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Restoration of R117H CFTR folding and function in human airway cells through combination treatment with VX-809 and VX-770

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[1]*Marsico Lung Institute/Cystic Fibrosis Research Center, University of North Carolina, Chapel Hill, North Carolina;* [2]*Department of Cell Biology and Physiology. University of North Carolina, Chapel Hill, North Carolina; and* [3]*Department of Biochemistry, School of Medicine, University of North Carolina, Chapel Hill, North Carolina*

Gentzsch M, Ren HY, Houck SA, Quinney NL, Cholon DM, Sopha P, Chaudhry IG, Das J, Dokholyan NV, Randell SH, Cyr DM. Restoration of R117H CFTR folding and function in human airway cells through combination treatment with VX-809 and VX-770. *Am J Physiol Lung Cell Mol Physiol* 311: L550–L559, 2016. First published July 8, 2016; doi:10.1152/ajplung.00186.2016.—Cystic fibrosis (CF) is a lethal recessive genetic disease caused primarily by the F508del mutation in the CF transmembrane conductance regulator (CFTR). The potentiator VX-770 was the first CFTR modulator approved by the FDA for treatment of CF patients with the gating mutation G551D. Orkambi is a drug containing VX-770 and corrector VX809 and is approved for treatment of CF patients homozygous for F508del, which has folding and gating defects. At least 30% of CF patients are heterozygous for the F508del mutation with the other allele encoding for one of many different rare CFTR mutations. Treatment of heterozygous F508del patients with VX-809 and VX-770 has had limited success, so it is important to identify heterozygous patients that respond to CFTR modulator therapy. R117H is a more prevalent rare mutation found in over 2,000 CF patients. In this study we investigated the effectiveness of VX-809/VX-770 therapy on restoring CFTR function in human bronchial epithelial (HBE) cells from R117H/F508del CF patients. We found that VX-809 stimulated more CFTR activity in R117H/F508del HBEs than in F508del/F508del HBEs. R117H expressed exclusively in immortalized HBEs exhibited a folding defect, was retained in the ER, and degraded prematurely. VX-809 corrected the R117H folding defect and restored channel function. Because R117 is involved in ion conductance, VX-770 acted additively with VX-809 to restore CFTR function in chronically treated R117H/F508del cells. Although treatment of R117H patients with VX-770 has been approved, our studies indicate that Orkambi may be more beneficial for rescue of CFTR function in these patients.

cystic fibrosis; rare mutation; heterozygote; R117H; W1282X; VX-809; VX-770; Orkambi

CYSTIC FIBROSIS (CF) IS CAUSED by mutations in the Cl^- channel CFTR, a protein kinase A (PKA)-regulated anion channel (10, 30). CFTR functions in the apical membrane of various epithelia and is responsible for Cl^- and HCO_3^- transport that is vital for epithelial fluid and electrolyte homeostasis. Defective CFTR channel function in CF airways impairs mucous hydration and the ability to expel inhaled pathogens effectively (15, 30). This leads to chronic airway infection, inflammation, and lung fibrosis that is responsible for the majority of CF morbidity and mortality.

CFTR is a large polytopic membrane protein that contains two membrane spanning domains (MSD), two nucleotide binding domains (NBD), and a regulatory region (28). Assembly of CFTR into an ATP-gated ion channel is dependent on proper domain folding and interdomain assembly events that are facilitated by sequential action of and cytosolic heat shock protein (Hsp)70, Hsp40, and endoplasmic reticulum (ER) luminal calnexin (3, 21, 29). There are more than 2,000 disease-associated CFTR mutations that impact CFTR biogenesis and function (32, 35), and the activity of protein quality machinery influences the extent that mutant forms of CFTR are triaged between biogenic and degradation pathways (22, 39, 40). The most common mutation associated with CF is F508del, which destabilizes NBD1 and thereby arrests channel assembly. F508del CFTR is retained in the endoplasmic reticulum and selected for premature degradation by the Hsp70-dependent E3 ubiquitin ligases RMA1/RNF5 and CHIP (16, 22, 39).

F508del CFTR accumulates in a nonaggregated and detergent soluble state and can be brought back onto its folding pathway with small molecule folding correctors (25, 40). However, the assembly defect in F508del CFTR is severe and monotherapy with currently available drugs that suppress misfolding of CFTR is of low efficacy (7, 33). The activity of repaired F508del CFTR is also low because F508del causes a defect in the ATP-dependent gating of the channel (35). Therefore, use of a folding corrector (VX-809) in combination with a channel potentiator (VX-770) is being employed to restore CFTR function homozygous F508del CFTR patients. Around 70% of CF patients of European origin are homozygous for the F508del CFTR mutation, the aforementioned drug combination termed Orkambi was recently approved to treat this patient population (20).

Biochemical studies show VX-809 stabilizes MSD1 of CFTR, thereby increasing the efficiency of F508del CFTR folding (27). Action of VX-809 on MSD1 has broad benefits as this leads to allosteric stabilization of CFTR folding intermediates and thereby overcomes folding defects caused by F508del. Fortuitously, VX-809 action is not specific to F508del

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as it also acts on wild-type (WT) CFTR, and it restores proper folding and function to some, but not all, rare CFTR mutants (27, 34). Approximately 30% of the CF patient population has a compound heterozygous CFTR mutant genotype comprised of the F508del mutant in conjunction with a different, less common mutated CFTR allele (10). The ability of folding correctors to allosterically suppress folding inefficiencies in CFTR suggests that pharmacotherapies developed to act on F508del mutant can also be employed to restore proper hydration of epithelial cells in patients with rare CF-causing disease alleles (2, 35).

R117H is a relatively common CF-causing mutation that is associated with a moderate CF phenotype that has an early clinical manifestation (24). Sweat chloride levels are markers for CF and they range between 60 and 105 milliequivalents/l (meq/l). F508del/F508 del patients have sweat chloride of ∼100 meq/l, whereas F508del/R117H 5T patients have sweat chlorides of ∼75 meq/l (32). The R117H allele is also found in patients who have WT/R117H genotypes and do not have CF but present in the clinic with congenital absence of the vas deferens (14). The form of the R117H gene that is inherited by CF patients has a shortened polythymidine tract (9T to 5T) that causes variable skipping of exon 9 during processing of the CFTR mRNA (6). Exon 9 encodes the first 60 amino acids of NBD1, so its in-frame deletion results in the production of a CFTR variant that cannot fold or function properly and is rapidly degraded. In addition, the pools of R117H CFTR that are translated from properly spliced mRNA exhibit a 25% reduction in ion conductance and a >10-fold reduction in channel opening probability (41). R117H CFTR is associated with CF in ∼2,000 patients and since it has defective channel function its activity might be restored through monotherapy with the CFTR channel potentiator VX-770. However, restoration of lung function was modest in R117H patients who participated in a clinical trial with VX-770 (Kalydeco) (23).

