Perturbation of the Pore of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Inhibits Its ATPase Activity*

Received for publication, November 16, 2000, and in revised form, December 15, 2000
Published, JBC Papers in Press, December 21, 2000, DOI 10.1074/jbc.M010403200

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Mutations in the cystic fibrosis gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) lead to altered chloride (Cl−) flux in affected epithelial tissues. CFTR is a Cl− channel that is regulated by phosphorylation, nucleotide binding, and hydrolysis. However, the molecular basis for the functional regulation of wild type and mutant CFTR remains poorly understood. CFTR possesses two nucleotide binding domains, a phosphorylation-dependent regulatory domain, and two transmembrane domains that comprise the pore through which Cl− permeates. Mutations of residues lining the channel pore (e.g. R347D) are typically thought to cause disease by altering the interaction of Cl− with the pore. However, in the present study we show that the R347D mutation and diphenylamine-2-carboxylate (an open pore inhibitor) also inhibit CFTR ATPase activity, revealing a novel mechanism for cross-talk from the pore to the catalytic domains. In both cases, the reduction in ATPase correlates with a decrease in nucleotide turnover rather than affinity. Finally, we demonstrate that glutathione (GSH) inhibits CFTR ATPase and that this inhibition is altered in the CFTR-R347D variant. These findings suggest that cross-talk between the pore and nucleotide binding domains of CFTR may be important in the in vivo regulation of CFTR in health and disease.

Cystic fibrosis (CF) is a genetic, lethal disease that affects epithelial cells lining the duct-like passages of the respiratory tract, gastrointestinal tract, and the biliary and reproductive systems (1). Although the protein coded by the CF gene, the cystic fibrosis transmembrane conductance regulator (CFTR), has been implicated in multiple functions in epithelial tissues (2), it acts primarily as a nucleotide- and phosphorylation-regulated chloride channel (3–5).

CFTR belongs to the ATP-binding cassette (ABC) superfamily of membrane proteins (6). Other clinically important members of this family include P-glycoprotein (MDR1), the phosphatidylincholine translocase (MDR3), the multidrug resistance-related protein (MRP1), and the sulfonylurea receptor (SUR1; Refs. 7–10). Like CFTR, these family members possess two nucleotide binding domains (NBDs), which bind and hydrolyze ATP, and membrane-spanning domains (transmembrane domains (TMDs)), which form (or regulate, in the case of SUR1) a pathway for the movement of certain substrates through the membrane (3, 11).

Although the mechanism underlying CFTR channel activity remains unclear, it is generally thought that MgATP binding and hydrolysis by phosphorylated CFTR is required for normal regulation of channel gating from the open and closed conductance states (12–23). This current model is based on electrophysiological studies of channel gating and biochemical studies of ATPase activity. Several electrophysiological studies of the chloride channel function of phosphorylated CFTR show that MgATP, but not nonhydrolyzable ATP analogues, causes channel activation (3, 20–22). Furthermore, nonhydrolyzable ATP analogues and ATPase transition-state analogues like orthovanadate and berillium chloride disrupt normal transitions from the open to the closed configuration (20, 24). Our studies of purified, reconstituted CFTR revealed that CFTR, like other ABC proteins, possesses intrinsic ATPase activity and that, like channel gating, this catalytic activity is activated by phosphorylation (18, 25). Furthermore, chelation of magnesium, an essential cofactor of catalytic activity by other ATPases, inhibited ATPase activity and altered the rate of channel gating (18, 23). Moreover, the disease-causing mutation in the conserved Walker C motif of the first NBD of CFTR, i.e. CFTR-G551D, impaired both ATPase activity and the rate of channel opening (18). These studies show that the NBDs can transmit signals (conferred by nucleotide occupancy and/or hydrolysis) to the channel pore and regulate the gate that controls chloride flux through the pore of CFTR.

