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Review

Cystic fibrosis transmembrane conductance regulator: a chloride channel gated by ATP binding and hydrolysis

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Abstract: The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel that belongs to the ATP-binding cassette (ABC) transporter superfamily. Defective function of CFTR is responsible for cystic fibrosis (CF), the most common lethal autosomal recessive disorder in Caucasian populations. The disease is manifested in defective chloride transport across the epithelial cells in various tissues. To date, more than 1 400 different mutations have been identified as CF-associated. CFTR is regulated by phosphorylation in its regulatory (R) domain, and gated by ATP binding and hydrolysis at its two nucleotide-binding domains (NBD1 and NBD2). Recent studies reveal that the NBDs of CFTR may dimerize as observed in other ABC proteins. Upon dimerization of CFTR's two NBDs, in a head-to-tail configuration, the two ATP-binding pockets (ABP1 and ABP2) are formed by the canonical Walker A and B motifs from one NBD and the signature sequence from the partner NBD. Mutations of the amino acids that interact with ATP reveal that the two ABPs play distinct roles in controlling ATP-dependent gating of CFTR. It was proposed that binding of ATP to the ABP2, which is formed by the Walker A and B in NBD2 and the signature sequence in NBD1, is critical for catalyzing channel opening. While binding of ATP to the ABP1 alone may not increase the opening rate, it does contribute to the stabilization of the open channel conformation. Several disease-associated mutations of the CFTR channel are characterized by gating defects. Understanding how CFTR's two NBDs work together to gate the channel could provide considerable mechanistic information for future pharmacological studies, which could pave the way for tailored drug design for therapeutical interventions in CF.

Key words: ATP-binding cassette transporter; ion channel gating; cystic fibrosis; electrophysiology

囊性纤维化跨膜电导调节体：ATP 结合和水解门控 Cl^- 通道

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摘 要：囊性纤维化跨膜电导调节体(cystic fibrosis transmembrane conductance regulator, CFTR)是一种 Cl^- 通道，属于 ATP 结合(ATP-binding cassette, ABC)转运体超家族。CFTR 功能缺陷是高加索人种中普遍存在的致死性常染色体隐性遗传疾病囊性纤维化(cystic fibrosis, CF)发生的主要原因。这种疾病患者各组织上皮细胞内 Cl^- 转运失调。目前，与 CF 相关的不同突变超过 1 400 种。CFTR 调节(regulatory, R)域负责调控，核苷酸结合域(nucleotide-binding domains, NBDs) NBD1 和 NBD2 负责 ATP 结合和水解门控。近期研究发现 CFTR 的 NBDs 与其它 ABC 蛋白一样可以二聚化。二聚化过程中，NBD1 和 NBD2 首 - 尾相连，一个 NBD 上的 Walker A 和 B 模块与另一个 NBD 提供的标签序列(signature sequence)形成 ATP 结合袋(ATP-binding pockets, ABPs) ABP1 和 ABP2。ABPs 中与 ATP 结合相关的氨基酸突变实验揭示，ABP1 和 ABP2 在 CFTR 的 ATP 依赖门控中发挥不同作用。ABP2 由 NBD2 上的 Walk A 和 B 模块与 NBD1 提供的标签序列形成，它与 ATP 结合催化通道开放，而 ABP1 单独与 ATP 结合不能促进通道开放，只能稳定通道构象。有一些 CFTR 突变相关疾病的特征就是门控失调，进一步深入研究 CFTR 的 NBD1 和 NBD2 如何通过相互作用而达到通道门控，将为药理学研究提供更多所需的机制信息，有利于为 CF 治疗的药物设计铺平道路。

关键词：ATP 结合转运体；离子通道门控；囊性纤维化；电生理学
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1 Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel located in the apical membrane of many epithelial cells. It plays a critical role for chloride movement across epithelia. Defective function of CFTR is responsible for cystic fibrosis (CF), the most common lethal autosomal recessive disorder in Caucasian populations[1-3]. The disease is manifested in defective chloride transport across epithelial cells in various tissues such as respiratory, gastrointestinal, hepatobiliary and reproductive tracts[4]. More than 1 400 mutations have been identified so far as disease associated in the CF database (www.genet.sickkids.on.ca).

The CFTR gene was cloned in 1989[1]. One of the most characteristic features of this 1 480 amino acid long protein is the presence of two repeated motifs each consisting of six transmembrane domains and a cytoplasmic nucleotide-binding domain (NBD). Linking the two homologous motifs is the regulatory (R) domain, which contains several consensus sites for phosphorylation by protein kinase A and protein kinase C[1] (Fig.1). Based on these topological characteristics, CFTR is placed in the ATP-binding cassette (ABC) superfamily. Nearly all members of this family actively transport substances across the cell membrane using ATP hydrolysis as the main energy input[5]. Both NBDs of the ABC proteins contain highly conserved motifs: the Walker A (GxxGxGKS/T, where x represents any amino acid), involved in coordinating the β- and γ-phosphates of ATP, the Walker B (hhhhD, where h represents a hydrophobic amino acid), involved in coordinating Mg^{2+}, a cofactor for ATP hydrolysis, and the signature sequence (LSGGQ), whose function remains unclear. The R domain though is unique to CFTR.

