Thermally Unstable Gating of the Most Common Cystic Fibrosis Mutant Channel (ΔF508)  
“RESCUE” BY SUPPRESSOR MUTATIONS IN NUCLEOTIDE BINDING DOMAIN 1 AND BY CONSTITUTIVE MUTATIONS IN THE CYTOSOLIC LOOPS*

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Most cystic fibrosis (CF) cases are caused by the ΔF508 mutation in the CF transmembrane conductance regulator (CFTR), which disrupts both the processing and gating of this chloride channel. The cell surface expression of ΔF508-CFTR can be “rescued” by culturing cells at 26–28 °C and treating cells with small molecule correctors or intragenic suppressor mutations. Here, we determined whether these various rescue protocols induce a ΔF508-CFTR conformation that is thermally stable in excised membrane patches. We also tested the impact of constitutive cytosolic loop mutations that increase ATP-independent channel activity (K978C and K190C/K978C) on ΔF508-CFTR function. Low temperature-rescued ΔF508-CFTR channels irreversibly inactivated with a time constant of 5–6 min when excised patches were warmed from 22 °C to 36.5 °C. A panel of CFTR correctors and potentiators that increased ΔF508-CFTR maturation or channel activity failed to prevent this inactivation. Conversely, three suppressor mutations in the first nucleotide binding domain rescued ΔF508-CFTR maturation and stabilized channel activity at 36.5 °C. The constitutive loop mutations increased ATP-independent activity of low temperature-rescued ΔF508-CFTR but did not enhance protein maturation. Importantly, the ATP-independent activities of these ΔF508-CFTR constructs were stable at 36.5 °C, whereas their ATP-dependent activities were not. Single channel recordings of this thermally stable ATP-independent activity revealed dynamic gating and unitary currents of normal amplitudes. We conclude that: (i) ΔF508-CFTR gating is highly unstable at physiologic temperature; (ii) most rescue protocols do not prevent this thermal instability; and (iii) ATP-independent gating and the pore are spared from ΔF508-induced thermal instability, a finding that may inform alternative treatment strategies.

Cystic fibrosis (CF),2 a common genetic disorder among Caucasians, is caused by mutations in the CF transmembrane conductance regulator (CFTR). CFTR is a member of the ATP-binding cassette (ABC) transporter superfamily defined by having two transmembrane domains and two nucleotide binding domains (NBD1, NBD2) (1). Unlike other ABC transporters, CFTR functions as an ion channel that controls salt transport and water flow by conducting chloride ions across epithelial cell membranes (1–3). CFTR channel activation depends on both phosphorylation of its unique R domain (mainly through protein kinase A-mediated phosphorylation) and ATP binding at the two NBDs (4–6). It has been generally accepted that ATP-induced NBD dimerization is a key step in CFTR channel opening, although currently there is no high resolution structure of the entire CFTR protein. Recent structural studies of other ABC transporters (e.g. MsbA) have provided detailed information regarding how NBD dimerization promotes conformational changes of the translocation pathways of these transporters (e.g. from an inward facing (nucleotide-free) to an outward facing conformation (nucleotide-bound state)) (7–9). How R domain phosphorylation further regulates CFTR channel activity remains unclear.

CF is caused by loss-of-function mutations that reduce the amount and/or channel activity of CFTR (10–13). Deletion of phenylalanine at position 508 (ΔF508) in NBD1 is the most frequent and severe mutation that contributes to approximately 70% of observed CF (14, 15). The ΔF508 mutation results in the production of immature, misfolded CFTR protein that fails to pass endoplasmic reticulum quality control and escapes from the endoplasmic reticulum at very low levels. The majority of newly synthesized protein instead is rapidly degraded by the proteasome (16, 17), resulting in little ΔF508-CFTR protein on the cell surface (18, 19).

References:

1. The abbreviations used are: CF, cystic fibrosis; ABC, ATP-binding cassette; CFTR, CF transmembrane conductance regulator; NBD, nucleotide binding domain; TES, N-tris(hydroxymethyl)methyl-2- aminoethanesulfonic acid; TMD, transmembrane domain.

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3. Background: Thermal stability of the common cystic fibrosis mutant channel (ΔF508-CFTR) is unclear.

4. Results: ΔF508-CFTR channels inactivate at 36.5 °C in excised patches. Constitutive cytosolic loop mutations induce an ATP-independent activity in ΔF508-CFTR that is thermally stable.

5. Conclusion: ΔF508 mutation strongly disrupts ATP-dependent gating at physiologic temperature but spares ATP-free gating.

6. Significance: Our results may inform new treatment strategies.

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ΔF508 is a temperature sensitive mutation that can be partially rescued in vitro by culturing cells at low temperature (e.g. 27 °C (20)). However, low temperature-rescued ΔF508-CFTR has large defects in several perspectives, for example, a shortened residency time at the cell surface when the incubation temperature is raised to 37 °C (21–23) and strongly impaired channel activity (24–26) even at 22–23 °C. The latter indicates that low temperature-rescued ΔF508-CFTR fails to adopt a tertiary structure that supports normal channel gating.

