A Small-Molecule Modulator Interacts Directly with ΔPhe508-CFTR to Modify Its ATPase Activity and Conformational Stability

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ABSTRACT

The deletion of Phe-508 (ΔPhe508) constitutes the most prevalent of a number of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) that cause cystic fibrosis (CF). This mutation leads to CFTR misfolding and retention in the endoplasmic reticulum, as well as impaired channel activity. The biosynthetic defect can be partially overcome by small-molecule "correctors"; once at the cell surface, small-molecule "potentiators" enhance the channel activity of ΔPhe508-CFTR. Certain compounds, such as VRT-532, exhibit both corrector and potentiator functions. In the current studies, we confirmed that the inherent chloride channel activity of ΔPhe508-CFTR (after biosynthetic rescue) is potentiated in studies of intact cells and membrane vesicles. It is noteworthy that we showed that the ATPase activity of the purified and reconstituted mutant protein is directly modulated by binding of VRT-532 [4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol] ATP turnover by reconstituted ΔPhe508-CFTR is decreased by VRT-532 treatment, an effect that may account for the increase in channel open time induced by this compound. To determine whether the modification of ΔPhe508-CFTR function caused by direct VRT-532 binding is associated with structural changes, we evaluated the effect of VRT-532 binding on the protease susceptibility of the major mutant. We found that binding of VRT-532 to ΔPhe508-CFTR led to a minor but significant decrease in the trypsin susceptibility of the full-length mutant protein and a fragment encompassing the second half of the protein. These findings suggest that direct binding of this small molecule induces and/or stabilizes a structure that promotes the channel open state and may underlie its efficacy as a corrector of ΔPhe508-CFTR.

Cystic fibrosis is caused by mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene. CFTR is a member of the ATP binding cassette (ABC) superfamily of membrane proteins (Riordan et al., 1989). Together, ABC family members mediate the transport of a diverse range of substrates, with some family members working as exporters and some as importers. These activities depend on regulated interactions between their cytosolic nucleotide binding domains (NBDs) and membrane-spanning domains (MSDs). The NBDs of CFTR mediate ATP binding and ATPase activity like the other members of this family (Li et al., 1996; Ramjeesingh et al., 2008). However, CFTR is unique in that its membrane-spanning domains form a chloride-selective pore to mediate chloride conduction (Bear et al., 1992; Linsdell, 2006). ATP binding at the interface between NBD1 and NBD2 in CFTR is thought to promote opening of the channel gate, whereas ATPase activity promotes dissociation of the NBD heterodimer, leading to the closing of the channel (Lewis et al., 2004; Vergani et al., 2005a; Aleksandrov et al., 2007). Opening of the CFTR channel gate also requires phosphorylation of multiple serine residues within the unique "R domain" region by PKA, a modification that alters "R domain" interactions with other domains, notably NBD1 (Winter and Welsh, 1997; Seibert et al., 1999; Csanády et al.,

ABBREVIATIONS: CFTR, cystic fibrosis transmembrane conductance regulator; ABC, ATP binding cassette; NBD, nucleotide binding domain; MSD, membrane-spanning domain; PKA, protein kinase A; VRT-532, 4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol; BHK, baby hamster kidney; HA, hemagglutinin; MOPS, 3-(N-morpholino)propanesulfonic acid; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; FL, full-length; CFTRinh-172, CFTR inhibitor-172.

2005; Baker et al., 2007). Overall, interactions between multiple domains are necessary to communicate signals between cytosolic domains and the chloride channel gate in the membrane.

The major mutation found in CFTR results in the deletion of phenylalanine at position 508. This mutation leads to a serious folding defect, causing ΔPhe508-CFTR to be retained in the endoplasmic reticulum and quickly degraded (Cheng et al., 1990). The first clues to the structural basis for this trafficking defect came from protease susceptibility studies by Zhang et al. (1998). The full-length ΔPhe508-CFTR protein exhibited an enhanced susceptibility to digestion by lower concentrations of trypsin relative to the normal CFTR protein, supporting the idea that the mutant protein is conformationally altered and tryptic digestion sites are aberrantly exposed (Sharma et al., 2004; Du et al., 2005). More recent structural studies of protein fragments corresponding to NBD1 (Lewis et al., 2005) and molecular models of the full-length CFTR protein (Mornon et al., 2008; Serohijos et al., 2008) suggest that Phe508 resides at the interface between NBD1 and the MSD2 and that the deletion of this residue may perturb native intramolecular interactions. Chemical cross-linking studies, using wild-type and ΔPhe508-CFTR proteins lacking all 18 endogenous cysteine residues and possessing non-native cysteine pairs strategically inserted into different domains, support the idea that deletion of Phe508 perturbs domain-domain interactions (Serohijos et al., 2008). To date, evaluation of the consequences of Phe508 deletion in the context of the native CFTR protein sequence represents a significant experimental hurdle, preventing a comprehensive understanding of the molecular basis for cystic fibrosis disease.

The biosynthetic defect in ΔPhe508-CFTR is temperature-sensitive. Denning et al. (1992) have shown that incubating cells expressing the recombinant mutant protein at 27°C for 24 to 48 h partially rescues its biosynthesis and cell-surface expression. Several small molecules that are also effective in rescuing the mistrafficking defect exhibited by ΔPhe508-CFTR in cells have been defined through high-throughput screens (Pedemonte et al., 2005; Van Goor et al., 2006). After biosynthetic rescue and delivery to the cell surface, ΔPhe508-CFTR exhibits phosphorylation dependent and ATP regulated chloride channel activity, albeit with altered gating in mammalian cells (Wang et al., 2000). Other small molecules, such as genistein (Hwang et al., 1997), capsaicin (Ai et al., 2004), and, more recently, VRT-532 (Van Goor et al., 2006), were found to enhance or "potentiate" the open probability of ΔPhe508-CFTR after its biosynthetic rescue and phosphorylation at the cell surface. The latter compound was subsequently shown to also function as a "corrector" rescuing the surface expression of ΔPhe508-CFTR (Wang et al., 2006). Wang et al. (2006) suggested that VRT-532 may directly interact with the mutant protein to induce structural modifications that are both conducive to enhanced channel function and correct trafficking through the biosynthetic pathway. The purpose of the current study was to determine whether VRT-532 directly interacts with ΔPhe508-CFTR to alter its conformation and function. Toward this goal, we describe novel experimental approaches that enable us to study the structure-activity relationships for ΔPhe508-CFTR with its native primary sequence. These studies provide insight into the molecular lesion inherent in this mutant protein and the mechanisms underlying its repair.