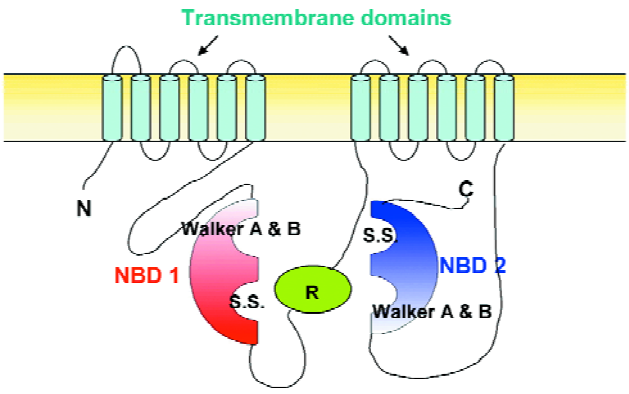

Fig.1. Cartoon depicting the domain structure of CFTR. It includes a cytoplasmic amino (N) and carboxyl (C) termini, two nucleotide-binding domains (NBD1 and NBD2), a regulatory (R) domain, and the transmembrane domains. The Walker A and B motifs of NBD1 and the signature sequence (S.S.) of NBD2 form the ATP-binding pocket 1 (ABP1). ABP2 is formed by the Walker A and B motifs of NBD2 and the S.S. of NBD1. The Walker A lysine K464 in NBD1 and K1250 in NBD2 coordinate the β- and γ-phosphates of the bound ATP. W401 in NBD1 and Y1219 in NBD2 interact with the adenine ring of the ATP molecule. E1371 serves as the catalytic base in NBD2 for ATP hydrolysis. In the S.S., there are two important disease-associated mutations, G551D in NBD1 and G1349D in NBD2. Both G551 and G1349 presumably interact with the bound ATP once the NBDs have dimerized.

Since most members of the ABC family are active transporters, it was initially very puzzling how the CFTR protein is related to the cAMP-activated chloride conductance seen in endogenous CFTR-expressing epithelial cells. For a while it was not clear if CFTR was a chloride channel itself or it somehow regulated another chloride channel. Although several reports have documented a regulatory role of CFTR in ion transport in epithelial cells[6,7], a series of studies have firmly established that CFTR itself is indeed a chloride channel. For example, expressing CFTR in cells that normally do not contain cAMP-activated chloride channels generated chloride currents activated by cAMP agonists[8-12]. Anderson *et al*.[8] mutated amino acids in the putative transmembrane domains, and showed that the ion selectivity was altered. Most convincingly, Bear *et al*.[13] showed that when purified CFTR was introduced into lipid bilayers, a chloride channel with similar characteristics to that observed in intact cells was detected.

As described above, in addition to being a chloride channel, there is evidence that CFTR may regulate the function of other ion channels such as Na^+ channels, K^+ channels, and other chloride channels. It is not clear though whether this modulation is due to a direct association of CFTR with the other channels, or indirect, via several protein-protein interactions or signaling pathways (for a review see reference[14]). More recently it has been shown that CFTR directly interacts with members of the SLC26 family of multifunctional anion exchangers, providing a possible explanation for the abnormal bicarbonate secretion in CF-affected tissues[15,16].

The focus of this review is to elaborate on CFTR gating studies from a historical perspective. The first part will be devoted to the experiments mostly performed before the first crystal structure of ABC proteins was solved, and their interpretations based more or less on sequence analysis of CFTR and its related proteins. Then we will glance through some of the high-resolution crystallographic studies of ABC proteins, including CFTR, and finally

discuss the most recent development in CFTR gating, with special emphasis on the role of ABP1.

2 Gating of CFTR channels

2.1 Gating studies before crystallization

The first piece of evidence for CFTR being an ATP-gated ion channel comes from patch-clamp experiments by Anderson *et al*[9]. They expressed human CFTR stably in NIH-3T3 or transiently in HeLa cells and demonstrated that in excised inside-out patches, phosphorylation by PKA is required for CFTR activation, and that hydrolysable nucleoside triphosphates (such as ATP, GTP, ITP, CTP and UTP) are needed to open the phosphorylated channel. These results were confirmed by Nagel *et al*.[17] for endogenous CFTR in guinea pig cardiac myocytes. These early data nicely provide hints for the functional roles of the R domain and the two NBDs.

Although there is no doubt that phosphorylation is required for CFTR function, the question as to how the R domain regulates the channel is still controversial. Rich *et al*.[10] showed that removing part of the R domain results in channels that conduct chloride without the presence of cAMP stimulation. In excised patches, this partially R domain-deleted CFTR can be opened by ATP without the need of prior PKA-dependent phosphorylation. Later studies on CFTR constructs with the R domain completely deleted confirm this phosphorylation-independent activity[18,19]. All these results were interpreted as that the R domain at least serves as an inhibitor for channel gating by ATP. However, how phosphorylation of individual serine residues in the R domain controls CFTR function remains poorly understood despite numerous mutagenesis studies done so far. Since the regulation of CFTR channels by phosphorylation has been covered extensively in other reviews, this subject will not be repeated here (for reviews see references[20,21]). Instead, we will focus our discussion on ATP-dependent gating of CFTR.

In the ABC transporter family, the NBDs are sites for ATP binding and hydrolysis. The role of ATP hydrolysis in CFTR gating was implied by Anderson's studies[9] since the CFTR channel failed to open in the presence of nonhydrolyzable ATP analogs such as AMP-PNP or ATPγS. Also Mg^{2+}-free ATP did not activate CFTR channels. It was then speculated that ATP hydrolysis might be required to open CFTR[22,23]. Interestingly, when AMP-PNP was applied in the presence of ATP, the open state of the channel was prolonged considerably[23-25], suggesting that hydrolysis may be also needed to close the channel. Based on all of these results, several gating models were proposed. In general, it was believed that two ATP hydrolysis events are involved: one is required for channel opening and the other one controls channel closing.

