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Orphan Missense Mutations in the Cystic Fibrosis Transmembrane Conductance Regulator

A Three-Step Biological Approach to Establishing a Correlation Between Genotype and Phenotype

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More than 1860 mutations have been found within the human cystic fibrosis transmembrane conductance regulator (*CFTR*) gene sequence. These mutations can be classified according to their degree of severity in CF disease. Although the most common mutations are well characterized, few data are available for rare mutations. Thus, genetic counseling is particularly difficult when fetuses or patients with CF present these orphan variations. We describe a three-step *in vitro* assay that can evaluate rare missense *CFTR* mutation consequences to establish a correlation between genotype and phenotype. By using a green fluorescent protein–tagged CFTR construct, we expressed mutated proteins in COS-7 cells. CFTR trafficking was visualized by confocal microscopy, and the cellular localization of CFTR was determined using intracellular markers. We studied the CFTR maturation process using Western blot analysis and evaluated CFTR channel activity by automated iodide efflux assays. Of six rare mutations that we studied, five have been isolated in our laboratory. The cellular and functional impact that we observed in each case was compared with the clinical data concerning the patients in whom we encountered these mutations. In conclusion, we propose that performing this type of analysis for orphan *CFTR* missense mutations can improve CF genetic counseling. ***(J Mol Diagn 2011, 13: 520–527; DOI: 10.1016/j.jmoldx.2011.05.004)***

Cystic fibrosis (CF) is the most common severe autosomal recessive genetic disorder in the white population, with an incidence of approximately 1 in 3500. It results from mutations in the CF transmembrane conductance regulator (*CFTR*) gene[1,2] that encodes a chloride channel normally expressed at the apical membrane of epithelial cells.[3,4] CFTR is a member of the ATP-binding cassette transporter family. It contains two repeated units composed of a membrane-spanning domain, which includes six transmembrane helices, and a nucleotide-binding domain, which harbors an ATP-binding and hydrolysis site.[5] Both halves are joined by a specific cytoplasmic regulator (R) domain, whose phosphorylation by protein kinase A activates the outward anionic channel activity.[6,7] To date, >1860 mutations identified within the human *CFTR* sequence are listed in the CF mutation database (*http://www.genet.sickkids.on.ca/Home.html*, last accessed March 8, 2011). These mutations can be divided into six classes, according to the mechanism that disrupts CFTR function[8,9]: absence of the protein at the apical plasma membrane because of i) defective protein synthesis or ii) impaired maturation leading to protein degradation, iii) defective regulation of CFTR channel activity, iv) altered ionic selectivity and conductance, v) lowered CFTR mRNA amount, and vi) decreased protein stability.

The most common mutation, F508del, is a class II mutation. It is carried by 90% of patients with CF, on at least one allele, and it leads to a severe CF phenotype. In our laboratory, we use the Elucigene CF30 Kit (Gen-Probe, Inc., San Diego, CA), which has been approved in the French national neonatal CF screening program for routine genetic diagnostics of patients with CF. This test has been designed to detect the 30 most frequently found mutations, including F508del, within the French population.[10] In a second step, we perform mutation analysis on *CFTR* whole coding sequences (27 exons) and their flanking intronic region by denaturing high-performance liquid chromatography (DH-

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PLC) and high resolution melting (HRM) techniques. The molecular and physiological consequences of most common mutations have been extensively studied,[11–15] but few data are available for rarer mutations isolated by systematic approaches. These mutations should be classified as familial or so-called orphan mutations because of their low incidence. Consequently, genetic counseling is particularly difficult when fetuses or patients with CF present these orphan mutations. Depending on the severity of the mutation found on the other allele for a compound heterozygous genotype, the clinical study of a patient presenting a rare familial mutation cannot provide sufficient information on the phenotype induced by this mutation. Biological investigations should be realized to determine the severity of this mutation to help genetic counseling in case of a new pregnancy in this family.

This study describes the cellular and functional consequences of six rare missense mutations of *CFTR*, of which five have been isolated in our laboratory during CF molecular diagnosis. In collaboration with technical platforms (Bioscreen for iodide efflux study and ImageUP for confocal microscopy; CNRS and Université de Poitiers, France), we have developed a robust three-step biological assay to characterize CFTR's mutation impact to establish a genotype-phenotype correlation. We previously used the expression of green fluorescent protein (GFP)–tagged CFTR in COS-7 cells to study interactions between CFTR and syntaxin 8.[16] Moreover, we observed that wild-type (WT)–CFTR and well characterized mutants, p.Phe508del and p.Gly551Asp (where p. indicates protein sequence), exhibit expected intracellular trafficking and pharmacological profile in these cells.[17]

In the presence of each of the six rare missense mutations, CFTR cellular localization was determined by confocal microscopy using several markers: calreticulin, which is a marker of the endoplasmic reticulum (ER) and cortical actin, which delimits the plasma membrane. By using Western blot analysis, we studied the CFTR maturation process by evaluating the relative amount of mature and nonmature CFTR (C and B bands), and we measured *CFTR* channel activity by automated iodide efflux assays. Two of these mutants exhibited null maturation and activity; one, impaired activation with a lowered maturation; two, decreased activity; and the last one, unaffected.

