Video Article

Functional Reconstitution and Channel Activity Measurements of Purified Wildtype and Mutant CFTR Protein

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Abstract

The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a unique channel-forming member of the ATP Binding Cassette (ABC) superfamily of transporters. The phosphorylation and nucleotide dependent chloride channel activity of CFTR has been frequently studied in whole cell systems and as single channels in excised membrane patches. Many Cystic Fibrosis-causing mutations have been shown to alter this activity. While a small number of purification protocols have been published, a fast reconstitution method that retains channel activity and a suitable method for studying population channel activity in a purified system have been lacking. Here rapid methods are described for purification and functional reconstitution of the full-length CFTR protein into proteoliposomes of defined lipid composition that retains activity as a regulated halide channel. This reconstitution method together with a novel flux-based assay of channel activity is a suitable system for studying the population channel properties of wild type CFTR and the disease-causing mutants F508del- and G551D-CFTR. Specifically, the method has utility in studying the direct effects of phosphorylation, nucleotides and small molecules such as potentiators and inhibitors on CFTR channel activity. The methods are also amenable to the study of other membrane channels/transporters for anionic substrates.

Video Link

The video component of this article can be found at https://www.jove.com/video/52427/

Introduction

Chloride transport across the apical membranes of epithelial cells in such tissues as the lung, intestine, pancreas and sweat glands is primarily mediated by the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), an ATP- and phosphorylation-regulated member of the ABC (ATP-Binding Cassette) C subfamily of membrane proteins (reviewed in[1]). Like other members of the ABCC subfamily, CFTR is a large, multi-spanning integral membrane protein that binds ATP at two nucleotide binding sites formed at the interface of its nucleotide-binding domains (NBDs), where it possesses modest ATPase activity at a single site. However, unlike other ABCC subfamily members, CFTR has evolved as a unique regulated Cl channel rather than as an active solute transporter.

Mutations in CFTR cause Cystic Fibrosis, a disease affecting multiple organs including the lungs, gastrointestinal tract, pancreatic and reproductive tract, leading to morbidity and mortality in young adults. Lung disease typically accounts for early mortality in Cystic Fibrosis and in most cases is caused by the loss of CFTR function on the surface epithelium of the conducting airways. The lack of CFTR chloride channel activity causes a reduction in both Cl^- and water movement across the surface epithelium to modify the fluid layer on the apical surface of the ciliated respiratory epithelium. This results in a viscous airway surface liquid that impairs the ability of ciliated respiratory epithelial cells to effectively clear pathogens out of the airways. As a consequence, most CF patients suffer from recurrent bouts of lung infection and lung damage due to inflammation.

As expected, studies of the mechanism of action of the normal CFTR protein focused primarily on detailed electrophysiological studies of its channel gating activity. Single channel studies have shown directly that CFTR functions as a PKA-dependent Cl^- channel which possesses an ATP regulated gate[2]. Detailed electrophysiological studies provide a great deal of information on single CFTR channels[1,3], however there may be concern as to whether the characteristics of any particular single channel that has been studied is reflective of the entire population of CFTR channels and therefore the single channel results should always be considered along with methods to study the macroscopic population. Direct assay of the population channel activity of purified CFTR has the potential to provide insight into the molecular defect associated with disease-causing mutations and to drive discovery of chemical modulators which repair mutant CFTR proteins. To date, there are in excess of 1,900 different mutations in CFTR thought to cause Cystic Fibrosis[4]. The major mutation, F508del-CFTR, found on at least one allele in approximately 90% of patients in North America and Europe leads to protein misfolding and retention in the endoplasmic reticulum[5]. F508del-CFTR also has other consequences, including defective channel activity[6-9]. The resultant absence of CFTR from the cell surface is associated with severe disease. G551D-CFTR, a less common mutation, is thought to be properly folded yet is dysfunctional as a chloride channel at the cell surface[6]. The development of small molecule correctors and potentiators has the goal of correcting folding and/or trafficking of mutants such as F508del-

CFTR to the cell surface, and potentiating or increasing the channel activity of mutations such as G551D when present on the cell surface, respectively. While the correctors VX-809 and VX-661 (are not yet approved for use in patients, the potentiator Kalydeco (ivacaftor; VX-770) is being used at 150 mg every 12 hr in CF patients >6 years with at least one G551D-CFTR mutation, and more recently for patients with one of G178R, S549N, S549R, G551S, G1244E, S1251N, S1255P and G1349D. Kalydeco is both safe and results in improvement of clinical measures of CF disease[10], however the mechanism of action of this small molecule was poorly understood at the time of FDA approval for use in patients.

A handful of CFTR purification methods have been described previously[2,11-18], many of which require a considerable length of time to complete. In a recent publication[19], a unique rapid purification and reconstitution method was described for CFTR overexpressed in the S*f*9 cell expression system, and this purified protein in defined lipid systems was used to develop a CFTR halide channel activity assay for a population of CFTR molecules. The assay recapitulates the known effects of phosphorylation, nucleotides and inhibitors on CFTR function. The system was used to interrogate the effects of the potentiator VX-770/Kalydeco on Wt- (wild-type), F508del- and G551D-CFTR and it was shown for the first time that the drug interacts directly with the CFTR protein to potentiate its channel activity in an ATP-independent manner, demonstrating the utility and applicability of these methods to the study of the interaction of CFTR and mutants with nucleotides and small molecules from a population perspective to answer clinically relevant questions about the protein. The methods have also been used to study other potentiator molecules and their derivatives[20], as well as the effects of a small molecule corrector on the activity of the protein[21].

Efflux assays have been used in many studies previously to investigate the activity of CFTR mutants and the effects of CFTR-modulatory compounds on its activity, including whole cell assays using electrodes, radioactive tracers and fluorophores[22,23], membrane vesicles with ion selective electrodes[24], and purified reconstituted CFTR with radioactive tracers[25]. However the use of ion selective electrodes to study purified reconstituted CFTR was first reported recently[19]. An adaptation of the current method has been used for reconstitution and functional characterization of two membrane proteins in *Pseudomonas aeruginosa*, a common CF pathogen. Reconstitution of purified AlgE outer membrane protein coupled with iodide efflux measurements were used to support a model for anionic alginate secretion through this transporter[26]. Reconstitution and iodide efflux measurements were applied to the purified Wzx protein, which allowed a model to be proposed that suggests an H^+-dependent antiport mechanism for lipid-linked oligosaccharide translocation across the bacterial inner membrane by this protein[27]. In both cases iodide was used as a surrogate for the anionic substrate, albeit at lower throughput than one might expect for a native substrate. The method may be suitable for adaptation to other proteins with cationic transport or conduction pathways for anionic substrates.

Here a rapid purification procedure is described for the CFTR protein and its reconstitution into proteoliposomes of defined lipid. The rapid reconstitution procedure can easily be tailored for use with CFTR purified by other methods, provided that the type of detergent used in the purification is amenable to removal by the methods used here or can be exchanged for a suitable detergent before the reconstitution procedure. The iodide efflux method for measurement of channel activity of purified and reconstituted CFTR protein is described in detail and some typical results that can be obtained from this method are presented.

