Increased folding and channel activity of a rare cystic fibrosis mutant with CFTR modulators

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Cystic Fibrosis (CF) is caused by mutations in the CFTR gene (19). The gene product is a PKA-regulated anion channel that is important for fluid and electrolyte transport in the epithelia of lung, gut, and ducts of the pancreas and sweat glands. The most common CFTR mutation, ΔF508, causes a severe, but correctable, folding defect and gating abnormality, resulting in negligible CFTR function and disease. There are also a large number of rare CF-related mutations where disease is caused by CFTR misfolding. Yet the extent to which defective biogenesis of these CFTR mutants can be corrected is not clear. CFTRV232D is one such mutant that exhibits defective folding and trafficking. CFTRΔF508 misfolding is difficult to correct, but defective biogenesis of CFTRV232D is corrected to near wild-type levels by small-molecule folding correctors in development as CF therapeutics. To determine if CFTRV232D protein is competent as a Cl− channel, we utilized single-channel recordings from transfected human embryonic kidney (HEK-293) cells. After PKA stimulation, CFTRV232D channels were detected in patches with a unitary Cl− conductance indistinguishable from that of CFTR. Yet the frequency of detecting CFTRV232D channels was reduced to ~20% of patches compared with 60% for CFTR. The folding corrector Corr-4a increased the CFTRV232D channel detection rate and activity to levels similar to CFTR. CFTRV232D-corrected channels were inhibited with CFTRinh-172 and stimulated fourfold by the CFTR channel potentiator VRT-532. These data suggest that CF patients with rare mutations that cause CFTR misfolding, such as CFTRV232D, may benefit from treatment with folding correctors and channel potentiators in development to restore CFTRΔF508 function.

corrector; potentiator; patch clamp

cystic fibrosis (CF) is caused by mutations in the CFTR gene (19). The gene product is a PKA-regulated anion channel and member of the large family of ATP-binding cassette transporters (18). Located in the apical membrane of various epithelia, this channel is responsible for Cl− and HCO3− transport, which 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 (8, 21). Consequently, the unrelenting airway infection and inflammation result in progressive lung disease and are responsible for the majority of CF morbidity and mortality.

More than 1,000 disease-associated CFTR mutations have been identified. The most common mutation, CFTRΔF508, causes protein misfolding and channel gating defects, which together result in a severely reduced level of apical epithelial anion transport (21). Around 70% of CF patients of European origin are homozygous for the CFTRΔF508 mutation, but there remains a significant number of patients that are compound heterozygotes who inherit the ΔF508 allele along with a different rare mutant allele (3).

An approach to treat CF is correction of the conditional misfolding of CFTRΔF508 (4); yet drugs that accomplish this task are still in development (15). Interestingly, some disease-causing mutations in CFTR elicit folding defects that are less severe and much easier to correct than those caused by ΔF508 (9). Thus functional correction of the protein encoded by mutant alleles other than CFTRΔF508 in compound heterozygous CF patients might be an avenue to treatment.

The function of mutant CFTR can be restored, at least partially, with small molecules, termed “correctors,” which promote proper folding, or “potentiators,” which stimulate activity of mutant channels at the cell surface (23). Interestingly, some of the folding correctors that have been developed and are in use as tool compounds are not specific for the ΔF508 mutation and act to correct misfolding of a number of different CFTR mutants (9). However, the extent to which misfolding of rare CFTR mutant alleles can be corrected and the activity of the corrected mutant channel are just beginning to be investigated.