For the mammalian ABC membrane proteins P-glycoprotein, MDR3 and MRP1, there is evidence supporting long-range signal transduction from the membrane-spanning domains to the nucleotide binding domains. Substrates that bind to residues in the transmembrane domains of these transporters induce changes in the ATPase activity by the NBDs (26–29). In fact, ATPase activity of P-glycoprotein stimulated by certain substrates has been shown to correlate well with transport, since the turnover numbers are similar (26). Inhibitors that bind to the membrane domain to block the transport of these substrates also cause a change in the catalytic activity (30, 31). Furthermore, modification of certain cysteine residues strategically engineered into the transmembrane domains of P-glycoprotein inhibited its catalytic activity, and this effect could be protected by substrates of P-glycoprotein (30–32). Therefore, cross-talk between the pore and NBDs is an important means

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To date, there are several clues that signals originating from the transmembrane domains of CFTR may regulate the catalytic activity of this protein. For example, diphenylamine-2-carboxylic acid (DPC), an inhibitor that permeates the chloride channel pore of CFTR, not only causes a change in the rate of chloride ion flux but also the rate of channel gating (33). Furthermore, certain disease-causing mutations in the transmembrane domains of CFTR have been shown to cause a change in gating (33–35).

In the present work, we found that perturbations of the pore either by the channel blocker DPC or through mutagenesis inhibited ATP turnover rate by purified CFTR protein. These results provide direct biochemical evidence suggesting that pore properties can signal feedback inhibition to the NBDs. Furthermore, low millimolar concentrations of GSH appear to inhibit CFTR ATPase activity via a similar pathway, revealing the potential physiological significance of this regulatory mechanism in health and disease.

EXPERIMENTAL PROCEDURES

Production and Purification of CFTR-His Proteins—Procedures describing productions and purification of CFTR-His proteins were published previously (36). Briefly, Sf9-baculovirus expression system was used for large scale production of wild type or mutant CFTR proteins. Crude plasma membranes from frozen Sf9 cell pellets expressing recombinant CFTR-His proteins were obtained and solubilized in 8% pentadecafluorooctanoic acid, 25 mM phosphate, pH 8.0. Purification of CFTR-His proteins was performed using nickel affinity chromatography. The solubilized and filtered samples were applied to a freshly generated nickel column at a rate of 2 mL/min. A pH gradient (pH 8.0–6.0) was then applied to the column using fast protein liquid chromatography, and 5-mL fractions were collected.

Identification and Analysis of Immunopositive Fractions—Dot blot was used for analyzing the fractions eluted from the column for the presence of CFTR protein. Immunopositive fractions were selected, and the corresponding fractions were subjected to 6% SDS/polyacrylamide gel electrophoresis (PAGE) and silver-stained to enhance chemiluminescence (Amersham Pharmacia Biotech). The solubilized and filtered samples were applied to a freshly generated nickel column at a rate of 2 mL/min. A pH gradient (pH 8.0–6.0) was then applied to the column using fast protein liquid chromatography, and 5-mL fractions were collected.

Statistical Analysis—Results are expressed as the mean ± S.E. Statistical significance was assessed using one-way analysis of variance (ANOVA; with or without trend, as appropriate) or by two-tailed Student’s t test. For comparison of drug treatment to 100% control, one population Student’s t test was used.

RESULTS

The Pore Blocker DPC Inhibits ATPase Activity of Wild Type CFTR—As reported previously (19), purified and reconstituted CFTR protein displays low level ATPase activity unless phosphorylated by exogenous PKA (Fig. 1A). Electrophysiological studies have shown that PKA phosphorylation is also required for the chloride channel function of CFTR (4, 40). As seen in Fig. 1B, the open channel blocker DPC inhibited the catalytic activity of phosphorylated CFTR at concentrations of 2 and 10 mM. This inhibitory effect was evident only for phosphorylated protein (Fig. 1C), as expected if DPC acts inside the open channel pore. A modest (but statistically insignificant) decrease in ATPase activity (~8%) in phosphorylated CFTR samples was also observed with 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), another channel blocker from the arylaminobenzolate family (data not shown). Kinetic analyses revealed that DPC inhibits ATPase activity through an effect on nucleotide turnover (Fig. 2). As shown in Fig. 2, DPC treatment caused a 4–5-fold decrease in the Vmax of CFTR-ATPase, from 0.84 (nmol of ATP hydrolyzed/2 h) to 0.18 (nmol of ATP hydrolyzed/2 h). On the other hand, DPC did not have any effect on ATP binding to the NBDs of phosphorylated CFTR, with a Kd of 0.4 mA (ATP) close to that typically observed in the absence of drug (0.3 mA (18)). These data suggest that DPC is unlikely to interact directly with the NBDs but, rather, decreases catalytic activity through its effect on the pore.
of action of these inhibitors is less clearly defined than for DPC (41). In contrast to our findings with DPC, interaction of these blockers with CFTR failed to cause a significant change in the ATPase activity of either the unmodified or PKA-phosphorylated CFTR protein at concentrations typically used to block CFTR chloride channel activity (Refs. 41 and 42; Fig. 3). In fact, both DIDS and glibenclamide tended to enhance (though not significantly) the ATPase activity of unmodified and PKA-phosphorylated forms of the protein relative to the effect of the vehicle alone (2.5% Me₂SO). These data suggest that the sites of action of DIDS and glibenclamide on CFTR differ from that of DPC.