Protocol

1. Purification of CFTR

NOTE: Please see Table of Materials and Equipment for a list of equipment and materials used in this protocol. A detailed protocol exists for overexpression of human Wt-CFTR and mutants in the S*f*9-baculovirus expression system[17,28]. Overexpress CFTR and prepare pellets of S*f*9 cells according to this protocol.

1. Crude membrane preparation
    1. Obtain a fresh or thaw a frozen S*f*9 cell pellet from a 500 ml culture of cells over-expressing wildtype-, F508del-, or G551D-CFTR protein with a C-terminal His_{10}-tag, grown by standard cell culture methods, as described previously[17,28]. Cell pellets will have a volume of approximately 5 ml and a wet weight of approximately 5 g.
    2. Resuspend S*f*9 cells in 20 ml of phosphate buffered saline (PBS) pH 7.4 with protease inhibitor cocktail (1 tablet per 50 ml) at 4 °C, ensuring the sample appears visually homogeneous and no clumps of cells remain.
    3. Lyse cells using a high pressure cell disruptor (Table of Materials and Equipment), reaching a minimum of 10,000 psi (preferably 15,000-20,000 psi) for 2 min per passage. Keep the sample at 4 °C during lysis.
        1. Collect the sample in a tube on ice after the lysis period then immediately reload the sample in the cell disruptor. Repeat lysis procedure 3 times. Examine under a microscope to ensure less than 10% remain intact.
        NOTE: other methods to lyse cells, such as glass beads, etc. have not been rigorously examined for this system, however a user may find such an alternate method suitable.
    4. Centrifuge cell lysis material at 4 °C in a high speed centrifuge at 2,000 x g for 20 min. Collect the supernatant and dispose of the pellet. Divide the supernatant among 20 tubes and centrifuge the samples for 1 hr at 4 °C at 125,000 x g in a table top ultracentrifuge.
    5. Remove and discard the supernatant, being careful not to disturb the pellet, and store pellets in the same tubes at -80 °C for less than 2 months until use, or retain on ice and continue with the purification immediately.
2. CFTR solubilization
    1. Obtain 1-4 fresh or frozen S*f*9 crude membrane pellets and resuspend each in 1 ml of Buffer 1 (2% *fos*-choline; see **Table 1** for complete recipe) at 4 °C on ice, using a 25 G needle and 1 ml syringe until the solution is homogeneous by eye without visible cell membrane clumps. Where more than one pellet is being solubilized, continue to treat each sample per the instructions for a single pellet below in parallel, pooling the samples at step 1.3.2.
    2. Dissolve 1 mini protease inhibitor tablet in 1 ml of Buffer 2 (10 mM imidazole; see **Table 1**) which lacks *fos*-choline 14 detergent (protease inhibitors are 10x more concentrated than their recommended dilution at this point) and add 100 µl to the resuspended crude membrane pellet (protease inhibitors are at their recommended dilution at this point). Set on ice for 45-60 min with mixing by inversion 5 times every 5 min.

3. Centrifuge resuspended crude membrane pellet in a table top ultracentrifuge for 25 min at 125,000 x g and 4 °C. Retain the supernatant, which contains solubilized CFTR and discard the pellet.

3. Column binding, phosphorylation and elution
    1. Wash 400 µl of nickel-NTA (nitrilotriacetic acid) agarose slurry or equivalent resin with 1 ml of Buffer 2 (10 mM imidazole; see **Table 1**) which lacks detergent, to remove solvent and buffer at 4 °C. Repeat wash twice more.
    2. Combine solubilized CFTR, remaining protease inhibitor (900 µl) and nickel resin (from 400 µl slurry) to a total of 10 ml with Buffer 2 (10 mM imidazole; see **Table 1**) which lacks any added detergent. The *fos*-choline 14 concentration is effectively diluted to 0.2% (w/v) at this step. If there are multiple tubes, pool all samples containing solubilized CFTR, protease inhibitors, nickel-NTA resin and 0.2%(w/v) *fos*-choline-14 at this point. Incubate for 60 min at 4 °C with gentle shaking.
    3. Pass CFTR-protease inhibitor-nickel NTA solution down a small screening column (**Table 1**) or an equivalent empty column.
    4. Wash the nickel NTA resin, which is retained in the column, with 4 ml per initial pellet of Buffer 3 (10 mM imidazole+DDM; see **Table 1**) which lacks *fos*-choline 14 but contains 1 mM DDM (dodecyl maltoside detergent), at 4 °C . Addition of DDM detergent does not significantly alter the pH of this buffer.
    5. Prepare PKA-MgATP solution by adding 25 µl (5 mM, final concentration) of MgATP, 2,500 U of cAMP-dependent protein kinase A, catalytic subunit (PKA) to 475 µl of Buffer 3 (10 mM imidazole+DDM; see **Table 1**) to the column. Phosphorylate protein by sealing the bottom end of the column with a cap or Parafilm, and adding 500 µl of PKA-MgATP solution for each initial pellet used.
    6. Incubate at room temperature (20 °C) for 20 min with gentle mixing and resuspension of the resin using a 1 ml pipettor every 2 min to ensure that PKA has come in contact with the entire surface of the nickel NTA resin.
    7. Remove the cap and elute the PKA/MgATP solution from the column. Wash the column with 2 ml of Buffer 3 (10 mM imidazole+DDM; see **Table 1**) at 4 °C.
    8. Multiply the number of pellets used in the experiment by 4. Wash the column with this number of ml of Buffer 4 (50 mM imidazole +DDM; see **Table 1**) at 4 °C, by adding 4 ml of buffer to the column, allowing it to flow through and immediately adding the next 4 ml aliquot to the column.
    9. Elute the protein by passing 1 ml of Buffer 5 (600 mM imidazole+DDM; see **Table 1**) at 4 °C per initial pellet used through the column, 1 ml at a time without a pause to allow the column to dry, and collect the entire eluate containing purified CFTR in a single tube.
    10. Concentrate protein sample to remove imidazole and low molecular weight degradation products using a centrifuge filter device (100,000 molecular weight cut-off) such as described in Table of Materials and Equipment or other similar device, in a total of 10 ml Buffer 6 (75 mM KI+DDM; see **Table 1**) or other buffer of choice containing 1 mM DDM (such as Buffer 7 for non-iodide efflux experiments, 25 mM HEPES+DDM; see **Table 1**), by centrifuging at 2,000 x g and 4 °C for about 20 min until the sample concentrates to 200 µl. Dilute sample to 10 ml and repeat the concentration step.
    NOTE: In our experience (unpublished), imidazole significantly inhibits the ATPase activity of CFTR and should be removed at this step by dilution and concentration at least twice if the sample will be used for functional measurements.
    11. Collect concentrated purified CFTR. Keep at 4 °C in the presence of DDM buffer until use within 1-2 days or continue on immediately to the reconstitution step. Do not freeze the purified protein, as in our experience we observe reduced activity.

