Molecular and Functional Characterization of CBAVD-Causing Mutations Located in CFTR Nucleotide-Binding Domains

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Key Words

CFTR • CFTR gene mutation • Chloride channel • Male infertility • Vas deferens • Cystic fibrosis

Abstract

Background: About 98% of male affected with cystic fibrosis (CF [MIM 219700]) are infertile due to bilateral absence of vas deferens (CBAVD [MIM 277180]), which makes up 1-2 % of all cases with male infertility. A previous screening of the entire coding region of the cystic fibrosis transmembrane conductance regulator gene (CFTR [MIM 602421]) in CBAVD patients identified three novel mutations: P439S is located in the first nucleotide binding domain (NBD1) of CFTR, whereas P1290S and E1401K are located in NBD2. **Methods:** We analysed the effects of these novel mutations on CFTR processing and chloride (Cl⁻) channel activity. **Results:** Although maturation patterns were not affected, total amounts of mature P439S-CFTR and P1290S-CFTR were reduced. Confocal microscopy showed correct membrane localisation of E1401K-CFTR, whereas P439S-CFTR and P1290S-CFTR mutants were located mainly in the cytoplasm. Iodide influx assay and whole-cell patch clamp demonstrated significantly reduced cAMP-de-

pendent anion conductances for all three mutants. **Conclusion:** Dysfunction of CFTR is caused by either defective CFTR trafficking (P439S and P1290S) or/and Cl⁻ channel function (P1290S and E1401K). Thus reduced Cl⁻ conductance caused by the three CFTR mutations affects normal development of vas deferens and leads to CBAVD, but the remaining function is sufficient to prevent other typical CF symptoms.

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Introduction

Cystic fibrosis (CF; MIM 219700) is the most frequent severe autosomal recessive genetic disorder in the Caucasian population, affecting approximately 1 in 2500 live births [1]. The gene responsible for CF encodes a 170 kDa glycosylated protein, named CF Transmembrane Conductance Regulator (CFTR), which functions as a cyclic adenosine 3, 5-monophosphate (cAMP)-regulated chloride (Cl⁻) channel in the apical membrane of epithelial cells [2]. It is essential for fluid transport in epithelial cells by allowing Cl⁻ secretion across the apical mem-

brane of epithelial cells [2]. Mutations in the *CFTR* gene, which lead to low levels of expression or function of CFTR cause viscous secretions that obstruct the lumen of pancreatic and biliary ducts, airways, sinuses, sweat glands, gastrointestinal tract and reproductive tissues [3].

CFTR is a member of the ABC transporter superfamily and consists of two similar motifs, each containing a membrane spanning domain (MSD) and a cytoplasmic nucleotide-binding domain (NBD) [2]. The two parts are linked by a cytoplasmic hydrophilic regulatory (R) domain, which contains a number of consensus sites for phosphorylation by protein kinases [2]. In contrast to other ABC transporters which actively pump substrates, binding of ATP to CFTR-NBDs is required for gating of the channel that allows Cl^- flow passively down its electrochemical gradient [4, 5]. High-resolution structures are not yet available for full-length eukaryotic ABC protein including human CFTR, but have been obtained for prokaryotic Sav1866 [6] and BtuCD [7] transporters and for isolated NBDs [8-10]. NBDs reveal the same basic fold, with highly conserved sequence motifs that bind ATP [8-10]. Sequence alignment of CFTR's NBDs showed only 30% homology, however they share crucial and highly conserved sequences: Walker A and B motifs, the ABC signature sequence and the Q- and H- loops that provide glutamine and histidine residues critical for ATP binding [11]. Anion flow through CFTR occurs after gating of the channel by ATP binding to cytoplasmic NBDs [12-15].

Upon phosphorylation of the R domain and ATP binding both NBDs dimerize, which is required for opening of CFTR channels. Intramolecular heterodimerization may cause a signal that is transmitted through cytoplasmic-linking domains to open the channel [14]. According to structural information from a bacterial ABC transporter, hydrolysis of ATP at NBD2 would separate both NBDs and close the channel [14]. In case of CFTR both NBDs have different properties, which seem to have evolved to allow hydrolysis of only one bound ATP, while the other remains unaltered during several gating cycles [14].

To date, more than 1500 *CFTR* mutations have been reported (Cystic Fibrosis Mutation Database, <http://www.genet.sickkids.on.ca/cftr/>) with only a minority being characterized at the protein level. *CFTR* mutations may affect function by causing defects in (I) biosynthesis, (II) processing, (III) regulation, (IV) conductance and (V) by reducing the amount of functional CFTR. Classes I, II and III have been associated with typical severe multi-organ disease [16]. In contrast, class IV and V mutations produce sufficient functional CFTR that causes