To further understand factors that limit the ability of VX-770 to restore R117H CFTR function in lung airways we examined the biogenesis of R117H CFTR in primary human bronchial epithelia cells and in model epithelial cell lines. We report that mutation of R117H causes a kinetic defect in the folding of nascent R117H CFTR that leads to its accumulation in large pools within the endoplasmic reticulum of polarized epithelial cells. Remarkably, unlike F508del, the folding defect caused by R117H is highly correctable. By itself the folding modulator VX-809 is sufficient to restore significant levels of R117H CFTR function at the cell surface. The activity of R117H CFTR in VX-809-treated cells is further increased through acute treatment with the potentiator VX-770. Additive increases in activity of CFTR in R117H/F508del human bronchial epithelial (HBE) cells are also detected in response chronic treatment with the combination of VX-809 and VX-770. This is of relevance for treatment of CF patients carrying the R117H mutation, because currently these patients have access to VX-770 (Kalydeco) but not to VX-809 and VX-770 combination treatment (Orkambi).

METHODS

Culture of primary HBE cells. Human lung tissue was procured under The University of North Carolina Office of Research Ethics Biomedical Institutional Review Board Approved Protocol No. 03–

1396. Primary HBE cells were harvested and cultured using established procedures previously described in detail (11, 13).

Production of UNCCF7T cell lines that express inducible WT and mutant CFTR. UNCCF7T cells were created by lentiviral addition of Bmi-1 and hTERT to primary HBE cells from a CFTR G542X homozygous donor as previously described for non-CF and F508del homozygous donors (12). These cells retain the ability to polarize and differentiate as previously reported for the other genotypes. Due to the double stop G542X mutation the fragment of CFTR synthesized is degraded prematurely and the cells serve as a CFTR negative backdrop for functional and biogenic studies on WT and mutant CFTR.

To express CFTR in UNCCF7T, the parental cells were expanded to P7 and then seeded into treated Costar polystyrene plate (Corning, Corning, NY). Cells were grown for 3 days in bronchial epithelial growth medium (BEGM) to ∼40% confluence. Cells were washed with PBS and then lentivirus that was packaged with pTRIPZ-CFTR or pTRIPZ-R117H-CFTR was added to 1 ml of conditioned media that contained 4 μg of polybrene (Sigma-Aldrich, St. Louis, MO) and the mixture was added directly to cells. Cells were incubated in viral solution for 6 h before being washed twice with PBS and incubated in BEGM for 48 h. Transduced cells containing the pTRIPZ vector were selected with 1 μg/ml puromycin (Sigma-Aldrich) and expanded on polystyrene plates.

CFBE cell lines that express inducible form of WT and mutant CFTR. The immortalized CF bronchial epithelial CFBE41o- (provided by J.P. Clancy, Univ. of Cincinnati) was seeded at a density of ∼20% onto treated Costar polystyrene plates (Corning, Corning, NY). Cells were grown for 2 days in DMEM media (Life Technologies, Carlsbad, CA) and washed with PBS. Then, 500 μl of lentivirus that harbors pTRIPZ-CFTR, pTRIPZ-F508del-CFTR, or pTRIPZ-R117H-CFTR were mixed with 1 ml of conditioned of DMEM was added to cells. Cells were incubated the virus enriched media for 6 h before being washed with PBS and then incubated in DMEM for 48 h. Transduced cells containing the pTRIPZ vector were selected with 1 μg/ml puromycin and expanded on polystyrene plates.

Air liquid interface culture of stably transduced CFBE and UNCCF7T cell lines. Cells were removed from polystyrene plates with trypsin and reseeded into the apical compartment of 0.4-μm pore size Snapwell (Corning Costar, Cambridge, MA) or Millicell inserts (Millipore, Bedford, MA) coated with placental type IV collagen (Sigma-Aldrich) at a density of 0.2 × 10⁶ cells/insert. Cells were grown with air liquid interface (ALI) media in the basolateral and apical compartment for ∼7 days, until confluent. Cells were then maintained at an ALI. Three times per week, the apical surface was washed with PBS and medium was replaced only in the basal compartment. Cells were grown at ALI for ∼2–3 wk. Before experimentation, cells were grown in ALI media containing 3 μg/ml doxycycline (Sigma-Aldrich) for 48 h to induce expression of CFTR. Cells were then incubated in the presence or absence of 5 μM VX-809 (Selleckchem, Houston, TX) and doxycycline for an additional 48 h.

Western blot analysis of endogenous CFTR expression in polarized HBEs. Indicated HBE cultures that were treated as described in the figure legends and were dissolved in NP-40 lysis buffer containing protease inhibitors. CFTR was immunoprecipitated from cleared lysates with rabbit anti-CFTR polyclonal antibody 155 and protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). Immune precipitated material was eluted from the agarose beads and separated by SDS-PAGE and CFTR was detected by Western blot with primary CFTR antibodies (596, 217) or actin and then fluorophore-conjugated secondary antibodies. Proteins were visualized using a LI-COR Odyssey scanner.

Measurement of CFTR activity in Ussing chambers. Ion transport measurements with HBEs were performed in modified Ussing chambers (Physiologic Instruments) under voltage clamp-conditions using Acquire & Analyze (version 2.3) software (Physiologic Instruments). Reference measurements [potential difference (P_d)] and transepithelial resistance (R_t) were obtained for each culture. Short circuit current

(I_{sc}) was measured every 20 s and recorded digitally after bilateral equilibration at pH 7.4 for 10 min in 5 ml of pH 7.4 Krebs bicarbonate Ringers (KBR; 115 NaCl mM, 25 NaHCO$_3$ mM, 2.4 K$_2$HPO$_4$ mM, 1.2 CaCl$_2$ mM, 1.2 MgCl$_2$ mM, 0.4 KH$_2$PO$_4$ mM, and 5 D-glucose mM). Chamber temperature was maintained at 36°C ± 1°C by a circulating water bath and KBR was bubbled with 95% O$_2$-5% CO$_2$ throughout the experiment.

For measurements of CFTR currents in polarized CFBE cells, the cultures were equilibrated in bilateral KBR for a minimum of 5 min, and I_{sc} was measured in a 5- to 120-mM Cl$^-$ gradient. The gradient was generated by replacing the apical buffer with a high-potassium, low-chloride buffer (HKLC, pH 7.4) that contained the following: 15 mM gluconate, 95 mM gluconate, 25 mM NaHCO$_3$, 2.4 mM K$_2$HPO$_4$, 1.2 mM CaCl$_2$, 1.2 mM MgCl$_2$, and 0.4 mM KH$_2$PO$_4$.