High throughput screening studies identified small compounds (correctors) that can partially rescue the ΔF508 mutant by improving its processing and cell surface expression and increasing Cl− transport in ΔF508 epithelial cells (27–29). Many other small molecules and compounds (potentiators) have been reported to activate or potentiate the channel activity of ΔF508-CFTR after it has reached the cell surface by low temperature rescue or corrector treatment (25, 30–32). The working mechanisms of CFTR correctors and potentiators remain largely unknown. One important question is whether ΔF508-CFTR channel activity that has been increased by corrector or potentiator treatment is stable at physiologic temperature.

Recent mechanistic studies of CFTR folding have revealed that the ΔF508 mutation disrupts domain interactions among NBD1, cytosolic loops 1 and 4, and NBD2 (33–35), the last of which compromises NBD2 folding (36). The packing of CFTR transmembrane helices also has also been argued to be affected by this mutation (37); consequently, ΔF508-CFTR has a globally disrupted tertiary structure that is inadequate for endoplasmic reticulum export and for optimal ATP-dependent channel gating. Several second site suppressor mutations in NBD1 that not only rescue the processing and surface localization of ΔF508 but also restore its channel activity have been identified (38–44). In the absence of the phenylalanine side chain at position 508, these uniquely located suppressor mutations (e.g. nearby signature motif or distal to Phe-508) may allow them to compensate for or mimic a certain conformation that is essential for CFTR channel maturation and function (e.g. by stabilizing interdomain contacts) (34, 38). However, not all of these mutations can fully correct ΔF508-CFTR at a functional level. For example, Hegedus et al. have shown that eliminating two arginine-based motifs (RXR) from ΔF508-CFTR (e.g. R29K and R555K) promotes maturation of ΔF508, but channel activity in lipid bilayers is highly thermally unstable (i.e. inactivates at physiologic temperature) (42). This disparity indicates different folding requirements for CFTR maturation and for channel function. In contrast, Aleksandrov et al. have recently shown that deletion of the regulatory insertion from NBD1, a unique feature of CFTR (45) among ABC transporters, restored both maturation and channel activity of ΔF508-CFTR and stabilized channel activity in lipid bilayers at physiologic temperature (46). The role of regulatory insertion in CFTR function and how its deletion improves processing and channel function are unknown.

Although ΔF508-CFTR channel activity at the cell surface is detectable after incubation at low temperature or with CFTR correctors, the thermal stability of rescued ΔF508-CFTR and the mechanism of how ΔF508 disrupts channel gating remain unclear. The low cell surface expression and greatly reduced channel activity of ΔF508-CFTR make such investigation difficult. In a recent study we showed that two cytosolic loop mutations (e.g. K190C and K978C) promote ATP-independent CFTR channel activity, allosterically increase ATP and PKA sensitivity, and also significantly restore the function of mutant CFTR channels that cannot be activated by ATP binding or NBD dimerization (e.g. G551D and Δ1198, which lacks NBD2) (47). One intriguing issue is whether these constitutive mutations have any impact on the maturation or channel activity of ΔF508-CFTR. In the present study we address two questions: (i) are ΔF508-CFTR channels whose expression has been rescued by low temperature, suppressor mutations, or correctors or whose channel activity has been enhanced by CFTR potentiators thermally stable in excised membrane patches and (ii) do constitutive mutations in the cytosolic loops rescue maturation and/or improve the function and thermal stability of ΔF508-CFTR channels in excised patches. Our results show that ΔF508-CFTR channels that have been delivered to the cell surface in response to low temperature incubation or corrector treatment rapidly inactivate at physiologic temperature in excised patches. Conversely, second site suppressor mutations in NBD1 stabilize ΔF508-CFTR channel activity at physiologic temperature. The cytosolic loop mutations (K978C and K190C/K978C) do not improve ΔF508-CFTR processing but do increase the ATP-independent channel activity of ΔF508 channels that are delivered to the cell surface by incubation at low temperature. Importantly, this ATP-independent activity of ΔF508-CFTR channels is much more stable at physiologic temperature as compared with the rapid inactivation of ATP-dependent gating. Our results indicate that thermal inactivation of ΔF508 channel function is limited to ATP-dependent (presumably NBD dimerization-dependent) channel gating. The pore is spared as is the ATP-independent activity that can be induced by mutations in the cytosolic loops. The latter may be worth considering as targets for developing new strategies to enhance ΔF508 mutant function.

