

Structure-function analysis of a double-mutant cystic fibrosis transmembrane conductance regulator protein occurring in disorders related to cystic fibrosis

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Received 30 April 1999

Abstract A number of disorders related to cystic fibrosis have been described since the cloning of the cystic fibrosis gene, including infertility due to the congenital bilateral absence of the vas deferens. We have identified, in several patients, complex cystic fibrosis transmembrane conductance regulator genotypes like double-mutant alleles. We have now analyzed the structure-function relationships of one of these mutants, R74W-D1270N cystic fibrosis transmembrane conductance regulator, expressed in HeLa cells, to evaluate the contribution of each mutation in the phenotype. We found that R74W cystic fibrosis transmembrane conductance regulator appears to be a polymorphism, while D1270N cystic fibrosis transmembrane conductance regulator could be responsible for the congenital bilateral absence of the vas deferens phenotype. The combination of the two produced a more severe effect on the chloride conductance pathway as well as on the phenotype.

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Key words: Double-mutant; R74W-D1270N; Cystic fibrosis transmembrane conductance regulator; Heterologous expression

1. Introduction

Cystic fibrosis (CF) is the most common severe autosomal recessive genetic disorder in Caucasians. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a cAMP-regulated chloride channel [1]. The number of disorders associated with molecular defects in the CFTR gene has increased steadily since the cloning of the CF gene. These have included male infertility due to the congenital bilateral absence of the vas deferens (CBAVD) [2], disseminated bronchiectasis [3], nasal polyposis [4,5] and, more recently, chronic pancreatitis [6,7]. We and others have identified complex CFTR genotypes in these patients, including double-mutant alleles where two missense mutations are carried by the same chromosome [8]. It is diffi-

cult to evaluate the contribution of each mutation or polymorphism to the phenotypic expression of these conditions.

We have now investigated the structure-function relationships of one of these mutants, R74W-D1270N-CFTR, using a heterologous expression system. The first mutation lies in the intracellular N-terminus of the CFTR protein and the second within the second nucleotide binding domain (NBD2), replacing an arginine by a tryptophan at position 74 and an aspartic acid by an asparagine at position 1270 [9,10]. We postulated that the switch from a basic hydrophilic residue to a neutral hydrophobic one (Arg to Trp) would affect the processing of the CFTR channel in the N-terminus part of the protein and that the change from a conserved acidic to a neutral residue in a critical region for channel gating (NBD2) could be deleterious. We therefore tested these mutations separately and together to elucidate the contribution of each mutation to the phenotype.

The mutant genes were expressed in HeLa cells. We analyzed the protein biosynthesis by immunoprecipitation and pulse-chase experiments and chloride channel function by a halide-sensitive fluorescent dye assay (6-methoxy-*N*-ethylquinolinium (MEQ)) [11]. This assay was greatly improved by using a mammalian expression vector that allows visual detection of transfected cells containing the green fluorescent protein (GFP), which does not alter the function of wild-type CFTR expressed in HeLa cells. These assays showed that the R74W mutation should be considered to be a polymorphism and suggest that D1270N alone is sufficient to produce the CBAVD phenotype, while the combination of the two increases the chloride conductance pathway dysfunction and the phenotype.

2. Materials and methods

2.1. Plasmid construction

A plasmid vector for expressing the human CFTR gene in mammalian cells was constructed by placing the full-length CFTR cDNA encoding sequence (4.5 kb from nucleotide position 90 to 4578, obtained from pTG5960, a gift from Transgene) in the expression vector pTracer-CMV (Invitrogen), a vector designed for visual detection of transfected mammalian cells. The resulting plasmid was designated pTCFwt. Expression of the CFTR gene in pTCFwt is controlled by the CMV promoter, while synthesis of the GFP-Zeocin fusion protein is controlled by the SV40 promoter. Site-directed mutagenesis was performed on pTCFwt using the GeneEditor kit (Promega).