2. Reconstitution of CFTR

1. Lipid preparation
NOTE: CFTR has been successfully reconstituted into Egg PC, POPC, 2:1 (w/w) Egg PC:POPC (1:1 w/w) mixture, or a mixture of PE:PS:PC:ergosterol, 5:2:2:1 (w/w).
    1. For iodide efflux experiments, dry 5 mg of Egg PC (200 µl of 25 mg/ml stock, see Table of Materials and Equipment) under a stream of argon gas for 20 min at room temperature in a Pyrex or other sturdy (non-borosilicate) glass tube.
    2. Using absorbance at 280 nm, an ELISA assay or other method, estimate the concentration of the purified CFTR protein sample. For iodide efflux experiments with wildtype CFTR, use a protein:lipid ratio of 1:300-1:3,000 (w/w) to produce a sufficient signal. For G551D- and F508del-CFTR, use a protein:lipid ratio of approximately 1:200-1:1,200 (w/w) in order to detect efflux activity.
    3. Optimize the protein:lipid ratio for each particular system. Calculate the volume of purified protein required. Calculate the volume of buffer with 1 mM DDM required to make a final volume of 1 ml, *i.e.*, 1 ml – (volume of protein solution required) = volume of buffer.
        1. Add the calculated volume of the desired buffer system with 1 mM DDM to the dried lipid (but do not add the CFTR protein at this time) and cover the glass tube with Parafilm. For iodide efflux experiments, resuspend lipids in Buffer 6 (75 mM KI+DDM; see **Table 1**). For other experiments resuspend in Buffer 8 (25 mM HEPES+DDM, see **Table 1**) or another desired internal buffer containing 1 mM DDM.
        2. Vortex for 2 min at room temperature to aid in suspension of the lipid in the DDM buffer. Alternately, heat the sample to 37 °C for 1-2 min before vortexing.
    4. Suspend the lipid repeatedly at room temperature with a 1 ml syringe and 25 G needle until no lipid film is visible on the sides of the tube. Avoid injecting air into the solution which would produce fine bubbles and aid in oxidation of iodide and lipids.
    5. Sonicate the suspended lipid in the Parafilm covered tube for a 20 sec burst in a bath sonicator containing ice, and then transfer immediately to ice for 1 min. Repeat 3 times.
    NOTE: Intense sonication turns iodide solutions yellow due to oxidation. Therefore avoid excessive sonication. Monitor the sample for color changes. If a color change is noted, discard the sample and prepare again. The change in color due to oxidation of some of the iodide would result in oxidation of the lipids under the same conditions. Displacing the air from the tube with argon gas before sonicating the solution will help prevent oxidation of lipids and iodide. Intense sonication can break poor quality or scratched glass tubes resulting in loss of the sample.
    6. Add the volume of purified CFTR calculated in step 2.1.3 to the sonicated lipid sample to a achieve a final volume of 1 ml. Mix the protein with the lipid and detergent solution by resuspension 10 times with a 25 G needle and 1 ml syringe.
    7. Set lipid and protein sample on ice for 30 min with swirling of tube to mix 5 times every 5 min.

2. Detergent-binding column preparation

1. Obtain a single use commercial detergent-binding spin column (see Table of Materials and Equipment) with a 1 ml volume capacity and break the bottom tab to start the column flowing.
2. Centrifuge the column for 2 min at 2,000 x g to remove the storage buffer, and discard this buffer.
3. Add 5 ml of Buffer 7 (75 mM KI, see **Table 1**) to the column and then centrifuge at 200 x g for 4 min to elute the wash buffer. Repeat this step 2 more times for a total of 3 washes to remove all traces of the storage buffer. Discard all flow-through.
   NOTE: It is critical that the buffer used to wash the column DOES NOT contain DDM or any other detergent. The column has a finite binding capacity for detergent and if a buffer containing detergent is used, the column will be ineffective at removing the detergent from the protein-lipid-detergent sample.
4. Carefully aliquot all 1 ml of the lipid-detergent-protein sample onto the top of the column resin and incubate sample at the top of the column for 2 min at room temperature.
5. Centrifuge the sample at room temperature for 4 min at 2,000 x g and collect the cloudy proteoliposome sample in a clean 1.5 or 2 ml microfuge tube or a glass tube and place the sample on ice.
6. Collect remaining proteoliposomes in the column by adding 200 µl of Buffer 7 (75 mM KI, **Table 1**) or other buffer (without detergent) to the top of the column and repeat the centrifugation step for 2 min.
7. Add the second eluate to the proteoliposome sample if it looks cloudy.
8. Store the sample at 4 °C overnight or use immediately for iodide efflux measurements.

3. Iodide Efflux Measurements for Purified, Reconstituted CFTR

1. Generation of an iodide gradient across the proteoliposomal membrane
   1. Pre-swell Sephadex G50 beads in Buffer 9 (75 mM K-Glu; potassium glutamate, see **Table 1**), (approximately 5 g dry beads in 50 ml buffer) or desired external buffer at room temperature for at least 2 hr or at 4 °C overnight.
   2. Prepare 4 Sephadex G50 columns saturated in Buffer 9 (75 mM K-Glu, see **Table 1**) or other buffer in small screening columns by loading resin to at least ¾ full in the column.
   3. Centrifuge for 4 min at 2,000 x g to remove excess buffer from the resin. There should be approximately 2.25 ml of packed hydrated resin in the column at the end of the centrifugation step.
      NOTE: Resin should be lightly hydrated but not immersed in liquid and a subsequent brief spin should not elute significant volumes of buffer. It is critical to the successful elution of the proteoliposome sample that the column resin is neither too wet nor too dry and the user may need to experiment with the columns in order to become familiar with the most successful buffer exchange conditions.
   4. Carefully add 125-150 µl of proteoliposome sample to the top of each column and centrifuge for 4 min at 2,000 x g.
   5. Pool proteoliposome eluates together for immediate use in iodide efflux or other experiments.
      NOTE: The eluted volume from each column should be roughly 150 µl and the sample should be somewhat cloudy, but less cloudy than the original sample. Larger volumes or clear eluates suggest that the columns haven't been dried sufficiently, or have been dried too much, respectively, and will not result in a successful iodide efflux experiment.

