The intact CFTR protein mediates ATPase rather than adenylate kinase activity

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INTRODUCTION

The CFTR [CF (cystic fibrosis) transmembrane conductance regulator] is a member of the ABC (ATP-binding-cassette) superfamily of membrane proteins [1]. As for the other members of this family, its function in the membrane has been linked to its intrinsic ATPase activity [2,3]. However, unlike most family members, CFTR is not an ATP-driven transporter, but rather it is a chloride channel which is thought to utilize ATP binding and hydrolysis to promote conformational changes associated with channel gating [1,4–6]. A current model suggests that the opening of intact phosphorylated CFTR is coupled to ATP-induced dimerization of its two NBDs (nucleotide-binding domains), NBD1 and NBD2, and ATP hydrolysis by these domains initiates channel closure [6–8]. This model was developed on the basis of electrophysiological studies [6,8–10] together with biochemical measurements of ATPase activity by purified and functionally reconstituted CFTR protein [4,11,12].

The molecular basis for the ATPase activity of the CFTR remains unknown, but is likely to be conferred by the heterodimerization of its two NBDs (CF-NBDs) in a head-to-tail orientation, as revealed in crystal structures of prokaryotic ABC proteins [13–22]. Such a dimer of CF-NBDs results in two nucleotide-binding sites at the dimer interface. Interestingly, alignment with related ABC family members and biochemical (8-azido-ATP labelling) studies suggest that only one of these sites is catalytically active [23]. A glutamate residue proximal to the Walker B motif and thought to form the catalytic base in other family members [17,18,24] is conserved in only one of the two CF-NBDs: NBD2 (i.e., Glu1371). According to models based on the above prokaryotic ABC protein structures, Glu1371 in NBD2 of CFTR participates in ATP hydrolysis at an ATP-binding site comprising the Walker A motif on the same domain and the signature (L/GLGGQ) motif conferred by NBD1. The channel function of the CFTR-E1371Q mutant has been studied, and it exhibits a prolonged channel open time, consistent with the idea that ATPase activity promotes channel closure [6,7]. Yet, to date, the consequences of this mutation on catalytic activity have yet to be determined directly.

Two research groups have shown previously that the isolated NBDs of CFTR also exhibit adenylate kinase activity which mediates the reaction: ATP + AMP ↔ 2ADP [25–27]. The biochemical findings were incorporated into a novel model describing nucleotide-dependent gating of CFTR which invokes a role for intrinsic adenylate kinase activity. This model was based in part on patch-clamp studies of CFTR showing that ATP-dependent gating of phosphorylated CFTR is modified by the addition of AMP and Ap5A, an inhibitor of adenylate kinase. However, the authors of these papers acknowledge the importance of evaluating the adenylate kinase activity by using the intact, full-length CFTR protein in order to test their model and establish its physiological significance [27]. Therefore the purpose of this present study was to determine the relative adenylate kinase and ATPase activities of the full-length normal CFTR protein using a biochemical assay which can report both activities concurrently. Furthermore, the consequences of the disease-causing mutation G551D and the catalytic base mutation E1371Q on the enzymatic activity of the full-length protein were also evaluated.

Abbreviations used: ABC, ATP-binding-cassette; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DTT, dithiothreitol; H11470/1, putative metal-chelate ABC transporter encoded by H11470 and H11471 of Haemophilus influenzae; HA, haemagglutinin; Hb, haemolysin B; MaFGK2, maltose transport complex of MalF, MalG and MalK (1:1:2); ModB2C2A, molybdate transporter complex; NBD, nucleotide-binding domain; PKA, protein kinase A; wt, wild-type.