Materials and Methods

Materials

Baby hamster kidney (BHK) cells stably expressing the major mutant ΔPhe508-CFTR possessing an exofacial triple-HA tag on extracellular loop 4 were obtained from Dr. G. L. Lukacs (Du et al., 2005). The mouse monoclonal antibodies used in Western blotting, M3A7 and anti-HA, were obtained from Millipore (Billerica, MA) and Covance (Princeton, NJ), respectively. N-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin was purchased from Promega (Madison, WI), and soybean trypsin inhibitor was obtained through Sigma (St. Louis, MO).

Iodide Efflux Assays

Cell-Based Assay. BHK cells stably expressing the major mutant ΔPhe508-CFTR were grown in Dulbecco's modified Eagle's medium/Ham's F12 medium (50:50) supplemented with 5% fetal bovine serum and methotrexate (200 μg/ml; Sigma) to a density of approximately 10^6 cells/ml. Cells were incubated at 27°C for 24 h before measurement to increase cell surface expression of ΔPhe508-CFTR. In control studies, untransfected BHK cells were transferred to 27°C 24 h before measurement to be consistent with ΔPhe508-CFTR-expressing cells. Cells were loaded with NaI loading buffer [3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose, 20 mM HEPES, and 136 mM NaI] at 27°C for 1 h (Du et al., 2005). NaI loading buffer was aspirated and cells were subsequently washed twice with phosphate-buffered saline followed by iodide-free efflux buffer [3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 11 mM glucose, 20 mM HEPES, and 136 mM $NaNO_3$]. Cells were scraped in 500 μl of iodide-free efflux buffer and collected by centrifugation (350g for 3 min at 25°C). Iodide-free efflux buffer was removed, and the cell pellet was resuspended in 250 μl of fresh iodide-free efflux buffer. Iodide efflux was measured at room temperature using an iodide-sensitive electrode (Lazar Research Laboratories, Los Angeles, CA). ΔPhe508-CFTR at the cell surface was stimulated with 10 μM forskolin followed by addition of VRT-532 (10 μM), genistein (50 μM), or vehicle (DMSO). We quantified the maximal rate of iodide efflux as the 1-min interval associated with the largest positive slope during the 4- to 5-min time period after addition of the potentiator. Traces were recorded using the Digidata 1320A Data Acquisition System with Clampex 8 software (Molecular Devices, Sunnyvale, CA).

Vesicle-Based Assay. Iodide efflux from membrane vesicles was measured using a method described previously (Pasyk et al., 2009). In brief, inside-out vesicles were formed from the crude membrane pellets of ΔPhe508-CFTR-infected Sf9 cells in an iodide-containing buffer (150 mM KI and 20 mM MOPS, pH 7.4). Iodide was removed from the extravesicular solution by subjecting vesicle suspensions to Sephadex G-50 gel filtration columns saturated in an iodide-free external solution (150 mM potassium glutamate and 20 mM MOPS, pH 7.4). Valinomycin, a potassium-selective ionophore, was added (20 μM) to shunt changes in potential difference generated by CFTR-mediated iodide conductance. ΔPhe508-CFTR channels were activated with 1 mM MgATP and 200 nM of the catalytic subunit of PKA, followed by the addition of 10 μM VRT-532. All measurements of iodide efflux were made using an iodide-selective electrode as described above. Traces were recorded using the Digidata 1320A Data Acquisition System with Clampex 8 software (Molecular Devices). The method for analyzing for maximal rates of efflux was similar to that described for the cell-based assay.

Purification and Reconstitution of ΔPhe508-CFTR

A frozen Sf9 cell pellet from 0.5 liters of expression culture was thawed and resuspended in 50 ml of phosphate-buffered saline containing 2% Triton X-100, 40 units/ml DNase 1, 2 mM magnesium

chloride, 1 mM dithiothreitol, and protease inhibitors (Roche Diagnostics, Indianapolis, IN) while shaking at room temperature for 1 h. The mixture was then centrifuged for 2 h at 100,000g at 4°C to yield a Triton X-100 insoluble pellet. This pellet was solubilized for 2 to 4 h in 8% pentadecafluorooctanoic acid and 25 mM phosphate, pH 8.0. Procedures for purification, reconstitution, and phosphorylation of purified ΔPhe508-CFTR were similar to wild-type CFTR-His as described elsewhere (Ramjeesingh et al., 1997, 2008). Phosphorylated samples were pelleted using an airfuge, washed twice with 50 mM Tris-HCl, 50 mM NaCl, 2.5 mM MgCl$_2$, and 1 mM dithiothreitol at pH 7.5 and then dialyzed overnight against 4 liters of washing buffer.

ATPase Assay of Purified ΔPhe508-CFTR Protein

ATPase activity was measured as the production of $[\gamma^{32}]P_i$ from $[\gamma^{32}P]ATP$ as described by Gross et al. (2006). The ATP stock solution was prepared with or without the small-molecule compound in 50 mM Tris-HCl, 50 mM NaCl, 2.5 mM MgCl$_2$, and 1 mM dithiothreitol at pH 7.5. The ATPase reaction was initiated by mixing the ATP stock solution with the reconstituted protein (0.2–0.5 mg/ml) and incubating the mixture for 2 h at 37°C. The reaction was quenched, and P_i was separated from ATP by thin-layer chromatography performed using the conditions described by Gross et al. (2006).

