Two Mild Cystic Fibrosis-associated Mutations Result in Severe Cystic Fibrosis When Combined in Cis and Reveal a Residue Important for Cystic Fibrosis Transmembrane Conductance Regulator Processing and Function*

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The number of complex cystic fibrosis transmembrane conductance regulator (CFTR) genotypes identified as having double-mutant alleles with two mutations inherited in cis has been growing. We investigated the structure-function relationships of a severe cystic fibrosis (CF)-associated double mutant (R347H-D979A) to evaluate the contribution of each mild mutation to the phenotype. CFTR mutants expressed in HeLa cells were analyzed for protein biosynthesis and Cl− channel activity. Our data show that R347H is associated with mild defective Cl− channel activity and that the D979A defect leads to misprocessing. The mutant R347H-D979A combines both defects for a dramatic decrease in Cl− current. To decipher the molecular mechanism of this phenotype, single and double mutants with different charge combinations at residues 347 and 979 were constructed as charged residues were involved in this complex genotype. These studies revealed that residue 979, located in the third cytoplasmic loop, is critical for CFTR processing and Cl− channel activity highlighting the role of charged residues. These results have also important implications for CF, as they show that two mutations in cis can act in concert to alter dramatically CFTR function contributing to the wide phenotypic variability of CF disease.

The number of complex CFTR genotypes identified, including double-mutant alleles where two missense mutations are carried by the same chromosome, has been growing (2–6). It is difficult to evaluate the contribution of each mutation/or polymorphism to the phenotype as mutations in cis may act in concert to alter or reverse defective CFTR function and thus modify the CF phenotype (2).

The recent discovery of severe CF associated with a ΔF508/ R347H-D979A compound heterozygote genotype in two related patients suffering from pancreatic insufficiency and severe respiratory symptoms suggests that the R347H-D979A mutation has an important influence on CFTR processing and/or function (7). At least four CF-associated mutations have been identified in isolation at position 347 (R347C, R347H, R347L, and R347P) and two at position 979 (D979A and D979V), suggesting that Arg-347 and Asp-979 are important for CFTR structure and/or function. The mutation D979A was found in isolation in a patient with a congenital bilateral absence of the vas deferens (8) and the R347H mutation in CF patients with pancreatic sufficiency, congenital bilateral absence of the vas deferens, and no or mild pulmonary symptoms (7). As the R347H mutation is mostly associated with mild CF, it was suggested that the D979A mutation has a significant effect on CFTR function when combined in cis with R347H. Arg-347 lies within TM6 and is believed to line the pore (9, 10), whereas Asp-979 is located in CL3 connecting TM8 and TM9 in the C-terminal half of CFTR. The mutations R347H and D979A replace positively charged (Arg) and negatively charged (Asp) residues with ones that are uncharged (His and Ala, respectively) at physiological pH.

The present study investigates the structure-function relationships of the R347H-D979A double mutant, and as charged residues are involved in this complex genotype, single and double mutants with different charge combinations at residues 347 and 979 were constructed. All these mutants were transiently expressed in HeLa cells, and we analyzed CFTR processing by immunoprecipitation and chloride channel activity using the whole-cell patch-clamp technique.

Our data show that R347H is associated with defective chloride channel activity and that the D979A defect leads to misprocessing. The mutant R347H-D979A combines both defects for a dramatic decrease in Cl− current. These studies also revealed that residue 979 is a critical location for both CFTR processing and Cl− channel activity.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis, Cells, and CFTR Expression—CFTR mutants were constructed in the expression plasmid pTCFwt, a vector...
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**RESULTS**

