Functional Characterization of a Novel CFTR Mutation P67S Identified in a Patient with Atypical Cystic Fibrosis

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Key Words

CFTR • P67S mutation • Cystic fibrosis • Pancreatic insufficiency • *Xenopus laevis* oocytes • Chloride channel • Electrophysiology • Surface expression

Abstract

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CFTR serves as a cAMP-stimulated chloride channel in a wide range of epithelial tissues and its dysfunction is a hallmark of CF. Over 1400 mutations in the CFTR gene are known, but functional data exist only for a minority of the mutant channels. The aim of the present study was to functionally characterize a novel CFTR mutation identified in a patient with atypical CF. Full length sequencing of the patient's CFTR gene revealed a homozygous C to T transition at nucleotide position 331 (CCT>TCT), which results in a P67S amino acid substitution. Mu-

tant and wild-type CFTR were heterologously expressed in *Xenopus laevis* oocytes. CFTR whole-cell currents were studied using the two-electrode voltage-clamp technique. Channel surface expression was assessed by a chemiluminescence assay. Expression of P67S-CFTR resulted in functional CFTR chloride channels. However, the CFTR chloride conductance observed in oocytes expressing the mutant channel averaged only 24% of that in oocytes expressing wild-type CFTR. Similarly, surface expression of the mutant channel was reduced. In contrast, the mutation did not alter the anion selectivity of the channel, and Western blot analysis indicated a similar protein expression level of mutant and wild-type CFTR. Our findings indicate that the P67S mutation reduces CFTR chloride channel function by reducing channel surface expression. The mild disease phenotype of the patient indicates that the residual function of the mutant channel is sufficient to prevent the development of severe CF symptoms.

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Introduction

Cystic fibrosis (CF) is the most common lethal genetic disorder in Caucasian populations. It is an autosomal-recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1]. The disease is characterized by a chloride channel defect in epithelial tissues of a number of different organ systems, e.g. lung, gastrointestinal tract, reproductive organs, and sweat ducts. Approximately one person in 2000 newborns is affected, however, in some populations CF is less frequent, i.e. one in 10000 among British Orientals, one in 17000 US Africans, and one in 90000 in Hawaiian Orientals [2].

It is well established that CFTR functions as a chloride channel which is activated by the binding of adenosine 5'-triphosphate (ATP) and by the phosphorylation by cyclic adenosine monophosphate (cAMP) activated protein kinase A (PKA) [3]. Additional kinases including SGK1 [4, 5] and complex interactions with other ion channels [6-9] and regulatory proteins [10-13] are also thought to contribute to CFTR regulation and CF pathophysiology. In addition to its function as a secretory chloride channel numerous other functions of CFTR have been described and may be important in the pathogenesis of CF [7, 10, 13, 14]. To date, over 1400 variations in the CFTR gene have been identified (www.genet.sickkids.on.ca/cftr) of which more than 1000 are thought to cause CF [15]. However, only a minority of these mutations have been functionally characterized [16]. The phenotypic spectrum associated with mutations in the CFTR gene ranges from classic CF to mild monosymptomatic presentations [17]. Mutations in the CFTR gene can be classified in six classes according to their molecular mechanisms and consequences for different aspects of CFTR biogenesis, metabolism and function [16, 18, 19]: (I) defective protein synthesis, (II) abnormal processing and trafficking, (III) defective regulation, (IV) decreased channel conductance, (V) reduced synthesis, and (VI) decreased stability. Whereas class I, II, III, and VI mutations are usually thought to be associated with severe multiorgan disease, class IV and V mutations may result in a milder phenotype. However, a clear phenotype-genotype correlation has not yet emerged [16]. Moreover, the presence of compound heterozygotes further complicates the assessment of a phenotype-genotype correlation. Thus, at present it is still very difficult to predict a phenotype from a given genotype or *vice versa*. A systematic functional analysis of mutant CFTR channels in *in vitro* expression systems may lead to a better

understanding of the relationship between channel dysfunction and the severity of the disease in affected patients. Therefore, it remains an important challenge to identify novel CFTR mutations and to functionally characterize them.

