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Article

Identification of a NBD1-Binding Pharmacological Chaperone that Corrects the Trafficking Defect of F508del-CFTR

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SUMMARY

Most cases of cystic fibrosis (CF) are attributable to the F508del allele of CFTR, which causes the protein to be retained in the endoplasmic reticulum (ER) and subsequently degraded. One strategy for CF therapy is to identify corrector compounds that help traffic F508del-CFTR to the cell surface. Pharmacological chaperones, or correctors that bind specifically to F508del-CFTR and restore function, would be the most promising drug development candidates, but few pharmacological chaperones exist for F508del-CFTR. Using differential scanning fluorimetry (DSF), we have surveyed corrector compounds and identified one, RDR1, which binds directly to the first nucleotide binding domain (NBD1) of F508del-CFTR. We show that RDR1 treatment partially rescues F508del-CFTR function in both cells and in an F508del-CF mouse model. Thus, RDR1 is a pharmacological chaperone of F508del-CFTR and represents a novel scaffold for drug development.

INTRODUCTION

Cystic fibrosis (CF) is the most common autosomal recessive disease in Caucasians. It is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Riordan et al., 1989). CFTR is a multidomain cAMP-regulated chloride channel comprising two membrane-spanning domains (MSD1 and 2), two nucleotide-binding domains (NBD1 and 2), and a regulatory region that controls the gating of the channel (Sheppard and Welsh, 1999). The most common mutation is a deletion in NBD1 of the phenylalanine residue at position 508 (F508del), which causes misfolding of the full-length protein. Consequently, F508del-CFTR is retained in the ER and targeted for degradation rather than trafficked to the plasma membrane (Welsh et al., 1993). The trafficking of F508del-CFTR to the plasma membrane can be corrected by incubating cells expressing the mutant protein at reduced temperature (Denning et al., 1992). When properly localized to the plasma membrane, F508del-CFTR is functional; however, it has defects in gating (Dalemans et al., 1991), and a faster turnover at the cell surface than wild-type CFTR (Lukacs et al., 1993).

The gating defect of corrected F508del-CFTR has led to the discovery of compounds that can potentiate the channel's activity. Potentiators maximize CFTR channel activity when CFTR is phosphorylated (Hwang et al., 1997). Potentiators are thought to function via direct binding to the NBDs of CFTR to modulate NBD dimerization, ATP binding and hydrolysis (Cai et al., 2006; Vergani et al., 2005).

One approach to developing a potential treatment for CF is to identify small molecule correctors of the F508del-CFTR trafficking defect. Several high throughput screens for F508del-CFTR trafficking correctors have been performed to date (Carlile et al., 2007; Pedemonte et al., 2005; Van Goor et al., 2006); while several compounds have been identified as putative F508del-CFTR trafficking correctors, the mechanisms by which they restore trafficking to the plasma membrane, which is fundamental to further development as candidate therapeutics, remains elusive.

There are several possible mechanisms by which F508del-CFTR trafficking may be corrected. The cell's folding machinery, ER recognition, or degradation mechanisms could be altered to increase the proportion of F508del-CFTR that traffics to the cell surface. As these mechanisms alter proteostatic balance in the cell rather than F508del-CFTR itself, such correctors are termed proteostasis regulators (Balch et al., 2008). Indeed, several putative proteostasis regulators have been identified to date: Aha1 (Wang et al., 2006a), 4-phenylbutyrate (Rubenstein et al., 1997), glycerol (Brown et al., 1996), low temperature (Denning et al., 1992), histone deacetylase (HDAC) inhibitors (Hutt et al., 2010), phosphodiesterase 5 (PDE5) inhibitors (Dormer et al., 2005; Robert et al., 2008), glafenine (Robert et al., 2010), and miglustat (Norez et al., 2006). Since proteostasis regulators indirectly affect F508del-CFTR trafficking, these correctors may be nonspecific and may also correct the trafficking of other ER-retained mutations. Another possibility is that correctors could act specifically on F508del-CFTR as pharmacological chaperones by binding directly to F508del-CFTR and promoting the native fold such that the protein can evade ER quality control and degradation. Pharmacological chaperones would be expected to have fewer off-target effects and may be the most conducive to the development of an effective treatment for CF.

Pharmacological chaperones have been identified for other ER-retained trafficking diseases including Fabry disease (Fan et al., 1999), Gaucher disease (Sawkar et al., 2002), Tay-Sachs and Sandhoff disease (Tropak et al., 2004), nephrogenic diabetes insipidus (Morello et al., 2000), and many others. These compounds are generally active site inhibitors or substrate mimics hence they bind directly to the mutant protein. To date four correctors have been identified that might act as pharmacological chaperones of F508del-CFTR: VRT325, Corr4a and VRT532 (Wang et al., 2006b, 2007b), and benzo[c]quinolizinium (MPB) compounds (Becq et al., 1999; Dormer et al., 2001; Stratford et al., 2003). CFTR constructs containing only the two MSDs of CFTR acquire more glycosylation upon VRT325 and Corr4a treatment which suggests that these two correctors may bind elsewhere than to the NBDs (Wang et al., 2007c). Homology models of the CFTR protein constructed from the structure of the bacterial transporter Sav1866 show that F508 is located at an interface between the NBD1 and the fourth intracellular loop (ICL4) in MSD2 (Mornon et al., 2008; Serohijos et al., 2008). Based on this model, VRT325 and Corr4a may bind to the MSD side of the interface. VRT325 has been shown recently to decrease the affinity of partially purified F508del-CFTR for ATP (Kim Chiaw et al., 2010), which also suggests direct binding to F508del-CFTR. VRT532 may bind to a site at the interface between the NBDs and the MSDs since it increases channel open times but it does not alter the affinity of F508del-CFTR for ATP (Wellhauser et al., 2009). MPB compounds have been suggested to correct through their binding to the NBD1; however, the effective concentrations required for correction are very high (Becq et al., 1999; Dormer et al., 2001). Each of these pharmacological chaperones has some limitations, either in its effective concentration, toxicity profile, ability to correct trafficking in multiple cell types and models, or the location of the putative binding site lies outside the NBD1. Hence, there is a need to identify potent NBD1-binding pharmacological chaperones.

Differential scanning fluorimetry has been used successfully to identify ligands that stabilize proteins for structural studies (Niesen et al., 2007; Vedadi et al., 2006). We reasoned that this technique could also be used to identify pharmacological chaperones of F508del-CFTR that act directly on the mutated domain, NBD1. Here, we show that RDR1, a phenylhydrazone compound initially identified in our cell-based trafficking corrector assay, binds to and thermally stabilizes purified murine F508del-NBD1 in vitro, and partially corrects trafficking of F508del-CFTR in BHK cells and in human airway epithelial cells. This trafficking correction results in a significant increase in CFTR function in these two cell models, as well as in a mouse model of cystic fibrosis. RDR1 and structurally related compounds may thus provide new structural scaffolds for the development of therapeutics to treat CF.

