Walker Mutations Reveal Loose Relationship between Catalytic and Channel-Gating Activities of Purified CFTR (Cystic Fibrosis Transmembrane Conductance Regulator)†

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ABSTRACT: The cystic fibrosis transmembrane conductance regulator (CFTR) functions as an ATPase and as a chloride channel. It has been hypothesized, on the basis of electrophysiological findings, that the catalytic activity of CFTR is tightly coupled to the opening and closing of the channel gate. In the present study, to determine the structural basis for the ATPase activity of CFTR, we assessed the effect of mutations within the "Walker A" consensus motifs on ATP hydrolysis by the purified, intact protein. Mutation of the lysine residue in the "Walker A" motif of either the first nucleotide binding fold (CFTRK464A) or the second nucleotide binding fold (CFTRK1250A) inhibited the ATPase activity of the purified intact CFTR protein significantly, by greater than 50%. This finding suggests that the two nucleotide binding folds of CFTR are functioning cooperatively in catalysis. However, the rate of channel gating was only significantly inhibited in one of these purified mutants, CFTRK1250A, suggesting that ATPase activity may not be tightly coupled to channel gating as previously hypothesized.

The cystic fibrosis transmembrane conductance regulator (CFTR) functions as a chloride channel in the apical membrane of epithelial cells to promote fluid transport across certain tubular or ductular organs (*1*). In cystic fibrosis (CF), the lack of CFTR channel function is thought to cause disease, in part, because it causes increased viscosity of the luminal contents of these organs (*1*). Most naturally occurring mutations in the CFTR gene cause disease by inducing an alteration in protein biosynthesis or by leading to defective channel function (*1*). Of the mutations which cause defective function, many do so by interfering with the opening and closing of the channel gate through which chloride fluxes. Hence, an understanding of the structural basis for channel gating will provide a framework with which to understand the molecular basis for disease in these CF patients.

Like other ABC (ATP Binding Cassette) superfamily members, CFTR possesses membrane-spanning domains which are thought to mediate substrate translocation and nucleotide binding folds (NBFs) that contain the Walker A and B motifs as well as a "signature motif" with the consensus sequence LSGGQ (*2*, *3*). The membrane-spanning domains of CFTR form a pore through which chloride ion fluxes, and it has been suggested that the NBFs of CFTR utilize ATP to regulate access through this membrane pore (*4*−*10*). Electrophysiological studies, employing transition-state analogues and magnesium chelators, support models wherein CFTR utilizes the energy released by ATP hydrolysis to directly open and/or close the channel gate (*5*−*7*, *10*). Unlike other ABC transporters, however, ATP dependent chloride flux requires that CFTR be phosphorylated by PKA (*4*, *8*).

We have shown in a recent publication that purified, reconstituted intact CFTR protein is capable of hydrolyzing ATP and, like channel gating, this activity is also dependent on phosphorylation (*11*). Furthermore, we found that a disease-causing mutation in the "signature" motif of NBF1, namely, G551D, abrogated this activity (*11*). However, we did not determine whether one or both NBFs are required for ATPase activity by CFTR. In order to assess the relative roles of the two NBFs of the related protein, the human multidrug transporter (MDR1), several research groups targeted the conserved lysine residue within the Walker A motifs in mutagenesis studies. As mutation of the Walker A lysine residue in either NBF abolished ATPase activity by purified MDR1 protein (*12*, *13*), it has been suggested that both NBFs of MDR1 function cooperatively to hydrolyze ATP (*14*). Recently, the catalytic activity of isolated fusion proteins which include either NBF1 or NBF2 of CFTR has been measured (*15*, *16*). While it was determined that the K464A mutation abrogated ATPase activity of the CFTR−NBF1 fusion protein (*15*), the effect of the K1250A mutation in the context of the CFTR−NBF2 fusion protein is not known. Therefore, to date, there is no information available on the relative role of each nucleotide binding fold in the catalytic activity of the whole molecule. In the present study, we analyzed the catalytic activity of purified, reconstituted CFTR protein containing the Walker lysine mutations in either NBF1 or NBF2. We also determined the channel-gating kinetics for each mutant purified protein reconstituted in planar lipid bilayers in order to reassess the extent of

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coupling between the catalytic and channel activities of CFTR.