These early gating models gain some further support from mutagenesis experiments. The Walker A lysine residues (K464 in NBD1 and K1250 in NBD2) drew a lot of attention because of their roles in ATP binding and hydrolysis in other ABC proteins (e.g. reference[26]). It was shown that mutating K464 in CFTR channels decreases the opening rate whereas equivalent mutations at the K1250 residue slow down channel closing, mimicking the effect of AMP-PNP[25,27]. Biochemical studies also showed that the ATP hydrolysis rate is decreased by mutations at these two residues[28]. It was therefore concluded that ATP hydrolysis at NBD2 closes the channel, while ATP hydrolysis at NBD1 is either coupled to channel opening or is required for channel opening. This early idea of how CFTR's two NBDs controlled the gating transitions was refuted by more recent studies (see below for more details). For example, the role of ATP hydrolysis in channel opening was disapproved when photolabeling studies with 8-azido-ATP showed that NBD1 does not appreciably hydrolyze ATP[29-31]. These biochemical results were also supported by structural studies of CFTR NBD1[32,33].

In addition to nonhydrolyzable ATP analogs, ADP was the other commonly used nucleotide for CFTR gating studies. Anderson *et al*.[9] showed that ADP by itself cannot stimulate the channel activity, but in the presence of ATP it inhibits CFTR activity[34]. They proposed that ADP competes with ATP for the binding site responsible for the opening of the channel because the inhibition is reduced with increasing [ATP]. Later, it was shown that ADP increases the closed time of the channels, with little effect on the mean open time[24,35,36]. On the other hand, Weinreich *et al*.[37] reported a decrease of the open time, obtained from macroscopic relaxation analysis, of channels opened in the presence of ATP and ADP. A definite kinetic mechanism for the ADP effect on CFTR channels was recently obtained by Bompadre *et al*.[19]. Single-channel kinetic analysis demonstrates the presence of an ADP-dependent closed state, unequivocally confirming the idea that ADP and ATP compete for a site for channel opening. It was also shown that a reduction of the mean open time of the channel by as much as 50% in the presence of ADP, confirming Weinreich's result[37] at the single channel level (Fig.2). This shortening of the open time by ADP was more evident in the hydrolysis-deficient mutants E1371S and D1370N[38]. The importance of this effect of ADP on the stability of the

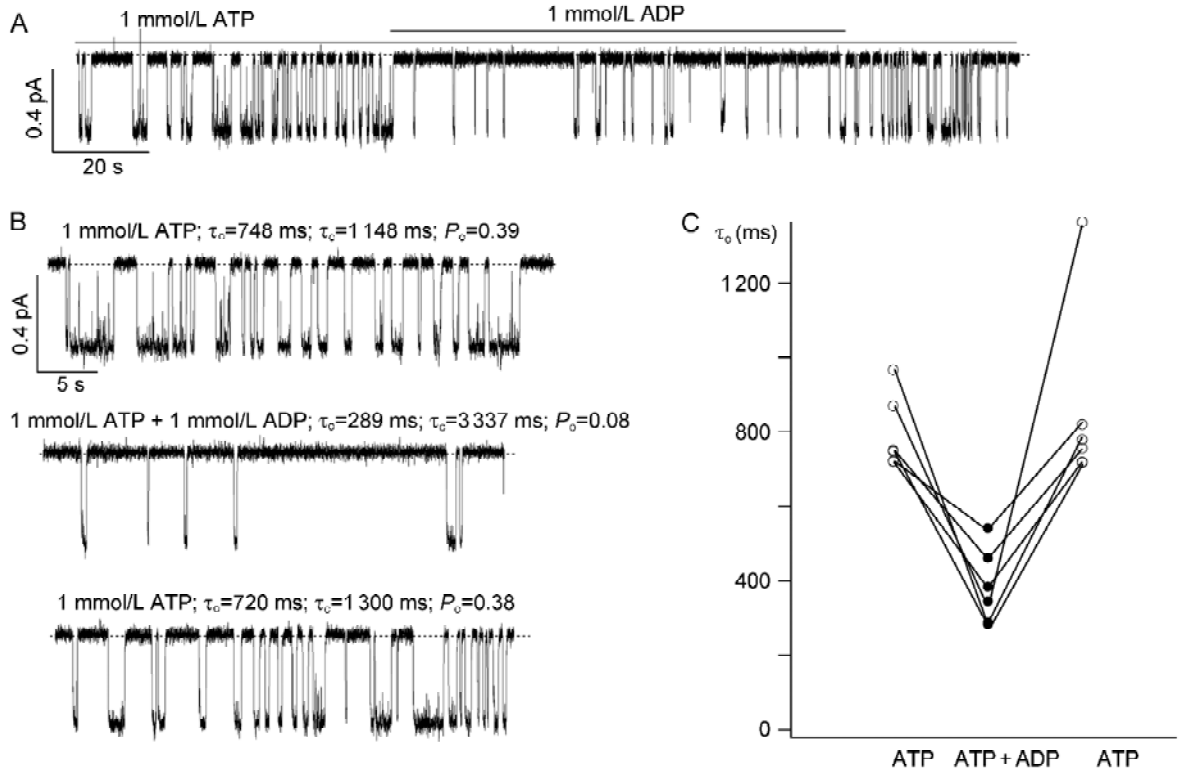

Fig.2. Effect of ADP on the mean open time (τ_o) of CFTR channels. *A*: Representative ΔR-CFTR current trace. *B*: Expanded traces with ATP alone or ATP + ADP from the trace shown in *A*. *C*: Reversible shortening of τ_o by ADP. Reproduced from Bompadre SG *et al*., the Journal of General Physiology 2005; 125: 361-375[19]. Copyright 2005 The Rockefeller University Press.

open state will be discussed after we provide a background introduction of the crystal structures of NBDs.