Materials and Methods

Patients

CFTR mutations were characterized in six white index patients, referred for mutation screening because of clinical features characteristics of CF disease: two for infertility, two for positive sweat test results associated with pulmonary or pancreatic pathological features, one for genetic counseling and prenatal diagnosis (familial history of death caused by CF), and one after systematic CF neonatal screening. We included each patient and his or her family members in this study after receiving their signed informed consent, according to French good medical practices. All but one of the missense variations described in this article were discovered in one family only, among >1200 families screened for *CFTR* mutations by our laboratory. The exception was p.Val562Ile (V562I), which we encountered in two unrelated families with distinct genotypes. For all patients, CF molecular diagnosis was performed on the 27 exons of the *CFTR* gene using denaturing high-performance liquid chromatography or denaturing gradient gel electrophoresis screening. *CFTR* microdeletions or microduplications were analyzed by multiplex ligation-dependent probe amplification.

Patient 1 was a 3-year-old girl when the CF diagnosis was established, with a genotype as follows: p.[Leu102Pro] + [Arg553X]. She presented an abnormal chronic cough and a positive sweat test result. Now, she is a 13-year-old girl who has a severe CF phenotype, notably with repeated bacterial lung colonization.

Patient 2 was a newborn girl with pancreatic failure and a positive sweat test result. The CF diagnosis was confirmed by *CFTR* molecular analysis, with a genotype as follows: p.[Phe508del] + [Leu167Arg]. Now, this patient is aged 16 years and has recurrent pancreatitis without respiratory symptoms.

Patient 3 was referred for CF testing during systematic CF neonatal screening performed by the Guthrie test. The genotype was as follows: p.[Asn1303Lys] + [Pro574Ser]. This 3-year-old girl had a normal sweat test result and is asymptomatic. For genetic counseling, we performed *CFTR* molecular diagnosis on two first cousins of this patient. The two boys had the same genotype: p.[Phe508del] + [Pro574Ser]); they presented with congenital bilateral aplasia of the vas deferens (CBAVD).

Patient 4 was a 31-year-old man who was referred for genetic counseling before performing an intracytoplasmic sperm injection (ICSI). He presented with CBAVD, and his spouse was heterozygous for the p.Phe508del mutation. *CFTR* mutational analysis revealed a complex genotype: p.[Arg74Trp;Val201Met;Asp1270Asn] + [Pro841Arg]. The *IVS8* polymorphism genotype was as follows: c.[1210-12T(7)] + [1210-12T(7)] (where c. indicates nucleotide coding sequence). Therefore, no CBAVD-related allele

Table 1. Sequences of Site-Directed Mutagenesis Primers

Mutation	Sense oligonucleotide sequences
L102P	5′-GTACAGCCTCTCTTACCGGGAAGAATCATAGCTTCC-3′
L167R	5′-AAGAAGACTTTAAAGCGGTCAAGCCGTGTTCTAG-3′
P574S	5′-GCTGATTTGTATTTATTAGACTCTTCTTTTGGATACCTAGATG-3′
V562I	5′-AGAATTTCTTTAGCAAGAGCAATATACAAAGATGCTGATTTG-3′
K696R	5′-CAGACTGGAGAGTTTGGGGAAAGAAGGAAGAATTCTATTCTC-3′
P841R	5′-GATATGGAGAGCATACGAGCAGTGACTACATGG-3′

Table 2. Summary of the Patients' Data, Concerning Genotype, Phenotype, and Protein Dysfunction

Patient no./sex/age at molecular diagnostics (years)	Genotype	Phenotype	Protein dysfunctions		
			Channel activity*	Maturation†	Intracellular localization‡
1/F/3	p.[**Leu102Pro**] + [Arg553X]	Positive sweat test result, bacterial lung colonization, no pancreatitis	++	++	++
2/F/newborn	p.[Phe508del] + [**Leu167Arg**]	Positive sweat test result, recurrent pancreatitis, no lung infection	++	++	++
3/F/3	p.[Asn1303Lys] + [**Pro574Ser**]	Normal sweat test result, asymptomatic	++	+	+
4/M/31	p.[Arg74Trp;Val201Met; Asp1270Asn] + [**Pro841Arg**]; c.[1210-12T(7)] + [1210-12T(7)]	CBAVD	+	Ø	Ø
5/F/37	p.[**Lys696Arg**]	Asymptomatic (prenatal CF diagnostics)	+	Ø	Ø
6/M/33	c.[274-6T>C(+)1210-12T(5)(+)1684G>A (=p.[**Val562Ile**])]	Infertility, no CBAVD, previously treated bilateral cryptorchidism	Ø	Ø	Ø

In the genotype column, the amino acid substitutions that we investigated are boldfaced. Clinical data and experimental results are displayed. The severity of the protein dysfunctions that we observed are rated according to specific criteria.
*++ indicates <40% of WT; +, approximately 50% of WT; Ø, nsd compared to WT.
†++ indicates no band C; +, decreased B/(B + C) ratio; Ø, nsd compared to WT.
‡++ indicates ER; +, ER plus cytosol; Ø, PM.
CBAVD, congenital bilateral aplasia of the vas deferens; nsd, not significantly different; PM, plasma membrane.

was presented. The outcome of this ICSI was previously described.[18]

Patient 5 was a 37-year-old pregnant woman referred for genetic counseling and prenatal CF diagnosis. The genotype was as follows: p.Lys696Arg (K696R). Her husband was heterozygous for the p.Phe508del mutation. Prenatal molecular diagnosis demonstrated that the fetus had inherited only the p.Phe508del mutation from his father.

Patient 6 was a 33-year-old man referred for 3 years of infertility and for the pre-ICSI assessment. The genotype was as follows: c.[274-6T>C(+)1210-12T(5)(+)1684G>A] [c.1684G>A equaling p.Val562Ile (V562I)]. There was no evidence of CBAVD. A medical history of bilateral cryptorchidism, treated during infancy by conventional hormonal administration, was noted.

