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Thermal Instability of ΔF508 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Channel Function: Protection by Single Suppressor Mutations and Inhibiting Channel Activity

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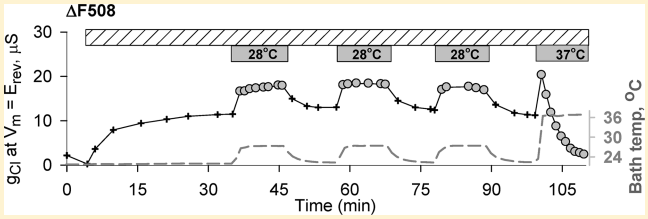
Supporting Information

ABSTRACT: Deletion of Phe508 from cystic fibrosis transmembrane conductance regulator (CFTR) results in a temperature-sensitive folding defect that impairs protein maturation and chloride channel function. Both of these adverse effects, however, can be mitigated to varying extents by second-site suppressor mutations. To better understand the impact of second-site mutations on channel function, we compared the thermal sensitivity of CFTR channels in *Xenopus* oocytes. CFTR-mediated conductance of oocytes expressing wt or ΔF508 CFTR was stable at 22 °C and increased at 28 °C, a temperature permissive for ΔF508 CFTR expression in mammalian cells. At 37 °C, however, CFTR-mediated conductance was further enhanced, whereas that due to ΔF508 CFTR channels decreased rapidly toward background, a phenomenon referred to here as "thermal inactivation." Thermal inactivation of ΔF508 was mitigated by each of five suppressor mutations, I539T, R553M, G550E, R555K, and R1070W, but each exerted unique effects on the severity of, and recovery from, thermal inactivation. Another mutation, K1250A, known to increase open probability (P_o) of ΔF508 CFTR channels, exacerbated thermal inactivation. Application of potentiators known to increase P_o of ΔF508 CFTR channels at room temperature failed to protect channels from inactivation at 37 °C and one, PG-01, actually exacerbated thermal inactivation. Unstimulated ΔF508CFTR channels or those inhibited by $CFTR_{inh}$-172 were partially protected from thermal inactivation, suggesting a possible inverse relationship between thermal stability and gating transitions. Thermal stability of channel function and temperature-sensitive maturation of the mutant protein appear to reflect related, but distinct facets of the ΔF508 CFTR conformational defect, both of which must be addressed by effective therapeutic modalities.



Cystic fibrosis is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride-selective channel that is widely expressed in human epithelial tissues. Like other ATP binding cassette (ABC) transporters, CFTR exhibits a modular domain architecture comprising two membrane spanning domains (MSD1 and MSD2), two nucleotide binding domains (NBD1 and NBD2), and a unique regulatory region (R) that connects the N- and C-terminal halves of the protein. The most common CF-causing mutation, present in 90% of patients, results in deletion from NBD1 of phenylalanine 508 (ΔF508), a residue which normally lies at the interface between NBD1 and the fourth intracellular loop (ICL4).[1] The structural integrity of this interface has been shown to be essential for the maintenance of CFTR channel function.[1−7]

Diminished apical chloride conductance in epithelial cells expressing the ΔF508 channel can be traced to folding defects that attenuate maturation, intracellular trafficking, and surface expression of the protein, and also result in impaired channel gating, manifest as reduced open probability (P_o).[8−13] In mammalian cells, the ΔF508 CFTR folding defect is partially suppressed at temperatures ranging from 26 to 28 °C, enabling a fraction of newly synthesized CFTR to reach the plasma membrane and restore chloride transport, albeit at a reduced level compared to its wild type (wt) counterpart.[11,14−17] Recent evidence indicates that low-temperature expression circumvents thermodynamic instabilities inherent in the ΔF508 channel and its constituent domains,[6,7,18−20] thereby allowing some of the mutant protein to escape degradation by endoplasmic reticulum quality control machinery. Consistent with this hypothesis, low-temperature rescued ΔF508 CFTR channels exposed to 37 °C exhibit a markedly reduced metabolic half-life ($t_{1/2}$ < 4 h versus $t_{1/2}$ > 24 h for wt CFTR[14−17,21]) and rapid thermal inactivation of chloride channel function.[5,22]

ΔF508 CFTR folding defects can also be suppressed to varying degrees by a variety of second-site mutations in NBD1.[4,8,18,23−30] I539T, occurring naturally in many CFTR orthologs, improved the maturation of ΔF508 CFTR at 37 °C,[4,25,30] but actually reduced open probability (P_o) determined in detached patches.[9] Another, R555K, modestly improved protein processing but also increased P_o of ΔF508 CFTR channels in detached patches. In contrast, the suppressor

Received: January 6, 2012
Revised: June 6, 2012
Published: June 8, 2012

   dx.doi.org/10.1021/bi300018e | *Biochemistry* 2012, 51, 5113−5124

mutation R553M was reported to be only modestly effective at correcting either defect.[4,23] Thus, while second-site mutations can confer different phenotypes on ΔF508 CFTR with respect to maturation and channel function, the precise mechanisms by which they impact intramolecular interactions within, and external to, NBD1 remain poorly understood.

The aim of the present study was to use the thermal stability of CFTR-mediated conductance to more precisely define the functional impact of individual, second-site mutations on ΔF508 CFTR channels. The *Xenopus* oocyte expression system is ideally suited for this purpose because oocytes are routinely maintained at 18−22 °C, temperatures that promote ΔF508 CFTR expression. The impact of mammalian physiological temperature on CFTR-mediated conductance can be assessed rapidly, and in real-time, by simply raising the bath temperature to 37 °C. We identified unique functional signatures for five second-site mutations, four in NBD1 (I539T, G550E, R553M, and R555K) and one in the fourth intracellular loop (ICL4, R1070W), and also investigated the relation of thermal stability to variations in channel gating brought about by intracellular cAMP, CFTR potentiators, and CFTR inhibitors. Consistent with previous studies, ΔF508 CFTR-mediated conductance, rescued by incubating oocytes at room temperature, decreased rapidly at 37 °C.[5,22] When ΔF508 CFTR was expressed in the context of single, second site mutations, however, results ranged from complete protection from thermal inactivation at 37 °C (R553M) to profound inactivation that was fully reversed upon returning the bath to room temperature (I539T). Unstimulated ΔF508 CFTR channels, and channels that were stimulated, but subsequently exposed to an inhibitor of channel function, $CFTR_{inh}$-172, were partially protected from thermal inactivation. These results, taken together with those of Wang et al.[22] and Aleksandrov et al.,[5] are consistent with the hypothesis that actively gating, ΔF508 CFTR channels are inherently unstable at 37 °C, but also indicate that even unstimulated ΔF508 CFTR channels exhibit an adverse response to elevated temperature. The effects of second-site suppressor mutations show that thermal stability of channel function correlates poorly with either the yield of NBD1 in a cell-based assay or the yield of CFTR protein at 37 °C in mammalian cells. Thermal inactivation of ΔF508 channels rescued to the cell surface by low temperature may be the earliest indication of thermally induced unfolding which triggers peripheral quality control[20] and should be a primary concern in the search for therapeutic small molecules.

MATERIALS AND METHODS

Mutagenesis and In Vitro Transcription. The methods used for mutagenesis and in vitro transcription were similar to those reported previously.[31−33] Briefly, CFTR mutants were generated using site-directed mutagenesis PCR. Ambion mMessage mMachine T7 Ultra transcription kit (Ambion) was used to generate the CFTR cRNAs for *Xenopus* oocyte injection. The sequences in the region of the mutation were confirmed by direct DNA sequencing.

Preparation and Microinjection of Oocytes. The preparation and microinjection of *Xenopus laevis* oocytes was performed using methods previously described in detail.[31,32] The follicular membranes were removed by mechanical agitation (1−2 h) in a Ca^{2+}-free solution containing (mM): 82.5 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES, pH 7.5, with 0.2 Wünsch units/mL Liberase Blendzyme 3 (Roche Molecular Biochemicals, Indianapolis, IN). Defolliculated oocytes were washed and maintained in a modified Barth's solution containing (mM): 88 NaCl, 1 KCl, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$ 0.41 $CaCl_2$, 2.4 $NaHCO_3$, 10 HEPES (hemi-Na), and 250 mg/L amikacin plus 150 mg/L gentamicin at pH 7.5. Stage V to VI oocytes were injected with 50 nL of CFTR cRNA plus cRNA encoding the human β_2-adrenergic receptor per oocyte. CFTR RNA concentration was adjusted so that the maximum steady state stimulated conductance is less than 200 μS (~12.5 to 250 ng/oocyte).

