Journal of Bioenergetics and Biomembranes, Vol. 29, No. 5, 1997

CFTR: Domains, Structure, and Function

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Received May 1, 1997; accepted July 1, 1997

Mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) cause cystic fibrosis (CF) (Collins, 1992). Over 500 naturally occurring mutations have been identified in CF gene which are located in all of the domains of the protein (Kerem *et al.*, 1990; Mercier *et al.*, 1993; Ghanem *et al.*, 1994; Fanen *et al.*, 1992; Ferec *et al.*, 1992; Cutting *et al.*, 1990). Early studies by several investigators characterized CFTR as a chloride channel (Anderson *et al.*; 1991b,c; Bear *et al.*, 1991). The complex secondary structure of the protein suggested that CFTR might possess other functions in addition to being a chloride channel. Studies have established that the CFTR functions not only as a chloride channel but is indeed a regulator of sodium channels (Stutts *et al.*, 1995), outwardly rectifying chloride channels (ORCC) (Gray *et al.*, 1989; Garber *et al.*, 1992; Egan *et al.*, 1992; Hwang *et al.*, 1989; Schwiebert *et al.*, 1995) and also the transport of ATP (Schwiebert *et al.*, 1995; Reisin *et al.*, 1994). This mini-review deals with the studies which elucidate the functions of the various domains of CFTR, namely the transmembrane domains, TMD1 and TMD2, the two cytoplasmic nucleotide binding domains, NBD1 and NBD2, and the regulatory, R, domain.

KEY WORDS: Chloride channels; CF; outwardly rectifying chloride channels; CFTR; review.

INTRODUCTION

Quinton suggested in 1983 that Cl^- transport is defective in CF and six years later, cloning of the CF gene and subsequent studies showed that CFTR is indeed a chloride channel (Anderson *et al.*, 1991b,c; Bear *et al.*, 1992) with a linear *I/V* relationship and a 9–11-pS single-channel conductance. The selectivity of the channel is $Br^- > Cl^- > I > F^-$. 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 CFTR. Results from the studies show that mutations can affect protein production, processing, channel conduction and regulation, and the ability of CFTR to interact with other ion channels. In most instances an individual mutation will have more that one effect. For example, the most common mutation in CF is the ΔF508 mutation. The primary effect of this mutation is to disrupt protein processing such that most of the protein is never fully processed to the apical cell membrane (Cheng *et al.*, 1990; Dalemans *et al.*, 1991; Denning *et al.*, 1992b; Drumm *et al.*, 1991). When studied in experimental cell systems, which are designed to partially overcome this processing defect, allowing Δ F508 to be processed to the plasma membrane, defects in channel regulation have also been identified. Thus, mutations in CFTR usually affect more than one process, sometimes making it difficult to pinpoint the influence of a mutation on a particular functional domain.

This mini-review deals with studies in which mutations were engineered into CFTR to elucidate the functions of the various domains (Fig. 1), namely the transmembrane segments, TMD1 and TMD2, the two cytoplasmic nucleotide binding domains, NBD1 and NBD2, the regulatory R domain, and how some of the mutations in these domains affect channel function.