a mild CF phenotype [16]. Mutations in CFTR were found to have an impact on human reproduction and consequently about 98% of male CF patients are infertile due to obstructive azoospermia, which is associated with congenital absence of the vas deferens (CBAVD; MIM 277180) [17, 18]. Mutations in *CFTR* gene have also been identified in patients with isolated CBAVD and in the absence of other clinical CF symptoms [19-24]. Thus CBAVD may represent an atypical incomplete (genital) form of CF [20]. The genetic link between CF and CBAVD has been confirmed by several studies that identified *CFTR* mutations in 80-100% of isolated CBAVD patients [24-26]. Clinical symptoms of CBAVD are characterized by azoospermia with low semen plasma volume (<1.5 ml) and low pH (<7.5), unilateral or bilateral non-palpable vas deferens, absence of the distal part of the epididymis and hypoplasia of the seminal vesicles [21]. Notably, *CFTR* genotypes in patients with CBAVD are markedly different from those found in CF patients [27]. CBAVD patients are usually heterozygous for a mild mutation in one and a severe mutation in the other allele (88%), or carry two mild mutations in both *CFTR* alleles (12%) [26]. In a previous study we described a spectrum of *CFTR* mutations causing CBAVD [24]. Here we analyzed the impact of three novel missense mutations on the CFTR processing, intracellular transport and Cl^- channel activity using a heterologous expression system.

Materials and Methods

Patients

Patients were identified during a previous study [24]. The three patients reported here are infertile due to congenital bilateral absence of the vas deferens (CBAVD). None of them had been reported to have pulmonary or gastrointestinal manifestations of typical CF and had sweat Cl^- values between 40-60 mmol/l. Diagnosis of CBAVD was based on azoospermia with low seminal fluid volume (<1.5 ml), pH (<7.2) and physical examination of the scrotum (presence of globus major and absence of palpable vas deferens). Transrectal and scrotal ultrasonography showed hypoplasia/aplasia of the seminal vesicles and a dilated epididymis head (globus major). Abdominal ultrasonography was performed to rule out renal abnormalities. Patients had normal serum hormone levels, normal 46,XY karyotypes and were tested negative for Y-chromosome azoospermic factor (AZF) and deleted in azoospermia (DAZ) microdeletions. At the diagnostic testicular biopsy, all patients showed conserved spermatogenesis with germ cell sloughing, suggestive of tubular obstruction. Sperm retrieval for infertility treatment was performed either by microsurgical epididymal sperm aspiration or open testicular sperm extraction.

Absence of the vas deferens and distal epididymis, and presence of epididymis globus major were confirmed during surgical procedures [28]. Genetic analyses were performed after receiving informed patient consent.

CFTR genotyping

DNA samples were obtained from peripheral blood lymphocytes using a salting-out method. Samples were screened for the 31 most common *CFTR* gene mutations within the Caucasian population with the Cystic Fibrosis Diagnostic System kit (Abbott, Wiesbaden, Germany) [23]. Characterization of the (TG)m(T)n polymorphic sequence was carried out by PCR amplification of the junction between intron 8 and exon 9 using: 5'TGA AAA TAT CTG ACA AAC TC3' and 5'ATT TGG GTA GTG TGA AGG G3', followed by automated DNA sequencing (ABI Prism BigDye Terminator Sequencing kit, ABI PRISM 310 DNA sequencer, Applied Biosystems, Foster City, CA, USA) [23, 29]. To further detect *CFTR* mutations, all 27 *CFTR* exons and bordering intron sequences were studied by Denaturing Gradient Gel Electrophoresis (DGGE) and denaturing High Performance Liquid Chromatography (dHPLC) as described previously [23, 24, 30, 31]. DNA samples with abnormal migration patterns were amplified by PCR and sequenced for identification of mutations.

Nomenclature and multiple sequence alignments

Nucleotide numbering was based on the A of the ATG translation start codon at position +133 (GeneBank NM-000492.2). Multiple sequence alignments were performed using ESPript 2.2 software (<http://esprict.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>). Full length CFTR sequences were obtained from Rhesus monkey (*Macaca mulatta*, AAC14011.1); Pig-tailed macaque (*Macaca nemestrina*, AAD46905.1); Olive baboon (*Papio cynocephalus anubis*, AAD46907.1); European rabbit (*Oryctolagus cuniculus*, AAF01067.1); Bovine (*Bos taurus*, AAA30772.1); Pig (*Sus scrofa*, AAS98211.1); Sheep (*Ovis aries*, AAA68600.1) and mouse (*Mus musculus*, AAA37417.1) (GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/>). For sequence and structural alignments, wtCFTR sequences were aligned with those of human F508del-CFTR NBD1 domain binding ATP (PDBid: 1XMJ) [32], mouse CFTR NBD1 binding ATP (PDBid 1ROX), the dimeric structure of MJ0796 (PDBid 1L2T) [33], Malk ATP-binding cassette dimer (1Q12) [34], ABC transporter from *Thermotoga maritima* (PDBid: 1JI0), Vitamin B12 transport ATP-binding protein BtuCD (PDBid: 1L7V) [7] and MJ1267 ATP binding cassette (PDBid: 1G6H) [35]. Three-dimensional structures of the SAV1866 protein (PDBid: 2HYD) [6] and phosphorylated mouse CFTR NBD1 with ATP (mNBD1, PDBid: 1ROZ) [36] were also used for the structural alignments. Images were obtained using PyMOL (<http://www.pymol.org>) and coordinates were obtained from the Protein Data Bank (PDB, <http://www.rcsb.org/pdb/home/home.do>).