Agonists and inhibitors used to modulate CFTR activity were purchased from Sigma Chemical. In the presence of amiloride (100 μM, apical), forskolin (10 μM) was applied bilaterally to induce cAMP activation of CFTR, and VX-770 (5 μM, apical) was applied to further activate CFTR. Currents detected were sensitive to the CFTR channel blocker CFTR$_{inh}$-172 (10 μM apical). As an internal control, UTP (100 μM) was used to activate calcium activated chloride channels (CaCC) at the conclusion of the experiment. Data were exported and analyzed in Microsoft Excel, and slope values were subtracted for accuracy when necessary. Mean, SD, and SE measurements were calculated for each culture. Statistical analysis was performed by an unpaired two-tailed Student's t-test. $P < 0.05$ was considered to indicate statistical significance. Line and Bar graphs were created using Origin 8.6 Software (OriginLab).

Microscopic analysis of R117H localization in UNCCF7T cells. UNCCF7T cells grown on millicell inserts were frozen in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA), and frozen sections were prepared as previously described (4). Fixation of cells for microscopy was performed using a method similar to that previously described (1). Frozen sections placed into −20°C acetone and incubated at room temperature for 5 min. Slides were then removed from acetone and dried at room temperature. Slides were placed in a humidified chamber and covered with 100 μl of the cross-linker bis[sulfosuccinimidyl] suberate (BS3; 100 μM) for 30 min. BS3 solution was washed from the coverslips and replaced with 100 μl of 1 M glycine for 15 min. Glycine was then removed, Slides were washed three times with PBS, and 100 μl of blocking buffer (PBS with 1% FBS, 1% milk) was put onto the coverslips for 1 h. Slides were incubated with primary antibody diluted in blocking buffer for 1 h. CFTR was immunostained with the mouse monoclonal antibodies 596 and 660 (9). Rabbit anti-Calnexin was obtained from Sigma-Aldrich. Slides were then incubated with Texas Red and Alexa Fluor 350-conjugated secondary antibodies (Life Technologies) diluted in blocking buffer with DAPI stain for 1 h. Slides were washed three times with PBS and mounted onto slides using mounting media. Samples were imaged with a IX81 motorized inverted microscope (Olympus, Center Valley, PA). Images were acquired using Meta-Morph (Molecular Devices, Sunnyville, CA).

Chronic CFTR rescue with VX-809 and VX-770. CF HBE cultures were maintained at ALI on 12-mm Millicell Cell Culture Inserts (Merck Millipore) for 20–28 days and then treated with 0.1% DMSO (vehicle) or 3 or 5 μM VX-809, 5 μM VX-770, or both compounds in 0.1% DMSO. Vehicle and/or compounds were added to ALI media and then exposed to cultures basolaterally (2 ml) and apically (20 μl) for 48 h before Ussing chamber measurements, with a fresh media plus vehicle or compound exchange after 24 h.

RESULTS

To test the concept that compound heterozygous CF patients can be treated with drugs developed to restore function of F508del CFTR, the action of VX-809 on function and folding of CFTR in patient cells with R117H/F508del mutations was explored. To calibrate the experimental system we compare results obtained with R117H/F508del to those obtained with HBE cells that harbor W1282X/F508del, F508del/F508del, and E60X/F508del. W1282X is a relatively common CFTR mutation allele that encodes a truncated mRNA that is subject to non-sense mediated decay and the protein product from W1282X mRNA cannot fold and is degraded prematurely (18, 35). E60X is a rare allele that produces a truncated mRNA and almost no CFTR protein product, so studies with W1282X/F508del and E60X/F508del enable us to estimate the contribution of the single copy of F508del to total CFTR activity R117H/F508del heterozygous HBEs.

HBEs are grown on semipermeable supports to promote polarized growth and then shifted to an ALI to drive differentiation of primary epithelial cells to a state that closely resembles that of lung airway epithelium (11). Fully differentiated HBEs were treated with 3 μM VX-809 for 48 h. and levels of folded CFTR, termed the C-form, and the nonnative and ER-localized pool, termed the B-form, of CFTR were measured by Western blot (Fig. 1A). Under control conditions, the B-form of CFTR was detected in all cells regardless of genotype. The C-form of CFTR was detected in R117H/F508del and was increased around 2-fold by VX-809. The levels of the C-form in E60X/F508del CFTR and W1282X/F508del were also increased to a lesser extent by VX-809. The twofold increase in the signal for folded CFTR in VX-809 treated R117H/F508del and the more modest increase in the C-form detected in E60X/F508del CFTR and W1282X/F508del HBEs suggest that biogenesis of R117H CFTR can be positively impacted by folding correctors.

Measurements of transepithelial short-circuit currents in Ussing chambers indicated that CFTR Cl- channel activity in R117H/F508del HBE cells was also increased above that detected in F508del/F508del HBE cells following 48 hrs. chronic treatment with 3 μM VX-809 (Fig. 1, *B* and *D*). Forskolin- mediated cAMP activation of CFTR-mediated currents reached 7 μA/cm^2 in R117H/F508del whereas currents in W1282X/F508del, E60X/F508del, and F508del/F508del (Fig. 1, *B* and *F–G*) were below 1 μA/cm^2. When we measured the forskolin-stimulated CFTR currents in Ussing chambers in quadruplicate for normal HBE cells that were derived from eight different individuals ($n = 32$) and grown under these same growth conditions, CFTR currents were on average 33 ± 2 μA/cm^2 (±SE). Uncorrected CFTR activity in R117H/F508del is therefore <20% of normal, but is increased to 33% of normal (11 μA/cm^2) by VX-809 (Fig. 1, *B* and *D*). Addition of the channel potentiator VX-770 increased CFTR activity in R117H/F508del an additional 30%. These results demonstrate that the combined use of VX-809 and VX-770 can effectively restore CFTR activity in HBEs from heterozygous R117H/F508del CF patients.

