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MULTI-AUTHOR REVIEW

# The gating of the CFTR channel

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**Abstract** Cystic fibrosis transmembrane conductance regulator (CFTR) is an anion channel expressed in the apical membrane of epithelia. Mutations in the CFTR gene are the cause of cystsic fibrosis. CFTR is the only ABC-protein that constitutes an ion channel pore forming subunit. CFTR gating is regulated in complex manner as phosphorylation is mandatory for channel activity and gating is directly regulated by binding of ATP to specific intracellular sites on the CFTR protein. This review covers our current understanding on the gating mechanism in CFTR and illustrates the relevance of alteration of these mechanisms in the onset of cystic fibrosis.

**Keywords** ATP binding and hydrolysis · Phosphorylation · Gating · Cystic fibrosis transmembrane conductance regulator (CFTR) · Nucleotide-binding domain

## Introduction

Cystic fibrosis transmembrane conductance regulator (CFTR) is a integral membrane protein encoded by the *CFTR* gene, which express in vertebrates [1]. CFTR belongs to the ATP-binding cassette (ABC) proteins (subfamily C, member 7; ABCC7), and conserves the general architecture of the ABCC sub-family (Fig. 1). This architecture involves four domains: two membrane spanning domains (MSD1 and MSD2) and two nucleotide binding domain (NBD1 and NBD2). Each MSD is by 6 transmembrane helices (TM1–TM6 and TM7–TM12). Every membrane spanning domain followed by a nucleotide binding domain. A distinctive characteristic of CFTR is the presence of an intrinsically disordered region, the regulatory domain (RD), located between NBD1 and MSD2.

In most ABC-proteins the ATPase activity of the NBDs utilize the energy of ATP binding and hydrolysis to energize the translocation of various substrates across membranes. Conversely, CFTR is a unique case of an ABC-proteins that forms an ATP-gated ion channel. CFTR is expressed in a varierty of organs and tissues including gut smooth muscle, tubular kidney cells and mucosal and secretory epithelia. The physiological role of CFTR in airways epithelial cells has been especially well characterised; CFTR conducts anions, mainly chloride and bicarbonate, across epithelial cells apical membrane. Mutations of the *CFTR* gene affecting chloride ion channel function lead to dysregulation of epithelial fluid and salt transport in the lung, pancreas and other organs, resulting in cystic fibrosis (CF).

Several techniques designed to evaluate ion transport in cells have provided insights into functional properties of CFTR [2–5]. The patch-clamp technique [6] remains the golden standard for the analysis of the molecular behaviour of the CFTR anion–channel. Measurements of the ion currents on cells heterologously expressing the wild type CFTR, and the study of the functional effects of disease-causing mutations has provided major insights into the relationships between the structure and function of the CFTR. In turn, this knowledge has our understanding of CF physiopathology, and has open new avenues for therapy, including pharmacological.

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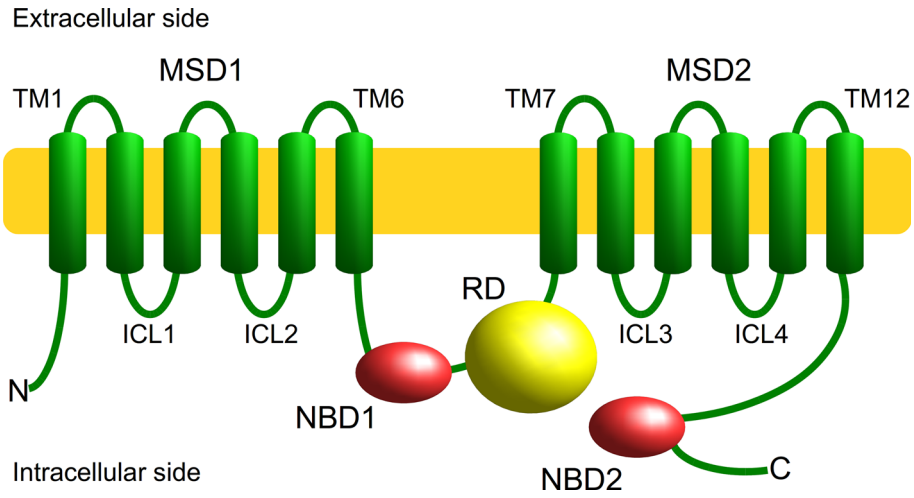