RESULTS

RDR1 Thermally Stabilizes F508del-NBD1

In order to identify F508del-CFTR pharmacological chaperones, the hits that we obtained from our cell-based high-throughput screen for CFTR correctors (Carlile et al., 2007) were tested for stabilization of purified murine F508del-NBD1 in vitro. Using differential scanning fluorimetry (DSF), we first tested a concentration series of ATP as a control (Figure 1A), given that residues in the Walker A, B, and ABC signature motif in NBD1 participate in two half-sites for ATP binding at the interface of the NBD1-NBD2 heterodimer (Lewis et al., 2004; Moran et al., 2005). We detected a dose-dependent increase in the thermal stability of F508del-NBD1 at concentrations down to 50 μM ATP, the lowest concentration tested, which is similar to the lowest K_d reported (88 ± 10 μM) for ATP binding to NBD1 (Qu et al., 1997). We next screened 224 hit compounds obtained from our corrector screen. Surprisingly, only one compound (RDR1) was found to thermally stabilize F508del-NBD1. RDR1 is a substituted phenylhydrazone from the Maybridge library (Figure 1B). We tested a range of concentrations of RDR1 and found a dose-dependent increase in F508del-NBD1 thermal stability that was detectable at concentrations of 5 μM or greater (Figure 1C). Interestingly, the thermal stabilization of F508del-NBD1 with 20 μM RDR1 was greater than that with 1 mM ATP (16°C versus 7°C, compare Figure 1C and Figure 1A).

We obtained compounds that were structurally related to RDR1 from Maybridge and ChemBridge and tested their ability to stabilize F508del-NBD1 in DSF. RDR2 is a hydrazone similar to RDR1, but it lacks the terminal phenyl ring (Figure 1B). A compound obtained from the Chembridge library, RDR3, retains this terminal phenyl ring, but it is a chlorosubstituted benzoic acid (Figure 1B). Of these two related compounds, RDR3 also showed thermal stabilization of F508del-NBD1 at concentrations down to 5 μM (Figure 1D). However, RDR2 did not thermally stabilize F508del-NBD1 even at concentrations up to 100 μM (Figure 1E). We chose to focus on RDR1 for the remainder of the study.

We next determined whether RDR1 was stabilizing F508del-NBD1 by binding to the ATP binding half-sites or elsewhere. We tested whether it competes with ATP for thermal stabilization of F508del-NBD1. A range of concentrations of ATP was added to F508del-NBD1 in the presence or absence of 10 μM RDR1 and the thermal stability of F508del-NBD1 was assessed (Figure 1F). In the presence of ATP, RDR1 stabilized F508del-NBD1 by an additional 6°C, which is consistent with the thermal stabilization observed by RDR1 alone (Figures 1C and 1F). This result suggests that RDR1 binds to a site other than the ATP-binding half-sites in F508del-NBD1.

RDR1 Treatment Corrects Trafficking of Human F508del-CFTR in BHK Cells and Human Airway Epithelial Cells

To examine the effect of RDR1 on human F508del-CFTR in cells, we tested its ability to correct trafficking in different cell types and at different concentrations. BHK cells stably expressing HA-tagged F508del-CFTR were treated with 10 μM RDR1 for varying durations to determine the earliest time at which correction could be detected (Figure 2). As shown in Figure 2, an increase in the amount of F508del-CFTR at the cell surface was observed after 4 hr after treatment, with a maximal increase (16%) detected after 18 hr of treatment. The onset of trafficking correction observed with RDR1 treatment occurred at approximately the same time as that detected with VRT325; however, the maximal RDR1 correction occurred earlier than that obtained

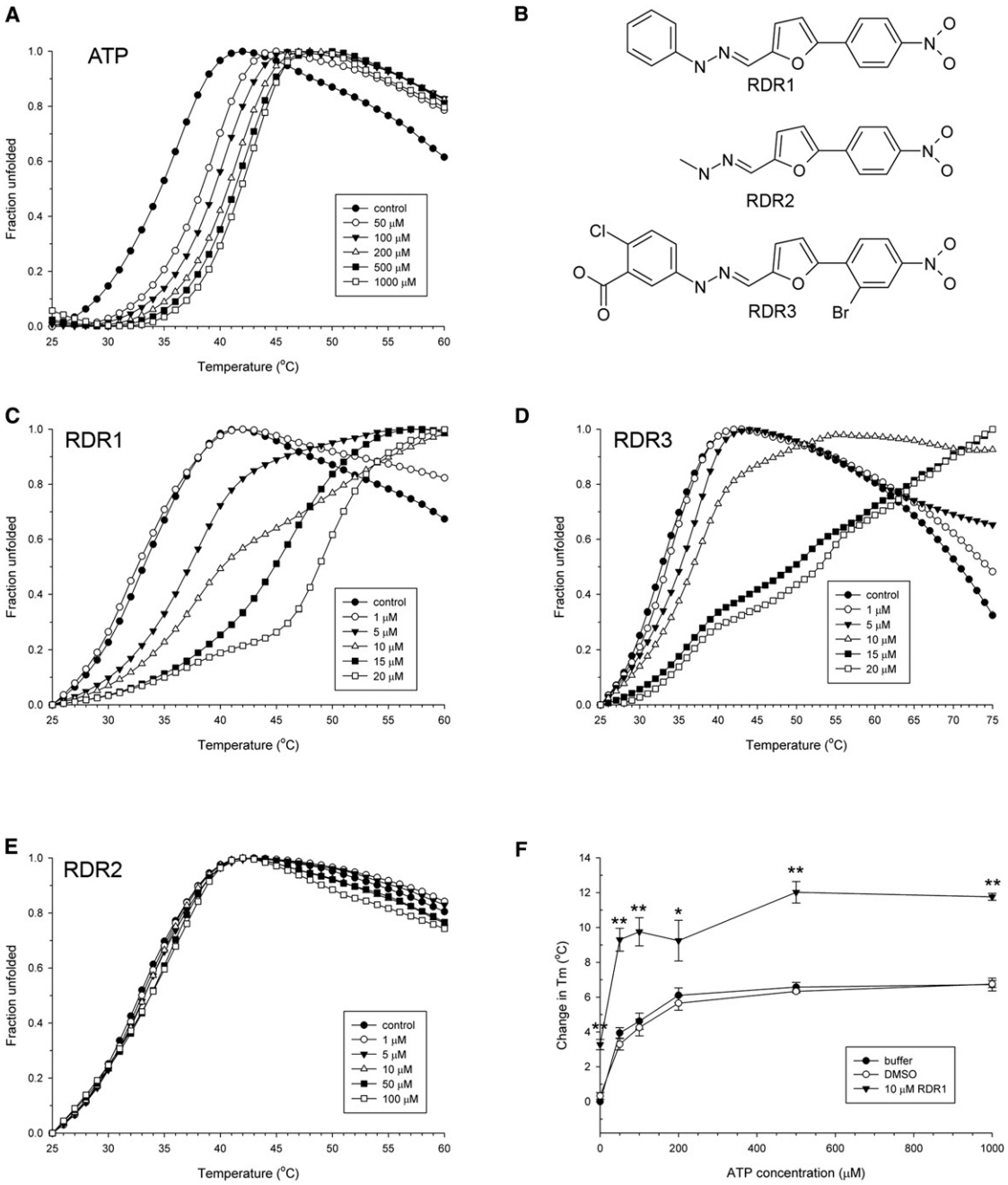


Figure 1. RDR1 Stabilizes F508del-NBD1 Dose-Dependently

(A) Representative fractional unfolding curves of murine F508del-NBD1 in the presence of ATP.