**Electrophysiology—**Whole-cell patch-clamp recordings were performed at room temperature (20–25°C) on isolated cells, 24 h after trypsinization and replating on plastic dishes at a low density. Before recording, the culture medium was replaced by the external solution to be used during the recording. This solution contained the following (in mM): 30 CsCl, 113 NaCl, 140 NaCl, 2 CaCl2, 1 MgCl2, 10 Hepes, 10 glucose, and 25 sucrose; its final pH value was adjusted to 7.3 with NaOH. Patch-clamp micropipettes were made from hard glass (Kimax 51); their shank was covered by Sylgard, and their tip was fire-polished. They were filled with an internal solution containing the following (in mM): 140 NaCl, 113 NaCl, 10 Hepes, 1 MgCl2, and 0.5 EGTA. The bath was connected to the ground via an agar bridge. The zero indicated on current traces and current plots is the absolute zero current level. To establish I-V curves of the response, an approximate membrane potential between −80 and +80 mV. CFTR CI− currents were activated with 200 μM 8-(4-chlorophenylthio)-ATP (Calbiochem). The resulting proteins were phosphorylated in vitro with 5 units of Plus reagent (Life Technologies, Inc.) with 2 μg of plasmid according to the manufacturer’s instructions. Confluent monolayers were harvested and used 48 h post-transfection for functional assays or immunoprecipitation.

**Immunoprecipitation/cAMP-dependent Protein Kinase Assay and Pulse-Chase Experiments—**The CFTR protein immunoprecipitation using the monoclonal antibody (mAb) 24–1 (R&D System), which recognizes the C terminus of CFTR, was already described (12). Briefly, cell lysates were mixed with 0.4 μg of mAb 24–1 and Pansorbin (Calbiochem). The resulting proteins were phosphorylated in vitro with 5 units of the catalytic subunit of cAMP-dependent protein kinase (Promega) and 10 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech), separated by 5% SDS polyacrylamide gel electrophoresis, dried, and autoradiographed. Radioactivity was quantitated by radioanalytic scanning (using a Molecular Dynamics PhosphoImager).

Pulse-chase experiments were performed by incubating cells for 30 min in Dulbecco’s modified Eagle’s medium lacking cysteine and methionine and then for 15 min in the same medium containing 100 μCi[s]labeled methionine and [35S]-labeled cysteine (Redive Pro-mix™ [35S]; Amersham Pharmacia Biotech). CFTR was immunoprecipitated from cell homogenates with the same mAb 24–1. 

**Processing of CFTR Mutants**—Many CF-associated mutations cause a loss of CFTR CI− channel function because of misprocessing of the mutant protein so that it does not reach the cell membrane. We first studied the maturation of CFTR in HeLa cells to determine why patients with the R347H-D979A-CFTR allele suffered from severe CF. HeLa cells were transiently transfected with cDNA encoding the wild-type and mutated CFTR proteins. The processing of CFTR can be assessed by examining its glycosylation. In our experimental conditions, electrophoresis of immunoprecipitated wild-type CFTR gave two bands (Fig. 1A). One was a diffuse band of an approximate molecular mass of 170 kDa (band C) that represented the mature, fully glycosylated protein that has migrated through the Golgi complex to the cell membrane. The second was a thin band of about 140 kDa (band B) that represented the core-glycosylated protein located in the endoplasmic reticulum.

Immunoprecipitation experiments show that both wild-type and CF-associated mutants R347H, D979A, and R347H-D979A-CFTR cells produced mature, fully glycosylated protein (Fig. 1A; band C), whereas none of the mock-transfected cells produced CFTR (data not shown). However, the D979A and R347H-D979A mutants produced significantly less band C (59 and 56% of the total CFTR; ratio C/(B+C) than wild-type and R347H-CFTR (92 and 91%; see Fig. 1B). This indicates that the R347H-D979A mutation caused a misprocessing defect. The turnover of immature and mature forms of wild-type and R347H-D979A-CFTR (results are representative of two independent experiments).

**FIG. 1. Production of mature protein by wild-type and mutants assessed by CFTR glycosylation states.** A, typical immunoprecipitation of cAMP-dependent protein kinase-phosphorylated (with [γ-32P]ATP) CFTR proteins using mAb 24–1. Bands B and C are indicated by arrows. WT, wild-type. B, quantitation of CFTR maturation efficiency calculated as the amount of mature CFTR (%C) relative to the total amount of CFTR produced (B+C). Data are the means ± S.E. of at least three independent experiments. *, significantly different from wild-type (WT; p < 0.01).

