Low Temperature and Chemical Rescue Affect Molecular Proximity of ΔF508-Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and Epithelial Sodium Channel (ENaC)

Background: Mutations in CFTR lead to CF, a lethal inherited disorder. Results: The rescue of mutated CFTR affects its interaction with ENaC. Conclusion: The mutated version of CFTR prevents its close association with ENaC unless ΔF508-CFTR is rescued. Significance: The nature of the CFTR-ENaC interaction is important for the management of the airway pathology, which is now the major cause of mortality for CF patients.

An imbalance of chloride and sodium ion transport in several epithelia is a feature of cystic fibrosis (CF), an inherited disease that is a consequence of mutations in the cftr gene. The cftr gene codes for a Cl⁻ channel, the cystic fibrosis transmembrane conductance regulator (CFTR). Some mutations in this gene cause the balance between Cl⁻ secretion and Na⁺ absorption to be disturbed in the airways; Cl⁻ secretion is impaired, whereas Na⁺ absorption is elevated. Enhanced Na⁺ absorption through the epithelial sodium channel (ENaC) is attributed to the failure of mutated CFTR to restrict ENaC-mediated Na⁺ transport. The mechanism of this regulation is controversial. Recently, we have found evidence for a close association of wild type (WT) CFTR and WT ENaC, further underscoring the role of ENaC along with CFTR in the pathophysiology of CF airway disease. In this study, we have examined the association of ENaC subunits with mutated ΔF508-CFTR, the most common mutation in CF. Deletion of phenylalanine at position 508 (ΔF508) prevents proper processing and targeting of CFTR to the plasma membrane. When ΔF508-CFTR and ENaC subunits were co-expressed in HEK293T cells, we found that individual ENaC subunits could be co-immunoprecipitated with ΔF508-CFTR, much like WT CFTR. However, when we evaluated the ΔF508-CFTR and ENaC association using fluorescence resonance energy transfer (FRET), FRET efficiencies were not significantly different from negative controls, suggesting that ΔF508-CFTR and ENaC are not in close proximity to each other under basal conditions. However, with partial correction of ΔF508-CFTR misprocessing by low temperature and chemical rescue, leading to surface expression as assessed by total internal reflection fluorescence (TIRF) microscopy, we observed a positive FRET signal. Our findings suggest that the ΔF508 mutation alters the close association of CFTR and ENaC.

Mutations in CFTR, the product of the cftr gene, lead to CF, a lethal autosomal recessive disorder. Clinically, CF is characterized by multisystem involvement; however, it is the airway involvement that is the leading cause of morbidity and mortality (1–3). The mutations lead to the disruption of the CFTR function as a Cl⁻ channel (4), which in turn interferes with proper airway hydration. Because chloride secretion and sodium absorption are responsible for the proper airway hydration, inadequate chloride secretion leads to sodium hyperabsorption. This sodium hyperabsorption, mediated by ENaC, is believed to result from CFTR mutations changing the ability of the protein to regulate sodium transport. The role of ENaC was further supported in mice that exhibited CF-like symptoms when the β-subunit of ENaC was overexpressed (5). We and others have shown previously that the two transport molecules are electrophysiologically coupled, where the presence of CFTR decreases the activity of channels formed by ENaCs.

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**ΔF508 CFTR and ENaC Link**

