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Analysis of cystic fibrosis–associated P67L CFTR illustrates barriers to personalized therapeutics for orphan diseases

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Introduction

Mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) lead to damage of respiratory, hepatic, pancreatic, and other exocrine tissues (1–3). CFTR functions as a chloride and bicarbonate ion channel, for which over 1,900 disease-associated mutations have been implicated. These defects are predominately classified into 1 of 6 groups, according to underlying molecular mechanism (abnormalities of CFTR ion channel gating, folding, premature truncation, aberrant splicing, etc.). Significant limitations of the current CFTR classification scheme have become increasingly evident. For example, approximately 90% of cystic fibrosis (CF) patients carry at least one copy of the prevalent F508del allele, which is classically associated with a protein biogenesis (class II) defect (3–5). More detailed analysis has established that F508del mediates disease through not only aberrant protein maturation, but also dysfunctional gating (class III) and plasma membrane instability (class VI) (6, 7). Mutations less common than F508del are poorly characterized, in part, due to the small numbers of patients involved. Moreover, access to new personalized treatments among patients with ultra-orphan genotypes has been limited by difficulty arranging phase III clinical trials, and off-label prescribing has been impaired by high drug cost and difficulty arranging third party reimbursement. Rare CFTR mutations such as P67L are emblematic of the challenges to “precision” medicine, including use of the best available mechanistic knowledge to treat patients with unusual forms of disease.

Emerging knowledge indicates the difficulty in categorizing unusual cystic fibrosis (CF) mutations, with regard to both pathogenic mechanism and theratype. As case in point, we present data concerning P67L mutation of the cystic fibrosis transmembrane conductance regulator (CFTR), a defect carried by a small number of individuals with CF and sometimes attributed to a channel conductance abnormality. Findings from our laboratory and others establish that P67L causes protein misfolding, disrupts maturation, confers gating defects, is thermally stable, and exhibits near normal conductance. These results provide one framework by which rare CF alleles such as P67L can be more comprehensively profiled vis-à-vis molecular pathogenesis. We also demonstrate that emerging CF treatments – ivacaftor and lumacaftor – can mediate pronounced pharmacologic activation of P67L CFTR. Infrequent CF alleles are often improperly characterized, in part, due to the small numbers of patients involved. Moreover, access to new personalized treatments among patients with ultra-orphan genotypes has been limited by difficulty arranging phase III clinical trials, and off-label prescribing has been impaired by high drug cost and difficulty arranging third party reimbursement. Rare CFTR mutations such as P67L are emblematic of the challenges to “precision” medicine, including use of the best available mechanistic knowledge to treat patients with unusual forms of disease.
modulators. The mechanistic category, therefore, can have far-reaching implications, and it is emblematic of the situation among many CF mutant alleles, for which precision treatments are being contemplated.

In the present study, we provide evidence that P67L CFTR exhibits deficient biogenesis and substantial channel gating abnormalities distinct from a previously assigned conductance defect. This includes a deficit in open probability and normal conduction as well as diminished maturation/trafficking. Furthermore, we show that P67L-related aberrations can be overcome using the FDA-approved CFTR modulators, ivacaftor (Kalydeco) and lumacaftor. Our findings illustrate the limitations of CF molecular phenotype as the primary determinant of therapeutic access. In CF and other conditions for which caregivers possess molecular and clinical tools to rationally evaluate a personalized intervention, significant challenges to achieving the goal of therapeutic “precision” remain.

**Results**

**Biogenesis of P67L.** Levels of mature, fully glycosylated P67L CFTR were markedly reduced compared with those of WT CFTR but were higher than those of F508del CFTR, which required longer exposure times for detection (Figure 1A). To further evaluate protein biogenesis, cells were incubated with lumacaftor, a CFTR modulator recently approved for clinical use. Protein expression of the mature, fully glycosylated P67L band C increased dramatically — with levels comparable to those of WT CFTR — following lumacaftor administration. This increase was tested further using biotinylation to verify P67L band C at the cell surface (Figure 1B) (10, 11). The data indicate that P67L is significantly misprocessed and suggest that the P67L pathogenic mechanism is, at least in part, a class II defect. Related biogenesis abnormalities (nucleotide binding domain 1 [NBD1] misfolding, defective cytosolic loop/NBD1 binding, plasma membrane instability [a class VI defect]) may also contribute to the findings shown here (4, 5, 11).