Limited Proteolysis of ΔPhe508-CFTR in Mammalian Membranes

Isolation of Crude Membranes. Crude membranes were isolated from BHK cells stably expressing ΔPhe508-CFTR containing a triple-HA tag in extracellular loop 4 as described previously (Aleksandrov et al., 2001). In brief, cells were homogenized in 10 mM HEPES, pH 7.2, and 1 mM EDTA in the presence of protease inhibitors, and unbroken cells were removed by centrifugation for 10 min at 500g. Crude membranes were isolated from the resulting supernatant after a 1-h spin at 100,000g.

Short-Term Treatment with VRT-532 and Trypsin Digestion of Crude Membranes. ΔPhe508-CFTR crude membranes (containing a total of 100 μg of protein) were resuspended in resuspension buffer (40 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$, and 0.1 mM EGTA). A final concentration of 5 μM VRT-532 in DMSO or an equivalent volume of DMSO was added to the membranes, sonicated, and incubated at 32°C for 20 min. Samples were cooled on ice for 5 min, and trypsin was added (at indicated concentrations of 5 to 50 μg/ml) (Du et al., 2005). Crude membranes were again sonicated and incubated at 4°C for 15 min; proteolysis was terminated with trypsin soybean inhibitor. Membranes were solubilized in modified radioimmunoprecipitation assay buffer [50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% (v/v) SDS, and 0.1% (v/v) Triton X-100] for 15 min, and the soluble fraction was analyzed by SDS-PAGE on a 4-to-20% gradient gel (Cui et al., 2007) and Western blotted with M3A7 or anti-HA antibodies to identify peptide fragments containing NBD2 or the exofacial HA tag in MSD2, respectively.

Quantification of Proteolysis Experiments. The mean pixel intensity of the full-length or proteolytic fragments resulting from the tryptic digestion of ΔPhe508-CFTR was measured using ImageJ software (http://rsbweb.nih.gov/ij/), which allowed for verification that the signal intensity was within the linear range. Mean pixel intensity of full-length ΔPhe508-CFTR after tryptic digestion was normalized to the intensity before treatment (0 μg/ml trypsin). The 60-kDa proteolytic fragment detected by both anti-HA and M3A7 antibodies was quantified as above, and the signal intensity at two trypsin concentrations were compared. Specifically, the protease sensitivity of this fragment was determined as the ratio of the band intensity measured at 10 μg/ml trypsin versus the band intensity at 5 μg/ml trypsin. These ratios were determined for control (DMSO-treated) cells and VRT-532-treated cells; the ratio in the presence of DMSO was assigned a value of 1 and the ratio obtained in the presence of VRT-532 was normalized relative to DMSO.

Statistics

Data are shown as mean ± S.E.M. of n observations. Statistical analyses, paired or nonpaired t tests as appropriate, were conducted using Prism 4 software (GraphPad Software, San Diego, CA), and P values of less than 0.05 were considered significant.

Results

The Small-Molecule VRT-532 Enhances Channel Activity of ΔPhe508-CFTR in Intact Cells and in Membrane Vesicles. An assay of cAMP-activated iodide efflux from iodide-loaded cells has been used routinely to monitor the channel function of CFTR within a population of mammalian cells (Schmidt et al., 2008; Seavilleklein et al., 2008). We modified this assay so that it could be used to monitor iodide efflux continuously rather than at 1-min intervals as in the original protocol. In Fig. 1A, the basic features of this modified iodide efflux assay have been shown using control, untransfected BHK cells. As expected, iodide-loaded BHK cells in suspension did not mediate iodide efflux after the addition of forskolin (10 μM), an activator of cAMP production, because they lacked CFTR expression. Furthermore, after a transient downward deflection, a deviation commonly observed after addition of solutions to the bath, there was no response to the addition of VRT-532 (10 μM), consistent with previous reports that this molecule specifically modifies the
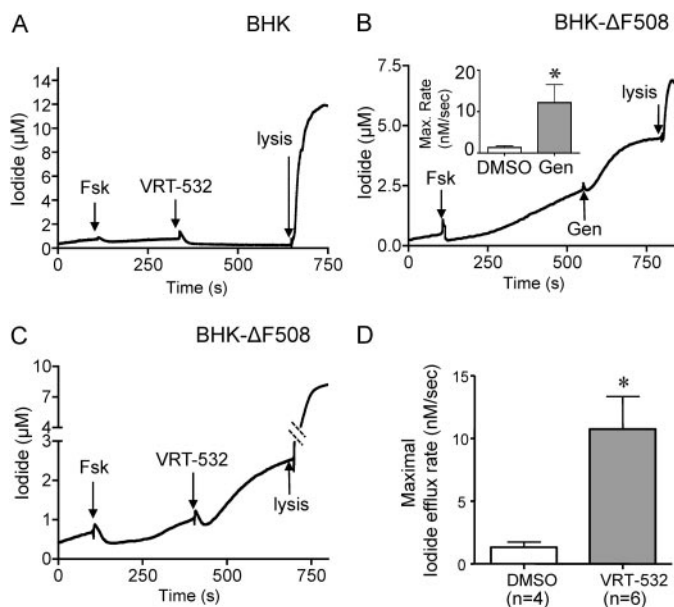
Fig. 1. VRT-532 stimulates channel function of ΔPhe508-CFTR in cells. A, representative trace showing that untransfected BHK cells, loaded with iodide, did not mediate iodide efflux in response to VRT-532 (10 μM). Cells were lysed with Triton X-100 [1% (v/v)] resulting in an increase in iodide release, confirming that the cells were effectively loaded. B, iodide efflux was stimulated by addition of genistein (50 μM) to a suspension of BHK cells stably expressing ΔPhe508-CFTR that had been subjected to temperature rescue and pretreated with forskolin (10 μM). Bar graph shows mean ± S.E.M. of maximal rates of iodide efflux (nanomolar per second) for genistein treated cell suspensions ($n = 3$) and vehicle (DMSO)-treated cell suspensions ($n = 3$). These groups are significantly different (∗, $p < 0.05$). C, tracing shows iodide efflux stimulated by addition of VRT-532 (10 μM) from BHK cells expressing ΔPhe508-CFTR, after temperature rescue and pretreated with forskolin (10 μM). D, bar graph shows that maximal efflux (mean ± S.E.M.) after the addition of VRT-532 ($n = 6$), was greater than maximal efflux after DMSO (1.3 ± 0.4 nM/s, $n = 4$) (∗, $p = 0.02$). Baseline efflux rates, attributed to leak and electrode drift, are subtracted from final efflux values.