In our recent work, we documented the close association between WT CFTR and ENaC subunits, showing with both co-immunoprecipitation (co-IP) and FRET that there is an interaction between these proteins (6). In this study, we investigated the association of ENaC subunits with the mutated variant of CFTR, ΔF508-CFTR. We found that all three ENaC subunits could be co-immunoprecipitated with ΔF508-CFTR. The results of our FRET findings, on the other hand, did not place ΔF508-CFTR and ENaC subunits in close proximity to each other. However, both chemical and low temperature rescue of ΔF508-CFTR led to an observable FRET signal, placing these two proteins in sufficiently close proximity to each other for a direct association to take place. Our biochemical findings using co-IP are suggestive of an overall association between mutated CFTR and ENaC subunits. In contrast, our FRET findings suggest that the ΔF508 mutation disrupts the close association of CFTR and ENaC, leading to excessive ENaC activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cell culture and maintenance were performed as described previously (6). Briefly, human embryonic kidney 293T (HEK293T) cells (kind gift of Drs. K. Kirk and W. Wang, University of Alabama at Birmingham) were maintained at 37 °C with 5% CO2, 95% air atmosphere in Dulbecco’s modified Eagle’s medium (Invitrogen) at room temperature for 5 min. In a separate 1 ml of Opti-MEM I (without fetal bovine serum and antibiotics). After a 5-h incubation at 37 °C with 5% CO2, 95% air atmosphere in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (HyClone, Logan, UT) and penicillin/streptomycin (100 IU/ml and 100 μg/ml, respectively; Invitrogen). Trypsin (Mediatech, Herndon, VA) was used to subculture the cells a day before the transient transfection. The cells were seeded on coverslips (0.13–0.17 mm thick; Fisher) coated with 1:10 diluted poly-L-lysine (Sigma).

**Transient Transfection**—Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommended protocol. Briefly, 1 μl of Lipofectamine 2000 reagent (the DNA/Lipofectamine 2000 ratio was 0.2 μg/1 μl) was incubated with 100 μl of Opti-MEM® I (Invitrogen) at room temperature for 5 min. In a separate Eppendorf tube, 0.2 μg of each DNA construct was incubated with 100 μl of Opti-MEM® I (Invitrogen). Following the 5-min incubation time, the diluted DNA constructs and Lipofectamine 2000 reagent were combined together and incubated for another 20 min. Then the transfection solution was added to the cells in Opti-MEM® I (without fetal bovine serum and antibiotics). After a 5-h incubation at 37 °C in a 5% CO2, 95% air incubator, the transfection solution was changed to the regular growth medium without antibiotics. Forty-eight hours post-transfection, the cells were rinsed with phosphate-buffered saline (PBS) (Invitrogen) and fixed for 15 min at room temperature with 4% paraformaldehyde (prepared from 20% EM Grade solution, Electron Microscopy Services, Hatfield, PA). After fixation, the cells were rinsed three times with PBS and mounted on glass microscope slides (Fisher) using 0.2% (w/v) n-propyl gallate (Sigma) in 9:1 glycerol/PBS (v/v). The same protocol was used to transfect the cells for co-IP experiments; the DNA/Lipofectamine 2000 ratio was 1 μg/2.5 μl.

**Generation of Fluorophore-tagged CFTR and ENaC cDNAs**—Generation of fluorophore-tagged CFTR and ENaC cDNAs was described previously (6, 7). The CFTR with the enhanced green fluorescent protein (EGFP-CFTR) fused to the N terminus was a kind gift of Dr. B. Stanton (Dartmouth Medical School) (8). To generate the CFTR construct tagged at its N terminus with the enhanced cyan or yellow fluorescent protein (ECFP-CFTR or EYFP-CFTR, respectively), the EGFP was exchanged with ECFP or EYFP following Nhel/Xhol (New England Biolabs (Ipswich, MA) digestion and ligation (6). The ΔF508 mutation was introduced into CFTR to generate ΔF508-CFTR N-terminally tagged with enhanced cyan fluorescent protein (ECFP-ΔF508-CFTR). The human chloride channel 1 (CIC-1) tagged N-terminally (9) with ECFP (ECFP-CIC-1) was a kind gift of Dr. Christoph Fahlke (Institut für Neuropysihologie Medizinische Hochschule Hannover, Hannover, Germany).

**ENaC Antibodies**—Rabbit polyclonal antibodies (Abs) were generated (6) against synthetic peptides in collaboration with Drs. Mark Knepper and Patricia A. Gonzales (National Institutes of Health, Bethesda, MD).

