

Suppressive interactions between mutations located in the two nucleotide binding domains of CFTR

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Abstract The S1235R locus in CFTR was studied in combination with alleles found at the M470V and G628R loci. While R628 caused a maturational defect, R1235 did not. The impact of R1235 was found to be influenced by the alleles present at the G628R and M470V loci. At the single channel level, R1235-V (R1235 on a V470 background) was characterized by an open probability significantly higher than V470-wildtype CFTR. M470, which on its own increases CFTR chloride transport activity when compared to V470-wildtype CFTR, suppressed the activity of R1235 in such a way that a protein with an open probability not significantly different from V470-wildtype CFTR was obtained. While R628-V CFTR had similar current densities as V470-wildtype CFTR in *Xenopus laevis* oocytes, R1235-V resulted in current densities that were more than twofold higher than those of V470-wildtype CFTR. However, the current densities generated by R1235/R628-V (R1235 and R628 on a V470 background) CFTR were significant lower than R1235-V or R628-V CFTR.

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1. Introduction

The CFTR gene encodes a cAMP and ATP dependent chloride channel that is present in the apical membrane of epithelial cells lining most exocrine glands [1]. Phosphorylation of the regulatory domain by protein kinase A, followed by binding and hydrolysis of ATP at both nucleotide binding domains, regulates the transport of chloride ions through the channel [2]. Absence, reduced levels, or malfunction of the CFTR protein results in cystic fibrosis, and diseases such as CBAVD (congenital bilateral absence of the vas deferens) [3,4], bronchiectasis [5] and chronic pancreatitis [6]. Since the discovery of the CFTR gene [7], more than 850 mutations and 120 polymorphisms have been identified ([8], CF Genetic Analysis Consortium; URL: <http://www.genet.sickkids.on.ca>). The majority of these mutations are rare and the relation to disease is not always clear. The S1235R amino acid alteration is such a mutation. It was first found in a cystic fibrosis (CF) patient who carried S1235R and G628R (g→c) on the same allele [9]. In compound heterozygosity with a severe mutation, the S1235R amino acid alteration alone might

also result in disease. However, varying degrees of symptoms are seen in the latter individuals, some of them are even unaffected. The partial penetrance of a mutation might be explained by polymorphisms [10,11], since the presence of polymorphisms on the same allele may interfere with, or mask, the functional defects caused by a particular amino acid alteration. For the M470V locus, we have shown that the two alleles interfere both with the processing and with the chloride transport activity of the CFTR protein [11]. In order to get better insight into the effect of the S1235R mutation, on different backgrounds, the maturational pattern and the electrophysiological properties of these mutant proteins were determined.

2. Materials and methods

2.1. Characterization of alleles at polymorphic loci

Screening for the alleles at the (TG)_m (located in intron 8), T_n (located in intron 8), M470V and S1235R loci was performed as previously described [9,11].

2.2. Construction of mutants

A *XhoI*–*KpnI* fragment containing the CFTR coding region was isolated from the prokaryotic vector pTG5960 (Transgene S.A., Strasbourg, France) and inserted in the eukaryotic expression vector pcDNA3 (Invitrogen BV, Leek, The Netherlands). In order to remove a potential ATG start codon derived from the multiple cloning site of pTG5960, the construct was digested with *KpnI* and *EcoRV* and the resulting linear plasmid was blunt-end ligated after T₄ DNA polymerase treatment. The CFTR coding region present in the resulting construct was characterized by dideoxy sequencing and one polymorphism, V470, was identified. The sequence also contained three neutral amino acid changes (t930c, a933g and t936c) that had been introduced in order to inactivate a cryptic prokaryotic promoter [12]. The different amino acid alterations, V470M, G628R and S1235R, were introduced using the Transformer Site-Directed Mutagenesis kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). The complete CFTR coding region of the different mutants was sequenced; no other mutations than the desired ones were found.

