Mutations That Change the Position of the Putative \(\gamma\)-Phosphate Linker in the Nucleotide Binding Domains of CFTR Alter Channel Gating*

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Allan L. Berger§, Mutsuhiro Ikuma‡, John F. Hunt¶, Philip J. Thomas, and Michael J. Welsh**

From the §Howard Hughes Medical Institute, Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, the ¶Department of Biological Sciences, Columbia University, New York, New York 10027, and the ‡Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390

The cystic fibrosis transmembrane conductance regulator (CFTR) Cl\(^-\) channel is an ATP-binding cassette transporter that contains conserved nucleotide-binding domains (NBDs). In CFTR, the NBDs bind and hydrolyze ATP to open and close the channel. Crystal structures of related NBDs suggest a structural model with an important signaling role for a \(\gamma\)-phosphate linker peptide that couples bound nucleotide to movement of an \(\alpha\)-helical subdomain. We mutated two residues in CFTR that the structural model predicts will uncouple effects of nucleotide binding from movement of the \(\alpha\)-helical subdomain. These residues are Gln-493 and Gln-1291, which may directly connect the ATP \(\gamma\)-phosphate to the \(\gamma\)-phosphate linker, and residues Asn-505 and Asn-1303, which may form hydrogen bonds that stabilize the linker. In NBD1, Q493A reduced the frequency of channel opening, suggesting a role for this residue in coupling ATP binding to channel opening. In contrast, N505C increased the frequency of channel opening, consistent with a role for Asn-505 in stabilizing the inactive state of the NBD. In NBD2, Q1291A decreased the effects of pyrophosphate without altering other functions. Mutations of Asn-1303 decreased the rate of channel opening and closing, suggesting an important role for NBD2 in controlling channel burst duration. These findings are consistent with both the bacterial NBD structural model and gating models for CFTR. Our results extend models of nucleotide-induced structural changes from bacterial NBDs to a functional mammalian ATP-binding cassette transporter.

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§ Associate of the Howard Hughes Medical Institute.

** Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Inst., University of Iowa College of Medicine, 500 EMRB, Iowa City, IA 52242. Tel.: 319-335-7619; Fax: 319-335-7623; E-mail: mjwelsh@blue. weeg.uiowa.edu.

† The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; ABC transporter, ATP-binding cassette transporter; CF, cystic fibrosis; NBD, nucleotide-binding domain; Pgp, P-glycoprotein; PKA, cAMP-dependent protein kinase; AMP-PNP, adenosine 5′-[(β-imino)triphosphate].

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The cystic fibrosis transmembrane conductance regulator (CFTR)\(^{1}\) is an epithelial Cl\(^-\) channel that belongs to the ATP-binding cassette (ABC) transporter family (1–3). The conserved features of this family are two membrane-spanning domains and two cytoplasmic nucleotide-binding domains (NBD). CFTR also contains a unique regulatory (R) domain. The membrane-spanning domains of CFTR show topological similarity to those of other ABC transporters, but there is little sequence similarity. In contrast, the NBDs show significant sequence conservation throughout the family.

In CFTR, the membrane-spanning domains form an anion-selective pore (4), and the activity of the cytoplasmic domains controls channel opening and closing (5, 6). Channel activity requires phosphorylation of the R domain. Then ATP binding and hydrolysis by the two NBDs determine gating.

Previous studies have probed the structure and function of the CFTR NBDs by examining the functional consequences of applying nucleotide analogs and introducing site-directed mutations. Those studies have revealed different roles for NBD1 and NBD2 in channel gating. Several studies have also highlighted the role of ATP binding and hydrolysis in CFTR gating (for reviews, see Refs. 5 and 6). Mutations of the conserved Walker A lysines (Lys-464 in NBD1 and Lys-1250 in NBD2) inhibit ATPase activity (7) and alter channel gating (5, 8–10). However, the effects of mutations in the two NBDs are not symmetrical; the K1250A mutation dramatically prolongs the burst duration, whereas the K464A mutation reduces the frequency of channel opening but does not change burst duration. More recent studies have suggested that ATP binding without hydrolysis may be sufficient to open the channel (11–14). Thus, the molecular mechanisms by which the ATP- and ADP-bound states of each of the two NBDs affect channel gating are not well understood.