Whole-Cell Recordings. Individual oocytes were placed in a recording chamber (RC-1Z, Warner) and continuously perfused with Frog Ringer's solution. The Ringer's solution contained (in mM): 98 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES-Hemi Na, at pH 7.4. CFTR channels were activated using 10 μM isoproterenol (a β-adrenergic agonist) and 1 mM IBMX (a phosphodiesterase inhibitor) as the stimulating cocktail (Isop + IBMX). The Oocyte 725 amplifier (Warner) and the pClamp 8 data acquisition program (Molecular Devices, CA) were used for whole-cell recordings. Oocytes were maintained in the open circuit condition and the membrane potential was periodically ramped from −120 to +60 mV over 1.8 s to construct the whole cell I−V plots.

Single-Channel Recording. Single-channel recordings were carried out using excised, inside-out patches as described previously.[34] The pipet solution contained (in mM) 142 NMDG-Cl, 2 $MgCl_2$, and 5 HEPES HemiNa, pH 7.4. Channels were activated by exposure to 65U of PKA (the catalytic subunit) after excision into intracellular solution (in mM) 146 NMDG-Cl, 2 $MgCl_2$, 0.5 Tris-EGTA, 5 HEPES HemiNa, and 1 Mg ATP, pH 7.4. NP_o-for-all-levels was obtained after single-channel searching using the event detection features in Clampfit 9 (Molecular Devices, CA) and N is the apparent number of channels in a patch while P_o is the open probability. Baseline drift that tended to occur after the temperature change was corrected using the features in Clampfit 9.

Temperature Control. A Dual Automatic Temperature controller (CL-200) and an in-line solution heater/Cooler (SC-20) (Warner Instruments, Hamden, CT) were used to apply acute temperature change under constant flow for whole-cell recordings. A thermo probe positioned near the oocytes was used to monitor the temperature in real time. The temperature was digitized and recorded using a USB Data Acquisition device (DI-158, DATAQ Instruments, Inc. Akron, OH). A TCbip Temperature controller and a preheater (HPRE2) (Cell Micro Controls, Norfolk, VA) were used to apply acute temperature change for single-channel recordings under constant flow. The pipet tip was placed at the tip of the outflow where the temperature was monitored in real time using the built-in thermistor. All experiments were conducted at room temperature (22−23 °C) unless specified.

Reagents. Isobutylmethyl xanthine (IBMX), isoproterenol (Isop), MgATP, and 2-mercaptoethanol (2-ME) were purchased from Sigma (St Louis, MO). PKA was purchased from Promega (Madison, WI). 2-(trimethylammonium)ethyl methanethiosulfonate, Bromide ($MTSET^+$) was purchased from Toronto Research Chemicals (Toronto, Canada). The following compounds were kindly provided by Dr. Robert Bridges (Rosalind Franklin University, Chicago, IL) and The Cystic Fibrosis Foundation (CFF): 4-[4-Oxo-2-thioxo-3-(3-trifluoromethyl-phenyl)-thiazolidin-5-ylidenemethyl]-benzoic acid ($CFTR_{inh}$-172 or CF172), 2-[(2−1H-Indol-3-yl-acetyl)-methyl-amino]-*N*-(4-isopropyl-phenyl)-2-phenyl-acetamide (PG-01 or P2), 4-Methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol

dx.doi.org/10.1021/bi300018e | *Biochemistry* 2012, 51, 5113−5124

(VRT-532 or P1). Genistein was purchased from TCI America (Portland, OR).

RESULTS

ΔF508 CFTR Chloride Conductance in *Xenopus* Oocytes Was Stable at 22 and 28 °C but Was Inactivated at 37 °C. To compare the thermal stability of wt and ΔF508 CFTR channels, we preincubated oocytes expressing either variant at room temperature (22 °C) and then warmed the superfusate to temperatures ranging from 28 to 37 °C for 10 min intervals while conductance was continuously recorded. This period was generally long enough to discern temperature-induced changes in conductance due to ΔF508 CFTR channels without evoking substantial increases in background conductance. CFTR-specific conductance was operationally defined as that sensitive to the CFTR inhibitor, $CFTR_{inh}$-172, referred to here as CF172.[35] Figure 1A illustrates temperature responses typical of oocytes expressing wt CFTR. Three successive increases in bath temperature from 22 to 28, 34, and 37 °C produced prompt increases in wt CFTR conductance, but in each case conductance returned to its original level when the bath temperature was returned to 22 °C. Figure 1B illustrates conductance changes recorded from an oocyte expressing ΔF508 CFTR. Three successive excursions to 28 °C, a temperature permissive for ΔF508 CFTR expression and channel function in mammalian cells, produced reversible increases in conductance similar to those seen with wt CFTR. A fourth increase from 22 to 37 °C, however, provoked a transient increase in conductance followed by a marked decline to near background level (referred to here as thermal inactivation) that occurred with a half-time of about 4 min.

Thermal inactivation of ΔF508 CFTR channels at 37 °C was partially reversed upon cooling the superfusate to 22 °C; however, after incubation at reduced temperature for about one hour, the conductance had recovered to only about 30% of its original value (Figure 1C). The conductance recovered after incubation at 22 °C was further increased by exposing the oocyte to the CFTR potentiator, PG-01, referred to here as P2[36] and reduced to near zero by subsequent application of the CFTR inhibitor, CF172, indicating that the recovered conductance reflects ΔF508 CFTR channels. To assay for the possible insertion of new channels during the recovery period, we expressed ΔF508 CFTR channels containing a cysteine at position 334 that is readily labeled by extracellular $MTSET^+$. We demonstrated previously that channels newly added to the oocyte surface can be detected by means of a "pulse labeling" protocol in which the surface channel population is labeled at $t = 0$ by a 40 s exposure to $MTSET^+$. A second exposure to $MTSET^+$ at a later time reveals any unlabeled (new) channels that have been added in the interim. These experiments, described in detail in the supporting text and Figure S1, revealed that the recovery of the thermally inactivated conductance at 22 °C was largely attributable to the stimulation (increase in open probability) of channels resident in the membrane throughout the temperature perturbation. However, as much as 17% of the recovered conductance could represent the addition of new channels to the cell membrane during the recovery phase at 22 °C (see Supporting text and Figure S1).

The summary contained in Figure 1D indicates that, upon warming to 37 °C, ΔF508 CFTR conductance increased transiently, declined to about 10% of its initial value at 22 °C, and then partially recovered after cooling to 22 °C. Also shown is the conductance increase induced by exposing the recovered

