The Second Half of the Cystic Fibrosis Transmembrane Conductance Regulator Forms a Functional Chloride Channel*

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The cystic fibrosis transmembrane conductance regulator (CFTR) consists of two transmembrane domains (TMDs), TMD1 and TMD2, two cytoplasmic nucleotide binding domains (NBDs), NBD1 and NBD2, and a regulatory domain. To elucidate the complex function of the CFTR, deletion constructs encompassing the second half of the CFTR distal to the first transmembrane domain were expressed in Xenopus oocytes and IB3 cells (a cystic fibrosis cell line). Constructs containing the regulatory domain, the second transmembrane domain, and the second nucleotide binding domain formed constitutively active channels, which were further stimulated upon the addition of cAMP. On the other hand, a construct encompassing the second transmembrane domain and the second nucleotide binding domain was stimulated to a small but noticeable extent upon the addition of cAMP. The selectivity of the second-half construct was the same for iodide and chloride, in contrast to the selectivity of wild-type CFTR, which is Cl\textsuperscript–> I\textsuperscript–. However, both constructs displayed single-channel conductances that were significantly smaller than those displayed by the first half of the CFTR. We conclude that regions of the second transmembrane domain may contribute to the overall channel of the pore, although the first half of the CFTR may confer its selectivity.

Quinton suggested in 1983 (24) that Cl\textsuperscript– transport is defective in CF,\textsuperscript3 and 6 years later, cloning of the CF gene and subsequent studies showed that CFTR is indeed a chloride channel (1, 2) with a linear current versus voltage (I/V) relationship and a 9–11-pS single-channel conductance. The selectivity of the channel is Br\textsuperscript–> Cl\textsuperscript–> I\textsuperscript–> F\textsuperscript–. Subsequent to the cloning of the CF gene, many investigators have used site-directed mutagenesis to create CFTR cDNAs containing both naturally occurring and artificial mutations. Studies on the consequences of these mutations have shaped our thinking about the structure and function of the protein. For example, a mutant CFTR (TNRCFTR) composed of the first transmembrane domain, the first nucleotide binding domain, and the R domain can form a functional chloride channel with characteristics approaching that of wtCFTR (3, 4). An additional construct composed of only the first six transmembrane-spanning segments of TMD1 also forms an ion channel with selectivity and single-channel conductance identical to that of wild-type CFTR (5). We have previously shown (6) that the ion selectivity and single-channel conductance of Δ259 CFTR, which contains membrane-spanning segments M5, M6, and the second half of the CFTR, are identical to those of wild-type CFTR. These studies suggest that the major structural components that allow the CFTR to select among different anions reside within TMD1, more specifically, distal to transmembrane segment M4.

Although TMD1 (7, 8) has been studied extensively, the role of the second half of the CFTR in ion conduction is still unresolved. To evaluate the functional contributions of this region of the protein, we generated deletion constructs encompassing the second half of the CFTR (Fig. 1). The cRNA for these mutants was then injected into Xenopus oocytes. Seventy-two h after injection, two-electrode voltage clamp experiments were performed to look for channel activity. The first construct contains the regulatory domain, the second transmembrane domain, and the second nucleotide binding domain of CFTR (RT2N2CFTR). A second construct containing the second transmembrane domain and the second nucleotide binding domain (T2N2CFTR) was also tested for cAMP-stimulated chloride channel activity. Both constructs included the first 159 bases of wtCFTR so as to include the Kozak methionine for translation initiation. Our results suggest that the second half of the CFTR does play a role in Cl\textsuperscript– conduction.