2.2 Crystallographic studies of ABC transporters

In the last ten years, high resolution crystal structures of several NBDs from different prokaryotic members of the ABC family[39-42], as well as the full transporters, BtuCD[43] and Sav1866[44] have been obtained. The first structural breakthrough occurred in 1998 when Hung *et al*.[45] solved the structure of HisP, the ATP-binding subunit of the ABC transporter histidine permease. They proposed a dimeric structure in a back-to-back conformation that placed the two ATP binding sites far from each other. This dimeric model is likely non-physiological since later studies repeatedly showed a head-to-tail dimer of NBDs[39-42], which can better explain numerous previous biochemical data. One important feature of the head-to-tail dimeric configuration is that the two ATP-binding sites are buried in the dimer interface with each bound ATP molecule interacting with the Walker A and Walker B regions of one NBD and with the signature sequence of the partner NBD. No closed dimers were found in the absence of ATP (e.g. reference[41]), suggesting that ATP binding initiates the dimer formation[5].

The ATP-driven dimerization of the NBDs was also shown biochemically by Moody *et al*.[46] in MJ0796 and MJ1267, two bacterial ABC transporters' NBDs. They showed that the NBDs can form dimers in the presence of ATP, when hydrolysis was abolished through the mutation of the Walker B glutamate. Moreover, neither ADP nor AMP-PNP promoted stable dimerization. Since dimers were not observed in wild-type NBDs in the presence of ATP, it was proposed that ATP binding induces the formation of the NBD dimer, and that ATP hydrolysis provides the free energy for the dissociation of the dimer[5].

As for CFTR, high resolution structures were obtained for mouse and human NBD1[32,33]. Although low-resolution structures of the whole CFTR protein from 2-D crystals have been solved[47], the immediate application of the low-resolution structure is unclear. Solving the high-resolution crystal structure of the CFTR protein remains an intractable challenge, but the mammalian over expression system developed for 2-D crystal formation in Riordan's group could be the first step toward accomplishing this important goal. CFTR gating studies will certainly benefit tremendously once the high-resolution

structure of the whole CFTR protein becomes available.

The major structural difference between CFTR NBD1 and the NBD1 of other ABC proteins is the presence of an insertion region (amino acids 415-432) and an extension at the C-terminus that includes several potential regulatory phosphorylation sites (amino acids 639-670). In the crystal structure, both the insertion and extension regions are positioned to obstruct the dimerization of the NBDs[32] but since both segments seem to have good mobility[32], they may move to allow for the dimerization to occur. It was speculated that phosphorylation in these regions may provide the necessary conformational changes to allow dimerization, but deleting part of the insertion or extension regions does not alter the requirement for phosphorylation, only the open time of the channels lacking the insertion region is slightly shortened by the mutation[48]. It was concluded then that these regions are not responsible for the regulation of the channel[48]. It should be noted that these results do not rule out the possibility that the regions may move, upon phosphorylation, to allow for the dimer formation. To disprove the hypothesis, one would have to show that once the regions are immobilized, the channel can still be activated by PKA-dependent phosphorylation.

The main difference observed between the mouse and human NBD1 structures is that in the mouse CFTR three residues (W401, L409, and F430) are in close contact with the adenine ring of the bound nucleotide. Interestingly, in human CFTR only W401 is shown to be in close contact with the adenine ring. This discrepancy may be due to the numerous mutations introduced into human NBD1 in order to obtain a crystal. Alternatively, simply a difference in the species could account for the structural differences. More interestingly, since crystal structures only provide snapshots of the protein, the different structures of NBD1 between human and mouse could reflect different conformations (or states) "frozen" in the crystals.

One important lesson learned from the structure of NBD1 is that it does not hydrolyze ATP. Although the crystal is formed in the presence of ATP, the γ-phosphate of the bound ATP remains intact in the crystal structure. This result supports several biochemical studies that show negligible ATP hydrolysis rate at NBD1[29-31]. This is now not surprising since two residues, a glutamate and a histidine, which are important for ATP hydrolysis[49,50] are replaced by serines in CFTR's NBD1. This new structure/function information about NBDs has not only changed our interpretations of many of the previous experimental results, but also help investigators design new experiments to tackle the molecular mechanism of CFTR gating by ATP binding/hydrolysis.

2.3 Gating studies after crystallization

The structural evidence obtained so far from the ABC transporters suggest that the NBDs form a head-to-tail dimer upon ATP binding, and it is believed that ATP hydrolysis provides the free energy for dimer dissociation. Soon after the model of the head-to-tail dimer of NBDs was proposed, it was used to explain data obtained from gating studies of CFTR. Powe *et al*.[51] showed that, in contrast to previous studies[25,27], the K464A mutation does not affect the channel opening rate, but it shortens the mean open time. This effect is not due to a change of the ATP hydrolysis rate since this mutation also destabilizes the AMP-PNP-locked open channels, which closes not through ATP hydrolysis, but through a nonhydrolytic mechanism. In addition, introducing the K464A mutation into the K1250A mutant whose ATP hydrolysis at NBD2 is diminished[25,27] dramatically shortens the stable open state seen in the K1250A mutation (Fig.3). The fact that mutations in NBD1 can affect the phenotype of NBD2 mutants compelled Powe *et al*.[51] to propose an interaction between CFTR's two ATP-binding sites in controlling gating (details will be elaborated in below). Taking one step further, Vergani *et al*.[52] proposed that binding of ATP to both ATP-binding sites initiates NBD dimerization and channel opening.