**Functional P67L rescue by ivacaftor and lumacaftor.** The capacity of ivacaftor and lumacaftor to restore activity of P67L CFTR stably expressed in polarized Fischer rat thyroid (FRT) cells and primary nasal airway epithelial cells was examined by Ussing chamber analysis. Short-circuit current (I_sh) following treatment with these compounds led to enhancement of CFTR-mediated chloride transport in cells expressing P67L or F508del CFTR (Figure 2, A and B). Primary airway epithelial cells encoding a P67L/F508del...
**Figure 2. Functional assessment of the P67L cystic fibrosis transmembrane regulator (CFTR) mutant in polarized Fischer rat thyroid (FRT) and primary airway epithelial cells.** Using chamber experiments were carried out in FRT (A and B) or primary airway cells from 3 cystic fibrosis patients (genotypes P67L/F508del, F508del/F508del, F508del/F508del, C–F) at 37°C following 24 hours of treatment with DMSO (control) or 3 μM lumacaftor. Data showing short-circuit current (Isc) after luminal stimulation with forskolin (10 μM, far left arrows) and ivacaftor (300 nM, middle arrows) and inhibition by CFTR inh172 (30 μM, a CFTR inhibitor, far right arrows) are depicted in A, C, and E. Summary data is shown as mean ± SEM in B, D, and F (diamonds, forskolin; circles, ivacaftor + forskolin; triangles, inh172); some error bars are hidden by symbols. Variable response to lumacaftor is characteristic of the two independent homozygous F508del CFTR mutant patient codes shown in C–F. Dot plots describe n = 3–4 filters (technical replicates for each cell line or patient sample) per condition (n = 2 for lumacaftor-treated WT/WT primary cells). *P < 0.05 for forskolin + ivacaftor stimulation; **P = 2 for lumacaftor-treated WT/WT primary cells). *P = 0.02 for inh172 inhibition (indicative of total constitutive + activated CFTR function), determined via 2-sample t test.

**Discussion**

Despite an advanced understanding of causative alleles in CF, establishment of precise molecular intervention remains problematic (5–7, 15). For example, comparatively straightforward pathogenic categories (gating, trafficking, conductance, etc.) have allowed CF defects to be classified in a fashion intended to predict clinical response by tailored or precision treatments (16). However, even for well-described CFTR abnormalities, such as F508del, personalized therapies must also account for non-CFTR gene modifiers, epigenetics, CFTR allelic polymorphism (17), somatic cell chimerism, and environmental exposures among other features. Any of these might influence clinical phenotype and/or therapeutic effect. Importantly, among patients with ultra-orphan alleles, identification of a causal (and fundamental) mechanism does not necessarily mean a specific patient will benefit from a precise treatment.

The complexity of this perspective and its relevance to personalized medicine is placed in relief by P67L CFTR, a mutation emblematic of hundreds of other rare CF alleles. First, P67L has sometimes been incorrectly grouped as a class IV (conduction) defect, perhaps based, in part, on decreased CFTR currents observed previously for a different mutation at the same position, P67S (18). The categorization as class IV argues against usefulness of ivacaftor or lumacaftor and might otherwise be taken as evidence to deter pharmaceutical trials seeking FDA approval and/or third party reimbursement with regard to available CFTR modulators. In addition, for patients suffering from an orphan disease — CF being one example — a limited market often leads to specialized but very expensive therapeutic agents. Although third party reimbursement may require FDA approval for the diagnosed indication, an insufficient number of subjects for clinical trial evaluation impedes the conventional drug approval process. Such barriers have been highlighted previously from an ethical perspective (19–22), and specific examples also include access to anticancer and anti-infective treatments (20, 23, 24).

Second, data from our laboratory and others indicate robust activity of clinically approved CFTR modulators for P67L CFTR in multiple cell systems and in vivo (Figures 1–3) (9, 12, 25). The present studies show
that the class IV designation for P67L is incorrect and that the mutation causes protein misfolding, impairs biogenesis, confers gating defects, and exhibits normal conductance. Considerations such as these illustrate the importance of thorough evaluation for any rare allele in terms of molecular pathogenesis and theratype. Based on emerging knowledge regarding more common CFTR defects, it is likely that hundreds of CFTR mutations have been placed in categories that are grossly oversimplified, i.e., a single mutation eliciting a single defect in CFTR appears to be an exception. This is particularly the case when one considers that even premature truncation alleles may be improved by lumacaftor or ivacaftor (26). The present results provide one means by which a more complete molecular profile for rare CF alleles can be obtained in the future.

