Biophysical Journal Volume 69 December 2443–2448

Structural and Functional Similarities between the Nucleotide-Binding Domains of CFTR and GTP-Binding Proteins

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ABSTRACT The opening and closing of the CFTR Cl^- channel are regulated by ATP hydrolysis at its two nucleotide binding domains (NBDs). However, the mechanism and functional significance of ATP hydrolysis are unknown. Sequence similarity between the NBDs of CFTR and GTP-binding proteins suggested the NBDs might have a structure and perhaps a function like that of GTP-binding proteins. Based on this similarity, we predicted that the terminal residue of the LSGGQ motif in the NBDs of CFTR corresponds to a highly conserved glutamine residue in GTP-binding proteins that directly catalyzes the GTPase reaction. Mutations of this residue in NBD1 or NBD2, which were predicted to increase or decrease the rate of hydrolysis, altered the duration of channel closed and open times in a specific manner without altering ion conduction properties or ADP-dependent inhibition. These results suggest that the NBDs of CFTR, and consequently other ABC transporters, may have a structure and a function analogous to those of GTP-binding proteins. We conclude that the rates of ATP hydrolysis at NBD1 and at NBD2 determine the duration of the two states of the channel, closed and open, much as the rate of GTP hydrolysis by GTP-binding proteins determines the duration of their active state.

INTRODUCTION

When the primary sequence of CFTR was deduced (Riordan et al., 1989), it was interesting to discover the presence of two putative nucleotide binding domains (NBDs). The NBDs of CFTR shared sequence similarity with a family of proteins called the ATP-binding cassette (ABC) transporters (Higgins, 1992) or traffic ATPases (Mimura et al., 1991) and to a lesser extent with other proteins that hydrolyze ATP. Based on the presence of the NBDs, on the presence of 12 putative transmembrane segments (a topological feature shared by other members of the ABC-transporter/traffic ATPase family), and on the fact that many members of this family use the energy of ATP hydrolysis for active transport, it was hypothesized that CFTR was a pump. However, when a number of experiments established that CFTR is a Cl^- channel (Anderson et al., 1991c; Kartner et al., 1991; Anderson et al., 1991b; Bear et al., 1992; Tabcharani et al., 1993), this immediately raised the question: why should a channel contain domains that hydrolyze ATP? Subsequent studies showed that once the R domain of CFTR has been phosphorylated, ATP regulates channel activity through interaction with the two NBDs. Several observations indicate that hydrolysis of ATP by the NBDs is involved both in opening and in closing the channel (Anderson et al., 1991a; Hwang et al., 1994; Baukrowitz et al., 1994; Carson et al., 1995), and our previous data suggest that hydrolysis of ATP bound at NBD1 opens the channel into a burst of activity, whereas hydrolysis of ATP bound at NBD2 terminates the burst, thereby closing the channel (Carson et al., 1995). Yet it remains puzzling what function ATP hydrolysis may serve in regulating activity of a channel, inasmuch as once a channel is open ions flow passively down a favorable electrochemical gradient.

Without crystallographic data for CFTR, attempts to understand the structure and the corresponding function of the NBDs have been based on biochemical and functional data. However, the crystallographic structures of some ATP- and GTP-binding proteins have been used to develop structural models for the NBDs of CFTR (Mimura et al., 1991; Hyde et al., 1990). Recently, Dearborn and Manavalan (1994) noted similarity in the amino acid sequence of CFTR NBDs and the sequence of heterotrimeric G proteins. They suggested that the NBDs of CFTR might have structural similarity to the regions in G proteins that interact with nucleoside triphosphate. Fig. 1 shows one of the areas of similarity. Within the ABC transporter/traffic ATPase family the sequence L-S-G-G-Q is highly conserved. This sequence is located in the NBDs just N-terminal to the conserved Walker B sequence. This motif appears to correspond to the consensus sequence D-X-[G/A]-G-Q found in several families of GTP-binding and hydrolyzing proteins, including the *ras* family and heterotrimeric G proteins (Fig. 1; Bourne et al., 1991).