MATERIALS AND METHODS

Generation of CFTRK464A and CFTRK1250A Constructs. Mutations were introduced into CFTR cDNA in the bluescript vector (pBQ6.2, kindly provided by Dr. J. M. Rommens, HSC) using the Transformer Mutagenesis Kit (Clontech, Mississauga, Ontario) and specifically designed oligonucleotides. A small cassette containing the specific mutation, i.e., a *Bsp*E1/*Sph*I fragment for the K464A mutation or a *Pm*1I/*Tth*111I fragment for the K1250A mutation was subcloned into a new pBQ6.2 and then sequenced to confirm the introduction of the mutations. Ultimately, K464A (as an *Xba*I/*Sph*I fragment) or K1250A (as a *Pm*1I/*Tth*111I fragment) was subcloned into pBlueBac4 (Invitrogen, Carlsbad, CA) for baculovirus expression.

CFTR Expression and Purification. Recombinant CFTR, CFTRK464A, and CFTRK1250A viruses were produced in Sf9 cells by cotransfection of the baculoviral transfer vector pBlueBac4 encoding CFTR, CFTRK464A and CFTRK1250A ORF with linear wild-type viral DNA (Bac-N-Blue DNA, Invitrogen, Carlsbad, CA). The viral supernatants generated from these transfections were used to infect cells, and recombinant events were detected as blue plaques. Following plaque purification, working stocks of recombinant virus were produced and titered as previously described (*17*, *18*).

The starting material for purification was an Sf9 cell pellet produced from a 1 L suspension culture of cells infected with recombinant baculovirus containing wild-type or mutant CFTR ORF over a 44 h time period. The cells (approximately 10^9) were resuspended in 100 mL of PBS containing 2% Triton X-100 and a cocktail of protease inhibitors (leupeptin 10 mg/mL, aprotinin 10 μg/mL, E64 10 mM, benzamidine 1 mM, DTT 2 mM, and $MgCl_2$ 5 mM) and DNase 1 (20 units/mL). The mixture was nutated for 1 h at room temperature, after which the insoluble material was centrifuged at 100000*g* for 2 h. The resulting pellets were treated with 200 mL of 2% SDS, 3% mercaptoethanol in 10 mM sodium phosphate, pH 7.2, and the mixture was stirred overnight at 4 °C. Insoluble material was centrifuged at 60000*g*, and the supernatant was filtered through a 0.22 μm filter before being applied at 1 mL/min to a ceramic hydroxyapatite column. After being washed, eluted and identified the CFTR-containing fractions from the ceramic hydroxyapatite column were concentrated and applied to a Superose 6 column as the final purification step. The purified protein was quantitated by amino acid analysis. NH_2-terminal sequencing and amino acid analysis were performed by the HSC-U of T-Pharmacia Biotechnology Centre. An identical procedure was used for the purification of the CFTR variants CFTRK464A and CFTRK1250A.

CFTR Reconstitution. Fifty micrograms of CFTR protein in 0.25% LiDS was concentrated with a Centricon 100 concentrator (Amicon) to a final volume of 100 μL. The protein was further diluted 10-fold with a buffer containing 8 mM HEPES, 0.5 mM EGTA, and 0.025% sodium azide, pH 7.2 (buffer A), and reconcentrated to 100 μL, yielding CFTR protein with a final LiDS concentration of 0.92 mM. The protein was then added to 200 μL (2 mg) of a liposome preparation (PE:PS:PC:ergosterol, 5:2:1:1 by weight) prepared by sonication in buffer A. The lipid−protein mixture was incubated at room temperature for 1 h, transferred to a dialysis bag (Spectra/Por membrane, molecular mass cutoff = 50 kDa), and dialyzed for 17 h against 2 L of buffer A containing 2.5% sodium cholate. This was followed by a 24 h dialysis against 4 L of buffer A and another 24 h of dialysis against buffer A prepared without sodium azide.

