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Characterization of the ATPase and Transport Activities of Purified CFTR

Christian J. Ketchum, Garnepudi V. Rajendrakumar, and Peter C. Maloney*

Department of Physiology, Johns Hopkins Medical School, Baltimore, MD 21205

Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR) functions *in vivo* as a cAMP-activated chloride channel. A member of the ATP-binding cassette superfamily of membrane transporters, CFTR contains two transmembrane domains (TMDs), two nucleotide-binding domains (NBDs), and a regulatory (R) domain. It is presumed that CFTR couples ATP hydrolysis to channel gating, and as a first step in addressing this issue directly, we have established conditions for purification of biochemical quantities of human CFTR expressed in Sf9 insect cells. Use of an 8-azido-[$\alpha^{32}\text{P}$]-ATP binding and vanadate-trapping assay allowed us to devise conditions to preserve CFTR function during purification of a C-terminal His₁₀-tagged variant after solubilization with lyso-phosphatidylglycerol (1%) and diheptanolyolphosphatidylcholine (0.3%) in the presence of excess phospholipid. Study of purified and reconstituted CFTR showed that it binds nucleotide with an efficiency comparable to that of P-glycoprotein, and that it hydrolyzes ATP at rates sufficient to account for presumed *in vivo* activity (V_{Max} of 58 ± 5 nmol/min/mg protein, K_{M} (MgATP) of 0.15 mM). In further work, we found that neither nucleotide binding nor ATPase activity were altered by phosphorylation (using Protein Kinase A) or dephosphorylation (with Protein Phosphatase 2B); we also observed inhibition (~40%) of ATP hydrolysis by reduced glutathione, but not by DTT. To evaluate CFTR function as an anion channel, we introduced an *in vitro* macroscopic assay based on the equilibrium exchange of proteoliposome-entrapped radioactive tracers. This revealed a CFTR-dependent transport of ^{125}I that could be inhibited by known chloride channel blockers; no significant CFTR-dependent transport of [$\alpha^{32}\text{P}$]-ATP was observed. We conclude that heterologous expression of CFTR in Sf9 cells can support manufacture and purification of fully functional CFTR. This should aid in further biochemical characterization of this important molecule.

The Cystic Fibrosis Transmembrane conductance Regulator (CFTR) is the cAMP-activated chloride channel encoded by the gene defective in patients with the disease [1,2]. As a member of the ATP-Binding Cassette (ABC) superfamily of membrane transporters, CFTR shares a conserved architecture consisting of two homologous halves, each containing a transmembrane domain (TMD) and a nucleotide-binding domain (NBD). In CFTR, unlike other ABC transporters, a third domain, termed the regulatory (R) domain, is located between the two half molecules. Current evidence suggests that the TMDs define the CFTR chloride channel, while the NBDs and the R domain mediate channel gating [3-11]

Although CFTR is glycosylated, there is currently no evidence indicating that the presence of carbohydrate affects CFTR structure or function [12]. Consistent with this presumption, expression of human CFTR in Sf9 insect cells results in appearance of the 140 kD core polypeptide - containing little or no glycosylation - that mediates a newly acquired anion permeability with the electrophysiological signature of CFTR [13]. Moreover, crude membranes from such cells have been used in vanadate-trapping experiments that demonstrate

*Email: pmaloney@jhmi.edu

a functional competence for the CFTR NBDs [14,15]. In this study, we focused on purification of human CFTR from this heterologous expression system as a way to provide amounts of CFTR that might support extended biochemical and biophysical work with purified material. We show that following reconstitution into proteoliposomes, purified CFTR retains its ability to undergo vanadate-trapping of bound nucleotide, that it exhibits a rate of net nucleotide hydrolysis sufficient to achieve maximal channel gating [16,17] and that it displays an anion transport function ($^{125}\text{I}^-$ equilibrium exchange) that can be inhibited by well-known chloride channel blockers.

MATERIALS AND METHODS

Sf9 membrane preparation

Recombinant baculovirus specifying CFTR with a C-terminal decahistidine tag (His₁₀) was a gift from Dr. Christine Bear (University of Toronto [18]). The recombinant virus specifying Pgp with the same C-terminal His₁₀ tag was a gift of Dr. Suresh Ambudkar (NIH). Growth of *Spodoptera frugiperda* (Sf9 ovarian) insect cells was according to the procedures described by Invitrogen (Carlsbad, CA), with reagents from Life Technologies (Gaithersburg, MD). Briefly, insect cells were maintained in Grace's insect media supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) at a density between 0.6 and 2 million cells/ml in Bellco (Vineland, NJ) suspension flasks (0.01 L - 1 L). For protein expression, cells were infected with recombinant baculovirus carrying the *CFTR* or *Pgp* genes (5 viral particles per cell) and harvested by centrifugation 60 hours later. Harvested cells were washed with PBS (pH 7.5), suspended in a one-tenth volume of buffer containing 50 mM Tris HCl (pH 7.5), 50 mM mannitol, 2 mM EGTA, 2 mM DTT, 2 µg/ml aprotinin, and 0.5 µg/ml leupeptin, swollen on ice for 30 min, and lysed by dounce homogenization (typically, 100 strokes). Debris was removed by a low speed centrifugation (3,000g × 5 min) and the secondary membrane pellet (100,000g × 60 min) was washed with an equal volume of 50 mM Tris HCl (pH 7.5), 50 mM KCl, 5% (v/v) glycerol, 1 mM EDTA, and 0.5 mM DTT containing freshly added protease inhibitors (1 µg/ml aprotinin, 0.5 µg/ml leupeptin, 1 µg/ml pepstatin, and 10 µg/ml pA-PMSF). All steps were performed at 4 °C or on ice. SDS-PAGE and quantitative immunoblotting with either a rabbit polyclonal anti-R-domain antibody (raised against amino acids 724-746 [19]) or a mouse monoclonal anti-His-tag antibody (Qiagen) were used to confirm expression and to determine the percentage of total membrane protein attributable to CFTR of Pgp (near 1%; data not shown). Membrane protein concentration was determined by the Bradford assay (Bio-Rad).