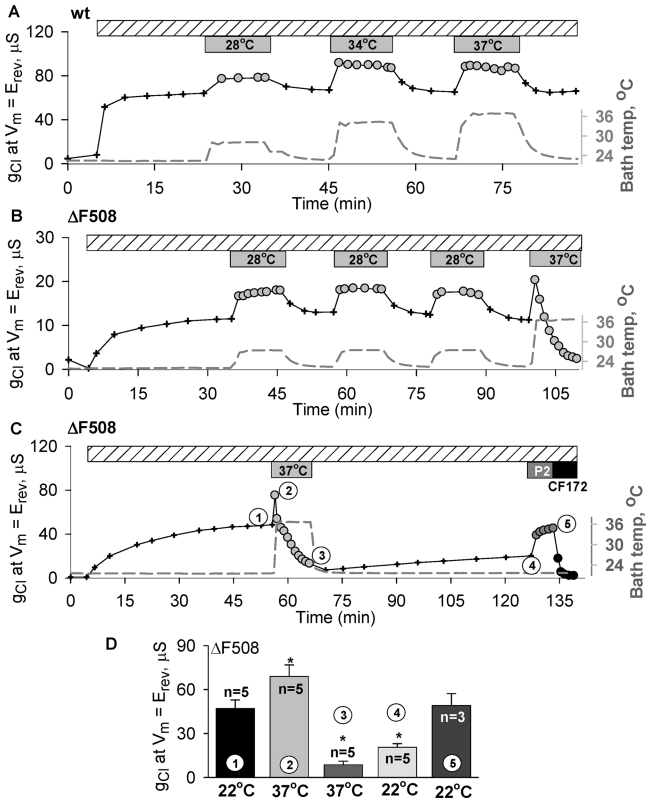


Figure 1. Differential effect of temperature on wt and ΔF508 CFTR channels. (A) Following stimulation (10 μM isoproterenol and 1 mM IBMX, hatched bar and crosshairs), an oocyte expressing wt CFTR was subjected to successive increases in bath temperature (gray bar and circles) for 10 min. (B) Following stimulation, an oocyte expressing ΔF508 CFTR was warmed to 28 °C three times and then to 37 °C for 10 min. (C) Following stimulation, an oocyte expressing ΔF508 CFTR was warmed to 37 °C, reducing the conductance to about 10% of its initial value and then was allowed to recover at 22 °C for about one hour when it was exposed to 2 μM CFTR potentiator, P2 (PG-01, dark gray bar and circles) and 10 μM CFTR inhibitor, CF172 (CF_{inh}-172, black bar and circles). Gray dashed lines indicate the actual temperatures measured in the bath close to oocyte. (D) Summary of temperature-dependent inhibition and recovery of ΔF508 CFTR conductance. ① represents the initial conductance following stimulation. ② represents the peak conductance at 37 °C. ③ is the conductance at the end of exposure to 37 °C. ④ is the conductance at 22 °C after 1 h wash with stimulatory cocktail. ⑤ is the conductance at 22 °C following stimulation by 2 μM P2. *P-value < 0.05 (t test).

oocytes to the CFTR potentiator, P2, at 22 °C. Additional covalent labeling experiments conducted with R334C/ΔF508 CFTR channels indicated that potentiation of the thermally inactivated conductance at 22 °C by P2 was not attributable to the addition of new channels to the membrane and must therefore reflect a P2-induced increase in channel open probability consistent with that reported by Pedemonte et al.[36] (see Supporting text and Figure S2). These results demonstrate that ΔF508 CFTR channels are rapidly inactivated at 37 °C in whole cells but slowly recover partial activity at 22 °C that can be enhanced by application of a CFTR potentiator.

Open Probability (P_o) of ΔF508 CFTR Channels Decreased at 35 °C. Figures 2 and 3 illustrate the behavior of single wt and ΔF508 CFTR channels recorded from inside-out patches detached from oocyte membranes at 22 °C and briefly warmed to 35 °C (patches were unstable at 37 °C). In the case of wt CFTR channels (Figure 2) warming the bath

dx.doi.org/10.1021/bi300018e | *Biochemistry* 2012, 51, 5113−5124

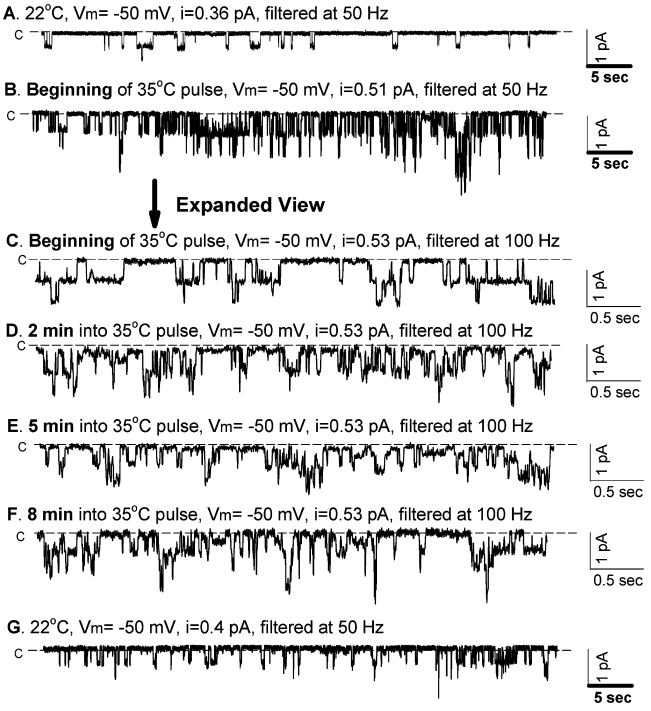


Figure 2. wt CFTR single-channels recorded from an inside-out patch using symmetric solutions (146 mM $[Cl]^-$) at pH 7.4. (A) 1 min recording following bath application of 65 U PKA and 1 mM ATP at 22 °C. (B) 1 min recording at the onset of the warming to 35 °C. (C–F) 5 s records at the onset, 2, 5, and 8 min into the warming to 35 °C. (G) 1 min recording after cooling to 22 °C.

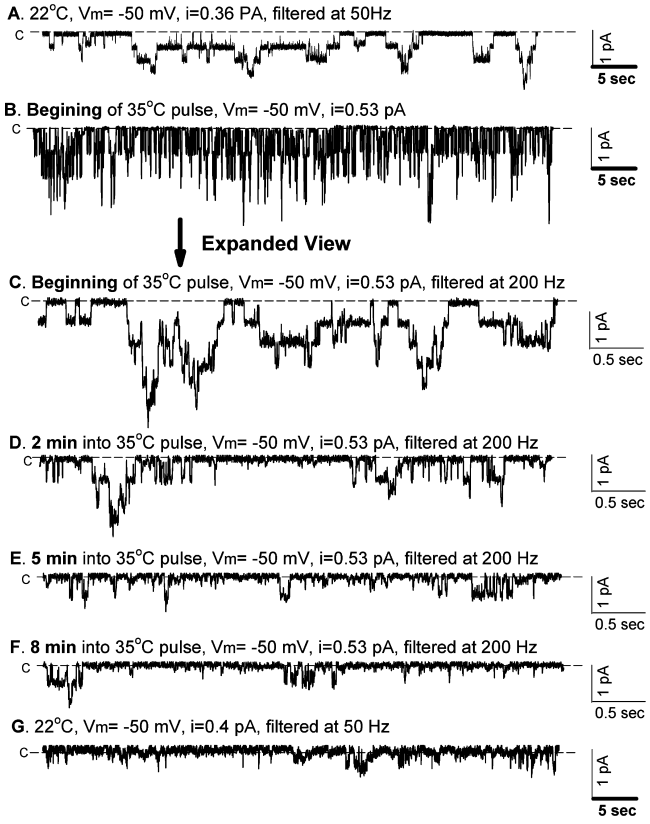


Figure 3. Time-dependent changes in single-channel activity of ΔF508 CFTR during warming to 35 °C. (A) 1 min recording following bath application of 65 U PKA and 1 mM ATP at 22 °C. (B) 1 min recording at the onset of warming to 35 °C. (C–F) 5 s records at the onset, 2, 5, and 8 min into warming to 35 °C. (G) 1 min recording after cooling to 22 °C. Although difficult to discern due to the time scale of this plot, single-channel current amplitude returned immediately to the prewarming level.

from 22 to 35 °C led to an essentially instantaneous increase in both single-channel conductance and the frequency of gating transitions that was sustained for the period of increased temperature. Single-channel conductance increased from about 7 pS to 11 pS, an increment of 57%. Analysis of the first minute of the wt single-channel record following warming to 35 °C revealed decreases in *both* mean closed time and mean open time (Table 1), that is, both the opening and closing rates were increased. The opening rate increased only slightly more than the closing rate so that P_o increased modestly, if at all. The temperature-induced changes in both single-channel conductance and apparent P_o for wt CFTR were reversed when the bath was cooled to 22 °C (Figure 2G). These results are consistent with those of Mathews et al.[37] who reported that warming from 23 to 37 °C increased single-channel conductance by 54%, from 8.3 to 12.6 pS ([Cl] approximately 155 mM). Csanády et al. (ref 38 and personal communication) noted a 57% increase in wt single-channel conductance, from 5.6 pS at 15 °C to 8.8 pS at 35 °C. Both of these reports also indicated that warming channels to 37 °C increased both the opening rate and the closing rate of wt CFTR channels, although both reported substantial (58% and 61%, respectively) increases in P_o, due to a greater increase in the channel opening rate.

Immediately following warming of patches detached from oocytes expressing ΔF508 CTFR channels from 22 °C (Figure 3A) to 35 °C (Figure 3B), single-channel conductance and the frequency of gating transitions increased, the latter a result of increases in both the opening rate and closing rate, such that P_o was roughly doubled during the first minute of recording. As with wt CFTR, ΔF508 CFTR single-channel conductance remained elevated throughout the warming period, but after 1 min at 35 °C ΔF508 CFTR channel activity, measured as NP_o (Figure 4B), had already begun to decline, and after 5 min NP_o was substantially reduced (Figure 3D–F). When the bath temperature was returned to 22 °C single-channel conductance returned to its room-temperature value essentially instantaneously (Figure 3G). Membrane patches subjected to warming eventually became unstable even at 22 °C so that we were unable to hold them for a period sufficient to determine if channel activity would partially recover with time.