**Fig. 1** Topology of the CFTR in the plasma membrane. *MSD1* and *MSD2*, shown in *green*, are the two membrane spanning domains composed by six transmembrane (TM) domains each. The four intracellular loops, *ICL1* to *ICL4*, are indicated. The nucleotide binding domains, *NBD1* and *NBD2*, are represented in *red*, and the regulatory domain, *RD* is depicted in *yellow*

## Activation of the CFTR

Activation of CFTR requires phosphorylation by protein kinase A (PKA) at multiple sites located at the regulatory domain (RD) [7, 8]. Because channel phosphorylation by PKA is mandatory for channel activity, CFTR channel is frequently defined as a "cAMP-activated channel". Protein kinase-C (PKC) dependent phosphorylation at multiple sites is necessary for complete PKA-dependent activation of CFTR [9, 10].

The gating of the phosphorylated CFTR channels is successively promoted by the binding of ATP at the NBDs [8, 11]. In intact cells, intracellular concentration of ATP ($\sim$2 mM) is high enough to ensure that most CFTR remain activated. During inside out of whole-cell patch-clamp recordings, it has been possible to study the relationship between intracellular ATP conecnatrtion and CFRT channel activity.

Phosphorylated CFTR channels show a single-channel activity that typically display bursts of openings, which lasts for hundreds of milliseconds. Duration and frequency of the bursts are strictly dependent on intracellular ATP concentration. In contrast to many other ion channel types, CFTR open probability is only moderately affected by the membrane potential [12]. Menbrane potantila depolarization slightly decreases both the duration of bursts and the interburst interval, while hyper polarization produce the opposite effects [12]. Within the physiological range of membrane potentials ($\sim -60$ to 0 mV) in epithelial cells the changes in channel kinetics and open probability are very small.

## Phosphorylation

The phosphorylation of CFTR in intact cells is obtained increasing the cytoplasmic concentration of cAMP, that in turn activates the endogenous PKA. In the intact epithelium in vivo, the production of cAMP is triggered by the activation of adenylate cyclase by a G-protein linked hormone pathway, such as glucagon, epinephrine or a beta-adrenergic agonist like isoproterenol, acetylcholine, the vasoactive intestine peptide (VIP) [13] or adenosine [14]. However, the intracellular cAMP concentration obtained by hormonal stimulation might be well below the levels necessary to achieve the maximal activation of CFTR. In ex vivo experiments, maximal CFTR activation can be obtained by pharmacological activation of adenylate cyclase with forskolin, or using permeant cAMP analogues, such as CPT-cAMP or 8-Br-cAMP [4]. Differently, when the intracellular side of CFTR is accessible, such as during inside-out patch clamp experiments, phosphorylation of CFTR can be obtained by direct application of the catalytic subunit of PKA.

The regulatory domain (RD) of CFTR is about 200 residues long, with 18 potentially phosphorylation sites (12 serines and 8 threonines). The sequence is highly conserved in mammals (>85 % identity), but less so in other vertebrates (from 60 to 35 % identity) [15]. Also the two putative phosphorylable serines, 660 and 670, located in the regulatory extension (RE) of the NBD1 (residues 638–670) have been considered as candidates for the CFTR regulation. Deletion of the RD, from amino acids 708–835, led to PKA-independent activity, indicating that this region of the R-domain encodes residues necessary for PKA-dependent activation [16–20]. In a fully phosphorylated protein, eight phosphoserines (residue positions 660, 700, 712, 737, 753, 768, 795 and 813) have been detected by mass spectrometry [21, 22] and NMR [23], and partial phosphorylation (approximately 60 %) of serine at position 670 [23].

Early models proposed that the unphosphorylated RD serves as an inhibitory particle that occludes the pore, much like the inhibitory 'ball' in Shaker potassium channels [16–18]. Nonetheless, in contrast to predictions,

attempts to add an unphosphorylated RD peptide to a CFTR in which the RD domain had been deleted and serine 660 was substituted by alanine did not inhibit activity, whereas a phosphorylated RD peptide stimulated activity increasing the rate of channel opening [24]. It follow the notion that phosphorylation of RD does favour the CFTR channel activity. In fact, the maximal channel opening rates were the same for wild type channels and split CFTR with no RD. Thus, it seems that the phosphorylated RD does not stimulate opening of CFTR channels; rather, the dephosphorylated RD inhibits them [25].