**Western Blot Analysis of CFTR and ENaC** (10, 11)—HEK293T cells were transfected with 2 μg of ECFP-CFTR construct. Forty-eight hours post-transfection, cells were lysed with radioimmunoprecipitation assay buffer (Pierce) complemented with Complete® protease inhibitor mixture (Roche Applied Science) at 4 °C. After centrifugation (15,800 × g for 10 min at 4 °C), non-soluble material was discarded. 100 μg of total cell lysate was subjected to 6% SDS-PAGE (Invitrogen), followed by transfer to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) that was probed with 1:10,000 diluted polyclonal Ab against the second nucleotide binding domain of CFTR (kind gift of Dr. J. Hong, University of Alabama at Birmingham Cystic Fibrosis Research Center). Detection was accomplished using a secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP) (Dako) and chemiluminescence. Western blot analysis of tagged ENaC constructs was as described previously (6).

**ΔF508-CFTR and ENaC Co-IP Experiments**—ΔF508-CFTR and ENaC co-IP experiments were performed following a protocol described previously (11). Briefly, the HEK293T cells were transfected with CFTR and appropriate combinations of ENaC subunits and incubated for 48 h. The cells then were lysed with 0.2% Triton X-100 in PBS with Complete® protease inhibitor mixture (Roche Applied Science) at 4 °C. The cell lysates were centrifuged at 15,800 × g for 10 min at 4 °C; the non-soluble material was discarded. The supernatant was incubated for 2 h with 1 μg of carboxyl-terminal CFTR monoclonal Ab clone 24–1 (R&D Systems, Minneapolis, MN) cross-linked to A/G-agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Then the beads were pelleted and rinsed three times in PBS with 1% Triton X-100. Immunoprecipitated CFTR and bound proteins were analyzed on SDS-PAGE and transferred to a PVDF membrane, and the samples were processed for Western blotting using Abs raised against ENaC and CFTR (R&D Systems).

**Surface Co-IP Assay**—A combination of surface biotinylation and co-IP (12) was used to identify changes in surface expression following low temperature rescue. HEK293T cells were co-transfected with ΔF508-CFTR and all three ENaC subunits (one of the three subunits in αβγ ENaC was tagged with EYFP-α-EYFP-βγ, β-EYFP-αγ, and γ-EYFP-αβ). The cells were incubated at 27 °C for 48 h after an initial 24-h incubation at 37 °C.
The cells were washed three times with ice-cold PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂ and incubated with sulfo succinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (Pierce; 1.5 mg/ml in ice-cold PBS) for 30 min at 4 °C. The cells were then washed twice with freshly prepared quenching solution (50 mM glycine in PBS) and lysed by radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 7.4) with protease inhibitor mixture tablets (Roche Applied Science). Cell lysates were homogenized by passing fifty times through a 22-gauge needle and centrifuged (13,200 rpm, 30 min at 4 °C). Protein concentration of the supernatant was measured using the bicinchoninic acid (BCA) protein assay (Pierce). Immunoprecipitation of the supernatant was measured using the biotinavidin beads (100 μg; Pierce) were added to the antibody-lysate complex and incubated overnight at 4 °C. After washing three times with lysis buffer, Protein G beads (100 μl; Pierce) were added to the antibody-lysate complex and incubated overnight at 4 °C. Beads were collected after centrifuging the lysates at 5000 rpm for 5 min and washed three times with lysis buffer. After removing residual buffer, bead-IP-CFTR interactions were disrupted by incubating the beads with 150 μl of 1% SDS for 2 h at 37 °C. The sample was centrifuged at 5000 rpm for 5 min, and the supernatant was mixed with 450 μl of lysis buffer. After washing three times with lysis buffer, streptavidin beads (100 μl; Pierce) were added to the samples and incubated overnight at 4 °C. The beads were collected as described above. After removing residual buffer, samples were heated at 95 °C for 6 min in 1× Laemmli sample buffer (25% glycerol, 2% SDS, 0.01% bromphenol blue, 10% β-mercaptoethanol, 62.5 mM Tris HCl, pH 6.8) and subjected to SDS-PAGE over 10% separating gels. Following transfer, membranes were blocked for 1 h with 5% bovine serum albumin (BSA) in Tris-buffered saline (100 mM Tris (pH 7.5), 150 mM NaCl), with Tween 20 (0.1%; Bio-Rad) (TBS-T) at room temperature and blocked for 1 h with 5% bovine serum albumin (BSA) in Tris-buffered saline (100 mM Tris (pH 7.5), 150 mM NaCl), with Tween 20 (0.1%; Bio-Rad) (TBS-T) at room temperature and incubated with appropriate constructs (see “Transient Transfection”). FLIM measurements were carried out on a Zeiss 710 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY) equipped with Becker & Hickl GmbH (Berlin, Germany) time domain FLIM system. The excitation source was a 405-nm picosecond pulsed diode laser with a 50-MHz repetition rate and a pulse width of around 70 ps integrated into the Zeiss confocal system. The fluorescence decay curves were analyzed using SPC Image software (Becker & Hickl). The apparent mean lifetime (Tₚₛ) of the donor was derived from double exponential fit of the decay curves. An apparent FRET efficiency (E) was calculated according to the equation, E = 1 – (Tₚₛ/Cₚₛ). The difference between groups was calculated using Student’s two-tailed test; the significance was set at p < 0.05.