Purification of CFTR and Pgp

For CFTR, Sf9 membranes were solubilized at 10 mg CFTR/ml for 20 min at 4 °C in a buffer containing 50 mM Tris HCl (pH 7.5), 50 mM KCl, 0.2 mM EGTA, 2 mM ouabain, 10 mM sodium azide, 2 mM MgCl₂, 1 mM NaATP, 20% (v/v) glycerol, 0.2% (w/v) *E. coli* phospholipid (Avanti Polar Lipids), 1% 1-palmitoyl-2-hydroxy-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (LPG; Avanti Polar Lipids), 0.3% (w/v) 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC; Avanti Polar Lipids), 10 mM imidazole, and protease inhibitors (as above). After removing insoluble material by centrifugation, the supernatant was mixed with Ni-NTA resin (Qiagen; 100 µl of 50% slurry per ml soluble crude extract) that had been washed in the solubilization buffer. The suspension was mixed for 3 hrs at 4 °C and then washed with 50 volumes (5 × 500 µl) of a buffer containing 50 mM Tris HCl (pH 7.5), 50 mM KCl, 2 mM MgCl₂, 1 mM NaATP, 20% (v/v) glycerol, 0.1% (w/v) *E. coli* phospholipid, 0.1% LPG, 0.3% DHPC, and 50 mM imidazole. Purified protein was eluted with 2 volumes of the same buffer lacking LPG, but containing 150 mM imidazole. An amido black 10B assay [20] was employed to determine protein concentration; purity was confirmed by silver-staining (Bio-Rad) and

immunoblotting of duplicate gels. Pgp was purified in the same fashion except that dodecylmaltoside replaced LPG.

Reconstitution

Purified protein was reconstituted by detergent dilution [21]. Liposomes were prepared at room temperature with a bath sonicator, using 25 mg hydrated *E. coli* phospholipid suspended in 0.5 ml of 50 mM Tris HCl (pH 7.5), 50 mM KCl. After a brief spin to clarify the suspension, the sonicated liposomes were transferred to an Eppendorf tube and octyl- β -D-glucopyranoside (CalBiochem) was added to 3.75% (w/v); one volume of the solubilized liposome mixture was then added to two volumes of solubilized protein. After a 20-min incubation at room temperature, proteoliposomes were formed by rapidly diluting the mixture about 150-fold into 50 mM Tris HCl (pH 7.5), 50 mM KCl (at room temperature), after which they were collected by centrifugation (180,000g $\times$ 60 min). This method is presumed to allow the random insertion of protein, generating proteoliposomes with an equal distribution of inside-out and right-side-out orientations [21-24]. Protein recovery was measured by an Amido Black 10B assay [20] and confirmed by quantitative immunoblot analysis.

Az-ATP binding and Vi-trapping

Membranes or proteoliposomes were suspended in a buffer containing 50 mM Tris HCl (pH 7.5), 50 mM KCl, 2mM MgCl₂, 0.1 mM EGTA, 2 mM ouabain, and 5 mM sodium azide supplemented with 5-50 μ M 8-azido-[α^{32} P]-adenosine-5'-triphosphate (Az-ATP; Affinity Labeling Technologies), in the absence or presence of 0.5 mM sodium orthovanadate (Vi; Sigma). The Vi was prepared as detailed by Goodno [25], and the Vi concentration of stock solutions determined by molar absorbance ($\lambda_{268\text{nm}} = 3600 \text{ M}^{-1}$). After incubation for 4 min at 37 °C to allow for nucleotide binding and trapping, 10 mM MgATP (pH 7.5) was added to reduce non-specific binding. Samples were then irradiated with UV light (365 nm) for 10 min at a fixed distance of 5 cm, after which labeled proteins were separated by SDS-PAGE, visualized by autoradiography (Kodak X-OMAT Film and/or a Fuji Phosphorimager) and quantified (arbitrary units) using a Fuji Phosphorimager and Fuji Image Gauge V3.3 imaging software.