2. Iodide efflux assay
   1. Wash an iodide selective micro electrode (Table of Materials and Equipment) extensively with de-ionized water, and then incubate for 20 min before the experiment in Buffer 10 (15 µM KI, see **Table 1**). Wash the electrode again extensively with de-ionized water.
   2. Add 150 µl of drug (20 µM typical concentration to produce a final concentration of 10 µM in the assay) in Buffer 9 (75 mM K-Glu, see **Table 1**) or vehicle in Buffer 9 to one well of a 96-well plate with a small flea stir bar.
      1. Ensure that there are no bubbles present. Use a pipette tip to remove stray bubbles. If a drug solution is being used, ensure that the final DMSO concentration in the well is less than 1% (v/v) (typically 0.1-0.2% (v/v)).
   3. Add 150 µl of reconstituted CFTR protein that was produced in section 3.1 in Buffer 9 (75 mM K-Glu, see **Table 1**) to the well with drug or buffer, to produce a total volume of 300 µl in the well of the plate.
   4. Place the 96-well plate on a stir plate and stir at 130 rpm (or at a moderate speed of approximately ¼ of the maximum speed).
   5. Insert the tip of the iodide selective electrode carefully into the well with the sample such that the tip is not touching the bottom of the well or being hit by the stir bar. Ensure that the reference electrode, if separate, is also in contact with the solution in the well.
   6. Record tracings using a home-made or commercial computer program that interfaces with the electrode (see Table of Materials and Equipment for details). Use a sampling rate of 2 Hz, with filtering of the signal through a low pass filter.
   7. Allow the sample to equilibrate for 5 min to produce a stable flat, or near flat baseline while recording.
   8. Add 3 µl MgATP (100 mM stock prepared in dH_2O and adjusted to pH 7 with KOH; 1 mM final concentration) to the sample to activate the protein. Allow ~5 min to reach a new stable baseline. Always adjust the pH of the 100 mM MgATP stock to neutral before use to avoid changes in the pH of the external buffer.
   9. Add 3 µl of valinomycin stock (2 µM; 20 nM final concentration) to the sample to shunt changes in potential difference generated by iodide-selective conductance through CFTR. Record the decreasing slope (decrease in mV) due to CFTR-mediated iodide efflux activity for at least 5 min.
   10. To ensure that the trapping of iodide in the proteoliposome lumen was sufficient, add 3 µl of 10% (v/v) Triton X-100 to the sample. Significant and immediate iodide release indicates that sufficient iodide has been trapped in the vesicles such that trapping was not the limiting factor for the changes in slope determined in 3.2.9 but rather the CFTR-mediated activity.
   11. Wash the electrode and stir bar thoroughly with de-ionized water after treatment of samples with drug or with Triton X-100 to ensure all traces of these reagents have been removed to avoid contamination of the next sample.
   12. Prepare a series of dilute KI concentrations in the micromolar to nanomolar range in Buffer 9 (K-Glu buffer, see **Table 1**). Record the mV response of the electrode to each concentration from 0 to the highest concentration prepared. Construct a standard curve where the probe response (mV) is plotted vs. log of the molar iodide concentration. Use the standard curve to convert the mV response of the probe during the efflux experiment to concentration of iodide released.
      NOTE: Prepare a calibration curve on a weekly basis until a user is sure of how quickly the response of the probe changes. There will be a drift in the response and sensitivity of the probe over time. As the probe ages, the response will degrade. This may be partially

abrogated by changing the internal solutions periodically and extensive washing of the probe tip in water after use, which likely limits adherence of molecules to the probe surface.

13. Determine the average slope of the line of the concentration vs. time plot for each proteoliposome sample for the last 30 sec before addition of MgATP, and after addition of valinomycin but before addition of Triton X-100. Subtract the baseline slope from the valinomycin to determine the CFTR-mediated iodide efflux activity for that sample.

Representative Results

Described in this written publication are methods to purify, reconstitute and measure regulated channel activity of the CFTR protein. **Figure 1a** shows the workflow for the purification, reconstitution and iodide efflux procedures. Methods for reconstitution and channel activity measurements by iodide flux are also shown in further detail in the associated video.

Purification and reconstitution of CFTR into proteoliposomes

CFTR can be functionally expressed at high levels in S*f*9 cells using recombinant baculovirus containing Wt or mutant CFTR cDNA[2]. While not a mammalian expression system, CFTR was expressed using the baculovirus system in S*f*9 cells to ensure a high level of expression. CFTR production can be as high as 1% of total cellular protein[17,28]. The S*f*9- baculovirus system has the advantage that the protein is core glycosylated and the quality control machinery in this system is relatively permissive such that CFTR and mutants can be produced on the cell surface. A ten histidine tag was engineered onto the carboxy terminus to allow purification by virtue of affinity of this tag for nickel-NTA. The C-terminal tag helps to prevent co-purification of truncated CFTR proteins and yields functional CFTR protein in ATPase, single channel and iodide efflux experiments[17,19,24,28,29]. However the purification protocol described here should be suitable for N-terminally tagged CFTR as well.

Previously, it has been shown that CFTR protein can be effectively extracted from S*f*9 plasma membrane preparations using NaPFO (sodium pentadecafluoro-octanoate) at room temperature[15]. However, the susceptibility of NaPFO to precipitate at 4 °C, a temperature conducive to preserving properly folded protein, and the lengthy dialysis required to remove PFO were not amenable to rapid purification and reconstitution methods intended to maximize the activity of the protein in subsequent assays of CFTR function. An evaluation of a panel of detergents (including dodecyl-β-D-maltoside (DDM), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), *fos*-choline 12 and *fos*-choline 14, showed that *fos*-choline 14 was most effective in solubilizing human CFTR-His_{10} from S*f*9 membranes, and that DDM was effective in maintaining the ATPase activity of CFTR in detergent solution (data not shown). Thus a *fos*-choline 14 detergent extraction step is performed, followed by exchange to DDM detergent to maintain the activity of the protein.

Following purification and concentration, a protein fraction is obtained in DDM detergent that contains a ~150 kDa band as its major component (**Figure 2**; Silver Stain). This molecular weight corresponds to human CFTR expressed in S*f*9cells and analyzed by SDS-PAGE, where it lacks the types of complex glycosylation characteristic of CFTR in mammalian cells. The 150 kDa species appears to be approximately 90% pure following SDS-PAGE analysis and silver staining. Western blotting using the M3A7 anti-CFTR antibody confirms that this mouse monoclonal directed against NBD2 reacts with the 150 kDa band (**Figure 2**; blot). In certain studies, trace amounts of lower molecular weight components are present as minor bands visible on the gel, at ~60 and ~80 kDa (data not shown). The M3A7 anti-CFTR antibody reacts with the 60 and 80 kDa bands, but does not react with other non-CFTR proteins (data not shown). This suggests that the lower molecular weight components are CFTR degradation products co-purified with CFTR via their C-terminal His tag. The purification is done in the presence of protease inhibitors and it appears that these components are present in the cells before the purification procedure is performed. The concentration step of the procedure removes virtually all of the contaminating lower molecular weight fragments. CFTR can be reconstituted at this point, or protein from another purification procedure can be used in the reconstitution protocol.

A liposomal flux based assay reports the functional reconstitution of a population of purified and reconstituted CFTR molecules as regulated, anion selective channels

In order to determine if a significant proportion of purified CFTR was reconstituted as a regulated chloride channel using the above methods, a proteoliposomal halide flux assay was developed which reports the activity of a population of reconstituted CFTR molecules (shown schematically in **Figure 1B**). If, in the worst case scenario, the majority of the reconstituted CFTR molecules are misfolded, these molecules will fail to confer a conductance, fail to discriminate between cations and anions and/or the gate will lack regulation by PKA phosphorylation and nucleotides. Lee *et al.* have published methods that allow estimation of percent functional reconstitution of a membrane protein[30].

Empty liposomes (lacking CFTR) or proteoliposomes (bearing reconstituted CFTR) are loaded with KI (75 mM) and extra-liposomal iodide exchanged with potassium glutamate (75 mM) by passing liposomes through a gel filtration column. The KI loaded proteoliposomes are added to a well containing K-Glu (potassium glutamate) to create an outward gradient for iodide and efflux is monitored as increases in extraliposomal iodide over time using an iodide sensitive microelectrode[24]. The efflux of negatively charged iodide results in a decrease in the mV signal (signal becomes more negative). When the concentration of iodide released over time is plotted, the slope is inverted (as in **Figure 3**) to show an increase in iodide in the external solution over time.

