Correctors of ΔF508 CFTR restore global conformational maturation without thermally stabilizing the mutant protein

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ABSTRACT **Most cystic fibrosis is caused by the deletion of a single amino acid (F508) from CFTR and the resulting misfolding and destabilization of the protein. Compounds identified by high-throughput screening to improve ΔF508 CFTR maturation have already entered clinical trials, and it is important to understand their mechanisms of action to further improve their efficacy. Here, we showed that several of these compounds, including the investigational drug VX-809, caused a much greater increase (5- to 10-fold) in maturation at 27 than at 37°C (<2-fold), and the mature product remained short-lived ($T_{1/2}$~4.5 h) and thermally unstable, even though its overall conformational state was similar to wild type, as judged by resistance to proteolysis and interdomain cross-linking. Consistent with its inability to restore thermodynamic stability, VX-809 stimulated maturation 2–5-fold beyond that caused by several different stabilizing modifications of NBD1 and the NBD1/CL4 interface. The compound also promoted maturation of several disease-associated processing mutants on the CL4 side of this interface. Although these effects may reflect an interaction of VX-809 with this interface, an interpretation supported by computational docking, it also rescued maturation of mutants in other cytoplasmic loops, either by allosteric effects or *via* additional sites of action. In addition to revealing the capabilities and some of the limitations of this important investigational drug, these findings clearly demonstrate that ΔF508 CFTR can be completely assembled and evade cellular quality control systems, while remaining thermodynamically unstable. He, L., Kota, P., Aleksandrov, A. A., Cui, L., Jensen, T., Dokholyan, N. V., Riordan, J. R. Correctors of ΔF508 CFTR restore global conformational maturation without thermally stabilizing the mutant protein. *FASEB J.* 27, 536–545 (2013). www.fasebj.org**

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Although secondary modifier genes influencing disease severity have been identified (1–3), cystic fibrosis is caused primarily by mutations in the single cystic fibrosis transmembrane conductance regulator (CFTR) gene (4), and restoration of mutant CFTR function has clinical efficacy (5, 6). The effectiveness of this strategy is most convincingly demonstrated by the small-molecule VX-770 (Kalydeco) that overcomes the dysfunction of G551D CFTR present in a significant subset of patients and improves their epithelial salt and fluid homeostasis and lung function (5). Progress also has been made in identifying small molecules that improve the biosynthetic assembly of CFTR with phenylalanine 508 deletion (ΔF508 CFTR), present in ~90% of patients with CF (7, 8). However, as yet, these so-called correctors have been much less effective in early clinical trials on patients with the ΔF508 mutation than VX-770 is on patients with the G551D mutation (5). The reason for this less than optimal influence of these compounds is unknown because neither their modes nor sites of action are known.

Recent mechanistic studies of how the absence of the F508 residue alters the folding, assembly, and stability of the mutant protein emphasize the crucial roles of both the destabilization of the first nucleotide binding domain (NBD1) and its interaction with other of the multiple CFTR domains (9–11). Therefore, assessment of the influence of first-generation correctors on these processes may illuminate the basis of their limited effectiveness. Using this approach, we have found that

Abbreviations: ΔF508, phenylalanine 508 deletion; ΔRI, regulatory insertion deletion; BHK, baby hamster kidney; C3, VRT-325, C4, Corr-4a; CFTR, cystic fibrosis transmembrane conductance regulator; HEK, human embryonic kidney; IR, infrared; M1M, 1,1-methanediyl bismethane-thiosulfonate; M8M, 1,5-pentanediyl bismethane-thiosulfonate; NBD1/2, nucleotide binding domain 1/2; CL2/4, cytoplasmic loop 2/4; WT, wild type

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some corrector compounds, including one tested in early clinical trials, VX-809, are effective in promoting the overall assembly of the multidomain protein, even though they do not restore thermal stability. VX-809 also promotes maturation of several disease-associated processing mutants in cytoplasmic loop 4 (CL4), and structure-based docking studies indicate that the NBD1/CL4 interface might be one binding site for the compound. However, its stimulation of maturation is strongly incremental with the effects of both second-site mutations that stabilize NBD1 and those that "patch" the NBD1/CL4 interface, suggesting that the compound has effects other than stabilization of NBD1 or its interface with CL4.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal CFTR antibodies to an N-terminal fragment (mAb 13-4, IgG1κ), regulatory insertion region (L12B4), NBD1 (mAb 660, IgG2b), and NBD2 (mAb 596, IgG2b) were generated as described previously (12). Goat anti-mouse IgG-IR800, IgG1-IR800, and IgG2b-IR680 were from Li-Cor (Lincoln, NE, USA).

Construction and expression of mutants

Various site-specific mutations were introduced into the CFTR construct in pcDNA3 vector by the Stratagene Quick Exchange protocol (Stratagene, La Jolla, CA, USA) as described previously (12). Point mutations were confirmed by automated DNA sequencing (University of North Carolina–Chapel Hill Genome Analysis Facility).

Human embryonic kidney (HEK)-293 cells were transiently transfected using Jet PEI transfection reagent (Polyplus Transfection, New York, NY, USA), according to the manufacturer's instructions. For stable expression, constructs were cotransfected with pNUT plasmid into baby hamster kidney (BHK)-21 cells, which were selected and maintained in methotrexate (500 μM) containing DMEM-F12. For drug treatment, 24 h after transfection, HEK cells were incubated in the absence or presence of correctors (see figure legends for doses) at 27 or 37°C for 24 h. BHK cells were treated for the same time period before harvesting for whole-cell lysate or membrane vesicle preparations. Cell lysates were prepared in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, and 1% deoxycholate, pH 7.4) plus protease inhibitor cocktail (1 μg/ml leupeptin, 2 μg/ml aprotinin, 3.57 μg/ml E64, 156.6 μg/ml benzamidine, and 2 mM Pefablock). Protein concentrations were measured using the bicinchoninic acid (BCA) assay (BCA reagent from Pierce Thermo Scientific, Rockford, IL, USA). Total proteins (20 μg) were loaded on 7.5% SDS-PAGE and subjected to Western blot analysis to determine CFTR protein expression and maturation.