**D979A-CFTR**

D979A-CFTR cells produced mature, fully glycosylated protein (Fig. 1A; band C), whereas none of the mock-transfected cells produced CFTR (data not shown). However, the D979A and R347H-D979A mutants produced significantly less band C (59 and 56% of the total CFTR; ratio C/(B+C) than wild-type and R347H-CFTR (92 and 91%; see Fig. 1B). This indicates that the D974H-D979A mutation caused a misprocessing defect. The turnover of immature and mature forms of wild-type and R347H-D979A-CFTR was further investigated by pulse-chase experiments (Fig. 1C). The kinetics of wild-type and R347H-D979A core-glycosylated and mature forms of CFTR were identical, whereas the efficiency of conversion to mature band C was lower for the mutant. Similar results were obtained with the D979A mutant (data not shown). This indicates a defect in the biosynthetic pathway, which accounts for the decreased amount of band C observed in the steady-state measurements.

Other mutants were generated to further characterize the D979A defect. Asp-979 was changed to Val (small hydrophobic residue; D979V), Arg (positively charged residue; D979R), or Glu (negatively charged residue; D979E). D979V (also a naturally occurring mutant) and D979R had impaired processing...
similar to D979A, whereas D979E permitted the complete maturation of the protein (Fig. 1B).

Altogether, these results indicate that D979A is responsible for the defective processing of R347H-D979A-CFTR and that a negative charge at 979 residue is necessary for proper CFTR processing.

Cl⁻ Channel Function of CFTR Mutants—We tested the cAMP-activated chloride channel activity of transfected HeLa cells using the whole-cell patch-clamp technique. None of the mock-transfected cells displayed a Cl⁻ current in either basal or cAMP-stimulated conditions (data not shown). By using 140 mM Cl⁻ (symmetrically) and 200 μM cAMP agonist, cells expressing wild-type CFTR produced saturating whole-cell currents for clamp potentials above/below ± 40 mV. We therefore reduced Cl⁻ (32 mM symmetrically by replacement with glutamate) and cAMP agonist (50 μM) to characterize wild-type CFTR current. As shown in Fig. 2A, there was little or no current under basal conditions, and application of CPT-cAMP activated large currents that reversed at the Cl⁻ equilibrium potential (close to 0 mV with the solutions used). The currents were glibenclamide-sensitive and DIDS- and TS-TM-calix[4]arene-insensitive as expected for CFTR Cl⁻ current (data not shown). These currents were activated a few seconds after stimulation and were slowly reversible after washout of CPT-cAMP. The current-voltage relationship exhibited a slight outward rectification (Fig. 2B), as described when internal Cl⁻ were replaced by large impermeant anions (14).

We separated the contributions of the R347H and D979A mutations to R347H-D979A-CFTR whole-cell Cl⁻ current production studying both single and double mutants. To maximize our ability to detect differences between mutants and to compare mutants with wild-type CFTR, we used 140 mM Cl⁻ and 200 μM CPT-cAMP agonist, and data were analyzed at 20 mV. Current-voltage relationships of naturally occurring mutants are shown in Fig. 2C. Mean changes in CAMP-activated currents recorded at 20 mV from R347H (29.4 ± 12.2 pA/pF; n = 8), D979A (67.9 ± 18.9 pA/pF; n = 5), and R347H-D979A (1.2 ± 0.8 pA/pF; n = 9) mutants were significantly different (p < 0.05) from wild-type (130.2 ± 34.7 pA/pF; n = 5), corresponding to 23, 52, and 1% of the wild-type Cl⁻ current (Fig. 2D). R347H, D979A, and R347H-D979A were also significantly different from each other (p < 0.01). As R347H processing is similar to wild-type, the small Cl⁻ current produced by R347H reflected defective channel properties, as demonstrated by single-channel studies (9). The decreased whole-cell Cl⁻ CAMP-dependent current observed with D979A probably reflected CFTR protein misprocessing, although we cannot firmly exclude altered channel properties. Thus these data indicate that the R347H-D979A double mutant combined at least D979A misprocessing and the R347H Cl⁻ channel defect to produce a very severe phenotype.

Charge-reversal Mutants—Taking into account the functional defects that result when Arg-347 and Asp-979 are each replaced with an uncharged amino acid such as His (uncharged at pH 7.3) and Ala (R347H and D979A), we constructed additional mutants with different charge combinations at residues 347 and 979, including the R347D-D979R double mutant in which the positive and negative charges were swapped.