Plasmids, cell culture, transient transfection

CFTR mutations (P439S, P1290S and E1401K) were generated (QuikChange site-directed mutagenesis kit, Stratagene, La Jolla, CA) in the eukaryotic expression vector pCMVCFTRNot6.2, according to the manufacturer's instruc-

tions. Primers used for mutagenesis are listed in Table 1. Complete *CFTR* coding regions of wild-type and mutant constructs were sequenced. HEK293 cells were kindly provided by Dr. T. Casals (Medical and Molecular Genetics Center-IRO, Barcelona, Spain) and maintained at 37°C / 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, Utah, USA), 100 µg/ml streptomycin, 100 U/ml penicillin and 2mM L-glutamine (Gibco). Cells were transiently transfected at a sub-confluent stage using PolyFect Transfection Reagent (Qiagen, Hilden, Germany) or Lipofectamine 2000 Reagent (Invitrogen). For Western blotting and patch-clamp analysis, HEK293 cells were co-transfected with a plasmid encoding enhanced green fluorescent protein (pEGFP-N1, kindly provided by Dr. T. Casals) at a ratio of 10:1 (pCMVCFTRNot6.2: pEGFP-N1). For iodide influx assay, cells were co-transfected with a plasmid encoding enhanced-yellow fluorescent protein (pEYFP-I152L) at a ratio of 2:1.

Western blotting and immunofluorescence

48h after transfection cells coexpressing EGFP and CFTR were quantified by flow cytometry (FACScalibur, Becton Dickinson, Oxford, UK) to evaluate transfection efficiency and cell viability. Protein was harvested by scraping and lysis of cells in sodium dodecyl sulphate (SDS) sample buffer (57.85 mM Tris pH 6.8, 2% w/v SDS, 10% v/v Glycerol and 0.004% w/v Bromophenol Blue). Protein concentrations were estimated using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein (80 µg) was separated by 7.3% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). CFTR was detected by mouse monoclonal antibody mAb M3A7 (Chemicon, CA, USA), which recognizes an epitope at the C-terminal end of NBD2, and a secondary rabbit polyclonal horseradish peroxidase-conjugated IgG (DakoCytomation, Glostrup, Denmark) antibody. The ubiquitous protein Golgin-97 was used as a loading control using a mouse monoclonal antibody (Molecular Probes, Eugene, OR, USA). Bands were visualized by enhanced chemiluminescence (Immun-Star HRP Substrate Kit; BioRad, CA, USA). For immunofluorescence assay, two protocols were performed in parallel based on membrane staining with Concanavalin A. In one protocol, cell surface was labelled with Concanavalin A before cell permeabilization, while in the other protocol membranes of cellular organelles were labelled with Concanavalin A after cell permeabilization. These protocols allowed better detection of CFTR protein in the cell surface and in the cytoplasm. CFTR was detected by a monoclonal mouse antibody MAB1660 (dilution 1:1000 in PBS, 1% BSA, 0.2% v/v Triton X-100; R&D Systems, Minneapolis, MN, USA), which recognizes an epitope in the R domain, followed by incubation for 1h at RT with Alexa Fluor 568 conjugated goat anti-mouse IgG secondary antibody (dilution 1:500 in PBS, 1% BSA, 0.2% v/v Triton X-100; Molecular Probes). Nuclei were stained with TO-PRO3 (dilution 1:3000 in PBS; Molecular Probes) for 1h at RT. Negative controls were obtained by replacing the primary or secondary antibodies with pre-immune serum. Immunofluorescence was detected by confocal laserscanning microscopy (MRC-1024ES; BioRad).

Primer	Sequence
P439S (forward)	5'-C TTC AGT AAT TTC TCA CTT CTT GGT ACT <u>TCT</u> GTC CTG AAA G-3'
P439S (reverse)	5'-C TTT CAG GAC AGA <u>AGT</u> ACC AAG AAG TGA GAA ATT ACT GAA G-3'
P1290S (forward)	5'-GCC TTT GGA GTG ATA <u>TCA</u> CAG AAA GTA TTT ATT TTT TCT GG-3'
P1290S (reverse)	5'-C CAG AAA AAA TAA ATA CTT TCT GTG <u>ATA</u> TCA CTC CAA AGG C-3'
E1401K (forward)	5'-GCT GAT TGC ACA GTA ATT CTC TGT <u>AAA</u> CAC AGG ATA GAA GC-3'
E1401K (reverse)	5'-GC TTC TAT CC T GTG TTT <u>ACA</u> GAG AAT TAC TGT GCA ATC AGC-3'

Table 1. Primers used for site direct mutagenesis. Two primers were used for each mutagenesis reaction. The altered bases, which encode the mutated amino acids are underlined.