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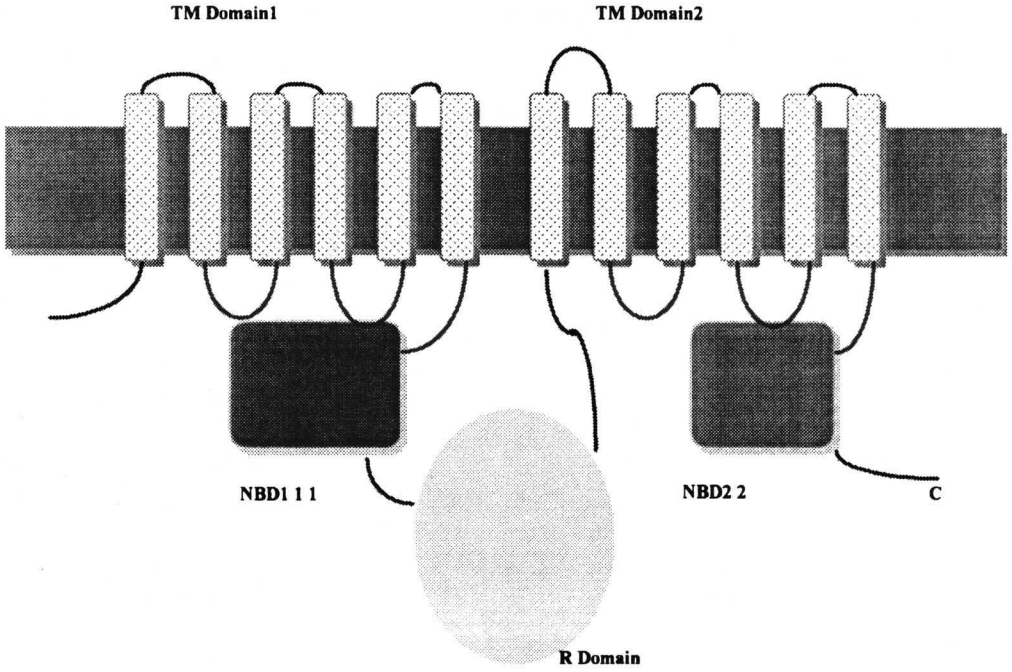

Fig. 1. Putative model of CFTR. NBD1 and NBD2 are the two nucleotide binding domains and R is the regulatory domain. TMD1 and TMD2 are the two transmembrane domains, each containing six transmembrane segments.

ROLE OF TRANSMEMBRANE DOMAINS 1 AND 2 IN CFTR CONDUCTION AND SELECTIVITY

The two TMDs are postulated to form the pore through which ions flow across the membrane in a manner analogous to other channel proteins. The role of the TMDs in determining the selectivity of CFTR is a well-investigated feature of the channel. Anderson *et al.* (1991) mutated several amino acids in the TMDs and evaluated their effects on CFTR channel function. They hypothesized that if 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^{95} in M1 and Lys^{335} in M6 were mutated to Asp and Glu respectively. HeLa cells were transfected with wt-CFTR or 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, mutation of Arg^{347} in M6 and Arg^{1030} in M10 did not induce major differences in halide selectivity compared to wt-CFTR. From these data, they concluded that residues in TMD1 play an important role both in the conduction pore and in the selectivity filter of CFTR.

Tabacharani *et al.* (1992) also provided evidence that M5 and M6 may line the channel pore. They demonstrated that CFTR is a multi-ion pore and mutating Arg^{347} in M6 alters both the conductance and converts CFTR to a single ion pore. Arg^{347} is also a naturally occurring site for mutations in patients with mild airway disease. (Sheppard *et al.*, 1993). These mutations may compromise CFTR function by affecting the channel pore directly. More recent evidence from the laboratories of Akabas *et al.* (Akabas *et al.*, 1994; Cheung *et al.*, 1996) also suggest that residues along M1 and M6 line the channel pore. They substituted each of the putative pore-lining segments with cysteines and expressed these mutants in Xenopus oocytes. Accessibility of these mutants to sulfhydryl reagents identified residues along M1 and M6 that lined the channel pore and were exposed to the water-accessible surface of the protein.

TRUNCATION MUTANTS IN TMD1

Many disease-causing mutations occur in TMD1 which are often associated with milder forms of CF. For example, the R117H mutation, a missense substitution of a histidine for an arginine at position 117, is

associated with mild pancreatic disease (Carroll *et al.*, 1995b). The R117H mutation is a semiconservative change which preserves some positive charge in the residue immediately preceding the second proposed membrane-spanning segment of the first transmembrane domain of CFTR. Although this mutation is in TMD1, the effects on channel properties are complex. Table I shows that the R117H mutation reduces both single-channel conductance and channel open probability without affecting channel selectivity.

To evaluate the function of TMD1 and the Kozak methionines that function in translation initiation, a series of 5′ truncations in TMD1 were made to coincide with residues that might serve as translation initiation codons (Carroll *et al.*, 1995a). The construct pSA306, in which the first 118 amino acids of CFTR were deleted, and ΔS118M, in which a methionine was introduced as the 118th amino acid, were expressed in Xenopus oocytes. Both these constructs exhibited similar *I/V* characteristics upon cAMP stimulation. The reversal potentials were consistent with chloride currents, and exhibited DIDS independence. These suggested that the currents were indeed generated by the truncated forms of CFTR.

