Cystic Fibrosis Phenotype Associated with Pancreatic Insufficiency Does Not Always Reflect the cAMP-dependent Chloride Conductive Pathway Defect

ANALYSIS OF C225R-CFTR AND R1066C-CFTR*

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We have previously screened the cystic fibrosis transmembrane conductance regulator (CFTR) gene and identified new disease-causing mutations. C225R and R1066C are both associated with pancreatic insufficiency, but the former mutation is associated with mild and unusual lung disease, whereas the latter is associated with severe lung disease. In the present study, we expressed these mutants heterologously in HeLa cells, and we analyzed protein synthesis by immunoprecipitation and chloride channel function by using a halide-sensitive fluorescent dye, 6-methoxy-N-ethylquinolinium. Immunoprecipitation and functional studies showed that cells transfected with C225R-CFTR exhibit cAMP-dependent chloride fluxes; C225R-CFTR protein is poorly expressed but fully glycosylated and can be compared with R117H-CFTR. R1066C-CFTR protein is not correctly processed and, unlike ΔF508-CFTR, this defect cannot be corrected by reduced temperature or overexpression in butyrate-treated cells; defective processing may occur at a different step in the biosynthetic pathway. These results point to two different mechanisms underlying the same pancreatic status and suggest that it is unwise to use pancreatic sufficiency and insufficiency to define mild and severe cystic fibrosis (CF) disease, respectively. Finally, the experimental model described here may be helpful to predict the pulmonary status of CF patients bearing mutations located in putative membrane-spanning domains of the CFTR protein.

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). Since the cloning of the CF gene, few missense defects affecting the transmembrane domain have been tested for their effects on chloride channel function in heterologous cells in vitro (2–5).

We have previously screened the entire CFTR coding regions and splice junctions to establish the spectrum of CF mutations in a sample representative of the French population and to identify new disease-causing mutations (6). While investigating these patients, we identified two missense mutations associated with pancreatic insufficiency (PI). The first lies within the fourth putative membrane-spanning domain and the second within the fourth intracellular loop of the CFTR protein, replacing a cysteine by an arginine at position 225 and an arginine by a cysteine at position 1066, respectively (6). We thought that the switch from a hydrophobic to a hydrophilic amino acid (Cys to Arg) or vice versa (Arg to Cys) would greatly affect the positioning and/or pore properties of the CFTR channel and, thus, decided to investigate the structure-function relationships of these mutants by using a heterologous expression system.

Immunoprecipitation and functional analysis using a halide-sensitive indicator showed that cells transfected with C225R-CFTR exhibit cAMP-dependent chloride fluxes; C225R-CFTR protein is poorly expressed but fully glycosylated. Cells transfected with R1066C-CFTR did not respond to cAMP stimulation, and R1066C-CFTR protein was not fully glycosylated, reflecting a defect in protein biosynthesis. Contrary to ΔF508-CFTR, the defect in R1066C-CFTR transfected cells was not corrected by overexpression or a lower growth temperature. These results point to two different mechanisms underlying the same CF phenotype.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Plasmid Construction—A plasmid vector for human CFTR expression in mammalian cells was constructed by placing the full-length CFTR cDNA coding sequence (4.5 kb from nucleotide position 90 to 4578, obtained from pTG5960, a gift from Transgene, Strasbourg, France) in the expression vector pECE (7). The resulting plasmid is designated pCFTrwt. Expression of CFTR in pCFTrwt was controlled by the simian virus 40 early promoter. Site-directed mutagenesis was performed on pCFTrwt by using either 2-step PCR or oligonucleotide-directed mutagenesis (Transformer™ kit, CLONTECH). Three mutants, C225R, R1066C, and R117H, were constructed. CFTR mutations were verified by DNA sequencing around the site of the mutation.

Cell Culture and Sodium Butyrate Treatment—Cells plated on Petri dishes (100 mm diameter) were grown at 37 °C or 24–26 °C with 5% CO2 in Dulbecco’s modified Eagle’s medium (Glutamax™, Life Technol-
ogy, Inc.) containing 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Life Technologies, Inc.). Sodium butyrate (Sigma) was made up as a 0.5 M stock solution in culture medium and was filter-sterilized (8). It was added to the cells at a final concentration of 5 mM, 18–20 h before analysis.