**RESULTS**

**Analysis of Fluorophore-tagged Constructs**—Western blot analysis demonstrated that the fusion of a 27-kDa fluorescent tag (ECFP or EYFP) to CFTR and ΔF508-CFTR did not affect our ability to immunoprecipitate or detect the protein (Fig. 1A). Also, fusion of the fluorescent tag to the WT CFTR did not interfere with its trafficking as evidenced by predominant intracellular channel function (6, 8). In contrast, tagged ΔF508-CFTR did not affect plasma membrane delineation of the staining (supplemental Fig. S1A) (6, 8) or Cl⁻ channel function (6, 8). In contrast, tagged ΔF508-CFTR constructs show predominant intracellular staining without plasma membrane delineation (supplemental Fig. S1B), suggestive of the impaired trafficking characteristic of this CFTR mutant (15).

We have previously shown that Western blot analysis of tagged ENaC revealed the expected bands matching the molecular weight of the ENaC subunits with the fluorescent tags (6). When tagged ENaC subunits were expressed in cells, microscopy showed a more prevalent ER staining; this pattern of staining is characteristic of ENaC constructs and indicates poor traf-
Nevertheless, functional currents with typical ENaC characteristics were recorded from cells transfected with tagged ENaC constructs (7, 16, 17).

ENaC Subunits Interact with ΔF508-CFTR by IP—The recent electrophysiological findings (18), co-IP data (6), and FRET results (6) have suggested a close association between WT CFTR and ENaC. Here, we assessed a physical interaction between the mutated version of CFTR (ΔF508-CFTR) and ENaC. HEK cells were transfected with wild-type or ΔF508-CFTR alone or in combination with ΔF508-ENaC, and then individual subunits were probed. No ENaC signal was detected when ΔFa, Δfb, or Δγ-ENaC or ΔF508-CFTR was expressed alone. The left panels show that ΔFa, Δfb, or Δγ-ENaC can only be detected when expressed in HEK-293 cells. Therefore, ENaC subunits are not endogenously expressed in those cells. These experiments were repeated at least three times, and similar results were obtained.

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Next, we explored the possibility that the random aggregation as a result of overexpression could lead to co-IP of ENaC with ΔF508-CFTR. The lysates of the cells expressing β-ENaC were mixed with the lysates of the cells expressing ΔF508-CFTR; no co-IP signal was observed, indicating that association of β-ENaC and ΔF508-CFTR did not occur postlysing (Fig. 3A). As another control, we performed the co-IP experiment with ΔF508-CFTR and another channel, ECFP-ClC-1. As Fig. 3B demonstrates, no co-IP signal was observed between ΔF508-CFTR and ECFP-CIC-1. The anti-GFP monoclonal Ab JL-8 (BD Living Colors), which also recognizes CFP, was used to confirm the expression of ECFP-CIC-1 in the cells (Fig. 3B). Our findings indicate that ΔF508-CFTR and ENaC can associate with