Recent work has described the three-dimensional crystal structures for the isolated NBDs of ABC transporters in the presence of ATP (15), ADP (16–19), or no nucleotide (17). These structures provide us with the opportunity to model the amino acid contacts between the CFTR NBDs and nucleotide and to evaluate structural models for channel gating. In this study, we have tested specific predictions of such models using site-directed mutagenesis to alter key residues and patch-clamp electrophysiology to examine the functional consequences.

EXPERIMENTAL PROCEDURES

Materials—The catalytic subunit of cAMP-dependent protein kinase (PKA) was purchased from Promega. Lipofectin was obtained from Invitrogen. All other reagents were obtained from Sigma.

Site-directed Mutagenesis and Transfection—CFTR mutants were
The apparent EC50 for ATP-stimulated CFTR activity was determined by analysis of data from multiple patches. A, examples of current from excised inside-out membrane patches containing single CFTR channels in the presence of 1 mM ATP and 75 nM PKA. Membrane potential was clamped at −80 mV. B, data from multiple patches. Asterisk indicates p < 0.05; n = 7 for WT, n = 4 for CFTR-Q493A, and n = 3 for CFTR-Q1291A.

FIG. 2. Single-channel gating of wild type CFTR, CFTR-Q493A, and CFTR-Q1291A. A, examples of current from excised inside-out membrane patches containing single CFTR channels in the presence of 1 mM ATP and 75 nM PKA. Membrane potential was clamped at −80 mV. B, data from multiple patches. Asterisk indicates p < 0.05; n = 7 for WT, n = 4 for CFTR-Q493A, and n = 3 for CFTR-Q1291A.

Switch Mechanism for CFTR NBDs

RESULTS

Potential Role of the γ-Phosphate Linker in NBD-dependent Gating of CFTR—Karpowich et al. (17) compared the crystal structure of NBDs in the ATP- or AMP-PNP-bound state (HisP and Rad50 in Refs. 15 and 16) with that of the ADP-bound or nucleotide-free NBD (LivG [MJ1267], Lod [MJ0796], MalK, and Tap1, in Refs. 16–19). In the ATP-bound state, a conserved Gln (Gln-100 in HisP) contacts the γ-phosphate of ATP (Fig. 1). Gln-100 lies at the amino-terminal end of a short segment of amino acids called the γ-phosphate linker. With ATP present, the γ-phosphate linker of the NBD stabilizes an α-helical subdomain in a fixed relationship to the ATP-binding core subdomain. In contrast, in the absence of nucleotides or with ADP bound (for example in LivG), the loss of contact with the γ-phosphate allows a conformational change to occur in the γ-phosphate linker, which facilitates outward rotation of the α-helical subdomain away from the ATP-binding core subdomain. Thus the presence or absence of the ATP γ-phosphate determines the position of the conserved Gln and hence the position of the α-helical subdomain via the γ-phosphate linker. As we note below, other structural interactions may also influence the position of the γ-phosphate linker.

Based on this model, we examined the effect of two sets of mutations in CFTR (Gln and Asn) that we predicted would have different effects on the γ-phosphate linker and hence the position of the α-helical subdomain. We mutated the conserved Gln (Gln-493 in NBD1 and Gln-1291 in NBD2) that connects the γ-phosphate linker to the γ-phosphate of ATP (Fig. 1). We hypothesized that these mutations should destabilize the ATP-bound conformation of the NBD. They might alter the affinity of ATP binding, thereby reducing channel activity. The mutations might disrupt ATP hydrolysis, thereby altering durations of the open and closed states. Or by interfering with the ability to “sense” the ATP γ-phosphate, they might disrupt ATP-dependent movement of the γ-phosphate linker and the α-helical subdomain, thereby uncoupling the structural consequences of ATP binding and hydrolysis.
We also mutated a highly conserved Asn (Asn-505 in NBD1 and Asn-1303 in NBD2 of CFTR). These align with Asn-102 of LivG, which mediates a set of hydrogen bonds with conserved residues in each conformation of the γ-phosphate linker. In the ATP-bound state, this conserved Asn (Asn-113 in HisP) connects via hydrogen bonds to the γ-phosphate linker. However, with ADP bound, hydrogen bond contacts with the γ-phosphate linker are disrupted, and hydrogen bonds form between a conserved Arg (Arg-166 in LivG) and the side chain of the Asn (Asn-102 in LivG) (17). These putative changes in bonding may favor an ADP-bound conformation in which the γ-phosphate linker is withdrawn from the nucleotide binding site (Fig. 1).