Cells

COS-7 cells were stably or transiently transfected using 3 μL of FuGene6 transfection reagent (Roche Diagnostics Corp, Indianapolis, IN) mixed with 2 μg of purified GFP-tagged CFTR construct containing either WT or mutated CFTR. Cell lines were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium with Glutamax-I (Invitrogen, Cergy Pontoise, France), supplemented with 10% fetal calf serum (Eurobio, Courtaboeuf, France), 100 IU/mL penicillin (Panpharma SA, Fougères, France), and 100 μg/mL streptomycin (Panpharma SA), under 1 mg/mL G418 (Sigma-Aldrich France, Saint-Quentin Fallavier, France) selection.

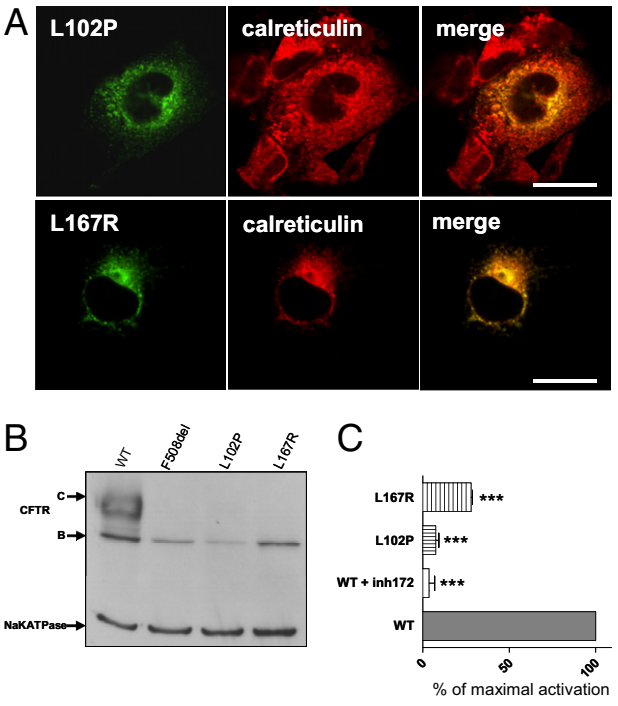

Figure 1. L102P and L167R amino acid substitutions impair CFTR protein maturation. **A:** Fluorescent confocal imaging of mutant GFP-tagged CFTR proteins (green) expressed in COS-7 cells. CFTR partially colocalizes with calreticulin (red). Scale bar = 20 μm. **B:** Maturation status of mutant CFTR visualized with GFP-specific Western blot analysis. **C:** CFTR channel activity measured by iodide efflux assays using stimulation cocktail (10 μmol/L forskolin and 30 μmol/L genistein). The null activation observed when the CFTR-specific inhibitor (10 μmol/L CFTR-inh172)[20] is added shows that the anionic efflux is because of CFTR channel activity. Data are presented as the mean ± SEM (n = 3 to 4 for each experimental condition). ***$P < 0.001$.

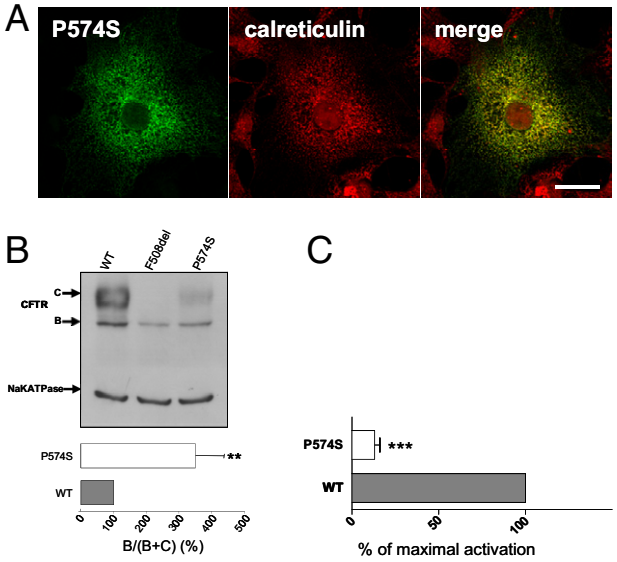

Figure 2. P574S amino acid substitution decreases CFTR protein maturation. **A:** Fluorescent confocal imaging of mutant GFP-tagged CFTR proteins (green) expressed in COS-7 cells. CFTR partially colocalizes with calreticulin (red). Scale bar = 20 μm. **B:** Maturation status of mutant CFTR visualized with GFP-specific Western blot analysis. Histogram showing the band B/(band B + band C) ratio. **C:** CFTR channel activity measured by the iodide efflux assay. Data are presented as the mean ± SEM (n = 3 to 4 for each experimental condition). $^{**}P < 0.01$, $^{***}P < 0.001$.

Construction of CFTR Mutants

GFP-tagged CFTR expression was performed using the pS65T/EGFP-C1/wt-CFTR construct, a gift from Bruce A. Stanton (Dartmouth College, Hanover, NH). The mutations were generated by oligonucleotide-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The DNA sequences for the sense oligonucleotides are summarized in Table 1. Mutations in individual clones were verified through plasmid sequencing on both strands using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Reactions were run on an ABI PRISM 310 automatic sequencer (Applied Biosystems).