MATERIALS AND METHODS

Cell Culture, Transfections, and Western Blotting—HEK293T cells were transiently transfected with wild-type (WT) or mutant CFTR cDNA using the Lipofectamine 2000 transfection kit (Invitrogen) as described (25, 32). In brief, the tested mutations were generated by PCR mutagenesis, verified by DNA sequencing, and subcloned into pcDNA expression vectors (Invitrogen). K978C and K190C/K978C mutations, which promote ATP-independent channel activity (47), were introduced into the ΔF508-CFTR construct. The ΔF508-CFTR construct with NBD1 suppressor mutations (G550E, R553M, R555K (3M/ΔF508)) was provided by Dr. Phillip Thomas (University of Texas Southwestern Medical Center, Dallas). Protein expression of all mutants was verified by immunoblotting (see below). Cells were cultured in DMEM (Mediatech) supplemented with 10% FBS and 1 mM penicillin/streptomycin. To improve cell surface expression of the ΔF508 constructs, cells were grown for 1–2 days at 27 °C (low temperature-corrected). Otherwise, these mutants exhibit low surface expression when cells are cultured at 37 °C.
Cells stably expressing ΔF508-CFTR were used for some micropatch experiments to record unitary currents with small tip pipettes. In brief, ΔF508-CFTR was expressed in HEK293F cells (Invitrogen) under the control of a tetracycline-responsive element as described previously (48). Genes encoding puromycin resistance and GFP were coexpressed as markers for selection. The ΔF508-CFTR-expressing cell pool was enriched by selecting for GFP expression via fluorescence-activated cell sorting in the presence of puromycin and doxycycline (to induce expression). Single cell clonal cultures were isolated and expanded. These stably expressing cells also were incubated at 27°C for 24–48 h prior to patch clamp analysis to optimize ΔF508-CFTR expression.

CFTR correctors (corr-4a and VRT-325 provided by CF Foundation) were used to improve cell surface expression of ΔF508-CFTR further. In brief, 24 h after DNA transfection, cells expressing ΔF508-CFTR were treated with corr-4a or a combination of corr-4a and VRT-325 (10 μM each) for 24–48 h at 27°C.

For immunoblotting, cells expressing the respective CFTR constructs were rinsed in PBS, then scraped and kept on ice for 1 h in a 1% Triton X-100 lysis buffer in PBS, supplemented with an EDTA-free protease inhibitor mixture (Complete; Roche Applied Science) and 1 mM PMSF. Lysates were cleared by centrifugation at 14,000 rpm for 15 min at 4°C, and the total protein content of supernatants determined by the MicroBCA protein assay (Pierce). The polypeptides were resolved by SDS-PAGE using Tris-glycine gels, transferred to PVDF membranes (Immobilon-P; Millipore), and probed with an anti-C terminus CFTR monoclonal antibody (R&D Systems) and a 1 mM PMSF. Lysates were cleared by centrifugation at 14,000 rpm for 15 min at 4°C, and the total protein content of supernatants determined by the MicroBCA protein assay (Pierce). The polypeptides were resolved by SDS-PAGE using Tris-glycine gels, transferred to PVDF membranes (Immobilon-P; Millipore), and probed with an anti-C terminus CFTR monoclonal antibody (R&D Systems, 24-1) at a dilution (Immobilon-P; Millipore), and probed with an anti-C terminus CFTR monoclonal antibody (R&D Systems, 24-1) at a dilution (Immobilon-P; Millipore), and probed with an anti-C terminus CFTR monoclonal antibody (R&D Systems, 24-1) at a dilution (Immobilon-P; Millipore), and probed with an anti-C terminus CFTR monoclonal antibody (R&D Systems, 24-1) at a dilution (Immobilon-P; Millipore), and probed with an anti-C terminus CFTR monoclonal antibody (R&D Systems, 24-1) at a dilution (Immobilon-P; Millipore), and probed with an anti-C terminus CFTR monoclonal antibody (R&D Systems, 24-1) at a dilution of 1:500. Blots were washed extensively in Tris (25 mM)-buffered saline plus 0.2% Tween 20, followed by a 1-h room temperature incubation with a goat anti-mouse IgG horseradish peroxidase-conjugated antibody (Immunopure® antibody; Pierce) at 1:10,000 dilution. Chemiluminescent detection was performed by incubation of blots for 5 min using the Supersignal West pico chemiluminescent substrate (Pierce) and exposure on x-ray film.

Patch Clamp Analysis—Macroscopic and unitary currents were recorded in the excised, inside-out patch configuration. Patch pipettes were pulled from Corning 8161 glass to tip resistances of 1–2 megohms (macroscopic recordings) or 9–12 megohms (unitary current recording). CFTR channels were activated following patch excision by exposure of the cytoplasmic face of the patch to catalytic subunit of PKA (110 units/ml; Sigma) and MgATP (1.5 mM). CFTR currents were recorded in symmetrical solution containing 140 mM N-methyl-D-glucamine-Cl, 3 mM MgCl2, 1 mM EGTA, and 10 mM TES (pH 7.3). Macroscopic currents were evoked using a ramp protocol from −80 to +80 mV with a 10-s time period. The patches were held at ±60 mV for unitary current recordings. An automatic temperature controller with chamber platform (TC-324B controller, PH-3 platform; Warner Instruments) was used for changing bath temperature. The bath temperature was calibrated by directly measuring its temperature using a probe in the chamber (Thermalert TH-5; Physitemp Inc); all indicated temperatures refer to the actual bath temperature. All control experiments were conducted at room temperature (22–23°C) prior to raising bath temperature to 36.5°C. Signals from macroscopic and unitary current recordings were filtered at 20 and 200 Hz, respectively. Data acquisition and analysis were performed using pCLAMP9.2 software (Axon Instruments). NP, and frequency of openings (openings/sec-patch) were estimated using pClampWin9.2 software. The averaged data are presented as the means ± S.E. Statistical comparisons were made by performing unpaired t tests unless otherwise indicated.