Figure 4 compares the time course of channel activity (NP_o) for wt and ΔF508 CFTR channels maintained at 35 °C for 8 min. ΔF508 channel activity was dramatically reduced with a half-time of about 1 min, whereas the elevated wt channel activity remained essentially constant. Changes in single-channel conductance were virtually identical for wt and ΔF508 channels, and in both cases the fractional increase (57%) exceeded that predicted from the temperature-dependence of the mobility of Cl^- in solution (33%, ref 39), a result that could reflect a change in channel geometry resulting from a stable, thermally induced conformational change in the outer vestibule of the pore of wt CFTR that we recently described.[40] More rapid thermal inactivation seen in detached patches likely reflects more rapid warming of channels in a detached membrane patch where the temperature could be changed instantly as compared to the whole-cell condition in which the intracellular side of the oocyte plasma membrane is thermally

dx.doi.org/10.1021/bi300018e | *Biochemistry* 2012, 51, 5113–5124

Table 1. Summary of Kinetic Analysis of Single-Channel Currents[a]

	T_b, ms	T_{ib}, ms	T_f, ms	A	P_o	i, pA
wt-22 °C (n = 3)	1072 ± 708	6032 ± 2013	18.4 ± 2.1	1.7 ± 0.5	0.14 ± 0.02	0.35 ± 0.03
wt-35 °C-1 min	135 ± 14	569 ± 214	29 ± 10	0.9 ± 0.4	0.18 ± 0.06	0.51 ± 0.04
wt-35 °C-5 min	84 ± 13	372 ± 92	20 ± 3	0.9 ± 0.2	0.15 ± 0.03	0.54 ± 0.02
ΔF508−22 °C (n = 4)	1106 ± 231	11531 ± 3303	27 ± 4	1.6 ± 0.6	0.1 ± 0.01	0.35 ± 0.00
ΔF508−35 °C-1 min	169 ± 27	528 ± 5	21 ± 5	1.1 ± 0.2	0.21 ± 0.03	0.56 ± 0.00
ΔF508−35 °C-5 min	67 ± 10[b]	2009 ± 568[b]	13 ± 3	0.9 ± 0.1	0.03 ± 0.01	0.53 ± 0.01[b]

[a]Kinetic parameters obtained using a three-state model (C ↔ C ↔ O) and the Fit program (Dr. László Csanády) at 22 and 35 °C (first minute and 5 min post temperature change). T_b = mean burst duration; T_{ib} = mean interburst duration; T_f = mean duration of short "flickery" closures; A = mean number of flickery closures per burst; P_o = open probability; i = single channel current at Vm = −50 mV. [b]P-value < 0.05 for t test between 1 and 5 min at 35 °C.

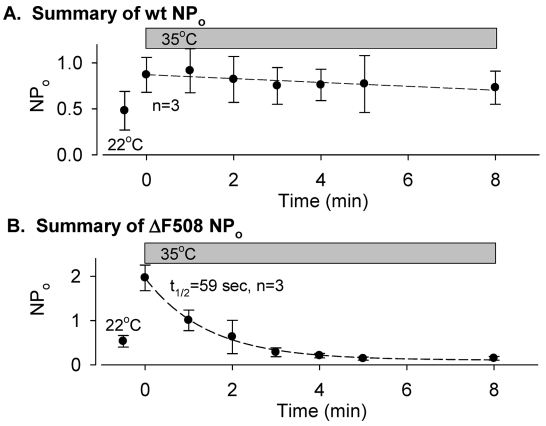


Figure 4. ΔF508 CFTR single-channel activity is reduced at 35 °C. (A) Summary of changes in NP_o for wt CFTR with time before and during warming to 35 °C. wt single-channel conductance increased from 8.5 ± 1.0 pS to 10.3 ± 0.7 pS (n = 3). (B) Summary of changes in NP_o for ΔF508 CFTR with time before and during warming to 35 °C. Single-channel conductance increased from 7.0 ± 0.1 pS to 11.1 ± 0.1 pS (n = 3). Half-times were estimated by fitting a single exponential curve to the data points.

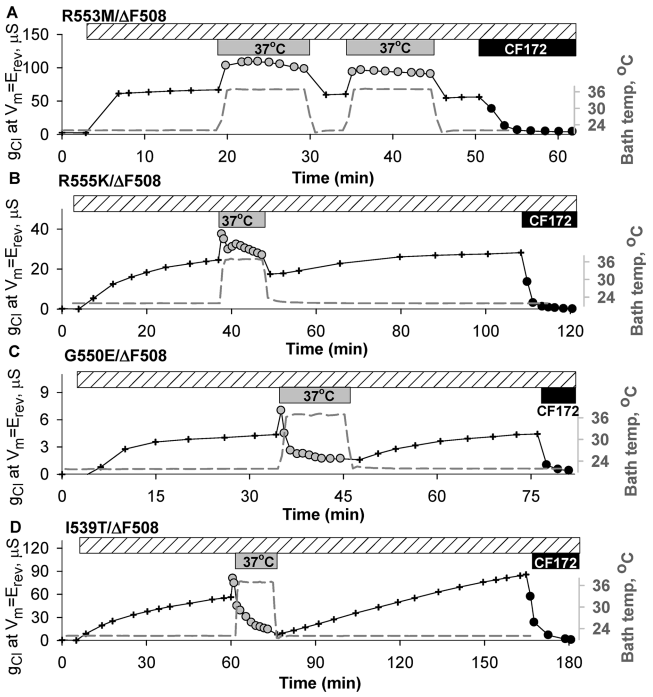


Figure 5. Differential protection of ΔF508 CFTR from thermal inactivation by 2nd site mutations. Representative experiments for (A) R553M/ΔF508 CFTR (n = 3). Following stimulation, an oocyte was warmed to 37 °C (gray bar and circles) for 10 min twice in succession. After cooling to 22 °C, the oocyte was exposed to 10 μM CF172 (black bar and circles). (B) R555K/ΔF508 CFTR (n = 5). Following stimulation, an oocyte was warmed to 37 °C for 10 min. After cooling to 22 °C, the oocyte was exposed to 10 μM CF172. (C) G550E/ΔF508 CFTR (n = 4). Following stimulation, an oocyte was warmed to 37 °C for 10 min. After cooling to 22 °C, the oocyte was allowed to recover for about 40 min and then was exposed to 10 μM CF172. (D) I539T/ΔF508 CFTR (n = 5). Following stimulation, an oocyte expressing was warmed to 37 °C for 10 min. After cooling to 22 °C, the oocyte was allowed to recover for about 90 min and then was exposed to 10 μM CF172. Not shown are the same second site mutations engineered on the wt background, all of which exhibited responses to warming identical to that of wt CFTR.

"buffered" by heat transfer to the remainder of the cell which functions as a partial "heat sink."

Table 1 summarizes the effect of temperature on single-channel kinetics of wt and ΔF508 CFTR channels, quantified by using the program, "Fit," developed by Dr. László Csanády[38] based on a three-state gating model (C ↔ C ↔ O). The estimated mean burst durations were comparable for wt and ΔF508 channels at 22 °C. Both were reduced to a similar extent after 1 min at 35 °C and were further decreased after 5 min at 35 °C. Interburst intervals were decreased during the first minute of exposure to 35 °C for both wt and ΔF508 channels, but after 5 min at 35 °C the interburst intervals for ΔF508 channels had lengthened significantly, whereas those of the wt channels were unchanged. This result is consistent with the notion that exposure to temperatures ranging from 35 to 37 °C caused ΔF508 CFTR channels to visit an inactivated state from which they were less likely to open.