2.2. Cells culture and transfection

HeLa cells (2.5×10^5 /well) were grown on six well dishes at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Glu-

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Abbreviations: CBAVD, congenital bilateral absence of the vas deferens; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; GFP, green fluorescent protein; MEQ, 6-methoxy-*N*-ethylquinolinium; NBD, nucleotide binding domain; PKA, cAMP-dependent protein kinase

tamax, Life Technologies) containing 10% fetal calf serum, 100 U/ml penicillin and 100 g/ml streptomycin (all from Life Technologies). Confluent cells (60%) were transfected by lipofection using 8 μ l LipofectAMINE and 6 μ l Plus reagent (Life Technologies) with 1 μ g of plasmid according to the manufacturer's instructions. Confluent monolayers were harvested and used 48 h post-transfection for functional assays, pulse-chase experiments or immunoprecipitation.

2.3. Immunoprecipitation/cAMP-dependent protein kinase (PKA) assay and pulse-chase experiment

CFTR was immunoprecipitated using the monoclonal antibody (mAb) 24-1 (Genzyme, Framingham, MA, USA), which recognizes the C-terminus of CFTR, according to the manufacturer's instructions. Briefly, cell lysates were prepared with two wells of confluent cells and mixed with 0.4 μ g mAb 24-1 and Pansorbin (Calbiochem). The resulting proteins were phosphorylated *in vitro* with 5 U of the catalytic subunit of PKA (Promega) and 10 μ Ci [γ - 32 P]ATP (Amersham), separated by 5% SDS-PAGE, dried and autoradiographed.

Pulse-chase experiments were performed by incubating cells for 30 min in DMEM lacking cysteine and methionine and then for 15 min in the same medium containing 100 μ Ci/ml methionine and cysteine 35 S-labelled (Redivue Pro-mix 35 S Amersham). CFTR was immunoprecipitated from cell homogenates with the mAb 24-1 from Genzyme and quantified using a Molecular Dynamics Phosphorimager.

2.4. MEQ fluorescence assay

Cells were grown on glass coverslips for 24 h and then loaded with the halide indicator fluorescent dye MEQ as previously described [11]. MEQ fluorescence was excited at 345 nm (filter 345/40 \times , Chroma) and emitted fluorescence was passed through the dichromatic mirror, DM 400 nm, and the barrier filter, BA 420 nm (Olympus U-MWB box). The transfected cells were identified by visual detection of GFP fluorescence before starting the MEQ fluorescence measurements. GFP fluorescence was excited at 440 nm/20 \times (Chroma) and the emitted fluorescence was passed through DM 500 nm and BA at 505 nm (Olympus). The cells were incubated at room temperature for 4 min in hypotonic medium. The medium contained 20 mM MEQ made by diluting isotonic solution (138 mM NaCl, 2.4 mM K_2HPO_4 , 0.8 mM KH_2PO_4 , 10 mM HEPES, 1 mM $CaSO_4$, 10 mM glucose and 20 μ M bumetanide (pH 7.4)) 5:1 with water. They were returned to isotonic buffer to recover. 15 min after loading, the coverslip was placed in a perfusion chamber, perfused continuously at 37°C with the isotonic chloride solution on the stage of an inverted microscope (IX70 Olympus, France) and intracellular MEQ fluorescence was measured. The chloride solution was replaced with nitrate solution after 2 min of perfusion. This was identical except that NO_3^- replaced Cl^- . Because nitrate does not interact with MEQ, the fluorescence increases as cell chloride flows from the cell through the anion conductive pathways if these pathways are functional in the plasma membrane. The changes in fluorescence of stimulated (stimulatory cocktail: 500 μ M 8-(4-chlorophenylthio)-cyclic AMP and 100 μ M 3-isobutyl-1-methylxanthine) and unstimulated cells were recorded. Excitation was at 350 nm and emission was at >440 nm. The fluorescence of single cells was measured with a digital imaging system and a CCD camera (Gen IV, Princeton Instruments, Trenton, NJ, USA/Paris, France). Results were analyzed using Metafluor 3.0 software (Universal Imaging, Media, PA, USA/Paris, France).

The results are expressed as relative fluorescence F/F_0 , where F is the change in fluorescence with time and F_0 is the minimum fluorescence Fig. 3. Cells were scored as positive when the rate of change in fluorescence with the stimulatory cocktail was greater than that in basal conditions. We then calculated the slope of the dequenching curve in basal (ΔF_{basal}) and stimulatory (ΔF_{stim}) conditions and derived the ratio $\Delta F_{\text{stim}}/\Delta F_{\text{basal}}$. This ratio was used rather than the absolute change in fluorescence as the latter may be influenced by dye loading.

