before each experiment. Except for those indicated in the text, all other chemicals were purchased from Sigma (Sigma-Aldrich China, Shanghai, China) in reagent grade. PyMOL software (http://www.pymol.org), a molecular visualization system, was used for preparing all structural figures.

Statistics
Results are expressed as means ± S.E.M. of n observations. In immunoblotting experiments, n represents the number of immunoblots and in electrophysiological experiments, n represents the number of individual membrane patches obtained from different cells. One-way ANOVA was used to analyze sets of data. Differences were considered statistically significant when \( P < 0.05 \).

RESULTS
The F508 residue is located at the end of the H3 helix in NBD1 of human CFTR and connects the H4 helix via a flexible loop structure (H3-H4 loop) (Fig. 1A, S1A) (18, 21, 26). Although deletion of this residue (\( \Delta F508 \)) severely impairs CFTR function (4), it shows small impacts on the crystal structure of wild-type NBD1, except residues 509-511 of the H3-H4 loop (Fig. 1A) (18, 26, 33). Interestingly, the NMR spectroscopy study (39) demonstrates that phosphorylation-induced conformational changes in wild-type and \( \Delta F508 \) NBD1 are different and that the differences extend beyond the immediate neighborhood of F508. These findings may suggest that if residues match at the level of the crystal structure, it does not necessarily indicate that they also match when protein flexibility is introduced. Therefore, differences in conformation and function of wild-type and \( \Delta F508 \) NBD1 may exist in vivo, contributing to \( \Delta F508 \)-CFTR dysfunction (e.g., the protein processing defect (4)). To test this hypothesis, we first asked whether residues near F508 play an important role in CFTR processing.

Effects of residue deletions or substitutions near F508 on CFTR processing
We began by individually deleting residues within positions 491 to 525 (Fig. 1A) and examining protein expression of these CFTR mutants (Fig. 1B, C). Our data demonstrate that \( \Delta V510 \) and \( \Delta S511 \)-CFTR expressed relatively abundant protein in the Band C form (\( \text{Band C }\% = \frac{C}{B + C}\% \), Fig. 1B, C), which may contain fully glycosylated CFTR present in the Golgi network and cell membrane as described previously (28). By
contrast, all other mutants including ΔF508-CFTR displayed proteins mostly in the Band B form (Fig. 1B, C), representing core glycosylated CFTR in the ER membrane (28). These data indicate that except for ΔV510 and ΔS511, residue deletions near F508 greatly impaired CFTR processing.

From a structural standpoint, removing a residue from a polypeptide chain may lead to two major alterations: shortening the peptide chain and removing side-chain interactions of the residue (13). Following this conceptualization, we used alanine substitutions at each residue from position 503 to 513 (Fig. 2A) to retain the length of the peptide chain while disrupting side-chain function of the residue for assessing their effects on CFTR processing. The data showed that Band C expression was profoundly decreased by the N505A mutation (Fig. 2A), moderately reduced by D513A, and little diminished by K503A, F508A and Y512A (Fig. 2A). Of note, Band C % of I507A-CFTR was 92.2 ± 0.5% (N = 6), similar to that of wild-type CFTR (P>0.05, one-way ANOVA). Interestingly, substitutions of the N505 residue with 8 other different residues all largely decreased Band C %. Thus, these data suggest that side chains of N505 and D513 residues are required for normal CFTR processing.

To better understand underlying mechanisms, we sought clues from side-chain interactions of these two residues with other regions in CFTR (Fig. 2C, S1B, S2C, S3E). The human NBD1 structure (Fig. 2C, S2C) (18, 21) reveals that the side chain of N505 interacts with the backbone of M498 and the side chain of R560. Therefore, to test whether intradomain interactions among these three residues are important for CFTR processing, site-directed mutations were made at these sites (Fig. 2D). We found negligible effects of M498A and M498F mutations on Band C % (Fig. 2D). By contrast, the M498P mutation, which might alter backbone conformation, greatly decreased Band C % (Fig. 2D). Moreover, profound reductions in Band C % were also observed in R560A-, R560N- and N505R/R560N-CFTR (Fig. 2D). These data suggest that precise intradomain interactions among these three residues N505, M498 and R560 may be required for normal CFTR processing.

In addition, the side chain of D513, which is located on the surface of NBD1 (Fig. 1A, S3E), weakly interacts with residues S511, Y512, R516 and Y563 at long distances (Fig. S1B, S3E) (18, 21) and is far from residues in other CFTR domains (Fig. S3E) (21). Thus, we hypothesized that negative charge at this position may contribute to the structural integrity required for protein processing. The data demonstrate that additional mutations of D513 with the negative glutamate (D513E), positive lysine (D513K) and neutral leucine residue (D513L) showed little, moderate and large reductions in Band C %, respectively (Fig. 2E). Band C % of D513K- (Fig. 2E) and D513A-CFTR (Fig. 2A) were similar, whereas both were smaller than that of D513E-CFTR (Fig. 2E; P<0.05, one-way ANOVA), consistent with our hypothesis.
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Taken together, these data suggest that normal CFTR processing requires integrity of all tested residues near F508 (Fig. 1) and proper side-chain interactions from D513 and N505 residues (Fig. 2). For those residues whose deletion, but not alanine substitution, strongly diminished CFTR processing (Figs. 1C, 2A), loss of backbone function at these positions might be a dominant factor contributing to the CFTR processing defect.

**ΔY512-CFTR processing is enhanced by low temperature and CFTR correctors**

Although our study identified deletion mutants near F508 affecting CFTR processing, it is unclear whether they have a similar mechanism of dysfunction as ΔF508-CFTR. We reasoned that if a deletion mutation impairs CFTR function by the mechanism similar to that of ΔF508, protein expression of this mutant may be enhanced by treatments well-known to rescue ΔF508-CFTR processing (Fig. 3), such as the low-temperature culture at 27 °C (40) and CFTR correctors C4 (compound 4a) (41) and C18 (25, 29).

Figure 3A demonstrates that after transient expression in HeLa cells for 40 h, ΔF508- and ΔY512-CFTR cultured at 27 °C displayed Band C % larger than that at 37 °C (Fig. 3A). Moreover, the low-temperature culture modestly enhanced Band C % of ΔV510- and ΔS511-CFTR, but slightly decreased Band C % of wild-type CFTR (Fig. 3A) and was without effects on other deletion mutants (Fig. 3A). Similarly, 24 h incubation with corrector C18 at 37 °C greatly increased Band C % of ΔF508- and ΔY512-CFTR (Fig. 3B) and enhanced that of ΔV510- and ΔS511-CFTR (Fig. 3B), compared to that without treatment (blank columns, Fig. 3B). These data suggest that ΔY512 and ΔF508 mutations may impair CFTR processing by similar mechanisms, which can be rescued with low temperature or corrector C18.