ATPase assay

ATP hydrolysis by CFTR was evaluated using proteoliposomes placed at 37°C in a buffer containing 50 mM Tris HCl (pH 7.5), 50 mM KCl, 2 mM ATP, 2mM MgCl₂, 0.1 mM EGTA, 2 mM ouabain, and 5 mM sodium azide, supplemented with 5-10 μ Ci [α^{32} P]ATP. At defined time-points, aliquots were removed, quenched by addition of 2.5% (w/v) SDS, and spotted on a polyethyleneimine-cellulose plate (J. T. Baker). Hydrolyzed [α^{32} P]ADP was separated from [α^{32} P]ATP by thin-layer chromatography in 1 M formic acid, 0.5 M LiCl. Radiolabeled nucleotides were visualized by a Fuji Phosphorimager and quantified (as above). To account for the spontaneous Mg-catalyzed hydrolysis of ATP, all values were corrected by comparison with parallel control samples containing liposomes (no protein). All assays were performed under conditions where ATP hydrolysis was linear with both time and protein content.

Transport assay

CFTR-dependent anion transport activity was measured by the equilibrium exchange of proteoliposome-entrapped radioactive tracers [26,27]. Proteoliposomes were formed by detergent dilution (as above) in a buffer containing 50 mM Tris HCl (pH 6.8), 50 mM KI, 50 mM potassium glutamate (pH 6.8) and 1mg/ml DTT in the presence of 100 μ Ci ¹²⁵I and 100 μ Ci ³H-glutamate. Proteoliposomes were collected by centrifugation, after which the surface of the pellet was rinsed with loading buffer (above) to which tracer had *not* been added. ¹²⁵I release was then initiated by resuspending the pellet in 1 ml of the same buffer. After a 1 h

incubation at room temperature, aliquots were collected by vacuum filtration onto presoaked Millipore GS filters (0.22- μ m pore size) and washed with tracer-free buffer (3×1 ml). Residual isotope remaining on the filter was used as the index of CFTR-mediated anion flux.

RESULTS

Overexpression of CFTR in Sf9 insect cells

It was shown earlier that coincident with expression of human CFTR in Sf9 insect cells there is appearance of a new anion permeability with features appropriate to a CFTR-mediated anion channel [13]. And by use of a vanadate (Vi)-trapping assay, CFTR-specific ATP binding and hydrolysis has also been observed in the crude membranes of such cells [14,15]. Indeed, because of its high sensitivity, we used this latter method to evaluate conditions suited to purification of human CFTR expressed in these cells. Vi is a structural analog of inorganic phosphate [28,29] and arrests the hydrolytic cycle of an ATPase at an intermediate stage, after nucleotide hydrolysis and release of phosphate, but before release of ADP (*e.g.*, CFTR•MgADP•Vi). In the presence of Vi, therefore, the entire population can be forced to accumulate in a single “trapped” state whose lifetime is considerably extended relative to the normal intermediate. If done in conjunction with a photolabile ATP analog, such as 8-azido-[α^{32} P]-ATP (Az-ATP), irradiation with UV light can cross-link (at low efficiency) the trapped nucleotide to the peptide backbone, generating a stable radioactive species whose presence reports the parent population. Figure 1 shows that crude Sf9 membranes harboring CFTR, but not those expressing β -galactosidase (β gal), display Vi-stimulated incorporation of radionucleotide into a protein migrating at a position corresponding to that of CFTR. This finding confirms that human CFTR can bind and hydrolyze ATP in this heterologous cell culture system.

Purification of CFTR from Sf9 membranes

Purification of membrane proteins such as CFTR requires their dissociation from other bound proteins and membrane lipids by solubilization with a compatible detergent. To determine which detergent(s) might be best for extracting functional CFTR from Sf9 membranes, we monitored solubilization using the Az-ATP binding and Vi-trap described above (Fig. 1). In such experiments, Sf9 membranes were subjected to detergent solubilization either *before* or *after* Az-ATP binding and trapping; in each case, the UV cross-linking of Az-ATP to the polypeptide backbone was performed last, after detergent solubilization. We found that when exposure to detergent occurred *before* Az-ATP binding and the Vi-trap, no CFTR-specific incorporation of the labeled nucleotide could be observed, despite significant extraction of protein (Fig. 2*b*). This suggests that detergent-solubilized material is unable to bind and/or hydrolyze nucleotide. By contrast, when detergent solubilization took place *after* Az-ATP binding and the Vi trap (but before cross-linking), the nucleotide incorporation observed in membranes was preserved to varying degrees according to the detergent(s) used. In these cases, we found that a detergent combination of LPG [30] and DHPC faired best, retaining > 85% of the CFTR-specific signal seen in crude membranes (Fig. 2). By contrast, detergents used previously as CFTR-solubilizing agents (*e.g.*, Triton X-100, SDS and NaPFO [31]) supported little or no retention of labeling, indicating that these agents gave at least a partial denaturation of the CFTR NBDs.

