Supporting Information

Benzbromarone Stabilizes ΔF508 CFTR at the Cell Surface.

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Contents

1. Experimental Procedures.

Construction and Expression of Mutants **-** Mutations were introduced into CFTR cDNAs containing an A52 epitope tag (*1*) by site-directed mutagenesis as described by Kunkel (*2*). A CFTR truncation mutant lacking the NBDs (TMD1+2) consisted of residues 1-388 (TMD1) plus residues 847-1196 (TMD2). The mutant CFTRs were transiently expressed in HEK 293 cells as described previously (*3*). HEK 293 cells were transfected with the cDNAs and the medium was changed four hours later to fresh medium (Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum) containing various concentrations of benzbromarone. Cells were harvested 24-40 h after the change in medium. Whole cell extracts of cells (from about 50,000 cells) expressing untagged or A52-tagged CFTRs were subjected to immunoblot analysis using 6.5% (w/v) acrylamide gels and a CFTR polyclonal antibody or monoclonal antibody A52. An equivalent amount of the sample was loaded onto 10% (v/v) SDS-PAGE gels and subjected to immunoblot analysis with a monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GADPH) (internal control). For treatment with endoglycosidase H, a one-tenth volume of 0.5 M sodium citrate, pH 5.5, was added to the solubilized cells followed by addition of 20,000 U/ml endoglycosidase H (New England Biolabs, Mississauga, ON, Canada). The sample was treated for 15 min at 20^{o}C and then subjected to immunoblot analysis.

Measurement of cAMP-stimulated Iodide Efflux – Equivalent numbers of BHK cells stably expressing wild-type, ΔF508, or H1085R CFTR proteins were grown in triplicate to 50% confluence in 6-well plates. The medium was then replaced with medium with or without 0.05

mM benzbromarone. After 40 h at 37 ^{o}C, the medium was removed and the cells washed to remove benzbromarone. Cells were then loaded with sodium iodide for 1 h at 37 ^{o}C. Iodide efflux was then monitored using an iodide-specific electrode (Analytical Sensors and Instruments, Sugar Land, TX) after stimulation with 0.01 □M forskolin in the presence of 0.03 mM genistein 0.2 mM 3-isobutyl-1-methylxanthine (IBMX), 0.01 mM VX-532, and 500 nM chlorophenylthio-cAMP. Each value is the mean ± SD (n = 9).

Cell surface labeling - Baby hamster kidney (BHK) cells stably expressing mutant ΔF508 CFTR were grown in DMEM medium containing 10% (v/v) calf serum for 24 h at 30 ^{o}C in the absence or presence of 0.05 mM benzbromarone to promote delivery of mature CFTR to the cell surface. The next day protein synthesis was stopped by addition of 0.2 mg/ml cycloheximide and the cells were incubated at 37 ^{o}C for 0-32 h. The cells were replenished with fresh media containing cycloheximide every 15 h. The cells were washed four times with phosphate buffered saline (pH 7.4) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBSCM) and then treated in the dark with PBSCM buffer containing 10 mM $NaIO_4$ for 30 min at 20 ^{o}C. The cell were then washed four times with PBSCM buffer and treated with sodium acetate buffer (100 mM sodium acetate buffer, pH 5.5, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$) containing 2 mM biotin-LC-hydrazide (Pierce, Rockford, IL. U.S.A) for 30 min at 20 ^{o}C. The cells were then washed twice with sodium acetate buffer and solubilized with Tris-buffered saline (100 mM Tris-HCl, pH 7.4 and 150 mM NaCl) containing 1% (w/v) Triton X-100 and protease inhibitors (Cocktail Set III, Calbiochem). Samples of the detergent extracts from equivalent numbers of cells were loaded onto 10% (v/v) SDS-PAGE gels and subjected to immunoblot analysis with a monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (cytoplasmic control). CFTR was immunoprecipitated from the cell extracts with monoclonal antibody A52, subjected to SDS-PAGE on 6% gels and biotinylated CFTR was detected with streptavidin-conjugated horseradish peroxidase and enhanced chemiluminescence. The gel lanes were scanned and the amount of labeled product was quantitated using the NIH Image program and an Apple computer. Results are expressed as mean ± SD of 3 measurements.