Immunofluorescence

COS-7 cells were plated on glass coverslips 24 to 72 hours before staining. Then, they were fixed in 3% paraformaldehyde and permeabilized in PBS and 0.5% Triton X-100 (Sigma-Aldrich). Unspecific antigen sites were blocked with PBS containing 0.5% bovine serum albumin (Sigma-Aldrich). Cells were incubated with monoclonal anti-GFP (1:100, clone GFP-20; Sigma-Aldrich) and polyclonal anti-calreticulin (1:200; Stressgen Bioreagents, Kampenhout, Belgium) for 1 hour at room temperature. Cells were then incubated with Rhodamine–Red-X–conjugated secondary antibodies (1:100; Jackson Immunoresearch Laboratories, West Grove, PA), Alexa 488–conjugated secondary antibodies (1:100; Fluoprobes, Montluçon), or phalloidin–tetramethyl rhodamine isothiocyanate (1:100; Sigma-Aldrich). Nuclei were stained in red

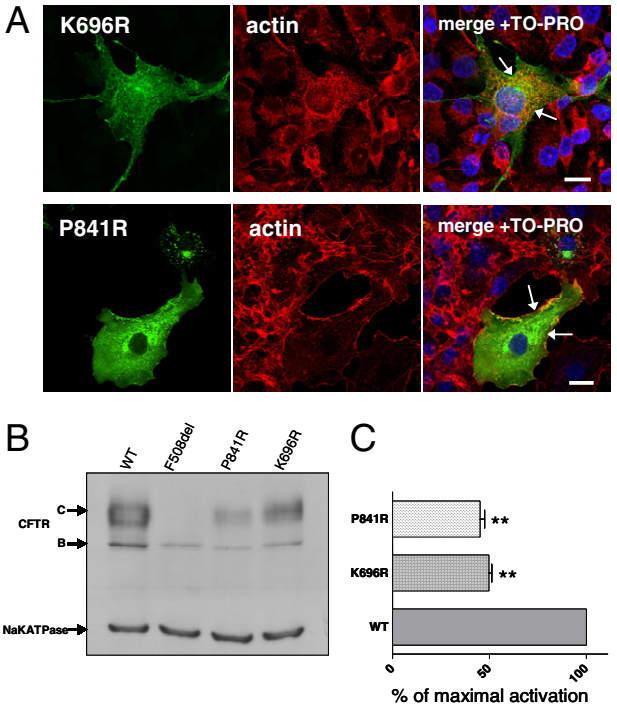

Figure 3. K696R and P841R amino acid substitutions affect CFTR channel activity. **A:** Fluorescent confocal imaging of mutant GFP-tagged CFTR proteins (green) expressed in COS-7 cells. Phalloidin staining (red) shows partial colocalization of CFTR with cortical actin (**arrows**). Scale bar = 20 μm. **B:** GFP-specific Western blot analyses. **C:** CFTR channel activity measured by iodide efflux assays. Data are presented as the mean ± SEM (n = 3 to 4 for each experimental condition). $^{**}P < 0.01$.

with TO-PRO−3 iodide (Invitrogen) over 1 hour at room temperature. Coverslips were mounted with Mowiol (Sigma-Aldrich) mounting medium. Fluorescence was examined with a spectral confocal station FV-1000 in-

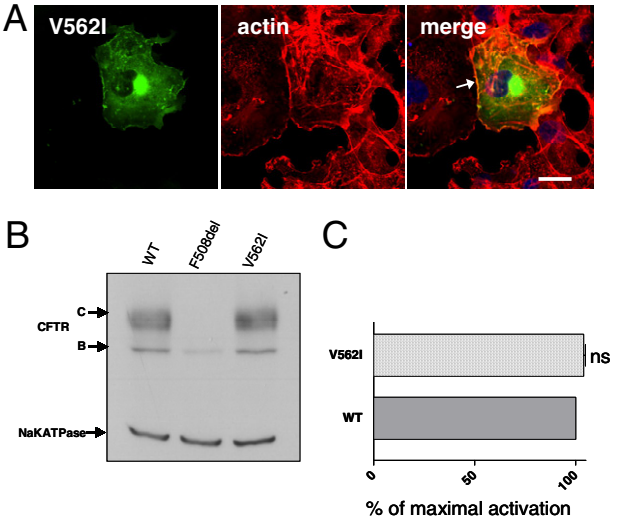

Figure 4. The V562I amino acid substitution does not alter CFTR phenotype. **A:** Fluorescent confocal imaging of mutant GFP-tagged CFTR protein (green) expressed in COS-7 cells. Phalloidin staining (red) shows partial colocalization of CFTR with cortical actin (**arrows**). Scale bar = 20 μm. **B:** GFP-specific Western blot analysis. **C:** CFTR channel activity measured by iodide efflux assay. Data are presented as the mean ± SEM (n = 3 to 4 for each experimental condition). ns indicates nonsignificant difference.

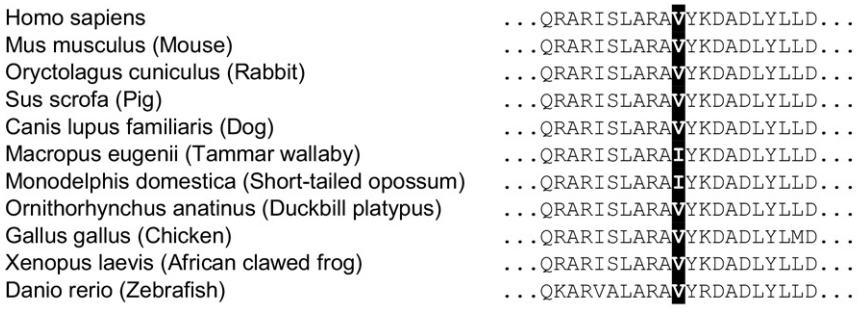

Figure 5. Local alignment, centered on the position 562 residue, of CFTR protein sequences from different animal species. We used the PolyPhen (now upgraded to PolyPhen-2) tool, designed to achieve polymorphism phenotyping.

stalled on an inverted microscope IX-81 (Olympus, Tokyo, Japan).