3. Results

3.1. Biochemical characterization of wild-type and mutated CFTR proteins

HeLa cells were transiently transfected with the gene encoding wild-type and the three mutated CFTR proteins. The

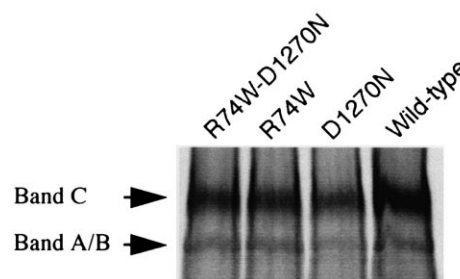


Fig. 1. Immunoprecipitation/PKA assay of wild-type and mutant CFTR proteins using mAb 24-1 (Genzyme).

CFTR protein production and function were assessed. The transfection efficiency was checked by spotting GFP expressing cells on the stage of an inverted microscope.

The immunoprecipitation/PKA assay (Fig. 1) showed that the fully glycosylated form of wild-type CFTR in HeLa cells showed as a diffuse band of an approximate molecular mass of 170 kDa (referred to as 'band C' [12]) on SDS-PAGE. The core-glycosylated and non-glycosylated forms were mixed and appeared as a thin band of about 140 kDa (band A/B) as expected from our previous study [11]. All three mutant proteins matured completely and the ratio (band C/band C+B+A=0.8) was identical, suggesting normal, complete processing.

The biosynthesis of wild-type and mutated CFTR proteins was further investigated by pulse-chase experiments (Fig. 2). The faint band A (non-glycosylated CFTR protein) and band B (core-glycosylated form) both appeared promptly at H_0 , whereas band C (fully glycosylated form) was only detected after 1 h of chase (H_1 , Fig. 2A). The mutated proteins had the same processing time-course as the wild-type CFTR for both biosynthesis and degradation (data not shown).

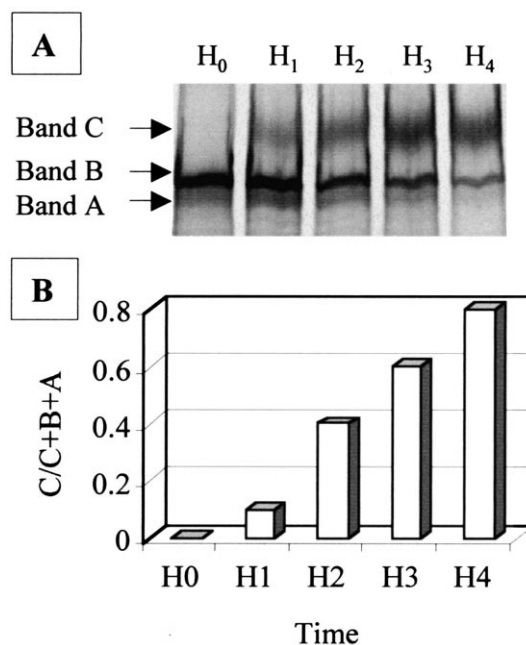


Fig. 2. Pulse-chase experiment of wild-type CFTR protein using mAb 24-1 (Genzyme), (A) typical immunoprecipitation of CFTR protein, (B) quantification of the amount of band C/band C+B+A.