The functional importance of the L-S-G-G-Q motif in CFTR is suggested by the observation that mutations within this region can cause CF: within the 548-L-S-G-G-Q-552 motif in NBD1, mutations of S549 and G551 are associated with CF, and within the 1347-L-S-H-G-H-1350 motif in NBD2, mutation of G1349 is associated with CF. The importance of this region is also highlighted by the finding that some mutations of residue R553, which sits immediately adjacent to this motif in NBD1, can partially suppress

Received for publication 26 July 1995 and in final form 31 August 1995.
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ABC transporter/ traffic ATPase	CFTR (N)	...LGEGGIT**LSGGQ**RARISLA...
	CFTR (C)	...LVDGGCV**LSHGH**KQLMCLA...
	MDR-1 (N)	...VGERGAQ**LSGGQ**KQRIAIA...
	MDR-1 (C)	...VGDKGTQ**LSGGQ**KQRIAIA...
	STE-6 (N)	...IGTGGVT**LSGGQ**QQRVAIA...
	STE-6 (C)	...TRIDTTL**LSGGQ**AQRLCIA...
	HisP	...QGKYPVH**LSGGQ**QQRVSIA...
	MalK	...LDRKPKA**LSGGQ**RQRVAIG...
Heterotrimeric G proteins	G_s	...FHMF**DVGGQ**R...
	G_o	...FKMF**DVGGQ**R...
	G_t	...FRMF**DVGGQ**R...
ras	H/K/N-RAS	...LDIL**DTAGQ**E...

FIGURE 1 Sequence alignment of portions of the NBDs of CFTR with proteins that bind and hydrolyze ATP and GTP. The leftmost column designates the protein family. For proteins with two predicted nucleotide binding domains, each is designated either N-terminal (N) or C-terminal (C). The leftmost column designates the protein family. Adapted from Mimura et al. (1991), Hyde et al. (1990), Bourne et al. (1991), and McGrath and Varshavsky (1989).

the effect of the most common CF-causing mutation, ΔF508 (Teem et al., 1993).

The significance of this motif in GTP-binding proteins has been demonstrated both by structural and by functional studies. Recent crystallographic analyses of $p21^{ras}$, $G_{i\alpha1}$, and $G_{t\alpha}$ have suggested that the conserved glutamine (Q61 in $p21^{ras}$, Q204 in $G_{i\alpha1}$, and Q200 in $G_{t\alpha}$) plays a key role in hydrolysis (Pai et al., 1990; Coleman et al., 1994; Sondek et al., 1994). During hydrolysis of bound GTP, the glutamine side chain moves into a position in which it can activate a bound water molecule, which mounts an in-line nucleophilic attack on the γ-phosphate of bound GTP. Functional studies support this interpretation. Mutation of Q61 in $p21^{ras}$ to any of 17 other amino acids promoted neoplastic transformation and decreased the rate of GTP hydrolysis approximately 10-fold, without altering GTP binding affinity (Der et al., 1986). Mutation of Q204 in $G_{i\alpha1}$ inhibited hydrolysis without altering nucleotide binding (Kleuss et al., 1994). Mutation of the corresponding glutamine in $G_{s\alpha}$ to valine or leucine decreased the rate of GTP hydrolysis, had little effect on the rate constants for dissociation of GDP (Masters et al., 1989; Graziano and Gilman, 1989), and was responsible for human malignancies (Landis et al., 1989).

If the L-S-G-G-Q motif in CFTR has a structural position analogous to the D-X-[G/A]-G-Q motif in GTP-binding proteins (Dearborn and Manavalan, 1994), then Q552 in NBD1 and H1350 in NBD2 would be appropriately positioned to participate directly in the hydrolysis of ATP, but they would not be absolutely required for hydrolysis. Based on this speculation, and our previous conclusions that the rate of ATP hydrolysis at NBD1 determines the rate of channel opening into a burst and that the rate of ATP hydrolysis at NBD2 determines the rate of closure from a burst, we made the following hypotheses. First, we predicted that mutation of Q552 in NBD1 would decrease the rate of hydrolysis at NBD1, which would cause a decrease in the rate of channel opening. Second, we predicted that mutation of H1350 in NBD2 to glutamine would increase the rate of hydrolysis, which would be manifested as shortened bursts of activity. To test these hypotheses we used the excised inside-out patch-clamp technique to study CFTR variants containing the Q552A, Q552H, H1350Q, and H1350A mutations.