R117H causes a folding defect in CFTR that is suppressed by VX-809. To evaluate the impact of R117H on CFTR biogenesis, independent of F508del, studies were conducted in transiently transfected HEK293 cells (Fig. 2, *A–D*). Based on molecular modeling, of the CFTR structure (31), R117 is predicted to be located at the tip of TM1 and be exposed in the ER lumen during biosynthesis of CFTR (Fig. 2A). With the use of the program Eris (38) to estimate changes in CFTR stability caused by R117H, R117H is predicted to change the delta-delta-G of CFTR by +1 kcal/mol. R117H does not dramati-
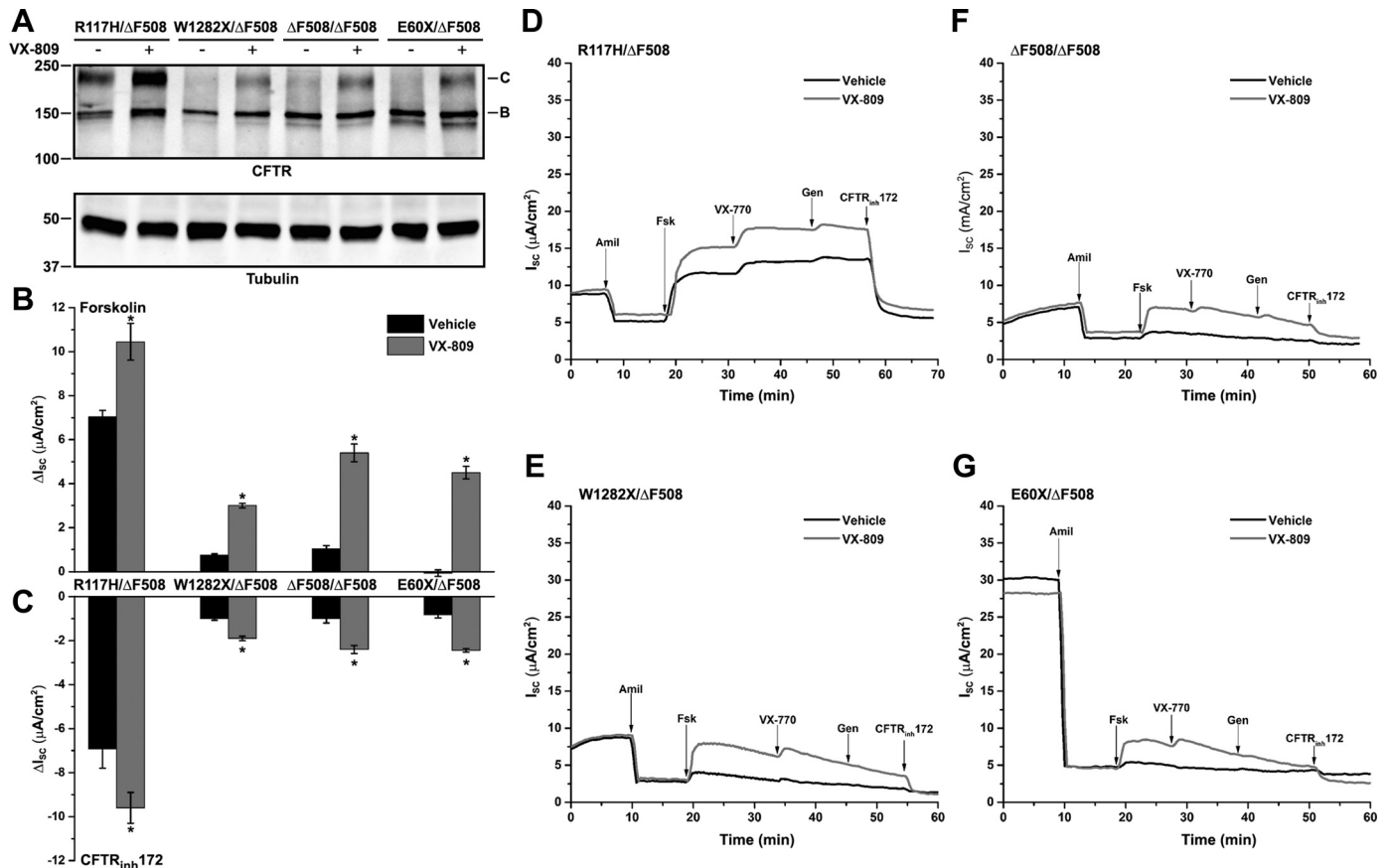
Fig. 1. Biochemical and functional response of cystic fibrosis transmembrane conductance regulator (CFTR) to corrector (VX-809) and potentiator (VX-770) in primary human bronchial cells (HBE) with heterozygous cystic fibrosis disease alleles. *A*: impact of VX-809 on accumulation of CFTR in HBE cells with the genotypes R117H CFTR 5T/F508del, W1282X/F508del, and E60X/F508del. HBE cultures were chronically treated for 48 h with 3 μM VX-809 or 0.1% DMSO (vehicle). Treatment with VX-809 resulted in an increase in CFTR protein levels. Bar graphs represent the mean net response (*n* = 4 cultures per genotype) to acutely added forskolin (*B*) and CFTR$_{inh}$172 (*C*) in Ussing chamber experiments. Representative traces (*D–G*) illustrate the response of each heterozygous mutation to the stimulation protocol. *Values that are significantly different than control, *P* < 0.05 by Student's t-test.

cally destabilize the final structure of properly folded CFTR but has the potential to cause subpopulations of CFTR folding intermediates to go off pathway. Indeed, R117H causes a defect in folding of newly synthesized CFTR: the C-form of R117H CFTR accumulates at 70% lower levels than the C-form of CFTR (Fig. 2*B*, *lane 1* vs. *2*). Accumulation of folded R117H CFTR was stimulated to different extents by addition of different folding correctors to the cell culture media (Fig. 2*B*). C18 and VX-809 are structurally related and were the most efficacious, driving accumulation of folded R117H CFTR to between 100 and 200% of WT CFTR levels (*lane 2* vs. *7* and *8*). C17 is a corrector that is structurally related to Corr4a and acts on MSD2 to suppress folding defects in CFTR (17). Addition of C17 to cell culture media restored R117H CFTR folding to 60% of WT levels (*lane 2* vs. *6*). R117H exhibits a biogenic defect that is effectively suppressed by action of VX-809 on MSD1. The lack of cooperation between VX-809 and C17 on R117H CFTR indicated that modulation of the conformation of MSD1 by VX-809 is sufficient to overcome folding defects caused by the R117H mutation.

To assess the impact of R117H on the kinetics of CFTR folding we took advantage of the fact that the efficiency of CFTR folding is limited by the basal activity of the cytosolic and ER associated molecular chaperones that scan the ER

membrane for proteins that contain non-native protein structure and selected them for proteasomal degradation (16, 39). Reductions in ERQC activity increase efficiency of CFTR folding, and increasing activity has reciprocal effects. Interestingly, inhibition of the proteasome with bortezomib resulted in a threefold increase in the accumulation of the ER-localized B-form of R117H CFTR (Fig. 2*C*), but a corresponding increase in the folded C-form was not detected. While the presence of bortezomib increases the pool of the B-form of CFTR, there was no dramatic increase in the efficacy of C18 and VX-809 at accumulation of the folded R117H CFTR (Fig. 2*C*).