The starting point of the present study was the tentative clinical diagnosis of atypical CF in a 14 year old female patient. Routine mutational screening did not reveal any typical CFTR mutation in this patient. However, full sequencing of the CFTR gene revealed a homozygous mutation, that leads to a substitution of a serine for a proline at position 67 (P67S) in the amino acid chain of the CFTR protein. The aim of this study was to investigate the functional effect of P67S in the *Xenopus laevis* oocyte expression system using whole-cell current measurements and a chemiluminescence assay to assess channel surface expression. We found that P67S largely reduces cAMP stimulated CFTR chloride currents and channel surface expression without altering the ion selectivity of the channel. Our findings indicate that P67S represents a class II mutation of CFTR associated with abnormal or reduced channel trafficking.

Materials and Methods

Patient's data

A 14 year old Turkish girl presented with recurrent episodes of upper abdominal pain lasting for 2-3 hours on each occasion. Abdominal pain was usually most pronounced in the morning and before meals. There was no relation to any particular diet. Nine months prior to presentation the girl's abdominal pain exacerbated and there was a weight loss of 3 kg. The patient's weight and height was between 3rd and 10th percentile. Bowel motions were regular, usually once per day. There was no diarrhoea and stool was not floating and not acholic. Urine was clear. The patient did not suffer from any respiratory signs. In particular there was no coughing or inflammatory phlegm seen in the patient. Pulmonary function test showed a vital capacity of 2.91 l which was 95% of the predicted value. Forced expiratory volume in the 1st second was 2.88 l (121% of predicted). X-ray of the thorax was completely normal showing no signs of chronic inflammation. No nasal polyps were present. There was no history of meconium ileus or rectum prolapse. The patient's family history was inconspicuous. In particular there was no history of CF or Mediterranean fever. Parents claimed not to be consanguineous.

Further examination of the abdominal situation presented the following results: Ultrasonography, computer tomography, small intestine contrast filling, and radionuclide imaging for Meckel diverticulum were normal. Gastroscopy and colonoscopy did not show any signs of inflammation or gastroesophageal reflux. *Helicobacter pylori* was negative and lactose free diet did not lead to an improvement of the abdomi-

nal symptoms. Celiac disease was excluded by serological examination. EEG monitoring during acute episodes of pain were not indicative of abdominal migraine. Finally the patient underwent laparoscopy which did not reveal any pathology.

Laboratory tests revealed a single pathological finding, i.e. a reduced pancreatic elastase which was below 50 µg/g stool on three occasions (<50, 20, 33µg/g). Chymotrypsin was also reduced. Total fat content of the stools was not measured. The reduced pancreatic elastase values suggested an exocrine pancreatic insufficiency [20] in the absence of morphological signs for pancreatic disease. As a rare cause for pancreatic insufficiency Shwachman-syndrome could be excluded by normal blood counts on several occasions. Similarly, Johanson-Blizzard-Syndrome could be excluded since the patient had no dysmorphic features and normal intelligence. Monosymptomatic CF was considered as another possible cause for exocrine pancreatic insufficiency. Consistent with mild CF were sweat chloride concentrations in the intermediate range on several occasions (40, 40, 50, 60 mmol/l on intervals between 2 and 4 weeks; normal < 30 mmol/l, CF-level > 60 mmol/l [15]). Nasal potential difference was not measured. With the findings of exocrine pancreatic insufficiency, intermediate sweat chloride concentrations and in the absence of any other convincing pathology a tentative diagnosis of atypical CF was reached in accordance with the criteria given by the European Diagnostic Working Group [15] and the Cystic Fibrosis Consensus Panel [17]. Therefore, genetic examinations were initiated.

Mutation Screening

Mutational analysis in the *CFTR* gene was carried out using genomic DNA of the patient and her parents. DNA was extracted from 10 ml of a venous blood sample by standard salting out procedure. Initial screening for common *CFTR* mutations was performed with the CF OLA Kit v.2.0 (ABBOTT, Wiesbaden, Germany) according to the manufacturer's instructions. Primers for all 27 coding exons of the *CFTR* gene were designed using Exon Locator and eXtractor (http://variation.swmed.edu/ex-lax/elxrdp_query.html) allowing amplification of all fragments under the same PCR conditions. PCR products were purified using 96 well filter plates (Millipore, Schwalbach, Germany) on a Miniprep™ robot (TECAN, Crailsheim, Germany). The fragments were sequenced using BigDye Version 3.1 (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's recommendations. Sequencing reaction cleanup was also performed on a Miniprep™ robot (TECAN) with the corresponding 96 well filterplates (Millipore). Purified sequences were separated on an ABI3730 sequencer (Applied Biosystems) and subsequently were analyzed using SeqMan Software (DNASTAR, Inc., Madison, USA).