activity of CFTR. A representative trace is shown in Fig. 1A, and we determined that there was no positive change in slope for 4 to 5 min after VRT-532 addition over three studies (mean slope = -0.96 ± 0.58 nM iodide/s.). We verified that iodide had indeed been trapped in these cells by the addition of Triton X-100. As expected, a large increase in iodide was detected in the bath after lysis, confirming that the cells had been loaded and supporting the claim that untransfected cells lack an endogenous response to cAMP and to VRT-532. We then evaluated the utility of this assay to monitor the efficacy of ΔPhe508-CFTR potentiation using the well known potentiator genistein as a positive control (Hwang et al., 1997). In these studies, BHK cells stably expressing ΔPhe508-CFTR were incubated at 27°C for 24 h to partially correct the ΔPhe508 trafficking defect and were treated with forskolin to activate the surface expressed protein (as described under *Materials and Methods*). The addition of genistein (50 μM) but not the vehicle (DMSO) caused a significant increase in the rate of iodide efflux (Fig. 1B). We quantified the maximal rate of iodide efflux as the 1-min interval associated with the largest positive slope during the 4- to 5-min time period after addition of the potentiator. The mean $\pm$ S.E.M. of three studies is shown in the bar graph in Fig. 1B. The difference between the DMSO and genistein treatment groups was statistically significant ($p = 0.01$).

As shown in Figs. 1C, addition of VRT-532 (10 μM) also caused potentiation of temperature-rescued and forskolin-pretreated ΔPhe508-CFTR channel activity. The maximal efflux rate after VRT-532 addition was significantly greater than the vehicle (DMSO) control (Fig. 1D, $p = 0.02$). This latter finding suggests that VRT-532 binds directly to the mutant protein at the cell surface to act upon its gating mechanism. Alternatively, VRT-532 could be acting to promote channel activity of ΔPhe508-CFTR by binding to cellular proteins that regulate CFTR function and/or endocytosis and recycling.

We developed a vesicle-based assay to study the effect of VRT-532 on ΔPhe508-CFTR function in a system devoid of most cellular regulatory proteins (Guggino and Stanton, 2006; Pasyk et al., 2009). ΔPhe508-CFTR was expressed in Sf9 insect cells to ensure a relatively high membrane expression of this mutant. Inside-out membrane vesicles were loaded with an iodide (KI)-containing buffer and placed into an iodide-free solution (potassium glutamate) to impose an outward gradient for iodide. Valinomycin (20 μM) was added to ensure that iodide flux was not limited by permeability of the counter-ion. As shown in Fig. 2A, the addition of the catalytic subunit of PKA (200 nM) along with MgATP (1 mM) caused no change in the rate of vesicular iodide efflux, consistent with a low level of functional expression of this mutant. The addition of VRT-532 (10 μM) caused a significant increase in the maximal rate of efflux relative to the effect of vehicle (DMSO) alone ($p = 0.03$; Fig. 2B). As in the studies shown in Fig. 1, this maximal efflux rate was determined as the greatest slope after addition of the potentiator compound during the 4- to 5-min evaluation period. The VRT-532 mediated activation is only observed in membrane vesicles expressing ΔPhe508-CFTR and not in membranes lacking CFTR. There was no increase in the rate of iodide efflux after addition of VRT-532 to membrane vesicles lacking CFTR (-0.1 ± 0.5 nM/s, $n = 3$). These findings are consistent with the hypothesis that this

small molecule interacts directly with the mutant protein to modify its conformation.

The Small Molecule VRT-532 Directly Modifies the ATPase Activity of Reconstituted ΔPhe508-CFTR. The flux-based assays described above support the electrophysiological studies by Van Goor et al. (2006), which showed that VRT-532 potentiates the channel activity of ΔPhe508-CFTR. Single-channel experiments in the above study suggested that potentiation occurred because of and increase in the mean channel open burst duration. These authors proposed that this effect may be mediated via inhibition of the intrinsic ATPase activity of ΔPhe508-CFTR, an activity that is associated with channel closure. Therefore, we were prompted to test this hypothesis directly.

The intrinsic ATPase activity of wild-type CFTR exhibits a very low $V_{\max}$ [3–5 (mol/μg)/h], comparable with the low rate of GTPases (Ramjeesingh et al., 2008). This low activity necessitates purification of the CFTR protein away from other cellular ATPases (Li et al., 1996; Ramjeesingh et al., 2008). Therefore, a similar strategy was employed to study the intrinsic ATPase activity by ΔPhe508-CFTR. As in the case of the wild-type protein, ΔPhe508-CFTR bearing a carboxyl-terminal polyhistidine tag was expressed in Sf9 cells to ensure high levels of expression. Unlike the purification procedure for wild-type CFTR protein, wherein CFTR was detergent-extracted from microsomal membrane preparations, ΔPhe508-CFTR was extracted from the entire cell lysate because its expression level is very low. Solubilized ΔPhe508-CFTR-His was bound to nickel nitrilotriacetic acid and specifically eluted using a pH gradient. As shown in Fig. 3A, ΔPhe508-CFTR protein was significantly enriched and could be visualized as the dominant silver-stained band migrating as expected for the 140- to 150-kDa protein in SDS-PAGE. As expected, this broad band was immunoreactive with CFTR-specific antibodies (including M3A7; Fig. 3A). The mutant protein can be purified to approximately 80% of the total silver-stained protein as quantitated using NIH Image (http://rsb.info.nih.gov/nih-image/). To date, efforts to further purify ΔPhe508-CFTR resulted in very low levels of protein, too low for subsequent analysis of ATPase activity.