(B) Chemical structures of RDR1 and related compounds. RDR1: 5-(4-nitrophenyl)-2-furaldehyde 2-phenylhydrazone; RDR2: 5-(4-nitrophenyl)-2-furaldehyde 2-methylhydrazone; RDR3: 5-(2-{[5-(2-bromo-4-nitrophenyl)-2-furyl] methylene} hydrazino)-2-chlorobenzoic acid.

(C) Representative fractional unfolding curves of F508del-NBD1 in the presence of RDR1.

(D) Representative fractional unfolding curves of F508del-NBD1 in the presence of RDR3.

(E) Representative fractional unfolding curves of F508del-NBD1 in the presence of RDR2.

(F) Effect of buffer, 1% DMSO, or 10 μM RDR1 on the stability of F508del-NBD1 in the presence of increasing concentrations of ATP. Error bars indicate SEM (n = 3). Asterisks indicate statistically significant differences with a Student's t test as compared with DMSO control (*$p < 0.05$, **$p < 0.001$).

See also Figure S1.

with VRT325 treatment (18 versus 24 hr). The maximal correction with RDR1 corresponds to approximately 50% of the correction obtained with 10 μM VRT325 at 24 hr.

We next used immunoblotting in BHK cells stably overexpressing untagged F508del-CFTR to assess the maturation of F508del-CFTR by its mobility in SDS-PAGE. F508del-CFTR

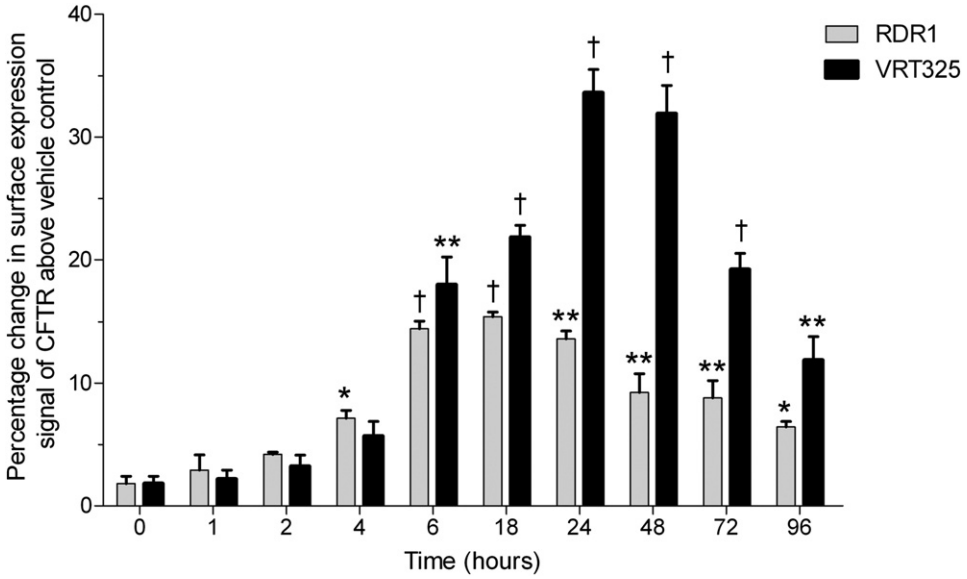


Figure 2. RDR1 Induces Trafficking of HA-Tagged F508del-CFTR to the Cell Surface in BHK Cells

A time course of F508del-CFTR trafficking in BHK cells treated with 10 μM RDR1 and 10 μM VRT325 treatments is shown. Error bars indicate SEM (n = 3). A difference in the means as compared with the respective 0 hr time point was tested for statistical significance using a Student's t test, *$p < 0.05$, **$p < 0.01$, † $p < 0.001$.

protein that has trafficked to the Golgi acquires terminal glycosylation (band C) and migrates more slowly in SDS-PAGE than the immature ER-localized glycoform (band B), or the nonglycosylated form (band A). We treated BHK cells with a range of concentrations of RDR1 and quantified the blots by densitometry (Figures 3A and 3B). Correction of F508del-CFTR trafficking was detectable at concentrations of 10 μM RDR1 or greater (Figure 3A, lanes 7 and 8). As positive controls, we treated the cells with the known corrector VRT325 (VRT325, lane 3) or incubated them at 29°C (lane 4). As shown in Figure 3B, a dose-dependent increase in total CFTR protein, band B or C is detected with increasing concentrations of RDR1, with 10 and 20 μM treatments showing a statistically significant difference in band C above the vehicle control. When the total amount of CFTR protein or band B was compared, 20 μM RDR1 treatment showed a statistically significant increase above the vehicle control. When the ratio of C band to B band was calculated, RDR1 concentrations of 10 and 20 μM showed an increase in the C/B ratio; however, these differences were not statistically significant ($p = 0.066$ and $p = 0.096$, respectively). When we treated nonpolarized CFBE41o- airway epithelial cells that stably overexpress F508del-CFTR with RDR1 for 24 hr, a similar dose-dependent increase in F508del-CFTR trafficking was observed (Figure 3C). In these cells, it appears that correction of F508del-CFTR reached a maximum at 10 μM RDR1. These results were quantified by densitometry (Figure 3D). Treatment with 10 μM RDR1 resulted in a 1.2-fold increase in total CFTR protein, but a 3.8-fold increase in band C as compared with the vehicle control (Figure 3C, lane 1); however, this difference was not statistically significant ($p = 0.204$).

To further elucidate the mechanism of RDR1 correction, we tested for additivity with low temperature incubation, a treatment that may correct trafficking by slowing down synthesis to allow folding of F508del-CFTR in the cell and also through proteostasis regulation (Denning et al., 1992; Jurkuvenaite et al., 2010). If RDR1 acts as a pharmacological chaperone, we would expect it to be additive with low temperature treatment due to their different mechanisms of action. BHK cells stably expressing F508del-CFTR were treated with increasing concentrations of RDR1 at 37°C or at 29°C. As expected, low temperature treatment corrected trafficking of F508del-CFTR (Figure 3A, lane 4). Treatment with RDR1 further increased the amount of correction detected at 29°C in a dose-dependent manner (Figure 3A, compare lanes 9–11 to lanes 5–7). Quantification of these results by densitometry shows that 10 μM RDR1 treatment doubled the C/B ratio obtained with low temperature alone (Figure 3E). These results confirm that RDR1 and low temperature treatment act through complementary mechanisms.

RDR1 Treatment Corrects Human F508del-CFTR Function

We next determined whether the correction we detect following RDR1 treatment results in functional F508del-CFTR channels at the cell surface. We first tested RDR1 in halide efflux assays using BHK cells stably expressing F508del-CFTR (Figure 4A). Treatment of BHK cells with 1 or 10 μM RDR1 resulted in a 1.5- and 1.7-fold increase in functional F508del-CFTR, respectively, as compared with the vehicle control (Figure 4B). As a positive control, VRT325-treated cells resulted in a 3.3-fold increase in functional channels. As expected, BHK cells stably expressing wt-CFTR had 6.8-fold more functional channels than F508del-CFTR cells treated with vehicle alone.