We hypothesized that mutation of this conserved Asn would be more destabilizing to the ADP conformation and therefore favor the ATP conformation. In this case, the Asn mutations would be expected to reduce the rate of entry into the ADP conformation and subsequent nucleotide dissociation.

We introduced the mutations at the indicated sites, expressed the variant CFTR in HeLa cells, and examined CFTR channel activity in excised, inside-out patches of membrane. Because the two NBDs have different functions in CFTR gating (reviewed in Refs. 5 and 6), we first show the effect of these mutations at NBD1 and then NBD2. Earlier models have suggested that ATP binding and/or hydrolysis at NBD1 are important for initiating channel activation and that ATP binding and hydrolysis at NBD2 are important in the opening and closing in a normal gating cycle.

**Switch Mechanism for CFTR NBDs**

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**FIG. 3.** Wild type CFTR, CFTR-Q493A, and CFTR-Q1291A had similar dose response curves for ATP-stimulated Cl− current. CFTR was first phosphorylated with 75 nM PKA in the presence of 1 mM ATP. Patches were then exposed to varying concentrations of ATP at −40 mV, and the ATP-stimulated current was measured. Data were normalized to the current measured with 1 mM ATP. The apparent EC50 was 176 ± 67 μM for wild type CFTR, 217 ± 55 μM for CFTR-Q493A, and 159 ± 70 μM for CFTR-Q1291A. n = 4–5 for each data point.

**FIG. 4.** Wild type CFTR, CFTR-Q493A, and CFTR-Q1291A showed similar cation requirements for Cl− channel activity. A, examples of current from patches containing multiple CFTR channels. Membrane potential was clamped at −40 mV. Patches were incubated with 1 mM MgATP, Na2ATP, and CaATP during the times indicated by bars. Without divalent cations the solution contained 1 mM EDTA and 1 mM EGTA to eliminate excess Mg2+ and Ca2+. B, data from multiple patches. There was no difference between the three constructs in the response to divalent cations.
the burst duration (Fig. 2). This result is consistent with the hypothesis that removal of the conserved Gln reduces either its affinity for ATP or its ability to induce the conformational change in the γ-phosphate linker required for channel activation, resulting in a lower frequency of channel opening. It could also be explained by a reduced rate of ATP hydrolysis (see "Discussion").

In both HisP and Rad50, the conserved Gln side chain contacts the γ-phosphate of the bound nucleotide through a water molecule (15, 23). This arrangement suggested that mutating Gln might impair ATP binding. Because ATP binding may open the CFTR Cl− channel (13, 14), thereby reducing the interburst interval, we asked if the prolonged interburst interval in Q493A was consistent with attenuated binding. To test this, we examined the effect of varying the ATP concentration. Wild type CFTR and CFTR-Q493A had the same apparent EC50 for ATP (Fig. 3). These data suggest that the conserved Gln is not critical for ATP binding.

The Gln side chain also contacts Mg2+ in Rad50 (23). In HisP, which was crystallized in the absence of Mg2+, the Gln side chain contacts a H2O molecule that was assumed to take the place of Mg2+ (15). If the conserved Gln in CFTR is a critical contact for the divalent cation, we expected that a Gln mutation would alter the functional consequences of changing the divalent cation. In wild type CFTR, the relative order of current stimulation is CaATP > MgATP > Na2ATP (13, 24).
Moreover, mutation of the NBD2 Walker B Asp, which also contacts the Mg$^{2+}$, abolishes differential effects of Mg$^{2+}$ and Ca$^{2+}$ compared with ATP alone (13). Fig. 4 shows that varying the divalent cation generated similar effects for wild type CFTR and the Q493A variant. These results suggest that contact between the conserved Gln and the divalent cation is not critical to this function.

ADP inhibits and pyrophosphate (PPi) stimulates CFTR Cl$^-$ current (9, 21, 25–27). These effects appear to be mediated predominantly through an interaction at NBD2. The Q493A mutation did not alter the response to either agent (Figs. 5 and 6).