Isolation of membrane vesicles and limited trypsin digestion

Membrane vesicles were isolated from BHK cells expressing variants of CFTR, as described previously (12). For limited trypsin digestion, BHK cells were treated with 100 μM cyclohexamide for 4 h before harvesting to stop new protein synthesis and deplete the immature forms of CFTR. Membranes were resuspended at 1 mg protein/ml in a buffer containing 40 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, and 0.1 mM EGTA. Membranes were incubated with various concentrations of trypsin (see figure legends) for 10 min on ice, and digestion was stopped by adding excess protease inhibitor cocktail. CFTR tryptic fragments separated on 4–20% gradient SDS-PAGE (Bio-Rad, Richmond, CA, USA) were detected by Western blot probed with antibodies recognizing different epitopes (12). For dual antibody labeling, isotype-specific secondary antibodies labeled with different infrared (IR) dyes were used. Specifically, goat-anti-mouse IgG1-IR800 was used to detect 13-4, and IgG2b-IR680 to detect mAbs 660 and 596.

Disulfide cross-linking in whole cells

Disulfide cross-linking of cysteine pairs introduced at NBD2/CL2 and NBD1/CL4 interfaces was carried out, as described previously (13). Briefly, HEK cells transiently expressing CFTR grown on 35-mm tissue culture dishes were harvested, washed twice in PBS, and resuspended in 60 μl PBS. Cell suspension (20 μl) was mixed with 40 μl PBS with DMSO as a vesicle control or PBS containing 300 μM cross-linkers to yield a final concentration of 200 μM. Bifunctional cross-linkers from Toronto Research Chemicals (North York, ON, Canada) were used: 1,1-methanediyl bismethane-thiosulfonate (M1M) and 1,5-pentanediyl bismethane-thiosulfonate (M8M). After 15 min of incubation at room temperature, the cross-linking reaction was stopped with Laemmli sample buffer with or without DTT. Samples (30 μl) were loaded on 7.5% SDS-PAGE, and anti-CFTR mAb 596 was used to probe Western blots. CFTR was detected with secondary antibodies labeled with IR dyes (IgG2b-680) using the Odyssey IR scanner (Li-Cor).

Metabolic pulse chase

BHK cells stably expressing ΔF508 CFTR with or without I539T were treated with VX-809 (3 μM; Vertex Pharmeceuticals, Cambridge, MA, USA) for 24 h at 27°C. Cells expressing wild-type (WT) CFTR were grown at 37°C. Long-term pulse-chase experiments were performed in the absence (WT CFTR) or the presence of VX-809 (ΔF508 and ΔF508/I539T CFTR) to follow the lifetime of mature CFTR (14). Briefly, BHK cells stably expressing WT CFTR, ΔF508 CFTR, and ΔF508/I539T CFTR were grown in 60-mm-diameter dishes and labeled for 8 h in 1.5 ml methionine-free medium supplemented with 10% normal growth medium, 10% FBS, and 66 μCi/ml ^{35}S-methionine (Perkin-Elmer, Waltham, MA), at 27 or 37°C. Cells were then washed 2 times and chased at 37°C with growth medium supplemented with 10 mM unlabeled methionine. The chase was stopped by washing 2 times with cold PBS and solubilizing the cells with RIPA buffer containing the protease inhibitor cocktail. CFTR was immunoprecipitated using mAb 596. The immunoprecipitated proteins were then run on a 7% SDS-PAGE, fixed with acetic acid-methanol, and soaked in 1 M sodium salycilate for radiography. Band intensity was quantified using Packard Instant Imager (Packard Instruments, Meriden, CT, USA).

Planar bilayer-based single-channel measurement

Planar lipid bilayers were prepared by painting a 0.2-mm hole drilled in a Teflon cup with a phospholipid solution in *n*-decane containing a 3:1 mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (Avanti Polar Lipids, Alabaster, AL, USA). The lipid bilayer separated 1.0 ml of solution in the Teflon cup (*cis* side) from 5.0 ml of a solution in an outer

glass chamber (*trans* side). Both chambers were magnetically stirred and thermally insulated. Heating and temperature control were established by a temperature control system (TC2BIP; Cell Micro Controls, Norfolk, VA, USA).

Membranes were prepared from BHK cells stably expressing CFTR variants and resuspended in a buffer containing 250 mM sucrose, 5 mM $MgCl_2$, 0.5 mM EGTA, and 10 mM HEPES (pH 7.4). Brief (3×20 s) bath sonication was used to generate vesicles of uniform size for single-channel measurements. To maintain uniform orientation and functional activity of CFTR channels, 2 mM ATP, 50 nM PKA, and 10 μl of membrane vesicles at 1 mg/ml total protein concentration were added to the *cis* compartment only. CFTR ion channels were transferred into the preformed lipid bilayer by spontaneous fusion of membrane vesicles containing CFTR variants in symmetrical salt solution (300 mM Tris/HCl, pH 7.2; 3 mM $MgCl_2$; and 1 mM EGTA).

Single-channel currents were measured at −75 mV under voltage-clamp conditions using an Axopatch 200B amplifier (Axon Instruments, Sunnyvale, CA, USA). For analysis, the single-channel current was digitized (Digidata 1322; Axon Instruments) with a sampling rate of 500 Hz and analyzed using pCLAMP 9.2 software (Axon Instruments). Origin 7.5 software (Origin Lab, Northampton, MA, USA) was used to fit all-points histograms by multipeak gaussians. Single-channel current was defined as the distance between peaks on the fitting curve and used for the calculation of the single-channel conductance. The single-channel open probability (P_o) was calculated as a ratio of the area under the peak for the open state to the total area under both peaks on the fitting curve. The transport capacity of the structural unit was defined as $\langle\gamma\rangle = \gamma P_o$ for mutants with a single conductive state and stable gating kinetics. The transport capacity of the structural unit $\langle\gamma\rangle$ for ΔF508/I539T CFTR with unstable gating kinetics and variable conductive state at 35°C was estimated as total charge transported in 10 min (area under the trace), divided by the potential difference applied and normalized per second so as to be an exact analog of γP_o used for the channels with stable and well-defined open state.