The two mutants were characterized further at the single-channel level and exhibited a smaller conductance and open probability when compared to wt-CFTR. The mutants also retained the wild type selectivity of $Br^- > Cl^-$. Thus, these mutants, which completely lack the M1 segment, can still form chloride channels with characteristics approaching that of wt-CFTR. To address the question of whether methionines other than the first can function as translation initiation sites within CFTR, were constructed a series of deletions as shown in Fig. 2. MIV (in which the first methionine was substituted for a valine). Δ119CFTR (the first 118 amino acids were deleted creating the next functional methionine at Met^{150}), and MIV–M150V (both methionines at positions 1 and 150 were

Table I. Single Channel Characteristics of CFTR Channels in Oocytes

	pS	P_0	Selectivity
WT	9.3±0.4(4)	0.65±0.02(7)	Br > Cl > I(4:3)
R117H	6.5±0.5(4)*	0.49±0.01(7)*	Br > Cl > I(5:4)

* Denotes significantly different from wild type (WT). (*n*) is the number of patches, which displayed only one level of channel activity. For ion selectivity experiments (*n:n*) represents the number of individual selectivity measures for Br and I respectively.

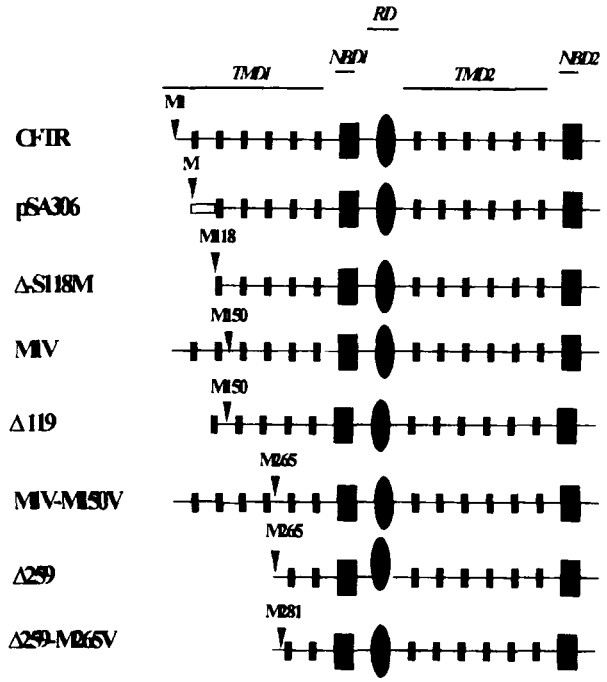

Fig. 2. Mutations in TMD1. Schematic representation of WT and mutant forms of CFTR. Each small rectangle represents a transmembrane segment while the straight lines are the intra- or extracytosolic loops. Bigger rectangles are NBDs and the oval is the R domain. The flat white rectangle at the beginning of pSA306 represents the 26 amino acid "flag" inserted in this sequence. Methionines at Kozak consensus sites are marked with an arrow (taken from Carroll *et al.*, 1995a).

substituted with valine) were constructed. cRNA for these mutants was injected into Xenopus oocytes and cAMP-stimulated chloride currents measured two to three days later. Water-injected, wild type CFTR, as well as the mutants, exhibited small baseline currents prior to stimulation. All these mutants generated chloride currents upon cAMP stimulation with wild type characteristics and exhibited time and DIDS independence. The reversal potential showed a positive shift upon shifting to low chloride solution and was consistent with a chloride current. These results demonstrated that the first methionine is not unique for translation initiation of CFTR and that downstream codons can also function as translation initiation sites. Generation of Cl^- currents by the double mutant (MIV–M150V) suggests that methionines beyond amino acid 150 may also be able to initiate translation initiation of CFTR.