Later Vergani *et al*.[53] provided experimental evidence supporting the hypothesis that the CFTR's two NBDs may form a dimer in the head-to-tail configuration, as seen in many NBD dimers of other ABC transporters. Using mutant cycle analysis they showed that the highly conserved residues R555 (a residue right after the signature sequence of NBD1) and T1246 (a residue in the Walker A motif of NBD2) interact only when the channel is open, and they do not interact when the channel is closed, clearly linking the dimer association with the opening of the channel. Latest biochemical experiments by Mense *et al*.[54] using sulhydryl reagents to crosslink engineered cysteines at the dimer interface further confirmed this idea that CFTR's two NBDs indeed can form a head-to-tail dimer similar to the one seen in the crystal structures of other ABC transporters.

Based on the dimer model, each ATP-binding pocket (ABP) is formed by regions from both NBDs: the Walker A and B motifs of one NBD, and the signature sequence of the other NBD. Therefore, it seems more accurate to think of ATP-binding sites, at least for the open state, in terms of individual binding pockets consisting of components from both NBDs instead of the individual NBD. We have recently defined the ABP1 as the ABP formed by the Walker

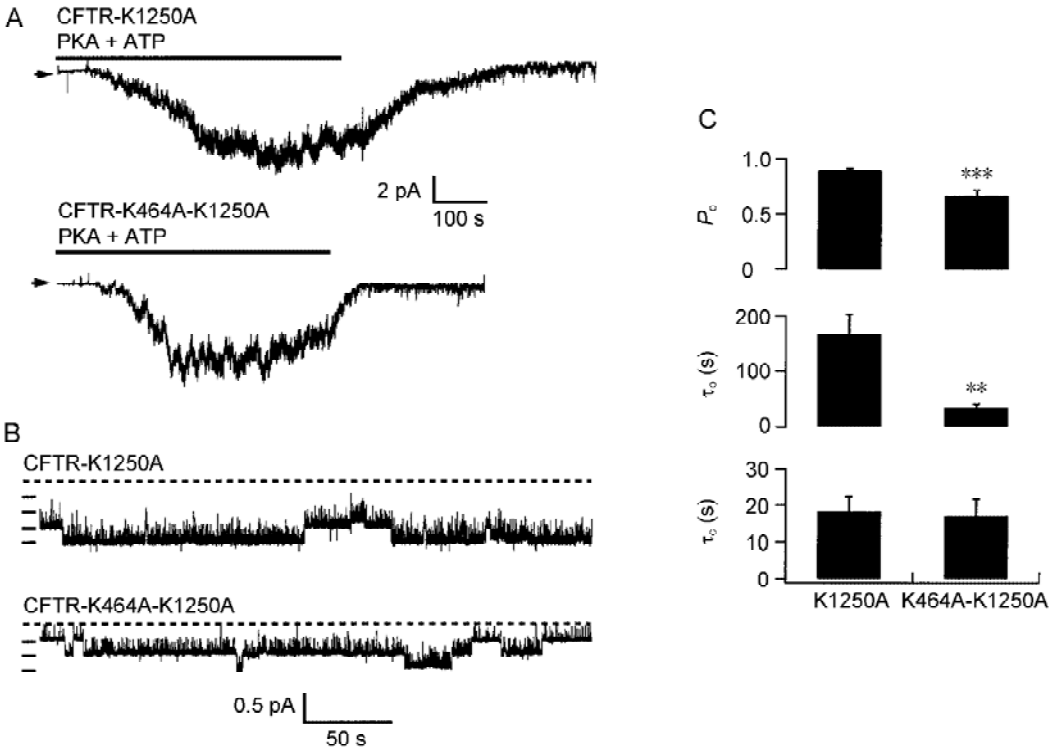

Fig.3. The K464A mutation shortens current relaxation of K1250A-CFTR. *A*: Representative traces for the current relaxation of K1250A-CFTR and K464A/K1250A-CFTR upon withdrawal of ATP and PKA. *B*: Current traces in patches containing few channel for the same mutants. *C*: Summary of the steady-state open probability (P_o), open time (τ_o) and closed time (τ_c) for both mutants. $^{**}P<0.01$, $^{***}P<0.005$ *vs* K1250A. Reproduced from Powe *et al.*, the Journal of Physiology 2002; 539: 333-346[51], with permission of the Journal of Physiology, Blackwell Publishing.

A and B motifs of NBD1 and the signature sequence of NBD2 and ABP2 as the ABP formed by the Walker A and B motifs of NBD2 and the signature sequence of NBD1[55]. It should be noted that the crystal structures of isolated NBDs show that ATP molecules bind to the Walker A and B motifs, but not to the signature sequence[32,40,43]. The ATP molecule may come in contact with the signature sequence only upon the dimerization of the NBDs. Thus, if the hypothesis that the physical movement upon NBD association/dissociation is coupled to opening and closing of the channel gate is correct, ATP interacts with the Walker A and B motifs in the closed state of the channel, and the signature sequence only comes into play when the dimer is formed. This concept is important since it will impact how functional data are interpreted in the context of structures. For example, mutations that change the apparent K_d of ATP for the opening rate likely are localized to those amino acids that interact with ATP before dimer formation (e.g. reference[56]). On the other hand, mutations of the signature sequences likely affect kinetic steps after ATP binding (e.g. reference[55]).