Docking

Putative binding poses for VX-809 in the pocket formed in the interface of NBD1 and CL4 were generated using MedusaDock (15, 16). Employing a new random seed for each iteration, a total of 1000 docking simulations were performed, thereby generating ~4500 poses. Side chains of residues within 10 Å of the docking boundary were repacked during the docking simulations. Poses generated using MedusaDock were rank ordered based on energy after applying the correction function, as described previously (15, 16).

RESULTS

Small-molecule correctors are most effective at subphysiological temperature

The conformational maturation and cellular trafficking of ΔF508 CFTR is known to be temperature sensitive, such that a modest level of maturation occurs below ~30°C (17, 18). Although it is widely assumed that this reflects the demonstrated influence of F508 deletion on NBD1 folding, clearly the overall cellular conditions for folding are very different at the lower and higher temperatures (19). It is not yet known whether small-molecule correctors discovered in cell-based screens act directly on the CFTR protein or on the folding conditions or both. In either case, it is important to know whether and to what extent correctors have a thermostabilizing influence. Therefore, we have analyzed and compared their actions at physiological and subphysiological temperature.

When ΔF508-CFTR-expressing BHK cells were treated with different correctors at either 37°C or 27°C, a very pronounced difference was observed in the extent of maturation at the two temperatures (**Fig. 1*A***). Although a small increase in the ratio of amount of the mature C band to the immature B band was evident at 37°C, there was a very much larger increase at 27°C with each of the compounds tested, including VX-809. This remarkable difference was displayed over the entire effective dose range of VX-809, as well as that of its analog, 1(benzo[*d*] [1,3]dioxol-5-yl)-*N*-(5-[(2-chlorophenyl)(3-hydroxypyrrolidin-1-yl)methyl)thiazol-2-yl]cyclopropanecarboxamide (C18; Fig. 1*B*).

Because a relatively large amount of mature ΔF508 CFTR is formed in VX-809-treated cells maintained at 27°C, we next determined whether the protein had been stabilized in terms of its lifetime in cells (**Fig. 2*A***). Comparison of its rate of decay ($T_{1/2}$~4.5 h) with that of the mature WT ($T_{1/2}$~14 h), in metabolic pulse-chase experiments, revealed that the corrector had not extended the lifetime beyond that of the low-temperature-rescued ΔF508 CFTR, which has a half-life of ~6 h (20, 21). Furthermore, even when the modestly effective second-site suppressor mutation, I539T (22, 23) also was present in VX-809-treated ΔF508-CFTR-ex-

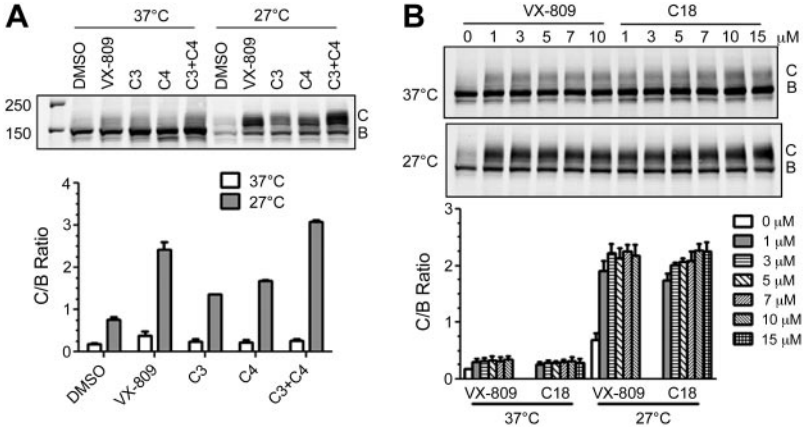

Figure 1. Small-molecule correctors have much greater effect on ΔF508 maturation at 27°C than at 37°C. *A*) BHK cells expressing ΔF508 CFTR were treated with VX809 (3 μM), VRT-325 (C3; 10 μM), Corr-4a (C4; 10 μM), or C3 + C4 (10 μM each) for 24 h at 27 or 37°C. Cell lysates in RIPA buffer with equal amounts of protein were subject to 7.5% SDS-PAGE and Western blot analysis using CFTR antibody mAb596. Intensities of mature C band and the immature B band were quantified using a Li-Cor Odyssey imaging system, and the C/B ratios were calculated and presented as means ± SD for each condition; $n = 3$. *B*) Dose response of VX-809 (1–10 μM) and CF-106951 (C18; 1–15 μM) on ΔF508 CFTR rescue at 27 and 37°C. Cell treatment, sample processing, and data analysis were carried out as in *A*.