Third, under the current pharmaceutical testing paradigm and third party reimbursement policies, patients with P67L (like many other rare CFTR mutations) are unlikely to be prescribed drugs such as ivacaftor or Orkambi (an FDA-approved treatment containing ivacaftor and lumacaftor). Notably, the preclinical results in Figures 1–5 are supported by observations in a patient given ivacaftor (9). From this perspective, one can argue that, for rare and poorly described mutations, novel approaches to the complex issue of drug registration should be entertained. For example, based on a need to thoroughly characterize
“personalized” interventions for unusual CF defects, development of innovative preclinical model systems that predict patient responsiveness could represent a valuable adjunct during the drug registration process for orphan CF alleles. Moreover, in the same manner that antibiotics, analgesics, chemotherapy, antihypertensives, and most drug therapies are commonly administered and monitored for clinical effect, formal mechanisms that allow physicians (within FDA-specified guidelines) to prescribe CF modulators might be considered. Given the difficulty arranging phase III clinical studies for very small CF patient sub-groups, innovative protocols designed to evaluate efficacy could help improve access among patients with rare alleles. In this context, theoretically, if FDA permission could be obtained, a 3- to 4-week clinical trial that shows improved FEV1 might justify continued treatment using a CFTR modulator. Establishing meaningful thresholds for provisional drug administration is not trivial, and patient eligibility for such a proposal would obviously require compelling evidence of safety in human subjects and strong data in support of genotype-specific effectiveness. That being said, formalizing access mechanisms of this sort could allow establishment of rigorous phase IV clinical trials to confirm patient benefit and a more systematic evaluation of emerging precision interventions. In either case, arrangements such as these would empower physicians in the clinic to offer the best available therapies, facilitate third party reimbursement, and favor patients who — at present — have inadequate access to potentially effective treatments.

The findings presented here are concordant with those in earlier studies. For example, Kraus et al. noted diminished ion transport attributable to P67L. Based, in part, on abnormal current/voltage relationship in the closely related P67S defect, P67L may have been incorrectly assigned class IV status (18). Other data from large surveys of rare CF alleles also point to significant processing and/or transport abnormalities associated with P67L (12, 25, 27). These previous reports furnish additional support for complexity of the molecular mechanism underlying P67L CFTR and reinforce the analysis described above.

In summary, patients with rare CFTR mutations such as P67L often cannot gain access to leading edge CF compounds unless they possess an FDA-approved genotype, and third party payers have struggled with the complexity of providing reimbursement for individuals carrying uncommon alleles until FDA registration is available. The biochemical, monolayer, and single-channel findings shown here indicate that individuals with P67L CFTR could potentially benefit from ivacaftor or Orkambi, if in vivo confirmation and regulatory approval were in place. Thorough evaluation of rare CFTR alleles, together with an approach that allows physicians more opportunity to evaluate and treat patients based on well-validated preclinical or clinical endpoints and FDA approval, would serve many individuals with rare forms of the disease.
Methods

**FRT cell line generation.** FRT cells were provided by M. Welsh (University of Iowa, Iowa City, Iowa, USA). Cells were modified by our laboratory to express WT or mutant CFTR on an isogenic background using the Flp-in system (Thermo Fisher). Briefly, \(CFTR\) cDNAs (WT, \(F508del\), \(P67L\)) were cloned into NheI and XhoI sites of the pcDNA5/FRT expression vector according to the manufacturer’s instructions (Thermo Scientific). Cells were cotransfected with plasmids containing \(CFTR\) and a flp recombinase construct (pOG44) and cultured in media with hygromycin (100 \(\mu\)g/ml) to select site-specific recombinants. Hygromycin-resistant clonal lines were isolated and screened for loss of \(\beta\)-galactosidase. Clonal cells were found to have \(CFTR\) mRNA levels that varied less than by 50% compared with cells expressing WT \(CFTR\), in contrast with more variable levels of mRNA in FRT lines tested previously (25). Stability of expression in the new FRT models was verified to at least passage 31. All new lines were validated by Western blot and Isc measurements with and without lumacaftor or ivacaftor. The P67L biogenesis defect and responses to lumacaftor were also shown following transient transfection in human embryonic kidney (HEK) 293 cells (ATCC, catalog CRL-3216; E.J. Sorscher, unpublished observations).