We also noted that DMSO (0.1%) alone at 37 °C significantly enhanced Band C % of ΔV510- and ΔS511-CFTR about 1.2- and 1.3-fold (blank columns, Fig. 3A, B, P<0.05, one-way ANOVA). We speculate that DMSO enhanced Band C % of ΔV510- and ΔS511-CFTR possibly by acting as a chemical chaperone (42, 43).

To investigate possible differences between processing defects of the ΔF508- and ΔY512-CFTR, we performed combination treatments on these and additional deletion mutants (Fig. 3C, D). The data demonstrate that compared to the vehicle control of 0.1% DMSO at 37 °C, low temperature or the combination of low temperature and corrector C18 showed no apparent increases in Band C % of ΔI506-, ΔG509-, ΔV510-, ΔS511- and ΔD513-CFTR (grey and green columns, Fig. 3C). Since the individual treatment of low temperature or corrector C18 at 37 °C enhanced Band C % of ΔV510- and ΔS511-CFTR (Fig. 3A, B), these data suggest that DMSO, low temperature and corrector C18 might have enhanced protein processing of these two mutants by
A loop abnormality in ΔF508-CFTR similar mechanisms (Fig. 3A, C), whereas corrector C18 exerted additional effects on these two mutants at 37 °C (Fig. 3B, C).

Conversely, low temperature and corrector C18 either alone or together enhanced Band C % of ΔF508- and ΔY512-CFTR (Fig. 3C). By close inspection, we found that the combination treatment promoted an additive increase in Band C % of ΔF508-CFTR, compared to individual treatments (Fig. 3C). However, the combination treatment only caused a small synergistic enhancement in Band C % of ΔY512-CFTR (Fig. 3C). It is also important to highlight that Band C % of ΔY512-CFTR was higher than that of ΔF508-CFTR at 37 and 27 °C with or without 0.1% DMSO in the culture medium (Fig. 1C, 3A, 3C, 3D, P<0.05, one-way ANOVA). Thus, these findings suggest that subtle differences exist between ΔF508- and ΔY512-CFTR in response to low temperature and corrector C18.

Recent studies indicate that the F508 residue mediates NBD1-MSDs interactions (Fig. S2F) (20, 21), which are disrupted by the ΔF508 mutation (14, 16, 19) but restored by corrector C18 in ΔF508-CFTR (25). However, the F508 residue is unchanged in ΔY512-CFTR and the Y512 residue mostly participates in intradomain interactions (Fig. S1C, S3D) (21). Therefore, it is possible that corrector C18 may rescue ΔY512-CFTR processing by another mechanism related to low temperature-mediated correction.

To further investigate differences in the processing defect between ΔF508- and ΔY512-CFTR, we tested these two mutants with CFTR correctors C4 and C18 together at 27 ºC (Fig. 3D). Our data demonstrate that at 27 ºC, correctors C4 and C18 caused additive increases in Band C % of both mutants (Fig. 3D). However, corrector C18 exhibited stronger effects than corrector C4 in ΔF508-CFTR (green and black columns, Fig. 3D, P<0.05, one-way ANOVA), whereas correctors C4 and C18 alone caused similar stimulations of Band C % for ΔY512-CFTR (Fig. 3D). Different responses to correctors C4 and C18 between ΔF508- and ΔY512-CFTR (Fig. 3D) suggest that the processing defect of these two mutants may share common mechanisms, although to varying degrees.

We next asked whether channel gating of ΔY512-CFTR may display defects similar to those of ΔF508-CFTR (6-8).

**ΔY512-CFTR gating exhibits low Pₒ and prolonged IBI**

We studied the single-channel activity of 4 deletion mutants: ΔF508-, ΔV510-, ΔS511- and ΔY512-CFTR (Fig. 4A) that had sufficient protein expression in the cell membrane at 37 or 27 ºC for patch-clamp experiments.
A loop abnormality in ΔF508-CFTR (Figs. 1B and 3A). As described in the Methods, we made prolonged, uninterrupted recordings at room temperature, where ΔF508-CFTR channel activity is stable (e.g., 44), to accurately determine channel number and acquire sufficient transitions to quantify channel gating. The single-channel current amplitude (i) of these 4 deletion mutants were similar to that of wild-type CFTR (Fig. 4B). However, the open probability (Pₒ) (Fig. 4C) and mean burst duration (MBD) (Fig. 4D) of ΔV510-CFTR were moderately lower than those of wild-type CFTR. Importantly, the Pₒ of both ΔF508- and ΔY512-CFTR were greatly decreased (Fig. 4C) due to profound prolongations of the interburst interval (IBI) (Fig. 4E). The data showed that deletions in the H3-H4 loop that links the F508 and Y512 residues (Fig. 1A, S1A) may alter CFTR gating. These data also revealed that ΔF508 and ΔY512 mutations caused similar gating defects of CFTR.

The similarity of CFTR processing and gating defects between ΔF508- and ΔY512-CFTR (Fig. 3 and 4) suggests that the H3-H4 loop might be dysfunctional in these two mutants. In support of this idea, only those CFTR mutants with deletions in the loop, including ΔV510-, ΔS511- and ΔY512-CFTR except for ΔG509-CFTR, showed improvement in protein processing by low-temperature culture or corrector C18 (Fig. 3A and B). Previous studies (18, 26) also demonstrate that the orientation of residues 509-511 in ΔF508 NBD1 is dramatically different from that of wild-type NBD1. Thus, it is possible that abnormality of the H3-H4 loop may offer a common defect to both ΔF508- and ΔY512-CFTR, although ΔF508 and ΔY512 mutations may impair loop structure and function differently.

Deletion of a residue in the H3-H4 loop alone may not play a major role because removing either the V510 or S511 residue only moderately reduced CFTR processing (Fig. 1C) and was without effects on IBI (Fig. 4E). The data suggest that additional defects from loop residues may impair function of ΔF508- and ΔY512-CFTR. To test this hypothesis, we replaced loop residues G509, V510 and S511 in ΔF508- or ΔY512-CFTR with alanine or glycine to alter properties of these loop residues (Fig. 5A, B). Notably, here we assume that ΔF508- and ΔY512-CFTR defects are associated with conformational changes of amino acid residues in the loop, regardless of other factors such as the effect of the mRNA sequence on protein processing (45).