Iodide Efflux Experiments

CFTR chloride channel activity was assayed by measuring the rate of ^{125}I efflux from cells, as previously described.[19] All experiments were performed with a MultiPROBE IIex robotic liquid handling system (Perkin Elmer Life Sciences, Courtaboeuf, France).

Western Blot Analysis

Cell proteins were extracted with a lysis buffer (10 mmol/L Tris, pH 7.4; 1% Nonidet P-40; and 0.5% deoxycholate) containing protease inhibitors [20 μmol/L leupeptin, 0.8 μmol/L aprotinin, 10 μmol/L pepstatin, and 2 mmol/L 4-(2-aminoethyl)benzene sulfonyl fluoride hydrochloride; Sigma-Aldrich France]. Cell lysates were incubated on ice for 20 minutes and clarified by centrifugation at 15,000 $\times$ *g* for 5 minutes at 4°C. Total proteins were quantified using the bicinchoninic acid protein assay reagent (Sigma-Aldrich France). Then, 10 to 25 μg of protein was separated on a 5% polyacrylamide gel and subjected to Western blot analysis, as previously described,[16] using GFP-specific monoclonal antibody (1:1000, clone GFP-20; Sigma-Aldrich France) and Na^+/K^+ ATPase α1-specific monoclonal antibody (1:1000, clone C464.6; Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase–conjugated anti-mouse IgG (1:3000; GE Healthcare, Little Chalfont, UK) was used as a secondary antibody and revealed with ECL Western Blotting Detection Reagent (GE Healthcare). Bands were quantified by densitometry using Scion Image software, and CFTR maturation status was estimated by the band B/bands (B + C) ratio.

Statistics

The results for iodide efflux are expressed as the mean $\pm$ SEM of *n* observations. To compare sets of data, we used the Student's *t*-test. Differences were considered statistically significant when $P < 0.05$. All statistical tests were performed using GraphPad Prism version 4.0 software for Windows (Graphpad Software Inc., La Jolla, CA).

Results

Defective Activation and Maturation of L102P and L167R CFTR Mutants

Details of the six patients, their genotypes and phenotypes, and characteristics of their *CFTR* mutations are provided (Table 2). Among the six missense *CFTR* variants that we investigate herein, two affect membrane-spanning domain 1: p.Leu102Pro (L102P) and p.Leu167Arg (L167R). Fluorescent confocal imaging (Figure 1A) shows that none of these CFTR mutants localize to the plasma membrane; instead, they are retained in a perinuclear compartment, where they colocalize with calreticulin. Thus, L102P and L167R mutants appear unable to escape the ER. Similarly, Western blot analysis evidence suggests that each mutant lacks the fully glycosylated mature form (band C). Only the ER core-glycosylated form (band B) is present similarly to the well-characterized F508del mutant (Figure 1B). We used the iodide efflux assay to compare the channel activity of each mutant with WT-CFTR, when it is stimulated by a forskolin plus genistein cocktail (Figure 1C). When 100% activation is attributed to the maximal activation peak of WT-CFTR, activation is reduced to 7.45% $\pm$ 3.76% ($n = 4$) for L102P and to 27.84% $\pm$ 1.96% ($n = 4$) for L167R. Taken together, these data strongly suggest a maturation defect at the ER level for both of these mutants. This defect causes an absence of the CFTR protein at the plasma membrane and, thus, sharply reduced channel activity. They should be classified as class II mutations. This suggests a severe phenotype when these mutations occur in *trans* of another severe one.

Mixed Phenotype with P574S CFTR Variant

The missense substitution p.Pro574Ser (P574S) lies within nucleotide-binding domain 1. No clear plasma membrane staining is displayed by confocal fluorescent imaging for this mutant (Figure 2A). However, the localization of this variant CFTR appears more widespread within the cell, compared with the perinuclear localization observed for the two mutants previously described.

Moreover, the P574S mutant does not fully colocalize with the ER marker and seems to escape this cellular compartment because a strong green stain can be observed, which clearly differs from the calreticulin red signal. After immunoblotting (Figure 2B), we quantify the ratio of immature core-glycosylated CFTR/total CFTR [band B/(band B + band C)]: attributing 100% to WT-CFTR, this ratio increases to 349.5% ± 88% ($n = 3$) for P574S. Thus, the maturation of this mutant does occur but is less efficient than that of WT-CFTR. Moreover, the iodide efflux assay exhibits a dramatic inhibition of CFTR channel activity: when 100% activation is attributed to the maximal activation peak of WT-CFTR, activation is reduced to 13.07% ± 5.23% ($n = 4$) for P574S (Figure 2C). The lowered maturation of P574S suggests an attenuated class II mutation. Nevertheless, considering the amount of the remaining fully glycosylated form observed using Western blot analysis, higher channel activity would be expected for the protein. These data should be compared to the patient genotype to evaluate the pathogenicity of the P574S mutation.