The processing of R347D was similar to that of R347H and the wild-type (Fig. 3, open bars). Thus, Arg-347 is not critical for processing, as the replacement of Arg by His or Asp permitted complete maturation of the protein. Surprisingly, the proc-
Figure 3: Maturation of charge mutants at 347 and 979 residues.

Data are obtained as in Fig. 1. Charge is indicated in brackets for each amino acid residue. Data are the means ± S.E. of at least three independent experiments. *, significantly different from the other CFTR proteins studied (p < 0.01).

In conclusion, this study highlights the importance of structure-function analysis of naturally occurring mutants for deciphering complex genotype and identifying residues important for CFTR processing and/or chloride channel activity. These results also have important implications for CF, as they show that two mutations in cis can act in concert to alter dramatically CFTR function. This may contribute to the wide phenotypic variability of CF disease and points to the need to screen for all mutations.

Manavalan et al. (15) observed significant amino acid homology and length conservation in the CL regions between CFTR and other transporter proteins. It was then suggested that CLs may be important for channel function and/or the folding and processing of the CFTR protein, and extensive studies of CF-associated mutations in the four CLs have revealed channel properties and/or processing defects (16–20). Our data strongly suggest that mutation D979R alters the properties of the chloride channel, and mutational analysis of 979 residue also confirmed that the requirements for channel processing and function are different, consistent with data for other residues (17, 18, 21). Many mutations in the CLs impair CFTR processing (16–20), suggesting that the correct folding of CLs is important for proper maturation of the whole molecule. Our data support this view, as replacing Asp-979 by Ala, Val, or Arg significantly impaired processing. Based on mutational analysis of 979 residue, Seibert et al. (18) proposed that the “influence on folding may depend more on the location of the altered residue than the specific residue change.” We observed that the negatively charged Asp-979 could be replaced only by the negatively charged Glu-979 without affecting processing, indicating that a negative charge is important at that location. Moreover, Asp-979 is highly conserved throughout CFTR evolution (22, 23) and belongs to a consensus sequence shared with other ABC transporters (15). These observations all indicate a crucial role for Asp-979 and thus raised the following question: how might removal of the negative charge at position 979 affect CFTR processing? Little is known about how the CLs contribute to CFTR processing. Considering the highly hydrophilic composition and length of CFTR CLs (53–61 residues) (15), they could take part in physical interactions with other cytoplasmic domains of CFTR, solvent-accessible TM residues, or cytoplasmic proteins. First, several lines of experimental evidence indicate that there is probably no direct salt bridge between Arg-347 and Asp-979: (i) removal of either the positive charge at position 347 (R347H and R347D) or the negative charge at position 979 (D979A, D979V, and D979R) has different effects on CFTR processing; (ii) the double-neutral (R347H-D979A) and reversed-charged (R347D-D979R) replacements for Arg-347 and Asp-979 do not lead to the recovery of wild-type processing. Second, Asp-979 might interact with an as yet unidentified positively charged residue located either in a cytoplasmic part of CFTR or in a solvent-accessible part of a TM domain, stabilizing CL3 at a distance from residue 347. Removal of the negative charge at position 979 would disrupt this interaction and could direct CL3 to residue 347 and thus allow a charge-dependent interaction with residue 347. This is supported by the decrease in the processing efficiency of D979R mutants combined with positive, neutral, and negative charges at residue 347. Furthermore, a single charge can form salt bridges with several opposite charges. Given the highly hydrophilic nature of CLs, multiple ion pairs could maintain a conformation of the CLs favorable to proper maturation of the protein, which is supported by the multiple charge disruption mutations in CLs affecting CFTR processing (17–20). It may thus be difficult to identify a single partner for Asp-979. However, interactions between charges exposed on the protein surface may do little to stabilize proteins, unlike buried ion pairs (24). Rather than being engaged in intramolecular interactions, Asp-979 might interact with cytoplasmic proteins. Several proteins have recently been shown to interact directly with CFTR cytoplasmic domains (25–28). It would be interesting to see whether Asp-979 participates in such binding.
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