In pulse-chase studies, the conversion of the B-form of R117H CFTR to the folded C-form occurred at low efficiency (Fig. 2*D*). VX-809 was able to suppress kinetic defects in R117H CFTR biogenesis because the drug increased the pool of the newly synthesized B-form and promoted its relatively efficient conversion to the C-form of R117H CFTR (Fig. 2*D*, compare *lane 1* of respective *bottom left* and *right*). Thus R117H causes a kinetic defect in CFTR folding that is suppressed by folding modulators. These data help explain why VX-809 strongly stimulates accumulation of the C-form of CFTR in R117H 5T/F508del HBE cells (Fig. 1*A*).
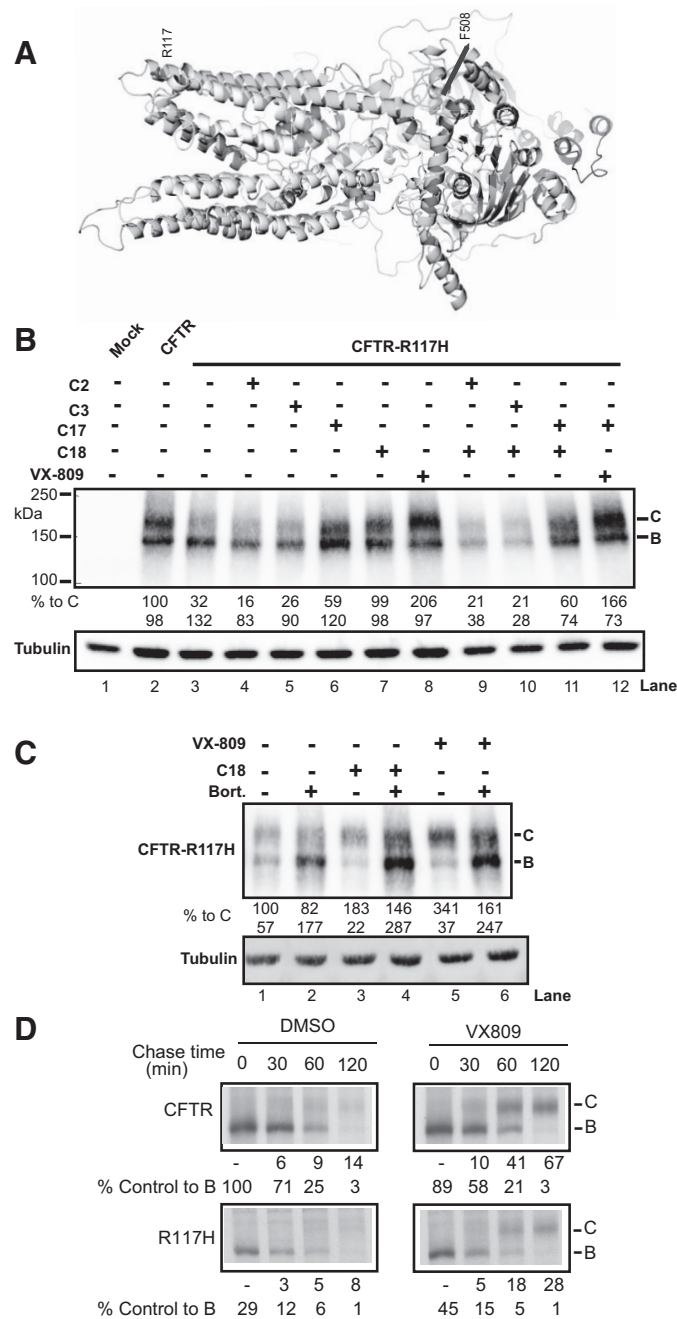
Fig. 2. R117H CFTR exhibits a folding defect that is corrected by VX-809. *A*: model for CFTR structure showing the location of R117 and F508. *B*: the impact of folding modulators on the accumulation of the folded C-form and ER localized B-form of CFTR in HEK293 cells. *C*: the impact that inhibition of the ERAD pathway with Bortezomib has on the ability of folding modulator to promote accumulation of the folded C-form of R117H CFTR. *D*: analysis of kinetic defects in R117H CFTR folding as determined by pulse-chase analysis. HEK293 cells were transfected with the indicated form of CFTR and incubated with the indicated folding modulator at a concentration of 5 μM. Cells were lysed and levels of CFTR were measured by western blot 18 h after addition of the specified folding modulator. For pulse-chase studies HEK293 cells were transfected with CFTR or R117H CFTR and treated with DMSO or 5 μM VX-809 for 1 h before the 20-min labeling period with ^{35}S-translabel. CFTR was immuneprecipitated from cells extracts after the indicated incubation time and ^{35}S-CFTR was detected by SDS-PAGE and autoradiography.

VX-809 suppresses intracellular accumulation of R117H CFTR. The aforementioned pulse-chase studies suggest that a population of newly synthesized R117H CFTR goes of pathway and is degraded before it can fold. Therefore, the extent that R117H leads CFTR to be retained within intracellular membranes of polarized human lung epithelial cells was evaluated (Fig. 3). To conduct these experiments, we employed 593X CFTR UNCCF7T cells that are generated from human bronchial epithelial cells (12). 593X CFTR UNCCF7T cells are devoid of CFTR because they are homozygous for 593X CFTR, but the truncated 593X mRNA is CFTR degraded by nonsense mediated decay; hence no CFTR signal is detected by fluorescence microscopy in these cells (Fig. 3, *row 1, left*). 593X CFTR UNCCF7T cells are therefore functionally null hosts for CFTR and permit study of WT CFTR and R117H CFTR biogenesis in native-like differentiated epithelial cells. When we expressed CFTR in UNCCF7T, we saw successful trafficking of it to the cell surface (Fig. 3, *row 2*). In contrast, large intracellular pools of R117H CFTR were detected in locations that overlap with calnexin, and detection of R117H CFTR on the cell surface was difficult (Fig. 3, *row 3*). Calnexin staining is used a marker for the ER membrane, and the ER of polarized UNCCF7T is present throughout the cell, so calnexin staining is wide spread. Notably, the presence of VX-809, caused a shift in localization of R117H CFTR to the surface of polarized UNCCF7T cells (Fig. 3, *row 4*). These data support the conclusion that R117H CFTR has a highly correctable trafficking defect that limits its cell surface accumulation in polarized human epithelial cells.