MATERIALS AND METHODS

Site-directed Mutagenesis—The double-stranded mutagenesis method (Stratagene) was used to generate all of the necessary mutations. Both constructs (RT2N2CFTR and T2N2CFTR) were constructed using a wtCFTR plasmid (PBluescript CFTR) as the template. A SstI site (AGGCCCT) was created at position 160 using the oligonucleotide CGCCTCTGGAAAAAGGCCTGCGTTGTTGTCACCAAAC, and another SstI site was created at positions 2125 (for RT2N2CFTR) and 2523 (for T2N2CFTR) using oligonucleotides CCTAATGCAGCCCTTACCGTCTTCTCATTAAGAGG and CGAAAAGTGCTGCTGGGCCCTCAGGCAAACCTTG, respectively. The selection primer used for this method of mutagenesis was one that eliminated the KpnI site on the vector backbone (CGAACCTAGGGCGGCGGCGATCTACGCTACGCGTTGCC). The three oligonucleotides were phosphorylated and added to a mixture of wtCFTR plasmid and mutagenesis buffer, followed by denaturation by boiling at 100 °C for 5 min. The mixture was cooled on ice for 5 min and then at room temperature for 30 min to facilitate the annealing of the primers to the single-stranded template. The oligonucleotides were extended using DNA polymerase and deoxynucleotide triphosphates, followed by ligation with DNA ligase. Any wild-type plasmid that remained was eliminated by digestion with KpnI for 2 h. A mutS strain was then transformed with the digestion mix and spread onto LB-agar plates overnight. Plasmid DNA was extracted from the colonies and redigested with KpnI to eliminate the wild-type plasmids. The remaining undigested plasmids were then retransformed into XL1 supercompetent cells. Plasmids were then extracted from individual colonies and screened for the mutation of interest. Plasmids carrying

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The abbreviations used are: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; TMD, transmembrane domain; NBD, nucleotide binding domain; R, regulatory; wt, wild-type; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

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**RESULTS**

**Chloride Channel Studies in Xenopus Oocytes**—To evaluate the functions of the second transmembrane domain of CFTR, cRNA for wtCFTR and the two second-half mutants was expressed in Xenopus oocytes. Water-injected oocytes exhibited little or no current activity in the absence ($I = 9.06 \pm 6.88 \text{nA}$, $n = 9$ at 60 mV; Fig. 2) or presence of cAMP agonists ($I = 92.24 \pm 7.44 \text{nA}$, $n = 9$ at 60 mV), wtCFTR-injected oocytes expressed linear currents upon cAMP stimulation that were DIDS insensitive and exhibited linear $1/V$ characteristics and a reversal potential consistent with a chloride current. The current magnitude was reduced for T2N2CFTR, $I_{\text{Cl}} = 534.67 \pm 59.7 \text{nA}$ at 60 mV ($n = 8$) and $I_{\text{Cl}} = 410.1 \pm 39.7 \text{nA}$ at 60 mV ($n = 8$); Fig. 3). Upon switching to an iodide Ringer solution, consistent with the selectivity profile demonstrated for wtCFTR ($CI^{-} > I^{-}$; Ref. 1).

On the contrary, oocytes injected with RT2N2CFTR ($I = 470.52 \pm 84.51 \text{nA}$ at 60 mV) exhibited a large baseline constitutive current compared with those injected with wtCFTR ($I = 115.06 \pm 9.16 \text{nA}$, $n = 8$ at 60 mV; Fig. 4). The current was further increased upon the addition of DIDS agonists.
(RT2N2CFTR, I = 676.03 ± 87 nA at 60 mV, n = 9; wtCFTR, I = 534.67 ± 39.7 nA at 60 mV, n = 8). The current magnitude was unchanged upon switching the Ringer solution from one in which the predominant anion was chloride to one in which the predominant anion was iodide. This was in contrast to wtCFTR-injected oocytes, in which a decrease in current magnitude was observed upon switching to an iodide-containing Ringer’s solution.

Oocytes injected with cRNA for T2N2CFTR exhibited a small baseline current similar to that exhibited by wtCFTR-injected oocytes (Table I, I = 90.06 ± 6.88 nA, n = 9; cAMP-stimulated current, I = 92.24 ± 7.44 nA, n = 9).

**Chloride Channel Studies in IB3-1 Cells**—To assess the Cl− channel functions of CFTR, paired 36Cl− efflux assays without and with a CAMP agonist mixture (2.5 μM forskolin and 250 μM 8-chlorophenylthio-cAMP or CPT-cAMP) were performed in IB3-1 cells. IB3-1 cells are a CF bronchial epithelial cell line used extensively to study CFTR function. The experiments were performed as an initial screen to assess whether an individual CFTR mutant could restore CAMP-stimulated efflux of labeled chloride. Parental IB3-1 cells and mock-transfected IB3-1 cells failed to respond to CAMP agonists (Table II), which is typical of CF cells. Wild-type-, Δ259-M265-, RT2N2CFTR-, and T2N2CFTR-transfected cells, however, all responded to CAMP agonists as predicted from oocyte recordings (Table II). Surprisingly, cells transfected with T2N2CFTR also demonstrated an increase in efflux upon CAMP stimulation that was similar to that exhibited by cells transfected with wtCFTR.