Genistein, curcumin (Sigma), NBBP-AM (25), and VRT-523 were used to potentiate channel activity of ΔF508 where indicated. Stacks of all compounds were made in dimethyl sulfoxide.

RESULTS

Low Temperature-rescued ΔF508-CFTR Irreversibly Inactivated in Excised Patches at Physiologic Temperature—Fig. 1 shows the effect of raising the bath temperature on the channel activities of WT- and ΔF508-CFTR in excised, inside-out membrane patches. The macroscopic currents mediated by both WT- and ΔF508-CFTR were stable at room temperature (22–23°C), although the absolute ΔF508-CFTR current was much lower than WT-CFTR due to lower cell surface expression and partially disrupted channel gating (18, 19, 24–26). Raising the temperature initially increased the macroscopic currents for WT- and ΔF508-CFTR, which is consistent with previous evidence for an endothermic CFTR gating process (49). However, whereas WT-CFTR currents were stably increased upon raising the temperature to physiologic temperature (36.5°C), ΔF508-CFTR currents irreversibly decreased within minutes of the temperature increase. The decline in the ΔF508-CFTR current at 36.5°C could be fit to a single exponential with a time constant of 5–6 min (Fig. 1C). The magnitude of the decrease in ΔF508-CFTR current following the 10 min of warming the bath was greater than 90% of the control current at 22°C (Fig. 1D). Importantly, the reduced ΔF508-CFTR channel activity following incubation at 36.5°C could not be restored by cooling the bath to room temperature or by adding fresh ATP, PKA, or CFTR activators (e.g. curcumin) at 36.5°C (data not shown). This indicates that the thermal inactivation of ΔF508-CFTR channel activity is irreversible.

Fig. 2 shows the effects of raising the bath temperature on the single channel properties of ΔF508-CFTR. The increase in bath temperature dramatically increased the dynamics of channel gating (increases in both opening and closing rates) with a modest increase in P0 as reported previously for WT-CFTR (49). With increasing time at 36.5°C ΔF508-CFTR channel activity markedly diminished due in large part to a reduction in the frequency of channel openings (see mean data in Fig. 2B). As expected, the magnitude of the unitary currents increased moderately upon warming the bath to 36.5°C (∼50%), which remain unchanged as the activity waned. No unusual conductance state appeared during thermal inactivation of ΔF508-CFTR as might occur if misfolding compromised the permeation pathway.

ΔF508-CFTR Channels That Are Rescued to the Cell Surface by Pharmacologic Corrector Treatment Also Inactivated at 36.5°C—Several CFTR correctors have been shown to increase the maturation and cell surface expression of ΔF508-CFTR in...
A logical question is whether the channel activity of corrector-rescued ΔF508-CFTR is thermally stable. To address this question we incubated ΔF508-CFTR-transfected cells with or without correctors at low temperature for 24–48 h. The immunoblot results in Fig. 3A show that the amount of mature ΔF508-CFTR was proportionally increased by incubating the cells with corr-4a alone or with the combination of VRT-325 and corr-4a. This additive effect of two correctors on ΔF508-CFTR maturation is consistent with previous reports that they have different mechanisms for promoting ΔF508-CFTR maturation (50). We then examined the thermal stability of corrector-rescued ΔF508-CFTR in excised patches by raising the bath temperature from 22 °C to 36.5 °C. As expected, the macroscopic currents at 22 °C were much higher for patches excised from cells that were incubated with the correctors versus cells cultured at reduced temperature alone (Fig. 3, B–D). These data are consistent with the immunoblot results in Fig. 3A. However, like low temperature-rescued ΔF508-CFTR, ΔF508-CFTR channels that had been rescued by the pharmacologic correctors inactivated nearly completely at 36.5 °C when these correctors were added directly to the bath, although VRT-325 modestly stimulated the channel activity of low temperature-rescued ΔF508-CFTR at 22 °C (data not shown). Our data support previous reports that these correctors enhance the cell surface expression of ΔF508-CFTR by promoting its maturation (27–29), but they also indicate that ΔF508-CFTR channels that have been rescued by these compounds have not adopted a wild-type structure that is essential for stable channel activity at physiologic temperature.