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EXPERIMENTAL

Purification and renaturation of CF-NBDs

Sf9 cell cultures which were grown in suspension (0.5–1 litre) were infected with baculovirus containing CF-NBD1 (residues 380–660) or HA (haemagglutinin)-tagged CF-NBD2 (residues 1201–1446), each bearing polyhistidine (His$_{10}$) tags, for 40 h at 26°C. The generation of the baculoviruses containing these proteins as well as their purification was performed as described previously [28]. Following purification, the CF-NBD proteins were dialysed in Spectra/Por dialysis membrane (12–14 kDa cut-off; Spectrum Laboratories) overnight at 4°C against 4 litres of washing buffer [50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 2.5 mM DTT (dithiothreitol) and 50 μM EGTA]. Dialysis was continued in 4 litres of 10% (v/v) glycerol, 50 mM Tris/HCl (pH 7.4), 50 mM NaCl, 25 μM DTT and 1 μM DTT at 4°C with two buffer changes. Renatured protein samples were concentrated 4-fold using a Centricron YM-10 concentrator (Amicon) and protein enzymatic activity was determined immediately. SDS/PAGE, Western blotting and $^{32}$P-32P electrophoresis were performed as described previously [4]. For Western blotting, the following antibodies were used: anti-NBD1 antibody (L12B4, 1:2000 dilution; Chemicon) and an anti-HA antibody (1:1000 dilution; Sigma). The secondary antibody used was an HRP (horseradish peroxidase)-conjugated goat anti-mouse IgG antibody (1:5000 dilution; Pierce).

Purification and reconstitution of wt (wild-type), CFTR-G551D and CFTR-E1371Q

Detailed protocols regarding the generation of wt and mutant CFTR–His$_{10}$ proteins are described elsewhere [29,30]. A frozen Sf9 cell pellet from a 1 litre expression culture was thawed and solubilized in PBS. Cells were then disrupted using a French press set at 1000 p.s.i. (1 p.s.i. ≈ 6.9 kPa) and centrifuged at 500 g for 20 min at 4°C. The supernatant containing crude membranes was centrifuged at 38,000 rev/min (Ti 70.0 rotor, Beckman) for 2 h at 4°C to yield a plasma-membrane- and microsome-enriched fraction. Membranes were solubilized overnight in 8% (v/v) PFO (pentadecanfluorooctanoic acid) and 25 mM sodium phosphate (pH 8.0). Procedures for purification, reconstitution and phosphorylation of purified CFTR–His$_{10}$ were performed as described previously [29,30]. Phosphorylated samples were centrifuged using an airfuge at 100,000 g for 30 min at room temperature (22°C) to pellet the samples, washed twice with washing buffer [50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 2.5 mM MgCl$_2$ and 1 mM DTT] and then dialysed overnight at 4°C against 4 litres of washing buffer.

Adenylate kinase and ATPase measurements

ATPase activity was measured by determination of the production of $\gamma$-32P]Pi, from $\gamma$-32P]ATP as described previously [27]. Adenylate kinase activity was measured by determination of the production of $\beta$-32P]ADP from $\gamma$-32P]ATP activity in the presence of unlabelled ATP (500 μM) and AMP (400 μM) [27]. The assay was carried out in a reaction mixture (50 μl final volume) containing 1 μg of NBD protein, 10% (v/v) glycerol, 50 mM Tris/HCl (pH 7.4), 50 mM NaCl, 2 mM MgCl$_2$, 0.1 μM dodecyl maltoside and 8 μCi of $\gamma$-32P]ATP (3000 Ci/mmol; PerkinElmer) and unlabelled ATP and AMP at pH 7.5. For assays with the full-length protein, the reaction mixture (25 μl final volume) contained 1 μg of reconstituted protein, 50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 2.5 mM MgCl$_2$ and 1 mM DTT. P$_i$ and ADP were separated from ATP by TLC using the same elution conditions as described by Gross et al. [27].

Analyses

The ATP dose–response for ATPase activity by the full-length protein was fitted with the Michaelis–Menten function using the curve-fitting programs in the GraphPad Prism program. Comparison between three or more groups was performed using a one-way ANOVA program on GraphPad Prism software (version 4.0a; GraphPad, San Diego, CA, U.S.A.) with secondary analysis of data pairs using the Tukey test and P < 0.05 was considered significant.

RESULTS

Isolated CF-NBDs exhibit ATPase and adenylate kinase activity

Previously, we and others determined that the NBDs of CFTR, NBD1 and NBD2, individually exhibit very low levels of ATPase activity relative to that demonstrated by the full-length CFTR protein [28]. Adenylate kinase activity by the isolated NBD1 [27] and NBD2 [26,27] of CFTR has also been measured. The specific ATPase and adenylate kinase activities of these domains appears to be variable between laboratories, and this may reflect differences in the expression system used, the presence of mutations to improve protein solubility and/or the domain boundaries employed for protein expression.