Single, Second-Site Mutations Can Suppress Thermally Induced Inactivation of ΔF508 CFTR Channels. Representative results shown in Figure 5 reveal a continuum of thermal inactivation phenotypes resulting from the addition of single, second-site mutations to ΔF508 CFTR. These ranged from complete protection from thermal inactivation to little or no protection, followed by full recovery after cooling the bath to 22 °C. Of the four NBD1 suppressor mutations tested only one, R553M, fully restored wt thermostability to ΔF508 CFTR channels. In contrast, pairing ΔF508 with R555K, a mutation that has been reported to be somewhat more effective than R553M at improving NBD1 folding and protein maturation,[4,6,24] resulted in a channel that, although unable to sustain the initial increase in conductance evoked at 37 °C, was inactivated only slightly and returned to its prewarming level relatively rapidly when superfusate temperature was returned to 22 °C. More pronounced inactivation was seen in G550E/

dx.doi.org/10.1021/bi300018e | Biochemistry 2012, 51, 5113−5124

ΔF508 CFTR and I539T/ΔF508 CFTR, but in both cases the conductance decrease at 37 °C was followed by complete recovery at 22 °C. Complete recovery of conductance at 22 °C suggested that, in the double mutants, even a profound inactivation at 37 °C was fully reversible. There was no apparent correlation of the functional phenotype of the double mutant channels at 37 °C with the improvements reported for NBD1 folding and protein maturation,[4,6] but the partial protection from thermal inactivation by R555K and G550E suggested that the effects might be correlated with the induction of increased P_o.[8,24] R553M, however, had been reported by Teem et al.[24] not to increase P_o of ΔF508 channels (34−36 °C), so we investigated the behavior of the double mutant in inside-out patches. We found that R553M/ΔF508 CFTR, like wt, exhibited a stable increase in P_o at 35 °C (Figure 6). P_o of R553M/ΔF508 CFTR channels was comparable to that of ΔF508 channels at 22 °C and increased similarly during the first minute of exposure to 35 °C. Over the ensuing 5 min, however, unlike ΔF508 channels, P_o of the double mutant was maintained, suggesting that this second-site mutation acts, at least in part, by stabilizing channel conformation(s) visited during the gating cycle that are rendered thermally unstable by the deletion of phenylalanine 508.

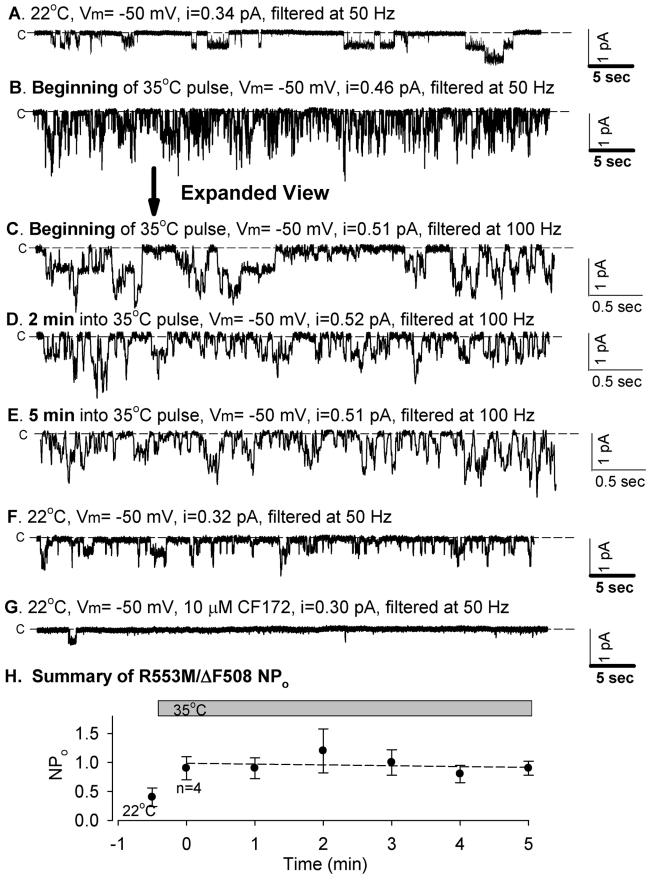


Figure 6. R553M/ΔF508 CFTR single-channels recorded from an inside-out patch using symmetric solutions (146 mM $[Cl]^-$) at pH 7.4. (A) 1 min recording following bath application of 65 U PKA and 1 mM ATP at 22 °C. (B) 1 min recording at the onset of warming to 35 °C. (C−E) 5 s records at the onset, 2 and 5 min into warming to 35 °C. (F) 1 min recording after cooling to 22 °C. (G) 1 min recording after 2 min inhibition by 10 μM CF172 at 22 °C. (H) Summary of changes in NP_o for R553M/ΔF508 CFTR with time before and during warming to 35 °C. Single-channel conductance increased from 7.3 ± 0.2 pS to 10.3 ± 0.6 pS (n = 4).

Protection from thermal inactivation by mutations that increased the open probability of ΔF508 CFTR channels led us to examine the thermal behavior of R1070W/ΔF508 CFTR. The substitution of this aromatic side chain in ICL4 increased the open probability of the double mutant [7] and was proposed to act, at least in part, by repairing defective coupling between NBD1 and ICL4.[6,7] Results depicted in Figure 7A show that

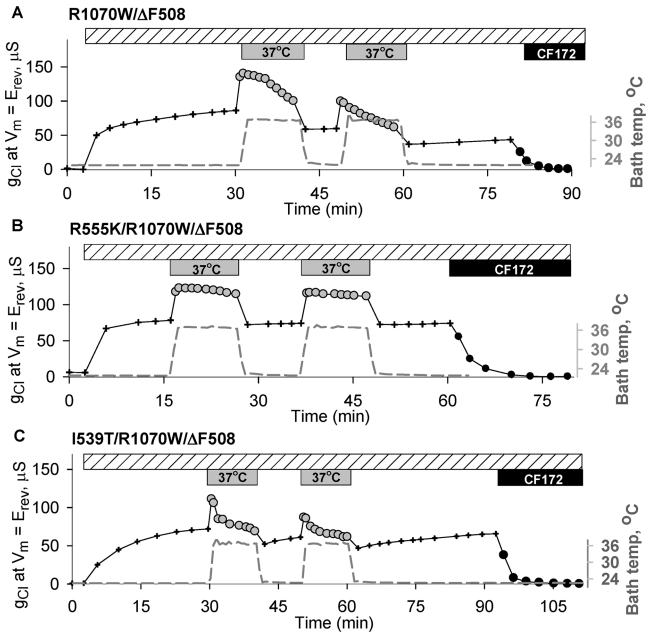


Figure 7. Protection of ΔF508 CFTR from thermal inactivation by R1070W. (A) Following stimulation, an oocyte expressing R1070W/ΔF508 CFTR (n = 4) was warmed to 37 °C (gray bar and circles) for 10 min twice in succession. After cooling to 22 °C, the oocyte was exposed to 10 μM CF172 (black bar and circles). (B) Following stimulation, an oocyte expressing R555K/R1070W/ΔF508 CFTR (n = 4) was warmed to 37 °C for 10 min twice. After cooling to 22 °C, the oocyte was exposed to 10 μM CF172. (C) Following stimulation, an oocyte expressing I539T/R1070W/ΔF508 CFTR (n = 4) was warmed to 37 °C for 10 min twice. After cooling to 22 °C, the oocyte was exposed to 10 μM CF172.

pairing ΔF508 with R1070W improved the thermal stability of the double mutant, but did not restore wt-like thermal stability. The initial increase in conductance was not sustained, but, compared to Δ*F*508 CFTR, conductance declined much more slowly following warming to 37 °C. After two exposures to the elevated temperature, recovery, although evident, was much slower than that seen with G550E/ΔF508 or I539T/ΔF508 CFTR. Pairing R1070W and a second NBD1 suppressor, R555K, with ΔF508, however, resulted in thermal stability that was indistinguishable from that of wt CFTR (Figure 7B). In contrast, combining ΔF508 with R1070W and I539T resulted in channels that could not sustain the elevated conductance seen immediately after warming to 37 °C but were nevertheless able to sustain a substantial conductance at 37 °C (Figure 7C). Supplemental Figure S3 compares the time course of the responses of all of the constructs bearing second-site mutants to 37 °C on an expanded scale.

Unstimulated ΔF508 CFTR Channels Are More Thermostable than Actively Gating Channels. Studies of a variety of proteins suggest an inverse relationship between

activity and thermostability.[41−46] To explore the possible relationship between ΔF508 CFTR channel activity and thermostability, we compared the effect of warming the oocyte superfusate before and after channels were stimulated by exposing the oocyte to isoproterenol and IBMX (Figure 8).