Molecular Biology

A human *CFTR* clone in pGEM containing a hemagglutinin (HA) epitope at amino acid N900 was kindly provided from Dr. Blanche Schwappach (ZMBH, Heidelberg, Germany) and was the same clone as used in previous studies [21, 22]. Starting from this clone a M470V polymorphism was generated by site-directed mutagenesis (QuikChange® Site-Directed Mutagen-

esis Kit, catalog no. 200519, Stratagene, Amsterdam, Netherlands; forward: *CAA GAC TTC ACT TCT AAT GGT GAT TAT GGG AGA ACT GGA G*; reverse: *CTC CAG TTC TCC CAT AAT CAC CAT TAG AAG TGA AGT CTT G*) as it was found in the patient. This clone is referred to as wild-type *CFTR* in this study. In a second step a P67S mutation was inserted into the wild-type *CFTR* clone (forward: *AGC TGG CTT CAA AGA AAA ATT CTA AAC TCA TTA ATG CCC TTC G*; reverse: *CGA AGG GCA TTA ATG AGT TTA GAA TTT TTC TTT GAA GCC AGC T*). In the same way a P67L mutation was generated (forward: *GCT GGC TTC AAA GAA AAA TCT TAA ACT CAT TAA TGC CCT TCG*; reverse: *CGA AGG GCA TTA ATG AGT TTA AGA TTT TTC TTT GAA GCC AGC*). All mutations were controlled by sequence analysis. Linearized plasmids were used as templates for cRNA synthesis using T7 RNA polymerases (mMessage mMachine, Ambion, Austin, TX).

Western blot analysis

20-30 oocytes per group were homogenized with a 27 gauge needle in PBS buffer supplemented with protease inhibitor ('Complete Mini EDTA-free' protease inhibitor cocktail tablets, Roche Diagnostics, Mannheim, Germany). Debris was removed by centrifugation at 1500g for 10 minutes. The supernatant was filled up with PBS and centrifuged at 100000g for 45 minutes at 4°C. The resulting pellet was suspended in 25 µl PBS plus 0.1% Triton X-100 and incubated on ice for 30 minutes. Protein concentration was measured. After incubation at 60°C for 7 minutes in loading buffer 130 µg of protein per lane were loaded and separated by SDS-PAGE (8%) at 20 mA for about 100 minutes and transferred to a PVDF-membrane as a semi-dry blot at 100 mA for 90 minutes. Blots were blocked for 60 minutes in TBST (Tris-buffered saline with 0.1% Tween 20 (Sigma, Taufkirchen, Germany)) containing 5% milk powder at room temperature, incubated over night at 4°C with a primary anti-HA antibody (Anti-HA High Affinity rat monoclonal antibody, clone 3F10, cat. no. 1867423, Roche, Penzberg, Germany) 1:1000 in TBST with 3% milk powder and finally for one hour in a secondary anti-rat antibody (Peroxidase-conjugated AffiniPure Goat Anti-Rat IgG F(ab')₂, Fragment specific, Jackson Immuno Research, Dianova, Hamburg, Germany) 1:10000 in TBST with 3% milk powder. Detection was performed with the ECL Plus Western Blotting Detection Reagent (Amersham Biosciences Europe GmbH, Freiburg, Germany).