### Transient Expression of CFTR in HeLa Cells—60% confluent cells were transfected by lipofection using LipofectAMINE™ (Life Technologies, Inc.) with 3 µg of plasmid according to the manufacturer instructions. Confluent monolayers were harvested for Northern blot analysis, functional assays, or immunoprecipitation 48 h posttransfection.

### Northern Blot Analysis—10 µg of total cellular RNA was isolated with the RNeasy system (Qiagen, Inc., Hilden, Germany). Hybridization was performed using the 1.5-kb EcoRI-EcoRI fragment of CFTR-cDNA labeled by random priming (High Prime, Boehringer Mannheim).

**6-Methoxy-N-ethylquinolinium Fluorescence Assay—**Cells were grown on glass cover slips for 24 h and then loaded with the halide indicator fluorescent dye 6-methoxy-N-ethylquinolinium (MEQ). MEQ was home-made (9) and had a Stern-Volmer constant in KCl solution of 149 M⁻¹. The cells were incubated in hypotonic medium containing 20 mM MEQ made by diluting isotonic solution containing 138 mM NaI, 2.4 mM K₂HPO₄, 0.8 mM KH₂PO₄, 10 mM Hepes, 1 mM CaCl₂, 10 mM glucose, and 20 µM bumetanide (pH 7.4) 1:1 with water, at room temperature for 4 min (10), and then 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 iodide solution on the stage of a inverted microscope (Diaphot, Nikon, France), and measurements of intracellular MEQ fluorescence were initiated. After 2 min of perfusion, the iodide solution was replaced with nitrate solution, which was identical except that NO₃ replaced I⁻. Because nitrate does not interact with MEQ, fluorescence increases as cell iodide flows from the cell through the anion conductive pathways if these pathways are functional in the plasma membrane. Changes in fluorescence were recorded in stimulated (stimulatory mixture: 500 µM CPT-cAMP and 100 µM IBMX) and unstimulated conditions. Excitation was at 350 nm, and emission was at >440 nm. Fluorescence in single cells was measured with a digital imaging system and a CCD camera (Photonics Sci, UK). Results were analyzed using Imstar software (Paris, France).

The results were expressed as relative fluorescence $F/F_0$, where $F$ is fluorescence as a function of time and $F_0$ minimum fluorescence. Cells were scored as positive when the rate of change in fluorescence with the stimulatory mixture 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 $ΔF_{stim}/ΔF_{basal}$. We chose this ratio rather than the absolute change in fluorescence as the latter may be influenced by dye loading. When appropriate, results are expressed as means ± S.D.

**Immunoprecipitation/ PKA Assay—**Immunoprecipitation was performed using the monoclonal antibody (mAb) 24–1 (Genzyme, Framingham, MA), which recognizes the C terminus of CFTR, according to the manufacturer instructions. Briefly, cell lysates were prepared with 2 × 10⁶ butyrate-treated or 4 × 10⁶ untreated cells and immunoprecipitated with 1 µg of mAb 24–1 and Pansorbin (Calbiochem). The resulting proteins were phosphorylated in vitro with 5 units of the catalytic subunit of PKA (Promega) and [γ³²P]ATP and were then separated on 5% SDS-PAGE, dried, and autoradiographed. RESULTS

Three mutated CFTR proteins (C225R-, R1066C-, and R117H-CFTR) and wild-type CFTR were transiently expressed in HeLa cells and analyzed at the mRNA, protein, and functional levels. Wild-type and R117H-CFTR were used as controls since both proteins are processed to the plasma membrane and function as cAMP-regulated chloride channels (3, 11).

**Biochemical Characterization of Wild-type and Mutant CFTR Protein—**To check the transfection efficiency, we first used Northern blot analysis to verify that transcripts for wild-type and mutated CFTR were present in transfected cells (Fig. 1A, 5 lanes from left). Then, we performed immunoprecipitation/PKA assay to determine the protein glycosylation pattern.

