Stimulatory and Inhibitory Functions of the R Domain on CFTR Chloride Channel

Jianjie Ma

**CFTR** is a chloride channel whose gating process involves coordinated interactions among the regulatory (R) domain and the nucleotide-binding folds (NBFS). Protein kinase A phosphorylation of serine residues renders the R domain from inhibitory to stimulatory and enables ATP binding and hydrolysis at the NBFS, which in turn control opening and closing of the chloride channel.

In 1989, the gene responsible for cystic fibrosis was isolated, and the protein product of this gene was named cystic fibrosis transmembrane conductance regulator (CFTR; Ref. 12). The amino acid sequence of CFTR was used to predict a structure, which consists of two sets of six membrane-spanning domains (MSDs), two nucleotide-binding folds (NBFS), and an intracellular regulatory (R) domain (Fig. 1A). This structure is similar to that of the ATP-binding cassette (ABC) family of transporters, but the R domain is unique to CFTR. Research during the last decade has identified CFTR as a multifunctional protein, which provides the pore of a linear conductance chloride channel and also functions to regulate other membrane proteins. Maturations in CFTR leading to defective regulation or transport of chloride ions across the apical surface of epithelial cells are the primary cause of the genetic disease cystic fibrosis (15).

As a chloride channel, CFTR is regulated by two cytosolic pathways. For the channel to open, the protein must first be phosphorylated by a cAMP-dependent protein kinase (PKA), and then intracellular ATP must bind to the NBFS and subsequently be hydrolyzed. The use of compounds that alter the ATP hydrolysis cycle of CFTR, such as AMP-PNP, pyrophosphate, and vandate, provides evidence that hydrolysis of ATP is required for both channel opening and closing transitions. Structure-function studies have suggested that ATP hydrolysis at NB1 is responsible for opening of the chloride channel and that ATP hydrolysis at NB2 terminates a burst of open events (5). Since cells normally contain high intracellular levels of ATP, regulation in the intact cell of CFTR is by phosphorylation. The R domain contains the consensus phosphorylation sites for PKA that are the basis for physiological regulation of channel opening (12).

**Patch-clamp technique vs. lipid bilayer reconstitution of CFTR channel**

The CFTR chloride channels are located in the apical membrane of epithelial cells. To study the function and regulation of the CFTR channel, two electrophysiological methods are commonly used: the patch-clamp technique and lipid bilayer reconstitution. These studies are carried out with either the primary or immortal cultures of epithelial cells expressing the endogenous CFTR proteins or the heterologous cell lines that either stably or transiently express CFTR.
the recombinant CFTR proteins. With both methods, a small current (~10^{-12} A) reflecting the movement of chloride ions through an aqueous pore formed by the CFTR molecule is measured under a given electrochemical potential, and the channel opening and closing transitions are monitored at various physiological conditions (i.e., PKA phosphorylation, ATP binding and hydrolysis, and so forth). Compared with the patch-clamp technique, the bilayer reconstitution system has several advantages. First, the lipid bilayer is formed across two aqueous compartments of milliliter-size dimensions. With this system, it is easy to change the ionic composition of both intracellular and extracellular solutions and to add modulators to either side of the CFTR channel (Ref. 8; see also Figs. 3 and 4). Second, reconstitution methods are easily implemented. Bear et al. (2) used lipid bilayer reconstitution to demonstrate conclusively that the purified CFTR protein is capable of functioning as a PKA- and ATP-dependent chloride channel. The reconstitution system also allows the study of CFTR proteins that are synthesized and retained in the endoplasmic reticulum membranes, i.e., processing mutants of CFTR such as ΔF508 and N1303K (15). These misprocessed CFTR channels are inaccessible to the patch-clamp electrode. Third, the system allows control of membrane phospholipid composition. For example, various surface charge densities and different thicknesses of the bilayer membrane can be achieved by changing the lipid compositions, which can be used to study the effect of membrane structure on the gating and conduction properties of the CFTR channel. The bilayer reconstitution system, however, has one major disadvantage compared with the patch-clamp technique, which is the excess amount of electrical noise associated with the large capacitance of the bilayer membrane. Thus, to enhance the signal-to-noise ratio, one would need to remove the high-frequency noises by using a low-pass cutoff filter. This does not seem to be a problem, since the CFTR channel has a characteristic slow open-close gating process (2, 5, 8).