MATERIALS AND METHODS

Chemicals and solutions

The catalytic subunit of PKA was from Promega Corp., Madison, WI. Adenosine 5′-triphosphate (ATP; disodium salt), adenosine 5′-diphosphate (ADP; sodium salt) and all other reagents were from Sigma Chemical Co., St. Louis, MO.

For experiments with excised, inside-out membrane patches, the pipette (extracellular) solution contained (in mM): 140 NMDG (N-methyl-D-glucamine), 100 aspartic acid, 35.5 HCl, 5 $CaCl_2$, 2 $MgCl_2$, 10 HEPES (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid), pH 7.3 with NaOH. The bath (intracellular) solution contained (in mM): 140 NMDG, 135.5 HCl, 3 $MgCl_2$, 10 HEPES, 4 Cs, and 1 EGTA, pH 7.3 with HCl ($[Ca^{2+}]_{free}$ $< 10^{-8}$ M).

Cells and transfection procedure

CFTR mutants were transiently expressed in HeLa cells using the vaccinia virus/bacteriophage T7 hybrid expression system as previously described (Fuerst et al., 1986; Elroy-Stein et al., 1989; Carson et al., 1993).

Patch-clamp technique

Methods for excised, inside-out patch-clamp recording are similar to those previously described (Carson and Welsh, 1993; Hamill et al., 1981; Anderson et al., 1991a). An Axopatch 200 amplifier (Axon Instruments, Inc., Foster City, CA) was used for voltage clamping and current amplification. A microcomputer and the pClamp software package (Axon Instruments, Inc.) were used for data acquisition and analysis. Data were recorded on videotape following pulse code modulation with a PCM-2 A/D VCR adapter (Medical Systems Corp., Greenvale, NY). Patch pipettes were fabricated as described (Carson et al., 1993), with pipette resistances of 5–15 MΩ and with seal resistance routinely greater than 5 GΩ. Voltages are referenced to the extracellular side of the membrane. Time course experiments were performed with the membrane potential clamped at -40 mV; single-channel data were recorded at a holding potential of -80 mV. P_o is voltage independent within this range. Experiments were conducted at 34–36°C with a temperature-controlled microscope stage (Brook Industries, Lake Villa, IL).

For excised macropatch data, replayed records were filtered at 1 kHz by a variable eight-pole Bessel filter (Frequency Devices Inc., Haverhill, MA) and digitized at 2 kHz. Average currents were determined as mean current during 1 s, with one point collected every 5 s. The average current was determined as the last 12 data points per intervention. To compensate for any channel rundown between interventions, all experimental interventions are bracketed with 1 mM ATP, and intervention current is compared with the average of preintervention and postintervention currents.

For single-channel analysis, replayed data were filtered at 1 kHz with a variable eight-pole Bessel filter, digitized at 5 kHz, and digitally filtered at 500 Hz. Idealized records were created by use of a half-height transition protocol; transitions less than 1 ms in duration were not included in the analysis. Data shown in Fig. 2 were also digitally filtered at 500 Hz.

Burst analysis was performed as previously described (Carson et al., 1994; 1995), with a t_c (the time that separates interburst closures from intraburst closures) of 20 ms. This value was derived from analysis of wild-type CFTR closed-time histograms of excised inside-out membrane patches containing a single channel studied in the presence of 1-mM ATP and PKA and by the method of Sigurdson et al. (1987). Closures longer