CFTR's NBDs show considerably structural asymmetry; not only do they share little sequence homology, except in those conserved motifs, they also seem to function differently. For example, only NBD2 can hydrolyze ATP[29-31]; NBD1 may have a higher ATP-binding affinity than NBD2[29-31,57,58]. It is pertinent to ask then, what is the role of each ATP-binding site in the gating of CFTR channels? A straightforward way to answer this question is to mutate amino acids in the Walker A and B motifs, or the signature sequence of each ABP.

Vergani *et al*.[52] proposed a model for CFTR gating in which both ABPs have to be occupied by ATP before the channel could open. This conclusion was reached after finding that the ATP dose-response relationships of the Walker A mutants K464A and K1250A and the Walker B mutant D1370N were shifted towards higher [ATP] compared to the ATP dose-response curve for wild-type channels. Later Berger *et al*.[59] used a different approach to answer the same question. They introduced bulky amino acids in either ABP and found that the opening rate of these mutants is decreased, concluding that ATP binding at both

ABPs is required for channel opening. It is worth noting that from this approach, as it was presented, it is not clear whether the introduction of bulky entities decreased the binding affinity or their side chains hindered the formation of the NBD dimer. It should also be noted that the dose-response shifts reported by Vergani *et al*.[52], as described above, were not seen in our studies[38,51].

Zhou *et al*.[56] tackled this issue using a somewhat different approach. They looked for amino acids that may affect only the binding, but not the hydrolysis of ATP. Based on sequence analysis and the crystal structure of CFTR's NBD1, they identified the amino acids that interact with the adenine ring of ATP. As mentioned previously, in the crystal structure of NBD1 from human CFTR, the aromatic residue W401 was shown interacting directly with the adenine ring of ATP in a stacking mechanism[32]. Using the structure of NBD1, they made a homology model of NBD2, and together with sequence analysis, identified the equivalent residue of W401 in NBD2 (i.e., Y1219). The mutation Y1219G shows an ATP dose-response relationship shifted more than 50-fold towards higher [ATP], however more conservative mutations (Y1219I, Y1219F) show smaller shifts, indicating the importance of the nature of the side chain in the interaction with the ATP molecule. Single channel analysis indicates that the Y1219G mutation reduces the opening rate of the channel while not affecting the open time (i.e. this mutation probably does not affect ATP hydrolysis in ABP2 like K1250A or D1370N). Interestingly, the equivalent mutation in ABP1, W401G, does not change the opening rate of the channel but instead increases the closing rate (Fig.4). This same effect was observed previously for K464A by Powe *et al*[51]. Since both amino acids are believed to be in contact with ATP, the mutations likely reduce the nucleotide binding affinity. However, their main effect seems to be the destabilization of the dimer (or reduced open time), instead of reducing the opening rate of the channel. This study suggests that while ATP binding at ABP2 is critical for catalyzing channel opening, ATP binding at ABP1 is not.

The idea that binding of ATP at ABP1 stabilizes the open state can help explain the observation that ADP shortens the open time of CFTR channels[19,37,38]. Likely ADP, a nucleotide smaller than ATP, does not bind as tightly to this binding site, thus its binding energy contribution is smaller, leading to a less energetically stable dimer. Another piece of evidence consistent with the idea that ATP binding at ABP1 may stabilize the open state is the presence of different open states observed by Bompadre *et al*[38]. The distribution of the open time depended on the ATP concentrations, suggesting that the lifetime of the open channel conformation was dependent on whether one or both binding sites are occupied[38].