Multiple phosphorylation sites in the R domain

Ten consensus phosphorylation sites for PKA and two for protein kinase C (PKC) are identified in the R domain, and only six of these sites seem to be heavily used in the intact CFTR molecule (Fig. 1B). These sites appear to be redundant and to have additive effects on CFTR function, since mutation of any one of them, or up to three of them, does not affect the maximal activation of the CFTR channel (11). But mutation of more than four major sites reduces the activation by PKA and the open probability of the channel (3). Phosphorylation apparently increases channel openings by adding negative charges, because the substitution of negatively charged amino acids like aspartate for the serines at the consensus sites results in an open channel without phosphorylation, provided that more than six residues are so substituted (11). On the other hand, not all phosphorylation sites are equivalent. Some are inhibitory, whereas others are stimulatory (13). S737 and S768 appear to be inhibitory sites for the channel because mutations S737A and S768A increase the basal activity of the CFTR channel. Even CFTR with all 10 consensus phosphorylation sites for PKA mutated maintains residual PKA-dependent openings (3), suggesting that other cryptic phosphorylation sites (either inside or outside the R domain) also contribute to the overall function of the CFTR channel.

Although there are two PKC consensus sites in the R domain (S686 and S790) and phosphorylation of these sites occurs in vivo and in vitro, PKC stimulation alone does not activate the CFTR channel. Prephosphorylation by PKC seems to be essential for the PKA-dependent activation of the CFTR channel since it potentiates the effect of PKA, but once CFTR has been fully activated by PKA channel activity is not increased by application of PKC (7).

Effects of deleting the R domain on CFTR function

Sequence alignment of CFTR with P-glycoprotein and other members of the ABC transporter family reveals that CFTR contains an extra 128 amino acids in the R domain (residues 708–835; Fig. 1C). Deletion of this portion of the R domain from CFTR, ΔR(708–835), removes the requirement for PKA phosphorylation to open the CFTR channel (10). Unlike the wild-type (wt) CFTR, which opens in a strictly PKA-dependent manner, the ΔR(708–835) channel opens without PKA phosphorylation, and, furthermore, its open probability does not change with PKA phosphorylation (Fig. 2A). The ΔR(708–835)
channel also exhibits altered response to AMP-PNP and pyrophosphate, compounds that prolong burst duration of the wt CFTR channel but fail to do so in the DR(708–835) channel (9). On the basis of these studies, it has been proposed that this portion of the CFTR molecule contains the putative gating particle of the chloride channel (10).

Compared with the wt CFTR, open probability of the DR(708–835) channel is significantly lower (Fig. 2B). This suggests that some stimulatory property of the R domain has been lost in the DR(708–835) mutant or that removal of these 128 amino acids from the R domain introduces structural changes that could affect the function of the NBFs.

### Interaction of exogenous R domain peptide with the CFTR channel

Further insights into the function of the R domain in the CFTR channel are obtained through reconstitution studies of the interaction of the exogenous R domain protein with a single CFTR channel captured in the lipid bilayer or in excised membrane patches. The R domain peptide (amino acids 590–858) interacts specifically with the wt CFTR channel in a phosphorylation-dependent manner (8). When applied to the intracellular side of the channel, the unphosphorylated R domain peptide inhibits opening of the wt CFTR channel, but once the R domain peptide is phosphorylated, the inhibitory effect is relieved and the channel reopens (Fig. 3A). Interestingly, addition of the unphosphorylated R domain peptide has no effect on the DR(708–835) channel. However, when the exogenous R domain protein is phosphorylated, significant stimulation of the DR(708–835) channel occurs (Ref. 9 and 14; Fig. 3B). The results indicate that the R domain does not function solely as an inhibitor that keeps the channel closed, so it is not simply an “on-off” switch for the channel. The function of the R domain differs mechanistically from the “ball-and-chain” model for the Shaker potassium channel, in which the amino-terminal “ball” is thought to physically obstruct the ion conduction pathway for potassium ions (6).
The data suggest the following putative model for CFTR function. CFTR forms a chloride channel in which parts of the transmembrane domains constitute the pore and the R domain functions as a channel inhibitor until it is phosphorylated by PKA and undergoes a conformational change to make the pore accessible to chloride ions. Phosphorylation of the R domain has two effects on CFTR: the first could be permissive, releasing a steric hindrance on the channel; the second might be stimulatory, facilitating interaction of ATP with the NBFs. Once the R domain is phosphorylated, binding and hydrolysis of ATP take place at NBF1, which leads to opening of the chloride channel. Subsequent binding and hydrolysis of ATP at NBF2 closes the channel. The closed state of the channel can be secured by dephosphorylation of the R domain.