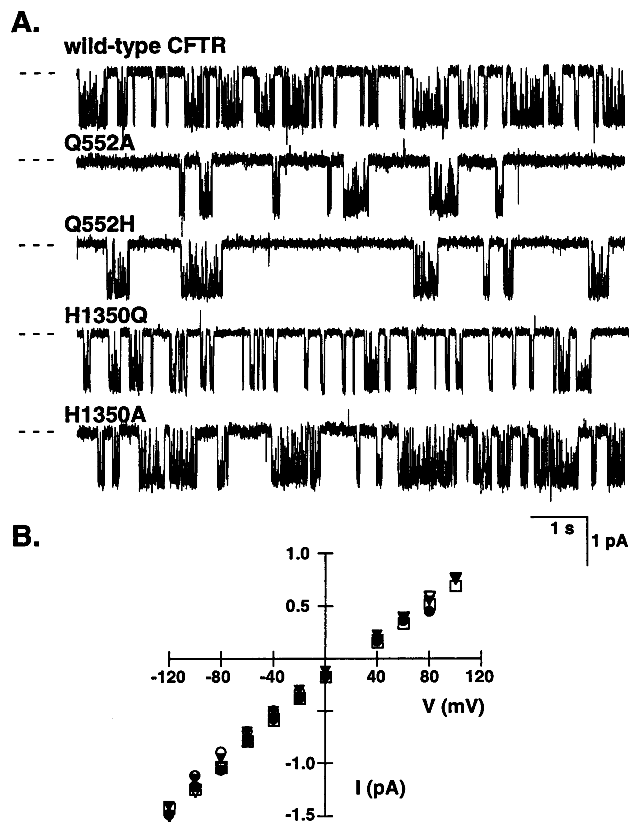

FIGURE 2 Effect of Q552 and H1350 mutations on CFTR Cl^- channels. *A*, Single-channel recordings of wild-type and mutant CFTR. Traces are from excised inside-out patches of membrane studied in the presence of 1 mM ATP and 75 nM PKA, with membrane potential clamped at -80 mV. Dotted lines show closed state and downward deflections correspond to openings. Single-channel current-voltage relationship of wild-type and mutant CFTR channels. Open circles, wild-type; open triangles, Q552A; open squares, Q552H; filled circles, H1350A; filled triangles, H1350Q. $N = 1-6$ observations for each data point; when appropriate, error bars are ±SEM and are hidden by data points in most cases.

than 20 ms were considered to define interburst closures, whereas closures shorter than this time were considered gaps within bursts. Burst data were from patches containing three or fewer active channels; bursts in which there were no superimposed openings and that were separated from other bursts by greater than 20 ms were included in the analysis. Some burst data were acquired in the presence of 100-μM ATP, in the continuous presence of 75-nM PKA. It was previously demonstrated that CFTR burst duration is independent of ATP concentration (Winter et al., 1994) and that inclusion of bursts from membrane patches with more than one active channel does not produce a discernible bias relative to burst data from patches containing only a single channel. We calculated the mean closed time between bursts (T_c) from the equation $P_o = T_b/(T_b + T_c)$. P_o, mean burst duration, and open probability within a burst were determined directly from experimental data. T_b was defined as the mean burst duration × the open state probability within a burst. Thus, the mean closed time (T_c) represents the mean closed time between bursts. None of the mutations altered open-state probability within a burst. For either wild-type or mutant channels, data from single and multichannel patches gave similar results.

RESULTS

All four mutants produced functional CFTR Cl^- channels when transiently expressed in HeLa cells. Fig. 2 *A* shows examples of single-channel traces. All the mutant channels showed a pattern of gating characteristic of CFTR, with short bursts of activity (in which the channel flickers open and closed) separated by longer closings. None of the mutations altered the linear single-channel current-voltage relationship or Cl^- selectivity (Fig. 2 *B*). These results suggest that the mutations did not alter the ion conduction pathway. However, these mutations altered the kinetics of channel gating.

At NBD1 we found that mutation of Q552, to either histidine or alanine, decreased single-channel open-state probability (P_o) (Fig. 3A). To quantify how these mutations altered the duration of channel closed and open states we calculated the mean closed time between bursts of activity and the duration of bursts as described in the Materials and Methods section. Fig. 3 *B* shows that both NBD1 mutations increased the closed time between bursts approximately fourfold. That is, Q552A and Q552H decreased the rate at