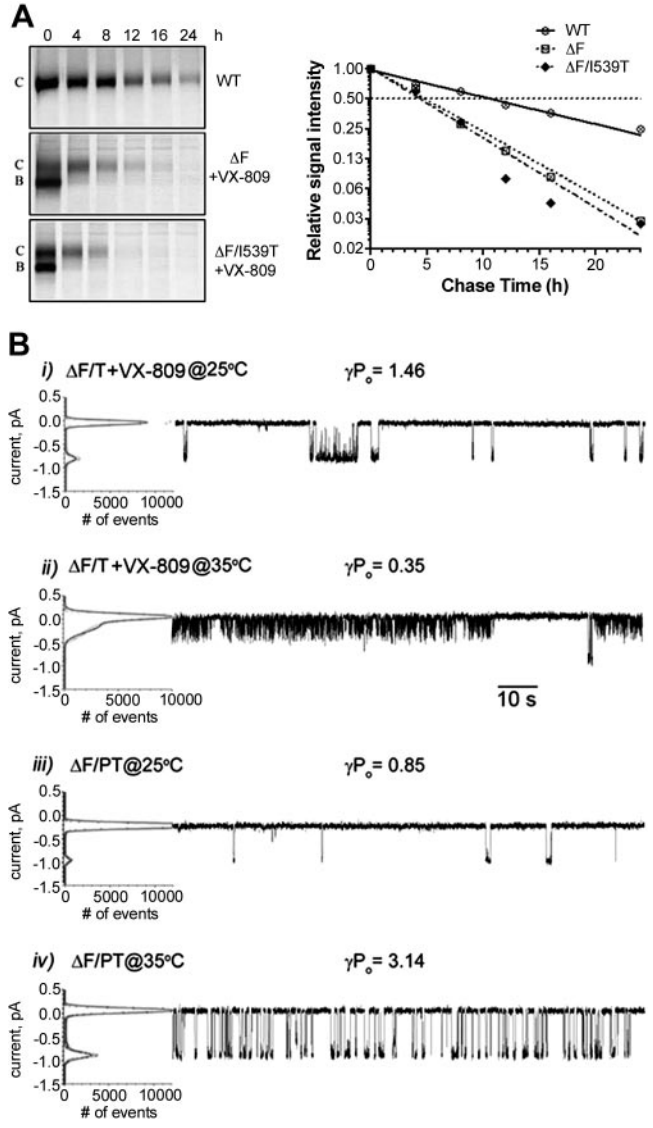

Figure 2. VX-809 does not restore the metabolic lifetime or thermal stability of ΔF508 CFTR. *A*) BHK cells stably expressing ΔF508 CFTR with or without I539T were treated with VX-809 (3 μM) for 24 h at 27°C. Cells expressing WT CFTR were grown at 37°C. Long-term pulse-chase experiments were performed in the absence of VX-809 (WT CFTR) or presence of VX-809 (ΔF508 and ΔF508/I539T CFTR) to follow the lifetime of CFTR, as described in Materials and Methods. Intensity of mature C band was quantified with a Packard Instant Imager to calculate the half-life of mature CFTR (n=2). *B*) Single-channel recordings of ΔF508/I539T CFTR (ΔF/T) rescued by 3 μM VX-809 at 35°C (*i*) and 25°C (*ii*) and of ΔF508/I539T/S492P CFTR (ΔF/PT) as an example of an already known (24) alternative type of ΔF508/I539T, thermally stabilized by proline substitutions at 35°C (*iii*) and 25°C (*iv*). Six independent experiments of 38 min total time were used to estimate transport capacity $\langle\gamma\rangle = 1.46 \pm 0.28$ for ΔF508/I539T at 25°C; data are shown as means ± SE. Five independent experiments of 34 min total time were used to estimate $\langle\gamma\rangle = 0.35 \pm 0.14$ for ΔF508/I539T at 35°C. Two sets of 4 independent experiments of 35 and 38 min total time were used to estimate transport capacity $\langle\gamma\rangle = 0.85 \pm 0.26$ at 25°C (*iii*) and $\langle\gamma\rangle = 3.14 \pm 0.32$ at 35°C (*iv*) for ΔF508/I539T/S492P CFTR. All point histograms are shown on the left of each trace to estimate open state conductance and open probability. Type of mutant and recording temperature is shown above each histogram. Transport capacity (γP_o) as a measure of functional ability is shown above each trace.

pressing cells, the turnover of the protein was still similarly rapid as that of its counterpart without VX-809 treatment (24).

While these lifetimes as reflections of the stability of the protein in cells do not necessarily equate to the functional thermal stability of its ion channel activity, only very transient channel activity with low open probability was observed in ΔF508/I539T-CFTR-expressing cells treated with VX-809 (Fig. 2*B*). Thus, the shortened functional lifetime of the ΔF508-CFTR channel described previously (14, 25, 26) is not extended by the I539T substitution. We used the ΔF508/I539T-CFTR variant as it, in contrast to the ΔF508 CFTR (14), has reasonably stable functional activity at 25°C, although it inactivates at higher temperatures (24). Therefore, it serves as a basis for the comparison of the influence of the VX-809 compound with that of a known stabilizing second-site mutation, S492P (24). For VX-809 treatment, the ΔF508/I539T CFTR variant was continuously exposed to the compound during cell growth, membrane vesicle isolation, and channel assay. As a result, nearly WT CFTR transport capacity (γP_o) was observed at 25°C, but this was strongly diminished when the temperature was increased to 35°C (Fig. 2*Bi*, *ii*). This behavior strongly contrasted that of the ΔF508/I539T variant with the stabilizing S492P mutation added, where transport capacity increased, and full conductance state persisted up to 35°C (compare tracings in Fig. 2*Biii*, *iv*). Thus, VX-809 did not appear to have an influence similar to that of a verified stabilizing amino acid substitution in NBD1 that served as an important positive control in this experiment.

Global assembly of ΔF508 CFTR can occur with or without thermal stabilization

As shown above, in contrast to the thermolability of ΔF508 CFTR rescued by correctors at reduced temperature, the mutant protein rescued by several different second-site modifications in NBD1 has thermostable channel activity (14, 24, 27, 28). The extent to which the full-length multidomain mutant protein has achieved a compact globally assembled state can be assessed by its susceptibility to limited protease digestion, with the unassembled ΔF508 mutant being cleaved at many more sites than the fully folded WT (12, 29, 30). We employed this assay to compare the folded state of ΔF508 CFTR that had matured under the influence of the strongly NBD1-stabilizing 4PT modification (prolines introduced at 4 mobile sites: S422, S434, S492, and A534 plus I539T); or exposure of cells to VX-809 at 27°C. Notably, in both cases, cells were pretreated with cycloheximide for 4 h to inhibit protein synthesis before the isolation of membranes for protease digestion, so that mature CFTR rather than the newly synthesized immature form was predominant (**Fig. 3*A***, left panel).

Employing antibodies recognizing four different epitopes across the protein sequence (12), the fragments produced by increasing concentrations of trypsin