R117H CFTR function in polarized bronchial epithelial cells is restored by combination treatment with VX-809 and VX-770. To assess the extent that functional defects reported for R117H CFTR can be restored in polarized epithelial CFBE41o- cells that only express this CFTR variant, we measured cAMP-dependent CFTR channel activity in Ussing chambers (Fig. 4, *A–I*). Forskolin acts to increase intracellular level of cAMP and stimulated currents in cells that express R117H CFTR to around 30 μA/cm^2 (Fig. 4, *A* and *B*) vs. 5 for F508del (Fig. 4, *D–F*) and 100 for WT CFTR (Fig. 4, *G–I*). Treatment of cells with VX-809 for 24 h before assay lead to a large increase in accumulation of the C-form of R117H (Fig. 4*C*), and more modest increases in F508del (Fig. 4*F*) and WT (Fig. 4*I*). Increases in levels of R117H CFTR correlated with VX-809-dependent increases in its channel activity to around 100 μA/cm^2 (Fig. 4*A*). R117H CFTR currents were further increased by the channel potentiator VX-770 and were oblated by the CFTR inhibitor CFTR$_{inh172}$ (Fig. 4*B*), so R117H CFTR activity can be restored by VX-809 and VX-770 to level that surpass that of F508del CFTR. R117H CFTR has both folding and functional defects that are strongly suppressed by modulator and potentiator therapy in polarized CFBE cells.

Maximum CFTR function in R117H/ΔF508 HBE cells is achieved with chronic treatment using the combination of VX-809 and VX-770. Acute addition of VX-770 to HBEs treated with VX-809 for over 24 h maximally stimulates CFTR in F508del/F508del cells. Yet, long-term exposure of HBEs with VX-770 is reported to reduce F508del CFTR folding efficiency and decrease its stability at the cell surface (5, 36). Therefore, the efficacy of chronic treatment with of R117H 5T/F508del HBEs with VX-809 and VX-770 was examined (Fig. 5). Interestingly, CFTR activity in R117H 5T/ΔF508
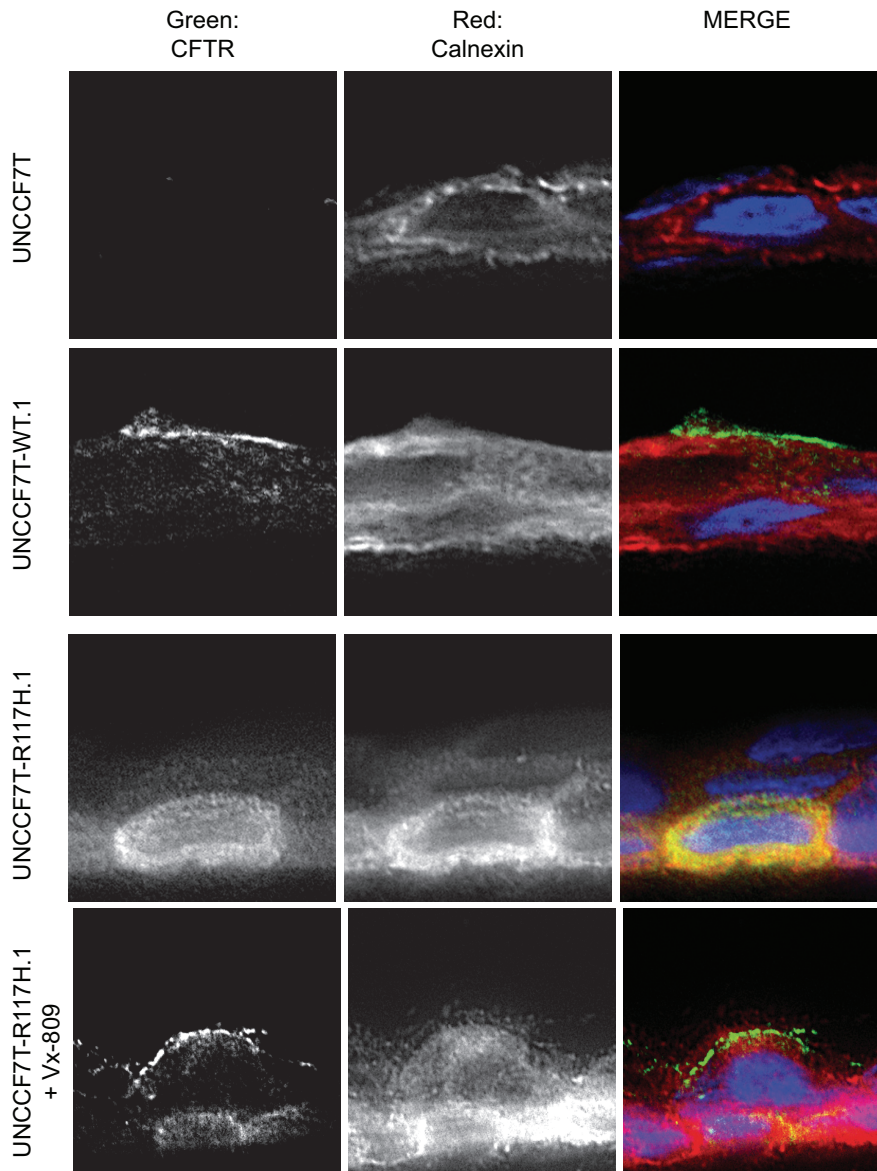
Fig. 3. VX-809 suppresses the intracellular accumulation of R117H CFTR in polarized bronchial epithelial cells. Fluorescence micrographs of frozen sections of UNCCF7T cells expressing wild-type (WT) or R117H CFTR were treated with VX-809 (5 μM) for 48 h. CFTR (green) and Calnexin (red) were immunostained. Nuclei were stained with DAPI (blue).

HBE cells was maximal when cells were treated chronically with 5 μM VX-809 and 1 μM VX-770 (Fig. 5, *A* and *B*). In addition, activity of CFTR in R117H 5T/ΔF508 HBEs was stable on the presence of VX-770 (Fig. 5, *A* and *B*), and this is in contrast to the activity of CFTR in F508del/F508del (5, 36). These data suggest that the R117H CFTR channel is stabile in the presence of VX-770 and VX-809, and combination therapy with these drugs could benefit patients with the R117H 5T/ΔF508 CFTR genotype.