**CFTR Potentiators Do Not Stabilize ΔF508-CFTR Channel Activity at 36.5 °C**—Many compounds such as genistein, NPPB-AM, VRT-523, and curcumin acutely enhance ΔF508-CFTR channel activity (29–32), although the precise working
mechanisms of these compounds are not clear. One of our interests was to determine whether any of these compounds can stabilize the channel activity of H9004F508-CFTR at 36.5 °C. Fig. 4 shows the strong stimulation of low temperature-rescued H9004F508-CFTR by four of these compounds when added to the bath at 22 °C. To our surprise, none of these compounds stabilized H9004F508-CFTR channel activity when the bath temperature subsequently was elevated to 36.5 °C. The mean data in Fig. 4 show that the four compounds increased macroscopic H9004F508-CFTR currents by 4–9-fold at room temperature, but there was no significant difference in the percentage decrease in current following warming the bath in the presence or absence of any of these compounds. Our results indicate that these CFTR potentiators do not correct the structural defect that underlines the thermal sensitivity of H9004F508-CFTR channels and predict that the potentiators tested here will not effectively enhance H9004F508-CFTR channel activity at physiologic temperature.

Suppressor Mutations in NBD1 Correct Misfolding and Stabilize ΔF508-CFTR Channel Activity at 36.5 °C—We have shown recently that three suppressor mutations (G550E, R553M, R555K (3M/H9004F508)) in NBD1 correct ΔF508-CFTR maturation and misfolding and markedly increase its channel activity in excised patches at room temperature (43). To determine whether these suppressor mutations correct the structural defect that underlies the thermal instability of ΔF508-CFTR channels, we tested the effect of raising the bath temperature to 36.5 °C on the currents mediated by 3M/H9004F508-CFTR channels. We first confirmed that the triple suppressor mutations greatly promoted maturation of H9004F508-CFTR by immunoblotting (more band C in Fig. 5A) and by macroscopic current recordings in excised patches (Fig. 5, B and C). Then we examined the thermal stability of 3M/ΔF508-CFTR by raising bath temperature. Fig. 5, B and C, shows that 3M/ΔF508-CFTR behaves very much like WT-CFTR in that its channel activity...
FIGURE 3. Corrector-rescued ΔF508-CFTR irreversibly inactivates at 36.5 °C. A, immunoblots showing the expression of WT and ΔF508-CFTR in the presence and absence of correctors (corr-4a and VRT-325) for cells cultured at 27 °C (for details, see “Materials and Methods”). B and C, temperature-induced inactivation of ΔF508-CFTR rescued by corr-4a (B) or a combination of corr-4a and VRT-325 (C). D, mean data showing increase of control (basal) channel activity by correctors at room temperature and fractional current loss after warming the bath to 36.5 °C. Numbers of patches are indicated in parentheses. There was no significant difference in fractional current loss for any of the three conditions (low temperature only data from Fig. 1D). *, p < 0.05 compared with control. Error bars, S.E.

FIGURE 4. CFTR potentiators increase ΔF508-CFTR channel activity but do not prevent temperature-induced inactivation. Low temperature-rescued ΔF508-CFTR channels were first stimulated by the indicated potentiators at room temperature, and then the effect of raising temperature on channel activity was examined. A–C, NPPB-AM (25), Genistein (30), and VRT-532 (28). D, mean -fold stimulation of current by each compound at room temperature and percentage of peak stimulated current left after heating in the absence or presence of the indicated compounds (numbers are indicated in parentheses, data in absence of compound from Fig. 1D).
increased in response to elevating the bath temperature and exhibited no time-dependent inactivation at 36.5 °C. Like for WT-CFTR, the 3M/ΔF508-CFTR-mediated currents returned to control levels when the bath temperature was reduced to room temperature. These data indicate that the tested suppressor mutations in NBD1 completely correct the structural defect that underlines the thermal instability of ΔF508-CFTR (41, 42).

Constitutive Loop Mutations Increase ATP-independent Channel Activity of ΔF508-CFTR, Which Is More Stable at Physiologic Temperature—Previously, we showed that several mutations in intracellular loop1 and loop3 (e.g. K190C and K978C) increase the ATP-independent channel activities of several CFTR constructs, including mutant forms that cannot be activated by ATP (e.g. G551D and Δ1198-CFTR, an NBD2 deletion mutant) (47). The enhancement of mutant CFTR function by these loop mutations motivated us to explore their effect on ΔF508-CFTR processing, function, and thermal stability. Fig. 6A shows that neither K978C nor K190C/K978C improved the maturation of ΔF508-CFTR when cells were grown either at 37 °C or 27 °C, as determined by immunoblotting. However, these mutations did increase the ATP-independent activity of low temperature-rescued ΔF508-CFTR channels in excised patches, as we observed previously for other CFTR constructs (47). On average, approximately 40 and 70% of the control current remained following adding an ATP scavenger (hexokinase plus glucose) for the single and double loop mutant, respectively (Fig. 7). As expected, the currents mediated by unmodified ΔF508-CFTR were virtually abolished by ATP removal.