For single channel measurements, the different mutants and wildtype CFTR cDNAs were transferred to the bicistronic green fluorescent protein (GFP) expression vector pCINeo/IRES-GFP [13]. For this purpose, wildtype CFTR cDNA derived from pcDNA3-CFTR was ligated as a T₄ DNA polymerase treated *XhoI*–*SacI* fragment in pCINeo/IRES-GFP. The latter had first been linearized with *NheI*, blunt-ended with T₄ DNA polymerase and dephosphorylated with bovine alkaline phosphatase. The region harboring the different studied mutations was subsequently removed from pCINeo/wtCFTR-IRES-GFP by complete digestion with *XbaI* and partial digestion with *BsrXI* and was replaced by the corresponding mutant CFTR fragments that had been obtained by complete digestion of the different mutant pcDNA3-CFTR constructs with *XbaI* and *BsrXI*.

2.3. Expression, pulse chase and immunoprecipitation of CFTR

The expression of mutant CFTR proteins, pulse chase and immu-

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noprecipitation experiments of CFTR were performed as previously described [14].

2.4. Physiological studies

In vitro transcription of mutant CFTR constructs, cRNA injection in oocytes, two electrode voltage clamp assays and single channel recordings were performed as previously described [14,15]. For single channel recordings, only one patch per cell was studied, therefore, the number of patches equals the number of cells studied. The open probability P_o was calculated from $P_o = I/iN$, where I is the averaged current during the whole stimulation period, i the single channel current obtained from amplitude histograms and N the number of channels estimated from overlapping events. All data were analyzed by Student's t -test. A value of $P < 0.05$ was considered significant. All data are reported as mean \pm S.E.M.

3. Results

3.1. Maturation of mutant CFTR proteins

The R628 mutation and the R1235 mutation, alone or in combination with the R628 mutation, were introduced into the CFTR coding region present in the eukaryotic expression vector pcDNA3. Since the M470 allele causes a delay in maturation and gives rise to a chloride channel with an increased open probability, the R1235 mutation was introduced either on a M470 or on a V470 background [11]. The different mutant constructs were transfected in COS-1 cells and the expressed proteins were characterized by means of pulse chase and immunoprecipitation experiments (Fig. 1). R1235 CFTR, either on a V470 or on a M470 background, was found to mature to the 190 kDa form. The R628 mutation, with or without the R1235 mutation on the same allele, gave rise to a protein of 150 kDa that was degraded; the mature 190 kDa form could not be detected.

3.2. Electrophysiological characteristics of mutant CFTR proteins

In order to study the effect of the different amino acid alterations on the electrophysiological characteristics of the corresponding chloride channels, cAMP inducible whole cell chloride currents in *Xenopus laevis* oocytes, injected with in vitro transcribed cRNA, were analyzed using the two electrode voltage clamp technique. The different studied CFTR chloride channels had similar permeability properties (Fig.

2A,B). The mutant R1235-V (R1235 on a V470 background) CFTR ($0.39 \pm 0.04 \mu\text{A}$, $n=8$) gave a significantly higher cAMP sensitive whole cell current, when compared to S1235-V (wildtype on a V470 background) CFTR ($0.16 \pm 0.02 \mu\text{A}$, $n=5$; $P < 0.001$) (Fig. 2C). A slightly lower whole cell current was observed for R1235-M (R1235 on a M470 background) ($0.36 \pm 0.06 \mu\text{A}$, $n=5$), compared to the R1235-V construct ($0.39 \pm 0.04 \mu\text{A}$, $n=8$); the difference, however, was not significant. The R1235-V ($0.39 \pm 0.04 \mu\text{A}$, $n=8$) current was significantly reduced when R628 was present ($0.11 \pm 0.01 \mu\text{A}$, $n=9$; $P < 10^{-5}$). Despite the fact that R628 causes a maturation defect, cAMP induced chloride currents measured for R628-V CFTR ($0.19 \pm 0.02 \mu\text{A}$, $n=4$) were not significantly different from V470-wildtype CFTR ($0.16 \pm 0.02 \mu\text{A}$, $n=5$). Indeed, overexpression of non-maturing CFTR proteins in *Xenopus* oocytes, grown at room temperature, can still result in the appearance of chloride channels at the cell surface. The amino acid present at residue 1235 affected the chloride transport activity of the resulting R628 channels since R628/R1235-V induced a significantly lower cAMP dependent chloride transport activity ($0.11 \pm 0.01 \mu\text{A}$, $n=9$) than R628/S1235-V CFTR ($0.19 \pm 0.02 \mu\text{A}$, $n=4$; $P < 0.001$). Since whole cell currents do not always correlate with single channel activities of CFTR proteins, but are also dependent on the number of chloride channels present in the cell membrane, single channel measurements were set up. For this purpose, COS-1 cells were transfected with bicistronic constructs expressing both CFTR and the green fluorescent protein from the same template. As a consequence, green fluorescent cells that thus express CFTR could be selected under the fluorescence microscope before they were patched. This selection method is very useful, especially when CFTR channels with low intrinsic activities are studied. Only mutant proteins with a normal maturation pattern, which are therefore expected to be present in the cell membrane, were analyzed. As a consequence the R628 mutants could not be analyzed at the single channel level. Representative channel recordings are shown in Fig. 3A. The different CFTR proteins had similar conductive properties (Fig. 3B). The open probabilities of the different CFTR proteins were determined (Fig. 3C). R1235-V was found to exhibit a significantly higher open probability (0.53 ± 0.09 , $n=4$) than S1235-V (V470-wildtype) CFTR