K-Glu was chosen as the impermeant counterion as it is one example of an anion that does not pass through the CFTR protein, does not appear to cause significant blocking of the pore under the conditions used, and does not cause interference with the iodide selective electrode. An assay could be optimized around another anion, provided that it meets these criteria. Seventy-five mM anion concentrations were selected empirically as concentrations that gave good trapping and sufficient signals for measurement in the assay under the conditions described. There will be essentially no iodide release from iodide-loaded liposomes lacking functionally reconstituted CFTR until the vesicles are permeabilized with detergent (**Figure 3**; control traces), whereas CFTR-reconstituted, iodide-loaded proteoliposomes mediate iodide release into the external solution after addition of MgATP (1 mM) to open the CFTR channel and valinomycin (20 nM) to prevent the development of a membrane potential (**Figure 3A**; see trace for protein:lipid mass ratio of 1:3,000 (w/w)).

Valinomycin is an ionophore that shuttles potassium specifically across the membrane, down its concentration gradient and the concentration of 20 nM was chosen empirically as the highest concentration which did not perturb integrity of empty liposome in the present system. This may need to be optimized for each particular system. Without addition of valinomycin, there is an abbreviated iodide release of a few nM that quickly reaches a plateau (data not shown). Although these conditions are known to otherwise maximally activate the anion channel activity of CFTR (as in **Figure 3A**), it appears that the lack of a significant response reflects the instantaneous increase in intraliposomal positive charge mediated by iodide conduction through the anion selective pore of CFTR, immediately limiting continuous iodide efflux where valinomycin is not included. This valinomycin-mediated response confirms that iodide efflux was limited in the previous experiments because of charge accumulation, supporting the anion selectivity of reconstituted CFTR. If CFTR lacked this selectivity, both potassium and iodide would have been able to efflux from proteoliposomes thereby abrogating the need for valinomycin to initiate flux.

The iodide concentrations measured at these initial time points were empirically chosen to fall within the optimal sensitivity range for the iodide sensing electrode employed. Use of other iodide electrodes may require optimization of these factors. After reaching a plateau, Triton X-100 is added to permeabilize the proteoliposomes and release any remaining trapped iodide. Unlike the initial iodide measurements (obtained between 1 and 2 min after valinomycin addition), the readings at these endpoints are not in the linear range of the electrode calibration curve (data not shown), thereby precluding an accurate evaluation of the fraction of liposomes from which CFTR protein is capable of mediating iodide efflux relative to the total number of liposomes in the assay, or its percent functional reconstitution.

The rate of iodide flux reflects the number of CFTR protein molecules, the open probability and unitary conductance of each CFTR channel. Hence, the rates of iodide efflux should be dependent on the number and open probability of each CFTR molecule. The prediction was tested in this system by increasing the number of CFTR molecules per liposome. The ratio of activated (phosphorylated and MgATP treated) CFTR protein to lipid was varied, with the ratios ranging from 1:300-1:3,000 (w/w) (**Figure 3A**). The greater the amount of reconstituted CFTR present in the proteoliposomes, the greater the rate of efflux (measured between 1-2 min after valinomycin addition), supporting the prediction that efflux rates are dependent on the number of CFTR molecules in each liposome. The efflux rate obtained using specific protein:lipid ratios are reproducible between experimental days and protein purifications, thus highlighting the rigor of this method.

The rate of iodide efflux appears to relate to the open probability of the CFTR channel in this reconstituted system. As phosphorylation by PKA is required for CFTR activation[31] (and review by[32]), it is expected that there will be a low rate of iodide efflux in vesicles containing CFTR that has not been phosphorylated by exogenous PKA treatment, where the open probability is low. In proteoliposomes that have been PKA treated, the open probability will be much higher, resulting in a much greater anticipated rate of efflux. In practice, for PKA-treated CFTR in the presence of 1 mM MgATP in this system, the rate of iodide efflux measured from one to two minutes after addition of valinomycin is approximately four fold the rate of CFTR protein subjected to MgATP but not PKA (**Figure 3B**). These findings support the hypothesis that the efflux rate relate to channel open probability. In practice it may be difficult to compare activity in this assay directly to open probability given that other factors such as time dependent changes in the electrochemical driving force will influence the observed efflux activity. The reader is cautioned that this assay is not a replacement for detailed single channel activity recordings.

Interestingly, there is typically observed a low rate of iodide efflux by “unphosphorylated” protein in the presence of MgATP which is significantly greater than efflux from liposomes lacking CFTR (**Figure 3B, C**). This low rate may reflect basal phosphorylation of CFTR in the S*f*9 cell expression system prior to purification[33]. CFTR purified from other expression systems may have different levels of basal phosphorylation and therefore different residual activity.

This rapid purification protocol yields Wt-CFTR from S*f*9 cells at near homogeneity, however for F508del- and G551D-CFTR, the method is better described as an enrichment procedure. Some contaminating bands are observed via SDS-PAGE[19], many of which reflect CFTR degradation products that are reactive to CFTR antibodies and appear to be present before cell disruption. The reconstitution and iodide flux methods are suitable for these enriched samples of clinically relevant mutants. As shown in **Figure 3D**, significant iodide efflux is measured for both F508del- (protein:lipid mass ratio of approximately 1:600) and G551D-CFTR (protein:lipid ratio of approximately 1:300). While both mutants have lower absolute activity than Wt-CFTR (protein:lipid mass ratio of 1:1,200) , it is difficult to compare the samples directly as quantitation cannot be as accurate for the mutants that are contaminated with CFTR degradation products. However both F508del-CFTR and G551D purified by these methods, reconstituted and subjected to efflux measurements respond as expected to small molecule potentiators (**Figure 4**), and show similar channel function to protein purified by the more rigorous PFO purification method[19].

The channel activity of purified and reconstituted CFTR can be modulated by small molecule inhibitors and potentiators

$CFTR_{inh}$-172[34] is the most specific CFTR inhibitor available that has been shown to inhibit the open probability of CFTR chloride channels in single channel conductance measurements[35] and to inhibit CFTR activity in other studies[22,36]. As shown in **Figure 3C**, the rate of efflux after valinomycin addition is significantly reduced by pretreatment with $CFTR_{inh}$-172. Hence, this assay of the channel activity of a population of purified and reconstituted CFTR molecules is effective in reporting activity of modulators such as the inhibitory ligand $CFTR_{inh}$-172.

The assay is sensitive and highly applicable to examination of the effects of small molecule potentiator treatment as well. As shown in **Figure 4**, all three CFTR genotypes tested are significantly potentiated by active small molecule potentiators such as Kalydeco/VX-770 or VRT-532 (**Figure 4C**; shown for G551D), while an inactive analog has no effect on the iodide efflux activity of CFTR (shown for F508del-CFTR; **Figure 4B**). The assay is applicable to the examination of potentiator concentration dependence, and the role of ATP and phosphorylation on potentiator-mediated CFTR activity.