Decreased Activity of K696R and P841R CFTR Mutants

Two missense mutations, p.Lys696Arg (K696R) and p.Pro841Arg (P841R), are situated within the cytoplasmic regulator domain of CFTR. In both cases, GFP fluorescence, visualized by confocal microscopy, assesses that a significant amount of mutant CFTR is trafficked to the plasma membrane (Figure 3A). Phalloidin staining shows that CFTR partially colocalizes with cortical actin beneath the inner side of the plasma membrane. After using Western blot analysis (Figure 3B), the proportions of core-glycosylated protein for K696R and P841R appear insignificantly different from that of WT-CFTR ($n = 3$, data not shown). Moreover, we observe by iodide efflux experiments that both mutations have similar functional consequence on CFTR activity: the maximum activation is reduced to 49.67% ± 2.61% ($n = 4$) for K696R and to 45.10% ± 4.94% ($n = 4$) for P841R (Figure 3C). Both of these mutants, exhibiting normal maturation but reduced activation, should be classified as class III or IV mutations. In either case, our data suggest that a mild phenotype should be induced by these mutations.

Normal Phenotype for the V562I CFTR Variant

The controversial V562I substitution lies inside nucleotide-binding domain 1. Fluorescent imaging exhibits normal trafficking to the plasma membrane of the protein, underlined by partial colocalization with cortical actin (Figure 4A). By using Western blot analysis, the maturation process of V562I-CFTR appears similar to the one visualized for WT-CFTR (Figure 4B). The proportion of core-glycosylated protein is found nonsignificantly different of WT-CFTR ($n = 3$, data not shown). We also observe unaltered channel activity of the protein by an iodide efflux experiment (Figure 4C): the maximum activation is estimated to be 103.92% ± 1.96% ($n = 4$), which is not significantly different from WT-CFTR activation. In our cell model, according to the three techniques that we used, V562I substitution behaves like a polymorphism with no evidence for cellular or functional impact.

We then used PolyPhen software (now upgraded to PolyPhen-2; *http://genetics.bwh.harvard.edu/pph2*, last accessed March 8, 2011), which predicts if an amino acid substitution may be a polymorphism by aligning protein sequences centered on this residue in various animal species (Figure 5). We found isoleucine instead of valine in two metatherian mammal species, whereas valine is present in that position of the CFTR protein sequence in a more archaic mammalian species (ie, duckbill platypus) and among bird, amphibian, and fish species. This confirms that V562I substitution should be a polymorphism with no structural or functional impact on the CFTR protein.

Discussion

Herein, we studied six rare *CFTR* mutations, of which five were first isolated in our laboratory. Three biological approaches were used to investigate the putatively deleterious consequences of these missense substitutions. The collected data include information concerning trafficking to the plasma membrane, the glycosylation process, and the anionic efflux triggered by the forskolin plus genistein activator cocktail of mutated CFTR. Our results are summarized in Table 2. This study intends to establish a correlation between genotype and phenotype, in an attempt to predict the severity for patients with CF of alleles bearing the studied *CFTR* mutations.

In our model, two missense mutations, L102P and L167R, exhibit an F508del-like phenotype: the proteins are blocked at the ER level, and their absence at the plasma membrane elicits reduced channel activity. Therefore, they should be considered nonfunctional alleles, resulting in poor CF disease prognosis. All of the patients from whom these mutations were discovered were composite heterozygous; the other allele was known as severe. One of these patients, with severe respiratory tract symptoms and long-term lung colonization, was diagnosed as having CF; his genotype is p.[Leu102Pro] + [Arg553X]. This finding is in accordance with the dramatically reduced activity that we found for L102P-CFTR protein in cell culture. Another patient, with chronic sinusitis and pancreatic failure, has the genotype p.[Leu167Arg] + [Phe508del]. The *L167R* allele exhibits, as described for L102P, a maturation and trafficking defect leading to a sharp decrease of CFTR activity compared with that of WT-CFTR. Nevertheless, iodide efflux experiments on L167R exhibit a residual activity slightly more elevated (27.84%) than that for L102P (7.45%). However, these data alone could not fully explain the less severe pulmonary phenotype presented by patient 2 compared with patient 1, notably because the iodide efflux technique provides no data about the kinetics and fine-tuning of CFTR activity at the single-cell or single-channel level. These data could be obtained using patch-clamp experiments. However, this technique

is difficult to perform and time-consuming; therefore, it is not suitable for the scope of this study. Rather, the robotized iodide effluxes that we used herein provide a global estimation of CFTR channel activity that allowed us to classify CFTR variants into the following three groups (Table 2): dramatically decreased, half reduced, or WT activities.

The results obtained for P574S are more complex to interpret. By iodide efflux, we measure low channel activity for the mutant. Contrary to L102P and L167R, P574S is not fully retained in the ER compartment because we can see the presence of the fully glycosylated mature form of CFTR by using Western blot analysis. This observation is sustained by confocal microscopic imaging clearly showing different staining patterns for P574S-CFTR and the ER resident calreticulin. Taken together, these results strengthen the idea that a small part of P574S could reach post-Golgi compartments and, thus, the plasma membrane. A neonate harboring this missense variation was tested for *CFTR* during CF neonate systematic screening performed on Guthrie cards. His genotype was p.[Asn1303Lys] + [Pro574Ser], and the allele in *cis* is considered a CF-causing mutation.[21] Five sweat test results were negative, and this patient is now a healthy 3-year-old girl, asymptomatic of CF disease. Her two first cousins were referred for CF molecular testing because both have CBAVD. Their genotype was p.[Phe508del] + [Pro574Ser]. These data clearly indicate that P574S induces a CF–related disorder phenotype (CBAVD) for males, consistent with a normal sweat test result and no symptoms of CF disease for the girl. However, if the results of our Western blot analysis indicate a mild phenotype, P574S–CFTR–impaired channel activation would suggest a severe CF phenotype. One possible explanation could be that iodide effluxes are not sensitive enough to precisely evaluate the CFTR chloride channel activation process; notably, CFTR channel open probability could not be studied directly by this method. Obviously, patch–clamp analysis would be useful to measure the real impact of P574S in CFTR channel gating. However, in our opinion, this technique cannot be used for reasonably rapid *in vitro* assays.