**Displacement of G509 in ΔF508-CFTR and the missing side chain of Y512 in ΔY512-CFTR caused the severe processing defect**

The amino acid sequence of the H3-H4 loop of ΔF508-CFTR could be considered the same as that of ΔV510-CFTR with two additional mutations F508G and G509V. Here we assume: ΔF508-CFTR = ΔV510-F508G/G509V-CFTR. In other words, we propose that the ΔF508-CFTR processing defect is generated from three structural alterations: ΔV510, F508G and G509V. Therefore, the huge difference in Band C % of ΔV510-
A loop abnormality in ΔF508-CFTR may be resulted from mutations F508G and G509V in ΔV510-CFTR. Furthermore, four structural changes in the residue by these two mutations were noted: (1) F508G removes the side chain of F508 and (2) inserts the special residue glycine; (3) G509V introduces the side chain of valine while (4) replacing the specific side chain (hydrogen) of glycine. Thus, we hypothesized that these four structural changes may lead to a ~ 63% reduction in Band C % of ΔF508-CFTR.

To test these four mechanisms, we used the mutation G509A that removes special properties of the glycine residue, such as the high conformational flexibility and helix breaker, at F508G (mechanism 2, lane 1, Fig. 5A) and V510A that eliminates interactions from valine’s side chain (mechanism 3, lane 2, Fig. 5A). The data indicate that G509A, V510A or the double mutation (lane 3, Fig. 5A) showed no apparent improvements in Band C % of ΔF508-CFTR, arguing against mechanism 2 and 3. To test mechanism 1, the F508 residue was introduced back to the normal position in ΔV510-F508G/G509V-CFTR to form ΔV510-G509V-CFTR, whose amino acid sequence is identical to that of ΔG509-CFTR. As ΔG509-CFTR exhibited a severe processing defect (Fig. 1B, C), mechanism 1 may not be supported by our data.

Therefore, we further examined mechanism 4 by introducing glycine back to the normal position (lane #4, Fig. 5A, the ΔF508-G509A/V510G mutation) and found that it markedly enhanced CFTR Band C % to about 57%, close to 67% in ΔV510-CFTR (Fig. 1C). These data suggest that moving the G509 residue back to its normal position in ΔF508-CFTR corrects a major part of the processing defect. We speculate that the rescue effect of G509A/V510G mutations is associated with benefits from alterations of loop properties or with alleviation of structural flaws brought about by the ΔF508 mutation.

Considering the marked difference in Band C % between ΔF508-G509A/V510A-CFTR (lane #3, Fig. 5A) and ΔF508-G509A/V510G (ΔF508/AG)-CFTR (lane #4, Fig. 5A), moving the glycine residue backward to the V510 position (V510G) in ΔF508-CFTR may play a critical role in improving protein expression. Two possible effects may exist: (1) the glycine residue increases flexibility of the local loop structure or (2) it restores formation of the normal H3 helix due to its specific function as the helix breaker. To test the first hypothesis, the loop flexibility was further increased by re-introducing G509 back to position 508 (lane #5, Fig. 5A, the ΔF508-V510G mutation), which actually decreased Band C %, compared to that of ΔF508/AG-CFTR (lane #4, Fig. 5A, \(P<0.05\), one-way ANOVA). These data suggest that glycine at position 508 in ΔF508-CFTR may disrupt protein processing. Moreover, adding another glycine at position 510 (S511G, lane #6, Fig. 5A) further decreased Band C %, compared to that at lanes #4 or #5 (\(P<0.05\), one-way ANOVA). By contrast,
A loop abnormality in ΔF508-CFTR adding V510G or V510G/S511G mutations to wild-type CFTR was without effects on Band C % (lanes #7-8, Fig. 5A).

These data suggest that increasing loop flexibility by glycine substitutions is not beneficial for ΔF508-CFTR processing, but also not harmful for wild-type CFTR expression. Therefore, restoring G509 position in the H3-H4 loop, but not increasing flexibility, may account for the enhanced Band C % of ΔF508/AG-CFTR. Furthermore, we speculate that the proper length or persistence of the H3 helix would be required for correcting the processing defect of ΔF508-CFTR.

On the other hand, the amino acid sequence of the H3-H4 loop of ΔY512-CFTR is the same as that of ΔV510-S511V/Y512S-CFTR. Therefore, the difference in Band C % of ΔY512- and ΔV510-CFTR (Fig. 1C) could result from alterations in side-chain interactions of S511 and Y512 residues. With alanine substitutions to remove side-chain interactions of the replaced residue, our analysis showed that V510A, S511A, V510A/S511A and additional G509A/V510A mutations in ΔY512-CFTR did not promote Band C % (lanes #1-4, Fig. 5B).

Conversely, restoration of Y512 back to its normal position creates ΔV510-S511V-CFTR, the same as ΔS511-CFTR, which showed protein processing similar to that of ΔV510-CFTR (Fig. 1B, C). Because the Band C % of Y512A-CFTR was relatively good (Fig. 2A), these data suggest that the side chain of Y512 is important for CFTR processing only when the loop is shortened by deletion of a residue. As the phenyl ring of Y512 weakly interacts with the side chain of I507 in the H3 helix and E514 and Y515 in the H4 helix (Fig. S1C, S3D) (21), we speculate that these side-chain interactions may be important for maintaining the structural integrity of NBD1 while the loop is shortened.

Since low temperature and corrector C18 enhanced CFTR processing only for some deletion mutants in the H3-H4 loop (Fig. 3A, B), we next asked whether loop mutations alter their effects on ΔF508- and ΔY512-CFTR processing.

**Loop mutations alter the rescue effect of low temperature and corrector C18 on ΔF508- and ΔY512-CFTR processing**

To test this hypothesis, we examined the effects of low temperature, corrector C18 or the combination treatment on Band C % of ΔF508/AG-CFTR and ΔY512-V510A/S511A (ΔY512/2A)-CFTR (Fig. 5C). We found that low temperature and corrector C18 additively enhanced Band C % of ΔF508/AG-CFTR (Fig. 5C), similar to that in ΔF508-CFTR (Fig. 3C). Because of a greatly improved Band C % of ΔF508/AG-CFTR (Fig. 5A), the
A loop abnormality in ΔF508-CFTR enhancement (Fig. 5C) or fold increase (Fig. 5D) in Band C % of ΔF508/AG-CFTR by two different rescue methods was much lower than that of ΔF508-CFTR (P<0.05, Fig. 3C, 5D). Of note, the combination treatment enhanced Band C % of ΔF508/AG-CFTR to about 91% (Fig. 5C), close to that of wild-type CFTR (~ 96%) (Fig. 5A). These data suggest that G509A/V510G mutations together with low temperature and corrector C18 rescued most processing defects of ΔF508-CFTR.