To verify that additional methionines in CFTR can function as translation initiation sites, further deletions were created. These include Δ259CFTR and Δ259–M265V CFTR. Δ259CFTR has the first 259 amino

acids deleted and the next functional methionine is at amino acid M265. This mutant generates currents similar to wt-CFTR upon cAMP stimulation. The currents exhibit time and DIDS independence, and a positive shift of reversal current upon switching to low chloride. The Δ259–M265V CFTR mutant has its next initiation methionine at amino acid M281. However, this nonfunctional mutant fails to generate Cl^- currents in *Xenopus* oocytes.

This suggests that Δ259CFTR includes the smallest amount of TMD1 that can form a functional chloride channel and exhibit near wild type characteristics. The single-channel conductance of Δ259CFTR shown in Table II was less than that of wild type CFTR while the selectivity of wt CFTR was retained ($Br^->Cl^-$). Thus, removal of a large portion of TMD1 including segments M1–M4 does not alter the ability of CFTR to conduct Cl^-. Further removal of portions of CFTR beyond M4 produces a nonfunctional Cl^- because as demonstrated by Tabcharani *et al.* (1992) critical components of the channel pore are located in M5 and M6. Because deletions of segments M1 to M4 reduce the single-channel conductance of CFTR only modestly (the maximum effect is a 30% reduction when all four domains are removed) and do not affect ion selectivity, it is clear that although segments M1–M4 may line distant portions of the pore, they do not participate in establishing CFTR's ion selectivity.

The mutant R117H, where a change in amino acid occurs in the first putative exofacial loop of CFTR, and all of the truncation mutants when activated by PKA and ATP have reduced open probabilities compared to wild type. This suggests that portions of segments M1 to M4 may play a minor role in channel gating but clearly are also not critical for activation of CFTR by PKA and ATP. One likely explanation for an effect on channel gating is that when residues in segment M1 to M4 are either mutated or deleted there are allosteric effects on another domain of CFTR. This would explain why a mutation in an exofacial residue such as in the R117H mutation could affect processes of gating normally associated with intracellular domains.

Table II. Single-Channel Characteristics of CFTR Deletion Mutant Channels in Oocytes

	pS	P_0
WT CFTR	9.3±0.4	0.65±0.02
pSA306	7.8±0.4	0.48±0.08
Δ-S118M	8.7±0.8	0.51±0.06
Δ-259	6.7±0.3	0.40±0.08

The second set of transmembrane segments in TMD2 of CFTR has been far less investigated compared to those in TMD1. A large number (19 thus far) of missense mutations associated with CF are located in the fourth intracellular loop (ICL4) between segments M10 and M11 of CFTR (Ferec *et al.*, 1992; Fanen *et al.*, 1992; Mercier *et al.*, 1993; Ghanem *et al.*, 1994; Savov *et al.*, 1994). One residue in particular, R1066, is associated with four separate CF mutations. Cotten and Colleagues (Cotten *et al.*, 1996) constructed several of these mutants and expressed them in heterologous cells to study their effects on protein processing and function. Examination of the glycosylation states of these mutants revealed that all of them produced protein. However, the relative amounts of Band C (mature protein) varied widely among them. At the whole cell level, 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 also resembled that of wt-CFTR, suggesting that ICL4 does not contribute directly to the conduction pore. The mutants, however, did have varied influences on the open state probability and burst durations. The R1066C mutant displayed longer closed times between bursts of activity whereas the R1066H mutant increased the P_0. A mutation in the adjacent residue A1067T caused a decrease in the P_0 with a corresponding decrease in the burst duration. Since these mutants produce effects similar to those seen with mutations in the NBDs (Kerem *et al.*, 1990; Anderson *et al.*, 1991; Carson *et al.*, 1995), the authors speculate that ICL4 mutations might disrupt or modify some aspect of NBD-mediated gating of the Cl^- channel pore.

Siebert and colleagues (Siebert *et al.*, 1996) conducted similar experiments on the third intracellular loop of CFTR and concluded that this region may be involved in maintaining stability of the channel in the open state. McCarty *et al.* (McCarty *et al.*, 1993; McDonough *et al.*, 1994) studied the diphenylamine-2-carboxylic acid (DPC) binding properties of residues within the putative chloride permeation pathway of CFTR. Residues in CFTR, which when mutated exhibit altered DPC binding properties, may line the pore. Using site-directed mutagenesis and by measuring both the binding affinity for DPC (K_D) and the electrical distance of the DPC binding site within the pore (Θ), these investigators have shown that residues on both M6 and M12 may line the channel pore.