We next examined the thermal stability of this ATP-independent channel activity by elevating the bath temperature. Fig. 7A shows that K978C/ΔF508-CFTR exhibited a partial decrease of current when the bath temperature was elevated to 36.5 °C, but on average 30–40% of this current remained after warming the bath (see Fig. 7D). Interestingly, for approximately 50% of the patches the current slightly recovered when the temperature returned to room temperature, indicating that some of the current decrease for K978C/ΔF508-CFTR was reversible. These results indicate that K978C partially protects ΔF508 channel activity from thermal inactivation. We then added hexokinase/glucose to remove ATP to determine whether the remaining current is ATP-independent. As shown in Fig. 7A, most of the current that remained following warming the bath was insensitive to removal of ATP, indicating that the ATP-independent activity of K978C/ΔF508-CFTR is far more stable at 36.5 °C compared with that of ΔF508-CFTR alone (Fig. 1B). To follow up this result we examined the thermal stability of the K190C/K978C/ΔF508 construct that has a greater relative ATP-independent channel activity. Fig. 7B shows that the K190C/K978C double mutation greatly protected the channel activity from thermal inactivation with nearly all of the remaining current following warming the bath being ATP-independent. As a control we also tested the thermal stability of the ATP-independent channel activity of G551D-CFTR channels with the K978C mutation. Fig. 7C shows that raising the temperature did not inactivate the K978C/G551D-CFTR current, indicating that the ATP-independent channel activity of K978C/G551D is thermally stable.

The results of Fig. 7 imply that the ATP-independent activity of the ΔF508-CFTR construct is far more stable at physiologic temperature than the ATP-dependent activity. To explore this further, we isolated the ATP-independent currents by adding hexokinase/glucose followed by bath perfusion to remove the ATP and then examined the effect of raising temperature on ATP-independent channel activity. Fig. 8, A and B, shows that...
the ATP-independent channel activity of K978C/ΔF508-CFTR and to a greater extent K190C/K978C/ΔF508-CFTR was resistant to temperature-induced inactivation. Nearly all of the ATP-independent current of the ΔF508-CFTR channel with the double loop mutations survived at 36.5 °C (see Fig. 8C). These findings indicate that the pore and its gate are largely spared during thermal misfolding of the ΔF508 mutant whereas the conformation of the NBDs or their coupling to the transmembrane domains is not. To examine this idea further we recorded the unitary currents mediated by single K978C/ΔF508-CFTR channels in micro-patches obtained with small tip pipettes. The ATP-independent current was isolated by adding hexokinase/glucose followed by bath perfusion as above. Fig. 8E shows that the amplitudes of the single channel currents for ΔF508 after incubation at 36 °C were similar to those for WT- and K978C-CFTR (47), indicating that the conformation of the pore for K978C/ΔF508 is not obviously compromised at physiologic temperature.

**DISCUSSION**

Our data indicate that ΔF508-CFTR channels in excised patches rapidly inactivate at physiologic temperature. These results support previous findings of thermal instability of ΔF508-CFTR with respect to folding and to channel function in lipid bilayers (22, 23, 42). Suppressor mutations in NBD1 corrected both maturation in cells and thermal instability of ΔF508-CFTR gating in excised patches, suggesting there may exist some mechanism that can both restore biosynthetic folding and stabilize channel gating at physiologic temperature. However, this could not be achieved by culturing cells at low temperature or by the CFTR correctors and potentiators that we tested here, despite the fact that these physical and chemical approaches promoted cell surface expression and increased macroscopic ΔF508-CFTR currents. We found that constitutive mutations in cytosolic loop1 and loop3 (i.e. K978C and K190C/K978C) increased ATP-independent channel activity of low temperature-rescued ΔF508-CFTR. This ATP-independent channel activity is thermally stable, whereas the ATP-dependent channel activity of ΔF508-CFTR is irreversibly inactivated at physiologic temperature. Our data provide new insights into the mechanism of thermal inactivation of ΔF508-CFTR and a potential therapeutic strategy for circumventing reduced ΔF508-CFTR channel activity in CF patients.

**ΔF508 Mutation Destabilizes Channel Activity at Physiologic Temperature**—Phe-508 in NBD1 is essential for the normal folding, trafficking, and channel activity of the CFTR protein.
Recent studies have demonstrated that proper domain interactions (e.g. between NBD1 and MSD1) are crucial for CFTR assembly (36) and indicate that Phe-508 plays a key role in the contacts that mediate domain interactions and proper folding (51, 52). The /H9004 F508 mutation causes misfolding and inefficient trafficking to the cell surface and disrupts channel gating at room temperature. Growing cells expressing /H9004 F508-CFTR at low temperature (e.g. 27 °C) can partially rescue /H9004 F508-CFTR maturation and trafficking to the cell surface (20–22), although the underlying mechanism is not clear. The channel gating of low temperature-rescued /H9004 F508-CFTR is still compromised at room temperature (24–26), which indicates that the restoration of /H9004 F508-CFTR expression by low temperature does not satisfy all of the requirements for normal channel gating. This raised the question of whether the gating of low temperature-rescued /H9004 F508-CFTR is stable at physiologic temperature.

