Characterization of mutations located in exon 18 of the *CFTR* gene

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Abstract In order to get a better insight into the function of amino acid residues located in the second transmembrane domain of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, all exon 18 mutations found in cystic fibrosis (CF) patients were characterized at the protein and at the electrophysiological level. Of the different mutations present in transmembrane helix 12 (M1137V, M1137R, I1139V and ΔM1140), and the intracytoplasmic loop connecting TM12 and NBD2 (D1152H and D1154G), only M1137R interfered with the proper maturation of the protein. Permeability studies performed after injection of the different wild-type and mutant cRNAs in *Xenopus laevis* oocytes indicated that the mutations did not alter the permeability sequence of the CFTR channels. The whole cell cAMP activated chloride currents, however, were significantly reduced for M1137V, I1139V, D1152H and D1154G and close to zero for ΔM1140, indicating that these mutations interfere with the proper gating of the chloride channels.

Key words: Cystic fibrosis transmembrane conductance regulator; Maturation; Electrophysiology

1. Introduction

Cystic fibrosis (CF), an autosomal recessive disorder characterized by alterations in transepithelial salt transport [1], is caused by mutations in the *cystic fibrosis transmembrane conductance regulator* gene (CFTR) [2–4]. This gene encodes a symmetrical multidomain protein, each half composed of a transmembrane domain (TMD) and a nucleotide binding domain (NBD), separated by a highly charged regulatory (R) domain. The protein forms a channel through which chloride ions permeate downwards an electrochemical gradient. Activation of the chloride channel occurs via different steps [5]. First, the R domain becomes phosphorylated by protein kinase A (PKA). In a second step, ATP is bound and hydrolyzed by the first nucleotide binding domain such that the channel opens and chloride ions are transported. Binding and hydrolysis of ATP at NBD2 will close the channel. How these different events are linked to each other is not completely clear yet. Mutagenesis studies of amino acid residues located in the different cytoplasmatic loops connecting the transmembrane helices indicated that these residues were important for the opening of the channel and that they might form the link between the nucleotide binding domains and the conducting pore [6–10].

The anion selectivity filter itself seems to be formed by the transmembrane helices [11], since mutagenesis of lysine 95 to aspartate and of lysine 335 to glutamate changed the anion selectivity of the channel. These data could, however, not be confirmed [12]. Deletion constructs, lacking the first four helices, gave rise to functional cAMP regulated chloride channels with ion selectivities identical to wild-type CFTR [13]. These findings indicated that only the fifth and the sixth helix of TMD1 were essential components of the conductive pore and the selectivity filter of CFTR. Scanning-cysteine-accessibility analysis, however, substantiated the findings of Anderson et al. [11]: amino acids residing in the first and sixth transmembrane helix take part in the formation of the ion pore. Moreover, residues located in the region of amino acids 351 and 353 seem to determine the anion-cation selectivity of the CFTR chloride channel [14].

When both halves of the protein (TMD1-NBD1-R and TMD2-NBD2) are expressed alone, no functional chloride channel can be detected using SPQ halide efflux assays [15]. When more sensitive techniques like patch clamp analysis were used, however, cAMP dependent chloride channels could be detected in cells expressing TMD1-NBD1-R alone [16,17]. When both halves of the protein were co-expressed, chloride transport could already be detected using SPQ assays and co-immunoprecipitation studies indicated that both halves interacted with each other. For this interaction, the presence of TMD2 and only the first two transmembrane helices of TMD1 were sufficient. These findings indicated that TMD1 and TMD2 may mediate association between the two halves of the protein [15].

In contrast to TMD1, the importance of TMD2 in ion pore formation and ion selectivity has not been studied in detail. In order to get a better insight into the structure and function of TMD2, we studied disease causing mutations located in TM12 and in the loop connecting TM12 and NBD2, both at the protein and at the electrophysiological level.

2. Materials and methods

2.1. Plasmid construction

A *Xho*I-*Kpn*I fragment containing the CFTR coding region was isolated out of the prokaryotic vector pTG5960 (Transgene) and inserted in the eukaryotic expression vector pcDNA3 (Invitrogen). Six different mutations: a3541g (= M1137V), t3542g (= M1137R), a3547g (= I1139V), deletion of atg from 3550 (= ΔM1140), g3586c (= D1152H) and a3593g (= D1154G) were introduced using the Transformer Site-Directed Mutagenesis kit (Clontech). The complete CFTR coding region of the wild type and the six mutant constructs was characterized by dideoxy sequencing. The different constructs only contained the introduced mutations.