There is a plethora of reported experiments substituting putative phosphorylation sites in RD, but the delineated picture of the role of each phosphoserine in the modulation of CFTR activity is quite complex. When serine at position 660, 737, 795, and 813, were substituted by alanine, CFTR that can still be phosphorylated by PKA to yield an activated chloride channel [17]. The substitution of serine 660 and 670 by alanine in the regulatory extension of the NBD1 have shown that phosphorylation of these serine residues do not have influence on the CFTR activity [26]. Substitution of serine at position 700, 795 and 813 decreased the channel open probability [17, 27, 28], whereas mutations at position 737 and 768 increased the channel open probability [28]. In general, when serine of RD is mutates in alanine, the resulting differences in channel open probability are due to differences in mean closed time. When serine at position 737, 795, and 813, as well as all of the other phosphorylation consensus sites were substituted (serine 686, 700, 712, 768, and threonine 788) activity was further reduced [27, 28].

Substitution of aspartate for consensus PKA phosphorylation sites in the R domain mimicked the effect of phosphorylation: mutants containing six or more serine-to-aspartate substitutions generated chloride channels that are active in the absence of PKA phosphorylation [17]. Thus, the effect of phosphorylation on the RD is correlated to the negative charges introduced by the phosphate group in phosphoserine. Circular dichroism, X-ray scattering and NMR experiments have demonstrated that phosphorylation induces a conformational change on the RD [23, 29–32].

A mutant for of the negative segment of RD (amino acids 817–838) in which the phosphorylation site was removed, completely eliminates the PKA dependence of channel activity. This observation demonstrated that this region is crucial in regulating CFTR activity, perhaps providing the structural link between RD phosphorylation and regulation of the channel [33]. On the other hand, phosphorylation of the RD increases the rate of channel opening by enhancing the sensitivity to ATP [24], indicating that phosphorylation of one domain stimulates the interaction of ATP with another domain, thereby increasing activity.

To investigate how phosphorylation controls activity, the single channel properties expressed by constructs with RD mutations either high or low activity values were measured as a function of the ATP concentration in excised patches. With 1 mM ATP, channel open probability was similar to that observed in cell-attached patches, but with 10 mM ATP, all constructs tested showed elevated channel open probability values. ATP-dependent increases in channel open probability were due to reductions in mean closed time [28]. Split channels with no RD are highly active without phosphorylation, with higher apparent ATP affinity, and less tight binding of 5′-adenylyl-beta,gamma-imidodiphosphate (AMP-PNP), than for WT [25]. However, the action of AMP-PNP is restricted to highly phosphorylated CFTR channels, which, in the presence of ATP, display a relatively high open probability, but is not seen in partially phosphorylated CFTR channels, which have a low open probability in the presence of ATP [34]. These results indicate that incremental phosphorylation of RD phosphorylation regulates the interactions between nucleotides and the two nucleotide binding domains of CFTR and not the subsequent steps of hydrolysis and channel opening [28, 34]. A model for the control of the CFTR activation proposes that RD phosphorylation, in a site-dependent manner, alters equilibrium between forms of CFTR with low and high affinities for ATP [28].

## ATP-dependent gating cycle

The most accepted molecular model of CFTR gating proposes that binding of ATP promotes the “dimerisation” of the NBDs, leading to a conformational change at the level of the MSDs, that in turn leads to channel opening [35–38]. The hydrolysis of ATP by the enzymatic activity of the NBDs terminates the activity cycle, releasing ADP. Interestingly, this activity cycle is not reversible (no ATP can be synthesised from ADP). Another key features is that the energy liberated by the hydrolysis of ATP is not used to transport chloride [38, 39] as illustrated by the fact that CFTR channels can be gated open by non-hydrolysable ATP analogues, such as AMP-PNP [11, 40].

The NBDs of ABC proteins are known to bind and hydrolyse ATP. The primary structure of the NBDs is well conserved in all ABC-protein subfamilies [41, 42]. In CFTR, NBD1 interacts with NBD2 in the mode of a labile nucleotide sandwich. This architecture is conserved in the NBDs of most ABC proteins (for review, see [43]). All the NBD structures reveal the same basic “tail- to-head” fold, with highly conserved sequence motifs positioned to interact with bound ATP. There are several evidences of the ATP-driven dimerization of NBDs. Small-angle X-ray scattering experiments show that the interaction of

recombinant NBD1 and NBD2 is modified by the presence of ATP, acquiring a tighter conformation, compatible with the "tail- to-head" fold [44, 45]. Sulfhydryl-specific cross-linking has been used to directly examine the cysteines' proximity, demonstrating that CFTR NBD1 and NBD2 interact in a head-to-tail configuration analogous to that in homodimeric crystal structures of nucleotide-bound prokaryotic NBDs [46]. Interestingly, phosphorylation of split CFTR by PKA strongly promoted both cross-linking and opening of channels, firmly bound together head-to-tail NBD1-NBD2 association to channel opening [46].