To address this issue, we screened a collection of CFTR mutants that have single amino acid substitutions in different subdomains for correction of misfolding by the small molecule N-(2-5-chloro-2-methoxyphenylamino)-4’-methyl[4,5′-bithiazolyl-2′-yl]-benzamide (Corr-4a) (9). Corr-4a had modest effects on misfolding of most mutants tested but could correct misfolding of CFTRV232D to wild-type levels in human embryonic kidney (HEK-293) cells. CFTRV232D is a rare CF-causing mutation located in the fourth transmembrane (TM)-spanning domain of the channel protein and is prevalent in CF patients of Spanish origin (2). The folding defect caused by the V232D mutation appears to be due to the introduction of a charged residue into a region of CFTR that is embedded in the lipid bilayer of the endoplasmic reticulum (ER) membrane (17, 24). Since the folding defect in CFTR caused by the V232D mutation is correctable to wild-type levels, CF patients with this allele may benefit from treatment with folding correctors. However, for this to be the case, the corrected protein must function effectively as a PKA-activated anion channel.

Utilizing the patch-clamp technique for direct measurement of the mutant protein’s single-channel behavior, we report on the functional properties of CFTRV232D. We found that the folding defect exhibited by CFTRV232D is not stringent and that a small portion of this protein can fold, accumulate, and...
function at the cell surface. Treatment with Corr-4a increased CFTRV232D protein expression and channel activity to levels that were similar to those of CFTR. Moreover, 4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol (VRT-532), a channel potentiator (27), reversibly increased CFTRV232D channel activity in perfused inside-out patches. Thus CF caused by defective folding of rare CFTR mutants might be treated with existing CFTR modulators.

METHODS

Plasmids, antibodies, and reagents. CFTR expression plasmids pcdNA3.1(+)–CFTR and pcdNA3.1(+)–CFTRV232D have been described previously (9). Antibodies used in this study were as follows: α-CFTR MM13-4 (Millipore, Billerica, MA) and α-tubulin (Sigma, St. Louis, MO). Corr-4a and VRT-532 were provided by the Cystic Fibrosis Foundation and Dr. Robert J. Bridges (Rosalind Franklin University, Chicago, IL).

Cell culture and transfection. HEK-293 cells were obtained from Agilent (Cedar Creek, TX; formerly Stratagene) and maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Invitrogen) at 37°C in an atmosphere of 5% CO₂. Cell transfections were performed using Effectene reagent (Qiagen, Valencia, CA).

Analysis of CFTR steady-state levels. Steady-state levels of CFTR and CFTRV232D protein were determined by Western blot analysis. HEK-293 cells were transiently transfected with pcdNA3.1(+)–CFTR or pcdNA3.1(+)–CFTRV232D. Transfected cells were allowed to recover for ~18 h before addition of DMEM (10% FBS and antibiotics) supplemented with VRT-532 (10 µM), Corr-4a (5 µM), or vehicle [0.1% (vol/vol) DMSO]. The harvested cells were diluted with 2× SDS sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.05% bromophenol blue, 20% glycerol, complete protease inhibitor cocktail, and β-mercaptoethanol; Roche, Indianapolis, IN), sonicated, and heated at 37°C, then the proteins were resolved on SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose membranes, which were probed with α-CFTR antibody. α-Tubulin antibody was used to indicate loading controls.

Fluorescence measurements. Macrophagic function of CFTR and of the indicated mutants was evaluated in Cos-7 cells using the 6-methoxy-(3-sulfopropyl)quinolinium (SPQ) fluorescence assay (12). Cells were seeded into a six-well plate at a density of 2 × 10⁶ cells/well and transfected with 1 µg of CFTR pcdNA3.1 constructs using Effectene transfection reagents. Transfected cells were replated into a clear-bottom, black 96-well plate and treated with 5 µM Corr-4a (18) for 18 h. Cells were loaded with 10 mM SPQ (Sigma) via hypotonic bath solution containing (in mM) 67.5 NaNO₃, 0.5 Ca(NO₃)₂, 1.2 K₂HPO₄, 0.3 KH₂PO₄, 1 MgSO₄, 5 glucose, 2.5% DMSO (vol/vol), and 10 HEPES, pH 7.4, for 20 min at 37°C. The cells were loaded with iodide using bath solution containing (in mM) 135 NaCl, 2.4 K₂HPO₄, 0.6 KH₂PO₄, 2 MgSO₄, 1 CaCl₂, 10 glucose, and 20 HEPES, pH 7.4, for 5 min at 37°C. Iodide buffer was replaced by halide-free bath solution containing (in mM) 135 NaCl, 2.4 MgSO₄, 2.4 K₂HPO₄, 0.6 KH₂PO₄, 10 glucose, and 20 HEPES, pH 7.4, and incubated for 5 min. CFTR was activated by the addition of 125 µM forskolin, 1 mM IBMX, and 500 µM dibutyryl-cAMP. Fluorescence (excitation wavelength = 326 nm, emission wavelength = 455 nm) was measured at 0 s and up to 240 s post-CFTR activation in 60-s intervals using a microplate reader (FlexStation-3, Molecular Devices, Sunnyvale, CA).