Like ΔY512-CFTR, ΔY512/2A-CFTR also showed a large increase in Band C % with corrector C18 (Fig. 5C). The fold increases in Band C % by corrector C18 in these two mutants were similar (black columns, Fig. 5D). However, low temperature strikingly promoted Band C % of ΔY512/2A-CFTR (Fig. 5C) and the fold increase in Band C % (Fig. 5D) to about 69% and 9.0-fold, respectively, larger than about 50% and 4.4-fold in ΔY512-CFTR (P<0.05, Fig. 3C, 5D). Moreover, corrector C18 at 27 ºC only showed a small synergistic increase in Band C % of ΔY512/2A-CFTR (Fig. 5C), similar to that of ΔY512-CFTR (Fig. 3C). Therefore, these data suggest that removing side chains of V510 and S511 from ΔY512-CFTR magnified the low temperature-mediated correction of the protein processing defect. All data support our hypothesis, suggesting that residue properties of the H3-H4 loop are associated with the rescue effect of low temperature and corrector C18 on ΔF508- and ΔY512-CFTR processing.

Next, we asked whether the gating defect of ΔF508- and ΔY512-CFTR could be alleviated by loop mutations.

**IBI prolongation of ΔF508- and ΔY512-CFTR is attenuated by loop mutations**

When compared to wild-type CFTR, ΔF508/AG- and ΔY512/2A-CFTR showed no alterations in the single-channel current amplitude (i) and MBD (Fig. 6A, B, D). However, the $P_o$ of ΔF508/AG- and ΔY512/2A-CFTR were moderately decreased (Fig. 6C) due to an approximately 2-fold prolongation of IBI (Fig. 6E). Importantly, the IBI of ΔF508/AG- and ΔY512/2A-CFTR (Fig. 6E) were shorter than those of ΔF508- and ΔY512-CFTR, respectively (P<0.05 for both mutants, Fig. 4E). As a result, the channel activity (Fig. 6A) and $P_o$ (Fig. 6C) of ΔF508/AG- and ΔY512/2A-CFTR appeared greater than those of ΔF508- and ΔY512-CFTR, respectively (Fig. 4A, C; P<0.05 for $P_o$ for both mutants). Because G509A/V510G mutations strikingly rescued ΔF508-CFTR processing and gating, the data suggest that they are revertant mutations similar to those described previously (e.g., 46-51). These data further suggest that displacement of the G509 residue in the H3-H4 loop may be a pivotal defect that underlies functional abnormalities of ΔF508-CFTR.
We next asked whether the rescue effects of G509A/V510G mutations may be associated with alterations in the structure and dynamics of NBD1. The crystal structure of ΔF508/AG NBD1 was modeled from ΔF508 NBD1 (PDB: 2PZF) and then subjected to replica-exchange molecular dynamics (REMD) simulation (Fig. 7A) and the secondary structure analysis (Fig. 7B, Table 1).

**G509A/V510G mutations enhance protein stability of ΔF508 NBD1 and persistence of the H3 helix**

Figure 7A presents the profiles of the root mean square fluctuation (RMSF) obtained from three simulations of ΔF508/AG NBD1, compared to similar profiles obtained for wild-type (modeled after PDB: 2PZE) and ΔF508 NBD1 as previously described (38). The data indicate that the dynamics of residues, measured by the RMSF value, varied modestly among the three NBD1 constructs (Fig. 7A). Interestingly, two regions corresponding to residues 502-505 and 510-511 (vertical grey shading, Fig. 7A) showed consistent differences in the RMSF values across the three simulations with the order wild-type < ΔF508 < ΔF508/AG and wild-type < ΔF508/AG < ΔF508, respectively. Further analysis indicated that 25% of the residues (57 residues) in ΔF508/AG NBD1 displayed lower RMSF values than those in ΔF508 NBD1, whereas only 3% of the residues (6 residues) showed higher values. These data suggest that G509A/V510G mutations reduce the dynamics of specific residues in ΔF508 NBD1 or in other words, they enhance the structural stability of ΔF508 NBD1. Moreover, the previous study (38) has demonstrated that the protein stability of ΔF508 NBD1 is lower than that of wild-type NBD1. Therefore, we speculate that mutations G509A/V510G may rescue the functional defects of ΔF508-CFTR partly by stabilizing NBD1 structure.

To investigate the molecular basis for above alterations in protein stability of NBD1, further analysis of ΔF508/AG NBD1 revealed apparent increases in residual interactions around regions near residues 509-510. First, H-bond formation between residues 511 and 563 (Fig. S3C) occurred in 53% of simulations with ΔF508/AG NBD1, but only 36% in ΔF508 NBD1. Second, H-bonds between K503 and D537 (Fig. S2A) and between R560 and N505 (Fig. 2C and S2C) in ΔF508/AG NBD1 were maintained across 27% and 18% of total simulations, respectively, in contrast to 17% and 8% in ΔF508 NBD1.

Since the helix is important for stabilizing the tertiary structure of the protein (52), we next analyzed the time evolution (Fig. 7B) and the percentage of time (%) (Table 1) in which residues 491-525 adopt specific secondary structures during the simulation of wild-type, ΔF508/AG and ΔF508 NBD1. Our data demonstrate that the helicity of the H4 helix is well maintained in all constructs throughout the simulations (Fig. 7B, Table 1). However, the persistence of the H3 helix formed by residues 502-507 was disrupted in ΔF508 NBD1, compared to that in wild-type NBD1 (blue color, Fig. 7B). This is evident by observing that almost all residues
A loop abnormality in ΔF508-CFTR making up this helix spent less time in an α-helix secondary structure (Table 1). In contrast, the persistence of the H3 helix in ΔF508/AG NBD1 appeared to be higher than that in ΔF508 NBD1 (Fig. 7B, Table 1). However, Table 1 also indicates that the length of the H3 helix in ΔF508 NBD1 was not increased upon the introduction of G509A/V510G mutations since the helicity of residue I507 was seemingly reduced in this construct.