**FIG. 2. Current characteristics of water-injected oocytes.** Representative two-electrode voltage clamp current recordings of water-injected oocytes. Basal, CAMP-stimulated and I/V current characteristics are depicted. The voltage protocol used consisted of a stepwise depolarization, in steps of 20 mV, from −80 to +60 mV with a holding potential of −40 mV. The CAMP stimulation mixture consisting of 10 μM forskolin and 1 μM 3-isobutyl-1-methylxanthine was added 15 min after recording the baseline current. Average whole cell currents at +60 mV are as follows: basal, I = 90.06 ± 6.88 nA, n = 9; cAMP-stimulated current, I = 92.24 ± 7.44 nA, n = 9.
This could be attributed to a larger number of channels being formed on the plasma membrane.

Whole cell current patch-clamp recordings were carried out to assess the Cl\(^-\) channel function of wild-type CFTR and the second-half mutants in IB3-1 cells, a CF cell line. Mock-transfected cells failed to exhibit cAMP-stimulated chloride currents (Fig. 6A). However, cells transfected with wtCFTR exhibited cAMP-stimulated currents that were DIDS insensitive (Fig. 6B). Cells transfected with RT2N2CFTR exhibited a larger baseline compared with those transfected with wtCFTR. When stimulated, RT2N2CFTR-transfected cells generated currents comparable to those of wtCFTR-transfected cells (Fig. 6C). On the other hand, cells transfected with T2N2CFTR exhibited a small baseline current comparable to that of wtCFTR-transfected cells and were stimulated to a smaller extent upon the addition of cAMP agonists (Fig. 6D). Single-channel analysis of the mutant constructs in excised inside-out patches also revealed chloride channels with significantly smaller conductances than that of wtCFTR (Table III; Fig. 7), whereas wtCFTR had a conductance of 10.2 pS, compared with a conductance of 4 pS for RT2N2CFTR and 3.8 pS for T2N2CFTR.

Interestingly enough, both constructs were nonselective for the halides chloride or iodide, in contrast with wild-type CFTR, which was more selective for chloride than iodide.

**DISCUSSION**

The importance of TMD2 for the normal functioning of the CFTR is evident in the large number (19 thus far) of missense mutations associated with CF located in the fourth intracellular loop (ICL4) between segments M10 and M11 of CFTR (10–14). One residue in particular, R1066, is associated with four separate CF-causing mutations. Cotten et al. (15) constructed several of these mutants and expressed them in heterologous cells to study their effects on protein processing and function. These mutants expressed chloride currents upon cAMP stimulation, which had close to wild-type characteristics. The single-channel conductances and anion selectivity of these mutants resembled that of wild-type CFTR, suggesting that ICL4 does not contribute directly to the conduction pore. However, the mutants did have varied influences on the open state probability and burst duration. The authors speculate that ICL4 mutations might disrupt or modify some aspect of gating.
of the Cl⁻ channel pore but not the conduction of anions through the pore. Siebert et al. (16) conducted similar experiments on the third intracellular loop of CFTR and concluded that this region may be involved in maintaining the stability of the channel in the open state.

Fluorescence assays on peptides corresponding to the individual transmembrane segments revealed that membrane-spanning regions in the second half were able to conduct chloride and could thus line the pore in the wild-type protein. McCarty et al. (17) and McDonough et al. (18) studied the diphenylamine-2-carboxylic acid binding properties of residues within the putative chloride permeation pathway of CFTR. Residues in CFTR that exhibit altered diphenylamine-2-carboxylic acid binding properties when mutated may line the pore. They showed that residues in both M6 and M12 may line the channel pore, suggesting that regions of the second half of the CFTR may line the channel pore.