We interpreted these data (Fig. 2) as indicating that nucleotide (*e.g.*, MgATP) must be included during solubilization to ensure recovery of functional CFTR. Accordingly, we used the detergent combination noted above (LPG/DHPC), along with 1 mM ATP, as the starting point for purification of CFTR by Ni-agarose affinity chromatography [32] (see *Methods*). The final preparation was of high purity (> 95%) as judged by silver staining (Fig. 3), with a net yield of 1 μ g CFTR/ml culture (1.6×10^6 cells), corresponding to a final recovery of about 10-15%

of solubilized CFTR. In part this low yield was due to poor binding of solubilized CFTR to the Ni-agarose column (see Fig. 3). Preliminary work now indicates that yield may be increased to 50-70% of the solubilized material by either reapplication of unbound material to Ni-agarose or by use of increased amounts (2 to 3-fold) of resin (Ye & Maloney, unpublished). These steps, however, lead to a somewhat reduced final purity (80-90%), and the experiments reported here used the highly purified material obtained by a single batch exposure to the resin (as in Fig. 3). A second factor contributing to reduced yield was inefficient disruption of cells from large culture volumes (> 200 ml). This may be related to use of homogenization to disrupt cells, since solubilization was markedly increased when membranes were dispersed using high pressure lysis (14,000 p.s.i) (Ye & Maloney, unpublished).

Nucleotide processing by purified and reconstituted CFTR

Purified membrane proteins are best studied after the detergent(s) used for solubilization has been replaced by phospholipid. In our work, this was accomplished by reconstitution of CFTR using a detergent dilution method [21,33] that yields proteoliposomes suited to characterization of nucleotide processing by ABC transporters (see [34]). Our initial trials used the Vi-trap of ^{32}P -labeled Az-ATP, as in earlier work. These experiments documented the expected dependence on divalent cation (Mg^{2+}), as well as the stimulation afforded by a Vi trap (Fig. 4*ab*). Of interest is the significant incorporation of labeled nucleotide in the absence of a Vi trap (Fig. 4*ab*). This behavior, noted by others in studies of crude membranes [14,15], has not been reported for other ABC transporters [14,15,33] and may be a distinctive feature of CFTR.

We also evaluated recovery of functional CFTR in a quantitative manner by comparing nucleotide trapping of CFTR in parallel with that of its well-characterized relative, Pgp, which had been expressed in Sf9 cells and purified and reconstituted using conditions optimized for its own recovery [33,35-37,38] (Table I, Fig. 4*c-e*). In both instances, we observed Vi-stimulated nucleotide binding, although signals associated with CFTR were markedly reduced relative to that found for Pgp (Fig. 4*cd*). Because these two proteins each carried the same C-terminal His₁₀ tag, we used an anti-polyhistidine antibody in a quantitative immunoblot analysis (Fig. 4*e*) that indicated this differential reaction was entirely attributable to a reduced content of CFTR in target proteoliposomes. Indeed, when the data were expressed on a molar basis (with correction for protein content and molecular weight), we concluded that CFTR and Pgp bind nucleotide (Az-ATP) with approximately equal efficiency (109% vs. 100%, respectively). Since the biochemical characteristics of Pgp purified from Sf9 cells (*e.g.*, V_{max} , K_{M} , and substrate stimulation) match values cited in the literature for the fully functioning molecule [33,35-38] (Table I), we conclude that nucleotide processing by purified CFTR exhibits characteristics similar to that of Pgp.

In mammalian systems, R domain phosphorylation must occur before CFTR chloride channel function is observed. However, in Sf9 cells, the CFTR-mediated anion conductance appears without addition of exogenous stimuli that might activate intracellular protein kinases and promote R domain phosphorylation [13]. This suggests that in these cells, the R domain of CFTR either constitutively phosphorylated, or that, for unknown reasons, R domain regulation does not operate in these cells. Since the phosphorylation status of purified CFTR is unknown, we monitored the effects of both dephosphorylation and phosphorylation to determine whether *in vitro* treatment might affect nucleotide binding. Treatment with Protein Phosphatase 2B (PP2B), a relevant phosphatase *in vivo* [39], had no apparent effect on the SDS-PAGE mobility of CFTR, while exposure to cAMP-dependent Protein Kinase (PKA) caused a clear retardation in CFTR mobility (Fig. 4*fg*) (see Table II and [16] for methods), coincident with phosphorylation as documented by labeling by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (data not shown). Moreover, the mobility shift induced by PKA was readily reversed by subsequent exposure to PP2B (Fig.

4fg). But despite that CFTR could be phosphorylated and dephosphorylated *in vitro*, CFTR-dependent nucleotide binding remained unaffected (Fig. 4f). This is consistent with the *in vivo* observations made with Sf9 cells, raising the possibility that nucleotide binding is independent of R domain phosphorylation.