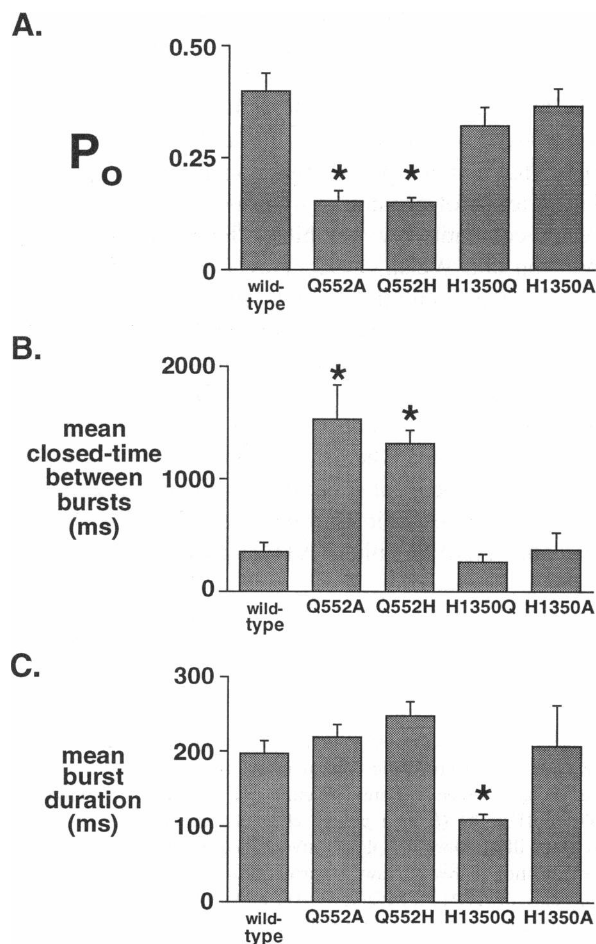

FIGURE 3 Effect of mutation of Q552 and H1350 on single channel activity. *A*, Open-state probability (P_o). Asterisks, $p < 0.0005$. *B*, Calculated mean closed time between bursts. Asterisks, $p \leq 0.001$. *C*, Mean burst duration. The asterisk indicates $p = 0.04$. Values are mean ± SEM; n = at least 7 for each variant. For all data, statistical significance was assessed with a one-way analysis of variance (ANOVA) with a post hoc Dunnett test.

which closed channels opened. This can also be appreciated from inspection of Fig. 2 *A*. These are the results predicted if both alanine and histidine are less potent at catalyzing the hydrolysis reaction at NBD1 that is required for opening the channel. A potency for promoting hydrolysis of $Q > H \approx A$ is consistent with previous studies in a number of GTP-binding proteins, which show that mutation of the conserved glutamine reduces the rate of, but does not abolish, GTP hydrolysis (Masters et al., 1989; Graziano and Gilman, 1989; Der et al., 1986). In contrast, neither mutation at Q552 altered burst duration (Fig. 3 *C*). The lack of effect on burst duration is also expected if, as we previously proposed, hydrolysis of ATP at NBD2, and not NBD1, is the major determinant of burst duration (Carson et al., 1995).

At NBD2 mutations of H1350 did not alter the time during which the channel remains closed between bursts, but they had interesting effects on burst duration. Mutation of H1350 to glutamine produced channels with a decreased mean burst duration. This is the predicted result if glutamine promotes a faster rate of hydrolysis than histidine, as it does in GTP-binding proteins, and if the rate of ATP hydrolysis at NBD2 is the primary determinant of the duration of a burst of activity (Carson et al., 1995). In contrast, mutation of H1350 to alanine had no effect on burst duration. Again, this was expected because data from GTP-binding proteins suggest that alanine may be functionally equivalent to histidine in terms of promoting hydrolysis.

ADP competitively inhibits CFTR channel activity (Anderson and Welsh, 1992; Travis et al., 1993), and we suggested that inhibition occurs through NBD2 because several mutations in NBD2 attenuated the ability of ADP to inhibit. For example, mutation of K1250 (the conserved lysine in the Walker A motif of NBD2) to methionine abolished ADP-dependent inhibition (Anderson and Welsh, 1992). To determine whether mutations of H1350 in NBD2 altered ADP-dependent inhibition, we examined the effect of ADP on macroscopic currents in excised patches. Fig. 4 shows that ADP inhibited ATP-supported current in the NBD2 mutants to an extent similar to that observed with wild-type CFTR.