We next tested for functional correction of F508del-CFTR in polarized CFBE41o- human airway epithelial cells overexpressing F508del-CFTR (Figure 5). Short-circuit currents were measured in Ussing chambers with an apical-to-basolateral

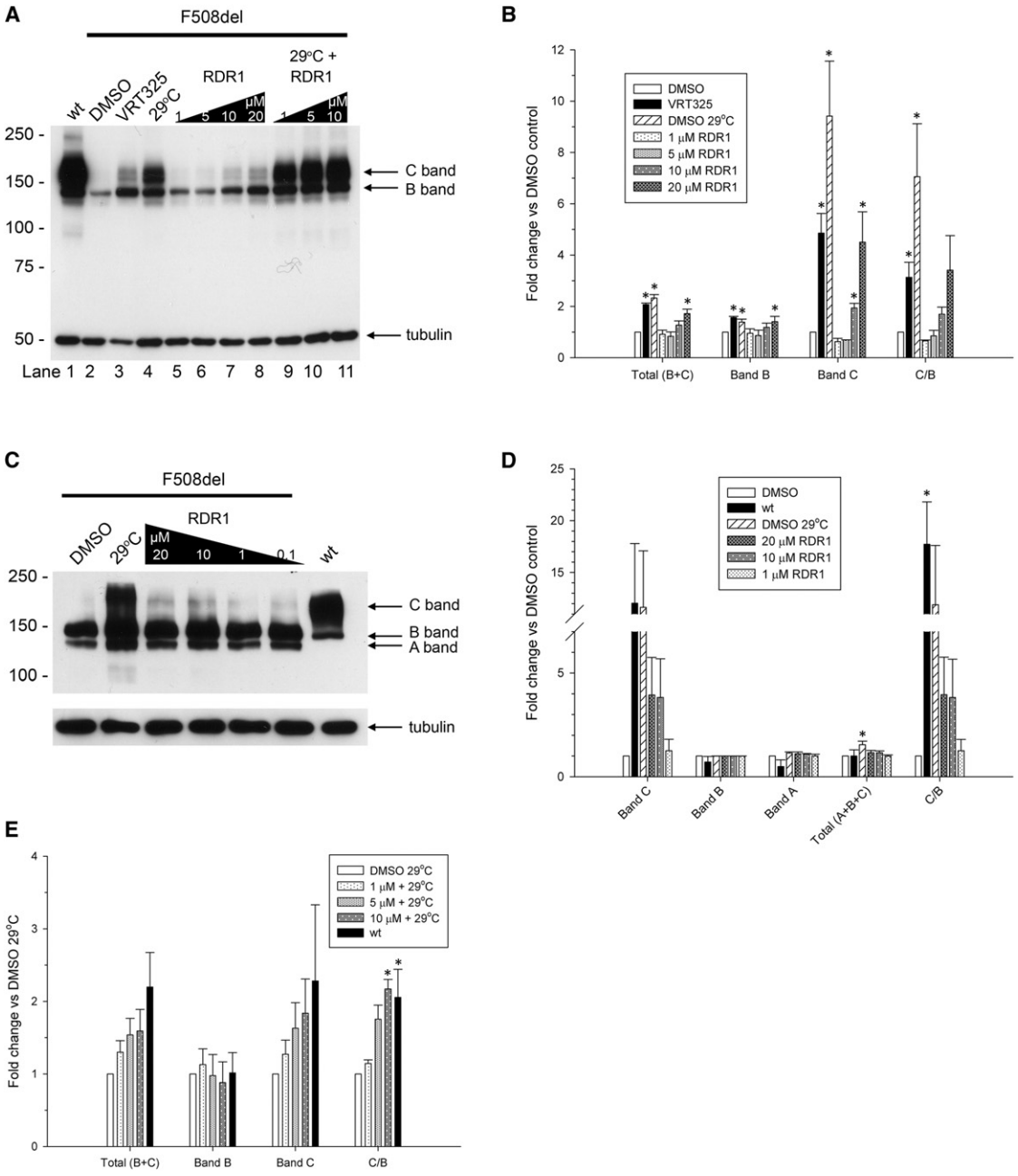


Figure 3. RDR1 Corrects Trafficking of F508del-CFTR in BHK Cells and Airway Epithelial Cells

(A) Immunoblot of BHK lysates stably overexpressing wild-type (WT) or F508del-CFTR (F508del) treated with RDR1 or 10 μM VRT325 at 37°C or 29°C, as indicated. Lanes are numbered beneath the blot. Triangles indicate increasing concentrations. CFTR C band, B band, and tubulin are indicated with arrows.

(B) Quantification of the band intensities for RDR1 treatments expressed as fold change of DMSO control. Error bars indicate SEM (n = 3). Asterisks indicate statistically significant differences with a Student's t test as compared with DMSO control ($p < 0.05$).

(C) Immunoblot of CFBE41o- lysates stably overexpressing CFTR wild-type (WT) or F508del-CFTR (F508del) treated at 37°C with RDR1 or treated at 29°C. Triangles indicate decreasing concentrations. CFTR C band, B band, A band and tubulin are indicated with arrows.

(D) Quantification of the band intensities for RDR1 treatments is expressed as fold change of the DMSO control. Error bars indicate SEM (n = 3). Asterisks indicate statistically significant differences with a paired Student's t test as compared with DMSO control ($p < 0.05$).

(E) Quantification of the band intensities for increasing RDR1 concentrations on low temperature rescued F508del-CFTR expressed as fold change of 29°C. Error bars indicate SEM (n = 3). Asterisks indicate statistically significant differences with a paired Student's t test as compared with the 29°C control ($p < 0.05$).

See also Figure S2.

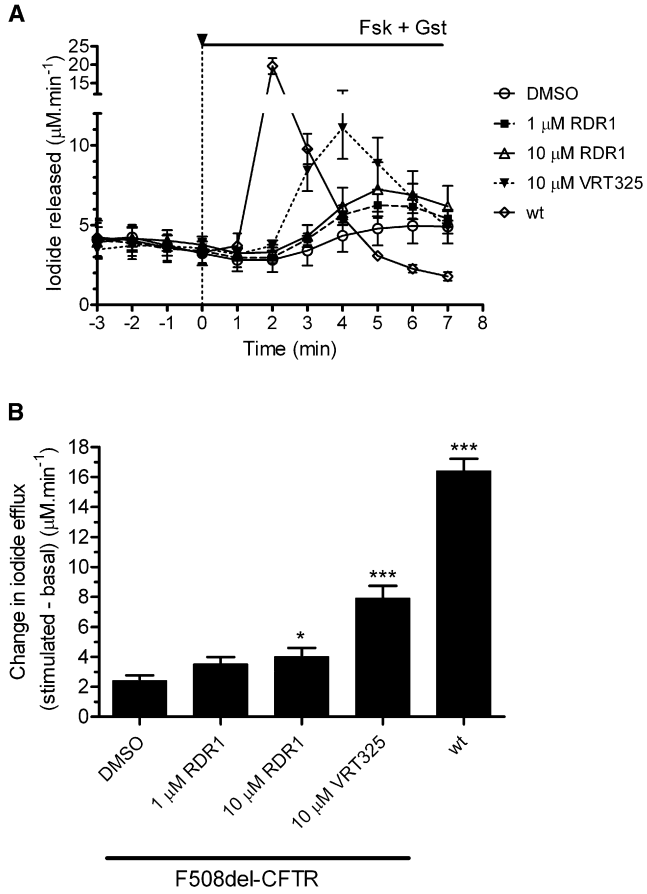


Figure 4. RDR1 Functionally Corrects F508del-CFTR in BHK Cells

(A) Iodide efflux assay measuring F508del-CFTR function at the plasma membrane in BHK cells treated with DMSO, 1 μM RDR1, 10 μM RDR1, or 10 μM VRT325. Iodide efflux in untreated BHK cells expressing wt-CFTR (wt) is also shown. An arrowhead and bar indicate addition and maintenance, respectively, of 10 μM forskolin (Fsk) and 50 μM genistein (Gst). Error bars indicate SEM (n = 3).