Our results suggest a mild phenotype for the K696R and P841R CFTR mutants: the trafficking and maturation rates appear normal, whereas activation is approximately half reduced. Other studies[21] indicated that mutations found in the cytoplasmic regulator domain of CFTR could induce variable consequences: some of them seem to have no effect, others affect protein maturation, and yet others lead to decreased function of the protein. The variant K696R was isolated from a familial study, in which we did not find any other CFTR mutation. We have no data about the phenotypic effect of this variant in patients. The P841R variant was carried by a patient with CBAVD, referred for pre-ICSI assessment; his spouse was heterozygous compound for p.[Phe508del]. His genotype was p.[Pro841Arg] + [Arg74Trp;Val201Met; Asp1270Asn]. The complex *CFTR* allele harboring three substitutions is considered responsible for a mild phenotype because a case of homozygosis has been reported for a patient exhibiting CBAVD, without severe CF features.[22] Because the genotype of the patient results in CBAVD, we can deduce that P841R is either a mild or a severe mutation. Therefore, in the case of ICSI, it was decided by a pluridisciplinary antenatal staff that, if the resulting fetuses carried P841R and F508del alleles, this couple would be offered a therapeutic abortion, given the high risk of CF for the evaluated offspring.[18] Our biological data are not fully in accordance with this conclusion because the P841R variant seems to not cause a severe CF phenotype because the CFTR protein could reach the plasma membrane and could be activated. Taken together, these data strongly suggest that P841R is more likely to be a mild than a severe CF mutation.

We found the V562I missense variation in a case of male infertility (genotype previously given). Because the patient does not have CBAVD, his sterility is unlikely to be a CF-related disorder. At the protein level, we showed that this substitution has no impact on CFTR trafficking, the maturation process, or channel activity. Moreover, the use of several CFTR activators in iodide efflux experiments suggests a pharmacological profile similar to that of the WT-CFTR protein (data not shown). Because valine and isoleucine are both hydrophobic amino acids, the folding and function of the mutated protein may be unaffected. Moreover, the *CFTR* gene harbors isoleucine instead of valine in that position in several mammal species. Our results are concordant with those previously obtained in baby hamster kidney cells overexpressing CFTR[23] (by using Western blot analysis, iodide effluxes, and inside-out patch-clamp experiments in which the V562I mutant behaves like WT-CFTR). However, V562I, which was first reported as a polymorphism,[24] has been reassigned as a non–CF-causing mutation, notably implicated in the CBAVD phenotype.[20] Because the V562I-CFTR protein appears unaffected, the mutation could only elicit dysfunction at the RNA level.

In this study, we used three *in vitro* biological assays to study rare *CFTR* missense substitutions found in patients and their impact on CFTR protein, rating the severity of each mutant protein dysfunction (Table 2). We conclude that we can predict a severe phenotype by our approach only when the three techniques simultaneously indicate maturation impairment, retainment in ER, and reduced channel activation for the mutant CFTR protein. Moreover, it appears that P574S, an allele exhibiting a lower severity for two of three criteria, is not a CF-causing mutation, despite strongly decreased estimated channel activity. That is why the three assays must be performed to conclusively identify mutations eliciting a severe phenotype for the patient. In the other cases, the amino acid substitution may be either a polymorphism or linked to a CF-related disorder (such as CBAVD for patient 6) whose phenotype depends on the genetic background of the patient.

The approach that we followed is not suited for rapid diagnosis every time a new missense mutation is encountered in a patient or a fetus. Rather, our purpose is to provide as much biological data as possible about the putative consequences of this substitution in case a new carrier of the mutation is detected. These extra data would enrich the pool of information that clinicians

could rely on to help for counseling. The three techniques that we used may not be practicable in every clinical laboratory performing routine CF genetic diagnosis; therefore, it is advisable to perform this analysis in collaboration with institutions or technical platforms skilled in these methods whenever a new missense *CFTR* substitution is found. Notably, such type of analysis should be useful if performed in a broad range of patients with CF (eg, the French cohort) in collaboration with clinicians to establish a relevant genotype-phenotype correlation.