Protein Detection. SDS−PAGE of purified wild-type CFTR, CFTRK464A, and CFTRK1250A protein was performed using 6% acrylamide gels (NOVEX, San Diego, CA), and protein was detected with silver stain, as previously described (*19*). For immunoblotting, protein was transferred to a nitrocellulose membrane and probed with an anti-CFTR mAb, M3A7 (*18*). Immunoreactive bands were visualized by chemiluminescence using the ECL system (Amersham, Oakville, CA)

Phosphorylation of Wild-Type and Mutant CFTR. Wild-type CFTR, CFTRK464A, and CFTRK1250A were phosphorylated in liposomes by the catalytic subunit of PKA as previously described (*11*). Protein-free liposomes were similarly treated as a control. In order to remove PKA after the phosphorylation reaction, CFTR proteoliposomes and protein-free liposomes were airfuged at 100000*g* for 30 min and then washed twice by sonication with buffer A and pelleted in the airfuge. Pellets were resuspended in the appropriate buffer for ATPase measurements (see below). For bilayer studies of channel function, CFTR was phosphorylated as described in the preceding paragraph, and PKA was separated from the proteoliposomes by microspin chromatography using Sephadex G-50.

Assay of Wild-Type and Mutant CFTR ATPase Activity. ATPase activity was measured as the production of [α-^{32}P]-ADP from [α-^{32}P]ATP by purified, reconstituted wild-type CFTR, CFTRK464A, and CFTRK1250A, as previously described (*11*). This ratio is corrected for spontaneous hydrolysis by subtracting the [α-^{32}P]ADP/[α-^{32}P]ATP ratio of control liposomes (no CFTR) from the experimental ratio. Radiolabeled ADP and ATP are separated by polyethyleneimine (PEI) cellulose chromatography (*11*). Unless otherwise stated, the assay was carried out in a 15 μL reaction mixture containing 100 ng of CFTR in phospholipid liposomes, 20 mM Tris, 40 mM NaCl, 5 mM $MgCl_2$, 1 μCi of [α-^{32}P]ATP (3 Ci/μmol), and 1 mM cold ATP at pH 7.5. Reaction mixtures were incubated at 30 ^{o}C for 4 h, and reactions were stopped by addition of 5 μL of 10% SDS. Samples (1 μL) were spotted on a PEI cellulose plate (Aldrich, WI) and developed in 1 M formic acid/0.5 M LiCl. The location and quantitation of the radiolabeled ATP and ADP were determined with a PhosphorImager. Data were analyzed using the ImageQuant software package (Molecular Dynamics, Sunnyvale, CA).

Assays of Wild-Type and Mutant CFTR Chloride Channel Activity: Concentrative Tracer Uptake Assay for Study of Chloride Channel Activity. A concentrative tracer uptake assay developed by Garty et al. (*20*) and modified by Goldberg and Miller (*21*) was used to characterize the chloride conductance properties of reconstituted CFTR. Proteoliposomes were preloaded with 150 mM KCl and centrifuged through Sephadex G-50 columns equilibrated with glutamate salts, K-glutamate (125 mM), Na-glutamate (25 mM), glutamic acid (10 mM), and Tris-glutamate (20 mM; at pH 7.6), to replace external chloride. Uptake was

initiated and quantified by addition of 1.0 μCi/mL of $^{36}Cl^-$. Intravesicular $^{36}Cl^-$ was assayed at various time points following separation of liposomes from the external media using a mini anion-exchange column (Dowex 1) (*22*).