(B) Change in iodide efflux upon stimulation with forskolin and genistein, defined as the peak iodide efflux after stimulation subtracted from the baseline response. Error bars indicate SEM (n = 3). A difference in the means as compared with the DMSO control was tested for statistical significance using paired t tests, *$p < 0.05$, ***$p < 0.001$.

chloride gradient after exposing monolayers to DMSO (Figure 5A), or 1 μM RDR1 for 24 hr (Figure 5B). Treatment with 10 μM VRT325 (Figure 5C) and low temperature (Figure 5D) served as positive controls. A 1.5-fold increase in current was detected following treatment with RDR1 as compared with the vehicle-treated control (Figure 5E, $p < 0.01$, n = 4). Similarly, VRT325 treatment induced an approximately 3-fold increase, and low temperature treatment induced a 20-fold increase in current above the vehicle-treated control (Figure 5E). These data show that RDR1 can rescue F508del-CFTR function in airway epithelial cells, albeit not as efficaciously as VRT325 or low temperature.

Since we observed functional correction by RDR1 treatment in multiple cell lines, we next tested for functional correction in an F508del *Cftr* mouse model (French et al., 1996; van Doorninck et al., 1995; Wilke et al., 2010). Mice were injected with 1mg/kg/day of RDR1 or saline for 48 hr and salivary secretion was monitored following beta-adrenergic stimulation. As shown in Figure 6, we detected a significant increase in total salivary secretion following treatment with RDR1 (67.54 $\mu g.g^{-1}$ for RDR1 versus 15.97 $\mu g.g^{-1}$ for vehicle controls, $p < 0.001$). This corresponds to approximately 7% of the salivary secretion observed with wild-type littermates.

RDR1 Is a Weak Potentiator of Human F508del-CFTR Function

Given that RDR1 binds to F508del-NBD1, we tested its ability to potentiate F508del-CFTR function. We incubated polarized CFBE41o- cells stably overexpressing F508del-CFTR at 29°C for 24 hr to rescue F508del-CFTR, measured short circuit currents following addition of 10 μM forskolin, and then added acutely DMSO, RDR1 or genistein (Figure 7). Representative traces are shown in Figure 7A. Treatment with 20 μM RDR1 resulted in a small increase in current (Figures 7A and 7B); however, 50 μM RDR1 treatment gave a statistically significant increase equivalent to approximately 9% of that detected with 50 μM genistein (Figures 7A–7C, $p < 0.01$, n = 3). The current declined to baseline following addition of $CFTR_{inh}$-172, which indicates that it is generated by F508del-CFTR.

DISCUSSION

We set out to find pharmacological chaperones for F508del-CFTR among the trafficking correctors identified in our cell-based screen (Carlile et al., 2007). Of over 220 correctors screened, only one, RDR1, thermally stabilized purified F508del-NBD1. The small number of pharmacological chaperones among our correctors could be attributed to the chemical libraries that were screened, the use of murine F508del-NBD1 as a screening tool, or alternatively, we may have missed those that bind elsewhere than the NBD1. We have shown that RDR1 thermally stabilizes isolated F508del-NBD1 in a manner similar to ATP, suggesting that it acts as a pharmacological chaperone of F508del-CFTR. RDR1 is the highest affinity NBD1-binding pharmacological chaperone found so far. RDR1 does not bind to the ATP binding site since ATP and RDR1 additively stabilize F508del-NBD1 against thermal denaturation. The structurally related compound RDR3 also stabilizes F508del-NBD1; however, RDR2, which lacks a terminal phenyl ring present in RDR1, does not thermally stabilize F508del-NBD1. These results suggest that this phenyl ring may be an important moiety for mediating the interaction with F508del-NBD1. We have shown that RDR1 treatment corrects F508del-CFTR trafficking in both BHK and CFBE41o- cells and in a mouse model of CF. Acute addition of RDR1 to polarized CFBE41o- cells shows that it is a weak potentiator of F508del-CFTR function, which is consistent with a mechanism of action that involves binding to F508del-NBD1 (Cai et al., 2006).

Several potentiators that are postulated to bind directly to the NBDs of CFTR have been identified to date, including genistein (French et al., 1997), MPB compounds (Becq et al., 1999; Galietta et al., 2001), phloxine B (Bachmann et al., 2000; Cai and Sheppard, 2002), capsaicin (Ai et al., 2004), and VRT532 (Van Goor et al., 2006; Wellhauser et al., 2009). However, none