**Mutating Asn-505 in NBD1 Can Increase Channel Opening.**—The N505C mutation had an effect opposite to that of Q493A; it reduced the interburst interval and increased $P_o$ (Fig. 7). As with Q493A, burst duration did not change. CFTR-N505A had gating kinetics similar to those of wild type; presumably the introduced Ala did not disrupt the structure to the same extent as when Cys was substituted for Asn. The effect of the Cys mutation can be explained by destabilization of the ADP conformation of the loop promoting faster entry into the channel-activating ATP conformation. Inhibition by ADP, stimulation by PPi, and the EC$_{50}$ for ATP simulation were not altered by the N505C or N505A mutations (data not shown). The opposite effects of the Q493A and N505C mutations on gating are consistent with the expectation that these mutations might have different effects on the γ-phosphate linker.

**Mutating Gln-1291 in NBD2 Reduced PPi Stimulation of CFTR Channel Activity.**—In contrast to the NBD1 Gln mutant, the NBD2 Gln variant Q1291A showed gating much like wild type (Fig. 2). These results indicate that the two NBDs in CFTR do not have equivalent functions in controlling gating; this conclusion is consistent with that drawn from earlier studies (9, 10, 13, 22). Previous studies have also suggested that termination of an open burst depends upon ATP binding and hydrolysis at NBD2. For example, the CFTR-K1250A mutant markedly prolongs burst duration (9, 10, 13, 22). Thus the normal burst duration in Q1291A suggests that mutation of the conserved Gln did not disrupt hydrolysis.

Wild type CFTR and CFTR-Q1291A had the same apparent EC$_{50}$ for ATP (Fig. 3) and the same response to variation of the divalent cation (Fig. 4). These results suggest that Gln-1291 is not a key factor in ATP binding or hydrolysis.

In the ADP-bound state, the conserved Gln of LivG MJ1267 (Gln-89), LolD MJ0796 (Gln-90), and TAP1 (Gln-586) does not have the opportunity to contact a γ-phosphate and makes no direct contacts with the β-phosphate of ADP (17–19). In CFTR, ADP inhibits channel activity, at least in part by competitively inhibiting ATP binding (27–29). This effect is abolished by some NBD2 mutations (21). The fact that the Gln does not interact with ADP bound to the model bacterial NBDs, coupled with our finding that the Gln mutations did not appear to inhibit ATP binding, predicted that the Gln mutations would not alter ADP-dependent inhibition of current. Fig. 5 shows that this was the case.

PPi and AMP-PNP applied with ATP lock channels open by prolonging the burst duration (22, 26, 30–32). It is thought that PPi and AMP-PNP bind tightly in an open-channel configuration and that the channel closes with dissociation of PPi or the non-hydrolyzable nucleotide. Several NBD2 mutations, but not equivalent NBD1 mutations, abolish PPi stimulation (9). This effect may be mediated at NBD2, although a recent report showed that the nucleotide base of AMP-PNP interacts with NBD1, suggesting more complicated explanations (33). Despite the lack of effect of the Q1291A mutation on other aspects of gating, it dramatically reduced PPi stimulation (Fig. 6). This result suggests that an interaction with the conserved NBD2 Gln is critical to either stabilizing the bound polyphosphate or transducing the signal generated by its binding.

**Mutating Asn-1303 in NBD2 Increased the Burst Duration and Decreased the Opening Rate.**—In addition to its role in determining structure, we were interested in Asn-1303 mutations because they are associated with CF (34). The single-channel gating of CFTR-N1303K, a relatively frequent CF--associated mutation, showed a long burst duration and a long interburst interval (Fig. 8). We observed similar kinetic effects when Asn-1303 was changed to another CF-associated mutation (N1303H), a sequenced mutation with undefined clinical consequences (N1303I), and an Ala (N1303A) (Fig. 8). These functional consequences are similar to those observed with mutations of Lys-1250, which disrupt ATP hydrolysis and probably binding (7, 13, 22). To further examine similarities

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2 M. R. Carson, A. L. Berger, and M. J. Welsh, unpublished results.
between CFTR-N1303K and CFTR-K1250A, we examined the effect of PP\textsubscript{i} and ADP. The N1303K mutation prevented PP\textsubscript{i}-dependent stimulation of current and eliminated ADP-dependent current inhibition (Fig. 9). Pyrophosphate caused only a 26.9 ± 18.6% (n = 4) increase in current for the CFTR-N1303K channel, and ADP caused only a 3.2 ± 6.8% (n = 3) inhibition in current. However, in contrast to the reduced EC\textsubscript{50} for ATP observed for mutations at Lys-1250 (21), the apparent EC\textsubscript{50} for CFTR-N1303A (253 ± 62 μM, n = 5) was not different from that of wild type CFTR (176 ± 67 μM, n = 5).