We expressed the two NBDs of CFTR as isolated fragments in Sf9 cells using the baculovirus expression system [28,31]. The domain boundaries for each domain (NBD1, amino acids 380–660 and NBD2, amino acids 1201–1446) were chosen on the basis of homology modelling studies and, in the case of NBD1 (for which crystal structures exist), our boundaries encompass the entire folded structure [32]. We focused on studies of the functional properties of these domains and hence the quantity of protein that we can purify per litre of Sf9 cell culture is suitable. Aside from the polyhistidine (His$_{10}$) tag inserted for purification purposes, the amino acid sequence of these proteins corresponds to the native sequence [28,31].

In this present study, we compared the relative adenylate kinase activity with the ATPase activity of each individual NBD. The generation of free P, was used to report ATPase activity from purified CF-NBDs and the production of [β-32P]ADP from the substrates, and the production of AMP and [γ-32P]ATP was used to assess adenylate kinase activity (ATP + AMP ↔ 2ADP). The radiolabelled products of these reactions were separated by TLC and quantified by phosphoimaging. In the right panel in Figure 1A we show that in the nominal absence of AMP (−) and presence of 500 μM MgATP, purified NBD1 conferred a modest ATPase activity, detected as an increased generation of radiolabelled P, from [γ-32P]ATP compared with the results without protein (Figure 1A, control, left panel). These findings are consistent with previous studies, documenting low levels of ATPase activity by isolated NBD1 [28]. Addition of both ATP (500 μM) and AMP (+, 400 μM) caused the generation of [β-32P]ADP, the product of intrinsic adenylate kinase activity by NBD1. The adenylate kinase activity of isolated NBD1 (in the presence of 500 μM ATP plus 400 μM ATP) is approx. 10-fold greater than the ATPase activity measured in the presence of ATP alone (Table 1). The dose-dependence of the adenylate kinase activity ([AMP]: 10$^{-3}$–10$^{-4}$ M) was analysed using a sigmoidal relationship ($I = 0.9$), yielding a maximal activity of 0.66 nmol/h per μg and an EC$_{50}$ of 30 μM (results not shown). These findings
Table 1 Enzymatic activity determined for CF-NBDs and CFTR

<table>
<thead>
<tr>
<th>Protein</th>
<th>Kinase activity</th>
<th>ATPase activity</th>
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<tbody>
<tr>
<td>NBD1</td>
<td>0.0068 ± 0.0006</td>
<td>0.0005 ± 0.0002</td>
</tr>
<tr>
<td>NBD2</td>
<td>0.0026 ± 0.0003</td>
<td>0.0012 ± 0.0002</td>
</tr>
<tr>
<td>NBD1 and NBD2 co-expressed</td>
<td>0.0040 ± 0.0002</td>
<td>0.0073 ± 0.0001</td>
</tr>
<tr>
<td>Full-length CFTR</td>
<td>0.0008 ± 0.0003</td>
<td>0.18 ± 0.01</td>
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CF-NBDs function predominantly as adenylate kinases in the presence of high micromolar concentrations of AMP.

However, the NBDs of CFTR proteins are likely to function as heterodimers in the full-length CFTR protein, with the catalytic active site(s) formed by both proteins at the dimer interface, as predicted on the basis of homology models using prokaryotic ABC proteins. Therefore we co-expressed both of the NBDs of CFTR in Sf9 cells in order to reconstitute such a heteromeric complex. In Figure 2A(i), we show that both NBD1 and NBD2 were purified. (B) Phosphoimage shows generation of radiolabelled free P, by co-expressed NBD1 and NBD2 (NBD1 + 2) in the absence (−) of AMP (i.e. ATPase activity) and the generation of radiolabelled ADP by co-reconstituted NBD1 and NBD2 in the presence (+) of 400 μM AMP (i.e. adenylate kinase activity) (right panel). Buffer alone served as a control (None, left panel) and these background measurements were subtracted from those obtained for NBD1 and NBD2 in calculations of specific activities. (C) The histogram shows fold increase in ATPase activity in co-purified NBDs. Result is mean ± S.E.M. (n = 3, *, P < 0.001) relative to NBD1 (absolute activities shown in Table 1) with ATP fixed at 500 μM. As in Figure 1, specific ATPase activities were determined following subtraction of control (buffer only). Fold increase in adenylate kinase (AK) activity was conferred by co-expressed CF-NBDs relative to NBD1. Result is mean ± S.E.M. (n = 3), with AMP and ATP fixed at 400 and 500 μM respectively.