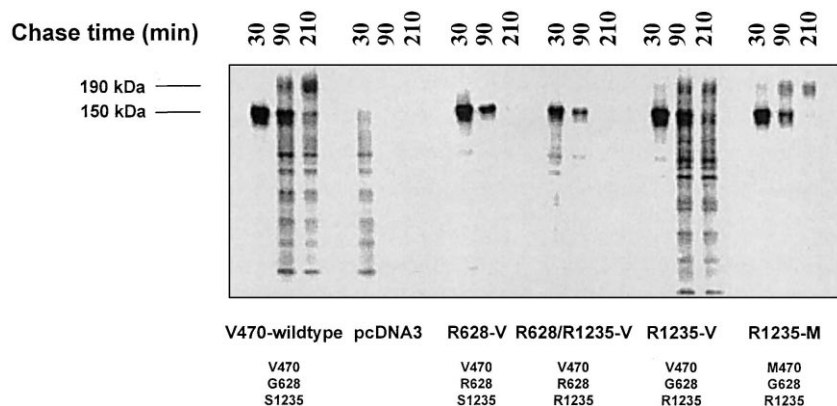


Fig. 1. Pulse chase and immunoprecipitation of CFTR from COS-1 cells transiently expressing V470-wildtype, pcDNA3 (empty vector), R628-V, R628/R1235-V, R1235-V or R1235-M CFTR. Proteins were labelled with [^{35}S]methionine and [^{35}S]cysteine, chased for the indicated time periods and CFTR proteins were immunoprecipitated with an antibody directed against the C-terminal part of CFTR. The immunoprecipitated proteins were separated on a 4–12% SDS gel and visualized by autoradiography. The core glycosylated 150 kDa and the mature 190 kDa forms are indicated.

(0.23 ± 0.01 , $n=4$; $P<0.01$). Since M470-wildtype CFTR (0.45 ± 0.05 , $n=6$) has a significantly higher open probability than V470-wildtype CFTR (0.23 ± 0.01 , $n=4$; $P<0.01$), and since R1235-V has still a higher open probability (Fig. 3), even higher P_o values were expected for R1235-M, compared to R1235-V and S1235-M (M470 alone). Surprisingly, R1235 on a M470 background (0.31 ± 0.04 , $n=6$) gave rise to chloride channels with open probabilities not significantly different from V470-wildtype (S1235) CFTR (0.23 ± 0.01 , $n=4$).

3.3. Frequency of R1235 in the general population

The R1235 mutation was analyzed by means of allele specific oligonucleotide hybridization. R1235 was found twice among 57 (0.035) normal *CFTR* genes derived from mothers of CF patients, while it was not found at all among 58 normal *CFTR* genes derived from fathers of CF patients. *CFTR* genes derived from random individuals not related to CF patients showed a frequency of 0.023 (3/128).