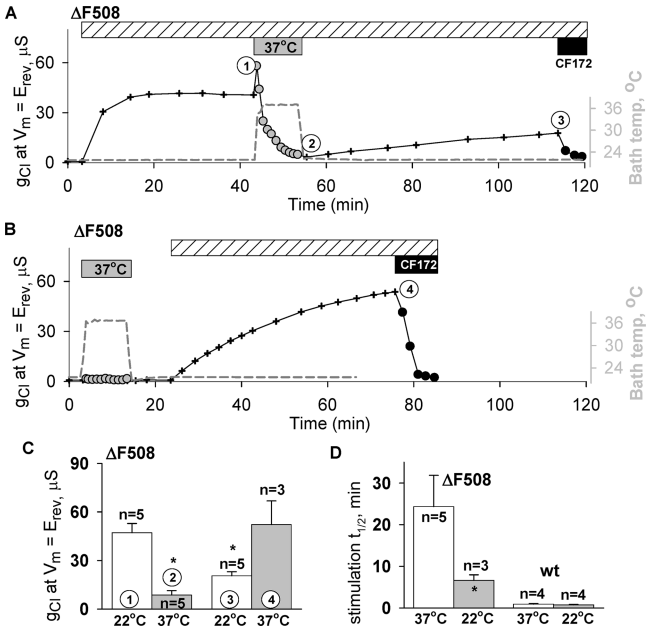


Figure 8. Unstimulated ΔF508 CFTR channels are partially protected from thermal inactivation. (A) After stimulation, an oocyte expressing ΔF508 CFTR was warmed to 37 °C for 10 min (gray bar and circles). After cooling to 22 °C, the oocyte was allowed to recover for about one hour (73 ± 8 min, n = 5) and then was exposed 10 μM CF172 (black bar and circles). (B) An oocyte was warmed to 37 °C for 10 min *prior* to stimulation. Afterward, it was stimulated at 22 °C. The conductance typically reached steady state within an hour (51 ± 1 min, n = 3) and the oocyte was then exposed to 10 μM CF172. (C) Summary of oocyte conductance after stimulation, with and without prestimulation warming. Circled numbers within the graph indicates time points for sampling conductance. (D) Summary of half-time for ΔF508 and wt CFTR stimulation with (open bars) and without (gray bars) prestimulation warming. *P-value <0.05 (t test). Half-times were estimated by fitting a single exponential curve to the data points.

Exposure of stimulated channels to 37 °C resulted in a rapid, 73% decrease in ΔF508 CFTR conductance (Figure 8A), as noted previously. In contrast, when the same temperature-challenge was administered to unstimulated ΔF508 channels expressed in paired oocytes, subsequent stimulation resulted in complete recovery of conductance (Figure 8B). The *time course* of subsequent stimulation in these oocytes, however, was markedly slowed (Figure 8D). In contrast, prestimulation exposure to 37 °C was without effect on either the magnitude or the time course of the stimulation of wt CFTR channels (Figure S4). This result suggests that nonstimulated ΔF508 CFTR channels are less thermally sensitive than stimulated ΔF508 CFTR channels but are nevertheless more thermally sensitive than wt channels. Nonstimulated ΔF508 CFTR channels, briefly exposed to 37 °C, appear to adopt a conformation that impairs channel stimulation by the subsequent elevation of intracellular cAMP at 22 °C, but this conformational change is completely reversible at the lower temperature.

K1250A/ΔF508/CFTR Channels Inactivate More Rapidly at 37 °C. The P_o of ΔF508 CFTR channels can be enhanced by substitutions for the "Walker lysine" in NBD2 (K1250) which slow the hydrolysis of ATP at composite site 2 of the NBD1/NBD2 dimer[47] such that channels exhibit prolonged open times at room temperature.[47] The results depicted in Figure 9 show that channel function of the double

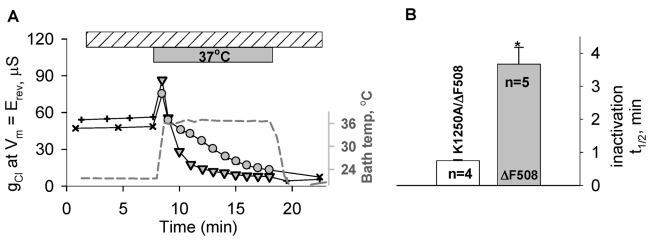


Figure 9. K1250A accelerated thermal inactivation of ΔF508 CFTR. (A) After stimulation, an oocyte expressing ΔF508 CFTR (gray bar and circles) or K1250A/ΔF508 CFTR (gray bar and downward triangles) was warmed to 37 °C for 10 min and then the bath was cooled to 22 °C. (B) Summary of the half-time of thermal inactivation of K1250A/ΔF508 and ΔF508 CFTR. *P-value < 0.05 (t test). Half-times were estimated by fitting a single exponential curve to the data points.

mutant (K1250A/ΔF508 CFTR) is even less stable than ΔF508 CFTR as judged by the increased rate of thermal inactivation. This finding is consistent with a model in which alterations in the structure of NBD2 or the NBD1/NBD2 interface that increase the stability of the open state at 22 °C may nevertheless increase susceptibility to thermally induced inactivation at 37 °C.

CFTR Potentiators Differentially Affect ΔF508 CFTR Channels. When ΔF508 CFTR channels were thermally inactivated at 37 °C and then permitted to recover at room temperature, application of the CFTR potentiator, P2, further increased the residual conductance (Figure 1C). This experiment, however, did not address potential effects of the potentiator itself on the stability of channel function at 37 °C. Therefore, we compared the effects of three potentiators, P2,[36] genistein,[47−49] and VRT-532 (ref 50 referred to here as P1), on thermal inactivation of ΔF508 CFTR. All three produced comparable increases in conductance of oocytes expressing ΔF508 CFTR channels at room temperature, but the *rate* of potentiation by P2 was significantly greater than that seen with either P1 or genistein (Figure S5).

The results compiled in Figure 10 indicate that none of the three potentiators protected ΔF508 CFTR from thermal inactivation, but one of them, P2, not only roughly doubled the rate of thermal inactivation, but also substantially increased the extent of thermal inactivation of ΔF508 CFTR conductance. In addition, *recovery* of conductance following cooling to 22 °C and in the continued presence of P2 was markedly attenuated compared to ΔF508 (compare Figure 1C and Figure 11B). This effect was specific to P2; neither P1 nor genistein altered either the rate or the extent of thermally induced inactivation of ΔF508 CFTR channels (Figure 10B,C). This result not only confirms the report of Wang et al.[22] that CFTR potentiators do not protect ΔF508 CFTR channels from thermal inactivation, but also suggests that such agents can actually *increase* susceptibility to thermal inactivation at 37 °C. The distinct difference in the impact of the three potentiators could imply that P_o, per se, is not the cause of

dx.doi.org/10.1021/bi300018e | *Biochemistry* 2012, 51, 5113−5124

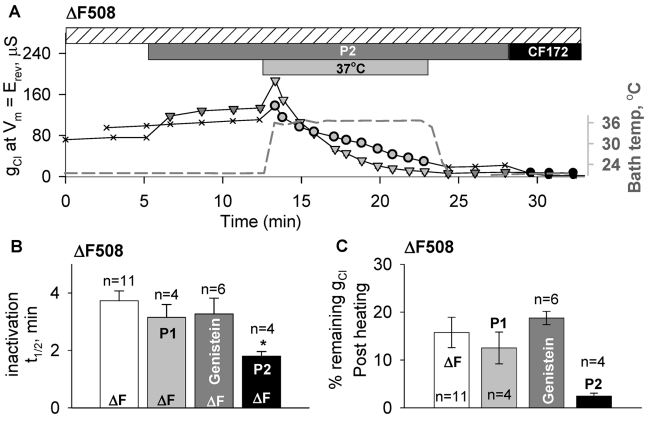


Figure 10. The CFTR potentiator PG-01 (P2) exacerbated thermal inactivation. (A) Oocytes expressing ΔF508 CFTR were stimulated (hatched bar and crosshairs). One was exposed to 10 μM P2 (dark gray bar and downward triangles) and then warmed to 37 °C in the continued presence of P2 for 10 min (gray bar and downward triangles). Another was warmed to 37 °C for 10 min in the absence of P2 (gray bar and circles). After cooling to 22 °C for about 5 min, oocytes were exposed to 10 μM CF172 (black bar and circles). (B) Summary of half-time of thermal inactivation in the absence and the presence of CFTR potentiators (10 μM P1, 50 μM genistein, 10 μM P2). (C) Summary of final CF172-sensitive conductance expressed as percent change relative to the initial conductance after warming to 37 °C for 10 min. *P-value < 0.05 (t test). Each potentiator has at least one paired control and the controls for all three potentiators were pooled. Half-times were estimated by fitting a single exponential curve to the data points.