**Figure 2** Co-expression of NBDs confers potentiation of ATPase activity rather than adenylate kinase activity

(A) Co-expression of NBD1 and NBD2 (1 + 2) leads to formation of a complex which can be co-immunoprecipitated using an NBD1-specific antibody, L1284 (α1). NBD1 was recognized by Western-blot analysis using α2 (upper panel) and NBD2 was recognized using anti-HA antibody (α2, lower panel) against the HA tag present. There is no band which is immunoreactive with α2 if NBD1 is expressed alone (1), demonstrating the specificity of the immunoprecipitation. Unfortunately, the converse experiment could not be performed as the NBD2-specific antibody (anti-HA antibody) did not effectively immunoprecipitate its target protein in our system. Molecular-mass markers are shown on the left in kDa (kD). (ii) NBD1 and NBD2 can be co-purified by Ni2+-nitrilotriacetate affinity following co-expression. The silver-stained band (SS) is likely to represent both NBD1 and NBD2, although differential migration of these two domains could not be resolved by SDS/PAGE. Immunoblotting using domain-specific antibodies, L1284 (NBD1) and an anti-HA antibody (NBD2) confirmed that both NBD1 and NBD2 were purified. (B) Phosphoimage shows generation of radiolabelled free P, by co-expressed NBD1 and NBD2 (NBD1 + 2) in the absence (−) of AMP (i.e. ATPase activity) and the generation of radiolabelled ADP by co-reconstituted NBD1 and NBD2 in the presence (+) of 400 μM AMP (i.e. adenylate kinase activity) (right panel). Buffer alone served as a control (None, left panel) and these background measurements were subtracted from those obtained for NBD1 and NBD2 in calculations of specific activities. (C) The histogram shows fold increase in ATPase activity in co-purified NBDs. Result is mean ± S.E.M. (n = 3, *, P < 0.001) relative to NBD1 (absolute activities shown in Table 1) with ATP fixed at 500 μM. As in Figure 1, specific ATPase activities were determined following subtraction of control (buffer only). Fold increase in adenylate kinase (AK) activity was conferred by co-expressed CF-NBDs relative to NBD1. Result is mean ± S.E.M. (n = 3), with AMP and ATP fixed at 400 and 500 μM respectively.
The enzymatic activity of full-length wt CFTR

The behaviour of these proteins as isolated fragments may not fully recapitulate their function in the context of the full-length protein. In fact, the activity of the NBD1 plus NBD2 complex was different from that of either NBD alone, arguing that the function of these domains is dependent on their molecular context. Therefore we evaluated the relative ATPase and adenylate kinase activities of the purified reconstituted full-length CFTR protein, a reconstitution system which has been shown to exhibit native CFTR channel function when phosphorylated by PKA (protein kinase A) [4,11,12,29]. In our previous work, we measured total enzymatic activity of the full-length PKA-phosphorylated protein as the generation of [α-32P]ADP from [γ-32P]ATP [29], a reaction which could reflect either ATPase and/or adenylate kinase activity. Using [γ-32P]ATP as the substrate, we show in the present study that the full-length protein functions as an ATPase almost exclusively (Figure 3, documenting the results of eight different purified protein preparations). The generation of P_i from radiolabelled ATP by purified CFTR (ATPase activity) was significant (*, P = 0.001) relative to the empty liposome controls, whereas there was no significant generation of radiolabelled ADP (adenylate kinase activity, P = 0.14) relative to the control. Interestingly, the addition of AMP (400 μM) to PKA-treated CFTR did not exert any significant effect on its ATPase activity (Figures 3A and 5B), a finding which is consistent with previous studies showing that the full-length enzyme exhibits a low affinity for AMP [33]. Analysis of the ATP dependence of the primary ATPase activity of reconstituted phosphorylated CFTR was fitted using the Michaelis–Menten equation as shown in Figure 3(C) and reveals a K_m (ATP) of 744 μM and V_max of 4.6 nmol/h per μg. These values are consistent with those published previously [29,34].