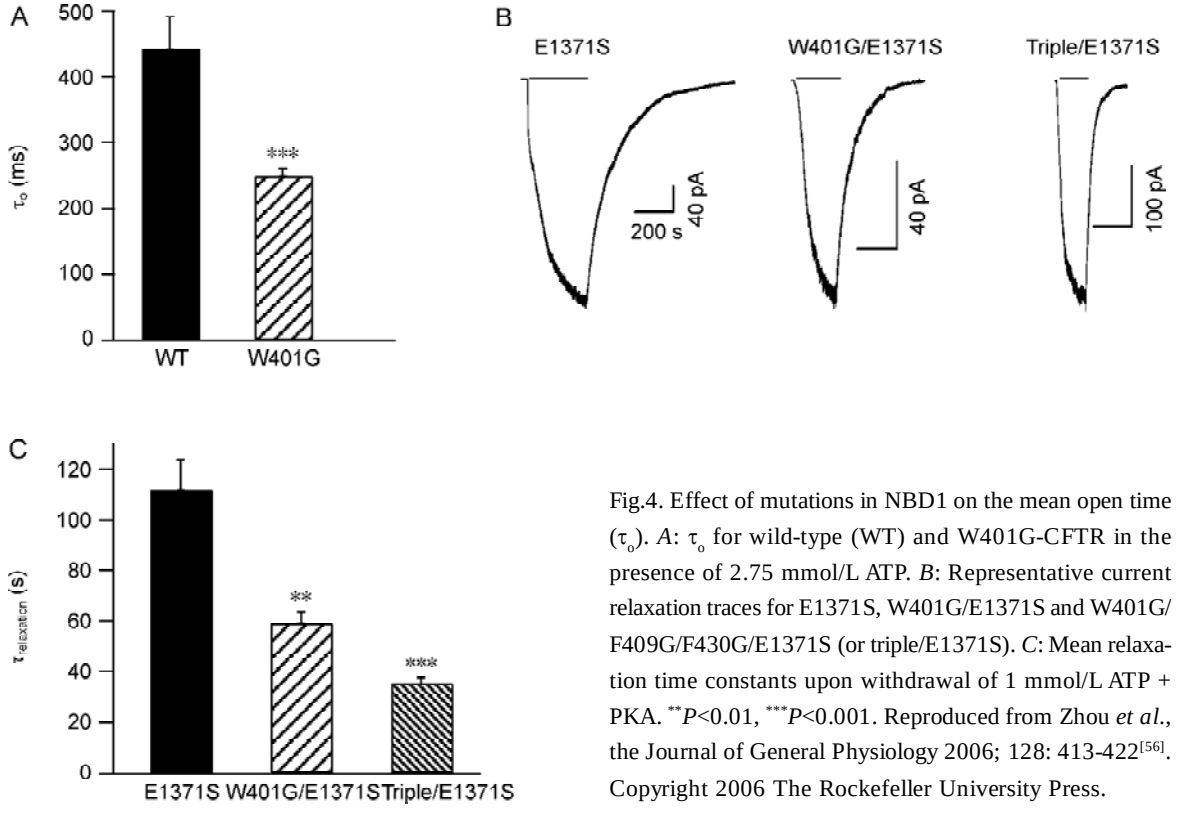

Fig.4. Effect of mutations in NBD1 on the mean open time (τ_o). *A*: τ_o for wild-type (WT) and W401G-CFTR in the presence of 2.75 mmol/L ATP. *B*: Representative current relaxation traces for E1371S, W401G/E1371S and W401G/F409G/F430G/E1371S (or triple/E1371S). *C*: Mean relaxation time constants upon withdrawal of 1 mmol/L ATP + PKA. $^{**}P<0.01$, $^{***}P<0.001$. Reproduced from Zhou *et al*., the Journal of General Physiology 2006; 128: 413-422[56]. Copyright 2006 The Rockefeller University Press.

Further evidence supporting the idea that the binding energy of the nucleotide contributes to the stability of the dimer comes from experiments using high-affinity ATP analogs. Zhou *et al*.[58] tested several ATP analogs that have been demonstrated to assume a higher binding affinity in other ATP-binding proteins[60], and identified N^6-(2-phenylethyl)-ATP (or P-ATP) as an ATP analog that exhibits ~50-fold higher potency than ATP in opening CFTR. Interestingly, P-ATP also prolongs the open time of wild-type CFTR and a hydrolysis-deficient mutant, E1371S-CFTR, indicating that the effect of P-ATP on the open time is not due to a perturbation of ATP hydrolysis. Using mutant cycle analysis (see details in reference[56]), Zhou *et al*.[56] concluded that P-ATP binds at ABP1 to stabilize the open state. In the same report, a potential role of F409 and F430, two aromatic amino acids interacting with the adenine ring of ATP in NBD1 crystal structure of mouse CFTR[32], in stabilizing the open state was also documented.

As described above, each ABP is composed of the Walker A and B motifs from one NBD and the signature sequence from the partner NBD. If two ABPs are playing different roles in controlling CFTR gating, one would predict that mutations at each of the two signature sequences in CFTR should affect CFTR gating differently. The importance of the signature sequence lies not only in the fact that they are part of the ABPs, but also in that many disease-associated mutations are found in this region of the protein. G551D, located in the signature sequence of NBD1, is the third most common CF-associated mutation (www.genet.sickkids.on.ca/cftr) and exhibits a much lower open probability (P_o) than wild-type channels[55,61,62]. Patients carrying the G551D mutation present a severe phenotype[63,64]. In contrast, the corresponding mutation in the signature sequence of NBD2, G1349D, is associated with a milder clinical phenotype[65]. Bompadre *et al*.[55] found that the G551D mutation (located at the ABP2) completely eliminates the ability of ATP to increase the opening rate of the channel (Fig.5). ADP does not inhibit G551D-CFTR currents and AMP-PNP does not lock the channel open. The observed low activity of G551D-CFTR channels likely represents the ATP-independent openings also seen with the wild-type channels[38,55]. This behavior corroborates with the idea that the occupancy of the ABP2 site by ATP is crucial for catalyzing channel opening[56]. Interestingly, the high affinity ATP analog P-ATP increases G551D-CFTR currents mainly by increasing the open time of the channel[66]. Introducing the mutation Y1219G (located at ABP2) in the G551D background, which reduces the ATP-binding affinity by more than 50-fold in wild-type channels[56], does not alter significantly the effect of P-ATP. However, the mutation W401G/G551D (W401G is located at ABP1) decreases remarkably the effect of P-ATP, suggesting that it is at this binding site (ABP1) where P-ATP binds to increase the G551D-CFTR activity[66]. These new results further affirm the idea that nucleotide binding at ABP1 stabilizes the open channel conformation. In contrast, the G1349D mutation retains some ATP dependence (Fig.5), with a maximal P_o approximately 10-fold lower than that of wild-type CFTR because of a reduced maximum opening rate. Although the exact mechanism for the functional defect of the G1349D mutation remains unclear, potential electrostatic repulsion between negatively charged side chain of D1349 and bound ATP may hinder NBD dimerization (i.e., a post-binding event).