FIGURE 1. Western blot analysis of tagged CFTR constructs. A, the blot was carried out using a polyclonal Ab raised against the NBD2 domain of CFTR and probed with an anti-rabbit HRP using chemiluminescence. ENaC subunits interact with both WT and ΔF508 CFTR. α-ENaC (B), β-ENaC (C), and γ-ENaC (D) could be co-immunoprecipitated using a C-terminal CFTR antibody when co-expressed with ΔF508-CFTR; αβγ-ENaC and ΔF508-CFTR were co-expressed, and then individual subunits were probed. No ENaC signal was detected when α-, β-, or γ-ENaC or ΔF508-CFTR was expressed alone. The left panels show that α-, β-, or γ-ENaC could only be detected when expressed in HEK-293 cells. Therefore, ENaC subunits are not endogenously expressed in those cells. These experiments were repeated at least three times, and similar results were obtained.

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each other in a specific manner when co-expressed in HEK293T cells.

**FRET Measurements by Acceptor Photobleaching**—Next, we utilized FRET imaging to assess the association between ΔF508-CFTR and ENaC. FRET can only be observed when fluorescent donor and acceptor proteins are less than 10 nm apart (20–22). In the acceptor photobleaching mode of FRET, bleaching of the acceptor will result in an increase in donor fluorescence if FRET was occurring prior to bleaching (13). The ratios of the donor (ECFP) fluorescence emission before and after selective photobleaching of the acceptor are used to calculate an apparent FRET efficiency (E).

As in our studies previously described (6), cells transfected with ECFP-linker-EYFP fusion protein served as a positive control, whereas cells transfected with separate ECFP and EYFP constructs served as a negative control for FRET (supplemental Fig. S2). A 514-nm laser was used to bleach the regions of interest. Images of ECFP and EYFP were taken before and after selective photobleaching of the acceptor. The ECFP-EYFP fusion construct (positive control) demonstrated ~30% FRET efficiency, whereas our negative control yielded less than ~2% (Fig. 4 and supplemental Fig. S2).

The HEK293T cells were transfected with ECFP-ΔF508-CFTR and all three ENaC subunits; one of the three subunits tagged at the C terminus with EYFP. The green box shows the area of bleaching. We did not find an increase in CFP fluorescence after selective bleaching of the acceptor; the energy transfer efficiency values (~3%) were not significantly different from those of the negative controls (~2%, p > 0.44). A bar graph with a summary of E values for all combinations of tagged ENaC and ECFP-ΔF508-CFTR is given in Fig. 4. These measurements indicate that FRET did not occur between ΔF508-CFTR and any ENaC subunits, suggesting that the misfolding of the ΔF508-CFTR prevents its close association with ENaC. As mentioned previously, we found an appreciable increase in CFP fluorescence after selective photobleaching with all combinations of ENaC and WT CFTR; the energy transfer efficiency values averaged ~7% (p < 0.05), but values as high as ~17% were observed (6).

Previous studies have shown that low temperature (19) and some chemical correctors (23–27) can partially improve the folding and consequently trafficking of the ΔF508-CFTR (28–32). Because our model, based on lipid bilayer studies, shows a functional interaction between the two proteins at the bilayer, intracellularly retained ΔF508-CFTR may not have a similar interaction with ENaC because the surface expressed ΔF508-CFTR. We therefore attempted further FRET experiments in cells transfected with ΔF508 and exposed to either low temper-
Ature or chemical correctors. Recently, Robert et al. (33) reported that glafenine, an anthranilic acid derivative, was able to partially correct ΔF508-CFTR misprocessing in several in vitro model systems. We therefore used glafenine for our ΔF508-CFTR and ENaC FRET experiments. We found that glafenine (10 μM in DMSO, 48-h incubation) resulted in a positive FRET signal (~11% versus ~3% in negative controls, p < 0.003) between ΔF508-CFTR and ENaC subunits (supplemental Fig. S4 and summary bar graph in Fig. 5A); this treatment, however, does not affect positive and negative FRET controls (Fig. 5A, bars 1 and 2). Next, we attempted FRET measurements between ΔF508-CFTR and ENaC following low temperature (27 °C for 48 h after an initial 24-h incubation at 37 °C following transfection) rescue of ΔF508-CFTR (supplemental Fig. S5 and summary bar graph in Fig. 5B). Again, we were able to measure a significant FRET signal (~13% versus ~2% in negative controls, p < 0.009) between ΔF508-CFTR and ENaC subunits (supplemental Fig. S5); this treatment does not affect positive and negative FRET controls (Fig. 5B).