ATP hydrolysis by purified and reconstituted CFTR

The Vi trap used to monitor nucleotide binding (Fig. 4) limits the analysis to a partial turnover of a binding domain. To study net ATP hydrolysis, we measured liberation of [$\alpha^{32}\text{P}$]ADP from [$\alpha^{32}\text{P}$]ATP [16] (Fig. 5). In our initial work, we found that CFTR hydrolyzes ATP at a maximal rate of 58 ± 5 nmol/mg/min, with a K_M for MgATP of 0.15 mM (Fig. 5a, Table II). These values are consistent with previous reports for CFTR [16,40] and also for several other ABC transport proteins, including Ste6p, ABCR, MRP1 and MRP2 [33,41-43]. Moreover, if one assumes that ATP hydrolysis is linked to channel gating, these values are sufficient to achieve the maximal rate of channel gating observed *in vivo* [16,17].

Using the CFTR ATPase assay (Fig. 5), we next explored the question of whether (R domain) phosphorylation affects the kinetics of the ATP hydrolysis. This work failed to show an effect of either dephosphorylation (with PP2B) or with phosphorylation (using PKA) on the CFTR ATPase (Table II), in contrast to an early suggestion that phosphorylation might increase the affinity for MgATP [16,40]. Instead, these more recent tests (Fig. 5, Table II), together with our findings described above (Fig. 4fg) indicate that R domain phosphorylation *in vitro* has little or no effect on either nucleotide binding (single turnover) or net hydrolytic rate (many turnovers).

Previous electrophysiological work [44] has suggested that CFTR channel function is modulated when reduced glutathione (GSH) is present at the cytosolic surface, and a more recent report indicates that this modulation may be evident at a macroscopic level as an inhibition of CFTR-mediated ATP hydrolysis [45]. Accordingly, the ATPase activity of purified CFTR was monitored (as above) in the presence of this putative modulator (Fig. 5). At a final concentration of 10 mM GSH, the maximal velocity of CFTR ATPase was reduced about 40% ($V_{\max} = 34 \pm 5$ nmol/mg/min), without significant change in nucleotide affinity (K_M for MgATP of 0.11). This reduction in maximal velocity was not observed in the presence of DTT (Fig. 5), suggesting GSH inhibits the ATPase activity of CFTR by a mechanism other than disulfide reduction.

CFTR-dependent transport activity

The work summarized above suggests that purified and reconstituted CFTR retains the features expected of nucleotide processing by an ABC transporter (Figs. 4 & 5). A necessary complement to this work is an evaluation of whether the CFTR anion channel function is also evident. For this purpose, we monitored anion transport using a macroscopic *in vitro* assay based on the equilibrium exchange of proteoliposome-entrapped radioactive tracers [26,27]. Thus, we reconstituted purified CFTR as for the ATPase assay described above except that proteoliposomes were formed in the presence of both a CFTR channel substrate ($^{125}\text{I}^-$) and an impermeant marker (^3H -glutamate). Efflux of proteoliposome-entrapped $^{125}\text{I}^-$ via CFTR as well as by non-specific routes, was initiated by resuspending proteoliposomes in buffer of exactly the same composition, but without addition of labeled tracers. In this way, $^{125}\text{I}^-$ might leave proteoliposomes in net exchange for external non-radioactive I^- , while ^3H -glutamate, which is not expected to pass through the CFTR at a significant rate, would act as an internal control whose loss would account for tracer release due to proteoliposome breakage. After extended incubation (ca. 60 min), proteoliposomes were isolated by filtration, and the net losses of entrapped $^{125}\text{I}^-$ and ^3H -glutamate were noted. It should be emphasized that such an assay does not monitor individual channel openings and closings, and that, in principle, a positive

finding may arise if a channel opens but then closes. With no added protein, both isotopes were lost to a comparable degree, corresponding to about 5-10% of their initial values, and this was taken as the base line for comparison with losses noted when CFTR was present. In these latter cases, as the amount of CFTR used during reconstitution was increased, a clear bias favoring loss of ^{125}I developed, so that the isotope ratio ($^{125}\text{I}/^3\text{H}$) decreased from its reference value of 1.0 to near 0.3, reflecting CFTR-dependent efflux of ^{125}I .

In experiments designed to probe the specificity of this response, we used an intermediate level of CFTR so as to obtain an isotope ratio ($^{125}\text{I}/^3\text{H}$) of about 0.5, allowing us to survey conditions that might increase or decrease anion flux (Table III). We found that loss of ^{125}I was significantly reduced by chloride channel blockers known to act on CFTR (glibenclamide, DPC, DIDS), confirming that this behavior reflects a CFTR-like anion channel. We also found that GSH reduced CFTR-dependent transport of ^{125}I , albeit to a lesser degree than did the chloride channel blockers (Table III). Finally, we asked if *in vitro* dephosphorylation (by PP2B) or phosphorylation (by PKA) affected anion flux. Indeed, while PKA had no effect on anion efflux, addition of PP2B appeared to eliminate a portion of the CFTR-dependent response (Table III), although this trend did not reach statistical significance.

CFTR-dependent transport of ATP?

There has been much debate concerning the possibility that ATP is transported by CFTR, driven either by ATP hydrolysis (as in other ABC transporters) or moving passively at low frequency via the embedded anion channel (for review see [46]). We were able to address this question directly with the *in vitro* transport assay by forming proteoliposomes in the presence of both ^3H -glutamate and $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, and in the *absence* of halide anions to avoid competition with other channel substrates. As shown in Table III, the isotope ratio ($^{32}\text{P}/^3\text{H}$) ratio was similar for both control and CFTR-containing proteoliposomes, suggesting ATP is not a transport substrate for CFTR.