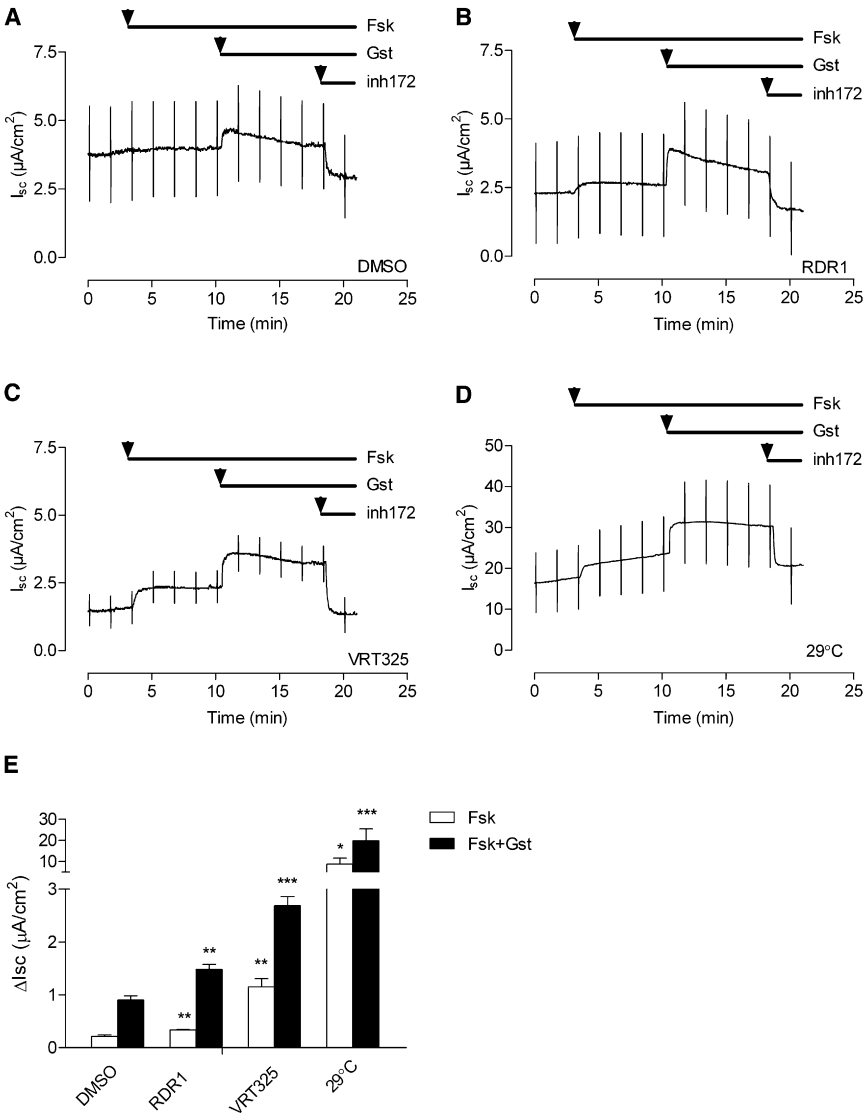


Figure 5. RDR1 Functionally Corrects F508del-CFTR in Airway Epithelial Cells

(A–D) CFBE41o- cells stably overexpressing F508del-CFTR were treated with (A) vehicle control (DMSO), (B) 1 μM RDR1, (C) 10 μM VRT325, or (D) vehicle control at 29°C for 24 hr and current was measured in an Ussing chamber. In (A)–(D), arrowheads and bars indicate addition and maintenance, respectively, of 10 μM forskolin (Fsk), 50 μM genistein (Gst), and 10 μM $CFTR_{inh}$-172 (inh172).

(E) Bar graph showing the change in I_{sc} (ΔI_{sc}) after adding forskolin (white) or forskolin + genistein (black), defined as the difference between the peak of the sustained phase of the current response after forskolin or genistein, respectively, and the baseline before stimulation. Mean currents for each treatment are shown. Error bars indicate SEM (n = 4). Means were tested for statistical significance using a Student's t test; $^{**}p < 0.01$, $^{***}p < 0.001$.

of the binding sites for these potentiators have been precisely located on CFTR. MPB compounds are postulated to interact with NBD1 via glycine 622, since mutation of this residue abolishes correction by these compounds (Norez et al., 2008). Genistein and many other potentiators are hypothesized to bind to the interface of the NBD1-NBD2 heterodimer with a surface involving the Walker A, B, and ABC signature of NBD1 and with the ABC signature motif of NBD2 (Moran et al., 2005). This hypothesis is consistent with the finding that the G551D and the V1293G mutations alter the affinity of these molecules for CFTR (Moran et al., 2005; Zegarra-Moran et al., 2007). Capsaicin likely binds to the same site since it competes with genistein (Ai et al., 2004). We tested genistein and capsaicin in our DSF screen and we were unable to detect thermal stabilization of F508del-NBD1 (data not shown). This result may indicate that both NBDs are required to bind to these potentiators. We also tested whether we could detect thermal stabilization with VRT532, a dual corrector/potentiator (Van Goor et al., 2006; Wang et al., 2006b, 2007b). Although we did see some stabilization of F508del-NBD1 with VRT532, it was only observed at very high (100 μM) concentrations (see Figure S1 available online). Interestingly, VRT532 is hypothesized to bind to a site between the NBD and MSD of F508del-CFTR rather than at the heterodimer interface (Wellhauser et al., 2009). This difference may explain why VRT532 can thermally stabilize F508del-NBD1 while genistein and capsaicin cannot. These findings suggest that RDR1 might bind to a site other than at the interface between the two NBDs.

Treatment with RDR1 caused a significant increase in F508del-CFTR function for all cell types presented here. In BHK cells, 10 μM RDR1 treatment resulted in 29% of the halide efflux observed with 10 μM VRT325 (Figure 4); in polarized CFBE41o- cells, 1 μM RDR1 treatment resulted in 25% and 2.5% of the functional correction achieved with 10 μM VRT325 and low temperature treatment, respectively (Figure 5). In the CF mouse, 1 mg/kg/day treatment of RDR1 resulted in approximately 7% of the salivary gland secretion observed in wild-type littermates (Figure 6). Conversely, treatment of BHK cells with RDR2, which does not thermally stabilize F508del-NBD1 in DSF, did not induce maturation of F508del-CFTR (Figure S2). Acute treatment of polarized CFBE41o- cells with RDR1 shows that it is a weak potentiator (Figure 7). This contrasts with VRT325, which has recently been shown to partially inhibit CFTR (Kim Chiaw et al., 2010). Taken together, these results indicate that thermal stabilization of F508del-NBD1 by RDR1 is correlated with an increase in F508del-CFTR maturation and function, which is consistent with the action of a pharmacological chaperone.

We have shown that RDR1 treatment is additive with low temperature correction, which is consistent with a mechanism of action that differs from proteostasis regulation. Interestingly,

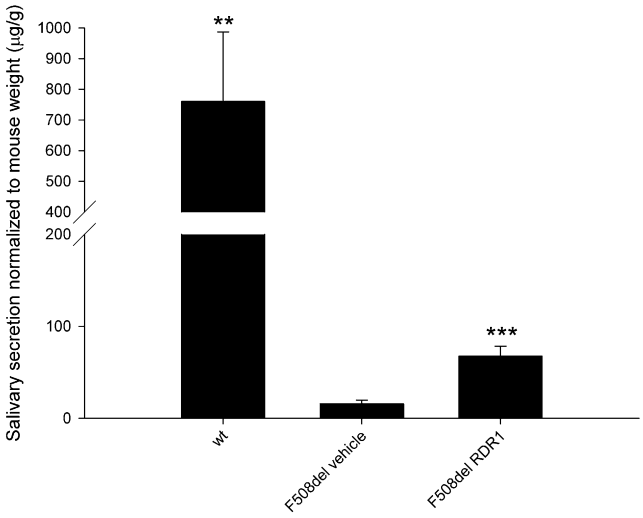


Figure 6. RDR1 Functionally Corrects F508del-CFTR in a Mouse Model of Cystic Fibrosis

Salivary secretion in wild-type mice (WT) or F508del-CFTR (F508del) mice treated with vehicle alone (vehicle) or 1mg/kg/day of RDR1 (RDR1) for 2 days was monitored for 30 min following stimulation. Results shown are for WT: n = 6, vehicle: n = 5, RDR1: n = 7. Means were tested for statistical significance using a Student's t test; **p < 0.01, ***p < 0.001 . Error bars indicate SEM.

See also Figure S3.