These observations can be explained if the structure of the NBDs resembles that of GTP-binding proteins. Such a structure indicates that the only interaction of H1350 with bound nucleotide would be in contact with the γ-phosphate of ATP via a bridging water molecule. By analogy with what is found in GTP-binding proteins (Der et al., 1986; Graziano and Gilman, 1989), alteration of this residue would have no predicted effect on either ATP or ADP binding affinity and therefore would have no effect on inhibition of current by ADP. In contrast, the structure of GTP-binding proteins places the lysine that corresponds to K1250 in a position where it interacts directly with nucleotide. In c-H-*ras*, the side chain of K16 interacts directly with the β-phosphate of GTP and GDP (Milburn et al., 1990); mutation of this lysine to an asparagine reduced affinity for nucleotide 100-fold (Sigal et al., 1986), and this might explain why a similar mutation in CFTR abolished ADP inhibition.

DISCUSSION

Two groups have proposed models for the NBDs of the traffic ATPase/ABC transporter family. Both Hyde et al. (1990) and Mimura et al. (1991) based their modeling on the topology of adenylate kinase. Because adenylate kinase does not have an L-S-G-G-Q motif, each of those models positioned that motif in different places. Recently, Dearborn and Manavalan (1994) noted sequence similarity between the NBDs of CFTR and GTP-binding proteins and proposed that the two types of protein may have structural similarity; specifically, the L-S-G-G-Q motif may form part of the structure that interacts with ATP. Their suggestion is in good agreement with the model of Hyde et al. (1990) but differs substantially from the model of Mimura et al. (1991), which placed the L-S-G-G-Q motif a considerable distance

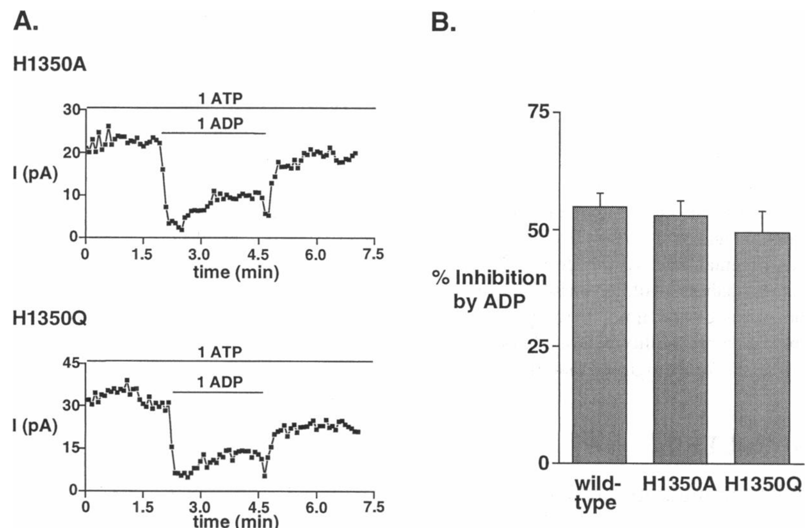

FIGURE 4 Effect of ADP on H1350 mutants. *A*, Time course of macroscopic current in excised membrane patches from cells expressing either H1350A (*top panel*) or H1350Q (*bottom panel*) channels. For the purpose of illustration, the plot is inverted so that upward deflection represents inward current. Bars indicate the presence of ATP (1 mM) and ADP (1 mM) cytosolic (bath) solution. Activation of current with 75-nM PKA and 1 mM-ATP are not shown. *B*, Average inhibition of current by 1-mM ADP, expressed as percent of current supported by 1-mM ATP. There was no difference between groups ($p > 0.3$ for all, $n = 4$, 3, and 3 for wild-type, H1350A, and H1350Q, respectively).

from the nucleotide-binding site. Until this study, there have been no functional data that addressed the proposed difference between these models. Recent crystallographic (Pai et al., 1990; Coleman et al., 1994; Sondek et al., 1994) and functional (Der et al, 1986; Kleuss et al., 1994; Masters et al., 1989; Graziano and Gilman, 1989) data from GTP-binding proteins indicate that the terminal glutamine residue in the D-X-[G/A]-G-Q motif participates in the hydrolysis reaction. With this information, we predicted that the terminal glutamine in the L-S-G-G-Q motif of CFTR may be in an appropriate position to catalyze the hydrolysis of the γ-phosphate of ATP. Our results are consistent with this hypothesis and suggest that the NBDs of CFTR may have structural and mechanistic similarities to GTP-binding proteins.