**Cell culture.** FRT cells were maintained in Coon’s modified Ham’s F12 (Sigma-Aldrich), supplemented with 5% fetal bovine serum and grown at 37°C in a humidified incubator containing 5% CO\(_2\)/95% O\(_2\). Primary human nasal epithelial cells were collected from non-CF, homozygous \(F508del\), and \(P67L/F508del\) individuals. Lower airway epithelial \(F508del/F508del\) monolayers were also evaluated. At 80%–90% confluency, primary cells were seeded onto 6.5 ml permeable supports (~0.5 \(\times\) 10\(^6\) cells per filter; Corning) and coated with NIH 3T3 fibroblast-conditioned (ATCC, catalog CRL-1658) media. Cells were then propagated in differentiation media containing DMEM/F12 (Invitrogen), 2% Ultroser G (Pall), 2% Fetal Clone (Hyclone), 2.5 \(\mu\)g/ml insulin (Sigma–Aldrich), and 0.25% bovine brain extract (Lonza). 20 nM hydrocortisone, 500 nM triiodothyronine, 2.5 \(\mu\)g/ml transferrin (Invitrogen), 250 nM ethanolamine, 1.5 \(\mu\)M epinephrine, 250 nM phosphoethanolamine, and 10 nM retinoid acid were included in the media until terminally differentiated (~4 weeks). For rescue and delivery of \(F508del\) and \(P67L\) CFTR to the plasma membrane, cells were incubated with 3 \(\mu\)M lumacaftor (Selleck Chemicals) for 24 hours.

**Antibodies and chemicals.** Anti-CFTR mouse monoclonal antibodies included 10B6.2 anti-human NBD1 monoclonal (http://www.cfrfolding.org/reagentRequests10B62.asp), UNC570 R domain (recognizes CFTR aa 731–742, code no. A2), 596 NBD2 (aa 1204–1211, code no. A4), and 24-1 anti–C-terminus monoclonal antibodies (http://www.unc.edu/~tjjensen/CFADP/) (all provided by J. Riordan, University of North Carolina, Chapel Hill, North Carolina, USA).

Polyclonal anti–\(\beta\)-actin antibody was purchased from Sigma-Aldrich (catalog A5441). HRP-conjugated goat anti-mouse IgG and anti-rabbit IgG were obtained from Bio-Rad (1706516 and 1721034, respectively). SuperSignal West Pico chemiluminescence substrate and CFTR potentiator (ivacaftor) were from Thermo Forma. All other chemicals were purchased from Sigma-Aldrich, Thermo Scientific, or as described below.

Figure 5. Thermal stability of mutant P67L cystic fibrosis transmembrane conductance regulator (CFTR) following lumacaftor rescue. Macroscopic currents obtained using inside-out excised patches were evoked by a ramp protocol from –80 to +80 mV. Membrane patches were exposed to ATP (1.5 mM) and PKA (110 U/ml) in the bath at room temperature (control conditions). (A) P67L currents shown for an extended period with temperature cooling. (B) P67L CFTR during elevation of the bath to 37°C. In contrast to the CFTR mutant F508del (14), lumacaftor-rescued P67L CFTR channels remain active at physiological temperature. This experiment has been repeated with similar results. inh172, an inhibitor of CFTR.
**Western blotting.** Cells were washed with PBS, pelleted, and lysed with radioimmunoprecipitation buffer containing protease inhibitor cocktail and EDTA for 45 minutes on ice. Protein concentration was measured using a Bicinchoninic acid assay (Thermo Scientific). Briefly, 40 μg cell lysate from each sample was resolved by SDS-PAGE and transferred to PVDF membranes, followed by blotting with specific primary antibody and HRP-labeled secondary antibody. The membrane was developed using chemiluminescent substrate (SuperSignal West Pico, Thermo Scientific) and signal was quantified by a ChemiDoc XRS System (Bio-Rad). Densitometry was performed using ImageJ software (NIH).

**Cell surface biotinylation.** The cell surface expression of P67L and F508del CFTR was measured after biotin labeling using EZ-link Biotin-LC-hydrazide as described previously (10). Following surface biotinylation of cells, P67L and F508del CFTR were immunoprecipitated using the C-terminal CFTR antibody (24-1, R&D Systems) and then blotted with Avidin D-HRP (10).