Gly551 is located in the signature motif of NBD1 [1] and is thought to exist at the interface through which NBD1 and NBD2 interact to form the catalytic site for ATP hydrolysis [4,7]. The mutation, CFTR-G551D, is associated with a severe form of CF. We reported previously that the enzymatic activity of purified PKA phosphorylated CFTR-G551D, measured as the generation of [α-32P]ADP from [α-32P]ATP, was severely abrogated [4]. In this present study, using [γ-32P]ATP as the substrate, this purified mutant CFTR exhibited markedly reduced ATPase activity (Figure 4C). Furthermore, as for the wt protein, there was no measurable adenylate kinase activity stimulated by the addition of the substrate, AMP (results not shown).

It has been proposed that the conserved glutamate residue proximal to the Walker B motif in NBD2 (i.e. Glu1371) functions as the catalytic base in the hydrolysis reaction [7,35,36]. However, this role for Glu1371 has not yet been tested directly in the full-length protein. Therefore we expressed the full-length mutant protein CFTR-E1371Q and purified it from Sf9 cells (Figure 4A) for the purpose of studying its ATPase activity. The silver-stained gel shown in Figure 4(A) indicates that the mutant CFTR protein has been purified to near homogeneity. Interestingly, mutation of the putative catalytic base did not abolish ATPase activity completely, as residual catalytic activity could be detected as the generation of P_i from radiolabelled ATP in excess of that detected in the liposome control (Figure 4B). However, the specific ATPase activity of the mutant protein was significantly less than that determined for the wt protein (Figure 4C; P < 0.001). As for the wt and CFTR-G551D proteins, there was no measurable adenylate kinase activity stimulated in CFTR-E1371Q by the addition of 400 μM AMP (Figure 4B).
Ap5A stimulates ATPase activity and reduces chloride flux through full-length CFTR

Previous studies showed that the adenylate kinase inhibitor Ap5A (0.2–1.0 mM) modified CFTR channel gating in cell membranes, suggesting that intrinsic adenylate kinase rather than ATPase activity is important for the regulation of CFTR channel activity [26]. These findings are difficult to reconcile with the results of this present study where the enzymatic activity of purified and reconstituted CFTR is primarily that of an ATPase rather than an adenylate kinase. Therefore we were prompted to confirm the effect of Ap5A on CFTR channel activity on preparations of purified and reconstituted CFTR. CFTR channel activity was assessed using a proteoliposomal flux assay rather than single channel analysis. Although single-channel gating analyses provide detailed information regarding the rates of channel opening and closing, the flux-based assay permits the simultaneous evaluation of populations of channels exposed to multiple conditions. As originally described by Garty and colleagues and modified by Goldberg et al., an inward electrical driving force on chloride uptake can be created in proteoliposomes containing a high chloride concentration [37,38].

In the presence of a low chloride bath, chloride ions move out of the proteoliposome through a purified chloride channel due to a chemical driving force. This chloride ion movement leaves a net positive charge behind, thereby generating the electrical driving force for the cumulative uptake of $^{36}$Cl (Figure 5A, inset). As established in our previously published work, half-maximal uptake is achieved at approx. 60 min [29]. Given the low protein/lipid ratio employed in this reconstitution system, differences in the cumulative uptake of $^{36}$Cl at this time point should primarily reflect differences in channel open probability.

As expected, we found that $^{36}$Cl electrodiffusion into proteoliposomes bearing reconstituted and phosphorylated CFTR was stimulated by the addition of ATP (500 μM, black bar) promoted enhanced uptake relative to untreated proteoliposomes (n = 3 and *, P < 0.04) (Figure 5A). The further addition of AMP (400 μM), a substrate for adenylate kinase activity, caused no significant increase in relative $^{36}$Cl uptake. These findings suggest that adenylate kinase activity does not contribute significantly to the regulation of CFTR chloride channel activity. However, Ap5A, an adenylate kinase inhibitor, added at a comparable concentration to that used in the studies by Randak and Welsh (0.1 mM) [26], caused inhibition of chloride flux through CFTR relative to the flux in the presence of ATP plus AMP (n = 4, P < 0.05) (Figure 5A). These apparently contradictory findings may be reconciled if Ap5A is not only an inhibitor of adenylate kinase activity, but also modifies the ATPase activity of CFTR.