Additionally, we attempted the FRET measurement between ΔF508-CFTR and ENaC subunits following the rescue by a combination of Corr3a and Corr4a (supplemental Fig. S6 and summary bar graph in Fig. 5C). Corr3a (quinazoline compound) and Corr4a (thiazole compound) are small molecules that act as pharmacological chaperones, promoting the folding and trafficking of ΔF508-CFTR (28–32). However, the interpretation of these FRET findings was complicated by the fact that with these “specific” correctors, we observed increased FRET efficiency in our negative FRET controls (~10% compared with <2% without the correctors). This interesting finding is probably due to the specificity and mechanism of action of these correctors, suggesting that perhaps a GFP-based FRET assay might be useful to screen/identify novel correctors.

Next, we examined the specificity of our positive FRET signal between ΔF508-CFTR and ENaC subunits following partial correction of ΔF508-CFTR by low temperature and chemical correctors. For this purpose, we repeated our low temperature and chemical correction experiments with ECFP-CIC-1 and ENaC subunits. Supplemental Fig. 7A demonstrates the life-time images of cells expressing positive (ECFP and EYFP fusion construct; left) and negative FLIM controls (ECFP and EYFP constructs co-expressed separately; right); the decrease in the mean ECFP lifetime to 0.8 ns (left) from 2.5 ns (right) indicates the energy transfer from ECFP to EYFP. Supplemental Fig. 7B demonstrates the lifetime images of cells expressing CIC-1 tagged with ECFP. In the absence of ENaC tagged with EYFP, the mean lifetime of ECFP tagged to CIC-1 averaged around 2.3 ns before (supplemental Fig. S7B, far left panel labeled 37 °C) and following low temperature and chemical correction (supplemental Fig. S7B, panels labeled 27 °C, glafenine, Corr3a, and Corr4a). We did not observe a decrease in the donor lifetime, an indication of FRET taking place, when ECFP-CIC-1 was co-expressed with any ENaC subunit tagged with EYFP (supplemental Fig. S8, A–C). The mean ECFP lifetime tagged to CIC-1 (in the presence of any EYFP-tagged ENaC) was 2.2–2.5 ns, not significantly different from that in the absence of EYFP (p >

![Figure 4](image-url) Apparent FRET efficiencies, E, obtained after acceptor photo-bleaching of HEK293T cells co-transfected with vectors encoding ECFP-ΔF508-CFTR and αβγ-ENaC (in αβγ-ENaC, one each of the three subunits tagged at the C terminus with EYFP). Also, E is shown for control constructs. Error bars, S.D. of at least five cells imaged.

![Figure 5](image-url) Apparent FRET efficiencies, E, obtained after acceptor photo-bleaching of HEK293T cells co-transfected with vectors encoding ECFP-ΔF508-CFTR and αβγ-ENaC (in αβγ-ENaC, one each of the three subunits tagged at the C terminus with EYFP) following the partial correction by glafenine (A), low temperature (B), and combination of Corr3a and Corr4a (C). Also, E is shown for control constructs. Errors bars, S.D. of at least five cells imaged.
FIGURE 6. Apparent FRET efficiencies, $E$, obtained following fluorescence lifetime measurements of HEK293T cells co-transfected with vectors encoding ECFP-CIC-1 and $\alpha\beta\gamma$-ENaC (in $\alpha\beta\gamma$-ENaC, one each of the three subunits tagged at the C terminus with EYFP) with no treatment (A) and following partial correction by low temperature (B) and glafenine (C). Not shown are the results of the partial correction with Corr3a and Corr4a. Also, $E$ is shown for control constructs. Error bars, are S.D. of at least three cells imaged.