Isolation of oocytes and injection of cRNA

Adult female *Xenopus laevis* were anaesthetized in 0.2% MS222 (Sigma) and oocytes were obtained by a partial ovariectomy. The oocytes were isolated from the ovarian lobes by enzymatic digestion at 19°C for 3-4 hours with 700 U/ml type V collagenase from *Cl. histolyticum* (Sigma) dissolved in a solution containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, and 1 mM HEPES (pH 7.4 with Tris). Defolliculated stage V-VI oocytes were injected (Nanoject automatic injector, Drummond, Broomall, PA) with 0.25 ng cRNA of wild-type *CFTR* or P67S mutant or P67L mutant. The cRNAs were dissolved in RNase-free water and the total volume injected was 46 nl. Injected oocytes were stored at 19°C in modified Barth's solution (85 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM Ca(NO₃)₂, 0.4

Fig. 1. CFTR gene mutation analysis. Sequencing analysis of CFTR exon 3 at the level of genomic DNA revealed a homozygous C>T transition at nucleotide position c.331 in the patient. This transition resulted in the substitution of a serine for a proline at position 67 (p.P67S). Both parents were found to be heterozygous carriers of the mutation.

mM CaCl₂, 0.8 mM MgSO₄, 10 mM HEPES, pH 7.4 with Tris) supplemented with 100 units/ml sodium penicillin and 100 µg/ml streptomycin sulphate to prevent bacterial overgrowth.

Two-electrode voltage-clamp

Oocytes were studied 2 days after injection using the two-electrode voltage-clamp technique as described previously [21, 22]. The oocytes were placed in a small experimental chamber and were constantly superfused with ND96 (NaCl 96 mM, KCl 2mM, CaCl₂ 1.8 mM, MgCl₂ 1 mM, Hepes 5 mM, pH 7.4 with Tris) at a rate of 2-3 ml/min at room temperature. CFTR was activated by application of 3-isobutyl-1-methylxanthine (IBMX 1mM, Sigma) and forskolin (1µM, Sigma) to increase intracellular cAMP. At the end of each experiment 1 mM diphenyl-amine-carboxylate (DPC, Sigma) was applied as a chloride channel blocker. Voltage-clamp experiments were performed using an OC-725C amplifier (Warner Instruments Corp., Hamden, USA) interfaced via a LIH-1600 (HEKA, Lambrecht, Germany) to a PC running PULSE software (HEKA) for data acquisition and analysis. For continuous whole-cell current recordings oocytes were routinely clamped at a holding potential of -60 mV. Starting from this holding potential of -60 mV voltage-step protocols were performed intermittently using consecutive 400 ms step changes to potentials ranging from -120 mV up to 60 mV in 20 mV increments. The average current values reached during the last 100 ms of the voltage steps were used for the current-voltage (I-V) relation plots. In the experiments in which the anion selectivity of CFTR was investigated, the oocytes were superfused with a solution containing K-gluconate 2mM, Ca-gluconate 1.8mM, Mg-gluconate 1mM, HEPES 5mM, and 96mM of either NaCl, NaBr, NaI, or Na-gluconate (pH 7.4 with Tris). To avoid artefacts by liquid junction potential differences when the chloride in the bath solution was replaced by other anions, a 3M KCl flowing junction electrode was used as voltage sensing bath electrode in these experiments.

Surface labelling of oocytes

Experiments were performed essentially as described [21, 23, 24] using 1 µg/ml rat monoclonal anti-HA antibody (clone 3F10, Roche Molecular Biochemicals) as primary antibody and 2 µg/ml peroxidase-conjugated affinity-purified F(ab)2 fragment goat anti-rat IgG antibody (Jackson Immuno Research) as secondary antibody. Individual oocytes were placed in a white 96 well plate with U-bottom and 50 µl of SuperSignal ELISA Fento Maximum Sensitivity Substrate (Pierce, Rockford, IL) were added to each oocyte. Chemiluminescence was quantified with a Tecan GENios microplate reader (TECAN, Crailsheim, Germany) with an intergration time of 150 ms and a gain of 100. Results are given in relative light units (RLU).

Statistical methods

Data are presented as mean +/- S.E.M. Student's unpaired t-test was used to assess statistical significance. N indicates the number of different batches of oocytes, n the number of individual oocytes studied.

Results

Identification of a novel mutation in CFTR

To confirm the clinical diagnosis of atypical CF we initially screened the genomic DNA of the patient for 31 known CF mutations. Since this did not show any positive result, full sequencing of the CFTR gene was performed. PCR products containing each exon of the CFTR gene were amplified and sequenced from genomic DNA. Sequencing of exon 3 revealed a homozygous C to T transition at nucleotide position 331 (CCT>TCT), which results in a substitution of a serine for a proline at position 67 (P67S). Although the parents of the patient stated not to be consanguineous, both were found to be heterozygous carriers of the substitution (figure 1). In order to analyze whether the identified alteration may be a non deleterious polymorphism, exon 3 was amplified and analyzed in 25 Turkish control persons. None of the 50 control alleles tested showed the sequence variation (data not shown). Therefore, we conclude that P67S is a novel CFTR mutation. However, genetic detection of a mutation does not prove that this mutation really accounts for a disease. To test the functional relevance of the mutation, we heterologously expressed wild-type and P67S mutant CFTR in *Xenopus laevis* oocytes.