This conclusion leads us to consider possible parallels in the function of the NBDs of CFTR and GTP-binding proteins. It is well accepted that GTP-binding proteins do not couple hydrolysis of bound GTP to activation of their downstream effectors (Gilman, 1987). Instead, the rate of GTP hydrolysis determines the duration of the sojourn in the active state. Thus, hydrolysis serves to change the *state* of the G protein—from active to inactive. In CFTR, the rate of ATP hydrolysis at NBD1 appears to determine the rate at which the channel will open from the closed state; once the channel is open, the rate of hydrolysis at NBD2 is a primary determinant of how long it will remain open (Carson et al., 1995). Thus hydrolysis determines the *state* of CFTR—closed or open. However, unlike G proteins, which have only one site for GTP binding and hydrolysis, CFTR appears to bind and hydrolyze ATP at both NBDs during one cycle of gating (Carson et al., 1995).

Although the structure of the NBDs and the mechanism of hydrolysis may be similar to that of G proteins, the functional consequences of hydrolysis are different in each NBD. In fact, the distinct separation of the effects of NBD1 and NBD2 mutations on the closed time between bursts and burst duration, respectively, supports previous conclusions that each NBD has a distinct function in channel gating (Hwang et al., 1994; Baukrowitz et al., 1994; Carson et al., 1995). The functional parallel to a G protein is more obvious for NBD2 than for NBD1. First, NBD2 mutations at K1250 that are predicted to inhibit hydrolysis prolong the duration of bursts (in contrast to the H1350Q mutation, which shortens bursts) (Carson et al., 1995). This effect is analogous to oncogenic *ras* mutations that inhibit hydrolysis and prolong the duration of the active state. Second, the nonhydrolyzable ATP analog AMP-PNP, in the presence of ATP and PKA, prolongs the duration of channel bursts (Hwang et al., 1994; Carson et al., 1995), similar to the way in which GMP-PNP prolongs the active state in G proteins. The functional consequences of hydrolysis at NBD1 seem to be less clearly analogous to those of G proteins because hydrolysis appears to be required for the channel to open (Anderson et al., 1991a; Baukrowitz et al., 1994; Carson et al., 1995).

The parallels between GTP-binding proteins and the NBDs raise the intriguing possibility that other parts of the CFTR molecule or other cellular proteins may modulate NBD function in a manner analogous to the way in which GTPase activating proteins or guanine nucleotide exchange factors regulate GTP-binding proteins. A likely candidate is the R domain. Phosphorylation of the R domain does not affect ATP binding (Travis et al., 1993) but does influence activity, perhaps by influencing hydrolysis (Hwang et al., 1994; Carson et al., 1995).

What are the implications of these considerations for other members of the ABC transporter/traffic ATPase family? Just as CFTR appears to utilize ATP hydrolysis at two NBDs during one cycle of gating, other ABC transporters may utilize ATP hydrolysis at both NBDs for one cycle of active transport of substrate. For example, data from Mimmack and Higgins (1989) suggested that the maltose transporter complex hydrolyzes two molecules of ATP for one molecule of maltose transported. We speculate that, in ABC transporters, ATP hydrolysis may drive the protein back and forth between two different states or conformations, with the substrate binding site alternately exposed to opposite sides of the membrane. Such states might be mechanistically similar to the open and closed conformations of CFTR.

In summary, these data suggest that the NBDs of CFTR may have structural similarities to GTP-binding proteins and support the idea that there may be functional analogies between the two types of protein. Just as the rate of GTP hydrolysis serves a timing function in signal transduction, the rate of ATP hydrolysis at the NBDs of CFTR may serve a timing function to control the amount of time during which the CFTR Cl^- channel remains in the closed and in the open states.

We thank Drs. Partha Manavalan, Dorr Dearborn, and Alan Smith for sharing their model before publication. We also thank Pary Weber, Lisa DeBerg, and Theresa Mayhew for excellent assistance. This work was supported by the National Heart, Lung and Blood Institute and the Howard Hughes Medical Institute. M. J. Welsh is an Investigator of the Howard Hughes Medical Institute.

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