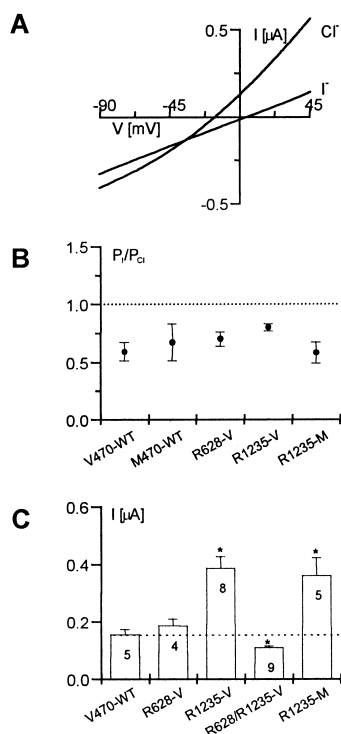


Fig. 2. Properties of whole cell chloride currents measured in *Xenopus laevis* oocytes expressing wildtype CFTR and mutant CFTR proteins. *Xenopus laevis* oocytes were injected with in vitro transcribed wildtype CFTR (V470-WT and M470-WT) and mutant CFTR RNA (R628-V (V470/R628/S1235), R1235-V (V470/G628/R1235), R1235-M (M470/G628/R1235), R628/R1235-V (V470/R628/R1235)) and the cAMP induced chloride currents were measured by the two electrode voltage clamp technique 72 h later. A: $I-V$ relationships obtained from oocytes expressing wildtype CFTR in ND-96 solution or iodide substituted ND-96 solution after stimulation. B: Comparison of the permeability ratios of wildtype and mutant CFTR after anion substitution ($n=3$ for each group). C: Whole cell chloride currents after stimulation of oocytes expressing wildtype and mutant CFTR. Mutants that showed a significantly different cAMP dependent chloride current ($P<0.05$), compared to wildtype CFTR, are marked with an asterisk and the number of cells analyzed is indicated.

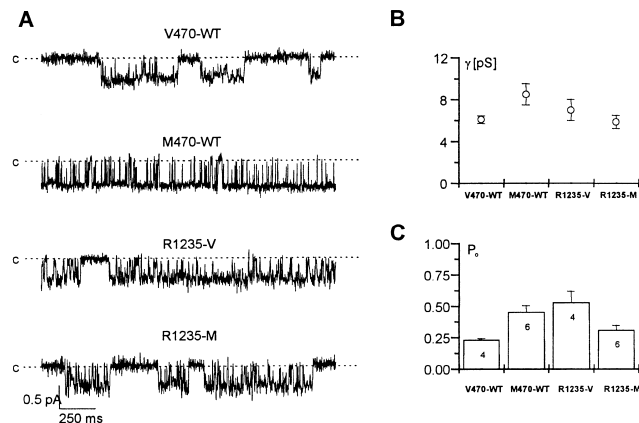


Fig. 3. Single channel properties of wildtype and mutant CFTR chloride channels expressed in COS-1 cells. COS-1 cells were transfected with a bicistronic construct of green fluorescent protein and wildtype CFTR (V470-WT and M470-WT) or mutant CFTR (R1235-V (V470/G628/R1235), R1235-M (M470/G628/R1235)) cDNA. Cells expressing the green fluorescent protein were selected and used for single channel measurements. A: Single channel recordings of wildtype and mutant CFTR in the cell attached configuration at a holding potential of -60 mV. The dotted line indicates the closed level of the single channel. B: Comparison of channel conductances between wildtype and mutant CFTR at -60 mV (the number of cells patched is indicated in C). C: Pooled data to compare open probabilities between wildtype and mutant CFTR at -60 mV. The number of channels was estimated by overlapping sweep events. The number of cells patched is indicated.

3.4. R1235 haplotype backgrounds

The haplotype backgrounds of six R1235 *CFTR* genes were determined. Two R1235 *CFTR* genes were found on the normal *CFTR* alleles of CF mothers, one was found in a CF patient who carried a second missense mutation G628R (g→c) on the same allele [9], one was from a patient with borderline sweat chloride values who was compound heterozygote for R1235 and $\Delta F508$, and two R1235 *CFTR* genes were found in random individuals. All R1235 *CFTR* genes carried a 12-7-M haplotype background, built up of the alleles found at the (TG)_m, T_n, and M470V polymorphic loci respectively.