![Fig. 1. Expression of wild-type and mutant CFTR at the mRNA and protein levels, with and without sodium butyrate treatment.](image-url)

**TABLE I**

| Cell type | Wild-type | R117H | C225R | R1066C | C225R + NaB | R1066C + NaB | pECE |
|-----------|-----------|-------|-------|--------|------------|------------|------|
| All responding | 60 (30) | 9 (16) | 8 (8) | 0 | 11 (22) | 0 | 0 |
| Fast | 30 (50) | 3 (33) | 0 | 0 | 3 (27) | 0 | 0 |
| ΔF_{stim}/ΔF_{basal} | 13 ± 6.8 | 8 ± 1.7 | | | 22.3 ± 7.1 | | |
| Range | 6–27 | 6–9 | 0 | 0 | 16–30 | | |
| Slow | 30 (50) | 6 (66) | 8 (100) | 0 | 8 (73) | | |
| ΔF_{stim}/ΔF_{basal} | 3.4 ± 1.0 | 2.4 ± 0.5 | 2.9 ± 1.4 | 3.4 ± 1.0 | | |
| Range | 2–5 | 2–3 | 1.5–5 | 1.5–5 | | |
| Total | 200 | 56 | 100 | 100 | 50 | 50 | 60 |

a Percentage of all cells.
b Percentage of positive cells.
c Mean values ± S.D.
d NaB, pretreated with sodium butyrate.
Fig. 1B shows that the fully processed form of wild-type CFTR protein was detected in HeLa cells as a diffuse band of approximate molecular mass of 170 kDa (band C) and that the core-glycosylated form appeared as a thin band of about 140 kDa (band B) on 5% SDS-PAGE; the pattern was almost identical for R117H-CFTR. C225R-CFTR expression showed a faint band C or B, indicating that some mutant protein matured and reached the cell surface. R1066C-CFTR produced a low level of core-glycosylated CFTR as a faint band B, suggesting a low level of synthesis and defective biosynthesis (Fig. 1B).

To increase the sensitivity of the assay, CFTR protein expression was increased with 5 mM sodium butyrate treatment for 18–20 h. Sodium butyrate stimulates the transcriptional activity of several viral and nonviral genes (12) and acts on the SV40 promoter of the expression vector used in this study. The intensity of bands C and B of wild-type, R117H- and C225R-CFTR increased markedly, and the ratio of bands C/B was similar in treated wild-type, R117H- and C225R-CFTR cells. Although synthesis of R1066C-CFTR increased markedly with sodium butyrate (strong band B), we did not detect a mature band C, suggesting that this mutant cannot function as a cAMP-regulated chloride channel (Fig. 1B). To determine if R1066C was a temperature-sensitive mutant of CFTR, cells were grown at 24–26 °C for 48 h and harvested for immunoprecipitation-PKA assay; ΔF508 was used as control as it has been shown that a reduced growth temperature permits maturation and delivery to the plasma membrane (13). No fully glycosylated CFTR (band C) protein was detected when R1066C-CFTR transfected cells were grown at 24–26 or 37 °C, whereas band C appeared when ΔF508-CFTR cells were grown at 24–26 °C (Fig. 1C). Thus, neither butyrate treatment nor low temperature rescued R1066C-CFTR.

Functional Analysis of Wild-type and Mutant CFTR Protein—To test cAMP-activated chloride conductance activity, transfected cells were analyzed using the MEQ fluorescence assay. None of the mock-transfected cells (n = 60) showed changes in fluorescence in either basal or cAMP-stimulated conditions. Transiently transfected cells were divided into responsive and nonresponsive cells on the basis of cAMP-responsive anion conductance. Responsive cells were further divided into fast (ΔFstim/ΔFbasal > 5) and slow (ΔFstim/ΔFbasal ≤ 5) responsive cells. 30% of wild-type transfected cells (n = 200) were responsive, and 50% of these positive cells were fast responsive (Table I). Similar results were obtained with R117H-transfected cells (33% being fast responsive and 66% slow responsive cells) supporting the immunoprecipitation data, which suggested that wild-type and R117H-CFTR activate a cAMP-regulated chloride pathway. When exposed to the stimulatory mixture, 8% of C225R-CFTR cells showed an increased rate of change in MEQ fluorescence, indicating the activation of a CAMP-dependent anion pathway. All these cells were slow responsive. None of the R1066C-CFTR cells (n = 100) exhibited an increase in MEQ fluorescence when exposed to the stimulatory mixture (Fig. 2C), supporting the notion that only fully glycosylated CFTR is functional.