The secondary structure of the H3-H4 loop varied greatly among three constructs in residues 509-511 (Fig. 7B, Table 1). G509 and V510 in wild-type NBD1 mostly maintained the structure of a bend or a turn (Table 1). For ΔF508/AG NBD1, residues 509-511 spent more time in the bend structure (Table 1). For ΔF508 NBD1, only V510 frequently occupied the bend conformation, whereas G509 and S511 resided more often in the random coil structure (Table 1). These data might suggest that maintaining a bend and turn conformation for residues 509-511 is associated with structural stability required for normal CFTR function.

Taken together, these data suggest that the revertant mutation G509A/V510G might correct the ΔF508-CFTR defects partly by altering the NBD1 structure, leading to increased protein stability, persistence of the H3 helix and maintenance of bends and turns in residues 509-511 in the H3-H4 loop.

DISCUSSION

Table S1 summarizes treatments and results obtained for key CFTR mutations tested in this study. Our study revealed that side chains of N505 and D513 and the backbone of residues from the H3 helix (residues 502-508), H3-H4 loop (509-512) and H4 helix (513-524) in CFTR NBD1 (53) are structurally important for normal CFTR processing. We also found that the position of the G509 residue in the polypeptide chain is the major factor that distinguishes Band C expression between ΔF508- and ΔV510-CFTR. Data obtained for loop mutations G509A/V510G in ΔF508-CFTR and V510A/S511A in ΔY512-CFTR suggest that conformational changes of residues in the H3-H4 loop and/or persistence of the H3 helix may contribute to the processing and gating defects of ΔF508- and ΔY512-CFTR. We speculate that these local structural changes near F508 may alter the stability of the H3 helix, local peptide folding, surface topology of NBD1 and side-chain interactions.

The role of the backbone and side chain of residues near F508 in CFTR processing

It has been a question for many years how the most prevalent CF mutation ΔF508 deletes only a single residue in CFTR, but causes multiple dysfunctions. Other CF mutations at NBD1, such as ΔI507, S549I, R560T and A561E (28, 50) also severely impair Band C expression of CFTR. Recent studies suggest that the ΔF508 mutation may alter NBD1 properties by affecting its folding efficiency (13, 17, 18, 54) and folding kinetics (55,
A loop abnormality in ΔF508-CFTR with a reduced thermodynamic stability of the native state (57). The ΔF508 mutation also impairs CFTR assembly (58) partly due to abnormal domain-domain interactions (14-16, 18). CFTR correctors and potentiators may rescue ΔF508-CFTR defects by targeting some of these abnormalities (23, 25, 59).

Surprisingly, most single-residue deletions near F508 in this study caused pronounced CFTR processing defects that could not be attributed to side-chain removal alone nor could they be rescued by low temperature or corrector C18. As the backbone of F508 plays a role in proper NBD1 folding (13), it is possible that abnormal folding may account for the processing defects of these deletion mutants. Following this idea, side chains of residues 503-513 mostly interact with other residues in NBD1, except that F508 and G509 also interact with ICL4 (Fig. S2, S3) (21). Moreover, since the H3-H4 loop makes a turn with residues 508-513 (Fig. 1A and S1A), deletion of residues in the middle of the loop, such as V510 and S511, may have minimal effect on folding since other residues could re-adjust their positions to compensate for the deletion (Fig. S3B, C). Our data support this hypothesis as these deletions only moderately disrupted CFTR processing (Fig. 1B, C). In contrast, deletion of residues at either end of the H3-H4 loop, including F508, G509, Y512 and D513, may significantly disrupt folding interactions (Fig. S2F, S3A, D, E) and indeed they strongly disrupted CFTR Band C expression (Fig. 1B, C).

We also found that intradomain interactions between residues N505 in the H3 helix, M498 in the Q loop and R560 in the H4 helix, contribute to structural integrity required for normal CFTR expression (Fig. 2, S2C). REMD simulation analysis in this study also showed that H-bond interactions between N505 and R560 in ΔF508/AG NBD1 occurred more frequently than that in ΔF508 NBD1. Recent work suggests that M498 and R560 structurally locate at the interface between NBD1 and ICL4 by the F508 residue (S2C) (20, 21, 60). Moreover, the exposed surface of these two residues is seemly largely extended in ΔF508-CFTR (61). Predicted by structure-based virtual screening, several novel small-molecule compounds for promoting ΔF508-CFTR expression may dock at the site containing M498 and R560 residues (62). These data suggest that interactions from the side chain of N505 may be important for NBD1 stability and could be a target for drug therapy.

**G509 plays an important role in the processing defect of ΔF508-CFTR**

Our data suggest that G509 shifted one position forward in the ΔF508-CFTR polypeptide chain contributes primarily to the processing defect. Our molecular analysis data (Fig. 7, Table 1) suggest that G509 in ΔF508-CFTR may destabilize the H3 helix and affect the bend and turn conformation of residues 509-511 in the H3-H4 loop. This is consistent with the well-known property of glycine as a helix breaker. Similarly, F508G-CFTR
A loop abnormality in ΔF508-CFTR like ΔF508-CFTR shows very little Band C expression (13, 15, 25). However, with the F508 residue unchanged, the ΔG509, but not G509A mutation profoundly reduced Band C expression of CFTR (Fig. 1C, 2A). Moreover, the F508A mutation exerted only small (Fig. 2A) or moderate reductions in Band C % of CFTR (13, 15, 63). The moderate Band C protein level of F508A- and G509A-CFTR suggests that when the length of the polypeptide chain remains unchanged, removing interdomain interactions from the side chain of F508 or disrupting the helix-breaker function of G509 has minor impacts on CFTR processing. Therefore, in ΔF508-CFTR, structural constraints generated by deletion of the F508 residue may be a primary cause of the defect, and together with G509 displacement greatly impair CFTR processing.

Recent studies (14, 16, 19) demonstrate that functional defects of ΔF508-CFTR are associated with abnormal interactions of NBD1 with ICL4 in MSD2, such as interactions between F508 and R1070 (Fig. S1A, S2F) (21). Restoring or enhancing these NBD1-ICL4 contacts have been shown to promote ΔF508-CFTR processing (14, 16, 19, 25, 64). Based on this mechanism, it is possible that the revertant mutation G509A/V510G-induced enhancements in the predicted stability of NBD1 and persistence of the H3 helix in ΔF508-CFTR (Fig. 7, Table 1) may facilitate NBD1-ICL4 interactions for promoting CFTR processing.