Patch-clamp electrophysiology. Channel activity from cell-at-tached and excised inside-out patches configured from HEK-293 cells pretreated with Corr-4a (24–30 h), 5 µM, and PKA agonist cocktail (0.2–1 h). Patch perfusion and solution exchange were achieved using a commercial solution changer (Warner Instruments, Hamden, CT) with exchange time (t₀ – t₅₀%) = 70 ms, as previously described (5). Corr-4a, VRT-532, and CFTRinh-172 (Sigma) stock solutions (in DMSO) were diluted to the indicated concentrations in bath solution and used within 3 h of preparation. DMSO [final concentration 0.01% (vol/vol)] was included in all patch bath solutions. Macroscopic currents were measured from inside-out macropatches configured with large-diameter pipettes (resistance ~5 mΩ). The time course of the potentiator-modulated current was fitted with a single-exponential model equation for a reversible drug-receptor interaction (11)

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I = I_0 + I_{p0}(1 - e^{-t/r})
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where J is macroscopic current, I₀ is baseline current, Iₚ₀ is potentiator-stimulated current, and r is time constant. Model parameters were given starting estimates, and data convergence was performed automatically using the Levenburg-Marquardt minimization of nonlinear least-squares algorithm (Table Curve 2D, SPSS).

Statistics. Results are reported as means ± SE, with n = number of experiments, unless otherwise stated. Statistical comparisons of SPQ fluorescence and patch-clamp data were made using ANOVA followed by Tukey’s test for evaluating the effect of genetic construct and drug on the mean data. Statistics were computed with SigmaStat.
CFTRV232D protein expression was studied in transiently transfected HEK-293 cells. A representative Western blot analysis of cell extracts is shown in Fig. 1A. Band B depicts the low-molecular-weight, immature protein product after CFTR core glycosylation in the ER. Band C depicts the high-molecular-weight mature CFTR protein that accumulates after complex glycosylation in the trans-Golgi network. Compared with CFTR, only a small pool of CFTRV232D accumulated as band C protein (Fig. 1A). Yet treatment of cells with Corr-4a (24 h, 5 μM) led to accumulation of CFTRV232D band C to levels comparable to CFTR. In related studies, we showed in pulse-chase experiments that Corr-4a increased accumulation of CFTRV232D due to an increase in folding efficiency and not increased expression (9). In addition, we found that VRT-532, a compound identified as a potentiator of CFTR Po, was associated with a modest increase in CFTRV232D band C expression, as was reported for CFTRΔF508 (27). Importantly, the CFTRV232D trafficking defect was surmounted by Corr-4a treatment, with the folded protein achieving CFTR expression levels in HEK-293 cells.

