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Benzbromarone Stabilizes ΔF508 CFTR at the Cell Surface

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Supporting Information

ABSTRACT: Deletion of Phe508 from the first nucleotide-binding domain of the CFTR chloride channel causes cystic fibrosis because it inhibits protein folding. Indirect approaches such as incubation at low temperatures can partially rescue ΔF508 CFTR, but the protein is unstable at the cell surface. Here, we show that direct binding of benzbromarone to the transmembrane domains promoted maturation and stabilized ΔF508 CFTR because its half-life at the cell surface was ∼10-fold longer than that for low-temperature rescue. Therefore, a search for small molecules that can rescue and stabilize ΔF508 CFTR could lead to the development of an effective therapy for cystic fibrosis.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-regulated chloride channel that is located on the apical surface of epithelial cells that line lung airways and ducts of various glands (reviewed in ref 1). Its physiological role is to regulate salt secretion and reabsorption to maintain normal salt and water homeostasis in epithelial tissues.

Cystic fibrosis is a genetic disease caused by mutations in the CFTR gene.[2] The most common defect is deletion of Phe508 (ΔF508 CFTR) from the first nucleotide-binding domain (NBD1). ΔF508 CFTR is rapidly degraded in the endoplasmic reticulum[3] and is unstable at the cell surface when rescued by low temperatures.[4] The ΔF508 mutation appears to alter the folding kinetics of NBD1[5] and disrupts interactions between NBD1 and intracellular loop 4 in the second transmembrane domain (TMD2).[6,7] Disruption of NBD1−TMD2 interactions leads to defective packing of the TM segments.[8] The lack of functional CFTR in the airways causes the accumulation of viscous mucus and colonization with microorganisms that cause inflammation and loss of function.[1]

A potential treatment for cystic fibrosis would be to promote folding of ΔF508 CFTR to increase the amount of protein delivered to the cell surface. Indirect approaches such as low-temperature rescue,[9] expression in the presence of "chemical chaperones" such as glycerol and DMSO,[10,11] perturbing CFTR−chaperone interactions with agents such as thapsigargin,[12] and inhibiting ER-associated degradation[13] were shown to yield mature ΔF508 CFTR at the cell surface that retains some functional activity. The ΔF508 CFTR rescued with these approaches is ∼5−10-fold less stable at the cell surface than wild-type CFTR.[14]

An alternative approach to rescuing ΔF508 CFTR is to mimic the direct drug-rescue approach described for CFTR's sister protein, the P-glycoprotein (P-gp) drug pump. It has been found that direct binding of drug substrates to the transmembrane domains of P-gp processing mutants stabilizes and promotes maturation of the protein, resulting in an active molecule at the cell surface.[15,16]

Compounds that specifically rescue ΔF508 CFTR would result in fewer side effects as other metabolic pathways would not be affected. The bioavailability of these compounds would be enhanced if they were not substrates of drug pumps such as P-gp.

There are a number of potential advantages for targeting the transmembrane domains of CFTR. For example, the TM domains are predicted to be a good target for rescue because packing of the TM segments during folding appears to be a rate-limiting step and occurs post-translationally.[17] In addition, many of the TM segments of mammalian ABC transporters such as CFTR and P-gp are unstable during synthesis.[18] Finally, it appears that disruption of domain−domain interactions by mutations such as ΔF508 in mammalian ABC proteins could yield TM domains with altered topologies.[19] Although it is possible to promote maturation using compounds that bind to NBD1,[20] it is possible that they may not stabilize CFTR because they are still deficient in NBD1−TMD2 interactions because of the lack of Phe508 at this critical interface.

It was recently reported that the stability of ΔF508 CFTR at the cell surface approached that of wild-type CFTR when NBD−TMD2 interactions were restored.[21] It was shown that introduction of a V510D mutation into NBD1 promoted the maturation and stability of ΔF508 CFTR by forming a a salt bridge with Arg1070 of TMD2.[21] Similarly, maturation of ΔF508 CFTR was promoted by a R1070W suppressor mutation in TMD2.[5] These suppressor mutation results suggested that direct binding of a compound to the TMDs may promote the maturation and stability of ΔF508 CFTR. To test this prediction, we tested whether benzbromarone that is thought to bind to the TMDs[22] could promote maturation of ΔF508 CFTR and increase its stability at the cell surface.