2.2. Expression, pulse chase and immunoprecipitation of CFTR

Twenty μg of plasmid DNA was electroporated (Bio-Rad Gene Pulser, Bio-Rad Laboratories, Hercules, CA, USA) into $1.5–3 \times 10^7$ COS1 cells. The transfected cells were cultured for 48–72 h at 37°C in DMEM F12 (Life Technologies, Inchinnan, Scotland, UK) supple-

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mented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and subsequently selected with G418 disulfate (480 mg/l; Duchefa, Haarlem, The Netherlands). After 2 weeks of selection, the cells were starved for 30 min in RPMI 1640 medium (Life Technologies, Inchinnan, Scotland, UK) without methionine and cysteine, labelled during a 30-min pulse in RPMI 1640 supplemented with 100 mCi/ml [^{35}S]methionine and [^{35}S]cysteine (ICN Pharmaceuticals, Costa Mesa, CA, USA) and finally chased for different time periods in DMEM F12 supplemented with 10% fetal bovine serum. Cells were scraped and subsequently lysed by sonication in ice cold IPPA-buffer (20 mM Tris, 150 mM NaCl, 1% Na-desoxycholate, 1% Triton X-100, 0.1% SDS, pH 7.4) supplemented with protease inhibitors (20 mg/ml soybean trypsin inhibitor, 1 mg/ml leupeptin, 1 mg/ml antipain, 1 mg/ml chymostatin and 1 mM phenylmethylsulfonylfluoride). The lysate was precleared with protein A-Sepharose CL-4B beads (Pharmacia Biotech, Uppsala, Sweden) and CFTR proteins were affinity purified by incubation for one hour with an anti-CFTR monoclonal antibody directed against the C-terminal part of CFTR (Genzyme Diagnostics, Cambridge, MA, USA). The immunocomplexes were purified on protein A-Sepharose CL-4B beads and after addition of loading buffer (1% β-mercaptoethanol, 16 mM Tris-HCl, pH 6.8, 4% SDS, bromophenol blue and 10% glycerol) loaded on a 4–12% SDS gel (Novex, San Diego, CA, USA). After drying, the gel was exposed at −70°C to a light sensitive film for 3–4 h.

2.3. In vitro transcription

The different *CFTR*-constructs were in vitro transcribed using the T_7 RiboMAX Large Scale RNA Production System (Promega, Madison, WI, USA), according to the protocol of the manufacturer. The quality of the RNA was determined by means of formaldehyde-agarose (1%) gel electroporesis.

2.4. RNA injection in oocytes

Female toads (*Xenopus laevis*) were anaesthetized in ice cold water containing 2 g/l 3-aminobenzoic acid ethylester (Sigma-Aldrich, Bornem, Belgium) and oocytes were subsequently collected via abdominal excision. The oocytes were incubated in a calcium-free solution containing 2 mg/ml collagenase A (Boehringer Mannheim, Mannheim, Germany) for 1 h. The oocytes were injected with 50 nl in vitro transcribed RNA (5 μg/μl).

2.5. Two-electrode voltage-clamp assays

Three days after RNA injection, oocytes were mounted in a perfusion chamber, impaled with two electrodes and voltage clamped. In order to block endogenous Ca^{2+}-activated chloride currents, the perfusion solution (96 mM NaCl, 5 mM HEPES, 2 mM KCl, 1.8 mM $CaCl_2$ and 1 mM $MgCl_2$; pH 7.5) contained 100 μM niflumic acid. CFTR-currents were activated through addition of 10 μM forskolin and 1 mM IBMX. Chloride currents were not activated in non-injected, in water-injected or in oocytes injected with RNA derived from in vitro transcribed pcDNA3. In order to determine the anion permeability of the channel, chloride ions were substituted in the perfusion solution by equimolar amounts of bromide or iodide. The anion permeability relative to that of Cl^- was calculated from shifts in reversal potential, using the equation $P_x/P_{Cl} = ([Cl^-]_e \cdot \exp(\Delta E_{rev} \cdot F/R \cdot T) - [Cl^-]_{res})/[X^-]_e$ in which $[X^-]_e$ represents the extracellular iodide or bromide concentration, $[Cl^-]_e$ and $[Cl^-]_{res}$ the intracellular and 'residual' extracellular concentrations (present in $CaCl_2$ and $MgCl_2$) and ΔE_{rev} is the measured shift in reversal potential. All data were analyzed by Student's *t*-test. A value of $P < 0.05$ was considered as significant. Data are reported as mean ± S.E.M.