Anderson et al. (1) mutated several amino acids in the TMDs and evaluated their effects on CFTR channel function. They hypothesized that if the charged amino acids that lined the channel were mutated, they would disrupt the wild-type electrical forces in the pore, thereby influencing ion selectivity. Lys<sup>345</sup> in M1 and Lys<sup>335</sup> in M6 were mutated to Asp and Glu, respectively. HeLa cells were transfected with wtCFTR or with the mutants. Analysis of whole cell cAMP-stimulated chloride currents showed that these lysine mutations altered wild-type ion selectivity from Br⁻ > Cl⁻ > I⁻ > F⁻ to I⁻ > Br⁻ > Cl⁻ > F⁻. In contrast, the mutation of Arg<sup>347</sup> in M6 and Arg<sup>1030</sup>
M10 did not induce major differences in halide selectivity compared with wtCFTR. From these data, they concluded that the residues in TMD1 play an important role in the selectivity filter of the CFTR.

The results from our studies suggest that regions of the second half of CFTR may line the channel pore and have important contributions to its chloride channel function, whereas the selectivity filter of CFTR is provided by charged residues on the sixth transmembrane segment (M6) of wtCFTR. We have shown that expression of the second half of CFTR distal to the first nucleotide binding domain generated functional chloride channels albeit with properties distinct from that of wild-type CFTR. RT2N2CFTR produces Cl\textsuperscript{−} currents that are stimulated by cAMP. On the other hand, T2N2CFTR generated a smaller increase in current upon cAMP stimulation. This could be due to the fact that T2N2CFTR has no consensus phosphorylation sites, whereas RT2N2CFTR retains most of the phosphorylation sites that lie in the R domain. In the case of the wild-type protein, phosphorylation of the R domain followed by ATP binding and hydrolysis causes the opening of the channel. In the case of RT2N2CFTR, the R domain is unable to keep the channel closed in the basal or unstimulated state. This could suggest that in the absence of the first transmembrane domain and the first nucleotide binding domain, the R domain is unable to form a regulated channel. Furthermore, the first two domains may be necessary for the R domain to keep the channel closed in the unstimulated state. The observation that T2N2CFTR channels...
were not stimulated significantly upon the addition of cAMP agonists is not surprising because this construct lacks any consensus PKA phosphorylation sites. Furthermore, NBD1 has been shown to be responsible for the opening of the channel, whereas NBD2 has been shown to be associated with channel closing. The presence of NBD2 in this construct could also suggest that the overall conductance of CFTR is provided by TMD1 and TMD2. TNRCFTR, which contains the first transmembrane domain, the first nucleotide domain, and the R domain, displays a conductance (8 pS) almost equal to that of wtCFTR, whereas the second-half constructs discussed above show much smaller conductances (RT2N2CFTR, 4 pS; T2N2CFTR, 3.8 pS). This could suggest that the major contribution to the size of the pore comes from the first half of the protein, whereas regions of the second half could contribute a smaller part.

The results summarized above suggest that the second half of CFTR has important functions in the overall tertiary structure of the channel. The regions of the second half distal to the second nucleotide binding domain may also play important roles in the regulatory interactions of CFTR with the outwardly rectifying chloride channel, the epithelial sodium channel, and other potassium channels. Finally, the discovery of proteins that facilitate interactions between membrane proteins via interactions at the amino and carboxyl termini could add importance to the second half of CFTR. There is growing evidence that proteins with PDZ domains do interact with other potassium channels (19, 20). Expression of proteins containing these domains causes a clustering of ion channels and receptors at the plasma membrane. For instance, coexpression of PSD-95 (a PDZ domain-containing protein) results in clustering of voltage gated K⁺ channels and N-methyl-d-aspartate receptors at the plasma membrane (19). The PSD-95 protein recognizes a specific sequence present at both the carboxyl termini of the Shaker voltage gated K⁺ channel and the NR2 subunit of the N-methyl-d-aspartate receptor. Similar domains have also been identified recently for CFTR (21). Furthermore, several such proteins have been discovered that interact with the car-

**Table III**

| Construct | \( \text{Conductance (pS)} \) | \( \text{Po}^a \) | Selectivity |
|-----------|-----------------|-----------------|-------------|
| wtCFTR    | 5.0 ± 0.1       | 0.56 ± 0.07     | Br⁻ > Cl⁻ > I⁻ |
| RT2N2CFTR | 4.0 ± 0.5       | 0.43 ± 0.05     | Br⁻ = Cl⁻ = I⁻ |
| T2N2CFTR  | 3.8 ± 0.6       | 0.15 ± 0.04     | Br⁻ = Cl⁻ = I⁻ |

* Po, probability.
boxyl-terminal of CFTR, rendering this region of the protein to be of greater importance than previously thought (21–23). 2

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