The ATPase activity of this partially purified ΔPhe508-CFTR protein was measured after its reconstitution in phospholipid liposomes and phosphorylation by PKA as described in our previous work with wild-type CFTR (Ramjeesingh et
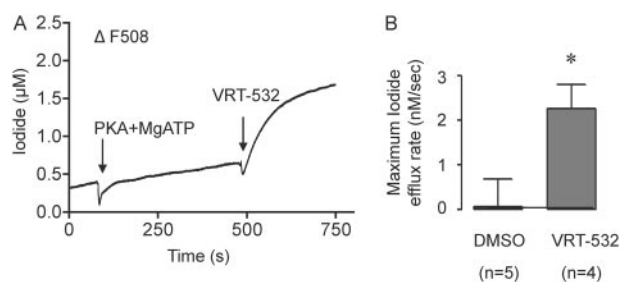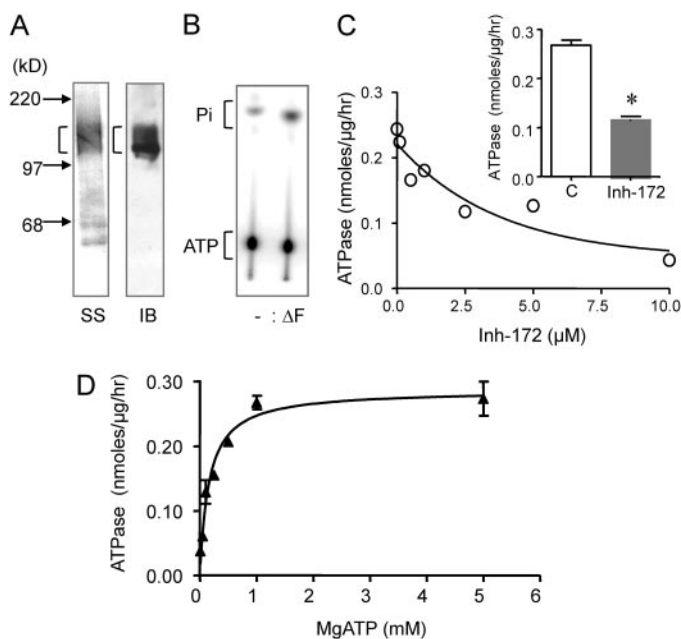

Fig. 2. VRT-532 stimulates channel function of ΔPhe508-CFTR in inside-out membrane vesicles. A, representative tracing of iodide efflux from inside-out membrane vesicles expressing ΔPhe508-CFTR, stimulated first by addition 1 mM MgATP and 200 nM PKA, followed by 10 μM VRT 532. B, the bar graph shows mean $\pm$ S.E.M. of maximal efflux rates from Sf9 membrane vesicles expressing ΔPhe508-CFTR and treated with DMSO or VRT-532 (10 μM). The maximal efflux rates are significantly different between this pair ($*$, $p = 0.03$). Baseline efflux rates, attributed to leak and electrode drift, are subtracted from final efflux values.

al., 2008). The production of ^{32}P-labeled P$_i$ from ^{32}P-labeled ATP by reconstituted ΔPhe508-CFTR (or phospholiposomes lacking reconstituted protein as a control) was quantitated by filmless autoradiographic analysis after separation of the product from its substrate by thin-layer chromatography. As shown in Fig. 3B, the reconstituted ΔPhe508-CFTR conferred ATPase activity (ΔF), apparent as the enhanced production of inorganic phosphate from ATP relative to phospholiposomes lacking protein ($-$).

To confirm that the ATPase activity associated with partially purified and reconstituted ΔPhe508-CFTR is conferred by the mutant protein directly and not by a contaminant possibly copurifying with it, we assessed its ATPase activity after pretreatment of a specific CFTR inhibitor (CFTRinh-172) (Ma et al., 2002; Taddei et al., 2004). As shown in Fig. 3C, we found that the ATPase activity of reconstituted ΔPhe508-CFTR was inhibited by CFTRinh-172 in a dose-dependent manner, with an IC$_{50}$ of approximately 2.6 μM, close to that observed for its inhibition of wild-type CFTR channel activity (Fig. 3C). The extent of this inhibition was statistically significant at 2.5 μM ($n = 3$, $p = 0.001$), supporting our claim that ATPase activity is directly mediated by reconstituted ΔPhe508-CFTR.

The ATP dependence of ΔPhe508-CFTR mediated ATPase activity can be described by Michaelis-Menten kinetics, with

an apparent affinity (or K_m) for ATP of 0.2 mM and a V_{max} of 0.3 nmol/μg h^{-1} (Fig. 3D). Although the estimate of K_m (ATP) for the mutant protein is somewhat lower, the value is still within range of the wild-type CFTR protein (0.7 mM ATP) (Ramjeesingh et al., 2008). Unfortunately, a direct comparison of the V_{max} determined for the ATPase activity of ΔPhe508 and wild-type CFTR proteins was not possible because the two proteins were purified differently, and the yield of functionally reconstituted protein may be different. However, the present findings suggest that ΔPhe508-CFTR is capable of binding and hydrolyzing ATP.

Potentiation of ΔPhe508-CFTR chloride channel activity by VRT-532 in intact cells and in isolated membrane vesicles prompted us to study the effect of this small molecule on the ATPase activity of reconstituted ΔPhe508-CFTR. We found that treatment with 10 μM VRT-532, the same concentration effective in modulating its channel function, caused a significant decrease in its ATPase activity (Fig. 4A). Furthermore, VRT-532 treatment also reduced the ATP dependence of this activity as observed through a 3-fold decrease in V_{max} (from 0.30 to 0.12 nmol/μg h^{-1}) but only had a minor effect on the K_m for ATP (from 0.18 to 0.22 mM ATP) (Fig. 4, A and B). Inhibition of ATPase activity was statistically significant, and these findings are consistent with a model wherein VRT-532 binds directly to the mutant protein at a position distinct from the ATP catalytic site.