Planar Bilayer Studies of Single-Channel Activity. Planar lipid bilayers were formed by painting a 10 mg/mL solution of phospholipid (PE:PS at a ratio of 1:1) in *n*-decane over a 200 μm aperture in a bilayer chamber. Typically, the cis compartment of the bilayer chamber, defined as that compartment to which liposomes were added, contained 300 mM KCl, and the trans compartment, connected to ground, contained 50 mM KCl. As in our previous studies, proteoliposome fusion with planar lipid bilayers was facilitated and detected by the introduction of nystatin (120 μg/mL), a technique originally described by Woodbury and Miller (*23*). Single-channel currents were detected with a bilayer amplifier (custom made by M. Shen, Physics Lab., University of Alabama). Data were recorded and analyzed using pCLAMP 6.0.2 software (Axon Instruments Inc., Foster City, CA). Prior to analysis of dwell times, single-channel data were digitally filtered at 100 Hz.

Histogram Analysis. Open- and closed-time histograms were created with a logarithmic *x*-axis with 10 bins/decade and a lower limit of 10 ms from at least four different experiments (each >5 min in duration). The maximum-likelihood method was used to fit the data with one or two exponentials (pClamp 6.0 software, Axon Instruments). The "goodness" of fit was assessed using the log likelihood ratio test. Analysis of the biexponential fit of channel closed times histograms provided an estimate of the burst delimiter (tc) as described by Winter et al. (*24*). The tc corresponds to the nadir between two peaks of the relationship defining the first and second closed-time constants. As in our previous studies (*11*), a value for tc of 60 ms was determined as the mean for four different experiments using wild-type CFTR. The mean value of tc for five different studies using CFTRK464A protein was 53 ± 0.5 ms. For CFTRK1250A, the data from five experiments was combined to generate the closed-time histogram because channel openings were relatively infrequent. A value for tc of 90 ms was determined for CFTRK1250A. Burst analysis was then performed using the tc value appropriate for each CFTR variant.

Statistics. Results are expressed as means ±SD. Statistical significance was assessed using the log likelihood ratio test or by "two-tailed *t*" tests for parametric or nonparametric comparisons as appropriate.

RESULTS

Disruption of the ATPase Activity of the Intact Purified CFTR Protein by Mutation of the Walker A Lysine in either NBF1 or NBF2. In order to evaluate the contribution of the first nucleotide binding fold (NBF1) to the overall ATPase activity of the intact CFTR molecule, we assessed the effect of disrupting the Walker A motif in NBF1. We found that the ATPase activity of the purified CFTRK464A protein was significantly reduced compared with that of the purified wild-type protein (Figure 1). Figure 1 shows the ATP dependence of the ATPase activity of purified wild-type CFTR and purified CFTRK464A, as a function of increasing ATP concentration, prior to and following PKA phosphorylation. As we previously reported, phosphorylation of purified wild-type CFTR by PKA increased its ATPase activity by decreasing its apparent K_m for ATP (*11*). We observed a similar effect for CFTRK464A, whereby PKA phosphorylation caused a shift in the apparent K_m for ATP. These data were fitted by nonlinear regression using the Michaelis Menten equation to yield kinetic parameters. As the apparent *K*m's of PKA-treated wild-type CFTR and CFTRK464A proteins were 407 and 490 μM, respectively, mutation of the Walker lysine residue in NBF1 does not alter the nucleotide affinity of CFTR remarkably. On the other hand, the V_{max} of the ATPase activity of CFTRK464A (i.e., 3 nmol/mg/min) is significantly reduced compared to that of the wild-type protein (63 nmol/mg/min). These analyses indicate that disruption of the Walker A motif in NBF1 impairs the catalytic activity of CFTR and, further, that NBF1 contributes to the hydrolytic activity of the intact protein. As the catalytic activity of CFTRK464A is considerably less than 50% of the catalytic activity of wild-type CFTR, it is likely that NBF2 is not functioning independently to hydrolyze ATP in the context of the whole molecule or, alternatively, it is not as active an ATPase as NBF1.