The molecular structure of both, recombinant NBD1 and NBD2 of CFTR, has been solved by X-ray crystallography [47–49]. This peculiar head-to-tail conformation of the CFTR–NBD dimer confers two nucleotide binding sites at the dimer interface, but only one consensus catalytic site. In the 'NBD2 composite' site, NBD2 contributes its nucleotide-binding residues of the Walker-A and Walker-B motifs and the H-loop, and NBD1 contributes its LSGGQ signature motif. The other interfacial site, the 'NBD1 composite' site, includes the residues from NBD1 Walker B and switch motifs, as well as from the NBD2 signature sequence [15].

The gating of CFTR requires the binding of ATP at both NBD composite sites [50]. Nucleotide-photolabeling experiments were designed on CFTR channels to distinguish binding of ATP at NBD1 and NBD2 composite sites [51, 52]. These experiments showed that the two domains appear to act independently in the binding and hydrolysis of ATP. The non-hydrolysed nucleotide triphosphate remained tightly bound at the NBD1 composite site for many minutes and slowly hydrolysed [51, 53]. In contrast, at the NBD2 composite site, ATP is hydrolysed as rapidly as it is bound and the nucleotide diphosphate hydrolysis product dissociates immediately. As the open and close gating cycle, including hydrolysis, occurred in a tenfold faster time scale than binding in the NBD1 composite site, CFTR channel opening and closing is determined preponderantly by the binding of nucleotides at NBD2 composite site. Vanadate, a blocker that interrupts ATP hydrolysis, has a very small effect on hydrolysis at the NBD1 composite site [51, 53], but a marked effect on ATP hydrolysis at the NBD2 composite site, and significantly delayed termination of channel open bursts [52]. Also NBD2 mutants that get rid of hydrolysis prolonged the burst duration, while mutations that reduce ATP binding prevent the long bursts [50].

Further evidences of the dimerization of the NBDs during the CFTR gating were obtained from double-mutant cycles [54]. A double-mutant cycle involves wild-type protein, two single mutants and the corresponding double mutant protein. If the change in free energy associated with a structural or functional property of the protein upon a double mutation differs from the sum of changes in free energy due to the single mutations, then the residues at the two positions are coupled. Such coupling reflects either direct or indirect interactions between these residues.

To validate the model in which only small conformational changes occur at the NBD1 composite site during a gating cycle, three pairs of amino acid residues putatively forming NBD1 composite site, one on each NBD, were analysed. The lack of coupling between these three pairs during gating is consistent with the hypothesis that the protein structure around NBD1 composite site does not undergo large conformational changes along CFTR gating cycle [55]. Differently, residues of NBD1 and NBD2 forming the NBD2 composite site, are independent of each other in closed channels, but become coupled as the channels open [36], confirming the conformational changes of NBDs interactions during the gating cycle.

A schematic representation of ATP-dependent gating cycle of phosphorylated CFTR channels is shown in Fig. 2a. As ATP binds the NBD1 composite site ($C_0 \rightarrow C_1$ transition), it remains tightly bound or occluded for several minutes, during which time many closed–open–closed gating cycles occur. Indeed, the opposite transition ($C_0 \leftarrow C_1$) is significantly slower. ATP binding at the NBD2 composite site ($C_1 \rightarrow C_2$ transition) determines the formation of the intramolecular NBD1–NBD2 tight heterodimer ($C_2 \rightarrow$ Burst transition—square brackets). In the burst state, there are two channel conformations in fast equilibrium, a state in which the channel pore has not yet opened, and a truly open state (Open). The relatively stable open (bursting) state becomes destabilised by hydrolysis of the ATP bound at the NBD2 composite catalytic site and loss of the hydrolysis product, inorganic phosphate (Pi) [38]. The consequence is the alteration of the dimer interface, leading to the channel closure and the loss of the remaining bound ADP (Open $\rightarrow C_1$ transition). Thus, when a single channel current is recorded in an inside-out patch of membrane with a phosphorylated CFTR channel (Fig. 2b), channel activity is observed shortly after perfusion with ATP. The channel activity consist on groups of fast channel opening and closures named bursts, corresponding to the CFTR states with two bound ATPs in the dimerised NBDs, intercalated with long closures, that result on the inactivity of the channel when the NBDs dimer is disrupted upon ATP hydrolysis.