Fig. 2. The P67S mutant CFTR channel is functional but at a largely reduced level. **A:** Representative whole-cell current traces recorded at a holding potential of -60 mV from an oocyte expressing wild-type CFTR (upper trace) or the P67S mutant CFTR (bottom trace). CFTR was activated by IBMX (1 mM) and forskolin (1 μ M). Application of DPC at the end of each experiment led to a partial inhibition of the inwardly directed current. Voltage step protocols for current-voltage (I-V) plots were performed at times indicated by asterisks, but the complete current responses were omitted from the continuous current trace for clarity. **B:** Average I-V plots of IBMX/Fsk induced CFTR whole-cell currents ($\Delta I_{\text{IBMX/Fsk}}$) were obtained from experiments as shown in A using matched oocytes expressing wild-type CFTR (circles) or P67S mutant CFTR (diamonds) (N=7, n=52 per group). $\Delta I_{\text{IBMX/Fsk}}$ values were calculated by subtracting the current values measured before stimulation with IBMX/Fsk from those measured in the presence of IBMX/Fsk after stimulation had reached a plateau. The steeper slope of the wild-type CFTR I-V curve compared to the slope of the P67S mutant CFTR I-V curve indicates a reduced CFTR whole-cell conductance in oocytes expressing the mutant channel. **C:** Representative Western blot analysis to detect CFTR protein expression in oocytes injected with 2ng of cRNA coding for wild-type (wt) or P67S mutant CFTR or in non-injected control oocytes (n. i.). Wild-type and mutant CFTR show a similar protein expression level.

Substitution of serine for proline at position 67 reduces CFTR whole-cell currents

As previously described [21] we used a combination of 1mM IBMX and 1 μ M forskolin (IBMX/Fsk) to elevate intracellular cAMP and activate whole-cell CFTR Cl⁻ currents ($\Delta I_{\text{IBMX/Fsk}}$) in the oocyte expression system. Figure 2A shows typical whole-cell current recordings obtained from an oocyte expressing wild-type CFTR (upper trace) and from an oocyte expressing the P67S mutant CFTR (lower trace) at a holding potential of -60 mV. In both cases application of IBMX/Fsk caused a sustained stimulation of a characteristic inward current component which was not observed in non-injected or water-injected control oocytes (data not shown; [21]). Its partial inhibition by the Cl⁻ channel inhibitor DPC (1mM) confirmed that the IBMX/Fsk activated current was due to CFTR-mediated Cl⁻ efflux [25]. These findings indicate that expression of P67S mutant CFTR results in functional CFTR chloride channels. However, the CFTR whole-cell currents activated by IBMX/Fsk were much smaller in oocytes expressing the P67S mutant CFTR than in matched oocytes expressing wild-type CFTR. Results from seven different batches of oocytes are sum-

marized in the I-V plots shown in figure 2B. From these plots IBMX/Fsk activated whole-cell conductance ($\Delta G_{\text{IBMX/Fsk}}$) was calculated as chord conductance using the current values measured at 40 mV and 60 mV. $\Delta G_{\text{IBMX/Fsk}}$ averaged $31.8 \pm 4.3 \mu\text{S}$ (n = 52) in P67S mutant expressing oocytes and was significantly lower than $\Delta G_{\text{IBMX/Fsk}}$ in wild-type CFTR expressing oocytes ($131.9 \pm 12.5 \mu\text{S}$, n = 52, $p < 0.0001$). These results demonstrate that the P67S mutation does not abolish but largely reduces CFTR chloride channel function in the oocyte system. Western blot analysis demonstrated similar CFTR pro-

tein levels in oocytes expressing the wild-type or the mutant channel (figure 2C). Thus, the reduced channel function of the P67S mutant compared to wild-type CFTR is unlikely to be caused by reduced protein synthesis of the mutant channel.