VRT-532 Binding Induces a Change in Conformational Stability of the Carboxyl-Terminal Half of ΔPhe508-CFTR. Susceptibility of the ΔPhe508-CFTR protein in mammalian cell membranes to limited trypsin digestion has been used to monitor conformational changes induced by low temperature rescue (Denning et al., 1992; Sharma et al., 2004). The carboxyl terminus of ΔPhe508-CFTR was found to be "hypersensitive" to proteolytic digestion, and low temperature rescue led to partial protection of this region. Our findings that VRT-532 treatment inhibited the ATPase activity of reconstituted ΔPhe508-CFTR is consistent with its possible role in promoting a tight ATP-bound NBD heterodimer (Vergani et al., 2005b; Mense et al., 2006). Therefore, we were prompted to test whether a short-term treatment of ΔPhe508-CFTR with the compound would alter its trypsin susceptibility.

Microsomal membranes prepared from BHK cells stably

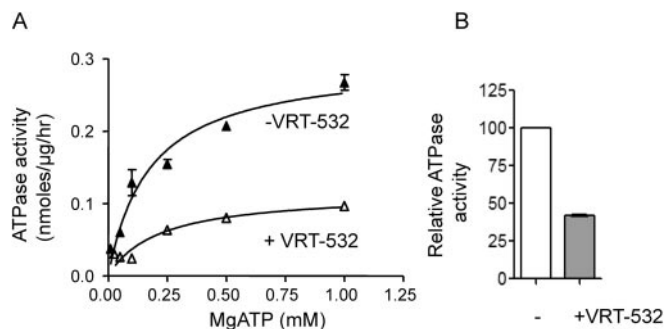Fig. 3. Partially purified and reconstituted ΔPhe508-CFTR exhibits CFTRinh-172 sensitive ATPase activity. A, silver-stained (SS) gel and immunoblot (IB) of partially purified ΔPhe508-CFTR that migrates as a diffuse 140-kDa protein (indicated by a bracket). This band corresponds to the core glycosylated form of the protein. B, ATPase activity of partially purified, reconstituted, and phosphorylated ΔPhe508-CFTR measured as the production of radioactive P$_i$ from radioactive ATP. Representative thin layer chromatography showed significant inorganic phosphate production with proteoliposomes (ΔF) over liposomes without protein ($-$). C, the ATPase activity of ΔPhe508-CFTR is inhibited by CFTRinh-172 in a dose-dependent manner. Activity was measured in the presence of 1 mM MgATP. Curve was fitted with an exponential decay function ($r^2 = 0.88$) yielding an IC$_{50}$ of 2.6 μM. Bar graph shows significant inhibition at 2.5 μM CFRTinh-172 relative to control (C) ($n = 3$ protein preparations, $p = 0.001$). D, ATP dose dependence of the ATPase activity; Michaelis-Menten analysis gave an apparent affinity K_m (ATP) of 0.176 mM ($r^2 = 0.95$); each point represents the mean of duplicate measurements using two different protein preparations).

Fig. 4. VRT-532 directly modifies ATPase activity of ΔPhe508-CFTR. Activity of reconstituted ΔPhe508-CFTR was measured in the presence or absence of 10 μM drug. A, ATP dose-dependence curves are fitted using Michaelis-Menten equation (+VRT-532, K_m (MgATP) = 0.22 mM, V_{max} = 0.12 (nmol/μg)/h, $r^2 = 0.9$; control (no drug), K_m (MgATP) = 0.18 mM, V_{max} = 0.30 (nmol/μg)/h, $r^2 = 0.95$, $n = 2$ different protein preparations). B, bar graph shows the relative ATPase activity of reconstituted ΔPhe508 after addition of 10 μM VRT-532 ($n = 3$ protein preparations, $p = 0.001$).

expressing ΔPhe508-CFTR containing an exofacial HA tag in MSD2 were treated acutely with VRT-532 (5 μM for 20 min) and subjected to limited proteolysis in the presence of varying concentrations of trypsin. The full-length and trypsin-digested fragments were analyzed by SDS-PAGE and probed using domain-specific antibodies. At first, we employed the monoclonal antibody M3A7 (which recognizes residues 1370–1380 in NBD2) because it has been effective at revealing changes in protease susceptibility of the carboxyl terminus of ΔPhe508-CFTR in previous studies (Du et al., 2005). In the presence of 5 μg/ml trypsin, two dominant bands are detected, one corresponding to the full-length protein and the other to the major proteolytic product that migrates as a ~60-kDa protein (indicated by an arrow and bracket, respectively, in Fig. 5A). An increase in the relative abundance of full-length (FL) protein at 5 μg/ml trypsin relative to its abundance before trypsin addition (0 μg/ml) was measured upon VRT-532 pretreatment. This protective effect of VRT-532 on the integrity of the full-length protein was small but significant as determined in three different preparations of BHK membranes (Fig. 5, A and B). VRT-532 treatment also induced protease resistance in the major fragment (the ~60-kDa band). The abundance of this 60-kDa fragment at 10 μg/ml trypsin relative to its abundance at 5 μg/ml trypsin was significantly greater after VRT-532 treatment than in DMSO-treated controls (Fig. 5C).

The size of the 60-kDa fragment is consistent with the idea that it encompasses both NBD2 and MSD2. To test whether this fragment contained both regions, we probed the proteolytic fragments using an anti-HA antibody to target the triple HA epitope in the fourth extracellular loop of MSD2 (Fig. 6). Indeed, a dominant 60-kDa fragment could also be detected in immunoblots of trypsin digests using this antibody (Fig.

6A). As in the case of the M3A7 immunoblots, the abundance of this fragment at 10 μg/ml trypsin relative to its abundance at 5 μg/ml (detected using the anti-HA antibody) was significantly greater in membranes pretreated with VRT-532 (Fig. 6, A and B). Together, the reduced susceptibility of MSD2 and NBD2 to proteolytic digestion suggests the carboxyl-terminal half of ΔPhe508-CFTR undergoes a structural change or alternatively, a more compact conformation is stabilized, upon binding of VRT-532.