DISCUSSION

In our recent study (6), we demonstrated the close association between WT CFTR and ENaC subunits using biochemical and FRET approaches. In this study, we probed the possible link between the most common disease-causing variant of CFTR, ΔF508-CFTR, and ENaC. Our co-IP findings are suggestive of a physical association between ΔF508-CFTR and ENaC subunits. However, our FRET findings indicate that the mutated version of CFTR prevents a close association between ΔF508-CFTR and ENaC unless ΔF508-CFTR is rescued by low temperature and chemical correctors.

The co-IP of the two transport molecules would, at first, suggest that there is some direct interaction between the two proteins. It has often been argued that in overexpression systems, the presence of an interaction as detected by co-IP is an artifact due to the superphysiological levels of the two proteins. However, as we showed previously with WT CFTR (6), the interaction of ΔF508-CFTR is specific for the ENaC subunits because we do not see an interaction with another transport protein, the chloride channel CIC-1. Because ΔF508-CFTR is poorly trafficked secondary to the ΔF508 mutation and it is well described that WT ENaC subunits are poorly trafficked (16, 34), it is possible that the co-IP interaction occurs in the ER or during targeting for degradation or retention. This idea is supported by the knowledge that CFTR expression alters ENaC trafficking.

TIRF Microscopy—We next assessed the trafficking of ΔF508-CFTR following low temperature and chemical rescue by utilizing TIRF microscopy. This approach allows the study of tagged molecules near or at the plasma membrane. Data shown in supplemental Fig. S9 show TIRF images of cells transfected either with WT (supplemental Fig. S9A) or ΔF508-CFTR (supplemental Fig. S9B) tagged on the N terminus with EYFP. The pattern of ΔF508-CFTR expression shows predominant cytoplasmic distribution indicative of various membrane-bound organelles (supplemental Fig. S9B; inset depicts the epifluorescence image of the same cell) as compared with a staining pattern closer to or at the plasma membrane of WT CFTR (supplemental Fig. S9A; inset depicts the epifluorescence image of the same cell). Rescue by low temperature and chemical correctors resulted in an improved membrane distribution of ΔF508-CFTR (supplemental Fig. S10); interestingly, the combination of the low temperature and chemical correctors had a more pronounced effect on ΔF508-CFTR (supplemental Fig. S10D) near or at plasma membrane expression. These TIRF results verify that low temperature and chemical rescue improves the trafficking of ΔF508-CFTR.

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The co-IP of the two transport molecules would, at first, suggest that there is some direct interaction between the two proteins. It has often been argued that in overexpression systems, the presence of an interaction as detected by co-IP is an artifact due to the superphysiological levels of the two proteins. However, as we showed previously with WT CFTR (6), the interaction of ΔF508-CFTR is specific for the ENaC subunits because we do not see an interaction with another transport protein, the chloride channel CIC-1. Because ΔF508-CFTR is poorly trafficked secondary to the ΔF508 mutation and it is well described that WT ENaC subunits are poorly trafficked (16, 34), it is possible that the co-IP interaction occurs in the ER or during targeting for degradation or retention. This idea is supported by the knowledge that CFTR expression alters ENaC trafficking. Although testing this would be challenging, it is a good example of the weaknesses of co-IP to either rule in or rule out a direct physical interaction. Although a positive signal validates that in these conditions the two proteins are in some complex together, it does not necessitate a direct physical interaction, and similarly a negative signal would not rule out the possibility that there is never a direct physical interaction.

Conversely, FRET cannot occur beyond the physical limit of 10 nm, with FRET efficiency changing dependent on the relative positions of the fluorescent tags (35). Our FRET findings did not place the fluorescent tags on ΔF508-CFTR in sufficiently close proximity to the fluorescent tags on ENaC for an association to occur, and the identity of ENaC subunits did not change the efficiency of energy transfer (Fig. 4). The simple interpretation of these data is that the deletion at residue position 508 prevents the close association of CFTR and ENaC. Indeed, this fits well with the pathogenesis of CF airway disease, which postulates that the mutation prevents the down-regulation of ENaC by CFTR (1). This CFTR-mediated inhibition of ENaC activity might involve the gating (18) of ENaC and/or the
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Protection of ENaC from proteolysis (36) that activates near silent ENaCs (37). These possibilities are not mutually exclusive; the close proximity or direct physical association of CFTR with ENaC conceivably might represent a step to protect the ENaC subunits from proteolysis and subsequent activation.