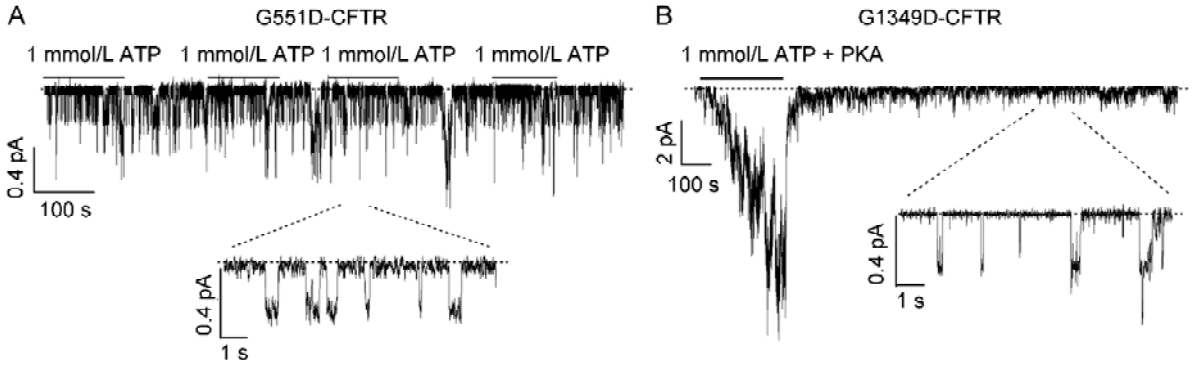

Fig.5. Gating of G551D-CFTR and G1349D-CFTR. *A*: Recording of G551D-CFTR current in an excised inside-out membrane patch. After washout of ATP the channels remain active. Repeated addition and removal of ATP did not result in changes in the activity of the channel. *B*: Recording of G1349D-CFTR current in an excised inside-out membrane patch. Upon removal of ATP and PKA, the current decays very rapidly. A small amount of current remains minutes after nucleotide removal. Reproduced from Bompadre SG *et al*., the Journal of General Physiology 2007; 129: 285-298[55]. Copyright 2007 The Rockefeller University Press.

Taken together, these results suggest that although CFTR channels can open in the absence of ATP with a very low opening rate (~0.006/s; from references[38,55]), it is ATP binding at ABP2 that catalyzes channel opening, while binding of ATP at ABP1 contributes to the stability of the dimer. For wild-type channels, hydrolysis precedes channel closing, while for the hydrolysis-impaired mutants the channel remains in a locked open state until the bound ATP dissociates from its binding site.

3 Future directions

Once the information from the structures of the different ABC proteins began to emerge, it became possible to form a picture of a more dynamic model of CFTR gating. This picture has helped address some important aforementioned issues on CFTR gating and to put the available functional data into structural perspectives. These structure/function models of CFTR gating will also help guide future studies. Despite the great progress made so far, we are still just beginning to understand a complex process. For example, the role of ATP binding at NBD1 in channel opening remains controversial. Although some groups reported that several NBD1 mutations do not affect the opening rate of the channel[28,51,56], we cannot rule out a possible role of ABP1 in channel opening since other groups have found a reduction of the opening rate in NBD1 mutants[25,27,52,59]. Since oftentimes different investigators use different expression systems and CFTR gating is subjective to modulations by differential phosphorylation of the R domain (and perhaps by other accessory proteins), the answer to this controversy perhaps lies in a system that can circumvent these complicating issues.

There are several questions regarding the dimerization process itself. For instance, how does ATP binding at the Walker A and B motifs catalyze the formation of the dimer? How is the signature sequence recruited to contact the bound ATP? We should also ask ourselves whether the dimerization of the NBDs is the only mechanism that leads to the opening of the channel. As we have mentioned before, CFTR channels open in the absence of ATP albeit with a very low open probability[38]. We certainly do not know whether this opening is linked to the dimerization of the NBDs or movement of the transmembrane domains, likely triggered by thermal fluctuations. Interestingly, a recent paper by Wang *et al*.[67] shows that the activity of CFTR channels whose NBD2 has been completely deleted can be enhanced with curcumin, bypassing ATP binding and dimerization of the NBDs.

Even if we just focus on NBD dimerization, questions remain to be answered include: after the NBDs have dimerized, how is the information transduced to the transmembrane domains, where the channel gate is located? How does phosphorylation of the R domain modulate the dimerization of the ATP-bound NBDs? For the open state (i.e., NBD dimer), do both ABPs have to be occupied? For channel closure, does it only happen when both ATP molecules dissociate?

Most importantly, the goal of understanding how CFTR works is to take us closer to developing drugs for therapeutical interventions in CF. We believe that gating studies should help us provide mechanistic and quantitative explanations for the different phenotypes exhibited in patients carrying the different disease-associated mutations. Many disease-associated mutations do not have trafficking defects, but the P_o of the channel is decreased. In these cases, understanding the mechanism of CFTR gating is of uttermost importance in order to develop a suitable potentiator that can restore the channel function. Numerous compounds that increase the CFTR activity include alkylxanthines, phosphodiesterase inhibitors, phosphatase inhibitors, isoflavones and flavones, benzimidazolones, and psoralens have been found (reviewed in reference[68]). Some agents modulate the activity of the protein kinases and phosphatases that regulate CFTR, whereas others interact directly with CFTR to control the channel activity[69]. However, a detailed understanding, at a molecular level, of how these compounds increase CFTR activity is still lacking.

Recently, Moran *et al*.[70] predicted three binding sites for genistein, one of the most studied CFTR potentiators, at the dimer interface using molecular simulations based on the crystal structure of NBD1. This interesting hypothesis was supported recently by mutational analysis of the putative binding sites[71]. Lately, our observation that P-ATP enhances G551D by binding to ABP1 implicates that ABP1 can potentially be a target for drugs to bind and increase the channel activity. With these new developments, we have reasons to be optimistic that soon clinically applicable CFTR potentiators will be identified and a vast number of CF patients will be benefited.

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