Here, we directly monitored the thermal stabilities of the gating of different /H9004 F508-based CFTR constructs in excised membrane patches. We found that low temperature-rescued /H9004 F508-CFTR channels inactivate rapidly (time constant of about 5 min; Fig. 1) and irreversibly at physiologic temperature. The irreversibility of the effect was evidenced by lack of current recovery after (i) reducing the bath temperature to 22–23 °C, (ii) adding fresh PKA and ATP (data not shown), and (iii) adding a CFTR potentiator. Our data are consistent with previous studies that have shown that /H9004 F508-CFTR channel activity in lipid bilayers is thermally sensitive (42) and that cells expressing low temperature-rescued /H9004 F508-CFTR exhibit reduced anion currents after the cells are warmed to physiologic temperature (53). These results also are in good agreement with a recent in vitro study of isolated NBD1 that showed that the /H9004 F508 mutation lowered the transition temperature for unfolding and promoted the formation of a partially folded, aggregation-prone domain (54). This occurs even though the /H9004 F508 mutation has only a modest impact on the global structure of NBD1 (55). Our findings also support the idea that low temperature-rescued /H9004 F508-CFTR has distinct biochemical and structural properties from WT-CFTR (22).

**CFTR Correctors and Potentiators Did Not Induce a /H9004 F508-CFTR Conformation That Supports Channel Activity at Physiologic Temperature**—CFTR correctors identified by high throughput screening have been shown to partially correct maturation and misfolding of /H9004 F508-CFTR and promote epithelial Cl− secretion (27–29). Several biochemical studies have show that the action of corr-4a on /H9004 F508-CFTR is TMD2-dependent and is ineffective at rescuing /H9004 F508-dependent folding defects in amino-terminal constructs (e.g. 873X/H9004 F508 (56). Both corr-4a and VRT-325 can improve /H9004 F508-CFTR maturation and stabilize /H9004 F508-CFTR on the plasma membrane (e.g. increase half-life) (50, 53) possibly by direct binding to the TMDs (e.g. between TMD6 and 7) (57). In addition, VRT-325 has been shown to correct membrane domain packing in /H9004 F508-CFTR (58) and also misfolding in loop4, a region that interacts with NBD1 near Phe-508, implying that VRT-325 may act as a linker to improve domain-domain interactions and thereby proper assembly (59). The fact that these correctors have additive effects on /H9004 F508-CFTR maturation supports the idea that CFTR correctors may work by different mechanisms,
and a single compound may lack the ability to rescue misfolding at all stages of CFTR domain synthesis and assembly. In the absence of detailed structural information for the entire CFTR polypeptide, however, it is unclear whether these small molecules truly correct F508-CFTR misfolding.

We confirmed that two CFTR correctors additively increase cell surface expression of F508-CFTR when combined with culturing the cells at low temperature by showing greatly increased band C levels by immunoblotting and higher basal macroscopic currents in excised macropatches. However, like for low temperature-rescued F508-CFTR, F508-CFTR channels that were treated with this combination of correctors inactivated with a similar time course at physiologic temperature, suggesting an incomplete functional rescue. These results indicate that low temperature incubation plus CFTR correctors do markedly enhance the cell surface expression of F508-CFTR, but do not stabilize the structure required for proper channel gating at physiologic temperature. This implies that F508-CFTR that has been corrected by these treatments still has a different tertiary structure compared with that of WT-CFTR. The effectiveness of future CFTR correctors needs to be functionally evaluated by multiple approaches, including patch clamp analysis at physiologic temperature.

Similarly, several CFTR potentiators that we tested (genistein, NPPB-AM, VRT-532, and curcumin) failed to stabilize F508-CFTR channel activity at physiologic temperature, even though these compounds strongly enhanced F508-CFTR channel activity at 22–23 °C. Our data indicate that if these compounds bind to the CFTR protein to promote channel activity at 22–23 °C, they do not induce a F508-CFTR structure that is thermally stable. These results also indicate that the ability of some CFTR potentiators to rescue the function of F508-CFTR could be overestimated if those potentiators cannot stabilize F508-CFTR channel activity at physiologic temperature.