4. Discussion

When the maturation patterns of the different mutant CFTR proteins (R628-V CFTR, R1235-M CFTR, R1235-V CFTR and R1235/R628-V CFTR) were compared, R1235 caused no aberrant processing, independent of the amino acid found at residue 470. R628-V gave rise to a protein that only reaches the 150 kDa form, indicating that it is retained in the endoplasmic reticulum. The allele present at the S1235R locus could not bypass this maturation defect.

When the different protein variants were analyzed at the electrophysiological level, R1235 induced a cAMP dependent whole cell chloride current which was significantly higher than wildtype CFTR, and this observation was independent of the amino acid residue found at position 470. Single channel measurements, however, indicated that R1235 induced a chloride channel with a significantly higher open probability than wildtype, when both carried V470. Very strikingly, when both R1235 and M470 were present, no difference with V470-wildtype was found. Previous studies [11] showed that M470 gives

rise to a channel with a significantly increased open probability, when compared to V470 CFTR. Thus, both variants (M470 and R1235) give rise to 'hyper'-active chloride channels when they are present on separate alleles, but induce a channel with V470 characteristics when they are found on the same allele. The 'hyper'-active effect of the two alleles (M470 and R1235) is thus suppressed when found in combination on the same allele. This might indicate that both amino acids, residue 470 located in the first nucleotide binding domain and residue 1235 located in the second nucleotide binding domain, interact with each other. They might therefore be involved in the cross-talk between the two nucleotide binding domains, a feature that plays a crucial role in the complex regulation of the CFTR chloride channel [2]. An interaction between both nucleotide binding domains might be supported by the whole cell data obtained for the alleles at the G628R locus. This amino acid alteration is located in the N-terminal part of exon 13, i.e. that part of exon 13 that has been proposed to form the C-terminal part of the first nucleotide binding domain [16–18]. The cAMP induced whole cell currents in *Xenopus* oocytes measured for R628 CFTR were dependent on the amino acid residue present at amino acid position 1235; R1235 induced an apparent decrease in whole cell current of R628-V CFTR. Since R628 causes a maturation defect in COS-1 cells, no confirmation of these data at the single channel level could be obtained. Therefore, the latter data have to be interpreted with caution.

In parents of CF patients, the R1235 mutation was only found on maternal chromosomes, and not on paternal ones. The difference, however, was not significant. The absence of R1235 on normal CFTR genes of fertile male CF carriers might indicate that it could cause CBAVD when found in compound heterozygosity with a severe CFTR mutation. It is therefore interesting to note that CBAVD patients that carry R1235 have been described [4].

Based on the functional studies, we have no evidence that R1235 causes disease, since R1235 CFTR proteins are able to transport chloride ions. We can, however, not exclude that the regulatory interaction of CFTR with other proteins [19,20] will be affected by R1235, in this way causing disease. Polymorphic loci, such as (TG)_m, T_n and M470V, have been shown to affect CFTR properties [11,21]. The six R1235 CFTR genes, derived either from patients or from healthy individuals, however, were found to have an identical haplotype background ([TG]₁₂-T₇-M470). We have therefore no evidence that R1235, on a particular haplotype background, affects chloride transport through CFTR and if it is involved in disease; however, only a limited number of R1235 genes were studied. The R1235 mutation was found to be associated with the M allele at the M470V locus, leading to normal chloride channel activity (Fig. 3). Of all T₇ alleles, the ones that harbor allele 12 at the (TG)_m locus will result in the lowest amount of functional CFTR due to a higher proportion of transcripts lacking exon 9 [11], which will not mature [22,23]. Individuals that harbor the R1235 allele may therefore also have a smaller number of CFTR channels, because of the alleles found at the (TG)_m and T_n loci. They might therefore be more susceptible to genetic and/or environmental modulators that affect the CFTR protein network and in this way cause disease.

In conclusion, an important finding of this study was that the effect of individual mutations/polymorphisms may not be

additive, but might even suppress each other. The defect caused by specific CFTR mutations found in patients might therefore be dependent on the alleles present at different polymorphic CFTR loci. In order to be able to explain the phenotypes associated with specific CFTR mutations, analysis of the defects caused by these mutations, at the maturational and electrophysiological levels, should be done on their proper genetic background. Regrettably, the genetic backgrounds are unknown for the majority of mutations.

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