Electrophysiological evidence supporting the claim that DPC binds in the open pore of CFTR was obtained in part through chloride competition studies (43, 44). McDonough et al. (44) have shown that the avidity of DPC blockade was dependent on the concentration of chloride ions on the external side of the membrane, with the IC₅₀ for blockade by DPC decreasing with a concomitant decrease in chloride ion concentrations (44). To determine whether the inhibitory effect of DPC on CFTR ATPase activity was also sensitive to changes in chloride ion concentrations, we initially assessed the effect of varying NaCl concentrations on CFTR ATPase activity. Surprisingly, we found that the catalytic activity was affected by changes in NaCl concentration alone in the absence of DPC. These findings made the above competition studies problematic given that the protein is randomly oriented in the inside-out and outside-out conformations in our proteoliposomes. We found that increasing the concentration of NaCl from 14 to 144 mM caused a gradual decrease in the ATPase activity of the PKA-phosphorylated but not the unphosphorylated CFTR protein (44). A similar trend was observed when phosphorylated CFTR was exposed to increasing concentrations (14 to 144 mM) of sodium gluconate or NMDG-Cl salts (Fig. 4B). Furthermore, there was no significant difference between absolute ATPase measurements obtained with NaCl, sodium gluconate, or NMDG-Cl salts at any given concentration (Fig. 4B). As all of these salt solutions evoked similar concentration-dependent effects on CFTR ATPase, we suggest that the response is not specific to chloride and may relate to changes in the ionic strength, affecting the conformation and function of phosphorylated CFTR protein. However, this hypothesis remains to be rigorously tested.

The Pore Mutant CFTR-R347D Exhibits Altered ATPase Activity—Direct evidence for communication between the chloride channel pore and the catalytic domains of CFTR came from assessments of the ATPase activity of a disease-causing variant of CFTR bearing an amino acid substitution (Arg to Asp) at position 347 within the putative pore region. This disease-causing variant has been shown to have altered chloride ion conduction through the pore of CFTR (45). As shown in Fig. 5A, basal specific ATPase activity (i.e., nonphosphorylated preparations) was essentially abolished in this mutant, but activity could be stimulated by PKA phosphorylation, as previously reported for wild type protein (18), suggesting that the purified mutant is not grossly misfolded and can undergo phosphorylation-dependent conformational changes important for catalysis. However, the specific activity of PKA-phosphorylated CFTR-R347D was...
FIG. 4. Effect of salt concentration on the ATPase activity of phosphorylated and nonphosphorylated wild type CFTR protein. Panel A, changes in CFTR ATPase activity in the presence of different concentrations of NaCl relative to 48 mM NaCl control were analyzed by one-way ANOVA ($p = 0.005$), with a post-test for linear trend ($p = 0.002$). Each value is shown as the mean ± S.E. Three protein preparations, studied in duplicates, are shown for each treatment group, except for 30 mM chloride, where each value represents a mean of duplicate values. Panel B, comparison of the effect of 14 mM (hatched bar) or 144 mM (open bar) NaCl, sodium gluconate (Na-Glc), or NMDG-chloride on the catalytic activity of phosphorylated CFTR. The effect of the different salts with the same concentration relative to each other on the catalytic activity of CFTR was analyzed by ANOVA. No statistical differences were found among the different treatment groups with the same salt concentration. However, inhibition of ATPase was seen in all groups at high salt concentration (*, unpaired Student’s $t$ test, $p < 0.02$). Three different protein preparations were studied independently with respect to the effect of NaCl (14 and 144 mM) or Na-Glc (144 mM). For Na-Glc (14 mM) and NMDG-Cl (14 and 144 mM), each value represents the activity for a set of duplicate values.