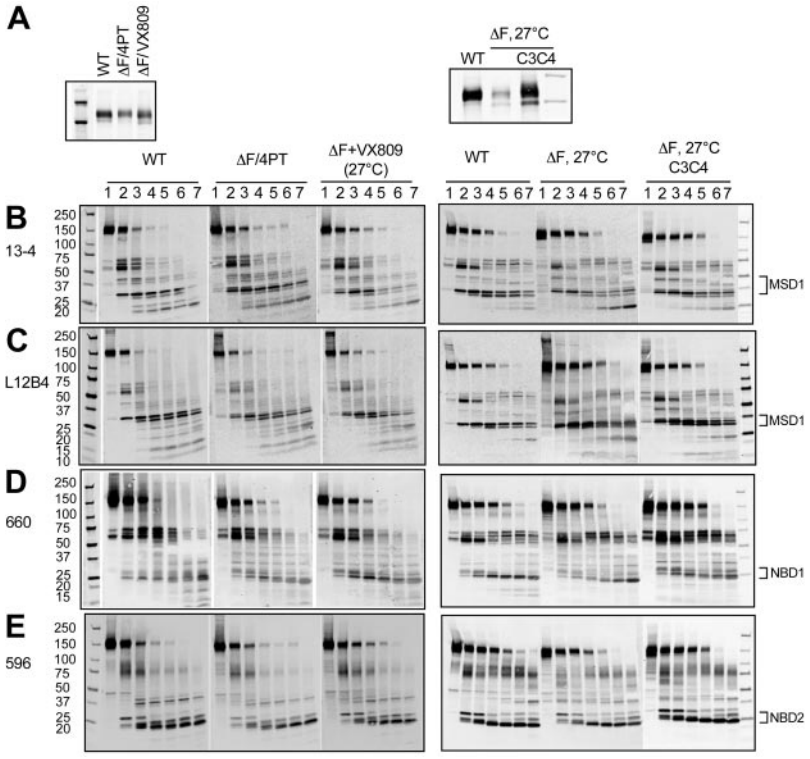

Figure 3. VX-809 restores the global structural assembly of ΔF508 CFTR. *A*) BHK cells expressing WT and ΔF508/4PT CFTR were grown at 37°C, and those expressing ΔF508 CFTR were treated with or without VX-809 (3 μM), C3 (10 μM), and C4 (10 μM) for 24 h at 27°C. Cells were treated with 100 μM CHX for 4 h to deplete immature forms of CFTR before harvesting for membrane isolation. Membrane vesicles (5 μg proteins) were resolved by 7.5% SDS-PAGE, and CFTR was detected by mAb 596. *B–E*) Membrane vesicles were incubated with various doses of trypsin for 10 min on ice. Digested CFTR fragments were resolved with 4–20% gradient SDS-PAGE and detected by Western blots probed with different antibodies: mAb 13-4 (*B*), mAb L12B4 (*C*), mAb 660 (*D*), and mAb 596 (*E*). Final trypsin concentrations: 0, 5, 15, 50, 125, 250, and 500 μg/ml for lanes 1–7, respectively. 4PT, S422P/S434P/S492P/A534P/I539T. $n = 3$ for WT, ΔF508/4PT CFTR, and VX-809-treated ΔF508 CFTR; $n = 2$ for low-temperature- and C3/C4-treated ΔF508 CFTR.

digestion were visualized in Western blots (Fig. 3*B–E*, left panels). Not unexpectedly, the fragment patterns of the mature ΔF508/4PT protein were virtually identical to those of the WT with all four antibodies. This is entirely consistent with the stability of the mature ΔF508/4PT construct reflected in its robust channel gating and WT-like lifetime at 37°C (24). Less expected was the observation that the protein that matured due to exposure to VX-809 at 27°C also exhibited very similar sensitivity to the protease as revealed by the fragments detected with each of the four antibodies. This finding indicates that the tertiary assembly of the mature CFTR that is achieved under different conditions, including those that have not restored thermal stability, is quite similar, at least at this relatively low level of resolution. This similarity also could be observed with correctors other than VX-809, such as the combination of VRT-325 (C3) and Corr-4a (C4) and even with ΔF508 CFTR that had matured in cells cultured at 27°C without any added corrector (Fig. 3, right panels). Even though the percent of maturation is quite low in the latter case, the overall conformational state does not appear to be different from that of the larger amounts formed due to chemical correctors or thermostabilizing second-site mutations. On this basis, the mature band C form of CFTR seems to be grossly similar, regardless of the conditions that have enabled the polypeptide to reach this state.

As a second measure of the global assembly promoted by the VX-809 corrector, cysteine cross-linking between domains known to occur in WT CFTR, but not in ΔF508 CFTR, was assessed (**Fig. 4**). Reversible cross-linking occurred between the crucial domain-swapped NBD1/CL4 and NBD2/CL2 interfaces when cysteine pairs were present in corrector-rescued Cys-less ΔF508 CFTR. Although only a relatively small proportion of the CFTR matured under the influence of the compound, virtually the entire amount of the mature form appeared to be cross-linked, as revealed by the mobility shift (Fig. 4 and ref. 11). Thus, the acquisition of both the resistance to proteolysis and native domain-domain

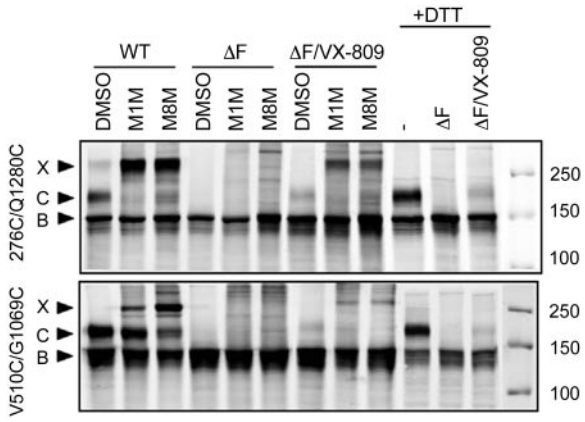

Figure 4. Restoration of NBD2/CL2 and NBD1/CL4 interfaces by VX-809. HEK293 cells were transiently transfected with Cys-less CFTR or Cys-less ΔF508 CFTR with Cys pairs 276C/Q1280C or V510C/G1069C, introduced at NBD2/CL2 and NBD1/CL4 interfaces, respectively (13). At 24 h after transfection, ΔF508 CFTR cells were treated with 3 μM VX-809 for 24 h at 27°C. Cells were harvested and resuspended in PBS and incubated with 200 μM M1M, M8M, or an equal amount of DMSO as vehicle control. Cell lysates in SDS-PAGE sample buffer with or without DTT, as indicated, were subjected to 7.5% SDS-PAGE and Western blot analysis with mAb 596. X, cross-linked CFTR; C, mature complex-glycosylated CFTR; B, immature core-glycosylated CFTR.

contacts under the influence of VX-809 at low temperature indicate that the mutant protein has achieved an overall conformational state similar to the WT.