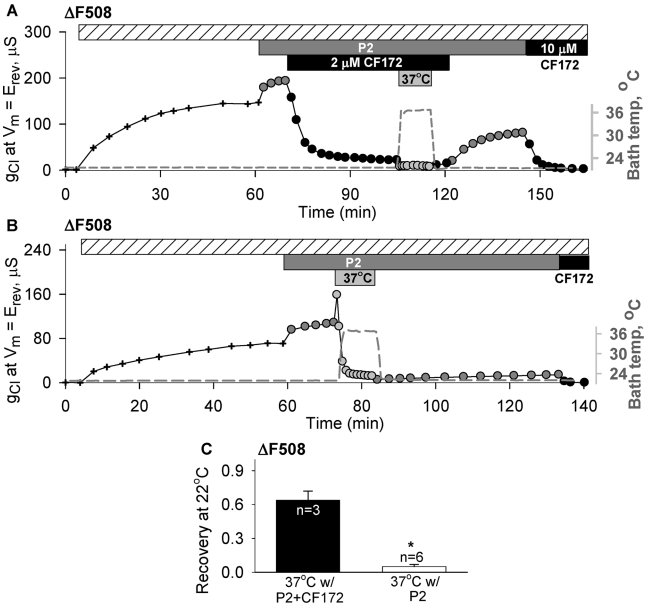


Figure 11. The CFTR inhibitor, CF172, protected ΔF508 CFTR from thermal inactivation. (A) After stimulation and exposure to P2 (10 μM, dark gray bar and circles), oocyte expressing ΔF508 CFTR was exposed to CF172 (2 μM, black bar and circles) in the continued presence of P2 at 22 °C. In the continued presence of P2 and CF172, the oocyte was warmed to 37 °C for 10 min (gray bar and circles) and then cooled to 22 °C. (B) After stimulation and exposure to P2 (10 μM), an oocyte expressing ΔF508 CFTR was warmed for 10 min 37 °C in the presence of 2 μM P2 and then cooled to 22 °C. (C) Average % recovery of CF172-sensitive conductance at 22 °C following 10 min warming to 37 °C in panels A and B. *P-value < 0.05 (t test).

ΔF508 CFTR thermal instability. However, this result is also consistent with the notion that individual potentiators achieve increases in P_o by way of different perturbations of ΔF508 CFTR channel structure which could in turn differentially impact thermal stability. A destabilizing effect of P2 was not seen with wt CFTR channels; thermostability of wt CFTR was unaffected by the potentiator (Figure S6).

Inhibition of Channel Activity by CF172 Increased Thermostability of ΔF508 CFTR Channels. A possible inverse correlation between channel activity and susceptibility to thermal inactivation suggested that an inhibitor might protect ΔF508 CFTR channels from thermal inactivation at 37 °C. In preliminary experiments we investigated possible effects of three CFTR inhibitors, GlyH-101,[51] glybenclamide,[52,53] and CF172[35] on thermal inactivation. Of these, only CF172 produced an apparent protective effect.

The protective effect of CF172 was demonstrable in the assay illustrated in Figure 11 in which oocytes expressing ΔF508 CFTR channels were first exposed to P2 in order to maximize the impact of the thermal challenge, and then warmed to 37 °C in the presence or absence of CF172. The results indicate that inhibition ΔF508 CFTR by CF172 resulted in substantial recovery of conductance at 22 °C in the presence of P2, whereas in the absence of the inhibitor little or no recovery of conductance was detectable. This result is consistent with a model in which inhibitor binding to ΔF508 channels stimulated by cytosolic cAMP and potentiated by P2 either induced or stabilized a protein conformation that exhibits reduced susceptibility to thermal inactivation.

DISCUSSION

Thermal Inactivation Provides a Novel Perspective on the Stability of ΔF508 CFTR. An abundance of previous studies, beginning with the work of Denning et al.,[11] have focused attention on the temperature sensitivity of the molecular phenotype associated with the common CF mutation, ΔF508. Temperature sensitivity is manifest in maturation of the protein,[11] lifetime of the protein in the plasma membrane,[14−17] and the thermodynamic stability of isolated NBD1.[6,29,30,54,55] The present work, along with the reports of Aleksandrov et al.[5] and Wang et al.,[22] reveals a previously unrecognized facet of the temperature-sensitive ΔF508 phenotype, namely, rapid inactivation of channel function at 37 °C. We confirm that the thermal sensitivity of channel function is a sensitive, real-time measure of the dynamic stability of ΔF508 CFTR channels rescued by low temperature to the plasma membrane of intact cells. The present studies, however, also reveal that the rate, extent, and reversibility of the temperature-induced decline in conductance due to ΔF508 CFTR is dependent on stimulation of the channel, second-site mutations and the effects of potentiators and inhibitors. This spectrum of temperature-sensitive, functional phenotypes appears to betray previously undetected subtleties, both in the nature of underlying structural defect that is induced by the ΔF508 mutation, and in the efficacy of experimental maneuvers designed to correct it. Taken together with our recent finding that wt CFTR can access additional, *stable* conformations associated with increased open probability at 37 °C,[40] the results presented here implicate unstable or quasi-stable, temperature-sensitive conformations of the ΔF508 CFTR channel that are associated with impaired channel function at human body temperature. The thermal behavior of ΔF508 CFTR channels reported here suggests that, at 37 °C,

dx.doi.org/10.1021/bi300018e | *Biochemistry* 2012, 51, 5113−5124

the mutant channel enters conformational states that are associated with markedly reduced open probability and from which escape, even at reduced temperature, is very slow. These low P_o states may represent thermally induced, partial denaturation of the ΔF508 CFTR channel which may be reversible or irreversible depending on the level of channel stimulation and the presence of second-site mutations.

The Impact of Second-Site Mutations on the Thermostability of ΔF508 CFTR Channel Function Correlates with Increased Open Probability Rather than Increased Yield of Folded Protein in Mammalian Cells. The most common measure of the efficacy of second-site mutations is the maturation of the ΔF508 protein in mammalian cells at 37 °C,[4,8,23−25,29,30] and two recent, complementary reports offer an integrated perspective. Mendoza et al.[6] and Rabeh et al.[7] proposed that significant improvement of ΔF508 CFTR maturation and delivery to the cell membrane at 37 °C requires that two elements of the molecular defect be repaired: subnormal folding efficiency (and thermodynamic stability) of NBD1 and malfunction of the coupling interface that mediates the interaction of NBD1 with the second membrane-spanning domain (MSD2) via the fourth intracellular loop (ICL4). From this perspective, therefore, it was somewhat surprising that a single, second-site mutation, R553M, reported to only modestly improve ΔF508 protein maturation at 37 °C,[4,6,23] nevertheless fully restored wt-like thermal stability to ΔF508 CFTR channel function. Similarly, G550E, R555K, and R1070W; when combined individually with ΔF508, improved protein maturation at 37 °C to at most 18% of wt,[4,6] but nevertheless significantly improved the thermal stability of the double mutant channels. In contrast, I539T, which also slightly improved ΔF508 CFTR maturation in mammalian cells, failed to protect ΔF508 CFTR channels from profound thermal inactivation at 37 °C, although the double mutant recovered fully when returned to room temperature. It is important to emphasize that we are comparing here the thermal stability of ΔF508 CFTR conductance in oocyte membranes with measurements of protein maturation and channel activity (see below) determined in mammalian cells. Previous studies, however, suggest that the two systems share many similarities, including the effects of mutations on protein processing[56] and the influence of correctors and potentiators on channel expression and open probability (unpublished observations).

The three NBD1, second-site mutations that fully or partially protected ΔF508 CFTR channels from thermal inactivation at 37 °C, R553M, R555K, and G550E, share a common effect on ΔF508 CFTR channel function. They either maintain (R553M, present work) or increase (G550E[8], R555K[24]) the open probability of ΔF508 CFTR channels at 37 °C. All three are also predicted to be localized within (G550) or near (R553, R555) the ABC signature sequence, LSGGQ.[1,57] Full or partial protection of ΔF508 CFTR channels from thermal inactivation by these closely grouped, second-site mutations suggests that the thermostability of channel function, as assayed here, is highly dependent on structural features of the protein that, although critical for the gating properties of the channel at 37 °C, may be less important for the recognition of the misfolded protein by cellular quality control mechanisms in mammalian cells. Mendoza et al.[6] reported that these three NBD1 suppressor mutations increased the yield of folded ΔF508 NBD1 in a cell-based assay from 0% (R553M) to 60% (R555K), although even a 60% increase represented less than 20% of the yield of wt protein under the same conditions. This stabilization of NBD1 could act to partially normalize gating-related NBD1-MSD2 interactions thought to be disrupted in the absence of Phe508,[1−5] despite the fact that the processing and delivery of folded, double mutant protein to the cell membrane remains markedly reduced at 37 °C in mammalian cells.