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References

1. Riordan JR, Rommens JM, Kerem BS, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, Drumm ML, Iannuzzi MC, Collins FS, Tsui LC: Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 1989, 245: 1066–1073
2. Rommens JM, Iannuzzi C, Kerem BS, Drumm ML, Melmer G, Dean M, Rozmahel R, Cole JL, Kennedy D, Hidaka N, Zsiga M, Buchwald M, Riordan JR, Tsui LC, Collins FS: Identification of the cystic fibrosis gene: chromosome walking and jumping. Science 1989, 245:1059–1065
3. Riordan JR: CFTR function and prospects for therapy. Annu Rev Biochem 2008, 77:701–726
4. Lommatzsch ST, Aris R: Genetics of cystic fibrosis. Semin Respir Crit Care Med 2009, 30:531–538
5. Linton KJ, Rosenberg MF, Kerr ID, Higgins CF: Structure of ABC transporters. ABC Proteins: From Bacteria to Man. Edited by IB Holland, S Cole, K Kuchler, CF Higgins. Academic Press, Waltham, MA 2003, pp 65–80
6. Tabcharani AT, Chang XB, Riordan JR, Hanrahan JW: Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene. Nature 1991, 352:628–631
7. Ostedgaard LS, Baldursson O, Welsh MJ: Regulation of the cystic fibrosis transmembrane conductance regulator Cl^- channel by its R domain. J Biol Chem 2001, 276:7689–7692
8. Welsh MJ, Smith AE: Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. Cell 1993, 73:1251–1254
9. Haardt M, Benharouga M, Lechardeur D, Kartner N, Lukacs GL: C-terminal truncations destabilize the cystic fibrosis transmembrane conductance regulator without impairing its biogenesis. J Biol Chem 1999, 274:21873–21877
10. Dequeker E, Stuhrmann M, Morris MA, Casals T, Castellani C, Claustres M, Cuppens H, des Georges M, Ferec C, Macek M, Pignatti PF, Scheffer H, Schwartz M, Witt M, Schwarz M, Girodon E: Best practice guidelines for molecular genetic diagnosis of cystic fibrosis and CFTR-related disorders: updated European recommendations. Eur J Hum Genet 2008, 17:51–65
11. Howell LD, Borchardt R, Cohn JA: ATP hydrolysis by a CFTR domain: pharmacology and effects of G551D mutation. Biochem Biophys Res Commun 2000, 271:518–525
12. Gregory RJ, Rich DP, Cheng SH, Souza DW, Paul S, Manavalan P, Anderson MP, Welsh MJ, Smith AE: Maturation and function of cystic fibrosis transmembrane conductance regulator variants bearing mutations in putative nucleotide-binding domains 1 and 2. Mol Cell Biol 1991, 11:3886–3893
13. Sheppard DN, Rich DP, Ostedgaard LS, Gregory RJ, Smith AE, Welsh MJ: Mutations in CFTR associated with mild-disease-form Cl^- channels with altered pore properties. Nature 1993, 362:160–164
14. Sheppard DN, Ostedgaard LS, Winter MC, Welsh MJ: Mechanism of dysfunction of two nucleotide binding domain mutations in cystic fibrosis transmembrane conductance regulator that are associated with pancreatic sufficiency. EMBO J 1995, 14:876–883
15. Xiong X, Bragin A, Widdicombe JH, Cohn J, Skach WR: Structural cues involved in endoplasmic reticulum degradation of G85E and G91R mutant cystic fibrosis transmembrane conductance regulator. J Clin Invest 1997, 100:1079–1088
16. Bilan F, Thoreau V, Nacfer M, Dérand R, Norez C, Cantereau A, Garcia M, Becq F, Kitzis A: Syntaxin 8 impairs trafficking of cystic fibrosis transmembrane conductance regulator (CFTR) and inhibits its channel activity. J Cell Sci 2004, 117:1923–1935
17. Melin P, Thoreau V, Norez C, Bilan F, Kitzis A, Becq F: The cystic fibrosis mutation G1349D within the signature motif LSHGH of NBD2 abolishes the activation of CFTR chloride channels by genistein. Biochem Pharmacol 2004, 67:2187–2196
18. Brugnon F, Bilan F, Heraud MC, Grizard G, Janny L, Creveaux I: Outcome of intracytoplasmic sperm injection for a couple in which the man is carrier of CFTR p.[R74W;V201M;D1270N] and p.P841R mutations and his spouse a heterozygous carrier of p.F508del mutation of the cystic fibrosis transmembrane conductance regulator gene. Fertil Steril 2008, 90:2004.e23-2004.e26
19. Norez C, Antigny F, Becq F, Vandebrouck C: Maintaining low Ca^{2+} level in the endoplasmic reticulum restores abnormal endogenous F508del-CFTR trafficking in airway epithelial cells. Traffic 2006, 7: 562–573
20. Thiagarajah JR, Song Y, Haggie PM, Verkman AS: A small molecule CFTR inhibitor produces cystic fibrosis-like submucosal gland fluid secretions in normal airways. FASEB J 2004, 187:875–877
21. Vankeerberghen A, Wei L, Jaspers M, Cassiman JJ, Nilius B, Cuppens H: Characterization of 19 disease-associated missense mutations in the regulatory domain of the cystic fibrosis transmembrane conductance regulator. Hum Mol Genet 1998, 711:1761–1769
22. Claustres M, Altiéri JP, Guittard C, Templin C, Chevalier-Porst F, Des Georges M: Are p.I148T, p.R74W and p.D1270N cystic fibrosis causing mutations? BMC Med Genet 2004, 5:19
23. Roxo-Rosa M, Xu Z, Schmidt A, Neto M, Cai Z, Soares CM, Sheppard DN, Amaral MD: Revertant mutants G550E and 4RK rescue cystic fibrosis mutants in the first nucleotide-binding domain of CFTR by different mechanisms. Proc Natl Acad Sci U S A 2006, 103:17891–17896
24. Fanen P, Ghanem N, Vidaud M, Besmond C, Martin J, Costes B, Plassa F, Goossens M: Molecular characterization of cystic fibrosis: 16 novel mutations identified by analysis of the whole cystic fibrosis conductance transmembrane regulator (CFTR) coding regions and splice site junctions. Genomics 1992, 13:770–776