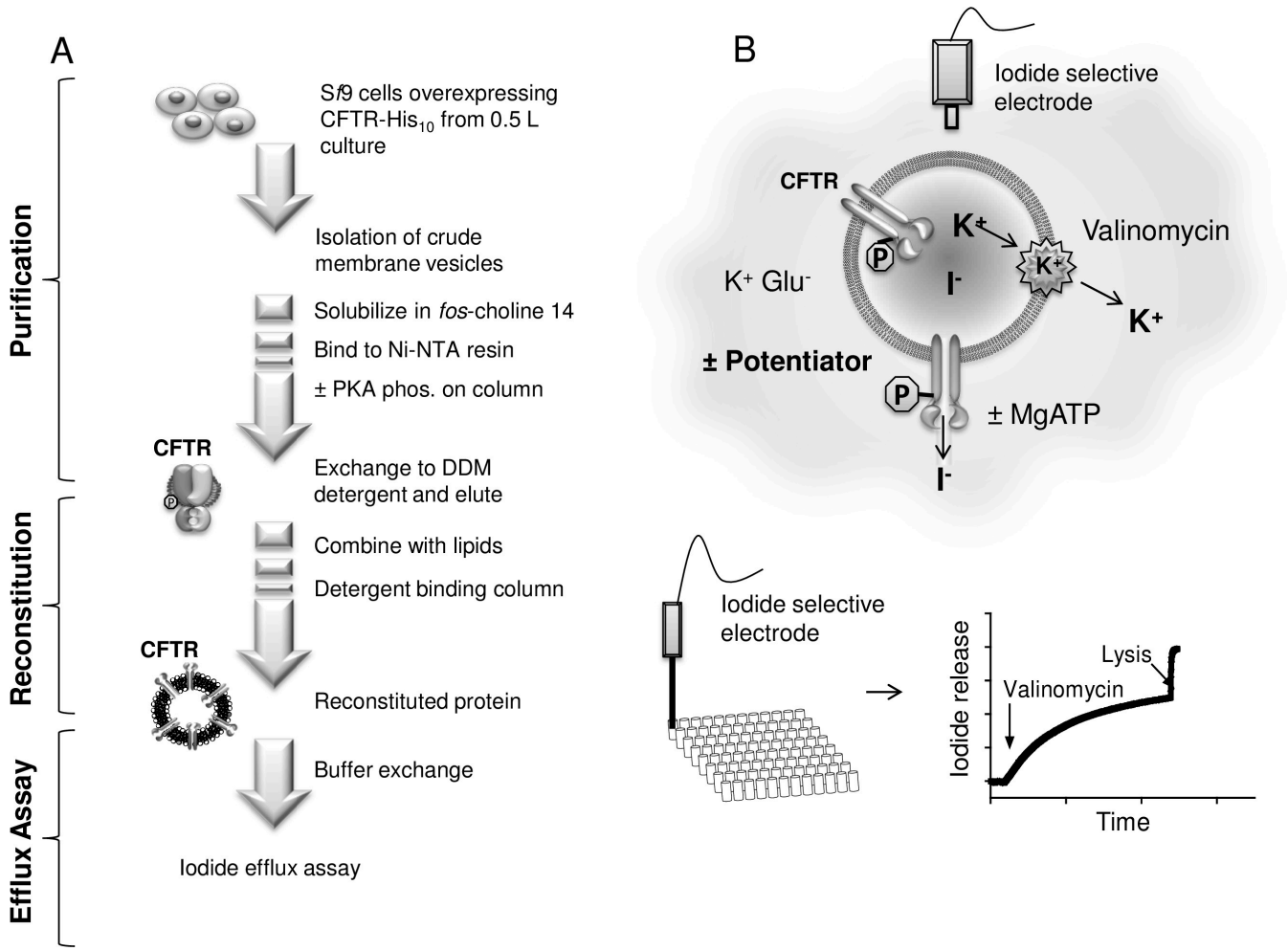

Figure 1: Assay design and workflow. (A) Workflow for the work described in this publication. **(B)** Schematic representation of the iodide efflux experiment. A modified version of panel B was originally published in The Journal of Biological Chemistry. Eckford, P.D.W.; Li, C.; Ramjeesingh, M.; and Bear, C.E. Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Potentiator VX-770 (Ivacaftor) Opens the Defective Channel Gate of Mutant CFTR in a Phosphorylation-dependent but ATP-independent Manner. 2012; **287:** 36639-36649. ©The American Society for Biochemistry and Molecular Biology.

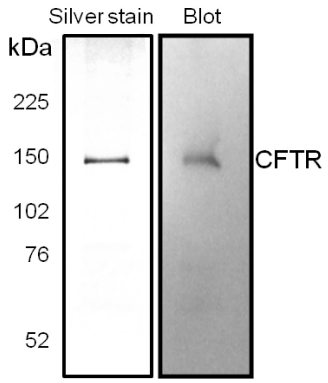

Figure 2: The CFTR rapid purification procedure yields purified Wt-CFTR protein. Silver stained SDS-PAGE gel and Western blot using the M3A7 CFTR antibody for purified human Wt-CFTR protein in DDM detergent (1 µg and 0.1 µg for the gel and blot, respectively), isolated from Sf9 cells over-expressing a $(His)_{10}$-tagged version of the protein. Wt-CFTR (>90% pure) is detected at 150 kDa, appropriate for this protein lacking complex glycosylation. This research was originally published in The Journal of Biological Chemistry. Eckford, P.D.W.; Li, C.; Ramjeesingh, M.; and Bear, C.E. Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Potentiator VX-770 (Ivacaftor) Opens the Defective Channel Gate of Mutant CFTR in a Phosphorylation-dependent but ATP-independent Manner. 2012; **287:** 36639-36649. ©The American Society for Biochemistry and Molecular Biology.