To determine if increased protein synthesis influenced the function of the C225R and R1066C mutants, we pretreated cells with 5 mM sodium butyrate for 18–20 h. R117H- and wild-type-CFTR were not tested since they were fully responsive in untreated conditions. After treatment with butyrate, 22% of C225R-CFTR cells were responsive; of these, 27 and 73% were fast and slow responsive, respectively (Table I). Thus, butyrate treatment increased the number of responsive cells and unmasked the full response. These results strengthen the conclusion that C225R-CFTR protein is correctly processed to the plasma membrane. Sodium butyrate treatment of R1066C-CFTR cells (n = 50) induced no change in MEQ fluorescence, indicating that butyrate treatment cannot overcome the defective function of this mutant. Representative data are shown in Fig. 2, A–C, and the mean values of slopes of fluorescence dequenching are shown in Table I.
**DISCUSSION**

We analyzed the structure-function relationships of two mutations, C225R and R1066C, that we had identified in CF patients with pancreatic insufficiency (6) and compared the properties of those mutations with those of wild-type-CFTR and R117H-CFTR. C225R was found in a compound heterozygote for the ΔF508 mutation and was associated with pancreatic insufficiency, normal lung function, and asthma. R1066C was first found in a patient bearing the ΔF508 mutation on the other chromosome. Six unrelated patients bearing the R1066C mutation have since been identified. Five patients had pancreatic insufficiency while the sixth had normal pancreatic function and was a compound heterozygote for a mild mutation (D110H) (14).

Six CF-associated mutations (P99L, R117H, P205S, R334W, R347P, and R347H) located in putative membrane-spanning domains that have already been analyzed for their functional properties (2–5) were all associated with a mild phenotype (pancreatic sufficiency, PS). To our knowledge, this is the first reported expression of the C225R-CFTR mutant and the first description of a transmembrane mutant associated with a severe phenotype (PI). Heterologous expression of this mutant in HeLa cells showed that C225R-CFTR protein elicited cAMP-dependent chloride fluxes. It thus seems that the pancreatic insufficiency associated with the C225R mutation cannot be fully explained by defective chloride conduction through the mutant CFTR protein.

The processing of C225R-CFTR resembles that of R117H-CFTR. This latter mutant, in which the substitution lies in the second putative membrane-spanning domain, is correctly processed and generates a reduced cAMP-activated apical membrane chloride current (2). Both mutants are associated with mild lung disease, but R117H is associated with PS whereas C225R is associated with PI. We think that it is unwise to use pancreatic sufficiency and insufficiency to define mild and severe CF disease, respectively.

Regarding the second mutant studied here, R1066C-CFTR, no cAMP-activated anion conductance was found in cells expressing this protein and no mature protein was detected. This probably reflects defective CFTR biosynthesis and/or incorrect delivery to the plasma membrane. During the course of this study, heterologous expression of R1066C-CFTR was reported by others using different expression systems (15, 16). Our results are in keeping with the report by Seibert et al. R1066C is a class II mutation in the classification proposed by Welsh and Smith (17). The functional defect of ΔF508-CFTR, another class II mutant, can be corrected by overexpression of the mutated protein (8). Unlike ΔF508-CFTR, R1066C-CFTR protein cannot bypass the “quality control” of the endoplasmic reticulum when grown at reduced temperature or when overexpressed in butyrate-treated cells; defective processing may thus occur at a different step in the biosynthetic pathway.

Taken together, the immunoprecipitation and functional assay results suggest that lipofection of a CFTR cDNA under the control of an SV40 promoter in HeLa cells mimics the biosynthetic pathway of human lung cells but not human pancreatic cells in vivo. This experimental model might be helpful to predict the lung status of CF patients bearing mutations located in putative membrane-spanning domains of CFTR protein.

At the molecular level, cysteine replacement, as in C225R and R1066C, may lead to the disruption or creation of disulfide bonds between cysteines and thereby change the channel properties. Thus, a new class of mutations may be defined on the basis of the presence (or absence) of these bonds.

The results of our study support Sheppard’s view that “it is not possible to predict the mechanism of dysfunction of CFTR based solely on the site of mutation” (5) and further demonstrate that it is difficult to predict the phenotype on the basis of the mechanism of dysfunction.

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