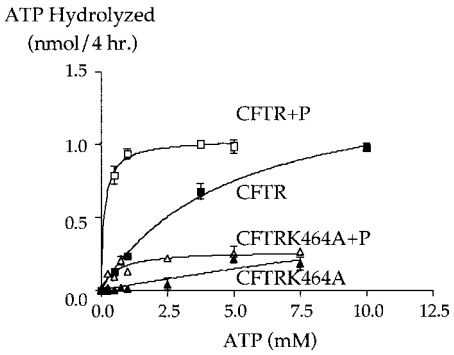

FIGURE 1: CFTRK464A exhibits decreased catalytic activity. The graph compares the ATPase activity of wild-type CFTR and CFTRK464A as a function of increasing MgATP concentrations. The ATPase activity was measured in both PKA-treated protein (wild-type, empty squares; CFTRK464A, empty triangles) and untreated protein (wild-type, solid squares; CFTRK464A, solid triangles). Each reaction mixture contained either 75 ng of wild-type CFTR protein or 500 ng of CFTRK464A protein and was incubated for 4 h before the reaction was terminated. Each point shows mean ± SD of triplicate experiments. The curves were fitted by nonlinear regression analysis, using the Michaelis Menten equation. The best curve fit was assessed as that fit which corresponded to the lowest SD. The software program used for regression analysis was Prism (Graph Pad, San Diego,CA).

Mutation of the conserved lysine residue in Walker A of NBF2, CFTRK1250A, also caused a profound reduction in ATPase activity by the intact, purified CFTR protein (Figure 2). In the presence of 1 mM MgATP, the untreated and the phosphorylated CFTRK1250A proteins exhibited ATPase activities of 0.58 ± 0.75 and 0.96 ± 0.96 (nmol/mg/min), respectively. These values are not significantly greater than control rates, i.e., hydrolysis by liposomes without CFTR protein. Further, the catalytic activity of CFTRK1250A was too low to reliably assess the ATP dependence of this function. However, as the low level of ATPase activity of CFTRK1250A could not be significantly increased even at the high ATP concentration of 20 mM (data not shown), we suggest that the catalytic activity of CFTRK1250A is probably severely impaired relative to the wild-type protein. Hence, NBF2 normally contributes significantly to the overall catalytic activity of the intact CFTR molecule. Furthermore,

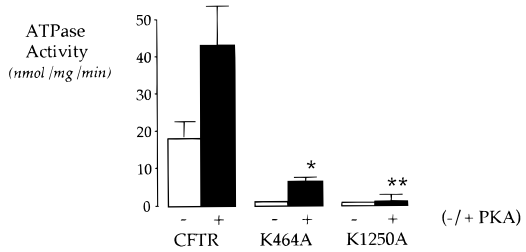

FIGURE 2: Comparative ATPase activity of Walker A mutants in NBF1 and NBF2 of purified CFTR. ATPase activity of 10 different preparations of wild-type CFTR, five preparations of CFTRK464A, and three preparations of CFTRK1250A purified protein, in the presence of 1 mM MgATP, have been compared. Mean ± SD has been shown. Treatment with the catalytic subunit of PKA caused significant stimulation of the ATPase activity of wild-type CFTR ($p < 0.02$) and CFTRK464A ($p < 0.04$). The ATPase activity of each of the PKA-treated mutants, CFTRK464A and CFTRK1250A, was significantly lower than that of the PKA-treated wild-type protein ($p < 0.01$ and $p < 0.03$, respectively*). The ATPase activities of PKA-treated CFTRK464A and CFTRK1250A are significantly different from each other ($p < 0.04$**).

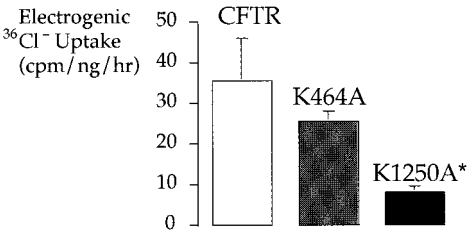

FIGURE 3: Comparison of the capacity of wild-type and mutant CFTR to mediate electrogenic flux. Cumulative uptake of $^{36}Cl^-$ over 1 h was measured for different preparations of purified, PKA-phosphorylated CFTR ($n = 4$), CFTRK464A ($n = 4$), and CFTRK1250A ($n = 3$). Mean ± SD has been shown. Each proteoliposome preparation contained approximately 50 μg of purified protein in 20 mg of phospholipids. There was no significant difference between the cumulative uptake mediated by wild-type CFTR and CFTRK464A ($p > 0.1$). However, there was a statistical difference in $^{36}Cl^-$ uptake between CFTR and CFTRK1250A (*, $p < 0.01$).