It is possible that the physical targeting of ΔF508-CFTR is different from that of ENaC subunits, which effectively prevents FRET. For example, ENaC might be predominantly at the surface while ΔF508-CFTR is retained intracellularly, but perhaps if both were at the surface, the two could interact directly and be observed by FRET. However, this is not the case because a fluorescent signal was clearly visible for both proteins inside the cell and at the surface (supplemental Fig. S3, A–C). The energy transfer efficiencies measured in delineated plasma membrane versus broad regions of interest across the cells were not significantly different from each other (Fig. 4 and supplemental Fig. S3, A–C). The lack of FRET signal with the ΔF508-CFTR (without the partial rescue by the low temperature and chemical correctors) also validates that the FRET signal between WT CFTR and ENaC was not due to overexpression, tight packing, or some other artifact as could have been conjectured with our prior work (6).

Of note, however, our prior work showed WT CFTR and ENaC to FRET intracellularly as well as at the surface (6). The lack of FRET shows that there is some significant difference between the interactions of the ΔF508-CFTR mutant and WT CFTR. The absence of these interactions could be very important for the design and implementation of therapeutics. These findings demonstrate that the ΔF508 mutation has the potential to disrupt the CFTR and ENaC link, a disruption that may account for the observed hyperabsorption of sodium in CF airways. Thus, correcting the trafficking of this mutant, while allowing for proper chloride transport, may still not fix the sodium transport problem. Perhaps proper trafficking of the mutant will not be sufficient to correct the pathophysiology of the end stage lung disease found in CF. More acutely, perhaps measures of ENaC function as noted by the effect of amiloride on nasal potential difference and recovery of CFTR function in the nasal epithelia (43). These data taken together would reiterate that ΔF508-CFTR has a different functional relationship from WT CFTR, as captured by the differences in our FRET efficiencies.

It may also be important to determine what led to the elevation of the negative control signal with the use of Corr3a/4a. Both Corr3a and Corr4a have been shown to improve the folding and trafficking of ΔF508-CFTR (30); it is noteworthy that Corr4a has shown more specificity for correction of ΔF508-CFTR than Corr3a (30). One possibility is that the elevation of negative control signal may indicate some potential off-target specificities of the correctors because folding and trafficking is a complex and multistep process; further studies will be needed to address this. Another possibility is that these correctors are capable of causing artificial cross-linking or complexing of the free fluorescent proteins, bringing them into close enough proximity to cause a positive FRET signal. This is very plausible because both the positive control of the fused YFP/CFP and the free floating YFP/CFP increase by approximately equivalent amounts (5%), as shown in Fig. 5C. Unlike our transport proteins, the FRET controls are not restricted to the membrane, and thus it is possible that these small molecule correctors are able to force the control fluorophores to complex in a manner in which the tethered transport protein tags are unable to. Regardless, the data clearly establish that with correction there is statistically significant FRET between ΔF508-CFTR and ENaC subunits. Further, it is noteworthy that our study has been carried out in a model system with recombinant ΔF508-CFTR and ENaC subunits; it remains to be explored if the same is true in in vivo circumstances. In synopsis, WT CFTR and ENaC subunits interact at the plasma membrane and internally,
as evidenced by our prior FRET studies. This study establishes that although ΔF508-CFTR and ENaC subunits appear to interact directly via co-IP, their cytoplasmic tails do not FRET intra-cellularly and only weakly FRET when trafficking is corrected. This regulation of Na⁺ transport, following the ΔF508-CFTR correction, probably occurs directly; however, our results cannot exclude the possibility of a role for an adaptor protein(s) in this association. Future work will need to examine which domains of these proteins are mediating this interaction and assess the nature of this interaction under dynamic conditions, such as when either transport protein is activated or inhibited. Understanding the structural and kinetic nature of this interaction may be critical to better managing the airway pathology that is the current life-limiting factor in patients afflicted with CF.

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