Contributions of the H3-H4 loop to the ΔF508-CFTR processing defect
Our data suggest that the H3-H4 loop in CFTR may play an important role in structural integrity required for normal protein processing and channel gating. Generally, loop structures form a connector between two rigid elements such as helices or β-sheets and works like a flexible hinge allowing for the folding of a peptide chain or like an allosteric regulator of domain-domain interactions (65). CFTR cryo-EM structures (20, 21) show that the H3-H4 loop interacts mostly with other intradomain residues in NBD1 (Fig. S2, S3), except for the residues F508 and G509 which are positioned close to the R1070 residue (Fig. S2F, S3A). Although it is unclear whether this loop exhibits dynamic and multiple conformations in native CFTR for domain-domain interactions, our data suggest that neither removing interactions from each residue’s side chain nor increasing loop flexibility by glycine substitutions altered Band C expression of wild-type CFTR (Fig. 2A, 5A).

By contrast, residue deletions in the loop displayed varying impacts on Band C % of CFTR (Fig. 1B, C). Because this loop turns the H3 and H4 helices to face each other for chelating the H5 helix in NBD1 (Fig. 2C, S1A), these data suggest that the loop-mediated helix-turn-helix structure, possibly affected by deletion-induced constraints, may be an important factor regulating CFTR processing. Consistently, our data indicate that the bend and turn secondary structures of residues 509-511 seemly occurred more frequently in wild-type and ΔF508/AG NBD1 than that in ΔF508 NBD1 (Fig. 7B, Table 1). Moreover, residues 502-524 covering the
A loop abnormality in ΔF508-CFTR

H3 helix, loop and H4 helix are highly conserved in CFTR among different species (Fig. S4). Key residues for maintaining normal CFTR processing such as N505, F508, G509, Y512 and D513 are also highly conserved in these CFTR orthologues (Fig. S4). These data may reveal the importance of these residues in structural integrity required for normal CFTR function.

Our data indicate that structural changes of residues in the shortened loop were associated with the low temperature-rescued processing defect in ΔF508- and ΔY512-CFTR. Low temperature may facilitate CFTR thermodynamic folding and enhance thermal stability (17, 23, 24, 54, 56, 57, 66-69), suggesting that protein stability of NBD1 may be associated with the functional defects of ΔF508-CFTR. Similarly, the revertant mutation G509A/V510G enhanced the predicted protein stability of ΔF508 NBD1 (Fig. 7A). Previous work using REMD simulations (38) demonstrates that the dynamics of some residues in the H3 and H5 helices were different between wild-type and ΔF508 NBD1 (Fig. 7A). Interestingly, several second-site revertant mutations that improve ΔF508-CFTR processing are found in the H5 helix, such as G550E, R553M, and R555K (46, 47, 50, 63, 70). Some of these mutations also increase both the protein (38) and thermal (57) stability of ΔF508 NBD1. Therefore, these data suggest that NBD1 instability may be associated with the ΔF508-CFTR defects.

Our study demonstrates that Band C expression of CFTR was largely reduced by all deletion mutations in H3 and H4 (Fig. 1) or by site-directed mutations at N505 and R560 residues in H3 and H5 helices, respectively (Fig. 2). Similarly, trivial Band C expression is observed in the CF mutant R560T- (30, 50, 71) and R560S-CFTR (72). Taken together, our own and previous data highlight the importance of H3, H4 and H5 helices in achieving normal CFTR processing. We speculate that loop mutations and other mutations near F508 may disturb the alignment of these three helices, resulting in abnormal NBD1 folding and stability, which could be recognized as a structural defect by quality control mechanisms in the ER (4), targeting CFTR for degradation.

Revertant mutations of the loop partially correct the CFTR gating defect

The gating defect of ΔF508-CFTR is characterized by a slow channel opening rate, which is shown as prolonged IBI in CFTR gating kinetics (Fig. 4E) (6-8). The channel opening rate of ΔF508-CFTR can be markedly enhanced by introducing revertant mutations near ATP binding sites (50), by mutations at domain-domain interfaces between NBD1 and MSD1, MSD2 or NBD2 (14, 16, 19, 73) or by adding potentiators to stimulate ATP-dependent CFTR gating (7, 74). Interestingly, those revertant mutations that correct domain-domain interactions between NBD1 and other regions in CFTR such as R1070W in ICL4 (14, 73) or that may affect ATP function in two NBDs such as G550E (50) all greatly stimulate the channel activity of ΔF508-CFTR,
A loop abnormality in ΔF508-CFTR but show little or no effects on CFTR processing (14, 16, 19, 50, 73). These data may suggest that the processing and gating defect of ΔF508-CFTR are generated by different mechanisms (19).

However, in this study loop mutations G509A/V510G greatly enhanced both the Band C expression (Fig. 5A) and channel activity of ΔF508-CFTR (Fig. 6A). Similarly, V510A/S511A mutations greatly enhanced the rescue effect of low temperature on protein processing of ΔY512-CFTR (Fig. 5C) and partially corrected its gating defect (Fig. 6E). Our data suggest that deletion of a residue and displacement of the G509 residue in the H3-H4 loop form pivotal defects of ΔF508-CFTR, possibly further inducing structural abnormalities that separately lead to either the processing or channel gating defect.

In conclusion, this study characterized the role of residues near F508 in CFTR processing. Our data suggest that abnormalities of the H3 helix and the H3-H4 loop may impair CFTR processing and gating, and contribute to dysfunction of ΔF508-CFTR. We also found that low temperature and corrector C18 stimulated protein expression of not only ΔF508-CFTR but also some other CFTR deletion mutants that miss a residue in the H3-H4 loop. Therefore, even if drug cocktails of CFTR correctors and potentiators provide a promising cure for people with CF (75), our data highlight the possibility that small molecules, which correct defects in the H3 helix and H3-H4 loop might rescue both the processing and gating defects of ΔF508-CFTR.
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Author contributions

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A loop abnormality in ΔF508-CFTR


A loop abnormality in ΔF508-CFTR


A loop abnormality in ΔF508-CFTR

A loop abnormality in ∆F508-CFTR

Table 1. Residence of residues 502-524 adopted specific secondary structure conformations calculated from three REMD simulations of wild-type, ∆F508/AG and ∆F508 NBD1.