Stimulatory and inhibitory functions of a short segment of the R domain

The R domain of CFTR contains two negatively charged regions, amino acids 725–733 (NEG1) and amino acids 817–838 (NEG2), that reside in close proximity to two PKA phosphorylation sites, S737 and S813, used in vivo (Fig. 1D). Two cystic fibrosis-associated mutations have been identified within the NEG2 region that result in the removal of negative charges, E822K and D836Y. The presence of these disease-causing mutations suggests the potential importance of the NEG2 region in CFTR function. Deletion of NEG1 from the R domain has no significant impact on the CFTR channel in terms of ion permeation and PKA-dependent gating. But deletion of NEG2 from the R domain produces a functional ΔNEG2-CFTR channel that opens without PKA, with open probability ~1/5 that of the wt CFTR (Fig. 4A). Moreover, addition of PKA up to 200 U/ml, four times the concentration required to fully activate the wt CFTR channel (8), does not increase the open probability of the ΔNEG2-CFTR channel (1). Thus removal of NEG2 from CFTR completely eliminates the PKA dependence of the chloride channel, although the ΔNEG2-CFTR still contains all 10 PKA phosphorylation sites. The synthetic 22-amino acid NEG2 peptide interacts with the CFTR molecule and exhibits both stimulatory and inhibitory effects on CFTR function (Fig. 4B). Additionally, covalent modification of a cystine residue at position 832, which resides within NEG2, by N-ethylmaleimide results in irreversible stimulation of PKA-phosphorylated CFTR channel activity, further emphasizing the importance of NEG2 in CFTR function (4).

These data show that the NEG2 region could confer both stimulatory and inhibitory functions of the R domain on the CFTR channel. When this region is deleted from CFTR, the resultant channel opens without PKA (loss of inhibitory function), but it never achieves open probability comparable with wt CFTR even when phosphorylated with PKA (loss of stimulatory function). This same NEG2 sequence, expressed as a peptide, results in stimulation of channel openings at lower concentrations and profound inhibition of channel activity at higher concentrations when added to the intracellular side of the CFTR channel. It seems likely that this sequence could interact with CFTR at different sites on the NBFs to either stimulate or inhibit channel openings.

Conclusion

The fact that the R domain has both stimulatory and inhibitory roles in CFTR channel function has important implications for the pharmacological and gene-therapeutic interventions of cystic fibrosis. First of all, understanding how the R domain works in the CFTR channel, i.e., by identifying the stimulatory interaction of NEG2, may facilitate the design of therapeutic reagents that stimulate CFTR opening to treat patients whose mutant forms of CFTR reach the cell surface. Furthermore, because gene therapy for cystic fibrosis has been plagued by inefficient delivery of the gene and inefficient expression of CFTR once the gene has been delivered, it would be desirable to have a form of CFTR that retains the absolute...
requirement for PKA activation (i.e., is regulated normally) and has increased open probability when fully activated. Since the conformation of the R domain is an important determinant of whether it functions in a stimulatory or inhibitory mode, and if the conformation of the R domain can be changed to enhance its activator function in the phosphorylated state without altering its inhibitory activity in the unphosphorylated state, the open probability of the phosphorylated channel might be increased while the channel remains under strict control of ATP and PKA. The wt CFTR channel, when fully activated, has an average open probability of ~0.30 (Fig. 2B). Therefore, there is potential to improve the overall function of CFTR to achieve a better vehicle for gene therapy of cystic fibrosis, either by mutating the R domain or the NBFs or by improving the intramolecular interactions among the three hydrophilic domains of CFTR: NBF1, R, and NBF2.

*This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-51770 and an Established Investigatorship from the American Heart Association to J. Ma.*

### References