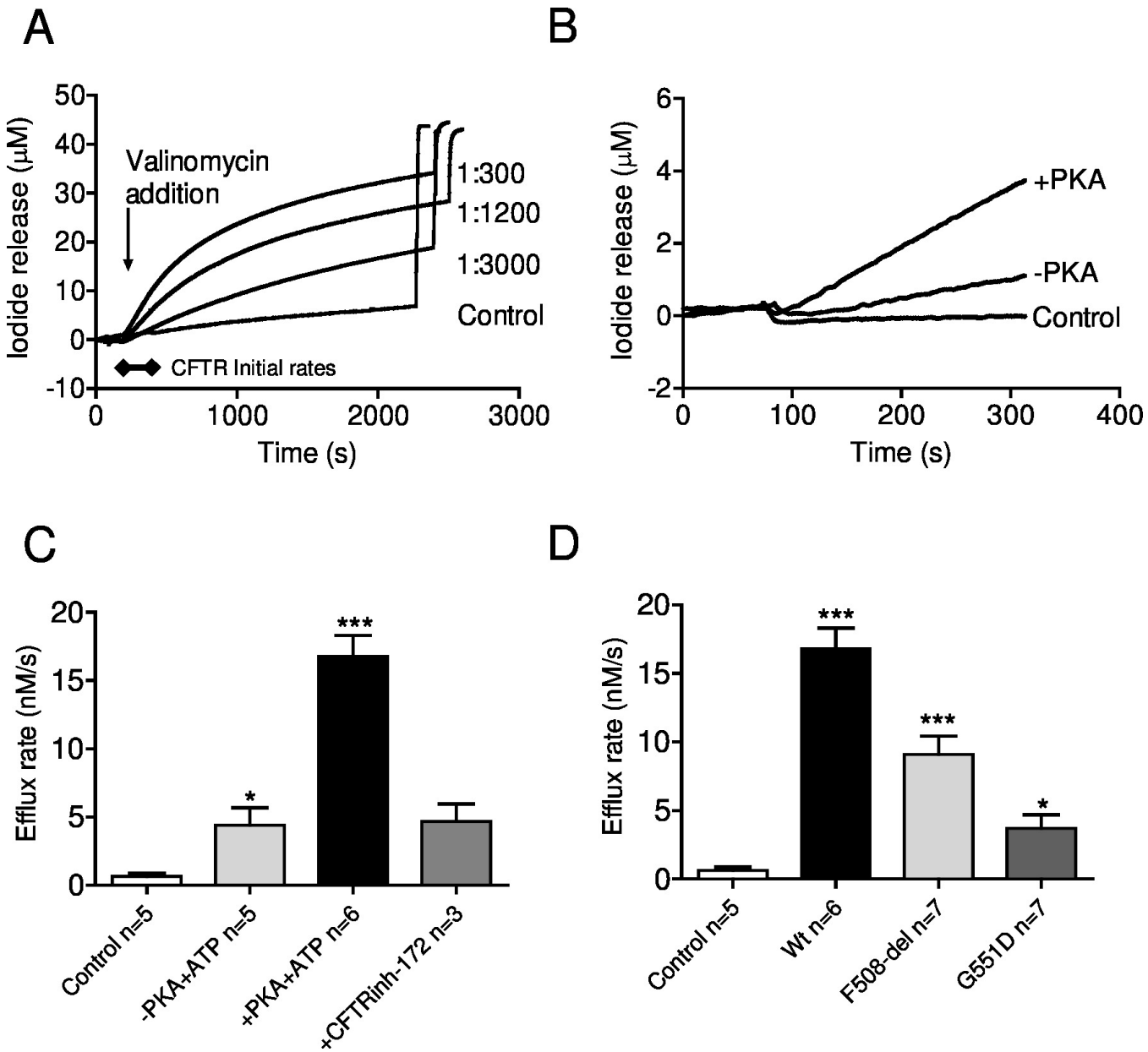

Figure 3: The iodide efflux assay reports regulated channel activity for the purified and reconstituted CFTR protein. (A) Purified and phosphorylated Wt-CFTR reconstituted at protein:lipid ratios of 1:300 (w/w), 1:1,200 (w/w) and 1:3,000 (w/w) mediates iodide efflux from the vesicle lumen to the bulk bath over time after addition of 1 mM MgATP and 20 nM valinomycin. The addition of 0.1% (w/v) Triton X-100 to permeabilize the proteoliposomes releases trapped iodide in the vesicle lumen at the end of the experiment. **(B)** Initial iodide efflux time course for reconstituted Wt-CFTR protein that had not been pre-phosphorylated. **(C)** Initial iodide efflux rates demonstrate the dependence of the observed activity on functional CFTR protein. PKA phosphorylation of the protein is required for significant channel activity, which is inhibited by the addition of the CFTR inhibitor, $CTTR_{inh}$-172 (20 μM). Data are means ± SEM; *p <0.05, ***p <0.0001. **(D)** Multiple genotypes produce regulated CFTR signals in the iodide efflux assay, including Wt-, F508del- and G551D-CFTR versus control. Data are means ± SEM; *p<0.05; ***p<0.0004 vs. control. This research (portions of data in panels A, C and D) was originally published in The Journal of Biological Chemistry. Eckford, P.D.W.; Li, C.; Ramjeesingh, M.; and Bear, C.E. Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Potentiator VX-770 (Ivacaftor) Opens the Defective Channel Gate of Mutant CFTR in a Phosphorylation-dependent but ATP-independent Manner. 2012; **287:** 36639-36649. ©The American Society for Biochemistry and Molecular Biology.

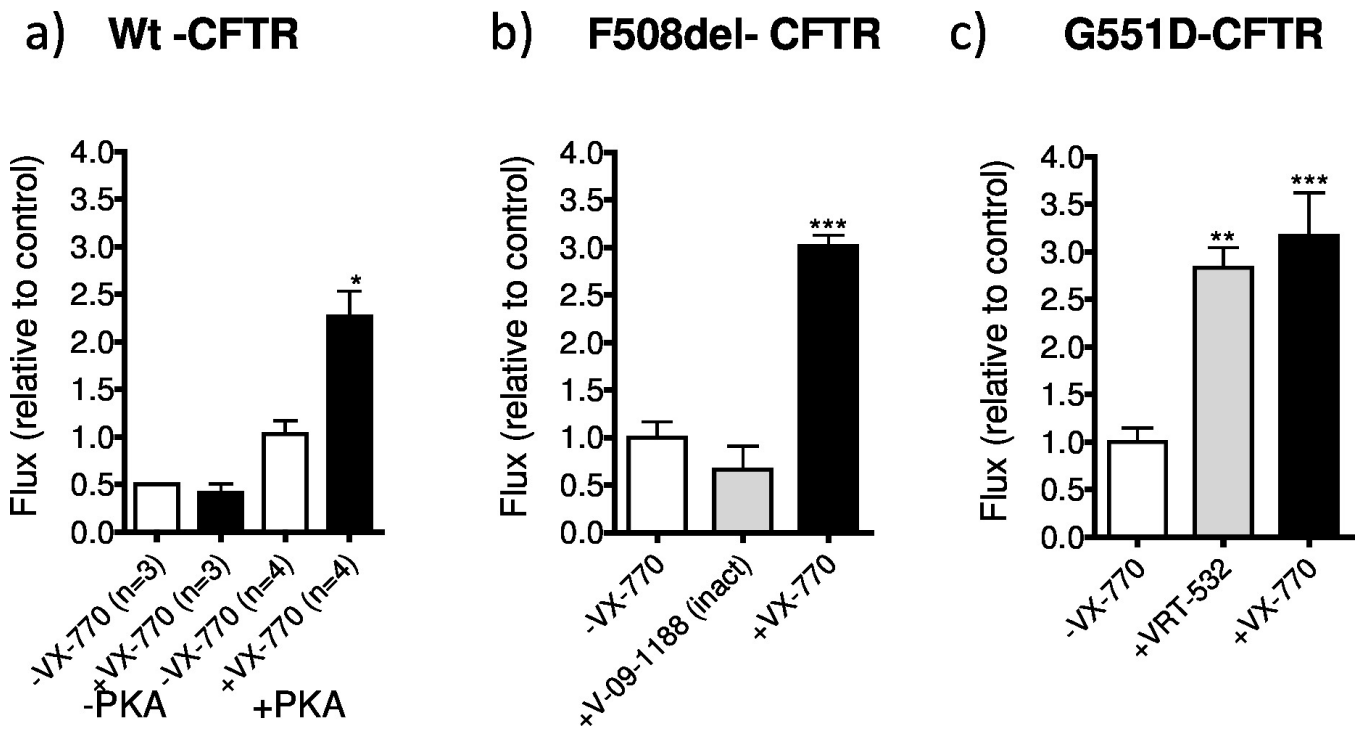

Figure 4: The phosphorylation-dependent potentiation of CFTR by small molecules can be interrogated using the iodide efflux system for Wt-, F508del- and G551D-CFTR. (a) Wt-CFTR is potentiated significantly by the potentiator VX-770 (10 μM), only in the PKA-phosphorylated state. Data are means ± SEM, *p <0.01 vs. +PKA-VX-770. **(b)** VX-770 (10 μM) but not an inactive analog, V-09-1188 (10 μM), significantly potentiates the iodide efflux activity of phosphorylated F508del-CFTR. Data are means ± SEM, n = 3, ***p <0.001 vs. –VX-770 control. **(c)** Potentiation of G551D-CFTR by 10 μM VRT-532 or VX-770. Data are means ± SEM, n = 5, **p <0.001, ***p <0.0001 vs. –VX-770 control. This research (panels a-c) was originally published in The Journal of Biological Chemistry. Eckford, P.D.W.; Li, C.; Ramjeesingh, M.; and Bear, C.E. Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Potentiator VX-770 (Ivacaftor) Opens the Defective Channel Gate of Mutant CFTR in a Phosphorylation-dependent but ATP-independent Manner. 2012; **287:** 36639-36649. ©The American Society for Biochemistry and Molecular Biology.