as the ATPase activity of CFTRK1250A is negligible, it is clear that NBF1 cannot function independently to hydrolyze ATP in the context of the whole molecule. Therefore, our analyses of CFTRK464A and CFTRK1250A suggest that both NBFs of CFTR probably function cooperatively to hydrolyze ATP.

Disruption of the Chloride Channel Activity of the Intact Purified CFTR Protein by Mutation of the Walker A Lysine in either NBF1 or NBF2. We assessed the consequences of each of the Walker lysine mutations, K464A and K1250A, on the chloride channel activity of CFTR using two assays. First, we employed an electrogenic flux technique which permits assessment of the capacity of a population of purified molecules, reconstituted in phospholipid liposomes, to mediate chloride electrodiffusion. Second, we used the planar lipid bilayer technique to study the detailed gating kinetics of individual mutant CFTR channels.

As we have previously shown, PKA-phosphorylated, but not untreated, CFTR proteoliposomes can mediate the electrodiffusion of chloride ion, monitored as the accumulation of $^{36}Cl^-$ in response to an inwardly directed positive potential difference (Figure 3) (*22*). We did not detect a significant reduction in the accumulation of $^{36}Cl^-$ mediated by PKA-phosphorylated CFTRK464A compared with phosphorylated, wild-type CFTR ($p > 0.1$). There was, however, a significant decrease in the $^{36}Cl^-$ electrodiffusion mediated by PKA-phosphorylated CFTRK1250A compared with the phosphorylated wild-type protein ($p < 0.01$). While the flux assay of electrogenic chloride transport provides only a gross assay of transport function, the results suggest that the single channel opening rate for CFTRK464A is close to that of the wild-type protein, whereas the opening rate for CFTRK1250A is impaired relative to the wild-type and CFTRK464A protein.

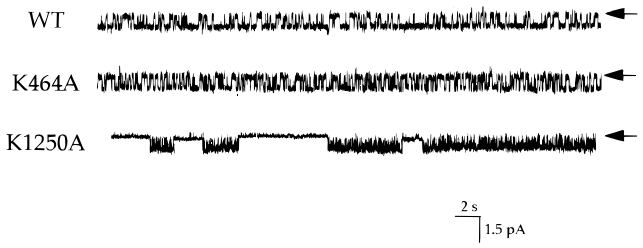

FIGURE 4: Single-channel activity of CFTR, CFTRK464A, and CFTRK1250A. Representative traces of channel activity exhibited by PKA-phosphorylated CFTR, CFTRK464A, and CFTRK1250A in the presence of 1 mM MgATP at the holding potential of −40 mV. The cis compartment of the bilayer chamber contained 300 mM KCl, and the trans compartment, 50 mM KCl. The arrows indicate the closed conductance state of the channel.

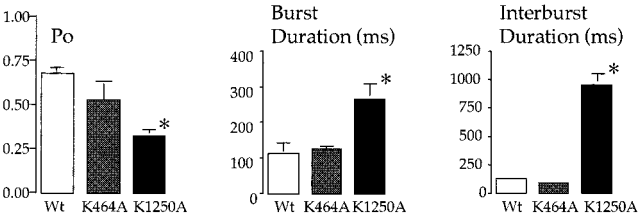

FIGURE 5: Kinetic analyses of CFTR, CFTRK464A, and CFTRK-1250A. The open probability, channel burst duration, and channel interburst duration were determined for CFTR, CFTRK464A, and CFTRK1250A on the basis of 8−10 min of single-channel recording. Mean ± SD has been shown. The differences between CFTR and CFTRK464A with respect to all of these parameters were not statistically significant. On the other hand, the open probability, burst duration, and interburst duration of CFTRK1250A channels were significantly different from those of wild-type CFTR channels (*, $p < 0.01$). The mean burst and interburst durations were determined using tc = 60 ms for CFTR, tc = 53 ms for CFTRK464A, and tc = 90 ms for CFTRK1250A.