Correctors act incrementally with thermostabilizing mutations

Because the correctors tested promoted maturation without providing stability of the mature product, it was of interest to examine their action on ΔF508 CFTR that already had been stabilized by established second-site changes in either NBD1 or at the NBD1/CL4 interface (14, 18, 24, 28, 31). **Figure 5*A*** shows the influence of VX-809 in combination with different NBD1-stabilizing substitutions in cells incubated at either 37 or 27°C. In all cases, the compound caused a further increase in maturation beyond the effect of the stabilizing mutations alone. VX-809 treatment of the combined NBD1 signature suppressor mutations together with the I539T substitution (ΔF/4S) caused substantial further enhancement of maturation at both temperatures. With the strongly stabilizing regulatory insertion deletion (ΔRI), the compound caused large increments that were of equivalent magnitude at both temperatures, and a similar effect was observed with the proline insertions in the context of I539T variant (4PT). Thus, when thermal stability has already been provided by these sequence changes, treatment with VX-809 results in a similar level of maturation at the higher as at the lower temperature.

Second site mutations at the NBD1/CL4 interface also improve ΔF508 CFTR maturation (18, 31). The R1070W substitution in CL4 may do so by contributing to interactions among a cluster of aromatic residues at the interface that is weakened by the absence of F508 from the NBD1 surface (11), whereas the V510D mutation was proposed to provide a salt bridge with R1070 (31). The V510D mutant on the NBD1 side of the interface is very sensitive to further enhancement of maturation by the compound, whereas that on the CL4 side (R1070W) responds only rather weakly (Fig. 5*B*). This difference may reflect the fact that the V510D substitution stabilizes isolated NBD1 (32) in the absence of the rest of CFTR, as well as influencing the interface, whereas R1070W has only the latter effect. One might speculate that the less substantial influence of VX-809 on R1070W/ΔF508 CFTR could possibly reflect similar effects of either the tryptophan residue or the aromatic small molecule to partially fill the void left by the absence of the F508 residue. Interestingly, as has already been observed by others (9), the influence of the combined V510D and R1070W substitutions is similar to that of V510D alone, which would not be expected if V510D were forming a salt bridge with R1070 but might be if V510D acted primarily to stabilize the NBD1 domain, as has been observed (32). In any case, the variant with both substitutions remains sensitive to further augmentation of maturation by the compound.

Since NBD1 and NBD1/CL4 mutagenic corrective changes are known to be strongly reinforcing of each other (9, 10), it was important to also evaluate how the compound influenced the ΔF508 protein with both

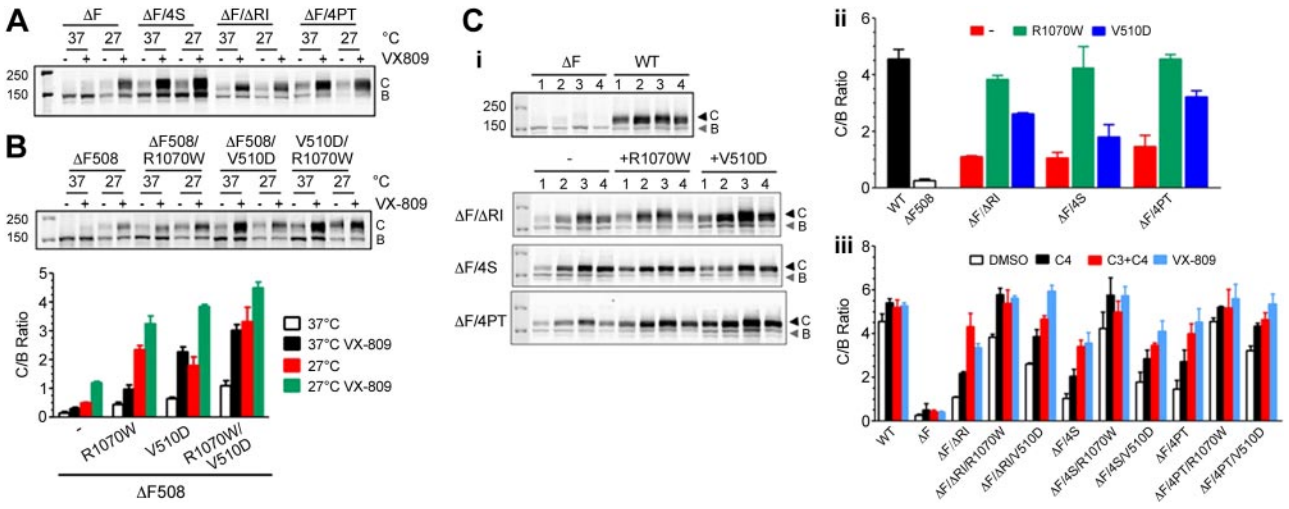

Figure 5. VX-809 promotion of ΔF508 CFTR maturation is incremental with the effects of NBD1-stabilizing second-site mutations and NBD1/CL4-patching mutations. BHK cells expressing ΔF508 CFTR with various mutations that promote its maturation were treated with VX-809 (3 μM), C4 (10 μM), or C3+ C4 (10 μM each) for 24 h at 27 or 37°C. Cell lysates in RIPA buffer with equal amounts of protein were subjected to 7.5% SDS-PAGE and Western blot analysis using CFTR antibody mAb 596. Intensities of mature band C and immature band B were quantified using a Li-Cor Odyssey Imager, and C/B ratios were calculated. *A*) ΔF508 with NBD1-stabilizing mutations: 4S, I539T/G550E/R553M/R555K; ΔRI, deletion of amino acid residues 404–435; 4PT, S422P/S434P/S492P/A534P/I539T. *B*) ΔF508 with NBD1/CL4 interface substitutions R1070W and/or V510D. *C*) ΔF508 with the combination of NBD1 stabilization and NBD1/CL4 patching mutations. *i*) Representative blots showing effect of small-molecule correctors on WT CFTR and ΔF508 CFTR with different combinations of NBD1 thermal stabilizers and NBD1/CL4 substitutions. Lane 1, DMSO control; lane 2, C4; lane 3, C3 + C4; lane 4, VX-809. *ii*, *iii*) Intensity of mature C and immature B bands was quantified with a Li-Cor Odyssey imaging system, and C/B ratios were calculated for DMSO control of each construct (*ii*) and corrector-treated samples (*iii*).

modifications. Figure 5*C* shows the results of experiments of this type using the correctors C3 and C4, as well as VX-809. Although the combinations of the two types of mutagenic modifications caused larger increases in maturation than each individually, those increased levels were elevated still further by the correctors. Interestingly, the patterns of enhancement by the compounds were remarkably similar for each of the three classes of NBD1 stabilizing mutations (ΔF/ΔRI, ΔF4S, and ΔF/4PT) with or without one of the interface substitutions (R1070W or V510D). Notably, this pattern was quite similar for the action of the correctors on WT CFTR, indicating that they are not entirely specific for ΔF508 CFTR. Overall, the data in Fig. 5 emphasize that these correctors have effects over and above those caused by known second-site mutations that improve maturation of ΔF508 CFTR.