DISCUSSION

Purification of functional CFTR

A major obstacle to the mechanistic link between structure and function for ABC transporters has been the difficulty in purifying biochemical quantities of fully active protein. In particular, whether from natural sources or after over-expression in a heterologous system, CFTR usually proves refractory to solubilization with non-denaturing detergents commonly used in purifying membrane proteins (*i.e.*, CHAPS, octylglucoside, dodecylmaltoside, *etc.* [33,35-38,47]). Accordingly, our first step was to establish an assay with which to gauge whether exposure to a test agent was compatible with long-term retention of the CFTR native state. For this purpose, we employed Az-ATP binding in the presence of a Vi-trap, and judged retention of the native structure by using UV light to cross-link Az-ATP still bound to CFTR after solubilization (Fig. 2). Based on these findings and on our other work with membrane proteins [33,48,49]), we established conditions for the solubilization of CFTR that include substrate stabilization (*i.e.*, bound nucleotide), a combination of nonionic detergents (LPG + DHPC), addition of exogenous phospholipid and use of the chemical chaperone, glycerol. Following purification by Ni-agarose chromatography [32,33], we then used reconstituted material [21,33] to document that the final product retains many of the attributes expected from earlier work. For example, use of a Vi-trap allowed us to show that purified CFTR displays nucleotide (Az-ATP) binding with an efficiency comparable to that of Pgp, a comparison we felt important to make directly, since the efficiency of UV cross-linking is low and may vary among laboratories. Indeed, for our work (Fig. 4), Pgp had been purified using conditions optimized for its own recovery, and the preparation showed a basal ATPase activity and response to substrate consistent with what one expects for the fully functioning molecule (Table I). As a

consequence, we conclude that nucleotide binding by CFTR is comparable to that shown by Pgp [36,38]. We also note that CFTR catalyzes a net hydrolysis of ATP (Fig. 5a and Table II) at rates comparable to several other ABC transporters, including Ste6p, ABCR, MRP1 and MRP2 [33,41-43]) and that this is sufficient to achieve the maximal rate of channel gating observed *in vivo* [16,17]. Finally, it is clear that purified CFTR mediates a halide-specific anion flux that can be inhibited by chloride channel blockers known to act on CFTR (Fig. 6 and Table III). Taken together, these results confirm that purification and reconstitution of functional CFTR can be achieved from an expression system capable of generating amounts of protein suited to extended biochemical and biophysical characterization.

Role of R domain phosphorylation

The cytoplasmic R domain, which distinguishes CFTR from other ABC transporters, contains 9 consensus sites (R/K-R/K-X-S/T) for phosphorylation by protein kinases, the most important of which appears to be PKA [3,4]. Because deletion of the R domain activates the CFTR Cl⁻ channel [4,50], it has been suggested that a dephosphorylated R domain may effectively occlude the conducting pore of CFTR, analogous to “ball and chain” inactivation of the Shaker K⁺ channel in *Drosophila* [51]. R domain phosphorylation may therefore cause a conformational change within the molecule, alleviating a steric blockade and thereby facilitating nucleotide-dependent channel regulation. It has also been proposed that phosphorylation of the R domain alters the kinetics of nucleotide binding [52]. The experiments supporting such models of R domain function rely largely on the *in vivo* analysis of channel activity, and we did not directly address such issues. We note, however, that our work with purified material failed to show significant phosphorylation-dependent effects on nucleotide processing, measured by either the Vi-trap of Az-ATP (Fig. 4d) or by net ATP hydrolysis (Table II). We cannot exclude the possibilities that CFTR was damaged during purification, causing an abnormal response to R domain phosphorylation, or that CFTR remains minimally phosphorylated (activated) following purification or after *in vitro* phosphatase treatment. Alternatively, if fully functional CFTR is recovered by our techniques, the simplest conclusion is that nucleotide binding and hydrolysis are not directly coupled to CFTR phosphorylation. This idea would not be compatible with early reports that R domain phosphorylation alters the Michaelis constant for ATP hydrolysis [16,40,52]. The origin of this discrepancy is not understood, but may be related to procedural differences. In particular, we note that the earlier experiments employed one or more detergents (Triton, NaPFO, SDS) that disrupt nucleotide binding during solubilization (see Fig. 2). It is feasible, and perhaps not unexpected, that phosphorylation of reconstituted protein might facilitate partial renaturation of one or both NBDs. While not compatible with this early view, our observations remain consistent with the R domain occlusion model noted above. It must be recalled that our test for CFTR-mediated anion transport is based on an equilibrium condition reached after an extended incubation. Such a test cannot monitor individual gating events or the global probability of opening, but gives a record of whether a channel opens at least once. Accordingly, binding and hydrolysis of ATP may occur with similar kinetics for both unphosphorylated and phosphorylated forms of CFTR, but only for the latter species would such nucleotide processing also affect channel gating. We note that our work may also suggest an alternative model in which the link between R domain phosphorylation and nucleotide binding and hydrolysis is mediated, *in vivo*, by elements that remain uncharacterized. This latter model should remain a speculation until further work is completed.