We next examined CFTRV232D channel function compared with CFTR, with and without Corr-4a treatment (Fig. 1B). Channel-mediated halide efflux was estimated in Cos-7 cells using the SPQ fluorescence assay (12). SPQ fluorescence measured 240 s after cAMP-dependent stimulation of CFTR channels was almost fivefold higher than CFTRV232D (Fig. 1B). Consistent with the low-level accumulation of mature glycosylated CFTRV232D protein (Fig. 1A), the SPQ fluorescence in cells that expressed CFTRV232D was not significantly different from background (pcDNA3.1). Strikingly, Corr-4a treatment increased CFTRV232D-mediated SPQ fluorescence to levels indistinguishable from that of CFTR in untreated cells (Fig. 1B). Corr-4a did not significantly increase CFTR-mediated fluorescence over the vehicle control, however.

The lack of functional evidence for CFTRV232D expression in the SPQ assay of untreated cells prompted us to use the patch-clamp technique to investigate the mutant protein’s single-channel characteristics. As shown in Fig. 2A, open channel gating transitions were detected in an excised patch from a HEK-293 cell that was pretreated with PKA agonist cocktail. Channel open transitions were measured over a wide range of voltages, and the unitary current amplitudes were not different from those of CFTR channels or from CFTRV232D channels previously exposed to Corr-4a (Fig. 2B). As we will show, channel activity from Corr-4a-treated channels was reversibly blocked by the CFTR inhibitor CFTR172-inh. Thus CFTRV232D is capable of folding and reaching the membrane surface, where it is functional and displays a Cl− conductance that is indistinguishable from CFTR.

However, when the extent to which CFTRV232D channels reached the membrane surface was evaluated, their frequency of expression was much reduced compared with CFTR. CFTR channel openings were detected in ~60% of patches (26 of 44 recordings), whereas CFTRV232D channel openings were found in only ~24% of patches (15 of 62 recordings; Fig. 2C). Treatment of cells with Corr−4a overnight increased the CFTRV232D channel detection frequency by nearly fourfold, to a level that was not significantly different from CFTR.

A representative current trace of channel activity recorded from a cell pretreated with Corr−4a is shown in Fig. 3A. Visual inspection of the record revealed multiple overlapping channel events, and analysis of the current histogram confirmed the presence of at least two channels in the patch (Fig. 3A). As summarized in Fig. 3B, CFTRV232D activity from Corr−4a-pretreated patches was increased by 3- to 17-fold over the...
activity of wild-type and CFTRV23D channels from untreated patches, respectively. Moreover, channel activity was blocked by the CFTR inhibitor CFTRinh-172. CFTRinh-172 (5 μM) perfused on the intracellular surface of inside-out patches reduced channel activity, as shown in Fig. 3C. CFTRinh-172 inhibition of channel activity was completely reversible. After 1 min of inhibitor washout, channel activity returned to baseline (Fig. 3C).

Summary data from these experiments are shown in Fig. 3D. CFTRinh-172 decreased channel activity by ~87%. Taken together, these results provide strong evidence that CFTRV23D is highly amenable to functional correction in HEK-293 cells.

Single-channel recordings (Fig. 2), which are far more sensitive than the SPQ assay (Fig. 1B), suggest that the small fraction of CFTRV23D protein observed to fold (Fig. 1A) exhibits bona fide Cl− channel activity. A number of control experiments indicated that Cl− currents detected in HEK-293 cells that express uncorrected CFTRV23D protein do, in fact, originate from this channel. HEK-293 cells express a voltage-regulated Cl− channel (10), but background channel transitions were detected infrequently in untransfected cells. In 2 of 11 cell-attached patch recordings of 3–10 min duration, a total of 4 channels with amplitude of ~0.4 pA and open dwell times of 1–5 s (NPo < 0.03) were detected (data not shown). Channel activity was transient, with detection occurring within the 1st min of seal formation and subsiding within 3 min of recording. Similar channel events were also detected in patches from untransfected cells pretreated with Corr-4a (data not shown).