<i>CFTR</i> mutation genotypes		[(TG)mTn] genotype		Patients (%)
<u>F508del</u>	<i>E1401K</i>	(TG)10T9	(TG)11T7	1 (2.2)
<u>R334W</u>	<i>P439S</i>	(TG)11T7	(TG)11T7	1 (2.2)
3272-26A → G	<i>P1290S</i>	(TG)11T7	(TG)10T7	1 (2.2)

Table 2. *CFTR* genotypes identified in three patients with congenital bilateral absence of the vas deferens. *CFTR* mutations: detected by the initial *CFTR* mutations screening (underlined); novel (italics). Genotype frequencies are given as numbers and, in brackets, as percentages of total cohort (45 patients).

Iodide influx

CFTR Cl⁻ channel activity was detected by I⁻ influx in HEK 293 cells co-transfected with *CFTR* and I⁻ sensitive pEYFP-I152L as described earlier [37]. Cells grown on glass coverslips were mounted in a perfusion chamber on the stage of an inverted microscope (Nikon) and perfused with a modified Ringer solution (125 mM NaCl, 0.4 mM KH₂PO₄, 1.6 mM K₂HPO₄, 5 mM D-glucose, 1 mM MgCl₂, 1.3 mM Ca²⁺-gluconate, 20 mM Na⁺-gluconate). I⁻ influx was detected after isotonic replacement of 20 mM Na⁺-gluconate by Na⁺-I⁻. Intracellular cAMP concentration was raised by 2 μM FSK and 0.1 mM IBMX (Sigma). I⁻ quenching of pEYFP-I152L fluorescence was compared in the same cell before and after stimulation using Metafluor software. *CFTR* was inhibited by 5 μM *CFTR*(inh)-172 (Sigma). All the experiments were performed at least in triplicates.

Patch-Clamp

Whole cell experiments were performed at 37°C in HEK293 cells coexpressing EGFP and *CFTR*, on the stage of an inverted microscope (IM35, Zeiss, Oberkochen, Germany). The bath was continuously perfused with Ringer solution (145 mM NaCl, 0.4 mM KH₂PO₄, 1.6 mM K₂HPO₄, 5 mM glucose, 1 mM MgCl₂, 1.3 mM Ca²⁺-gluconate) at a rate of 5-10 ml/min. Patch pipettes had a resistance of 2-4 MΩ when filled with a solution containing 30 mM KCl, 95 mM K-gluconate, 1.2 mM NaH₂PO₄, 4.8 mM Na₂HPO₄, 1 mM EGTA, 0.758 mM Ca-gluconate, 1.034 mM MgCl₂, 5 mM D-glucose, 3 mM ATP, Ca²⁺ 10⁻⁴ mM, pH 7.2. Access conductances were measured continuously and were between 60-140 nS. Currents (voltage clamp) and voltages (current clamp) were recorded using a patch-clamp amplifier (EPC 7, List Medical Electronics, Darmstadt, Germany), LH1600 interface and PULSE software (HEKA, Lambrecht, Germany). At regular intervals, cells were voltage clamped for 1 s in steps of

10 mV, from -50 to +50 mV. Conductance (Gm) was calculated by Kirchhoff's laws using total conductance and access conductance [38].

Structure analysis and statistics

Three-dimensional structures of the ABC transporters SAV1866 [6], MalK homodimer with its regulatory domains [34], MJ0796 NBD homodimer [33] and human NBD1 [32] were compared using PyMol program. The coordinates were obtained from Protein Data Bank ID codes (2HYD, 1Q12, 1L2T, 1XMJ) (<http://www.rcsb.org/pdb/home/home.do>). Detailed analysis of the exact location of each novel mutation in the *CFTR* protein was performed using the three-dimensional structure of the full-length ABC transporter SAV1866 [6]. The P439S mutation was evaluated using three-dimensional structures from MJ0796 dimer (PDBid: 1L2T), F508del-*CFTR* hNBD1 (PDBid 1XMJ), and *CFTR* mNBD1 (PDBid: ROZ). Unpaired and paired Student's *t* tests and analysis of variance (ANOVA) were used for statistical analysis. Linear regression analysis was performed by the method of least squares. *P*<0.05 was considered as statistically significant.

Results

Mutational screening and *CFTR* genotypes

Novel *CFTR* mutations were identified in three Portuguese CBAVD patients by DGGE and direct sequencing (Table 2) [24]. P439S (NBD1) was identified in a 34 year old patient who carried the R334W mutation on the other chromosome. P1290S (NBD2) was identified in a 45 year old patient heterozygous for the

Fig. 1. Multiple alignments of CFTR amino acid sequences from different species and localization of NBD1, NBD2 and three novel CFTR mutations (P439S, P1290S, E1401K).

Fig. 2. A) Western blotting of wt and mutant CFTR in transiently expressing HEK293 cells. NT = non-transfected, GFP = GFP transfected, WT = wtCFTR. Arrows indicate positions of core-glycosylated (band B) and fully glycosylated (band C) forms of CFTR. Equal loading is indicated by the housekeeping protein golgin. B) Densitometric analysis of the CFTR bands. Efficiency of CFTR maturation is shown as the ratio of mature fully-glycosylated (band C) to total CFTR (bands C+B). Data represent means $\pm$ SEM of three independent experiments and were normalized by expressing the data as a percentage of wt, designated as 100%. * Significant differences ($P < 0.05$) between mutant CFTR mutant and wt CFTR.

mild mutation 3272-26A $\rightarrow$ G [39]. E1401K (NBD2) was found in a 37 year old patient who carried the severe F508del mutation on the other chromosome. None of the novel mutations were detected in 100 healthy individuals, making these sequence variations rather unlikely to be polymorphisms. With one exception the mutated amino acids are well conserved among nine different species (Fig. 1).