Thermodynamic analysis of the CFTR gating cycle can elucidate molecular mechanisms by dissecting enthalpic and entropic contributions during transitions from one stable conformation to another. Analysis of temperature dependence of kinetic rates of the CFTR channel have shown that the opening of the channel was highly temperature dependent, while closing of the channel was only weakly temperature dependent [56]. However,

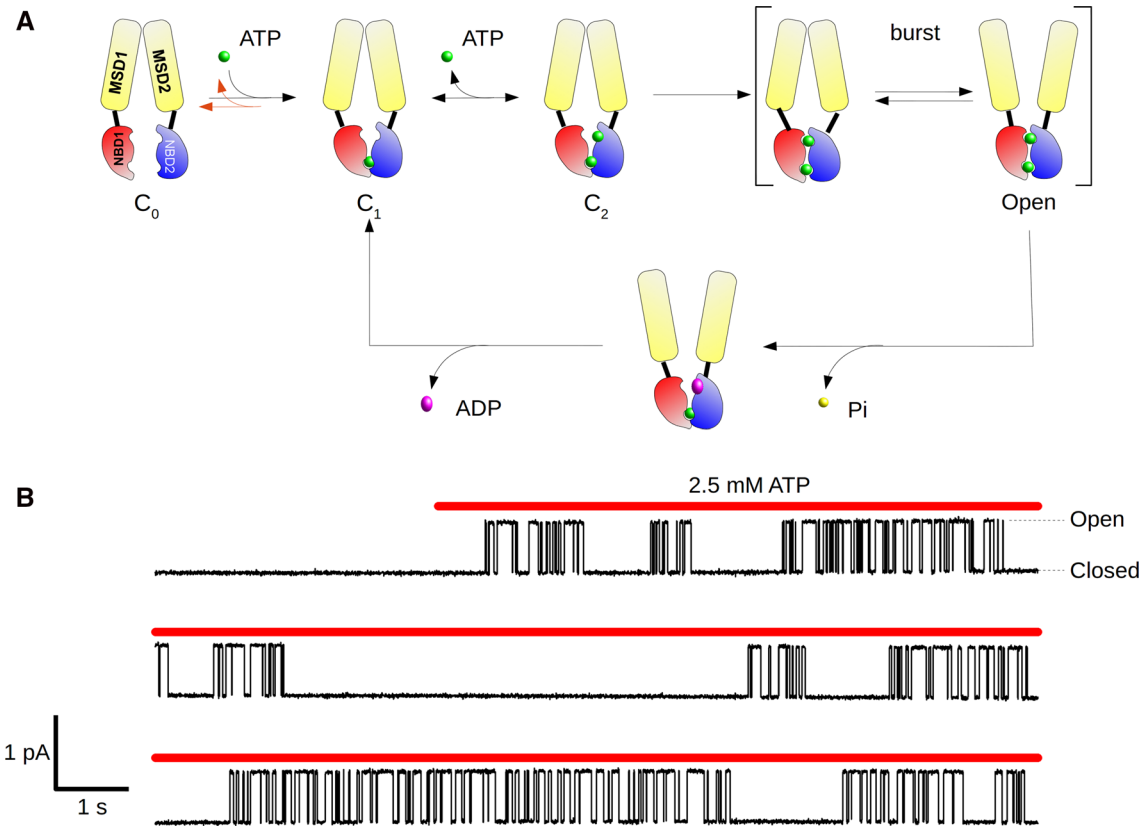


**Fig. 2** **a** Scheme representing the open channel and closed channel conformations of the MSDs, the dimerisation states and the ATP (or ADP) bound to the NBDs. The backward transition between states $C_0$ and $C_1$ is depicted in *red* because is very low probable. Notice that the transitions between the Open state and $C_1$ are not reversible. **b** Single channel record of a maximally phosphorylated CFTR channel. Channel activity initiates shortly after the application of ATP, and proceeds by bursts, intercalated by long closures