THE NUCLEOTIDE-BINDING DOMAINS

The CFTR chloride channel contains two predicted nucleotide-binding domains (NBD1 and NBD2). These contain the consensus sequences for Walker A and Walker B motifs (Walker *et al.*, 1982) and have sequence similarity to ATPases or ABC transporter family proteins such as MDR and Ste6 (Ames *et al.*, 1990; Hyde *et al.*, 1990). Members of this family utilize the energy released during ATP hydrolysis at the NBDs to actively transport substrate across the cell membrane. However, in the case of CFTR, ATP actually regulates the opening and closing of the channel via the NBDs (Carson *et al.*, 1995; Gregory *et al.*, 1991).

CFTR activation is a two-step process requiring both the phosphorylation by PKA and the binding of ATP to NBDs. A hypothesis regarding the nature of CFTR gating has emerged recently suggesting that the degree of phosphorylation of CFTR modulates an interaction between the two NBDs (Cheng *et al.*, 1991; Hwang *et al.*, 1994). ATP hydrolysis at NBD1 initiates a burst of activity associated with the opening of the channel, whereas hydrolysis at NBD2 terminates a burst. Using the patch clamp technique, Carson *et al.* (1995) demonstrated that CFTR variants which contained mutations in the conserved Walker A motif of either NBD1 (K464A) or NBD2 (K1250M and K1250A) decreased the open probability of the channel compared to wt-CFTR. Mutations in NBD1 alone decreased the open probability whereas mutations at NBD2 or simultaneously at both the NBDs (K464A/K1250A) prolonged the frequency of bursts of activity. These data point out convincingly that the two NBD's cooperate to control channel gating.

Besides their role in regulating CFTR's chloride channel function, the NBD's may also play a role in enabling CFTR to interact with other proteins such as the outwardly-rectifying chloride channel. In cells from CF patients, several laboratories showed that PKA regulation of ORCCs is defective when compared to normal epithelia (Hwang *et al.*, 1989). Complementation of CF airway epithelial cells with the wild-type CF gene corrects PKA regulation of ORCCs (Egan *et al.*, 1992; Gabriel *et al.*, 1993; Schwiebert *et al.*, 1994a). The biophysical properties of ORCCs include a large conductance (30–70 pS), a rectifying *I–V* relationship, sensitivity to DIDS, activation with depolarizing voltages, and a halide permselectivity of $I^- > Cl^-$ different completely from CFTR channel properties (Hwang *et al.*, 1989; Egan *et al.*, 1992; Gabriel *et al.*, 1993; Schwiebert *et al.*, 1994a). There is evidence that CFTR when activated by cAMP triggers the transport and release of the potent agonist, ATP, which then activates ORCCs via a purinergic receptor (Schwiebert *et al.*, 1995). Although the role of CFTR in ATP release is not well understood, there is some preliminary but compelling data that NBD1 plays a role. For example, two mutations, A455E and G551D, are associated with milder and more severe forms of CF, respectively. When these two mutations are studied in airway cells, both could still conduct Cl^-, however, only CFTR bearing the A455E mutation retained the function of regulating ORCC. These results indicate that CF mutations can affect CFTR functions differently and suggest that severity of pulmonary disease may be more closely associated with the regulatory function rather than the Cl^- function of CFTR. It also suggests that certain mutations in the NBD1 domain affect the ability of CFTR to facilitate the release of ATP. This intriguing result is a preliminary hint that the NBD1 domain may play an important role in allowing CFTR to regulate other processes.