Biosynthesis of CFTR mutants

Maturation patterns of wild-type (wt) CFTR and mutant CFTR (F508del-CFTR, P439S-CFTR, P1290S-CFTR and E1401K-CFTR) were compared by Western blotting. Maturation of different CFTR proteins was as-

sessed by examining its glycosylation patterns. One thin band with an approximate molecular mass of 140 kDa (band B) indicates the immature, partially glycosylated protein that is located in the endoplasmic reticulum, and a second diffuse band of an approximate molecular mass of 170 kDa (band C) represents mature and fully glycosylated protein, that migrated from the Golgi complex to the cell membrane (Fig. 2A). The severe mutant F508del-CFTR migrates as a single band B, while the three CFTR mutants exhibit maturation patterns, similar to those observed for wtCFTR. However, total amounts of mature P439S-CFTR and P1290S-CFTR were significantly reduced compared to wtCFTR, as indicated by densitometric analysis (Fig. 2B). Maturation efficiency

Fig. 3. Cellular localization of wt and mutant CFTR (red) in overexpressing HEK 293 cells assessed by confocal laser-scanning microscopy. (A) Membranes of organelles were labelled with Concanavalin A (CoA, cytoplasm, green) after cell permeabilization. (B) Cell membranes were labelled with CoA (Cell membrane, green) before cell permeabilization. wtCFTR and E1401K-CFTR were found in the plasma membrane, whereas F508del-CFTR is trapped in the cytoplasm. P439S-CFTR and P1290S-CFTR are detected predominantly in the cytoplasm but to some degree also in the plasma membrane. Sites of colocalization of CoA and CFTR proteins are in yellow colour (merged images). DNA is labelled with TO-PRO3 (blue). A mean number of 15 cells were examined in 3 independent experiments for each CFTR protein analysed.

was calculated as the amount of mature CFTR protein (band C) relative to the total amount of the CFTR (band B+C). All samples were normalized to the “housekeeping” protein golgin. Endogenous CFTR was not detected in HEK293 cells.

Cellular localization of mutant CFTR

Intracellular localization of wtCFTR and CFTR mutants was studied in HEK293 cells by immuno-

fluorescence. Confocal microscopy demonstrated predominant localization of wtCFTR protein in the plasma membrane (Fig. 3A-B). F508del-CFTR was only found in the cytoplasm but was not expressed in the cell membrane. The mutants P439S-CFTR and P1290S-CFTR were mainly expressed in the cytoplasm with little membrane expression, while E1401K-CFTR was clearly present in the cell membrane, similar to wtCFTR (Fig. 3A-B).

Fig. 4. A) Representative traces for I^- (20 mM) influx and fluorescence quenching in the absence or presence of stimulation with forskolin (2 μ M) and IBMX (100 μ M) of HEK 293 cells transiently expressing wt or mutant CFTR. B) Left panel: Histogram of the I^- influx (in arbitrary fluorescence units/second) measured before (white bars) and after (black bars) stimulation. Right panel: Comparison of cAMP (I/F) induced I^- influx and fluorescence quenching in HEK 293 cells, transiently expressing wt or mutant CFTR. I/F = IBMX/Forskolin. The number of cells analysed are indicated within brackets. * Significant differences ($P < 0.05$) between the fluorescence emission under stimulated and basal conditions. C,D) Original recordings and summary of the effects of cAMP on iodide quenching and inhibition by the CFTR inhibitor CFTR(inh)-172 (5 μ M). * Significant differences with $P < 0.05$. Number of experiments = 6.

Cl⁻ Channel activity of CFTR mutants

wtCFTR and mutant CFTR was coexpressed with iodide (I^-) sensitive YFP_{1152L} in HEK293 cells and activation of CFTR Cl^- conductance by stimulation with 3-isobutylmethyl-1-xanthine (IBMX, 100 μ M) and forskolin (FSK, 2 μ M) was detected by an increase in I^- quenching of the YFP_{1152L} fluorescence [37]. Exchange of 20 mM extracellular gluconate by I^- quenched YFP fluorescence. The rate of cAMP-stimulated I^- influx was quantified as arbitrary fluorescence units. Intracellular cAMP was enhanced by stimulation of the cells with 2

μ M FSK and 100 μ M IBMX, which largely increased the rate of I^- influx in HEK293 cells expressing wtCFTR, while non-transfected cells or F508del-CFTR expressing cells did not respond to stimulation (Fig. 4A-B). Expression of all three mutants P439S-CFTR, P1290S-CFTR and E1401K-CFTR allowed for cAMP-induced increase in I^- influx, although the rate of I^- influx was significantly lower than that for wtCFTR (Fig. 4A-B). Reduced I^- influx seems to be in accordance with the reduced levels of protein expression for two mutants P439S-CFTR, P1290S-CFTR. Although E1401K did not interfere with