DISCUSSION

Treatment of the entire population of CF patients requires the development of personalized therapeutic approaches because individuals inherit different combinations of disease alleles and disease phenotypes do not always correlate with a patient's genotype (8, 10, 32, 35, 37). Combination therapy with the folding corrector VX-809 and channel potentiator VX-770 for treatment of homozygous F508del/F508del patients was recently approved by the FDA. This advance

spawned interest in the treatment of compound heterozygous CF patients with both VX-809 and VX-770. Yet, individual disease alleles cause different defects in biogenesis and function of CFTR, so study of the efficacy of VX-809/VX-770 at restoring function of CFTR in heterozygous CF patients requires a case-by-case analysis. Two of the most common heterozygous CF genotypes, collectively representing ~4% of the CF population, are R117H CFTR 5T/F508del, and W1282X/F508del. Our studies show that W1282X/F508del HBEs respond poorly to VX-809 and VX-770, yet chronic treatment of R117H/F508del HBEs serves to restore CFTR folding and function to levels that were significantly above those detected in F508del/F508del cells. The FDA-approved VX-770 for CF patients with R117H mutation (23), and data presented in this study and by other groups suggest that restoration of R117H expression and folding will further increase R117H CFTR function as follows: *1*) R117H CFTR 5T mRNA is not processed efficiently, so level levels of the channel that are translated from properly processed transcripts
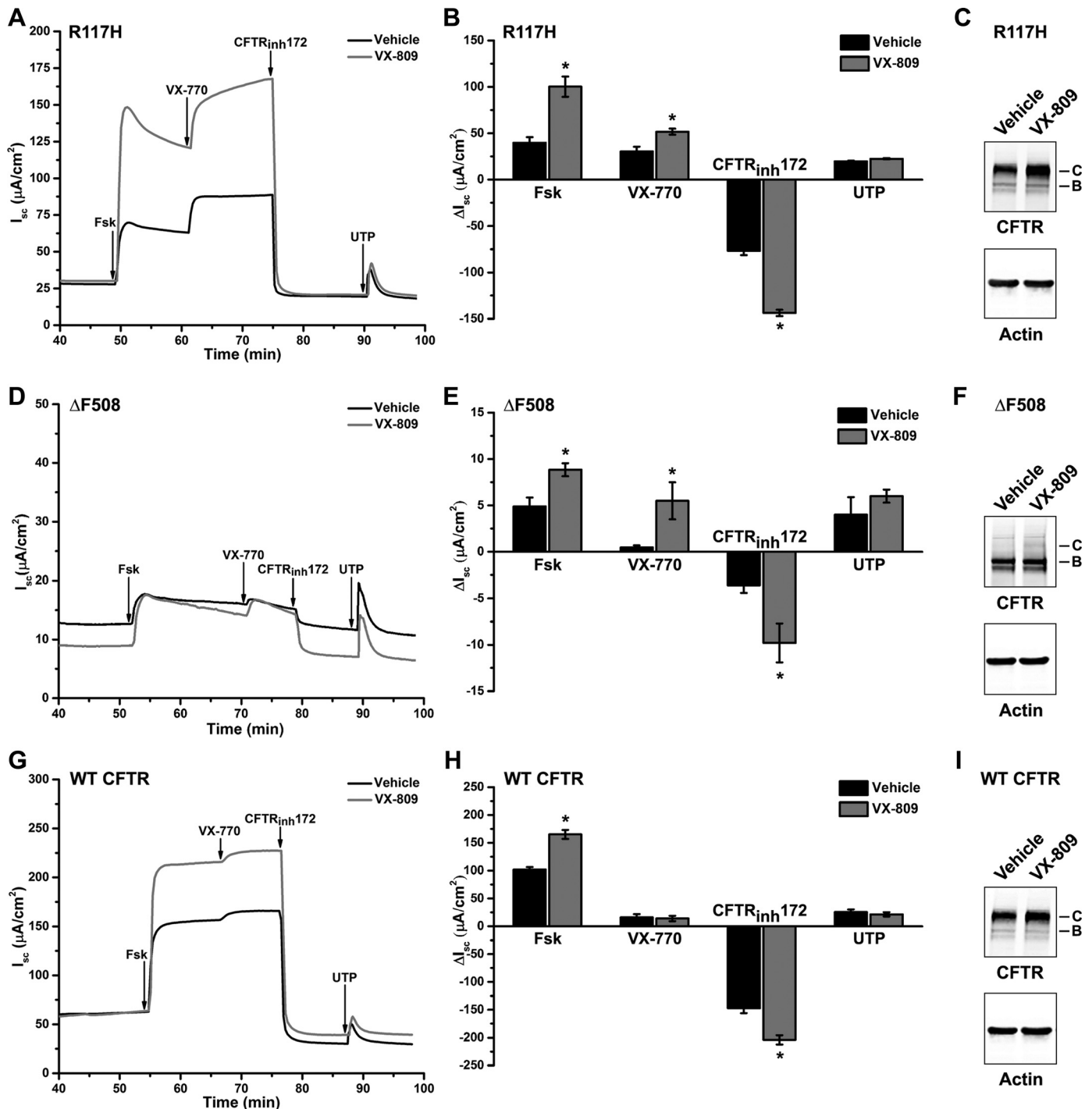
Fig. 4. R117H CFTR activity in polarized CFBE cells is increased several-fold by VX-809 and VX-770. CFBE41o- cells expressing R117H (*A–C*), F508del (*D–F*), or WT CFTR (*G–I*) grown at air liquid interface (ALI) were pretreated with doxycycline for 4 days and then treated with 0.1% DMSO (vehicle) or 5 μM VX-809 for 48 h. Short circuit current (I_{sc}) measurements of CFBE41o- cultures were recorded in Ussing chambers using agonists and inhibitors as described in METHODS. Representative I_{sc} traces of CFBEs expressing (*A*) R117H, (*D*) F508del, or (*G*) WT CFTR are shown. Mean responses (*n* = 3 cultures per genotype) are represented as bar graphs (*B, E,* and *H*). Treatment with VX-809 resulted in increased CFTR function. To visualize the amounts of CFTR protein present in treated cells, Western blot analyses were conducted in CFBE41o- cells expressing R117H (*C*), F508del (*F*), or WT CFTR (*I*). After treatment with vehicle or VX-809, cells were lysed and then proteins were analyzed by Western blot using antibodies against CFTR or actin. Treatment with VX-809 led to an increase in mature CFTR protein levels. *Values that are significantly different than control, $P < 0.05$ by Student's t-test.