3. Results and discussion

3.1. Maturation properties of the different mutants

Disease causing amino acid substitutions located in the membrane spanning sequences or in the intracytoplasmic loops can interfere both with the maturation and/or gating properties of the corresponding CFTR protein. The effect of the different mutations located in exon 18 was therefore first studied at the maturational level. To this effect, COS1 cells were transfected with the different mutant *CFTR* cDNAs, and the expressed proteins were analyzed using pulse chase experiments followed by immunoprecipitation. Fig. 1 shows that the different mutants, except M1137R, exhibit the same maturation pattern as wild-type CFTR. For M1137R, however, only the 150-kDa form was detected, even after 3 h and 30 min of chase time. This indicates that the core glycosylated M1137R translation product is degraded in the ER before it can be transported to the cell surface. This mutation introduces a strong positively charged amino acid in an aliphatic chain that spans the membrane. Therefore, it is very likely that this mutation interferes with the proper insertion of the translation product in the membrane and will give rise to a misfolded protein that is recognized and degraded by the ER control system. This hypothesis is substantiated by the finding that another mutation that affects the same amino acid residue, but that replaces it with an aliphatic one (M1137V), exhibits a 'wild-type' maturation pattern. Strikingly, deletion of amino acid M1140, located in the middle of transmembrane helix 12, does not affect CFTR conformation such that the protein is degraded by the quality control system.

3.2. Electrophysiological properties of the different mutants

Mutants that showed no maturation defect were subsequently analyzed at the electrophysiological level. *Xenopus laevis* oocytes were injected with the different mutant and wild-type cRNAs and whole cell currents were measured, before and after stimulation with forskolin and IBMX (Fig. 2A),

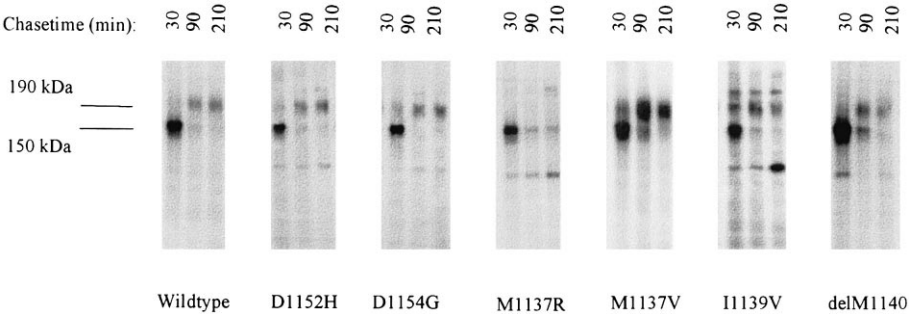

Fig. 1. Pulse chase and immunoprecipitation of wild-type and mutant CFTR proteins from transfected COS1 cells. COS1 cells transfected with wild-type, M1137V, M1137R, I1139V, ΔM1140, D1152H and D1154G CFTR were metabolically labelled, chased, CFTR was immunoprecipitated and separated on an SDS-PAGE gel. The core-glycosylated (150 kDa) and mature (190 kDa) CFTR protein bands are indicated. M1137V, M1137R, I1139V and ΔM1140 are located in transmembrane helix 12 and D1152H and D1154G are located in the intracytoplasmic loop connecting TM12 and NBD2.