Because of such few channel openings, we were unable to characterize unitary conductance. In contrast, channel activity from CFTRV23D-transfected cells was stable for the 3- to 10-min recording window (Figs. 4 and 5). Thus, given their paucity of detection and insensitivity to Corr-4a, background channels were deemed unlikely to confound interpretation of results on CFTRV23D functional expression.

Since CFTRV23D channels could reach the plasma membrane, we asked if channel activity could be stimulated by small-molecule potentiators of CFTR gating (25). As with the experimental protocol in Fig. 3C, we used a perfused patch assay, and cells were pretreated with Corr-4a to enhance channel detection efficiency. Figure 4A shows traces from a representative single-channel recording before, during, and after perfusion of an excised patch with VRT-532. CFTRV23D activity was readily increased within 40 s of VRT-532 exposure, and activity returned to near-baseline level upon potentiator washout. In contrast, Corr-4a was not found to stimulate CFTRV23D activity at a concentration comparable to that which promoted protein folding. Summary data from these experiments are shown in Fig. 4B. VRT-532 reversibly increased channel activity (NPo) 4.2-fold over the baseline level.

The fast stimulation and reversibility of VRT-532 action on CFTRV23D channel activity prompted us to measure the time course and magnitude of the potentiator-stimulated currents. Excised macropatches from Corr-4a-pretreated HEK-293 cells were configured using low-resistance (∼5 mΩ) pipettes. A representative current trace is shown in Fig. 5A. Baseline current was monitored for 40 s during patch perfusion. When the perfusate was switched to VRT-532-containing bath solution, a rapid increase in current was observed. The time course of the current change was fitted with a single-exponential equation (Eq. 2) with a time constant of 8 s. Once the current had stabilized (during ~45 s of VRT-532 exposure), the perfusate was switched to VRT-532-free solution and the current decayed back to the baseline level (time constant = 31 s). VRT-532 reversibly stimulated the CFTRV23D-mediated current 3.3-fold (Fig. 5B), which is in good agreement with current stimulation predicted from the NPo results. The monoexponential time course of the reversible stimulation of CFTRV23D current by VRT-532 is consistent with kinetics of a single drug-receptor interaction (11), although indirect effects (e.g., via drug-lipid partitioning) cannot be excluded (14). Taken together, CFTRV23D functional expression can be enhanced via folding correction in the ER and stimulation of channel activity at the cell surface.
Interestingly, we found that CFTRV232D Cl⁻ channel activity can be restored to high levels in HEK-293 cells by a small molecule, Corr-4a, that was identified in efforts to correct the misfolding of CFTRΔF508 (16). In addition, activity of CFTRV232D can also be enhanced by a potentiator, VRT-532, which is a member of a class of compounds developed to increase the activity of cell surface-localized CFTR gating mutants (26). Thus CF patients that harbor low-frequency mutations, such as V232D, might be treated with small molecules that correct CFTR misfolding or potentiate CFTR channel activity.

Why is it that Corr-4a has very modest effects on correction of CFTRΔF508 misfolding but can dramatically improve folding and increase the detection frequency and Cl⁻ channel activity of CFTRV232D to levels comparable to CFTR? The answer to this question is not clear, but it appears that the ΔF508 mutation causes global defects in CFTR that involve misfolding of nucleotide binding domain 1 and failure of nucleotide binding domain 1 to make proper intramolecular contacts required to stabilize the native CFTR structure (7, 13, 20, 22, 25). Studies on the mechanism of Corr-4a action suggest that it acts to stabilize the TM regions of CFTR (9, 28). In doing so, Corr-4a may prevent the V232D mutation from causing the aberrant hydrogen bonding between TM segments in the bilayer proposed to cause premature degradation of CFTR (24).
lize nascent CFTRΔF508, it appears incapable of correcting the global assembly defects caused by ΔF508 and is ineffective as a folding corrector for this mutant.