Reduced chloride conductance is a result of a reduced surface expression of mutant CFTR.

A decrease in chloride whole-cell conductance can be caused by a reduced single channel conductance of the mutant CFTR, by a decrease of its open probability, or by a reduction of its surface expression. Since the P67S mutation is not localized in the vicinity of channel regions thought to contribute to the pore of the CFTR channel or its gating mechanism [3], we hypothesized that the mutation was likely to affect channel surface expression. To investigate a possible effect of the P67S mutation on channel surface expression, we used HA-tagged CFTR constructs and a chemiluminescence assay as described previously to measure CFTR surface expression and whole-cell currents in parallel [21]. In three independent experiments using different batches of oocytes we found a large reduction of the surface expression of the P67S mutant channel compared to wild-type CFTR. Our results indicate that a reduced surface expression of the P67S mutant CFTR can fully explain the reduction of $\Delta G_{\text{IBMX/Fsk}}$ observed in oocytes expressing the mutant channel (figure 3).

The P67S mutation does not alter the anion selectivity of CFTR

It is well known that mutations in CFTR can influence the anion selectivity of the channel [26]. Therefore, we wanted to test whether the P67S mutation affects the anion selectivity of the IBMX/Fsk induced whole-cell conductance. When estimating the ion selectivity of a channel, one has to make a distinction between the permeability sequence obtained from reversal potential measurements and the conductance sequence obtained from current measurements [27]. Furthermore, to calculate the permeability the extracellular and the intracellular concentrations of an ion must be known. Since in large cells like *Xenopus laevis* oocytes the intracellular concentration of an ion is not known, the permeability cannot be accurately calculated. Therefore, we estimated the anion selectivity sequence by determining the whole-cell conductance in the presence of different anions in the bath solution, i.e. by replacing NaCl in the bath solution by an equimolar amount of Na⁺ salts of gluconate, I⁻ or Br⁻. With a similar experimental approach a whole-cell

Fig. 3. Surface expression of the P67S mutant CFTR channel is reduced. Surface expression (open columns) and IBMX/Fsk stimulated whole-cell conductance (closed columns) of HA-tagged wild-type and P67S mutant CFTR channels were assessed in matched groups of oocytes. To summarize data from three different batches of oocytes (N=3) the individual whole-cell conductance and surface expression values were normalized to the corresponding average value measured in oocytes expressing HA-tagged wild-type CFTR. In the surface expression experiments the background signal caused by non-specific antibody binding was determined in non-injected oocytes and was subtracted from the other values. Numbers in brackets indicate numbers of individual oocytes. *** p<0.001.

conductance sequence of Cl⁻ > Br⁻ > I⁻ has previously been determined for wild-type CFTR expressed in *Xenopus laevis* oocytes [28]. Figure 4A shows representative whole-cell current traces recorded in an oocyte expressing either wild-type (*upper traces*) or mutant CFTR (*lower traces*). Voltage pulse protocols were performed in the presence of different anions in the bath solution. For both, wild-type and mutant CFTR, outward currents were largest in the presence of bath Cl⁻ and were increasingly diminished in the presence of Br⁻, I⁻, and gluconate suggesting a whole-cell conductance sequence of Cl⁻ > Br⁻ > I⁻ > gluconate. Figure 4B summarises results from three similar experiments as shown in figure 4A. These results indicate that the P67S mutation does not alter the anion selectivity of CFTR.

The previously reported P67L mutation has a similar effect on CFTR function as the P67S mutation

As shown above the P67S mutation reduces $\Delta G_{\text{IBMX/Fsk}}$ by decreasing the surface expression of the mutated CFTR channels. Interestingly, another mutation at the

Fig. 4. Anion conductance sequence.