THE R DOMAIN

Research from several laboratories has shown that CFTR is regulated via phosphorylation of its regulatory domain (Tabcharani *et al.*, 1991; Berger *et al.*, 1993; Cheng *et al.*, 1991; Picciotto *et al.*, 1992). The R domain contains 8 of the 10 classic sites for PKA-mediated phosphorylation (Riordan *et al.*, 1989). Eight serines and one threonine are located within the R domain, and one serine is located just prior to NBD1. Site-directed mutagenesis of each of these serines individually to alanine and phosphopeptide mapping (Picciotto *et al.*, 1992) revealed some interesting results. *In vitro* studies show that only the 7 serine residues located within the R domain were phosphorylated. *In vivo,* however, only serines 660, 737, 795, and 813 were phosphorylated upon cAMP stimulation. Mutating any one of these four serines individually did not affect chloride channel activity (Cheng *et al.*, 1991). Using the SPQ fluorescence assay and whole cell recordings it was shown that mutating all four serines simultaneously, reduced but did not eliminate chloride channel activity. These data suggest that other phosphorylation sites could initiate a small amount of channel activity upon phosphorylation.

Rich and colleagues (Rich *et al.*, 1991) expressed CFTR lacking the R domain (CFTRΔR) in HeLa cells.

Whole cell and SPQ fluorescence showed that this mutant generated constitutive currents, which were further increased upon cAMP stimulation. This particular construct, however, did retain the serine at position 660. The same investigators (Cheng *et al.*, 1991) later showed that mutation of this serine to alanine in the same construct (CFTRΔRS660A) generated constitutive currents which did not increase further upon cAMP stimulation. These data suggest that the R domain functions to keep the channel closed while in the nonphosphorylated or inactive state. Phosphorylation of the R domain may cause a conformational change associated with the opening of the channel.

THE HALF MOLECULE—TNR-CFTR

In addition to wild-type CFTR mRNA, an alternately spliced form containing only the first transmembrane domain, the first nucleotide binding domain, and the regulatory domain (TNR-CFTR) is expressed in kidney (Morales *et al.*, 1996). Although missing the second set of transmembrane domains and the second nucleotide binding domain, when expressed in Xenopus oocytes, TNR-CFTR has cAMP- and PKA-stimulated single Cl^- channel characteristics and regulation of PKA activation of outwardly rectifying Cl^- channels that are very similar to those of wild-type CFTR (see Table III). TNR-CFTR mRNA is produced by an unusual mRNA processing mechanism and is expressed in a tissue-specific manner primarily in renal medulla.

In both *Xenopus* oocytes and in human airway cells, the TNR form of CFTR has single-channel properties surprisingly similar to wild type but its overall processing and plasma membrane expression in *Xenopus* oocytes and in mammalian epithelial cells is less efficient. Similar results were published by Sheppard *et al.* (1994) who created an artificial mutant CFTR missing TMD2 and NBD2 but containing the R domain (D836X). This mutant formed a regulated Cl^- channel probably by dimerization.

Table III. Single-Channel Characteristics of CFTR and TNR-CFTR Channels in Oocytes

	pS	P_0	n
WT CFTR	9.3±0.4	0.65±0.08	3
TNR-CFTR	9.5±0.8	0.65±0.20	3

Surprisingly, we observed that TNR-CFTR not only functions as a cAMP-stimulated Cl^- channel, but also corrects defective regulation of ORCCs when transfected into IB3-1 CF airway epithelial cells. These results also suggest that the components of CFTR necessary to perform regulatory interaction with ORCCs are contained within TNR-CFTR. Since mRNA for TNR-CFTR was detected in trachea and lung of rat, it is interesting to speculate that TNR-CFTR may also play a role in cAMP-stimulated Cl^- transport in the airway.

What is the functional significance of this variant? Wild-type CFTR, STE6, and MDR (Ames *et al.*, 1992; Higgins *et al.*, 1986) are present in the plasma membrane with two sets of TMDs and NBDs. However, several related members of the transport ATPase family including PMP70 (Valle *et al.*, 1993), TAP (Deversen *et al.*, 1990), and ALDP (Moser *et al.*, 1993) function as half molecules with the TMD1-NBD1 motif. One characteristic of these half molecules is that they each function in intracellular organelles. An intriguing possibility is that TNR CFTR may have a primary function in intracellular organelles rather than in the plasma membrane. This could explain its less efficient expression in generating the similar magnitude of whole cell currents in *Xenopus* oocytes and in mammalian epithelial cells.