**Ussing chamber analyses.** $I_w$ was measured under voltage clamp conditions with an MC8 instrument and P2300 Ussing chamber equipment (Physiologic Instruments) (28). Human primary epithelial and FRT CFTR monolayers were bathed on both sides with identical Ringer's solutions containing the following (in mM): 115 NaCl, 25 NaHCO3, 2.4 KH2PO4, 1.24 K2HPO4, 1.2 CaCl2, 1.2 MgCl2, and 10 D-glucose (pH 7.4). Solutions were vigorously bubbled by using 5% CO2/95%O2. One-second 3-mV pulses were imposed every 10 seconds to calculate resistance by Ohm's law. Where indicated, mucosal solutions were changed to a low Cl– buffer (1.2 mM NaCl and 115 mM Na+ gluconate, with other components as above). Amiloride (100 μM) was added to block residual Na+ current, followed by the CFTR agonist forskolin (20 μM) and potentiator ivacaftor (300 nM). Inh$_{23}$ (10 μM), an inhibitor of CFTR, was added to the apical solution at the completion of each experiment to block CFTR-dependent $I_w$. All chambers were maintained at 37°C during current recording.

**Single-channel activity.** FRT cells or primary nasal epithelial cells expressing CFTR (from non-CF or P67L/F508del individuals) were seeded on glass coverslips and mounted in flow-through chambers. Single-channel currents were recorded from inside-out patches using the Molecular Devices Axopatch 200B/pClamp 10 system (29). Currents were obtained under symmetrical Cl– at room temperature. Channels were activated with 1 mM MgATP/75 nM PKA (Sigma-Aldrich), and recordings filtered at 500 Hz for analysis and display.

**Thermal stability.** Thermal stability of P67L CFTR was evaluated by inside-out patch analysis (14). Patch pipettes were pulled from Corning 8161 glass to tip resistances of 1–2 MΩ (macroscopic recordings) or 9–12 MΩ (unitary current recording). CFTR channels were activated following patch excision by exposure of the cytoplasmic face of the patch to the catalytic subunit of PKA (110 units/ml) and MgATP (1.5 mM). CFTR currents were recorded in symmetrical solutions containing 140 mM N-methyl-D-glucamine-Cl, 3 mM MgCl2, 1 mM EGTA, and 10 mM TES (2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid; pH 7.3). Macroscopic currents were evoked using a ramp protocol from –80 to +80 mV with a 10-second time period. Patches were held at ±60 mV for 10 seconds to calculate resistance by Ohm’s law. Where indicated, mucosal solutions were changed to a low Cl– buffer (1.2 mM NaCl and 115 mM Na+ gluconate, with other components as above). Amiloride (100 μM) was added to block residual Na+ current, followed by the CFTR agonist forskolin (20 μM) and potentiator ivacaftor (300 nM). Inh$_{23}$ (10 μM), an inhibitor of CFTR, was added to the apical solution at the completion of each experiment to block CFTR-dependent $I_w$. All chambers were maintained at 37°C during current recording.

**Statistics.** Data were summarized as mean ± SEM. Replicates were treated as statistically independent in light of the fact that no comparisons were made across subjects or cell lines for any of the 3 patient samples studied: P67L/F508del, F508del/F508del, or F508del/F508del. Findings thus apply only to a specific cell line or subject evaluated, with 2-sample t tests used to assess statistical significance of observed differences in mean response across two conditions applied to a particular cell line or subject. No pooled patient data or subject-to-subject comparisons were analyzed (i.e., all comparisons involve only two conditions in the same individual). The findings, therefore, should not be taken to suggest that all primary airway cells encoding P67L or F508del CFTR would behave similarly. $P$ values of less than 0.05 were considered significant.

**Study approval.** The studies conducted for this paper were deemed “not human subject research” by the University of Alabama at Birmingham Institutional Review Board. All cells and cell models were obtained under regulatory supervision and fully deidentified.
Author contributions
Experiments were conducted and designed by CMS, WW, HW, CMM, WJC, and JSH, who also performed data acquisition/analysis. Reagents were furnished by KLK, JFC, JSH, MM, and EJS. Data was further analyzed and the manuscript written by CMS, JFC, JSH, KLK, and EJS.

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