A: Typical whole-cell recordings of two-electrode voltage-clamp measurements of a wild-type CFTR (top) and a P67S mutant (bottom) expressing oocyte after activation with IBMX/Fsk. Extracellular Cl^- was replaced by Br^- , I^- , or gluconate. To avoid bias from spontaneous non-specific changes of the whole-cell conductance, the bath solution was switched back to Cl^- each time after exposing the oocytes to another test anion. Voltage pulse protocols were performed as described in materials and methods from a holding potential of -60 mV down to -120 mV and up to 60 mV in 20 mV increments. Please note the different current scales for wild-type CFTR and the P67S mutant. **B:** Summary of results from three similar experiments as shown in **A**. Since extracellular anion substitution will mainly affect the outward current component carried by anion influx, the whole-cell conductance was calculated as chord conductance using the current values measured at $+40$ and $+60$ mV. Conductance values measured in the presence of a test anion were normalized to the mean whole-cell conductance measured in the presence of Cl^- before and after switching the bath solution to this test anion. There was no difference in the whole-cell conductance sequence of wild-type CFTR (open bars, $n=3$) and P67S mutant CFTR (grey bars, $n=3$).

same position in the CFTR protein has previously been described, P67L, and was reported to cause a dominant mild phenotype of CF [29], i.e. if a patient is heterozygous for this mutation, he suffers from a mild CF even when the mutation on the other allele is known to cause severe CF (e.g. ΔF508). However, so far no functional data are known for this mutation. Therefore, we decided to investigate the P67L mutation to compare its effect with the effect of the P67S mutation. In two different batches of oocytes we found that $\Delta G_{\text{IBMX/Fsk}}$ was similarly decreased in P67S CFTR ($30.2 \pm 2.8 \mu\text{S}$, $n=9$) and P67L CFTR ($14.3 \pm 4.1 \mu\text{S}$, $n=9$) expressing oocytes compared to $\Delta G_{\text{IBMX/Fsk}}$ of wild-type CFTR ($157.0 \pm 24.4 \mu\text{S}$, $n=9$, see figure 5) expressing oocytes. Thus, both mutants cause a similar reduction of $\Delta G_{\text{IBMX/Fsk}}$.

Fig. 5. IBMX/forskolin activated whole-cell conductance ($\Delta G_{\text{IBMX/Fsk}}$) of wild-type, P67S, and P67L CFTR. Experiments were essentially performed as described in figure 2 to determine $\Delta G_{\text{IBMX/Fsk}}$ in matched oocytes from two different batches expressing either wild-type CFTR, P67S or P67L CFTR ($N=2$, $n=9$, ** $p<0.01$, *** $p<0.001$).

Discussion

In the present study we identified and functionally characterized a novel mutation of CFTR in a patient diagnosed with atypical CF. The following findings of this study support the hypothesis that this mutation is indeed pathophysiologically relevant: 1) Expression of the P67S mutant CFTR in *Xenopus laevis* oocytes resulted in a CFTR whole-cell conductance that averaged only 24 % of that of wild-type CFTR. 2) The surface expression of the P67S mutant was also largely reduced compared to that of wild-type CFTR. 3) The mutation P67L, a previously described dominant mild form of CFTR [29], reduced the CFTR whole-cell conductance to about the same level as the P67S mutation. Therefore we believe that P67S is a novel CFTR mutation which in homozygous patients can cause a mild phenotype of CF characterized by abdominal pain and pancreatic insufficiency in our patient.

The combination of a reduced CFTR whole-cell conductance, decreased channel surface expression, and normal protein expression level indicates that the P67S mutation causes a defect in channel trafficking and corresponds to a class II mutation [16]. In the absence of single channel patch clamp data we cannot rule out the possibility that the mutation also affects single channel conductance or channel open probability. However, the observed reduction in channel surface expression can fully account for the observed reduction in CFTR whole-cell conductance. Therefore, it is unlikely that a reduced open probability or a reduced single channel conductance of the mutant channel contribute to the reduced CFTR whole-cell conductance. Interestingly, the P67S mutation appeared to reduce channel surface expression even more than CFTR whole-cell conductance (fig. 3). Thus, the few mutant channels that make it to the membrane may even have a slightly higher open probability and/or single channel conductance than wild-type channels. However, from structural considerations it is unlikely that the P67S mutation affects the channel pore or the gating mechanism [3, 30]. CFTR is composed of two motifs, each containing a membrane-spanning domain (MSD) usually formed by six transmembrane segments and a nucleotide-binding domain (NBD). Both motifs are connected *via* a regulatory R domain. The membrane-spanning domains contribute to the formation of the channel pore whereas the nucleotide-binding domains bind and hydrolyze ATP to regulate channel gating, and phosphorylation of the R domain controls channel activity. The intracellular N-terminus contains the first 80 amino acids of CFTR. Thus,

the P67S mutation is located distant from the channel pore or from domains likely to be involved in channel gating. Moreover, our finding that the P67S mutation does not affect the anion selectivity of the channel supports our conclusion that the mutation does not affect the channel pore. For both, wild-type and mutant CFTR we determined a whole-cell conductance sequence of $\text{Cl}^- > \text{Br}^- > \text{I}^- > \text{gluconate}$. In conclusion it is unlikely that the P67S mutation has a major effect on channel properties like single channel conductance or open probability.