Corr-4a can increase the cell surface expression and single-channel activity of CFTRV232D to levels that resemble those of wild-type CFTR. Data from comparison of corrected CFTRV232D and CFTR activity in whole cells, channel detection frequency, and the $N_{P_o}$ from excised patches configured from cells exposed to PKA agonist cocktail support this conclusion. However, it is not yet clear that the open and closed channel dwell times, PKA sensitivity, and ATP dependence on gating between CFTRV232D and CFTR are the same.

An interesting feature of CFTRV232D is that the folding defect it exhibits is not stringent and pools of it escape the ER and reach the cell surface of HEK-293 cells, where it is competent as a Cl$^{-}$ channel. The extent to which this occurs in epithelial cells in lungs of CF patients is not clear. Yet we found that the activity of CFTRV232D in excised patches was potentiated by VRT-532. Thus, if a sufficient quantity of CFTRV232D were to reach the apical surface of lung epithelia,

patients with this mutation might be treated with a potentiator of CFTR channel activity (1).

ΔF508 is the most common CF-causing mutant; yet up to 30% of CF patients are compound heterozygotes who inherit a copy of ΔF508 and a different less-common allele. Restoration of CFTRΔF508 function is clearly achievable; yet our studies on CFTRV232D indicate that less-common CF-causing mutants might be more amenable to functional correction. Therefore, it is important to identify additional CFTR mutants that behave similar to CFTRV232D and identify CF patient populations that might be treated with small molecules developed to correct CFTRΔF508 misfolding or potentiating channel activity (e.g., CFTRG551D). These data will open the door for individualized treatment of subpopulations of CF patients who inherit mutant forms of CFTR that are amenable to functional correction.

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Fig. 4. CFTRV232D activity is stimulated by the potentiator VRT-532. A: representative CFTRV232D single-channel current traces during bath perfusion of an inside-out patch. Baseline channel activity was recorded for 5 min. Baseline trace was obtained at 4.6–5 min. With exposure to 10 μM VRT-532 (20–40 s), as many as 3 open channels were detected. Only single-channel current transitions were detected following VRT-532 washout (Wash, 40–60 s). Patch perfusion of 2 μM Corr-4a (40–60 s) resulted in no discernable increase of channel activity. B: summary data of potentiator action on CFTRV232D activity. VRT-532 increased CFTRV232D $N_{P_o}$ 4-fold over baseline channel activity [from 0.2 ± 0.0 to 0.8 ± 0.1, n = 9 (**$P < 0.001$)] and was completely reversible (Wash $N_{P_o} = 0.2 ± 0.1, n = 8$). Channel activity measured in the presence of Corr-4a was not significantly different from baseline activity [Corr-4a $N_{P_o} = 0.1 ± 0.1, n = 6 (P = 0.43)$]. Channel recordings were made using HEK-293 cells, as described in Fig. 3C legend.

Fig. 5. Macroscopic kinetics and magnitude of the potentiator-stimulated CFTRV232D currents. Macroscopic Cl$^{-}$ currents from excised macropatches were measured. A: representative current trace recorded at −40 mV. After baseline current measurement (0–40 s), VRT-532 (10 μM) perfusion resulted in a rapid increase of the inward current, which stabilized over 60 s and then returned to baseline following potentiator washout. Gray curves depict single-exponential fits to the time course of current stimulation [mean time constant ($\tau_{on}$) = 6.2 ± 0.9 s, n = 8] and recovery after VRT-532 washout from the patch ($\tau_{off} = 24.0 ± 2.2$ s, n = 5). In contrast, Corr-4a (2 μM) was not found to stimulate current. B: summary of fold stimulation of current ($I_{fold-stim}$) achieved with CFTR modulators. $I_{fold-stim}$ was increased 3.3 ± 1.0-fold with VRT-532 [10 μM, n = 9 (**$P < 0.050$)] but was insensitive to Corr-4a [2 μM, n = 6 ($P = 0.143$)]. Currents were recorded using HEK-293 cells, as described in Fig. 4 legend.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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