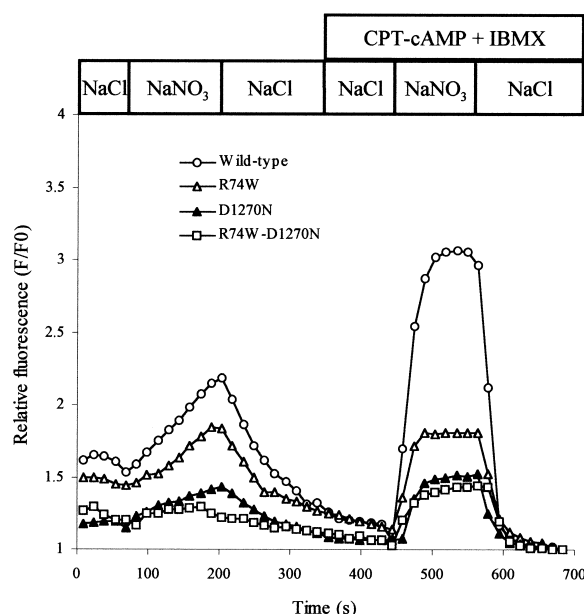


Fig. 3. Analysis of the wild-type and mutant CFTR function with the MEQ fluorescence assay. The sequence of buffer changes is indicated in the top inset. The relative fluorescence was measured over time. HeLa cells were transfected with either wild-type CFTR ($\Delta F_{\text{stim}}/\Delta F_{\text{basal}} = 12$), R74W-CFTR ($\Delta F_{\text{stim}}/\Delta F_{\text{basal}} = 11$), D1270N-CFTR ($\Delta F_{\text{stim}}/\Delta F_{\text{basal}} = 3$) or R74W-D1270N-CFTR ($\Delta F_{\text{stim}}/\Delta F_{\text{basal}} = 2$).

3.2. Function of wild-type and mutated CFTR proteins

We tested the cAMP-activated chloride conductance activity of transfected cells using the MEQ fluorescence assay. Our assay [11] has been greatly improved by the use of a new mammalian expression vector. The synthesis of the GFP allowed visual detection of transfected cells, therefore, all cells analyzed were CFTR-transfected cells. Also, placing the CFTR cDNA under the control of the human CMV immediate early promoter resulted in a great gene activity. Both cDNAs (GFP and CFTR) were contained in the same plasmid vector, but were placed under different promoters.

Table 1 summarizes the results of the MEQ fluorescence assay. None of the mock-transfected cells (pTracer, $n=28$) displayed changes in fluorescence in either basal or cAMP-stimulated conditions, which are believed to activate the CFTR chloride channel.

Transiently transfected cells were divided into responsive and non-responsive cells on the basis of their cAMP-respon-

sive anion conductance. Responsive cells were further divided into fast ($\Delta F_{\text{stim}}/\Delta F_{\text{basal}} > 5$) and slow ($\Delta F_{\text{stim}}/\Delta F_{\text{basal}} \leq 5$) responding cells. Most (85%) of the wild-type CFTR-transfected cells ($n=53$) were responsive and 47% of these positive cells were fast responders (Table 1). The majority (67%) of the R74W-CFTR cells showed an increased rate of change in MEQ fluorescence when exposed to the stimulatory cocktail, indicating activation of a cAMP-dependent anion pathway, 45% of these cells were fast responders. Lastly, 89% of D1270N-CFTR and 81% of R74W-D1270N-CFTR also had a cAMP-responsive anion conductance with different ratios between fast and slow responding cells (38% fast in D1270N-CFTR and 24% fast in R74W-D1270N-CFTR).

4. Discussion

We have analyzed the structure-function relationships of three CFTR mutations, R74W, D1270N and the R74W-D1270N double-mutant, and compared their properties with those of wild-type CFTR. R74W was first described in isolation [10], but has since been found in association with D1270N (Mireille Claustres, personal communication). D1270N was found in a CBAVD patient bearing the $\Delta F508$ common mutation on the other allele [9]. Finally, the R74W-D1270N double-mutant was first identified in a compound, $\Delta F508$, heterozygote with clinical features of CBAVD, rhinitis, recurrent respiratory infections and elevated sweat chloride concentrations [13]. We have since identified three unrelated patients bearing this complex genotype on one allele, two CBAVD patients and a 22 year old woman screened for CFTR mutations because she had severe nasal polyposis. All three patients had an abnormality in only one copy of the CFTR gene. The whole encoding sequence was screened together with the 5T allele, a variant of intron 8 that greatly reduces the amount of mature CFTR [14], and no other mutation was detected.

To our knowledge, this is the first reported expression of the R74W- and/or D1270N-CFTR mutant and the first functional description of a double-mutant associated with CBAVD, a disorder related to CF.

The processing of the three mutated CFTR proteins was normal and similar to that of the wild-type CFTR, given the presence of the fully glycosylated form of the protein and the ratio (band C/band C+B+A). This indicates that they are not class I or II mutations in the classification proposed by Welsh and Smith [15] (class I mutations produce no CFTR protein and class II ($\Delta F508$) fails to mature properly).