Fig. 5. Whole cell Cl^- currents measured in HEK 293 cells transiently overexpressing wt or mutant CFTR. Only cells expressing GFP were selected for patch clamp experiments. A) Original traces of whole-cell currents obtained by voltage clamping from -50 mV to +50 mV in steps of 10 mV. B) Summary of the calculated whole cell conductance (G_m) under baseline conditions (white columns) and after stimulation with forskolin (2 μM) and IBMX (100 μM) (black columns). GFP = green fluorescence protein, wt = wtCFTR, F508del = F508del-CFTR, P439S = P439S-CFTR, P1290S = P1290S-CFTR, E1401K = E1401K-CFTR. * Significant differences with $P < 0.05$. (Number of experiments). C) Effect of IBMX (100 μM) and forskolin (2 μM) on whole-cell currents of non-transfected cells and in cells expressing wtCFTR, F508del-CFTR and P1290S-CFTR. Current-voltage curves where mean whole-cell currents (in nA) were plotted against the V_c values (in mV), in non-transfected (black trace) and wtCFTR (red trace) expressing cells (left panel), and in cells expressing F508del-CFTR (black trace) or P1290S-CFTR (blue trace) (right panel). (Δ) control, ($\bullet$) IBMX/forskolin, ($\circ$) IBMX/forskolin in Cl^- 30mM. Results were reported as means. (Number of experiments).

the normal processing of the CFTR protein, it demonstrated a reduced activity. Moreover, cAMP induced increase in I^- influx was inhibited by the specific CFTR blocker CFTR(inh)-172, indicating I^- influx through CFTR Cl^- channels (Fig. 4C).

The functionality of the different mutants was further assessed in fast whole cell patch clamp experiments. Non-transfected control cells or HEK293 cells expressing F508del-CFTR did not increase their whole cell conductance when stimulated with IBMX/FSK. In contrast expression of wtCFTR caused a large increase in whole cell currents and conductance when stimulated with IBMX/FSK (Fig. 5). Similar to the results from I^- uptake studies, CFTR mutants P439S-CFTR, P1290S-CFTR and

E1401K-CFTR showed a reduced but significant current increase and activation of whole cell conductance. Activated whole cell conductances were partially inhibited by reducing extracellular bath Cl^- concentration to 30 mM, indicating stimulation of Cl^- conductance by IBMX/FSK. Figure 5C represents current-voltage (IV) curves, where mean whole-cell currents (in nA) were plotted against the V_c values (in mV), in non-transfected cells and HEK293 cells expressing wtCFTR (left panel), and in HEK293 cells expressing F508del-CFTR and P1290S-CFTR (right panel). In cells expressing wtCFTR ($N = 7$) (Fig. 5C), the addition of IBMX/FSK to the bath solution induced an increase in Cl^- cell conduction which was partially inhibited by reducing extracellular bath Cl^-

Fig. 6. Three-dimensional structure of ABC transporters and possible locations of mutations. The bound ATP molecules are represented as red sticks and locations of mutations are shown as red spheres. A) Full-length ABC transporter SAV1866, MJ0796 NBD homodimer, MalK homodimer with regulatory domains and human F508del NBD1. The models show membrane spanning domains (MSDs), regulatory domain (R) and nucleotide binding domains (NBDs) (PyMol models; coordinates were obtained from the protein data bank (ID codes: 2HYD, 1Q12, 1L2T, 1XMJ)). B) 3D structure of SAV1866 with bound ATPs and positions of the three CFTR mutations located in NBD1 and NBD2. The location of the P439S mutation is also presented on the 3D structures of the MJ0796 dimer (PDBid: 1L2T; the two subunits are represented in green and yellow) and mNBD1 (PDBid: ROZ; orange) superimposed with MJ0796 monomer (PDBid: 1L2TA; green). Highlights of the possible location of E1401K and P1290S mutations refer to the SAV1866 model.

concentration to 30 mM. Increase in Cl⁻ conductance was paralleled by a depolarization from -47.65 to -24.14 mV. Removal of bath Cl⁻ further depolarized the membrane voltage to -6.26 mV, indicating selectivity for Cl⁻.

Structural localization of mutated residues

We localized mutated residues to gain a better understanding of the molecular alterations introduced by these mutations. Because of the lack of structural information for human CFTR, location of the mutations reported in this study was predicted using multiple sequence and structural alignments. Figure 6A,B illustrates the association between NBDs, membrane spanning domains (MSDs) and R domain of ABC transporters, and locations of the mutations and the ATP

binding sites as discussed in the present paper. Each mutation was further investigated using other structures available in the Protein Data Bank (Fig. 6B). Figure 7 shows the sequence alignment of the two NBD sequences of human CFTR with those of other ABC transporters with known 3D structure. Sequence and structural alignment of NBDs from human CFTR with those of SAV1866 [6] and MalK [34] showed that all mutations appear to be far from the interaction with the R domain. While P439S and E1401K are close to ATP binding sites and therefore likely to affect NBD-dimerization and ATP binding, respectively, P1290S is located near the interface NBD/MSD, and may interfere with side-chain contacts within the membrane spanning subunit.