contradictory conclusions on the enthalpic and entropic contributions to the free energy differences between the open and closed sates were obtained by a different groups [56, 57], maybe because gating of CFTR is not a thermodynamic equilibrium process, and thus methods of classic thermodynamics cannot be applied. A more consistent thermodynamic description was obtained by the non-equilibrium thermodynamic analysis, allowing the reconstruction of the thermodynamic profile of gating of CFTR [58]. The large activation enthalpy ($\Delta H‡$) and activation entropy ($\Delta S‡$) for opening suggest that the transition may occur when NBDs have already dimerised, while the pore is still closed. The small $\Delta S‡$ for closure is appropriate for cleavage of a single bond (ATP's beta-gamma phosphate bond), and suggests that this transition state does not require large-scale protein motion and precedes the disruption of the dimer interface [58].

The molecular mechanism by which the dimerization of NBDs conditions the channel opening remains unknown. Molecular models of the CFTR based on prokaryotic ABC proteins clearly show close interactions between the NBDs and the intracellular loops [59–62]. Concurrently, single residue mutations at second intracellular loop (ICL2) and the fourth intracellular loop (ICL4) that alter the CFTR gating have been identified. Two residues of the ICL2, serine at position 263 that increases the CFTR current, and glutamate at position 267 that decreases current [63]. On the other hand, most ICL4 mutants disrupted the biosynthetic processing of CFTR, although not as severely as the most common ΔF508 mutation [64]. The remaining channel expression shows some altered gating behaviour, similar to those observed with mutations in the NBD [64]. Thus, it is reasonable to hypothesize that these two intracellular loops, ICL2 and ICL4, may act as transducers, to convert the conformational changes occurring when the NBDs bind 2 ATP molecules, to a major conformational change of the MSDs, probably modifying the tilting of the transmembrane helices [60], to open the ionic pathway of the channel.

## Gating defects in cystic fibrosis

More than 1600 CFTR mutations have been identified; these mutations are divided into 5 classes based on their functional alterations [65, 66]. It is noteworthy that about

one-third of the CFTR-mutations correlated with CF are located in the intracellular side of the protein (see http://www.genet.sickkids.on.ca), where the protein structures devoted to the CFTR channel gating are located [15]. Indeed, 9 out of the 10 more frequent mutations occur in the intracellular side of the protein [67]. Among these frequent mutations, the worldwide most common, present in at least one allele in about 89 % of CF patients, is the deletion of the residue phenylalanine 508 (F508del). Mutation F508del is classified as class II mutation, that causes a defect in CFTR maturation and targeting to the cytoplasmic membrane that produces the premature degradation of the mutant protein. Other mutations, such as the relatively frequent mutation G551D, severely impair CFTR gating (class III), reducing the ion transport. Of note, the F508del mutant presents both a maturation (class II) and a gating (class III) defect.

CFTR channels with class III mutations are characterised by a loss of chloride channel function by disrupting ATP binding, hydrolysis, and thus, channel gating [68–70]. The lessening of anion transport is correlated to the severity of the CF disease: G551D mutation has an observed channel activity ~100-fold smaller than wild type CFTR, and manifests a severe clinical case; conversely, mutation G1349D, that causes a milder from of CF, has a channel activity ~tenfold lower than WT. The quantitative analysis of channel gating reveals that these mutants have exceptionally slow opening rates and very fast closing rates when compared with those of wild type CFTR [70, 71]. It has been suggested that the loss of function in these mutants are due to a perturbation of the ATP-driven NBD dimerization during the CFTR channel gating cycle [71].

When the traffic defects of the F508del CFTR are overcome, the resulting chloride channel docked in the membrane presents a gating defect. The chloride transport is severely reduced as a result of a reduced open channel probability, caused by much greater closed times, [72, 73]. It has been proposed that the gating modification in the mutant F508del could be also related to the ATP-driven NBD dimerization [74]. In any case, one must consider that the traffic defect of F508del CFTR should have consequences on the folding that perhaps, even after rescuing, results a protein that is intrinsically different than the wild type [75].

Interestingly, these cited mutations, G511D, G1349D and F508del, are responsive to CFTR potentiators, that increase the chloride transport by CFTR, even in the presence of mutations. These predisposition to augment the channel activity by potentiators have allowed to develop a drug for the treatment of CF patients with the mutation G511D [76], and successively extended to patients carrying other class II mutations (G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, and S549R). Similar potentiaton is expected to be applied to F508del, as far as its traffic defect could be solved.

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