In order to examine the details of CFTR channel function, proteoliposomes containing the mutant molecules were fused with planar lipid bilayers, and single channel gating kinetics were analyzed. As expected on the basis of the electrogenic flux assay, the open probability of CFTRK464A single channels was only moderately reduced compared to the wild-type CFTR single channels, 0.68 ± 0.05 vs 0.53 ± 0.09, respectively (Figures 4 and 5). Further, as expected from the flux assay, the open probability of the CFTRK1250A single channels was markedly impaired relative to wild-type channels with a mean value of 0.34 ± 0.06 (Figures 4 and 5). While the single-channel gating of CFTRK464A was almost indistinguishable from that of the wild-type protein, the gating properties of CFTRK1250A were markedly altered (Figure 5). The decreased opening probability of CFTRK1250A is primarily due to an eightfold prolongation of the long channel closed time, or interburst duration from 142 ± 5 to 950 ± 110 ms. Hence, the opening rate to a burst is markedly impaired in CFTRK1250A single channels. The closing rate

of CFTRK1250A channels, determined as the inverse of the open burst duration (265 ± 50 ms), is also diminished by two- to three-fold in the case of CFTRK1250A, as previously reported in other studies (*9*, *10*, *25*).

DISCUSSION

The Two NBFs of CFTR Hydrolyze ATP in a Cooperative Manner in the Intact Molecule. Our studies suggest that the catalytic activity of CFTR is dependent on effective coupling between the two nucleotide binding folds, as disruption of the Walker A consensus site in either NBF significantly impairs overall ATPase activity of the whole purified CFTR molecule. While the concept of coupling between these domains is not new and has been invoked in previous models (*5*, *7*, *14*, *26*), this paper provides the first direct evidence that coupling is required for normal catalytic function of CFTR. Similar observations have been previously reported for the related, class I P-glycoproteins human MDR1 (*12*, *13*) and mouse MDR3 (*27*). Mutation of the Walker A lysine residue in either the first or second NBF of these molecules also causes complete abrogation of their overall ATPase activity. An "alternating site" model has been invoked to describe the catalytic activity by the two NBFs of P-glycoprotein (*14*), and the present data obtained with purified CFTR mutants is also consistent with this model.

Our results also show that the two NBFs may not be functionally identical in the context of the whole CFTR molecule. Whereas the NBF1 mutant, CFTRK464A, retained approximately 10% of wild-type ATPase activity, the NBF2 mutant, CFTRK1250A, retained barely detectable levels of ATPase activity (according to data shown in Figure 2). The double mutant, CFTRK464A/K1250A, also exhibits negligible catalytic activity, comparable to that of CFTRK1250A (data not shown), supporting our suggestion that the two Walker sites are not symmetrical. The apparent differences in the ATPase activity of the two Walker mutants may reflect differences in inherent nucleotide binding affinities, differences in the phosphorylation status of each mutant protein, and/or differences in the situation of the Walker A lysine residue in the context of the catalytic site at each NBF. Currently, we cannot discriminate between these possibilities.