FIG. 5. Characterization of the effect of CFTR-R347D mutation on the ATPase activity. Panel A, catalytic activity of phosphorylated and nonphosphorylated wild type (WT) and mutant CFTR proteins at 1 mM ATP. The mean ± S.E. is shown for nine phosphorylated and nonphosphorylated wild type CFTR preparations. For CFTR-R347D preparations, each bar represents the activity of duplicate values. Differences between either phosphorylated or nonphosphorylated wild type and mutant preparations were analyzed by unpaired Student’s $t$ test. The asterisk represents a statistically significant difference in ATPase activity of phosphorylated mutant relative to phosphorylated wild type CFTR ($p = 0.014$). PKA phosphorylation of liposomes alone, treated in the same manner as CFTR-R347D preparations, accounted for less than 8% of the ATPase activity observed for the phosphorylated CFTR-R347D protein (0.16 nmol of ATP hydrolyzed/2 h versus 2.03 nmol of ATP hydrolyzed/2 h). The effect of PKA phosphorylation was calculated by subtracting the basal ATPase activity of nonphosphorylated liposomes from PKA-treated liposomes. Panel B, MgATP dependence of the catalytic activity of purified and either phosphorylated or nonphosphorylated CFTR-R347D protein. Curve fitting was performed by nonlinear regression analysis (Prism software, San Diego, CA) using the Michaelis-Menten equation to yield parameters for the phosphorylated protein of $V_{\text{max}} = 1.1$, $K_m = 0.1$ mM and, for the nonphosphorylated protein, of $V_{\text{max}} = 0.2$, $K_m = 1.1$ mM. The mean ± S.E. is shown for each treatment group. Each sample contained ~8 ng of purified, reconstituted CFTR-R347D protein, and duplicate or triplicate samples were assessed. Panel C, effect of ionic strength on the ATPase activity of phosphorylated wild type CFTR and CFTR-R347D proteins. Differences in catalytic activity between the mutant (hatched) and wild type (open) proteins at the same salt concentration were assessed by unpaired Student’s $t$ test. The asterisk represents statistically significant difference ($p = 0.02$). Duplicate samples of wild type protein and triplicate samples of CFTR-R347D were studied.

clearly defective, exhibiting only 5–10% of the activity of phosphorylated wild type protein (1.17 ± 0.14, $n = 3$ and 15.8 ± 5.7, $n = 9$, $p = 0.01$, unpaired Student’s $t$ test). The increase in ATPase activity associated with PKA-treated CFTR-R347D samples was not due to PKA itself, since treatment with this enzyme conferred less than 8% of the total activity measured for the phosphorylated mutant (see legend to Fig. 5A for details).

The MgATP dependence of catalysis by phosphorylated mutant protein was well described using the Michaelis-Menten equation ($r^2 = 0.95$; Fig. 5B), and this analysis revealed that the decrease in specific ATPase activity caused by this mutation was primarily due to a decrease in $V_{\text{max}}$ of the enzyme. Whereas the $V_{\text{max}}$ of phosphorylated wild type CFTR protein is about 50 nmol/mg/min (18), the $V_{\text{max}}$ determined for phosphorylated R347D protein is 1.1 nmol/mg/min (Fig. 5B, Table 1). This reduced ATPase activity is comparable with that obtained for mutations of conserved residues thought to reside in the nucleotide binding pocket in the NBDs (e.g., G551D; (18)). Defective ATPase activity by CFTR-R347D mutant was not due to...
global misfolding of the nucleotide binding folds, as the apparent affinity of the phosphorylated mutant for MgATP was comparable, even somewhat higher, than previously reported for phosphorylated wild type protein ($K_m = 0.1$ mM for CFTR-R347D versus $K_m = 0.3$ mM for wild type protein, respectively (18)).