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Data show the percentage of time (%) measured from Figure 7B. Data are means ± S.E.M. (N=3 for all data) for wild-type (WT), ∆F508/AG (ΔF/AG) and ∆F508 NBD1. *, P<0.05 vs. wild-type NBD1, one-way ANOVA. #, P<0.05, ∆F508/AG vs. ∆F508 NBD1, one-way ANOVA. For further details, see Figure 7.
**FIGURE LEGENDS**

**Figure 1.** Localized deletions near the F508 residue severely impair CFTR processing. A) Comparison of NBD1 structures between human wild-type CFTR (grey; PDB: 2BBO) and ΔF508-CFTR (green; PDB: 1XMJ). Expanded figure on the right displays the aberrant H3-H4 loop (blue) in ΔF508-NBD1 vs. the normal (grey) in wild-type NBD1. Yellow dots indicate positions of residues tested in panel B. B, C) Immunoblotting image (B) and quantitative data (C) show protein expression of wild-type (WT) and mutant CFTR. Columns in grey represent the data of CFTR mutants with deletions outside positions 503-513. The percentage of Band C expression (\( \frac{C}{B+C} \)) was calculated by measuring protein band intensity of Band C vs. that of Bands B+C. Data are means + S.E.M. (N = 6-8). *, P<0.05 vs. wild-type CFTR, one-way ANOVA.

**Figure 2.** Side-chain function of N505 and D513 residues is required for normal CFTR processing. A–E) Effects of site-directed mutations on CFTR processing with alanine substitutions at residues from positions 503 to 515 (A) (N=6-8) and with substitutions of different residues for N505 (B) (N = 6-7), at N505, M498 and R560 (C, D) (N = 5-7) or at D513 (E) (N = 6-8). Data of wild-type (WT) CFTR were adapted from Figure 1C. Data are means + S.E.M. *, P<0.05 vs. wild-type CFTR, one-way ANOVA. See other details in Figure 1.

**Figure 3.** The processing defect of ΔY512-CFTR is rescued by low temperature and CFTR correctors. A–D) Band C % of wild-type (WT) CFTR or indicated mutants incubated at 27 °C (A), treated with the CFTR corrector C18 (10 µM) (B), treated with corrector C18 at 27 °C (C) or treated with correctors C18 and C4 (10 µM) at 27 °C (D). In (A), the control data were adapted from Figure 1C for each CFTR variant. The vehicle controls of 0.1% DMSO were tested at 37 °C (B, C) or at 27 °C (D). Data are means + S.E.M. (N = 6-10). *, P<0.05 vs. control, one-way ANOVA. #, P<0.05 between two sets of the data in ΔF508- or ΔY512-CFTR, one-way ANOVA.

**Figure 4.** Channel gating of ΔY512-CFTR exhibits prolonged IBI. A) Representative recordings show the single-channel activity of wild-type and mutant CFTR in the presence of ATP (1 mM) and PKA (75 nM). Dotted lines indicate the closed state and downward deflections correspond to channel openings. B–E) The single-channel current amplitude (i) (B), open probability (P_o) (C), mean burst duration (MBD) (D) and interburst interval (IBI) (E) of wild-type and mutant CFTR are shown. Data are means + S.E.M. (N = 6, except ΔF508-CFTR where N = 4). *, P<0.05 vs. wild-type CFTR, one-way ANOVA.

**Figure 5.** Differential effects of H3-H4 loop mutations on the correction of ΔF508- and ΔY512-CFTR processing. A, B) Effects of alanine or glycine substitutions at the H3-H4 loop on Band C % of ΔF508- (A) and
A loop abnormality in ΔF508-CFTR (B). Wild-type, ΔF508- or ΔY512-CFTR were used as controls (blank columns). In (A), the image of ΔF508-G509A/V510A-CFTR was acquired separately from the other data.  C) Effects of low temperature and corrector C18 on Band C % of ΔF508/AG (G509A/V510G)-CFTR and ΔY512/2A (V510A/S511A)-CFTR. Blank columns represent the data of the vehicle control of 0.1% DMSO at 37 °C. Notably, a protein band between Band B and C was evidently found in the immunoblot of ΔF508/AG-CFTR in the presence of corrector C18, but excluded from the analysis in this and other experiments (e.g., Fig. 3D) due to lack of the control.  D) The fold increase in Band C % of ΔF508-, ΔF508/AG-, ΔY512- and ΔY512/2A-CFTR by low temperature or corrector C18 vs. the control at 37 °C with no treatment (blank bars). Fold increase in Band C % = (Band C %-treatment) / (Band C %-no treatment control at 37 ºC). Dotted lines indicate the fold increase in Band C % = 1 for the control. All data are means + S.E.M. (N = 6-10); *, P<0.05 vs. the control (blank columns), one-way ANOVA; #, P<0.05 between two groups, one-way ANOVA.

**Figure 6.** The IBI of ΔF508/AG- and ΔY512/2A-CFTR are only mildly prolonged. A) Representative recordings show the single-channel activity of ΔF508/AG- and ΔY512/2A-CFTR in the presence of ATP (1 mM) and PKA (75 nM). Dotted lines indicate the closed state and downward deflections correspond to channel openings. B–E) The single-channel current amplitude (i), P_o, MBD and IBI of two mutants are shown. Data are means + S.E.M. (N = 6 for both mutants). *, P<0.05 vs. wild-type CFTR, one-way ANOVA.

**Figure 7.** The revertant mutation G509A/V510G enhances protein stability and persistence of the H3 helix in ΔF508 NBD1. A) Three REMD simulations of wild-type (blue), ΔF508/AG (green) and ΔF508 (red) NBD1 constructs. Two vertical grey shadings indicate the RMSF values of residues 502-505 and 510-511, respectively. Data of wild-type and ΔF508 NBD1 were retrieved from the previous publication (38) with permission. B) Representative DSSP plots show persistence of secondary structure elements for residues 491-525 in wild-type (top), ΔF508/AG (middle) and ΔF508 (bottom) NBD1.
Figure 1

A

Wild-type NBD1  ΔF508-NBD1

ATP

F508

B

(kDa)

WT  ΔC491  ΔS495  ΔP499  ΔK503  ΔE504  ΔN505  ΔI506  ΔF508  ΔG509  ΔV510  ΔS511  ΔI512  ΔD513  ΔY515  ΔQ525

Band C

Band B

C

\[
\frac{C}{B+C} \%
\]
**Figure 2**

(A) Western blot analysis of wild-type (WT) and mutant proteins. The gel shows the relative molecular weights (kDa) of the different variants.

(B) Quantification of the bands indicated by arrows in panel A. The graph shows the percentages of bands C/(B+C) for WT and mutant proteins.

(C) 3D structure of the protein domain showing key residues M498, H5, N505, R560, H3, and D513. The structure highlights the interactions and positions of these residues.