Corr4a and VRT325 treatment is also additive with low temperature correction (Pedemonte et al., 2010). Additionally, reports have suggested that Corr4a and VRT325 may bind to different sites on F508del-CFTR, as simultaneous treatment with these correctors had an additive effect on F508del-CFTR maturation (Pedemonte et al., 2010; Wang et al., 2007a). Given that constructs containing only the two MSDs of CFTR can acquire glycosylation upon VRT325 and Corr4a treatment (Wang et al., 2007c), these two correctors likely do not bind to the NBDs. Hence RDR1 is the most potent NBD1-binding pharmacological chaperone identified to date. Additionally, RDR1 treatment of human embryonic kidney cells for 24 hr did not inhibit the proliferation of these cells suggesting that it is not toxic to human cells at the concentrations that generate functional correction (Figure S3). This result contrasts the reported cytotoxicity of VRT325 (Robert et al., 2010). As the level of correction detected with a single corrector is quite small, a combinatorial approach (i.e., either two correctors, or a corrector and potentiator) will likely be necessary to generate a significant increase in CFTR function to ameliorate the symptoms of CF (Jurkuvenaite et al., 2010). RDR1 represents a promising lead for further development as a potential addition to a new generation of CF treatments.

SIGNIFICANCE

Although the most common mutation causing cystic fibrosis (CF), F508del-CFTR, has been known for over 20 years (Riordan et al., 1989), we still do not have a cure for this disease. F508del-CFTR is recognized by the cellular quality control machinery and degraded rather than being trafficked to the cell surface where it functions as a chloride channel. One approach to developing a cure for CF is to identify small molecules that correct the basic trafficking defect. The most specific small molecule trafficking correctors would be pharmacological chaperones that bind directly to the F508del-CFTR protein. Few pharmacological chaperones exist for F508del-CFTR that are not limited either in effective concentration, toxicity profile, ability to correct trafficking in multiple cell types and models, or the location of the putative binding site lies outside the NBD1. Our data suggest that RDR1 is the most potent NBD1-binding pharmacological chaperone identified to date that corrects trafficking in multiple cell models and that does not appear to be toxic to human cells and mice. However, estimates of the correction level required to ameliorate the symptoms of CF range between 10% and 30% of wild-type CFTR function (McKone et al., 2003; Zhang et al., 2009), a level that cannot be achieved by a single pharmacological chaperone. Hence, a combination of correctors with different mechanisms of action will likely be necessary to cure this disease (Jurkuvenaite et al., 2010; Van Goor et al., 2006).

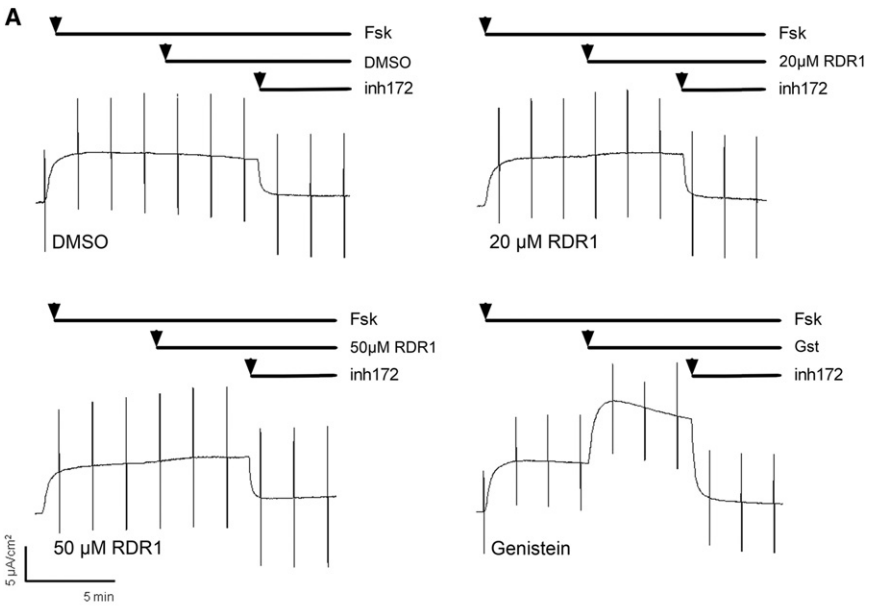
EXPERIMENTAL PROCEDURES

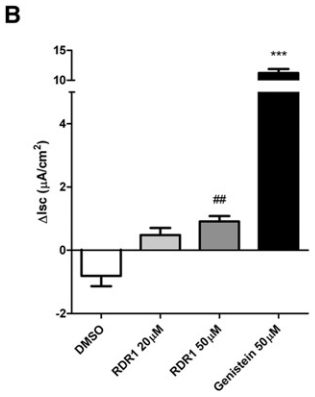
Cloning

Murine NBD1 (residues 389–673) and F508del-NBD1 constructs were generated by PCR using pET15-mCFTR as a template and appropriate primers (5′-TCCGGAACCACAGGCATAATCATGG-3′ and 5′-GGATCCTTAATCGTCTACTGAGAAC-3′) and subcloned into pETSUMO (Invitrogen). The SUMO-Histidine tagged proteins were expressed in *Escherichia coli* and purified according to published procedures with slight modifications (Lewis et al., 2004). In brief, proteins were purified on Nickel Sepharose Fast Flow resin (GE Biosciences) and eluted with an imidazole step gradient (20 mM Tris, 250 mM NaCl, 400 mM imidazole, 12.5% glycerol [pH 7.6]). ATP was added to eluted fractions to a final concentration of 2 mM. Fractions containing the SUMO-NBD1 were pooled and cleaved with a partially purified GST-ULP1 C-terminal conserved fragment kindly provided by Dr. Gregor Jansen (McGill University). The cleavage reaction was then separated on an S200 gel filtration column (GE Biosciences) equilibrated with the following buffer: 50 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM ATP, 2 mM 2-mercaptoethanol, 12.5% glycerol (pH 7.6). Fractions containing the cleaved F508del-NBD1 were pooled and concentrated with a 10,000-molecular weight cutoff spin filter column (Amicon).

Differential Scanning Fluorimetry

Differential scanning fluorimetry was performed using an Mx3005P instrument (Stratagene) as described (Vedadi et al., 2006). In brief, purified NBD1 or F508del-NBD1 was diluted in DSF buffer (100 mM HEPES, 150 mM NaCl [pH 7.4]) containing 5X Sypro Orange dye (Sigma-Aldrich) to a final concentration of 4 μM and aliquoted. DMSO or corrector compounds were added to the protein solution such that the final DMSO concentration was 1%. The solution was mixed gently and transferred to a 96-well plate (Stratagene) in duplicate. The plate was sealed with optical tape (Bio-Rad) and centrifuged at low speed to remove bubbles. The samples were then immediately run in the Mx3005P instrument using the excitation filter for carboxyfluorescein (FAM, 492 nm) and emission filter for Cy3 (568 nm) or Texas red (610 nm). Results were exported to Excel (Microsoft) and the apparent melting temperatures were obtained by fitting the curves using the Boltzmann equation and XLFit 4 software (IDBS). Fractional unfolding curves were generated with the following equation: $(F_T - F_{min}) / (F_{max} - F_{min})$, where F_T indicates fluorescence at temperature T, F_{min} indicates the minimum fluorescence and F_{max} indicates the maximum fluorescence. Experiments were performed at least three times.