Ap5A is a specific inhibitor of adenylate kinase activity at low micromolar concentrations; however, at higher concentrations (>100 μM), Ap5A has been reported to exert non-specific effects and to enhance the activity of the Ca$^{2+}$–ATPase [39]. Therefore we were prompted to investigate whether Ap5A could affect the ATPase activity of CFTR. As expected, low micromolar concentrations of Ap5A (5 μM) did not significantly affect the ATPase activity of purified CFTR (results not shown); however, Ap5A significantly stimulated the ATPase activity of reconstituted CFTR at a concentration of 100 μM (Figure 5B). These findings suggest that the effect of high concentrations of Ap5A on CFTR channel function as described in previous studies [26] is related to the effect of this nucleotide analogue on ATPase activity. These findings are consistent with the proposed role of the intrinsic ATPase activity of CFTR in promoting channel closure [7,40].

In order to understand its mechanism of action, we evaluated the effect of Ap5A (100 μM) on the ATP-dependence of ATPase activity. As shown in Figure 5(C), and consistent with the results shown in Figure 5(B), ATPase activity is enhanced by Ap5A (100 μM) when measured in the presence of 500 μM ATP. The ATP dose-dependence (0.01–1.0 mM) in the presence of Ap5A was fitted using the Michaelis–Menten equation (Figure 5Cii). ATP dependence of CFTR in the presence of Ap5A is shown and a comparison of the kinetic parameters (−−) Ap5A is defined above (see the Results section for details).
analogue on ATPase. As yet, the molecular basis for this complex effect is unknown and will form the basis for future studies.

**DISCUSSION**

The putative mechanisms of action of prokaryotic ABC transporters including MalFGK₂ [maltose transport complex of Haemophilus influenzae] [18], ModB:C₃:A (molybdate transporter complex) [22] and H11470/1 (putative metal-chelate ABC transporter encoded by H11470 and H11471 of *Haemophilus influenzae*) [17] are based on recently solved crystal structures. According to these mechanistic models, ATP binding to two nucleotide-binding sites located at the NBD dimer interface converts the transporter from the resting configuration to the transporting configuration [41]. ATPase activity by these transporters is thought to facilitate reversal of the ATP-dependent conformational changes and restoration of the resting state. CFTR is a chloride channel rather than a transporter. However, certain features of the above model have served as a template for understanding the mechanism of CFTR function, where ATP binding to one or both nucleotide-binding sites converts the protein to the active state with its channel gate in the open position, with subsequent ATPase activity facilitating closure of the channel gate [6,7,40]. Interestingly, recent biochemical studies of the ABC–NBD complex Rad50 [42] and the isolated NBDs of CFTR measured intrinsic adenylate kinase activity [26,27], prompting our investigation of the role of this function in the full-length CFTR protein.

The present study shows that the full-length CFTR protein functions as an ATPase in its channel-competent form. Therefore our results support a mechanistic model for CFTR gating which is similar to that proposed for the ABC family members discussed above, i.e. MalFGK₂ [18], ModB:C₃:A [22] and H11470/1 [17]. Interestingly, however, we confirmed the biochemical studies showing that, in isolation, each of the two NBDs of CFTR exhibit adenylate kinase activity rather than ATPase activity [26,27]. These findings suggest that the enzymatic activity of these domains is dependent on their molecular context and their native function requires correct assembly of this multi-domain membrane protein.

It is well known that the ABC NBDs and adenylate kinases share certain structural similarities [43]. Adenylate kinases possess a ‘P-loop’ (similar to CFTR, which binds ATP), an AMP-binding site which comprises three helical segments and a loop referred to as the ‘lid’ domain [44]. All three of these regions undergo large domain movements during substrate binding and the phosphoryl transfer reaction. The flexibility of adenylate kinase is essential for its function and, indeed, certain denaturants have been shown to increase intrinsic activity [45]. We speculate that, expressed separately, each NBD of CFTR may possess an AMP-binding site in addition to the P-loop, as well as the flexibility required for the phosphoryl transfer reaction. Alternatively, the well-known propensity for isolated NBDs to form non-physiological dimer interfaces [46] may also account for the adenylate kinase activity observed in preparations of isolated CFTR NBDs [26] and by Gross et al. [27].