Interestingly, another mutation affecting the P67 residue of CFTR has previously been reported to cause a mild CF phenotype [29]. This P67L mutation was found to confer a dominant mild effect, i.e. compound heterozygotes carrying the P67L mutation on one allele and a severe CFTR mutation (e.g. ΔF508) on the other allele have a mild phenotype. Gilfillan et al. [29] reported that none of the P67L compound heterozygotes they investigated showed consistently raised sweat chloride concentrations and 77% of the 13 patients investigated were pancreatic sufficient. Another study [31] reported one patient with a negative sweat test and pancreatic sufficiency, who was also heterozygous for P67L. To our knowledge no functional data have yet been reported for the P67L mutant. In our study we found that the P67L mutant had a similar inhibitory effect on the CFTR whole-cell conductance as the P67S mutant. These findings confirm the functional relevance of the P67 residue and support our conclusion that the newly identified P67S mutation can lead to a mild CF phenotype.

The most frequent mutation found in CF patients is ΔF508 , which usually leads to a severe phenotype in homozygous patients unless combined with a dominant mild mutation in compound heterozygotes. In a previous study [32] the ΔF508 mutation was shown to cause a 85% reduction of the CFTR whole-cell conductance compared to wild-type CFTR in the oocyte expression system. Thus, in the oocyte expression system the inhibitory effect of the ΔF508 mutation seems to be rather similar to that observed in the present study for the P67S and P67L mutant channels. This is surprising in view of the largely different CF phenotypes associated with these mutations. Interestingly, it has been reported that the ΔF508 mutation causes a temperature-sensitive stability defect with a decreased conformational stability of ΔF508 -CFTR at higher temperatures [33]. Since expression studies in *Xenopus laevis* oocytes are usually performed at a temperature of around 19°C, it is likely that at this temperature ΔF508 -CFTR is more stable than at 37 °C. Thus, it is likely that ΔF508 -CFTR shows a larger residual activ-

ity in the *Xenopus laevis* oocyte system than in mammalian tissues. Indeed, in several epithelial tissues of mice transgenic for $\Delta F508$ -CFTR [34-38] cAMP stimulated chloride currents are essentially absent which is consistent with the severe phenotype of this mutation. Thus, the temperature sensitivity of the $\Delta F508$ -CFTR may explain its more severe phenotype compared to the P67S or the P67L mutations. In any case, the mild CF phenotype of patients with the P67S or the P67L mutations suggests that the residual function of these mutant channels is sufficient to prevent severe symptoms. In addition, mutant CFTR channels with residual function may respond particularly well to novel pharmacological approaches designed to rescue channel function by using chloride channel openers [39] or chaperones [40, 41].

The P67S mutation in our patient was found on the background of a M470V polymorphism of CFTR. V470 shows a slightly lower IBMX/Fsk activated whole-cell conductance than the more frequent M470 polymorphism, when heterologously expressed in *X. laevis* oocytes [42]. It is conceivable that the combination of V470 and P67S leads to a mild phenotype of CF whereas the combina-

tion of M470 and P67S shows a normal phenotype. However, to clarify this question it would be necessary to identify persons with a M470 P67S genotype and to test for symptoms of CF. In addition we have to consider that the phenotype of the P67S-CFTR mutation may be influenced by modifier genes or other factors [43].

In summary we have identified a novel CFTR mutation in a patient clinically diagnosed with atypical CF. The P67S mutant channels can be stimulated by exposure to IBMX/Fsk and have normal anion selectivity. However, the mutation largely compromises overall channel function by decreasing channel surface expression. The residual function of the P67S mutant channel is consistent with the mild CF phenotype of the affected patient.

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