Table 1
Summary of the MEQ assay results

Cell type	Wild-type	R74W	D1270N	R74W-D1270N	PTracer
Total	53	30	27	26	28
Non-responding	8	10	3	5	28
All responding	45 (85%) ^a	20 (67%) ^a	24 (89%) ^a	21 (81%) ^a	0
Fast	21 (47%) ^b	9 (45%) ^b	9 (38%) ^b	5 (24%) ^b	0
$\Delta F_{\text{stim}}/\Delta F_{\text{basal}}$	13.3 ± 6.3^c	11.9 ± 8.7^c	7.7 ± 2.4^c	7.3 ± 2.7^c	
Range	5.4–26.3	5.9–34	5.6–12.8	5.2–12	
Slow	24 (53%) ^b	11 (55%) ^b	15 (62%) ^b	16 (76%) ^b	0
$\Delta F_{\text{stim}}/\Delta F_{\text{basal}}$	2.7 ± 1.1^c	2.5 ± 0.9^c	2.8 ± 0.9^c	3.2 ± 1.2^c	
Range	1.3–5	1.1–4.1	1.4–4.5	1.2–4.9	

^aPercentage of all cells.

^bPercentage of positive cells.

^cMean values \pm S.D.

Expression of these three mutant genes in HeLa cells showed that R74W-CFTR, D1270N-CFTR and R74W-D1270N-CFTR proteins elicited cAMP-dependent chloride fluxes. R74W-CFTR elicited a cAMP-responsive anion conductance at the same rate as wild-type CFTR. Half of the cells were fast responders, the other half were slow responders. On the basis of the results of the functional assay, we postulate that the R74W mutation is a sequence variation that has no deleterious effect alone.

D1270N and R74W-D1270N had abnormal responsive patterns. The ratio between fast and slow responder cells was different, especially for the double-mutant, since only 24% of the cells were fast responders. This fraction, as well as that of D1270N-CFTR (38%), resembles the pattern of responses of R117H-CFTR, another mild mutant associated with CBAVD [11]. The substitution is in the second putative membrane spanning domain of CFTR, the protein is correctly processed and generates a smaller cAMP-regulated apical membrane chloride current [16]. The R117H mutation produces different phenotypes, depending on the presence or absence of the 5T allele on the same chromosome [17]. When R117H occurs on a 5T background, the physiology of the vas deferens, lungs and sweat glands is impaired, defining a complete but mild CF. When R117H occurs on a 7T background, only the physiology of the vas deferens is impaired. The R74W-D1270N double-mutant was associated with a 7T background in the three patients studied. This implies that the amount of mutated mRNA synthesized *in vivo* was normal. On the other hand, the structure-function analysis performed *in vitro* showed that the protein is quantitatively and qualitatively correctly processed to the plasma membrane. Thus, this double-mutant strongly resembles the R117H mutation when it is associated with the 7T background. We therefore believe that the R74W-D1270N double-mutant is responsible for the CBAVD phenotype. The contribution of each mutant to this phenotype may be as follows: R74W is a polymorphism which may slightly reduce the normal amount of CFTR protein (67% of responding cells versus 81–89%) *in vivo* and D1270N has a cAMP-responsive anion conductance with different ratios between fast and slow responder cells, as for the R117H mutation. We infer that the combination of R74W enhances the effect of D1270N by reducing the number of fast responder cells, as a consequence of the defective regulation of the R74W-D1270N mutated protein or of a different turn-over of the protein at the cell surface *in vivo*. This is supported by the fact that the compound heterozygote $\Delta F508$ /D1270N described by Anguiano et al. occurred in a CBAVD patient with normal sweat sodium and chloride values, i.e. normal chloride secretion [9], whereas the compound heterozygote $\Delta F508$ /R74W-D1270N described by Casals et al. had a more severe phenotype and elevated sweat chloride concentrations suggesting an abnormal chloride secretion [13].

A recent review by Pilewski and Frizzell [18] pointed out the importance of the CFTR function for the normal organ function, underscoring the great susceptibility of the vas deferens

to CFTR mutations. Our results support this observation and point to the importance of a structure/function analysis of naturally occurring mutations for understanding how defects in chloride secretion affect CBAVD patients.

Acknowledgements: We thank Prof. Mireille Claustres for her support and advice and Marie Desgeorges for her kind cooperation. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (INSERM), Centre National de la Recherche Scientifique (CNRS) and Association Française de Lutte contre la Mucoviscidose (AFLM). The English text was edited by Dr Owen Parkes.

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