According to the crystal structures of intact ABC transporters described above, the NBDs associate in a head-to-tail orientation to confer two nucleotide-binding sites. The ABC signature motif of one domain is juxtaposed against the Walker A and B motifs from the other domain. In CFTR, the two nucleotide-binding sites are not symmetrical as the key motifs required for ATP hydrolysis are conserved only at the site comprising the signature motif of NBD1 and the Walker A and B motifs of NBD2. The glutamate residue at position 1371 (Glu¹³⁷¹), proximal to the Walker B motif in NBD2, is thought to be the primary catalytic base in the context of the full-length protein. Substitution of Glu¹³⁷¹ with a glutamine residue has been shown to delay the rate of channel closing, providing support for the idea that ATPase activity promotes closure of the channel gate [6,7]. It was recently suggested for the ABC transporter HlyB (haemolysin B) [13,47] and for the maltose transporter MalFGK₂ [18] that this conserved glutamate residue interacts via a hydrogen bond with a canonical histidine residue during the transition state of hydrolysis. Interestingly, the ATPase activity of CFTR was not completely inhibited in the CFTR-E1371Q mutant, which exhibited approx. 30% activity compared with wt. A similar, partial effect of the glutamate-to-glutamine residue substitution on ATPase activity has been cited for certain ABC proteins including HlyB [16] and GlcV (NBD of the ABC-type glucose transporter of *Sulfobolus solfataricus*) [48], but not for others including MJ0796 [24]. In the case of P-glycoprotein, Urbatsch and colleagues found that the glutamate-to-glutamine residue substitution at either of the two catalytic sites formed by the NBD1–NBD2 heterodimer disrupted drug-stimulated ATPase activity. However, vanadate trapping of 8-azido-labelled nucleotide could still be detected in the NBDs, arguing that the catalytic transition site could be obtained in these mutant proteins. These findings support their suggestion that steps after the transition state may be impaired in these glutamate mutants [49]. Clearly, the role of this glutamate residue has yet to be fully understood in ABC proteins in general.

The CF disease-causing mutant, CFTR-G551D, exhibits defective ATPase activity, a result which is consistent with the putative location of the signature motif (L¹⁸⁹⁵SGQQ²⁰⁵⁵) relative to ATP in the NBD dimer interface [7,21]. Unfortunately, the structure of the CF-NBD1–CF-NBD2 heterodimer remains unknown. However, mutation of a glycine to an aspartic acid residue in the signature motif may be predicted to alter the interaction between this residue, ATP and the other NBD partner. Such a change would be expected to alter the rate of hydrolysis, the interaction between the two domains and/or the release of the products of hydrolysis, Pᵢ and ADP. Clearly, the generation of structural models of the CF-NBD1–CF-NBD2 heterodimer is necessary to understand fully the molecular basis for the catalytic defect exhibited by this naturally occurring mutant.

Overall, our findings support the model wherein the binding and hydrolysis of ATP is an intrinsic function of CFTR [4,6,7,29,40] and this activity, rather than adenylate kinase activity, is coupled to channel gating. It has been suggested that coupling to ATPase activity would exact an insupportable cost to cells for CFTR channel activity [26]. This present study confirms that CFTR possesses a very low specific activity (approximately three orders of magnitude lower than that of the Na⁺/K⁺-ATPase) [50]. Therefore it is unlikely that the ATPase activity of CFTR will be costly to the cell. Rather, this low level of activity is consistent with the putative role of this activity as a molecular switch in regulating the slow burst-like gating of the CFTR channel.

This work was supported by an operating grant awarded to C.E.B. from the CFHF (Canadian Cystic Fibrosis Foundation) F.U. and F.L.L.S. were supported by Fellowships awarded through the CIHR (Canadian Institutes of Health Research) Strategic Training Program in the Structure of Membrane Proteins and Disease.

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