VX-809 action on other CF-associated mutants

Knowing that VX-809 influences the WT as well as ΔF508 CFTR, we tested its effect on other CF-associated mutants, beginning with several on the CL4 side of the interface in which F508 normally participates (33). We found that the maturation of five of these mutants, which occurs very little or not at all at either 37 or 27°C, is strongly promoted by VX-809 (**Fig. 6*A***). Unlike ΔF508, the enhancement of the maturation of these mutants is not less at the higher temperature than at the lower, consistent with the other indications that the compound appears not to act by increasing thermal stability.

In view of this lack of NBD1 stabilization and correction of several CL4 mutants, we wondered whether VX-809 might influence other processing mutants in NBD1 or in cytoplasmic loops other than CL4. As seen in Fig. 6*B*, mutants in each of the other three cytoplasmic loops were, like those in CL4, highly responsive to the compound. Although these disease-associated variants studied previously (34, 35) are all capable of a minimal level of maturation at physiological temperature, this level is increased substantially at 27°C and to an even greater extent by VX-809 at either temperature. In contrast, VX-809 clearly did not promote the maturation of other CF-causing variants in NBD1 (Fig. 6*C*). The ΔI507 mutant that also causes severe disease (36) was among those, as was the R560T missense mutation, prominent in some ethnic populations (37). In addition to their lack of response to the corrector, the maturation of these two mutants was not improved at reduced temperature (27°C).

DISCUSSION

The effect of the absence of the single F508 residue from NBD1 of the CFTR protein on its biogenesis and function is complex and still not fully understood, despite very extensive research over >2 decades. Fortunately, searches for small molecules able to promote the traffic to and function at the surface of cells expressing the mutant protein were instigated during the past decade, and significant progress has been made (8, 38, 39). Several classes of small-molecule correctors with varying degrees of efficacy have been found in cell culture models, with at least one progressing to clinical trials (6). This compound, VX-809, developed by Vertex Pharmaceuticals, has been reported to cause a small, but detectible, improvement in lung function of patients with the ΔF508 mutation (7). Ongoing trials are testing the influence of combined treatment of VX-809 and the U.S. Food and Drug Administration–approved drug Kalydeco, which does not improve ΔF508-CFTR folding or trafficking but may augment the channel activity of the VX-809-rescued population of molecules. However, there are intensive continuing efforts to discover compounds with greater corrector efficacy than VX-809 and to understand why it is not more effective.

In the present study, we have addressed the latter question by examining the ability of the compound to influence formation of the fully assembled multidomain structure at different temperatures, as well as its lifetime and channel function. In addition, the effect of the compound on genetically stabilized ΔF508 variants with different second-site mutations in NBD1 and the NBD1/CL4 interface was determined, as was the effect of the compound on several disease-associated processing mutants other than ΔF508. Overall, the results of these experiments provide considerable insight into the mode of action of the VX-809 corrector, including some clues as to why its effectiveness is limited. First, the compound most strongly promotes maturation

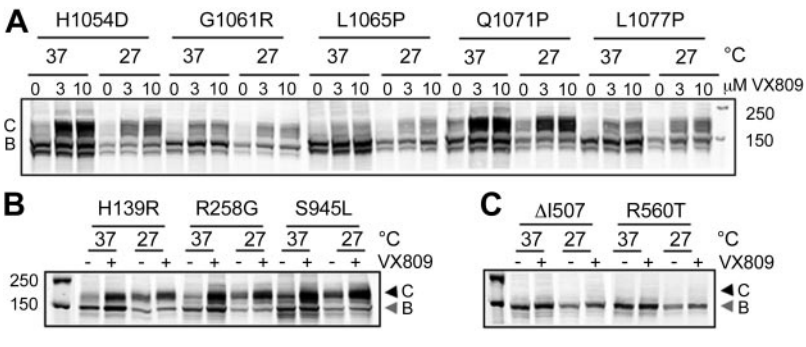

Figure 6. VX-809 promotes maturation of disease-associated misprocessing mutants in cytoplasmic loops. *A*) BHK cells stably expressing various CL4-processing mutant CFTRs were treated with VX-809 (3 and 10 μM) for 24 h at 27 or 37°C. *B, C*) HEK293 cells were transiently transfected with CFTR with misprocessing mutations located in CL1 (H139R), CL2 (R258G), and CL3 (S945L) (*B*), or in NBD1 (ΔI507 and R560T; *C*). At 24 h after transfection, cells were treated with 3 μM VX-809 and grown at 27 or 37°C for another 24 h. Cell lysates in RIPA buffer with equal amount of proteins were subject to 7.5% SDS-PAGE and Western blot analysis using CFTR antibody mAb 596.

when the thermodynamic instability of ΔF508 has been compensated for in cells maintained at reduced temperature (27°C). This observation in itself indicates that the compound is not thermostabilizing, and this conclusion is supported by the facts the VX-809-rescued mature form does not have an extended lifetime and loses channel activity when temperature is increased from 25 to 35°C (Fig. 2). Second, a notable finding in this study was that the mature full-length mutant protein could form a compact fully assembled structure regardless of whether thermal stability had been restored to NBD, which was evident from the achievement of similar sensitivity to limited proteolysis as the WT and the formation of interdomain cross-linking. Previous work from several laboratories had shown that NBD1 stabilization by different second-site modifications promoted assembly and trafficking of a portion of the full-length protein population (18, 40). These results might have been considered consistent with the idea that stabilization of the domain is necessary for the overall assembly of the full-length mutant protein.