**DISCUSSION**

We hypothesized that amino acid contacts with the NBD γ-phosphate linker are an important determinant of NBD function. By combining knowledge of the structure of related NBDs with measurements of CFTR gating, our results provide insight into the relationship between structure and function of CFTR and other ABC transporters in the region of the γ-phosphate linker.

Mutations of the conserved Gln and Asn did not alter the EC\textsubscript{50} for ATP-dependent current stimulation. These results suggest that disrupting the interaction of the γ-phosphate linker with ATP is not critical for ATP binding. Whether the Gln and Asn mutations disrupt hydrolysis by CFTR NBDs is not known. We can, however, compare the gating of the Gln and Asn mutants with the behavior of channels with Walker A lysine mutations, which are known to disrupt hydrolysis (7). The Asn-1303 mutations prolonged the burst duration and increased the interburst interval, gating changes similar to mutations of the NBD2 Walker A lysine (9, 10, 22, 35). The Gln-493 mutation increased the interburst interval without affecting the burst duration, similar to the behavior of channels
with mutation of the NBD1 Walker A lysine (9, 10, 22, 35). However, mutations of Asn-505 and Gln-1291 were not consistent with the gating effects expected of mutations that disrupt hydrolysis. Therefore, we surmise that the Gln and Asn mutations probably have, at most, modest effects on hydrolysis.

The conclusions that the Gln and Asn mutations did not inhibit ATP binding or abolish hydrolysis are consistent with other studies. In HisP, the Q100L mutation showed normal ATP binding although the rate of histidine transport was reduced (36). In P-glycoprotein (Pgp), mutation of the conserved glutamine in either NBD1 or NBD2 caused no change in the apparent affinity for ATP, the $M_g$ requirement, or transition state trapping. Moreover, these mutations only modestly reduced the ATPase rate (37). In LivG, mutation of the equivalent Gln residue did not change the $K_m$ for ATP and decreased hydrolysis only modestly. Thus, the γ-phosphate linker is probably not critical for either ATP binding or hydrolysis.

An ATP-induced change in the position of the γ-phosphate linker may be a key event, or one of several key events, that couples ATP binding and hydrolysis to gating of the channel. This mechanism has been proposed for Pgp, for which the conserved Gln residue coupled ATP binding and hydrolysis to transmembrane transport (37). In CFTR, the requirement for Gln-1291 to mediate the effects of an activating polyphosphate supports this hypothesis. We hypothesize that the γ-phosphate of ATP contacts the side chain of Gln-1291 (possibly through a water molecule), influencing the position of the γ-phosphate linker and α-helical subdomain, and thereby maintaining the channel in an open state.

Our finding that mutation of equivalent residues in the two NBDs produced different gating patterns is consistent with previous work indicating that the two NBDs have different functions in gating CFTR (9, 10, 22, 35) and in the function of some other ABC transporters (38–40). For example, in CFTR movement of the γ-phosphate linker in NBD1 may be part of an “activating” mechanism that allows NBD2 to play the predominant role in gating the channel (13). Although the details of how conformational changes in the NBDs cause opening and closing of the channel remain untested, the very recent crystal structure of the Escherichia coli MsbA transporter indicates that the NBDs are likely to transmit a conformational signal to the membrane-spanning domains, and are unlikely to interact directly with the channel pore (41).

Based on the structural differences in ATP-bound, ADP-bound, and nucleotide-free NBDs, Karpovich et al. (17) proposed that an ATP-induced alteration in the affinity between the two NBDs controls their interaction, thereby converting chemical into mechanical energy. Their analysis suggested that the position of the α-helical subdomain contributes to NBD-NBD interactions and hence the activity of the transporter. Specifically, when ATP is bound, inward rotation of the α-helical subdomain may promote NBD-NBD interactions that activate the transporter. Such a model is also consistent with a structural relationship between the transmembrane domain and NBD of MsbA (41).

Some of the mutations we studied have been observed in patients with CF. N1303K is a frequent CF-associated mutation that has been shown to affect channel processing (42). Our data indicate that this mutation also affects channel gating and reduces $P_c$. N1303H and N1303I also alter channel gating although their effect on $P_c$ was minor. Because these are rare variations, it is not clear whether or not they cause CF. Two relatively uncommon missense mutations have been described at Gln-1291, Q1291H and Q1291R (43), and a single chromo-
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