Accordingly, cells expressing ΔF508 CFTR were incubated in the presence of various concentrations of benzbromarone for 40 h. In agreement with a previous study of ΔF508 CFTR,[23] no increase in the level of mature protein was observed when cells were expressed in the presence of 0−20 μM benzbromarone (Figure 1A). A potential problem in the initial study was that benzbromarone is a relatively low-affinity CFTR channel blocker (K_i = 11.5 μM)[22] and higher concentrations would be needed to rescue ΔF508 CFTR. This was indeed the case, as mature ΔF508 CFTR could be readily detected when cells were expressed in the presence of 50−75 μM benzbromarone (Figure 1A). Higher levels appeared to be toxic as the relative amount of mature

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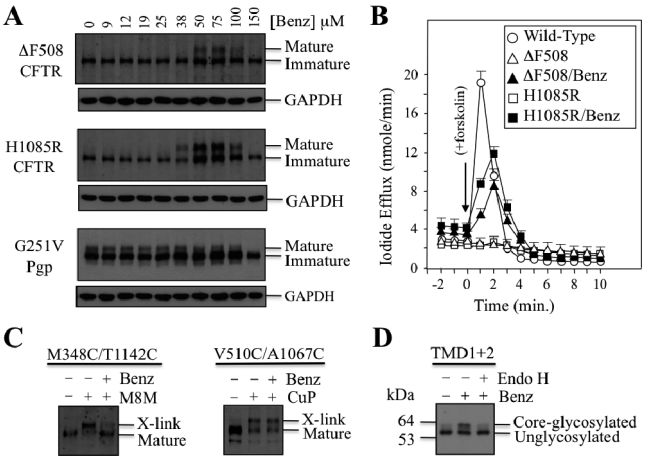

Figure 1. Effect of benzbromarone on maturation of CFTR and P-glycoprotein processing mutants. (A) Immunoblot analysis of cells expressing CFTR mutant ΔF508 or H1085R or the P-gp G251V processing mutant after treatment with the indicated concentrations of benzbromarone (Benz) for 40 h. (B) Iodide efflux assays performed on BHK cells stably expressing wild-type, ΔF508, or H1085R CFTR. Cells expressing mutant CFTRs were assayed after treatment with 0.05 mM benzbromarone for 40 h. (C) Effect of benzbromarone on cross-linking (X-link) between cysteines in TMD1 and TMD2 (M348C/T1142C) or NBD1 and TMD2 (V510C/A1067C).[7] (D) Immunoblot of cells expressing CFTR TMD1+2 in the absence (−) or presence (+) of 0.05 mM benzbromarone. Samples were treated with (+) or without (−) endoglycosidase H (Endo H).

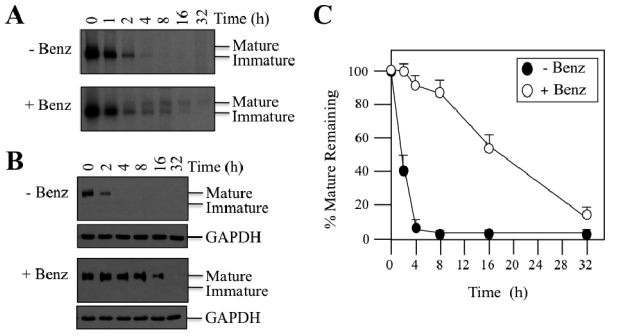

Figure 2. Effect of benzbromarone on the stability of ΔF508 CFTR. (A) Pulse−chase assays of ΔF508 CFTR performed in the presence (+) or absence (−) of 0.05 mM benzbromarone (Benz). (B) Cell surface labeling performed on cells expressing ΔF508 CFTR in the presence (+Benz) or absence (−Benz) of 0.05 mM benzbromarone. Cells were first incubated for 24 h at 30 °C in the presence (+Benz) or absence (−Benz) of benzbromarone to promote maturation of the protein. Cell surface labeling was performed after the indicated times at 37 °C in 0.2 mg/mL cycloheximide. (C) Amounts of mature labeled CFTR at each time point (B) quantitated and expressed relative to time zero.

CFTR decreased when cells were incubated in the presence of 100−150 μM benzbromarone.

We tested whether benzbromarone could rescue CFTR with a different processing mutation. The H1085R mutation is located in TMD2 within the intracellular loop (ICL) connecting TM10 and TM11.[24] Mature protein was observed when H1085R CFTR was expressed in the presence of 38−100 μM benzbromarone (Figure 1A). The yield of mature CFTR peaked in the presence of 50−75 μM benzbromarone and then decreased at higher concentrations.

CFTR's sister protein, the P-glycoprotein drug pump, was used to test for the specificity of benzbromarone rescue. The G251V processing mutant was selected as it shows an ∼15% maturation efficiency.[25] Figure 1A shows that benzbromarone did not promote maturation of G251V P-gp.