(D) Western blot analysis of additional mutant proteins. The gel shows the relative molecular weights (kDa) of these variants.

(E) Quantification of the bands indicated by arrows in panel D. The graph shows the percentages of bands C/(B+C) for these mutant proteins.
Figure 3

A  
27 °C

WT ΔK503 ΔE504 ΔN505 ΔI506 ΔG508 ΔV510 ΔS511 ΔV512 ΔD513

(kDa)

250 150 100

C

B+C(%)

WT ΔK503 ΔE504 ΔN505 ΔI506 ΔG508 ΔV510 ΔS511 ΔV512 ΔD513

Control (37 °C) 27 °C

C

B+C(%)

WT ΔK503 ΔE504 ΔN505 ΔI506 ΔG508 ΔV510 ΔS511 ΔV512 ΔD513

Control (37 °C) C18 (10 μM)

C

B+C(%)

WT ΔK503 ΔE504 ΔN505 ΔI506 ΔG508 ΔV510 ΔS511 ΔV512 ΔD513

Control (37 °C) 27 °C C18 (10 μM)

C

B+C(%)

WT ΔK503 ΔE504 ΔN505 ΔI506 ΔG508 ΔV510 ΔS511 ΔV512 ΔD513

C4 C18

ΔF508

ΔY512

C

B+C(%)

ΔF508 ΔG508 ΔV510 ΔS511 ΔY512 ΔD513

ΔC4 C18

ΔC4+C18

ΔF508 ΔY512

ΔC4 C18

ΔC4+C18

ΔF508 ΔY512
Figure 4

A

Wild-type

ΔF508

ΔV510

ΔS511

ΔY512

B

i (pA)

-0.8

-0.4

0

Wild-type  ΔF508  ΔV510  ΔS511  ΔY512

C

P₀

0

0.3

0.6

Wild-type  ΔF508  ΔV510  ΔS511  ΔY512

D

MBD (ms)

0

300

600

Wild-type  ΔF508  ΔV510  ΔS511  ΔY512

E

IBI (ms)

0

3000

6000

Wild-type  ΔF508  ΔV510  ΔS511  ΔY512

*  **
**Figure 5**

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### Graphs

- **Fold Increase in Band C %**
- **C / (B+C) (%)**

**Notes:**

- Δ indicates absence.
- + indicates present.
- * indicates statistical significance.
- # indicates another level of significance.
Figure 6

A

$\Delta F_{508/AG}$

$\Delta Y_{512/2A}$

0.5 pA

0.5 s

B

C

D

E

$\text{i (pA)}$

$\text{P}_o$

$\text{MBD (ms)}$

$\text{IBI (ms)}$

WT

$\Delta F_{508/AG}$

$\Delta Y_{512/2A}$

WT

$\Delta F_{508/AG}$

$\Delta Y_{512/2A}$

WT

$\Delta F_{508/AG}$

$\Delta Y_{512/2A}$

WT

$\Delta F_{508/AG}$

$\Delta Y_{512/2A}$

* *
Figure 7

A

RMSF (nm)

Residue number

 Wildcats NBD1

AF508 NBD1

AF508/AG NBD1

B

Coil

B-Bridge

Bend

Turn

$\alpha$-Helix

3-Helix

Residue

Wild-type NBD1

Secondary structure

Time (ps)

490

500

510

525

0

5000

10000

AF508/AG NBD1

AF508 NBD1

Residue

525

510

500

490

0

5000

10000

Time (ps)
A defective flexible loop contributes to the processing and gating defects of the predominant cystic fibrosis-causing mutation

SUPPLEMENTAL DATA

Table S1. Summary results of protein expression of key CFTR mutants tested in this study.

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| Deletion mutation | Δ | Δ | Δ | Δ | Δ | Δ | Δ | Δ | Δ | Δ | Δ |
| Expression scale | 37°C | + | + | + | + | + | + | ++++ | ++++ | + | + |
| 27°C | + | + | + | + | +++ | + | ++++ | ++++ | ++++ | + |
| 37°C + C18 | + | + | ++ | + | +++ | + | ++++ | ++++ | ++++ | ++ |
| 27°C + C18 | + | ++++ | + | ++++ | ++++ | ++++ | ++++ | ++++ | ++ |
| 27°C + C4 | +++ | +++ | +++ | ++++ | ++++ | +++ | ++++ | ++++ | ++++ |

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Note: Expression scale is defined as follows: +, Band C % < 10%; ++, < 20%; +++ < 30%; ++++, < 40%; +++++, ≥ 40%. N indicates the number of experiments. Blank spots indicate no experiment performed. In most experiments, cells were cultured at 37 °C except where shown at 27 °C.

*, means ± S.E.M. of Band C % are shown.
SUPPLEMENTAL FIGURE LEGENDS

**Figure S1.** Positions of key secondary structures and residues in NBD1 tested in this study. A) Positions of the H3, H4 and H5 helices, F508 residue (red) and H3-H4 loop (orange) are displayed in the cryo-EM structure of human wild-type CFTR (PDB: 5UAK) with MSD1 in blue color, NBD1 in grey and MSD2 and NBD2 in green. B, C) Residues that interact with side chains of the D513 (B) and Y512 (C) residue are shown in NBD1 of cryo-EM human wild-type CFTR.

**Figure S2.** Side-chain interactions of residues 503-508 in the cryo-EM structure of human wild-type CFTR. A-F) Side-chain interactions of K503 (A), E504 (B), N505 (C), I506 (D), I507 (E) and F508 (F) are shown. Only the shortest distance (< 6 Å) is drawn between two residues. Distance of two atoms is labelled for those polar chemical bonds. For the purpose of illustration, residues 515-516 were omitted from the structures. See other details in Figure S1.

**Figure S3.** Side-chain interactions of residues 509-513 in the cryo-EM structure of human wild-type CFTR. A-F) Side-chain interactions of G509 (A), V510 (B), S511 (C), Y512 (D) and D513 (E) are shown. For further details, see Figures S1 and S2.

**Figure S4.** Amino acid sequence alignment of CFTR orthologues for residues 501 to 553. Residues that differ from that at the same position in human wild-type CFTR are identified by blue letters and grey background. Percentage similarity was calculated using the equation: (number of matched residues) / (total number of CFTR orthologues [45]) * 100%.
A

Cryo-EM structure of human CFTR

B

Interactions of D513's side chain

Interactions of Y512's side chain
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% Similarity

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