Cooperative ATPase Function of the Two NBFs Is Loosely Linked to Opening of the Channel Gate. Our assessments of the channel function of purified phosphorylated wild-type CFTR and Walker A mutants, CFTRK464A and CFTRK-1250A, fail to support previous hypotheses which suggest that there is tight coupling between the catalytic activity of CFTR and channel gating (*5*, *9*). While our previous analyses of a CFTR mutation in the signature motif of NBF1, CFTRG551D, showed that *both* ATPase activity by the whole molecule and the rate of channel opening were severely impaired to less than 10% of those of wild-type CFTR (*11*, *26*), such strict concordance between these activities was not observed for the Walker A mutant in NBF1. In the current studies of purified, reconstituted protein, the CFTRK464A mutation caused only modest changes in channel activity. The present analyses of the gating kinetics of purified CFTRK464A compare reasonably well with other reports of the channel-gating kinetics of this mutant studied in biological membranes (*9*, *10*, *25*). While previous studies report a relatively minor, albeit significant, reduction in the rate of CFTRK464A channel opening to a burst, i.e. approximately twofold lengthening of the interburst duration (*9*, *25*), our studies of purified CFTRK464A failed to show significant differences in the interburst dwell time. On the other hand, the purified, phosphorylated CFTRK464A protein exhibited a near 10-fold decrease in ATPase activity from that measured for the purified, phosphorylated wild-type CFTR. Hence, for this NBF1 mutant, there appears to be some dissociation between its effects on the channel-gating and catalytic activity of CFTR.

Mutation of the Walker A lysine in NBF2, CFTRK1250A, caused a significant inhibition on both the rate of channel gating and ATP catalysis. Previous analyses of the gating kinetics of CFTRK1250A in biological membranes gave quite variable results (*9*, *10*, *25*). In general, however, the effects on channel gating were more severe for CFTRK1250A than for CFTRK464A. Carson et al. (*9*) showed that CFTRK1250A exhibited prolongation of both the channel interburst dwell time and the channel burst duration. We report similar results for purified CFTRK1250A in the present paper, wherein the gating of this mutant was very sluggish. The channel open probability was decreased from that of wild-type by about twofold, because the opening rate was retarded more than the closing rate from a burst, suggesting that a defect in channel opening rate is the predominant lesion in this mutant.

Our data obtained for the Walker A mutant, CFTRK464A, argues that the two activities of CFTR may be rather loosely coupled to one another and that there may be a low threshold for catalytic activity required for channel gating. Possibly, this threshold catalytic activity is attained by CFTRK464A but not by CFTRK1250A (or CFTRG551D). Alternatively, our data could be explained by a model in which normal ATP binding to the NBFs, rather than hydrolysis, is required for normal gating. According to our analysis, ATP binding to CFTRK464A is close to normal values, and this may account for the normal gating exhibited by this mutant. However, further comparative analysis of the nucleotide binding affinities of each of the Walker mutants must be carried out in order to assess the validity of this model.

The apparent loose association between ATPase and channel-gating activities observed in the case of the Walker mutants is unlikely to be due to inherent limitations to the methods used. Conceptually, significant differences in the percentage of properly folded and reconstituted mutant and wild-type CFTR protein could account for the large differences in overall ATPase activity expressed per milligram of total protein. On the other hand, it is likely that only properly folded wild-type or mutant CFTR molecules will confer 10−11 pS single channel currents upon fusion with planar lipid bilayers. Hence, we may be studying different populations of CFTR molecules with these two assay systems. However, the electrogenic chloride flux assay, like the ATPase assay, reflects the overall activity of successfully reconstituted protein relative to the total protein. Both the electrogenic flux assay and single channel gating studies of CFTRK464A support the claim that this NBF1 mutant exhibits channel activity which is close to that of the wild-type protein, despite a 10-fold decrease in catalytic activity.

In summary, on the basis of our analyses of both the ATPase activity and channel gating by the purified Walker A mutants of CFTR, CFTRK464A, and CFTRK1250A, we

propose a revision of the current model for the mechanism of action of CFTR. We propose that the coupling between the overall catalytic activity and channel gating is not very tight, as relatively profound changes in catalytic activity lead to more moderate changes in channel gating. As yet, it is not clear whether a similarly "loose" relationship between the turnover number for transport and ATP hydrolysis may exist for other ABC proteins. While Ambudkar et al. (*31*) and Eytan et al. (*32*) have reported that the turnover numbers for drug transport and ATP turnover by P-glycoprotein are comparable, Bishop et al. report that there appears to be a rather loose coupling between ATP hydrolysis and histidine transport with the stoichiometry of 5:1 for the histidine periplasmic permease (*33*).

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