Fig. 7. Alignment of NDB sequences of human CFTR and ABC transporters with known three-dimensional structure. The three *CFTR* missense mutations are indicated by red stars. Walker A and B motifs, ABC signature, and the Q- and H-loops are indicated. Protein data bank (PDB) identifiers at the end of each sequence.

Discussion

CBAVD Patients are compound heterozygous for CFTR mutations

Mutations in the *CFTR* gene impairs epithelial Cl⁻ secretion that may lead to either CF [1] or isolated CBAVD [20]. In the present study, we report biochemical and functional data on three *CFTR* missense mutations located in NBD1 (P439S) and in NBD2 (P1290S, E1401K). CBAVD patients carrying the three novel *CFTR* mutations present a *CFTR*-related disorder. They were diagnosed with obstructive azoospermia due to isolated CBAVD with low semen volume and lowered pH, hypoplasia/aplasia of seminal vesicles, dilated epididymis head and absence of concomitant renal anomalies. However, apart from CBAVD the patients are in good conditions

without pulmonary or gastrointestinal disease and with normal or borderline sweat test. The three CBAVD patients were compound heterozygous, carrying a novel *CFTR* mutation on one chromosome and another known mutation on the other chromosome (Table 2). P439S was identified in heterozygosity with the mild class IV R334W mutation. Previous structure and functional studies have shown that R334W is properly processed but produces reduced ion conductance [40]. P1290S was detected in a patient with additional 3272-26A→G mild mutation on the other chromosome. 3272-26A→G included into the class V accounts for about 2% of all CF alleles in Portugal and has been described in patients with a variable clinical phenotype that ranges from CBAVD to a severe CF phenotype [41-43]. E1401K was identified in compound heterozygosity with the severe F508del mutation.

F508del, present on at least one allele in 70-90% of all CF patients and in 21-40% of alleles in CBAVD patients depending on studies [21, 22, 27, 29], halts biosynthetic maturation and belongs to class II [44].

Mutation P439S was previously reported in a 10-year old Hispanic boy with the R668C mutation in the other chromosome and with somewhat atypical features of CF such as a sweat Cl^- level of 59 mmol/L, mild lung disease and pancreatic insufficiency [45]. Based on our present results and since P439S was associated with the very mild R688C mutation [30], it would not be expected a clinical phenotype of pancreatic insufficiency in that patient, as this is usually associated with the presence of severe *CFTR* mutations [1]. This finding thus suggests the additional contribution of other factors which would modify the severity of the disease, such as additional mutations in the *CFTR* gene, the action of modifier genes and/or environmental effects.

Mutations have different effects on CFTR

CFTR is known to be extremely susceptible to the ER quality control system [43, 46]. Densitometric analysis showed that the ratio of mature fully-glycosylated to immature core-glycosylated bands detected for P439S-*CFTR* and P1290S-*CFTR* was lower than those obtained for wt*CFTR*, indicating that P439S and P1290S mutations cause dysfunction of *CFTR* by decreasing total amounts of mature protein. Sub-cellular localization of the three *CFTR* mutants in HEK293 cells indicated dramatically reduced membrane staining for P439S-*CFTR* and P1290S-*CFTR* and suggests biosynthetic arrest of *CFTR* maturation similar to F508del-*CFTR* [43, 47]. In contrast, E1401K-*CFTR* was correctly localized in the cell membrane, similar to wt*CFTR*. Nevertheless, Cl^- currents generated by the three mutants were all significantly lower when compared to those of wt*CFTR*. Thus E1401K probably reduces the activity of *CFTR* Cl^- channels while P439S-*CFTR* and P1290S-*CFTR* reduce the number of *CFTR* channels in the membrane. Further studies using single channel patch clamp will assess if E1401K reduces whole cell conductance by affecting channel open probability or single channel conductance, and if P439S and P1290S also affect *CFTR* channel pore/gating mechanism.

Localization of CFTR-mutations

Structural localization of mutated residues was performed in order to get further insights into the molecular consequences of the corresponding mutations and predict their effects on protein function. NBD is bound to

the transmembrane subunit primarily through contacts between residues around the Q-loop [7]. P1290S (proline $\rightarrow$ serine) is located just before the Q-loop glutamine residue, near the interface between NBD and MSD (Figs. 6B, 7). This was compared to the ABC transporter from *Thermotoga maritima*, which has a proline residue in a similar position, and to the vitamin B12 transport protein [7], human F508del NBD1 [32] and mouse NBD [36], which have serines at that position. We suggest that this residue may not alter the orientation of the main chains, but may rather influence side chain interactions. The glutamine of the Q-loop was proposed to work as a sensor that triggers movement of the helical transmembrane domain upon ATP hydrolysis. Gamma-phosphate release during ATP hydrolysis leads to withdrawal of the glutamine from the active site, coupled to a bulk movement of the alpha domain relative to the NBD core [35,48]. Mutations in this region may therefore interfere with side-chain contacts between NBD subunit and the membrane spanning subunit, as well as influence the transmission of signals from NBD to MSD, which is important for channel gating.