In vitro transport by CFTR

Nucleotide processing reflects operation of the CFTR NBDs, while the CFTR chloride channel is assigned to the TMDs. To assess the functional status of CFTR-mediated anion transport, we exploited an *in vitro* assay based on the equilibrium exchange of proteoliposome-entrapped radioactive tracers (Fig. 6 and Table III). This assay, used earlier to study the bacterial chloride

channel, EriC [26,27], verified recovery of CFTR channel function with appropriate responses to chloride channel blockers. The more general utility of this assay is also worth comment, since we were able to use it to exclude the possibility of ATP transport via CFTR (Table III). We anticipate that similar tests can be used to evaluate CFTR-mediated movements of other anions, some of which have been suggested as substrates (*e.g.*, gluconate, pyruvate, formate, isothionate, bicarbonate and glutathione) [44,53,54]. We also note that the assay for anion transport uses the same experimental conditions as those used to monitor nucleotide processing. Thus, use of reconstituted CFTR provides a system with which one might study the functional linkage between these events (*i.e.*, coupling). An equivalent survey using Pgp [22,36] identified a new class of agents, the “chemosensitizers,” that stimulate ATPase activity without an effect on transport. A similar survey of CFTR might be useful in revealing novel modulators that affect coupling, either negatively or positively.

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Abbreviations

ABC, ATP-binding cassette
 ABCR, human retinal transporter
 Az-ATP, 8-azido-[$\alpha^{32}\text{P}$]-adenosine-5'-triphosphate
 βgal , β -galactosidase
 cAMP, adenosine-3',5'-cyclic monophosphate
 CFTR, Cystic Fibrosis transmembrane conductance regulator
 HisQMP₂, *E. coli* histidine permease
 LPG, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]
 DHPC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine
 DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid
 DPC, diphenylamine carboxylate
 MalFGK₂, *E. coli* maltose transporter
 MDR, multidrug resistance
 MRP, multidrug resistance protein
 NBD, nucleotide-binding domain
 Pgp, P-glycoprotein
 Pi, inorganic phosphate
 PKA, cAMP-dependent protein kinase
 PP2B, protein phosphatase 2B or calcineurin
 SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
 Sf9, *Spodoptera frugiperda* ovarian insect cells
 Ste6p, *S. cerevisiae* pheromone transporter
 TMD, transmembrane domain
 Vi, sodium orthovanadate.

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Figure 1. Vi-stimulated Az-ATP binding to CFTR in Sf9 membranes

Membranes prepared from Sf9 cells expressing either β -galactosidase (β Gal) or CFTR were treated with 8-azido- $[\alpha^{32}\text{P}]$ -ATP at a nominal concentration of $5\ \mu\text{M}$, in the presence or absence of $0.5\ \text{mM}$ Vi as described in *Methods*, before exposure to UV light to cross-link the reactive probe to CFTR. The top panel (*a*) gives the autoradiograph, while the bottom panel (*b*) shows an immunoblot of the same gel probed with a polyclonal antibody against the R domain.

Figure 2. The effect of detergent solubilization on Az-ATP binding by CFTR

As noted, Sf9 membranes containing CFTR were exposed to the indicated detergents before (#1) or after (#2) exposure to Az-ATP in the presence of Vi. UV cross-linking was performed last, as indicated. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. After recording signals using a Fuji Phosphoimager, CFTR was localized on the same nitrocellulose membrane by immunoblot using the R domain polyclonal antibody.

Figure 3. Purification of CFTR

Sf9 membranes (M) expressing CFTR were solubilized and mixed with Ni-affinity resin. The unbound (UB) fraction was collected, the resin washed (W), and purified protein eluted (E). For each preparation, a small aliquot of each fraction (0.04% of M and UB, 0.4% of W, and 4% of E) was loaded onto duplicate gels for silver-staining (*a*) and immunoblotting (*b*).

Figure 4. Vi trap of Az-ATP bound by purified and reconstituted CFTR

Sf9 membranes expressing CFTR, Pgp or βGal were solubilized, and purified protein (or the equivalent fraction for βGal-expressing cells) was reconstituted and subjected to Az-ATP binding and Vi-trapping. After SDS-PAGE and transfer to nitrocellulose, the nitrocellulose membrane was first exposed to a Fuji Phosphoimager screen (*a*) and then immunoblotted (*b*) using a monoclonal antibody directed against the C-terminal His₁₀ tags. The same techniques were used in a separate experiment using purified CFTR and Pgp (*c*, *d*). In this case, the relative amounts of CFTR and Pgp in proteoliposomes were also determined by quantitative immunoblot analysis (*e*), with band intensities quantified (arbitrary units) using Fuji Image Gauge V3.3 imaging software. Table II describes the characteristics of this preparation of Pgp. (*f*) In a third experiment, the effects of dephosphorylation by PP2B and phosphorylation by PKA were evaluated for purified CFTR, using methods described in Table IV. Az-ATP/Vi-trapping was evaluated as described above; (*g*) SDS-PAGE mobility was monitored with the monoclonal antibody reactive to the C-terminal His₁₀ tag.