are low (19). *2*) We report that nascent R117H CFTR has a folding defect that further limits its cell surface expression and activity. *3*) R117H CFTR has a gating defect that prevents properly folded R117H CFTR functioning as an ion channel to regulate the hydration of airways (41). VX-809 is able to restore folding of R117H CFTR toward normal and VX-770 can overcome gating defect caused by R117H. Yet, neither drug appears capable of suppressing defects in R117H 5T
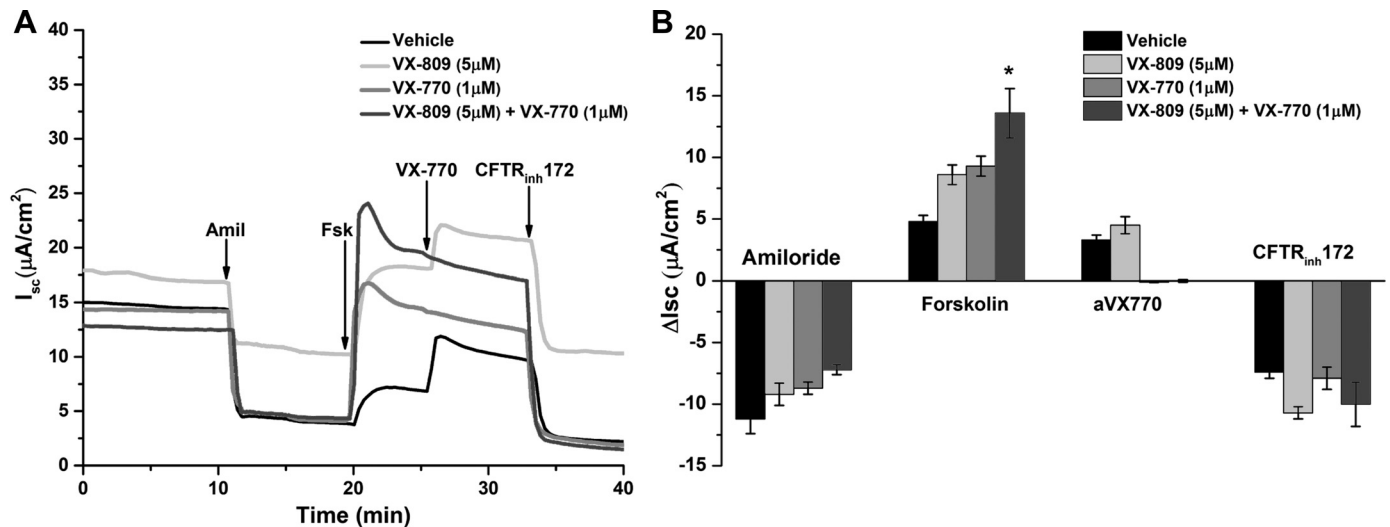
Fig. 5. Chronic treatment with VX-809 and VX-770 additively increases CFTR function in R117H CFTR 5T/F508del HBE cells. *A*: representative I_{sc} traces of HBE cells (R117H/F508del) recorded in Ussing chambers. Cultures were treated with vehicle (0.1% DMSO), 5 μM VX-809, 1 μM VX-770, or 5 μM VX-809 plus 1 μM VX-770 for 48 h. *B*: CF HBE cells chronically treated with VX-809 combined with VX-770 showed a significant increase in responses to forskolin compared with VX-809 or VX-770 alone. Primary CF HBE cultures were derived from 3 different individuals (R117H/F508del, 5T/9T), and 3–4 replicates were performed per individual for a total of 10 measurements per condition. *Values that are significantly different than control, $P < 0.05$ by Student's t-test.

CFTR mRNA processing. However, combination treatment with VX-809 and VX-770 additively restores CFTR function in R117H 5T/F508del cells to 12.5 μA/cm², which is >40% of WT CFTR function. R117H is associated with mild forms of CF, so there is potential for VX-809 and VX-770 to restore CFTR function in R117H 5T CF patients to clinically significant levels.

F508del CFTR is an unstable protein that has a short half-life in the ER and cell surface and these issues contribute to the low efficacy observed for VX-808/VX-770 combination therapy for F508del/F508del patients. An additional issue is suggested by observations that chronic treatment of F508del/F508del HBE cultures with VX-770 decreases the quantity of corrected F508del CFTR channels that accumulate when cells are treated with VX-809 alone (5, 36). This appears to occur because VX-770 destabilizes F508del CFTR and induces its turnover. Interestingly, R117H CFTR does not appear to be destabilized by VX-770 as chronic treatment of R117H/F508del HBEs with both VX-770 and VX-809 results in an additive increase in total CFTR activity. Since chronic combination treatment of R117H cells with VX-809 and VX-770 leads to even higher CFTR-mediated responses, VX-770 does not severely abrogate VX-809-mediated correction of R117H. These data illustrate the need to study the impact of potentiator and folding corrector therapies on mutant forms of CFTR on a case-by-case basis.

Insights into the mechanism for misfolding of nascent CFTR caused by the R117H mutation come from structural modeling of the folded channel (Fig. 2*A*; see METHODS for details). R117 is predicted to be located on the termini of the helix that extends from TM1 into the ER lumen and connects to TM2 through extracellular loop 1. TM1 and TM2 form a bundle of helices that assemble with TM9-12 to become one wing of the CFTR channel, whereas TM3-6 assemble with TM7-8 to form the second wing. H117 is predicted to be oriented differently than R117 and is rotated toward TM10 and this has the potential to decrease the efficiency by which TM1/TM2 are

assembled into a complex with TM9-12. The RMA1/RNF5 E3 and Hsp70/CHIP ubiquitin ligase complexes sense defects in the assembly of membrane domains of CFTR (16, 26, 39), so even subtle defects in the assembly of CFTRs TM segments caused by R117H will be detected by ERQC. VX-809 acts on MSD1 to suppress folding defects in CFTR caused by F508del and is thereby able to increase the efficiency of CFTR membrane domain assembly (27). Therefore, the ability of VX-809 to restore R117H CFTR folding to WT levels is consistent with the drug stabilizing MSD1 to increase the efficiency of intradomain protein assembly interactions to overcome folding defects imparted by R117H.

During the CF communities, initial characterization of CFTR mutation alleles, defects exhibited were grouped into distinct classes that included class I, defective expression; class II, defective folding/trafficking; class III, defective gating: class IV, defective conductance; class V, defective mRNA splicing; and class VI, defective cell surface stability. It is now becoming clear that the same mutation in the CFTR coding sequence and/or intron can cause more than one type of biogenetical and/or functional defect (35). R117H 5T is a complex disease allele is considered a class I, II, IV, and V mutant, whereas, F508del CFTR is a class II, III, VI mutant. The realization that mutations in CFTR can cause a broad spectrum of abnormalities will help design future therapies with different combinations of drugs that target specific defects in CFTR biology. There are over 2,000 disease-related CFTR alleles and many are inherited heterozygously. The studies presented provide and outline on how to study functional correction of mixed CFTR alleles in native and model cells. Future studies will be focused on expanding investigations of molecular mechanisms and pharmacological rescue to other CFTR rare alleles with the goal being to provide in vitro data that will guide the development of personalized approaches to treat CF patients with different genotypes.

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