Discussion

Studies of the ATPase Activity of Purified and Reconstituted ΔPhe508-CFTR Provide Insight into the Molecular Defect Caused by This Mutation. There have been several informative studies on the molecular lesion inherent in the major CFTR mutant, ΔPhe508-CFTR. Protease susceptibility of the ΔPhe508-CFTR protein (with its native sequence) was enhanced relative to the wild-type protein indicating that it exhibits a "hyper-protease-sensitive" conformation (Zhang et al., 1998). These authors suggested that deletion of Phe508 in NBD1 could lead to the perturbation of native domain-domain interactions in CFTR, thus accounting for the increased susceptibility to proteases observed for this mutant. This hypothesis was tested in chemical cross-linking studies employing "Cys-less" wild-type and ΔPhe508-CFTR proteins in which non-native cysteines were strategically introduced at putative domain interfaces (Loo et al., 2008; Serohijos et al., 2008). The results of chemical cross-linking studies in the modified "wild-type" CFTR supported the molecular models for CFTR based on the crystal structure of the prokaryotic ABC protein Sav1866 (Mornon et al., 2008; Serohijos et al., 2008). Sav1866 exhibits an interesting "cross-over" pattern of domain-domain interactions wherein the NBD in half of the molecule interacts primarily with the membrane spanning domain of the other half (Dawson and Locher, 2006). It is noteworthy that Loo et al. (2008) and Serohijos et al. (2008) showed that deletion of Phe508 *impairs* the chemical cross-linking that normally occurs between a non-native cysteine residue incorporated into NBD1, in the proximity of Phe508 (V510C), and a non-native cysteine introduced into the fourth intracellular loop (A1067C)
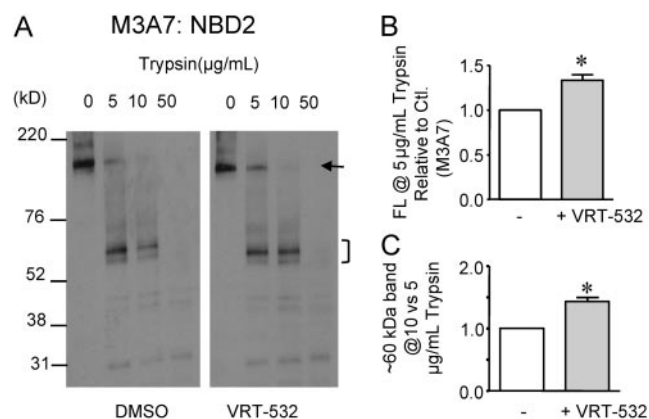
Fig. 5. VRT-532 binding induces conformational change in the carboxyl-terminal half of ΔPhe508-CFTR. A, Western blots of trypsin-digested crude membranes expressing ΔPhe508 CFTR using the M3A7 antibody against NBD2 after a pretreatment with vehicle (DMSO) or 5 μM VRT-532. Arrow indicates position of full-length mutant protein, Band B (core glycosylated form). B, the bars in B show significant protection of full-length (FL) mutant protein at 5 μg/ml trypsin (expressed as a ratio relative to undigested protein at 0 μg/ml trypsin) (∗, n = 3, p = 0.02). The intensity of the bands corresponding to full-length CFTR protein was quantified using ImageJ software. C, a proteolytic fragment (~60-kDa band indicated by a bracket), exhibited an increase in resistance to digestion (assessed as the ratio of band intensity at 10 relative to 5 μg/ml trypsin) after VRT-532 treatment versus vehicle (DMSO) treatment (n = 3; ∗, p = 0.02,). The ratio in the presence of DMSO assigned a value of one and the ratio obtained in the presence of VRT-532 normalized relative to DMSO. The intensity of the bands corresponding to this fragment was quantified using ImageJ software as above.
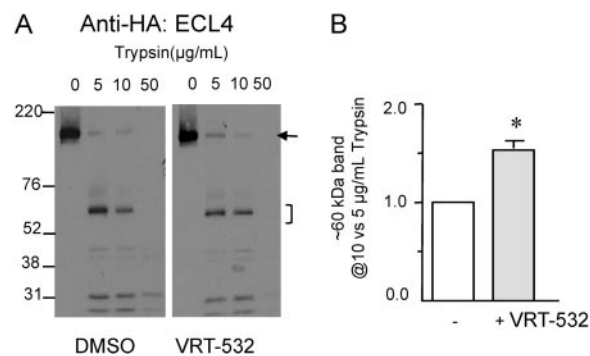
Fig. 6. VRT-532 binding induces conformational change in the carboxyl-terminal half of ΔPhe508-CFTR. A, a proteolytic band (~60 kDa and bracketed), is reactive to an anti-HA antibody that recognizes an epitope engineered into extracellular loop 4. This band was more resistant to trypsin digestion (ratio of abundance measured using ImageJ software at 10 μg/ml relative to 5 μg/ml trypsin) after VRT-532 treatment versus DMSO treatment. B, this protective effect was observed for VRT-532 in three trials (∗, p = 0.032). The arrow indicates the migration of the full-length protein, band B.

extending from MSD2. Loo et al. (2008) also found that deletion of Phe508 disrupted the chemical cross-linking that normally occurred between non-native cysteines of MSD1 (A274C) and NBD2 (Loo et al., 2008) and cross-linking between non-native cysteines introduced into transmembrane helices 6 (MSD1) and 7 or 12 (MSD2) (Wang et al., 2007). Together, these findings support the hypotheses that the ΔPhe508 mutation in NBD1 disrupts multiple domain-domain interactions within CFTR. The current study provides novel insight into the molecular defect conferred by the ΔPhe508 mutation in the context of the native CFTR sequence.