The pore blocker DPC exerted a similar inhibitory effect on the ATPase activity of phosphorylated CFTR-R347D, as it did on the ATPase activity of wild type protein (67.3 ± 6.1% of control versus 66.8 ± 3.6% of control, respectively). These results support previous reports suggesting that DPC interacts with a pore-lining residue other than Arg-347, probably Ser-341 (44), and that the R347D mutation does not disrupt DPC binding to this site by inducing global misfolding. Moreover, a similar inhibitory trend was observed for increasing salt concentration on the ATPase activity of R347D protein (Fig. 5C), as compared with the wild type protein. However, there was a significantly greater inhibition for the mutant protein at 144 mM NaCl, supporting the notion that electrostatic interactions are altered in this pore mutant (35) and that these interactions may be important in signaling from the membrane domains to the NBDs.

**Glutathione Inhibits the ATPase Activity of Wild Type and Mutant CFTR Proteins**—Linsdell and Hanrahan (46) have shown that GSH, the most potent antioxidant in cells, could permeate through the CFTR channel pore and block chloride flux. Oxidized glutathione (GSSG) could also interact with the pore but with lower affinity. Consequently, we reasoned that GSH and possibly GSSG may alter the catalytic activity of CFTR through modification of the pore. Furthermore, if this regulation occurs at physiological concentrations of GSH (1–10 mM), it is likely to be biologically important. As shown in Figs. 6, A and B, GSH within the concentration range of 5–10 mM significantly decreased the ATPase activity of phosphorylated CFTR protein (one-population Student's t test, $p < 0.03$). We have several pieces of evidence to support the notion that GSH is interacting with the open channel pore. First, the effect of GSH was only seen when the protein was phosphorylated, the form of the protein known to gate to the open channel configuration. Furthermore, physiological and high nonphysiological concentrations of GSSG (20 μM and 10 mM, respectively) did not significantly affect the catalytic activity of either phosphorylated or nonphosphorylated CFTR, consistent with its reported weaker affinity with the channel pore (46). Finally, we observed a significant difference in the inhibitory effect of GSH (10 mM) on the ATPase activity of wild type and CFTR-R347D proteins, 39 and 63% of untreated controls, respectively (Fig. 6C, $p = 0.04$). These results suggest that the mutated pore either exhibits altered interaction with GSH and/or an altered glutathione-mediated signal from the pore to the catalytic domains. However, our results do not rule out the possibility that GSH may also have a direct effect on the NBDs of CFTR, and we plan to address this possibility in our future studies.

### TABLE I

|                  | $K_m$ (mM) | $V_{max}$ (μmol/mg/min) |
|------------------|-----------|-------------------------|
|                  | PKA       | -PKA                    |
| Wild type       | 0.3       | 1.0                     |
| R347D           | 0.1       | 1.1                     |

* Data for wild type CFTR were reported in our previous studies (18).

### DISCUSSION

Although it has been known for quite some time that the catalytic function of other ABC family members, e.g., P-glycoprotein and MRP1, is regulated by compounds that specifically interact with their membrane domains (26–29), this particular regulatory mechanism has not been previously directly assessed for CFTR. The previous lack of such studies relates primarily to the requirement to purify and reconstitute large amounts of CFTR protein to study its low specific catalytic activity. Second, CFTR is known to possess very different “transport” functions than P-glycoprotein and MRP1. Unlike these related proteins, which are thought to act as pumps, CFTR has been shown to function as an anion channel that utilizes ATP to operate a gate through which permeant anions can flux. There is sparse evidence in the ion channel literature documenting the regulation of channel gating mechanisms by permeant ions. However, for certain members of the CIC family of chloride channels such as CIC-0, it has been determined that voltage-dependent gating may be due in part to concentration of the permeant anion and its translocation through the pore (47–49). With regards to CFTR, there is indirect evidence supporting interaction between permeation and gating, in that certain mutations in the transmembrane segments of CFTR, namely S1118F in TM11 (33) and the disease-causing mutant R117H (50), exhibit altered channel open times. In the present paper, we provide the first direct evidence that perturbation of pore-lining residues of CFTR by either open pore blockers or by mutagenesis can lead to long range conformational changes in NBDs affecting nucleotide hydrolysis. Furthermore, as this mechanism of interdomain signaling can be mediated via pore blockade by low millimolar concentrations of the potent antioxidant glutathione, we suggest that it is biologically important.