Structural analysis also suggests that E1401 is probably not exposed to the solvent. It belongs to a cluster of negatively charged residues that are around the phosphates of bound ATP. Thus a charge alteration as found in E1401K (glutamic acid $\rightarrow$ lysine) is likely to disturb ATP binding and therefore reduce *CFTR* Cl^- channel activity, as demonstrated here. Sequence alignment and 3D structures show that ABC transporters different to *CFTR* have a glutamic acid residue at a position similar to E1401 in *CFTR*. The side chain containing the glutamic acid is also part of a cluster of glutamine and glutamic acid residues located around ATP-phosphates (Fig. 6B). Therefore, this suggests that a positive charge introduced by E1401K will reduce ATP binding.

Mouse and human NBDs have 32 amino acid insertions (Fig. 7). In mouse, this insertion is located between beta strands S1 and S2 and consists of two alpha helices connected by a long and flexible loop [36]. It was reported that both strands belong to a beta sub-domain that covers the nucleotide binding site and that S2 contains an aromatic residue that stabilizes ATP [36]. Because P439 will break the beta strand, proline to serine replacement causes a structural change in NBD1 subunit, which seems to be able to disturb NBD-dimerization. This becomes obvious when comparing 3D structures of mouse and human NBDs with the dimeric structures of MJ0796 and MalK (Fig. 6).

The possible effect of these three missense changes

on protein function and splicing was also predicted using the Polyphen (<http://genetics.bwh.harvard.edu/pph/>) and Netgene2 (<http://www.cbs.dtu.dk/services/NetGene2/>) bioinformatics tools, respectively. Polyphen analysis indicated that while E1401K (PSIC 1.609) is “possibly damaging”, P439S (PSIC 2.396) and P1290S (PSIC 2.108) are “probably damaging” (deleterious) to protein function. High PSIC values may indicate that substitution is rarely or never observed in the protein family. Moreover, as the crystal structure of the CFTR NBD1 was already solved, this computational tool also remarked that the P439 amino acid residue is in closest contact with the ATP binding site, which further supported the data obtained by the structural analysis. The evaluation of potential effect on splicing predicted that the three amino acid changes would have no effect on splicing. Although, data obtained with the available bioinformatic tools must be interpreted with caution, these results confirmed experimental data, suggesting that P439S, P1290S and E1401K interfere with protein function.

In summary, the combination of reduced CFTR whole-cell conductance and normal protein expression detected for E1401K indicates that this mutation causes a defect in CFTR channel activity probably by reducing ATP binding, as suggested by structural analysis. Thus, E1401K might belong to class III (defective CFTR regulation). The other two missense mutations, P439S and P1290S, cause a decrease in the total amounts of the mature protein that might be attributed to a defective processing or an increased protein turnover. In fact, P439S seems to be able to impair the fold stability or folding of post-translational NBD1 subunit, which will disrupt the formation of the NBD1/NBD2 heterodimer. Conversely, P1290S appears to affect the NBD1-MSD interaction and thus the overall folding of the entire protein. Based on present data and in what has been established for the classification of different *CFTR* mutations, P439S and P1290S might be classified as class V mutations, associated with reduced levels of normally functioning CFTR. Nevertheless, future metabolic pulse-chase assays will allow to distinguish whether these CFTR mutants are inefficiently processed or, instead, have an increased membrane turnover, and therefore an alternative classification of these mutants within class II (defective protein processing) or

into the recently added class VI (decreased CFTR stability) [49]. In addition, P1290S is also likely to cause a defective CFTR activity by affecting signalling with the MSD, and therefore P1290S might also be classified as a class III mutation. This observation might explain the lower whole-cell Cl^- currents determined for P1290S-CFTR when compared with P439S-CFTR and E1401K-CFTR.

Finally, mutations analysed here do not abolish CFTR function, but lead to limited impairment of CFTR activity. The residual Cl^- conductance generated by these mutant channels, although sufficient to prevent development of CF, led to isolated abrogation of normal development of particularly susceptible tissues such as the vas deferens. This explains why the mutations here reported confer a CFTR related disease or mild CF phenotype, even when in compound heterozygosity with a severe *CFTR* mutation (e.g. F508del). The characterization of the novel *CFTR* mutations, whose effect was previously unpredicted, is thus relevant for a proper genetic counselling of patients bearing these mutations. Although P439S, P1290S and E1401K might not lead to a typical CF phenotype, the knowledge of the mechanism by which they affect CFTR, improve the ability to interpret and predict the clinical phenotype in patients carrying the studied mutations and may give important insights about the molecular consequences of similar sequence alterations that have been, or remain to be characterized. Moreover, the characterization of *CFTR* mutations might also be clinically valuable for the rational design of therapeutic strategies to correct the underlying molecular and/or functional CFTR defects.

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