However, the finding that assembly occurred in the presence of VX-809, even though the assembled product was not thermally stable, indicates that assembly and thermal stability are not tightly coupled. This realization is significant, since measurement of maturation and assembly, such as the appearance of the complex glycosylated band C form, are commonly used as assays of rescue. Assembly, which VX-809 and other correctors support, is necessary but would need to be combined with stabilization to provide more completely effective rescue. Such a requirement is illustrated by the additive effects of the corrector with second-site stabilizing modifications of NBD1 and the NBD1/CL4 interface (Fig. 5). Although selected for its ability to rescue ΔF508 CFTR (7), we found that VX-809 also was effective in promoting maturation of several disease-associated missense mutants in cytoplasmic loops of CFTR. Single examples were found in the first, second, and third loops. Most notable, however was the very effective correction of five different mutants in CL4 that normally form the crucial interface with the F508 region on the surface of NBD1. Interestingly, these mutations do not appear to be temperature sensitive, and VX-809 increased their maturation as effectively at 37 as at 27°C. Thus, this action of the compound also is consistent with it not influencing thermal stability. Two other severe disease-causing mutations in NBD1 (ΔI507 and R560T) that also were not temperature sensitive were unresponsive to VX-809.

The site of action of VX-809 on CFTR is unknown, and indeed, as yet, there is only circumstantial evidence that it interacts directly with the protein, perhaps during its assembly at the endoplasmic reticulum (7). However, its influence on variants with mutations on either side of the NBD1/CL4 interface suggested the possibility that this could be the location of one binding site for the compound. To test this speculation computationally, we performed flexible-receptor, flexible-ligand docking of VX-809 at the NBD1/CL4 interface as viewed in our 3D model of CFTR (see Materials and Methods and **Fig. 7*A***, box). While fully aware of the potential limitations of any homology model, some justification for its use for this specific purpose comes from the experimental cross-linking of several residues on either side of this interface (11) and the fact that the interface is identical in other independently derived molecular models of CFTR (41–43). We specifically targeted the hydrophobic pocket formed at the interface due to the absence of the F508 side chain in ΔF508-NBD1 (Fig. 7*B*). This hydrophobic pocket is formed by residues T1053, H1054, T1057, Y1073, L1077, and K1080 from CL4, and M498, P499, G500,

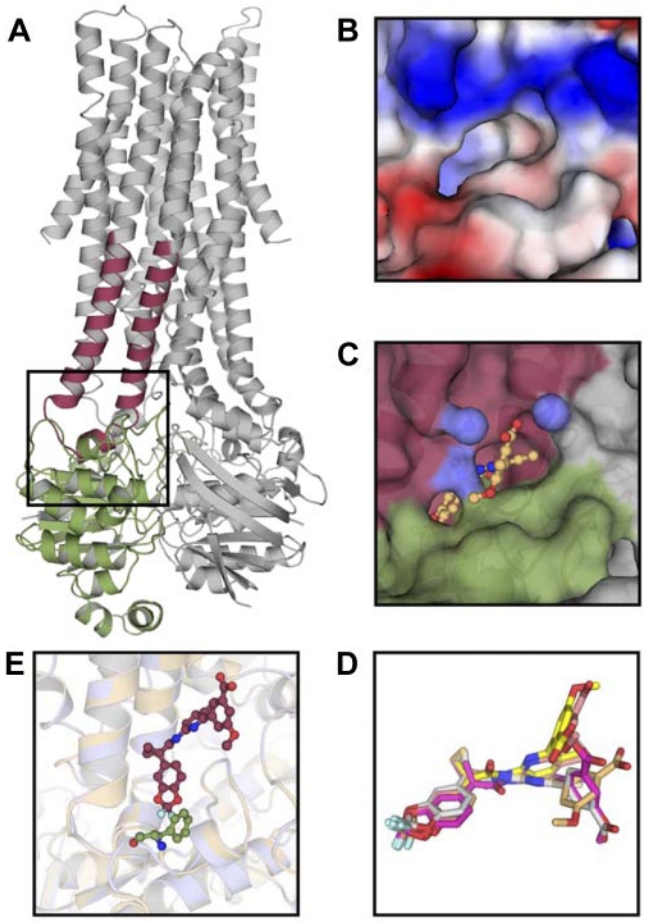

Figure 7. Putative binding pose for VX-809 in the NBD1/CL4 interface. *A*) Structural model of CFTR adapted from Serohijos *et al.* (11). WT NBD1 is replaced with ΔF508 NBD1 to generate the model of ΔF508 CFTR. The R domain is not represented in the model. NBD1 is shown in green, CL4 in red. Boxed region is represented in panels *B* and *C*. *B*) Hydrophobic pocket formed in the interface of NBD1 and CL4 due to the absence of the side chain of F508. *C*) Putative binding orientation of VX-809 in the pocket formed in the NBD1/CL4 interface of ΔF508 CFTR. Water molecules are shown in blue, VX-809 in light orange. *D*) Structural overlay of the binding poses from the top 5 structural models obtained using MedusaDock. *E*) Structural overlay of WT and ΔF508 CFTR, showing the relative positioning of the F508 side chain as compared to the bound orientation of VX-809. WT CFTR is shown in light blue, ΔF508 CFTR in light orange, F508 side chain in green, VX-809 in red.

E504, and E543 from NBD1. We performed extensive sampling of the possible binding poses for VX-809 (see Materials and Methods) and found that the fused aromatic ring structures in VX-809 energetically favor the predefined pocket formed between NBD1 and CL4 (Fig. 7*C*, *D*). The orientation of the compound in the binding pocket is such that it clashes with the F508 side chain if it were to be present (Fig. 7*E*). These results indicate that while other sites may be available, the NBD1/CL4 interface is one possible binding pocket for VX-809. Interestingly, this interface also has been targeted computationally in an effort to identify additional small-molecule correctors (44).

In summary, we find that VX-809 promotes the overall assembly but not the thermostability of ΔF508 CFTR. The additional rescuing effect of VX-809 on ΔF508 CFTR with all the NBD1-stabilizing mutants tested provide a rationale for the development of a strategy to identify reagents that thermally stabilize ΔF508 NBD1. The combination of ΔF508 NBD1-stabilizing reagents with VX-809 or similar assembly-promoting compounds may provide an effective structural and functional rescue of ΔF508 CFTR at physiological temperatures. FJ

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