Our work shows that only certain anions that bind to the pore-lining residues of CFTR can significantly alter its catalytic activity. The synthetic inhibitor DPC has been previously shown to interact with the open pore to block chloride ion flux (33, 44, 46). Our current data demonstrate that DPC can also inhibit CFTR ATPase activity. However, other open channel blockers such as DIDS, glibenclamide, or NPPB do not affect CFTR ATPase activity, at least at the concentrations employed in this study. Due to the nature of these studies and the requirement for large concentrations of purified protein, we did not perform complete dose response curves for each of these channel blockers. However, it is feasible that we may have seen significant effects on CFTR ATPase activity at higher concentrations of certain drugs. In fact, the incomplete inhibition of CFTR ATPase activity by DPC at 2 mM may reflect nonspecific sequestration of this lipophilic compound onto the liposomes (51). Furthermore, as there is no clear consensus regarding their relative potencies of blockade in the literature, with the available data it is difficult to assess whether a correlation exists between the efficacy of chloride channel block by these synthetic compounds and inhibition of CFTR ATPase activity. Future studies of the physical basis for the cross-talk from the pore to the NBDs of CFTR will benefit from a better understanding of the molecular basis for blockade by these inhibitors. Such detailed molecular mapping studies have been initiated by McCarty and co-workers (52) in studies of the voltage-dependent block by DPC and NPPB in wild type and mutant (S341A and T1134F) CFTR. These studies show that both drugs block the open pore, each inhibitor shows differential sensitivity to mutations in putative pore-lining residues, suggesting that the sites of interaction with the pore are overlapping but not identical (52).

Certain organic anions including GSH, GSSG, and gluconate have been previously shown to block chloride ion flux through
CFTR. However, in the present studies only GSH exerted a significant and specific inhibitory effect on the catalytic activity of CFTR. The relative effectiveness of GSH for blockade of CFTR ATPase activity appears to relate to its relatively high efficacy for blockade of chloride ion flux (46).

We found further evidence for direct communication between the pore domain and NBDs of CFTR when investigating the catalytic activity of CFTR-R347D, a disease-causing variant with a mutation in TM6 associated with mild disease (45). The R347D mutation led to inhibition of the ATPase activity of CFTR, suggesting that the region in which this arginine resides participates in the physical communication between the pore and the NBDs. This residue is thought to reside in the inner vestibule of the CFTR channel and has been implicated in anion binding within the pore (45, 50). Alternately, Cotten and Welsh (35) proposed recently that Arg-347 has an important role in stabilizing the pore architecture. Future studies are required to determine whether alterations in anion binding site occupancy and/or pore architecture caused by this mutation provide the trigger for long range changes in the NBDs.

Both the interaction of DPC with the pore and mutation of the putative pore-lining residue, R347D, induced similar changes in the catalytic activity of phosphorylated CFTR, namely, both of these perturbations caused a 3–5-fold decrease in the $V_{\text{max}}$ of the enzyme. Previous studies indicate that NBD1 and NBD2 of CFTR functionally interact in the whole protein, and in this context, NBD2 exhibited a higher rate of hydrolysis than NBD1 (16). Hence, we suggest that pore perturbation may decrease overall catalytic activity of CFTR by decreasing the relative contribution by NBD2 to this overall function. This hypothesis remains to be tested in our future studies.