Fig. 5. ATPase activity of purified and reconstituted CFTR

(a) ATP hydrolysis was measured as a function of MgATP concentration; mean $\pm$ SEM for 3-7 independent trials. V_{Max} and K_M were calculated from the best-fit linear regression of a Lineweaver-Burk plot (*inset*). (b) ATP hydrolysis was also monitored in the presence of increasing concentrations of GSH or DTT, as indicated.

Fig. 6. Transport activity of purified and reconstituted CFTR

Transport was monitored by the equilibrium exchange of proteoliposome-entrapped ^{125}I and ^3H -glutamate, using increasing amounts of purified CFTR during reconstitution; mean $\pm$ SEM for 3-7 independent trials.

Table I
ATPase of Purified Pgp Reconstituted in Proteoliposomes

	V_{Max}^a (nmol/min/mg)	K_M for MgATP ^b (mM)
No Addition	400 ± 25	0.15
+ 10 μ M Verapamil	2400 ± 125	0.14

^a Maximal rate of ATP hydrolysis after correction for spontaneous Mg-catalyzed hydrolysis in mock controls (no protein). Hydrolysis was determined at 37 °C as described in *Methods*. Data reflect mean ± SEM for 3 independent trials.

^b Calculated from the best-fit linear regressions of Lineweaver-Burke plots.

Table II**ATPase of Purified CFTR Reconstituted in Proteoliposomes**

	V_{Max}^a (nmol/mg/min)	K_M for MgATP ^b (mM)
CFTR + PP2B ^c	53 ± 6	0.10
CFTR	58 ± 5	0.15
CFTR + PKA ^d	54 ± 4	0.13
CFTR + GSH ^e	34 ± 5	0.11

^a Maximal rate of ATP hydrolysis after correction for spontaneous Mg-catalyzed hydrolysis in mock controls (no protein). Hydrolysis was determined at 37 °C as described in *Methods*. Data reflect mean values ± SEM for 3 - 9 independent trials.

^b Calculated from the best-fit linear regressions of Lineweaver-Burk plots.

^c Proteoliposomes were resuspended in 100 μ l of assay buffer (see *Methods*) to which was added 1 unit PP2B (Sigma), 2 units calmodulin, and 0.2 mM CaCl₂. Samples were placed at room temperature for 1 h, and then centrifuged and washed with 5 ml of assay buffer to remove components of the dephosphorylation mixture. ATP hydrolysis was then monitored as noted in *Methods*.

^d Proteoliposomes were resuspended in 100 μ l assay buffer (see *Methods*) to which was added 20 units PKA (Promega), 2 mM MgCl₂, and 0.1 mM NaATP. Samples were placed at room temperature for 1 h, and then centrifuged and washed with 5 ml of assay buffer to remove residual components of the phosphorylation reaction.

^e Glutathione used at a final concentration of 10 mM.

Table III

Transport Activity of Purified CFTR Reconstituted in Proteoliposomes

	$^{125}\text{I}/^3\text{H}$ Ratio ^a (%)	$[\alpha^{32}\text{P}]\text{-ATP}/^3\text{H-glutamate}$ ^b (%)
Control (no protein)	100	108 ± 10
CFTR	50 ± 8	89 ± 25
CFTR + glibenclamide ^c	95 ± 11	ND ^e
CFTR + DPC	84 ± 18	ND
CFTR + DIDS	90 ± 17	ND
CFTR + glutathione	72 ± 15	ND
CFTR + PP2B ^d	66 ± 9	ND
CFTR + PKA	48 ± 14	ND

^a As described in *Methods*; mean ± standard error for 3 - 4 independent trials.

^b Modified as follows: proteoliposomes were formed in a buffer containing 50 mM Tris HCl (pH 6.8), 50 mM Kglutamate (pH 6.8) 2 mM MgCl₂, 1 mM NaATP and 1 mg/ml DTT, in the presence of 100 μCi [$\alpha^{32}\text{P}$]-ATP; proteoliposome-entrapped [$\alpha^{32}\text{P}$]-ATP/ ^3H -glutamate counts were expressed as mean % of the mock control ± standard error for 3 independent trials.

^c Channel blockers were prepared as noted [11] and added to both sides of the membrane; final concentrations were 1 mM glibenclamide, 1 mM DPC, 5 mM DIDS, and 10 mM glutathione.

^d Prior to reconstitution, purified protein was incubated at room temperature for 1 h in the presence of either 1 unit PP2B (Sigma), 2 units calmodulin, and 0.2 mM CaCl₂ (*i.e.*, dephosphorylation) or 20 units PKA (Promega), 2 mM MgCl₂, and 0.1 mM NaATP (*i.e.*, phosphorylation).

^e Not Done