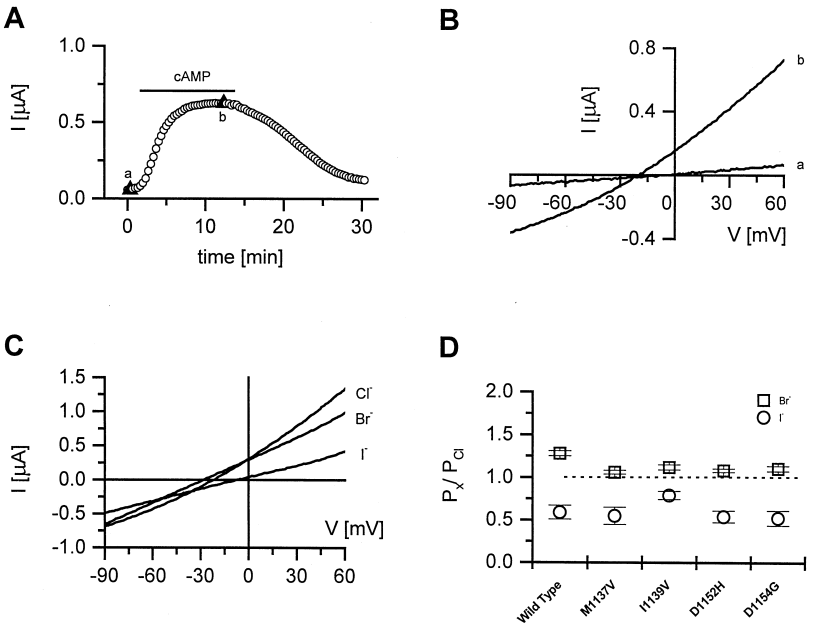

Fig. 2. The electrophysiological properties of CFTR injected *Xenopus laevis* oocytes. A: Time course of membrane currents at +50 mV in wild-type CFTR injected oocytes. 'cAMP' indicates the time period during which 10 μM forskolin and 1 mM IBMX was applied. B: Current voltage relationships before (a) and during (b) application of 10 μM forskolin and 1 mM IBMX measured at the time points (a and b) indicated by filled triangles in A. C: Anion selectivity of cAMP activated Cl^- currents in wild-type CFTR injected *Xenopus laevis* oocytes. I/V relationship was obtained from wild-type CFTR injected oocytes, incubated in ND-96 solution or anion-substituted ND-96 solution, after stimulation with 10 μM forskolin and 1 mM IBMX. D: Comparison of the permeability ratios of wild-type and mutant CFTR by anion substitution. Data are presented as mean ± S.E.M. ($n = 4$ for each group).

using the two electrode voltage clamp technique. The channels were characterized by a linear I-V relationship (Fig. 2B). Four mutants, M1137V, I1139V, D1152H and D1154G showed a significantly reduced current, when compared to wild type, and two other mutants, M1137R and ΔM1140 were not activated by cAMP (Fig. 3). The absence of cAMP sensitive chloride currents found for M1137R is most probably caused by a defect in maturation, as found in COS1 cells. The underlying mechanism by which the deletion of amino acid M1140 disrupts chloride transport remains completely unknown. Given the crucial location of M1140 in the middle of TM12, deletion of this amino acid might disrupt the ion pore such that ion passage is completely blocked.

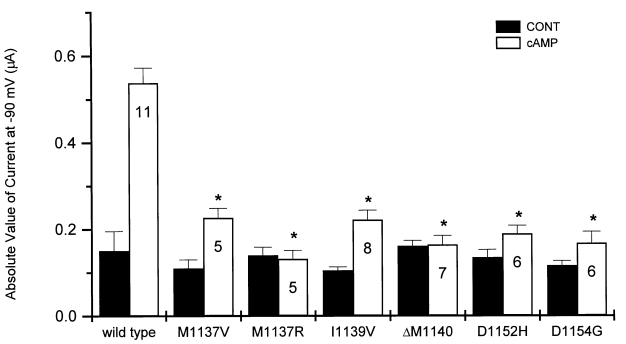

Fig. 3. Whole cell chloride currents measured in *Xenopus laevis* oocytes expressing wild-type and mutant CFTR proteins. *Xenopus laevis* oocytes were injected with in vitro transcribed wild-type and mutant CFTR RNA and the basal (black bar) and cAMP (white bar) induced chloride currents were measured by the two electrode voltage clamp technique 72 h later. Mutants that showed a significantly different cAMP dependent chloride current ($P < 0.05$), compared to wild-type CFTR, are marked with an asterisk and the number of oocytes analyzed are indicated.

The effect of mutations that only induced a reduction of the cAMP inducible chloride current was then analyzed for their influence on the permeation properties of the channel. Wild-type CFTR was characterized by the following permeation sequence: $Br^- > Cl^- > I^-$ (Fig. 2C). The same permeation sequence was found for the four mutants, M1137V, I1139V, D1152H and D1154G (Fig. 2D). Mutations D1152H and D1154G are located in the intracytoplasmic loop that connects TM12 and NBD2 and thus only affect the cAMP inducible whole cell currents. Since this loop connects the second nucleotide binding domain with the transmembrane spanning helices that form the pore of the channel, it might function in the cascade of events that connect the cycles of binding and hydrolysis of ATP to the opening and closing of the chloride channel. Single channel measurements of these mutants are needed to substantiate this hypothesis.

In summary, exon 18 *CFTR* mutations found in CF patients were functionally characterized. One mutation (M1137R) did result in failure of protein maturation while the remainder did affect gating of the chloride channel.

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