Earlier studies by Kopito and co-workers (53) show that oxidizing and reducing reagents alter the gating kinetics of CFTR channels. For example, treatment with the strong reducing agent dithiothreitol increased both the opening and closing rates of the channel. Such data imply that the redox potential in cells may also alter the catalytic activity of CFTR. We showed that GSH effectively inhibited the catalytic activity of CFTR in concentrations that are biologically relevant, supporting the notion that cellular redox potential might determine the catalytic function of this protein. However, the mechanism of action of dithiothreitol differs from that of glutathione, as we found that dithiothreitol caused a slight stimulation rather than inhibition of CFTR ATPase activity (data not shown), consistent with its stimulatory effect on channel gating reported by Harrington et al. (55). The site of action of dithiothreitol was not indicated in their studies. On the other hand, our data specifically support the suggestion originally proposed by Lindsell and Hanrahan that GSH interacts with the open pore of CFTR (46). First, GSH only inhibited the fully phosphorylated protein, known to be associated with channel activity with a high open probability. Second, mutation of the pore-lining residue R347D led to a change in the extent of blockade of CFTR ATPase activity by GSH.

Our findings show that the ATPase activity of CFTR is inhibited by certain small molecules like GSH, which interact with its membrane domain. The catalytic activity of CFTR has been correlated to channel open probability and conductance, i.e. the rate of chloride flux through the pore (16, 18). Hence, a reduction in catalytic function will be expected to lead to a net reduction in chloride ion flux across biological membranes. Since cellular glutathione levels can vary dramatically, e.g., during the cell cycle (increasing before cell division) and in response to inflammation and oxidative burden (54–57), cellular glutathione may constitute an important mechanism for the regulation of CFTR channel function in vivo.

Previous studies by Lindsell and Hanrahan (46, 58) show that organic anions such as gluconate, GSSG, and GSH can only permeate the CFTR pore from its cytosolic vestibule. The uni-directionality and energy dependence of this process prompted these authors to suggest that CFTR might mediate transport of certain large anions via a pump-like mechanism, similar to that proposed for P-glycoprotein, MDR3, and MRP1 (46, 58). As mentioned previously, substrates that are transported by these ABC proteins are thought to first bind to the membrane domain from which signals are transduced to the NBDs. This causes primarily, but not exclusively, the stimulation of ATPase activity by the NBDs in P-glycoprotein or to inhibition of ATPase activity, as in the case of MDR3. In our present studies, we found that GSH significantly inhibited ATPase activity by purified CFTR. Therefore, we suggest that this modulation may be consistent with the proposed role of

### Figure 6. Effect of glutathione on the catalytic activity of wild type and CFTR-R347D proteins.

Panel A, effect of reduced glutathione (GSH) or oxidized glutathione (GSSG) on the ATPase activity of phosphorylated (closed bars) or nonphosphorylated (open bars) CFTR. Differences in catalytic activity relative to no drug control were analyzed by one-population Student’s $t$ test. The asterisk (*) represents significant changes in ATPase activity ($p < 0.05$). The mean ± S.E. is shown for two to four protein preparations. For 10 mM GSSG, each point represents the mean activity for a set of duplicate values. Panel B, effect of increasing GSH concentrations on the catalytic activity of phosphorylated CFTR as a percentage of control (no GSH). Data were analyzed by ANOVA ($p = 0.0003$), with a post-test for linear trend ($p < 0.001$). Mean ± S.E. is shown for three to four protein preparations. Panel C, effect of 10 mM GSH on the catalytic activity of phosphorylated wild type (WT, open) and CFTR-R347D (hatched) proteins relative to control (no GSH). Differences in ATPase activity were analyzed by Student’s $t$ test. Statistical significance ($p < 0.05$) is marked by asterisk (*). Mean ± S.E. for quadruplicate samples is shown for each treatment.
CFTR in the energy-dependent transport of this anion across epithelial membranes. Clearly, further studies of this putative function using our reconstitution system for purified CFTR are required to substantiate this claim.

Finally, we determined by direct measurement of the ATPase activity of purified and reconstituted wild type and mutant CFTR proteins that perturbation of the pore signals long distance conformational changes to the NBDs and causes a change in the function of these domains. These findings support a novel mechanism for the regulation of this protein and potentially a novel molecular mechanism wherein disease-causing mutations may affect function.

Acknowledgments—We thank Dr. Mary Corey (Research Institute, Hospital for Sick Children) for the assistance with the statistical analysis and Dr. Johanna Rommens for providing us with cDNA coding for the mutant R347D (Research Institute, Hospital for Sick Children).

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