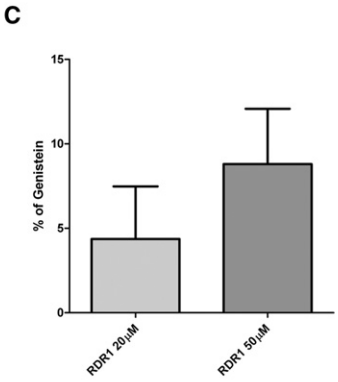


Figure 7. RDR1 Potentiates F508del-CFTR Function in Polarized Airway Epithelial Cells

(A) Representative traces from short circuit currents measured in Ussing chamber on polarized CFBE41o- cells overexpressing F508del-CFTR incubated at 29°C for 24 hr, stimulated with 10 μM forskolin, and then treated acutely with DMSO, 20 μM RDR1, 50 μM RDR1, or 50 μM genistein, followed by the addition of 10 μM $CFTR_{inh}$-172 (inh172), as indicated by arrowheads and bars.

(B) Bar graph showing the change in I_{sc} (ΔI_{sc}) after adding DMSO, RDR1, or genistein after the forskolin-stimulated I_{sc}, defined as the difference between the sustained phase of the current response after DMSO, RDR1, or genistein and the sustained phase of the current response after forskolin. Mean currents for each treatment (n = 3). Error bars indicate SEM, ***p < 0.001, ## p < 0.01.

(C) Currents generated by RDR1 treatment normalized to genistein treatment.

Cell Culture

BHK cells stably transfected with human F508del-CFTR were cultured under standard conditions in DMEM-F12 media supplemented with 5% FBS and 450 μM methotrexate. CFBE41o- cells stably transfected with F508del-CFTR were cultured under standard conditions in EMEM supplemented with 10% FBS and 1% L-Glutamax. Compounds for treatment were diluted from DMSO stocks into fresh media such that the final concentration of DMSO was 0.1%.

Western Blotting

Western blotting was performed according to published procedures (Carlile et al., 2007), with a mouse monoclonal antibody raised against the R domain of CFTR (Myriam Mirza, Veli-Pekka Määttänen and D.Y.T., unpublished data). The relative levels of each CFTR glycoform were estimated by densitometry using Photoshop (Adobe Inc.). The values reported are expressed as means ± SEM (n = 3). Data sets were compared by a Student's t test using SigmaPlot (Systat Software, Inc.).

Iodide Efflux Assays

Iodide efflux assays with stably transfected BHK cells expressing histidine-tagged wt-CFTR or F508del-CFTR were performed according to published procedures (Robert et al., 2010). CFTR-mediated iodide efflux was stimulated with 10 μM forskolin and 50 μM genistein at room temperature. Values reported are means ± SEM (n = 3). Data sets were compared with DMSO by paired t test using SigmaPlot (Systat Software, Inc.).

Ussing Chamber

We used the CFBE41o- airway epithelial cell line subsequently transduced with TranzVector lentivectors containing F508del-CFTR, which was generously provided by Dr. J.P. Clancy (University of Alabama at Birmingham, Birmingham, AL). Cells were seeded at a density of 2.5×10^5 cells/cm^2 onto 12 mm fibronectin-coated Snapwell inserts (Corning Incorporated). The apical medium was removed after 24 hr to establish an air-liquid interface, and then the cells were cultured for another 6–7 days. Cells were treated on both sides with optiMEM containing 1 μM RDR1, 10 μM VRT325, or DMSO (1/1000) at 37°C, or incubated at 29°C for 24 hr, before being mounted in EasyMount chambers and voltage clamped using a VCCMC8 multichannel current-voltage clamp (Physiologic Instruments). The apical membrane conductance was functionally isolated by permeabilizing the basolateral membrane with 200 μg/ml nystatin and imposing an apical-to-basolateral Cl^- gradient. The basolateral bathing solution contained 1.2 mM NaCl, 115 mM Na-gluconate, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 4 mM $CaCl_2$, 2.4 mM KH_2PO_4, 1.24 mM K_2HPO_4 and 10 mM glucose (pH 7.4). The $CaCl_2$ concentration was increased to 4 mM to compensate for the chelation of calcium by gluconate. The apical bathing solution contained 115 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2.4 mM KH_2PO_4, 1.24 mM K_2HPO_4, and 10 mM mannitol (pH 7.4). The apical solution contained mannitol instead of glucose to eliminate currents mediated by Na^+-glucose cotransport. Successful permeabilization of the basolateral membrane was obvious from the reversal of I_{sc} under these conditions. Solutions were continuously gassed and stirred with 95% O_2 and 5% CO_2 and maintained at 37°C. Ag/AgCl reference electrodes were used to measure transepithelial voltage and pass current. Pulses (1 mV amplitude, 1 s duration) were delivered every 90 s to monitor resistance. The voltage clamps were connected to a PowerLab/8SP interface for data collection. For correction experiments, 10 μM forskolin and 50 μM genistein were added sequentially to the apical side followed by an addition of 10 μM of $CFTR_{inh}$-172 to the apical side to demonstrate that measured currents were due to CFTR activity. UTP (100 μM) was added at the end of the experiment to the apical side to demonstrate the viability of the cells (data not shown). Transepithelial resistances of the epithelia were 300–400 Ohm/cm^2. For potentiation experiments, cells were incubated at 29°C for 24 hr, 10 μM forskolin was added to the apical side followed by acute addition of DMSO, 20 μM RDR1,

50 μM RDR1, or 50 μM genistein. The addition of 10 μM of $CFTR_{inh}$-172 to the apical side was performed to demonstrate that measured currents were due to CFTR activity.

Salivary Secretion Experiments

The methods used were similar to those described by Best and Quinton (2005). The procedures followed all CIHR regulations, and the protocol was approved by the Animal Care Committee of McGill University. In brief, 10- to 12-week-old homozygous F508del-CFTR mice (backcrossed on the FVB genetic background for more than 12 generations, *Cftr*$^{tm1\ Eur}$) (van Doorninck et al., 1995) and littermate wild-type controls weighing between 23 and 33 g were injected intraperitoneally with saline containing RDR1 (1 mg/kg/day) or vehicle alone (0.1% DMSO) for 2 days and monitored closely for possible adverse effects. The mice were anaesthetized with a combination of ketamine and diazepam on the day of the experiment and maintained on a flat surface in supine orientation with adhesive tape. The mouth was held open by cephalic retraction of the dorsal front teeth with a rubber band. An indirect heat lamp warmed the animals during the saliva collection period. Mice were pretreated with a subcutaneous injection of 50 μl (1 mM) atropine into the left cheek as a cholinergic antagonist. A 2 × 25 mm piece of Whatman filter paper was placed inside the previously injected cheek for approximately 4 min to absorb any saliva. Solution (100 μl) containing 100 μM isoprenaline and 1 mM atropine was then injected into the left cheek at the same site to induce secretion at time zero and the filter paper was replaced every 3 min for 30 min. Each piece of filter paper was immediately placed and sealed in a pre-weighed vial and the time of removal was recorded. The total salivary secretion was normalized to the mass of the mouse in grams. Results are expressed as the mean ± SEM of n mice.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2010.11.016.

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