A fourth NBD1 suppressor mutation, I539T, in contrast to G550E, R553M, and R555K, is predicted to lie within an unstructured linker connecting two α-helical portions of NBD1. Accordingly, its impact on the channel function of ΔF508 CFTR at 37 °C was also unique, namely, profound thermal inactivation that reversed completely at 22 °C. This complete reversal is in contrast to the behavior of ΔF508 CFTR channels which only recovered about 40% conductance after an hour at 22 °C. Recovery from partial inactivation was also seen with R555K and G550E, but unlike I539T, both of these second-site mutations also resulted in persistent, steady-state conductance at 37 °C. The basis for this difference may be apparent in the gating behavior of I539T/ΔF508 channels. Recently, Dong et al.[9] reported that the open probability of I539T/ΔF508 channels, studied at room temperature in patches detached from HeLa cells, was only about 20% of that of ΔF508 channels, due primarily to prolonged interburst intervals. This result indicates that I539T, although it appears to significantly improve the folding of ΔF508 NBD1 in a cell-based assay,[6] actually further reduces open probability of the double mutant channel, even at room temperature. This observation is consistent with the previous report of DeCarvalho et al.[25] that I539T/ΔF508, although it exhibited somewhat improved protein processing, was actually inferior to ΔF508 CFTR in a forskolin dose−response assay. Thus the failure of this second-site mutation to restore the thermal stability of channel function correlates with previously reported diminution of open probability.

The ICL4 mutation, R1070W, increased the thermal stability of ΔF508 CFTR channel function, although it did not fully restore the wt-like behavior at 37 °C. Like G550E, R553M, and R555K, this second-site mutation has been associated with increased open probability of the double mutant,[7] an effect attributed to a partial improvement in the interaction between NBD1 and ICL4.[29,57] Combining the ICL4 mutation with an NBD1 suppressor mutation on the ΔF508 background (R555K/R1070W/ΔF508), however, fully restored wt-like thermal stability at 37 °C, an "additive" effect similar to that reported by Mendoza et al [6] in their study of the effect of these mutations on NBD1 folding and ΔF508 CFTR protein yield. The rescue of wt thermal stability of channel function by the R553M mutation, however, indicates that the structural modifications introduced by combining ΔF508 with R1070W are not required for the thermal stabilization of channel gating. Mendoza et al.[6] also reported that combining R1070W with I539T, which alone increased the cellular yield of NBD1 to about 80% of wt, resulted in a yield of the I539T/R1070W/ΔF508 protein that was 76% of the wt level. We found that these channels, although apparently more thermostable that ΔF508 CFTR, nevertheless failed to exhibit full, wt-like thermostability of channel function, a result that is consistent with the adverse effect of the I539T second-site mutation on open probability.[9]

Channel Gating and Functional Stability of ΔF508 CFTR. Although second-site mutations that improve open probability of ΔF508 CFTR channels also appear to promote the thermal stability of channel function, the results presented

dx.doi.org/10.1021/bi300018e | Biochemistry 2012, 51, 5113−5124

here also suggest a negative correlation between the thermal stability of channel function at 37 °C and the stimulation of channel opening and closing, the nature of which is not readily apparent. On the one hand, when oocytes expressing ΔF508 CFTR channels were exposed to 37 °C prior to stimulating the conductance by raising cytosolic cAMP, the conductance increased more slowly than that of unwarmed controls, but eventually reached a similar steady-state value. This result suggests partial, but not complete, protection from thermal inactivation by virtue of the lack of stimulation by cAMP-dependent phosphorylation. Similarly, reducing channel open probability of stimulated ΔF508 CFTR channels with CF172 appeared to afford a degree of protection from thermal inactivation, although two other inhibitors (GlyH-101 and glibenclamide) did not. Likewise, two experimental maneuvers that increased channel open probability, a second site mutation in NBD2 (K1250A) and a CFTR potentiator (P2), actually exacerbated thermal inactivation, although two other potentiators (P1 and Genistein) did not. These observations are reminiscent of the recent report of Alexandrov et al.[58] who found that proline substitutions in NBD1 of ΔF508 CFTR which increased the thermostability of channel function also were associated with reduced open probability at 35 °C.

On the other hand, two of the three potentiators tested, P1 and genistein, although they increased conductance due to ΔF508 CFTR channels, presumably by increasing open probability, did not influence thermal stability of channel function. In addition, the slowing of the time course of stimulation of ΔF508 CFTR channels (lacking any second-site mutation) following a *pre*-stimulation thermal challenge is consistent with a model in which, at 37 °C, unstimulated ΔF508 CFTR channels that are rarely if ever transitioning to an open state, can nevertheless be driven into an inactivated state, but like I539T/ΔF508 CFTR, one from which they can recover spontaneously under stimulating conditions at 22 °C. It may not be possible, therefore, to identify the thermal instability of the ΔF508 CFTR channels with a particular gating state or ensemble of states that represent conducting or nonconducting conformations.

Drug Discovery Implications for Cystic Fibrosis. The recognition of the stimulation-dependent increase in susceptibility of ΔF508 CFTR channels to thermal inactivation, as well as the disparate effects of CFTR potentiators and second-site mutations, has implications for the search for therapeutic small molecules. Protein thermostability can be increased by the binding of small molecules, and recent studies indicate that this reflects, at least in part, ligand-induced *decreases in protein flexibility*.[42,43,59−61] The ligand binds selectively to the native (functional) form and alters the structure of the target protein (or selects for a stable conformer) and thereby protects the protein from thermally induced denaturation. Not surprisingly, these stabilizing ligands tend to be inhibitors, rather than activators.[60]

The variability in the effects of the three CFTR potentiators and three inhibitors examined in this study demonstrates that the relationship between open probability of the mutant channel and thermal stability is not absolute. There may be other compounds that could activate ΔF508 CFTR channels and also protect them from thermal inactivation. In any case the results reported here and by Wang et al.[22] and Aleksandrov et al.[5] strongly suggest that temperature is a critical variable in assays employed to screen compound libraries for CFTR modulators.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting text and Figures S1 and S2 document the lack of major insertion of new, unlabeled R334C/ΔF508 CFTR channels to the membrane during cAMP stimulation, recovery following warming (Figure S1) and potentiation by PG-01 (P2) following warming (Figure S2). Figure S3 depicts the normalized effects of second site mutations on thermal inactivation. Figure S4 documents stimulation-independent temperature response of wt CFTR. Figure S5 illustrates potentiator effects on ΔF508 CFTR conductance at 22 °C. Figure S6 depicts the lack of effect of P2 on thermal sensitivity of wt CFTR. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

Funding by CFF (Dawson08G0 [D.C.D.], Skach05XX0 [W.R.S.]) and NIH (DK045880 [D.C.D.], DK51818 [W.R.S.]).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors gratefully acknowledge the gift of CFTR potentiators and inhibitors from Drs. Alan Verkman and Robert Bridges and the Cystic Fibrosis Foundation (CFFT), without which this work would have been impossible. We are also grateful to the reviewers for insightful comments that greatly improved the paper.

■ ABBREVIATIONS USED

CFTR, cystic fibrosis transmembrane conductance regulator; IBMX, isobutylmethyl xanthine, Isop, isoproterenol; 2-ME, 2-mercaptoethanol; $MTSET^+$, (2-(trimethylammonium)ethyl methanethiosulfonate bromide; P2 (PG-01), 2-[(2−1H-indol-3-yl-acetyl)-methyl-amino]-*N*-(4-isopropyl-phenyl)-2-phenyl-acetamide); P1 (VRT-532), 4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol); CF172 ($CFTR_{inh}$-172), 4-[4-oxo-2-thioxo-3-(3-trifluoromethyl-phenyl)-thiazolidin-5-ylidenemethyl]-benzoic acid)

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