Evaluation of partially purified and reconstituted ΔPhe508-CFTR revealed that it retained measurable levels of ATPase activity, with a saturable ATP dependence that could be fitted by the Michaelis-Menten equation. The V_{max} determined for the ATPase activity of the mutant protein could not be compared with that of the wild-type CFTR, because these proteins were obtained using different purification protocols. The apparent affinity (K_{m}) for ATP binding to the catalytic site is independent of the number of functionally reconstituted protein molecules; thus, this parameter can be directly compared between the two genotypes. The apparent K_{m} (ATP) for ΔPhe508-CFTR is approximately 0.2 mM, which is less than that of the wild-type protein (~0.7 mM) (Ramjeesingh et al., 2008). This difference could reflect an increase in ATP affinity and/or a decrease in the rate of ATP hydrolysis upon deletion of Phe508. A more detailed understanding of the consequences of the ΔPhe508-CFTR mutation on ATP hydrolysis will require accurate quantitation of the number of functionally reconstituted molecules is a major goal for our future work. However, the present biochemical findings support the hypothesis that the chloride channel gating defect described for ΔPhe508-CFTR (Roxo-Rosa et al., 2006) may not be due to a defect in ATP binding per se but rather to a defect in the unknown conformational changes that occur subsequent to ATP binding and lead to the opening of the channel.

Molecular Mechanism of VRT-532 Action Revealed in Studies of the Functional and Structural Consequences of Binding. As shown in Figs. 1 and 2, VRT-532 is effective in potentiating the channel activity of ΔPhe508-CFTR after temperature rescue in intact BHK cells overexpressing the protein, as well as in membrane vesicles prepared from Sf9 cells (an insect cell expression system). The present experiments performed on membrane vesicles confirm previous electrophysiological studies showing that this small molecule potentiates the channel function of ΔPhe508-CFTR (Van Goor et al., 2006). These authors suggested that this potentiation effect was likely to be mediated by inhibition of the intrinsic ATPase activity of the mutant protein, an activity associated with channel closure. However, until the current study, it was not known whether ΔPhe508-CFTR retained ATPase activity and whether this activity was modulated by potentiators. The effect of VRT-532 on the ATPase activity of partially purified ΔPhe508-CFTR provides the most compelling evidence for direct binding to the protein and elucidates the mechanism underlying its potentiating effect on channel activity. VRT-532 specifically inhibited the intrinsic ATPase activity of ΔPhe508-CFTR not by preventing ATP binding to the NBD domains, but by reducing ATP turnover. As mentioned previously, ATP binding to the cat-

alytic site at the NBD1-NBD2 interface is thought to be permissive to opening of the channel gate, whereas ATP hydrolysis by CFTR permits the gate to return to its closed state (Gadsby et al., 2006; Aleksandrov et al., 2007). Therefore, the inhibitory effect of VRT-532 binding on ATPase activity would be predicted to prevent closing of the channel by stabilizing the opened/ATP bound state. The molecular basis for binding of VRT-532 to ΔPhe508-CFTR has yet to be determined, but our ATPase studies suggest it is probably not at the nucleotide binding sites formed from the NBD1-NBD2 heterodimer because there was little change in the apparent ATP affinity (K_{m}). Rather, it is possible that the binding site lies at an interface region between the NBDs and the MSDs, a region that is well positioned to modulate both the catalytic domains as well as the open gate structure in the membrane.

Molecular Mechanism of VRT-532 Action Shares Features with Mechanisms Proposed for Other Small-Molecule Potentiators of ΔPhe508-CFTR. Analyses of the single-channel activity of wild-type and ΔPhe508-CFTR have been instrumental in providing insight into the possible mechanism of action of such well known small-molecule potentiators as genistein and capsaicin (Ai et al., 2004). As in the case of VRT-532, both of these potentiators act to enhance the open time of ΔPhe508-CFTR single channels after prephosphorylation by protein kinase A. Ai et al. (2004) suggested that potentiators may act to stabilize the NBD1-NBD2 heterodimer, possibly by preventing ATP hydrolysis. The measurements of reduced ATP hydrolysis by ΔPhe508-CFTR after incubation with VRT-532 provide direct evidence to support this hypothesis.

The site at which potentiators (including VRT-532) bind to ΔPhe508-CFTR remains to be determined. The current findings suggest that it is unlikely that VRT-532 binds at the catalytic site because it does not inhibit the apparent affinity for ATP in this reaction. In agreement with this conclusion, Cai and Sheppard (2002) proposed that phloxine B, another potentiator that increases the channel open time and increases the affinity for ATP in ATP-dependent channel gating, interacted with the protein at a site distinct from the catalytic site. Molecular modeling and mutagenesis studies (Moran et al., 2005; Zegarra-Moran et al., 2007) also support a model wherein genistein and other potentiators act to stabilize the NBD1-NBD2 dimer interface by binding to a site proximal to, but distinct from, the primary catalytic site. Together, the biochemical assays described in the present study, previous single channel analyses, and molecular modeling studies support a common model wherein small molecule binding at a site distinct from the catalytic site, promotes enhanced stability of the NBD1-NBD2 heterodimer.

Finally, our studies of the protease susceptibility of ΔPhe508-CFTR expressed in mammalian membranes also suggest that binding of VRT-532 modifies intramolecular interactions within this multidomain protein. We showed that VRT-532 binding caused a structural change or stabilization of a particular conformation of ΔPhe508-CFTR in membrane vesicles. This effect was detected as a decrease in trypsin susceptibility of both the full-length protein and a large fragment encompassing both NBD2 and MSD2. These findings could indicate that VRT-532 binds to either NBD2, MSD2, or to a site where these domains interact. However, it is also possible that the compound binds at a distinct location

leading to the promotion of a more compact ΔPhe508-CFTR protein and protection of the second half the protein from trypsin digestion. These results are consistent with those of Zhang et al. (1998), who initially reported that "temperature-rescue" promotes a structural change associated with partial rescue of the protease susceptibility of the carboxyl terminus of ΔPhe508-CFTR.

In summary, the present study has demonstrated a direct interaction of a small-molecule modulator, VRT-532, with the full-length ΔPhe508-CFTR protein bearing its native sequence, with binding leading to direct modification of its structure. Furthermore, the development of methods for the partial purification and characterization of full-length ΔPhe505-CFTR permits future detailed investigations of the binding site for VRT-532. Such studies will enable an improved understanding of the structural basis for the mistrafficking of ΔPhe508-CFTR as well as future structure-based therapy design.

Acknowledgments

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