CONCLUSIONS

After the gene was cloned, research on CFTR focused on the structure and function of the predicted protein. The complex multi-domain structure of CFTR suggested more than one function for the protein and that several domains may be involved in regulating the different functions of the protein, both independently and via domain–domain interactions. Extensive mutagenesis and structure–function studies have demonstrated that the two transmembrane domains line the channel pore. Exactly which transmembrane segments line the water-accessible surface of the protein is yet to be determined although current evidence suggests that M5, M6, and M12 do line the pore. Data from several studies suggest that the two nucleotide-binding domains are involved in mediating the opening and closing of the channel pore via ATP binding and hydrolysis and that the regulatory R domain controls channel opening via its phosphorylation by PKA. The studies also suggest that the R domain keeps the channel closed in the dephosphorylated or inactive state possibly by

an interaction with the second nucleotide-binding domain. A domain–domain interaction between the R domain and the second nucleotide-binding domain could cause a conformational change accompanied by the closing of the channel. Upon phosphorylation by PKA the R domain probably undergoes a change in conformation, which could involve an interaction with the first nucleotide-binding domain, accompanied by the opening of the channel. The molecular dynamics of channel opening, closing, and gating may thus be governed by the domain–domain interactions between the three putative cytoplasmic domains of the protein as well as the highly conserved intracellular loops The challenge will be to determine the precise nature of the interactions between these domains which regulate the functions of the protein.

As reviewed above, CFTR regulates ORCC's and sodium channels and may also be involved in the transport of ATP. Thus, CFTR is both a Cl^- channel and conductance regulator. CF mutations affect CFTR function differently with varied effects on the conduction and conductance regulatory properties of CFTR. Mutations in the TMDs seem to affect chloride channel activity, whereas mutations in the NBDs affect both channel and regulatory functions. The fact that TNR-CFTR retains all of the functions of the wild type protein and is also expressed in the renal medulla suggests that components of CFTR necessary to perform its conductance-regulatory interactions may be contained within the first three domains.

The study of ion-channel interactions at the plasma membrane is currently under intense investigation for several different ion channels. There is growing evidence that proteins with PDZ domains do interact with other ion channels (Sheng, 1996). Expression of proteins containing these domains causes 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 NMDA receptors at the plasma membrane (Kim *et al.*, 1995,1996). 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 NMDA receptor. These studies could provide some insight into the mechanism of channel–channel interactions involved with CFTR. The mechanism whereby CFTR functions as a regulator will need to be investigated further because several key questions remain unanswered. Including: Does CFTR conduct ATP or does it mediate the transport of ATP? How does CFTR interact with ORCCs and sodium channels (Fig. 3). Answers to these questions may provide more insights into how to activate an alternate chloride transport pathway for patients with cystic fibrosis.

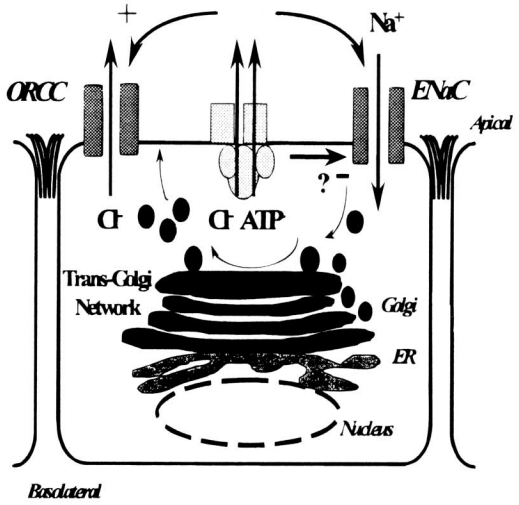

Fig. 3. Putative model of CFTR interactions with other channels. The model illustrates the interaction between CFTR and the outwardly rectifying chloride channel (ORCC) and the epithelial sodium channel (ENaC). The agonist mediating the interaction could be ATP. The arrow through CFTR indicates the flow of chloride ions through CFTR.

ACKNOWLEDGMENTS

This work was supported by the Cystic Fibrosis Foundation Research Development Program and National Institutes of Health Grants Nos. HL 51811, HL 47122, and DK 48977 to WBG.

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