**FIGURE 8. ATP-independent channel activity of constitutive loop mutations is resistant to raising temperature.** A and B, effect of raising temperature on ATP-independent currents of K978C/ΔF508- and K190C/K978C/ΔF508-CFTR. ATP was first removed by hexokinase/glucose and bath perfusion (Wash), and then the bath temperature was elevated to 36.5 °C, as described for Fig. 1. C, mean fractional loss of ATP-independent current for K978C/ΔF508 and K190C/K978C/ΔF508-CFTR (n = 4 for each construct). D, effect of raising temperature on unitary currents of K978C/ΔF508. A small pipette was used to reduce the number of channels in the membrane patch. Effect of raising bath temperature on K978C/ΔF508 current was monitored at the macroscopic level (ramp protocol), and then the unitary currents were recorded in the gap-free mode after reduction to room temperature.
Suppressor Mutations in NBD1 Fully Rescue ΔF508-CFTR—We found that three suppressor mutations in NBD1 not only correct maturation of ΔF508 (3M/ΔF508) but also stabilize channel activity of ΔF508-CFTR at physiologic temperature, implying that ΔF508-CFTR has adopted the correct structure. This full restoration of ΔF508-CFTR function by these suppressor mutations may include preventing NBD1 misfolding (43), restoring domain-domain interactions between the NBDs (36) and the loop interface (e.g. loop2, loop4) (35), and increasing the stability of ΔF508-CFTR on the cell surface. These data are consistent with previous studies (38, 39, 42–44) that have shown that second site suppressor mutations can correct both maturation and channel function of ΔF508-CFTR.

Constitutive Loop Mutations Induce an ATP-independent Channel Activity of ΔF508-CFTR That Is More Thermally Stable—It is now well accepted that low temperature-recued ΔF508-CFTR is markedly defective in channel gating (25, 26); however, questions remain such as (i) how does this mutation disrupt channel gating, (ii) is the channel activity of low temperature-recued ΔF508-CFTR stable at physiologic temperature, and (iii) does the ΔF508 mutation have any effect beyond ATP-dependent channel gating (e.g. ATP-independent channel gating or ion conduction through the pore of the misfolded protein). Regarding the latter point, it has been argued that the ΔF508 mutation affects packing of the CFTR TMDs; such an effect might compromise the structure of the CFTR pore in addition to affecting ATP-dependent gating (37).

To investigate the extent to which temperature-sensitive ΔF508 misfolding disrupts channel gating and permeation, we took advantage of constitutive mutations in the cytosolic loops that we showed previously to promote channel opening in the absence of ATP binding and NBD dimerization (47). These mutations map to the apparent symmetry axis of the channel where they destabilize the closed state and strongly promote ATP-free channel opening. Such gain-of-function mutations are characteristic of conventional ligand-gated channels for which ligand binding allosterically increases the probability of channel opening. Gain-of-function mutations for the latter reciprocally enhance ligand sensitivity because of allosteric coupling between the ligand binding domain and the pore. The constitutive loop mutations that we have characterized similarly increase the ATP (and PKA) sensitivity of CFTR gating. Thus, despite the fact that CFTR is a unique ligand-gated channel that usually consumes its ligand (ATP) by hydrolysis, its gating mechanism does share interesting features with conventional ligand channels. Earlier we showed that these constitutive loop mutations markedly increased channel opening rates for CFTR constructs that cannot respond to ATP (G551D-CFTR) or dimerize the two NBDs (i.e. an NBD2 deletion construct, Δ1198-CFTR). These findings motivated us to test here the effects of these gain-of-function mutations on ΔF508-CFTR processing and gating.

These mutations did not enhance ΔF508-CFTR biosynthetic processing. However, they substantially increased the ATP-independent channel activity of low temperature-recued ΔF508-CFTR at 22–23 °C. In addition, we observed that the constitutive loop mutations partially protected ΔF508-CFTR channels from thermal-induced channel inactivation (Fig. 8). Importantly, using an ATP scavenger we determined that ATP-independent channel activity is resistant to inactivation by increasing the temperature; the current deactivation exhibited in the compound mutants was completely attributable to the loss of ATP-dependent gating. This was further confirmed by testing the thermal stability of the ATP-independent channel activity of the compound mutants in macro- and micropatches at physiologic temperature after ATP was removed. The unitary currents for the compound mutants harboring the constitutive mutations were normal, indicating that the ΔF508 mutation has no obvious impact on the structure of the translocation pathway, whereas it disrupts ATP-dependent channel gating at both room and physiologic temperature. This thermal stability of the ATP-independent channel activity of ΔF508-CFTR could provide an opportunity for rescuing ΔF508-CFTR channel function at physiologic temperature (e.g. perturbation of loop regions by small molecules).

Based on our present results and previous reports (24–26, 42), we predict a simple mechanism for the ΔF508-CFTR gating defect. First, at room temperature in the presence of ATP and PKA, ΔF508-CFTR is unable to form the tight NBD dimer that is required for optimal channel gating. This is consistent with a recent study that showed a highly affinity ATP analog can strongly enhance ΔF508-CFTR channel activity by possibly stabilizing the NBD dimer (60). Second, the loose NBD dimer of ΔF508-CFTR is thermally sensitive and further disrupted at physiologic temperature (e.g. involving greater separation of the NBDs) that may lead to misfolding and/or aggregation. Further studies are needed to elucidate the mechanism of the ΔF508-CFTR channel gating defects at either temperature.

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