Buffer	Name	Contents	pH	Adjust pH using
Buffer 1	2% *fos*-choline	2% (w/v) *fos*-choline 14 detergent in 10 mM imidazole, 25 mM HEPES	7.4	Imidazole/HEPES
Buffer 2	10 mM imidazole	10 mM imidazole, 25 mM HEPES (no detergent)	7.4	Imidazole/HEPES
Buffer 3	10 mM imidazole+DDM	10 mM imidazole, 25 mM HEPES, 1 mM dodecyl-β-D-maltoside	7.4	Imidazole/HEPES
Buffer 4	50 mM imidazole+DDM	50 mM imidazole, 25 mM HEPES, 1 mM dodecyl-β-D-maltoside	7.4	Imidazole/HEPES
Buffer 5	600 mM imidazole+DDM	600 mM imidazole, 25 mM HEPES, 1 mM dodecyl-β-D-maltoside	7.4	Imidazole/HEPES
Buffer 6	75 mM KI+DDM	75 mM KI, 20 mM MOPS, 1 mM dodecyl-β-D-maltoside	7.4	KOH
Buffer 7	75 mM KI	75 mM KI, 20 mM MOPS	7.4	KOH
Buffer 8	25 mM HEPES+DDM	25 mM HEPES, 25 mM NaCl, 1 mM dodecyl-β-D-maltoside	7.4	HCl/NaOH
Buffer 9	75 mM K-Glu	75 mM K-Glutamate, 20 mM MOPS	7.4	KOH/Glutamic acid
Buffer 10	15 mM KI	15 mM KI, 20 mM MOPS	7.4	KOH

Table 1: Table of Buffers.

Discussion

There have been a limited number of purification protocols for full-length, functional CFTR isolation, from a variety of cellular overexpression systems. The method described here is advantageous as it allows rapid purification of Wt-CFTR or high enrichment of F508del- and G551D-CFTR in moderate quantities that is highly functional in assays including ATPase and direct measurements of channel function, including single channel measurements in planar bilayer systems and demonstrated measures of CFTR population channel function that are highly relevant to the study of small molecules[19-21]. The purification method is integrated into a rapid and effective reconstitution protocol, however purified protein from other methods may be coupled to this reconstitution protocol as well, provided that the purification detergent is amenable to removal similarly.

As opposed to complicated and painstaking single channel experiments from excised membrane patches, the methods described here allow a robust yet straightforward assessment of CFTR channel function, and uniquely this measure reports the function of a larger population of CFTR channels rather than one or a few molecules. Unlike techniques involving membrane patches, this method allows direct assessment of the effects of small molecule modulators on the channel in the near or complete absence of associated proteins, depending on the effectiveness of the coupled purification procedure.

Critical steps within the protocol

Although there are several critical steps in functional reconstitution of CFTR for the anion flux assay, as indicated notes in the Protocol, optimization of the protein:lipid ratio during CFTR reconstitution is key. Such optimization is required for each CFTR genotype.

Modifications and troubleshooting of the technique

The baseline mV response for the probe used here during the iodide efflux assay with samples prepared as described is typically -20 to -50 mV. Significant deviations from this range suggest there may be problems with the sample. Other probes sold by different manufacturers may differ from the guidelines provided here. If a stable baseline cannot be achieved, residual detergent or inactive, denatured, poor quality protein may be permeabilizing the vesicles to iodide.

Failure to detect a significant change in the rate of valinomycin dependent iodide efflux from liposomes containing phosphorylated CFTR in the presence of MgATP may reflect several issues, all of which should be investigated. These issues include: poor quality, partially denatured CFTR or impure CFTR; incomplete removal of detergent during reconstitution; non-optimal protein: lipid ratio in proteoliposomes; or insufficient valinomycin added, rendering charge dissipation incomplete.

The iodide efflux assays offers the flexibility to use pre-phosphorylated CFTR protein (as described in the protocol), or to phosphorylated CFTR during the assay. If the reconstituted protein was not phosphorylated during the purification procedure, the sample can be phosphorylated during the iodide efflux experiment at step 3.2.7, by addition of PKA with the MgATP. In this case ensure that the baseline is recorded for at least 5 min to ensure that the protein has been sufficiently phosphorylated by the PKA enzyme before measuring function with addition of valinomycin. Similar results are obtained by either method.

During the protocol, the modulator molecule is allowed to interact with the CFTR protein for 5 min before the measurement of activity by addition of MgATP and then Valinomycin. As most CFTR modulators are very lipophilic, it may take time between addition to the bath and equilibrium association with the CFTR protein. The assay cannot proceed without the addition of valinomycin, and thus no information is lost by performing the assay in this manner. In situations where the user is not concerned that there may be a lag time before interaction of the small molecule with the protein, the assay could be performed by the acute addition of modulator *after* the addition of valinomycin, while still in the initial rate phase of the measurement.

Limitations of the technique

As stated previously, this proteoliposomal flux based assay of regulated channel activity by a population of purified and reconstituted full-length CFTR molecules provides a unique method for interrogating ligands that directly modify activity. However, the method is limited in that it doesn't provide insight into the mechanism through which ligands change the rate of flux through CFTR. Ideally, the proteoliposomal flux assay of purified and reconstituted CFTR should be used in combination with planar lipid bilayer studies of single channel activity. This combination would provide insight into the state of the protein that binds the ligands, *i.e.* the open and/or the closed state.

Significance with respect to existing methods

There is currently a lack of methods for assessing the direct interaction of small molecules with the CFTR channel versus small molecule modulators that interact with another cellular component to indirectly alter channel activity, *e.g.*, through a modulatory or signaling pathway or disruption of important protein-protein interactions. The iodide efflux method demonstrates direct interaction with the protein of any small molecule that modulates the channel activity of CFTR. Despite the low throughput manner of the measurements, there remains considerable value in these unique methods for studying the direct interaction of CFTR with small molecule potentiators and correctors. Many small molecule modulators are under development by both academic groups and the pharmaceutical industry to treat CF patients clinically, as this is the major thrust of current CF research. It is anticipated that the methods described here will aid in the development and validation of the mechanism of action of the most promising of these molecules.

Future applications of the technique

The methods employed here are also highly amenable to adaption to other proteins of interest. Indeed a modification of the described methods was employed to study two *P. aeruginosa* membrane proteins implicated in the movement of anionic substrates across the membrane[26,27],

demonstrating the utility and versatility of these methods. Their use in the study of additional anion channels and transporters is anticipated in the future.

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