To test if the CFTR mutants were active after benzbromarone rescue, we performed iodide efflux assays on BHK cells stably expressing ΔF508, H1085R, or wild-type CFTR proteins. It was found that both ΔF508 and H1085R exhibited forskolin-activated iodide efflux after rescue with benzbromarone (Figure 1B).

It was shown that benzbromarone appeared to interact with the CFTR TMDs because 200 μM benzbromarone blocked cross-linking between cysteines introduced into TM segments 6 and 12 (M348C/T1142C).[23] This concentration of benzbromarone is now shown to inhibit maturation of CFTR (Figure 1A). We then tested if a benzbromarone concentration that promoted maturation of CFTR processing mutants (50 μM) also blocked cross-linking. Figure 1C shows that 50 μM benzbromarone inhibited cross-linking between TMD1 and TMD2 [M348C(TM6)/T1142C(TM12)] but not between NBD1 and TMD2 [V510C(NBD1)/A1067C(ICL4)].

It was possible, however, that benzbromarone interacted with the NBDs and inhibited cross-linking through long-range effects. To determine if benzbromarone could modulate folding of the TMDs alone, we tested its effect on glycosylation of a truncation mutant lacking both NBDs (TMD1+2). The mutant is inefficiently core-glycosylated (Figure 1D). Expression in the presence of benzbromarone, however, promoted core glycosylation of TMD1+2 (Figure 1D). The ability of benzbromarone to inhibit cross-linking between TM6 and TM12 (Figure 1C), promote glycosylation of TMD1+2 (Figure 1D), and inhibit CFTR chloride channel activity[26] suggests that it directly interacts with the TMDs.

Mature ΔF508 CFTR is ∼10-fold less stable than wild-type CFTR.[14] In BHK cells, we found that mature ΔF508 CFTR had a half-life of ∼1.5 h compared to a half-life of ∼14 h for wild-type CFTR.[8] To test the effect of benzbromarone on ΔF508 CFTR stability, we performed pulse−chase assays in the presence or absence of 0.05 mM benzbromarone. Benzbromarone promoted a slow maturation of ΔF508 CFTR that took ∼4−8 h to reach maximal levels (Figure 2A). The slow maturation was similar to what was previously observed with the V510D/ΔF508 CFTR suppressor mutant.[8] Maturation of mutant V510D/ΔF508 CFTR required ∼4−8 h (Figure 2A) compared to 1−2 h for the wild-type enzyme.[21]

The half-life of the mature ΔF508 CFTR in the pulse−chase assays was ∼16 h after rescue with benzbromarone (data not shown). To measure the stability of ΔF508 CFTR at the cell surface, cell surface labeling assays were performed. BHK cells expressing ΔF508 CFTR were incubated overnight at 30 °C in the presence or absence of 0.05 mM benzbromarone to promote maturation of the protein. The next day, protein synthesis was stopped by addition of 0.2 mg/mL cycloheximide. Turnover of ΔF508 CFTR from the cell surface was then monitored by performing cell surface labeling after various time periods at 37 °C (Figure 2B). In the absence of benzbromarone, ΔF508 CFTR had a short half-life of 1.7 ± 0.3 h (Figure 2C). Benzbromarone stabilized ΔF508 CFTR as its half-life increased to 18 ± 2 h (Figure 2C).

The results from this study suggest that benzbromarone directly interacts with the TMDs of CFTR to promote the

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maturation and stability of CFTR processing mutants. Benzbromarone rescue of CFTR resembles drug rescue of P-gp processing mutants. Drug substrates bind to the TMDs of P-gp processing mutants to increase the level of maturation, stability, and activity at the cell surface.[16,27] Benzbromarone stabilization of ΔF508 CFTR differs from that of compounds that act indirectly such as dynasore that stabilize CFTR by inhibiting endocytotic internalization.[28,29]

Benzbromarone rescue suggests that the TMDs of CFTR are potential target sites for corrector molecules. Corrector molecules have been identified using structure-based virtual screening[30] by docking at the interfaces between the cytoplasmic domains but not at the interface between the TMDs. A potential drawback of targeting the TMDs is that compounds like benzbromarone block the channel.

Could a corrector molecule bind to a region of the TMDs that does not block the pore? A recent study using a suppressor mutation of P-gp suggests that it may be possible to identify compounds that bind to intradomain sites outside of the pore to promote maturation.[31] It has also been proposed that CFTR contains a large intracellular vestibule.[32] The intracellular vestibule may be large enough to accommodate a corrector molecule without blocking the pore. Because benzbromarone is already used to treat hyperuricemia and gout (see the Supporting Information), it may be possible to identify analogues that rescue ΔF508 CFTR without blocking the channel.

■ ASSOCIATED CONTENT

Supporting Information. Details of experimental procedures and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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