Disulfide Cross-linking Analysis – HEK 293 cells were transfected with the double cysteine mutants V510A/M348C(TMD1)/T1142C(TMD2) or V510C(NBD1)/A1067C(TMD2) CFTR

cDNAs in Cys-less backgrounds and the cells were incubated for 4 h at 37 ^{o}C. The transfection medium was removed and the cells were incubated in fresh medium. The next day membranes were prepared from the transfected cells. Membranes were treated for 15 min at room temperature with or without 0.05 mM benzbromarone followed by cross-linking with 0.05 mM 3,6-dioxaoctane-1,8-bismethanethiosulonate (M8M) (V510A/M348C(TMD1)/T1142C(TMD2)) or 1 mM copper phenanthroline (CuP) (V510C(NBD1)/A1067C(TMD2)) for 5 min at 0 ^{o}C. Samples of the reaction mixtures were then subjected to SDS-PAGE (6.5% (w/v) polyacrylamide gels) and immunoblot analysis with a rabbit polyclonal antibody against CFTR (*4*). Intramolecular disulfide cross-linking between domains of CFTR can be detected because the cross-linked product migrates with a slower mobility on SDS-PAGE gels (*4*).

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2. Figures.

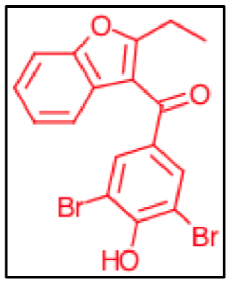

Figure S1. Structure of benzbromarone. Benzbromarone has been used for the treatment of hyperuricemia and gout for about 30 years (*5*). A plasma concentration of 0.025 mM benzbromarone could be achieved 2 hours after a single oral dose of 100 mg benzbromarone and the concentration varied depending on the patient's CYP2C9 genotype (*5*). Benzbromarone up to 200 mg/day is generally well tolerated with a small number (5-10%) of patients reporting adverse drug reaction such as dizziness and gastrointestinal effects (*6*). There have been a few reports of severe toxicity resulting in hepatic failure and the mechanism is likely due to reaction of benzbromarone metabolites with cytochrome CYP2C9 (*7*). This concern has led to its withdrawal from many markets but it remains available in some European countries as well as in Brazil and Japan. Other derivatives of benzbromarone have been synthesized (*8*) and may be useful as new therapies for gout and/or cystic fibrosis.

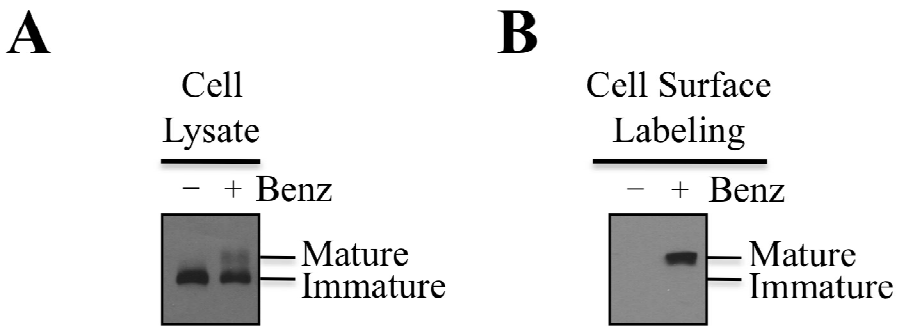

Figure S2. BHK cells expressing ΔF508 CFTR expressed in the absence (-) or presence (+) of 0.05 mM benzbromarone (Benz) were subjected to immunoblot analysis (A) or cell surface labeling with biotin LC hydrazide after periodate oxidation of the carbohydrate groups (B). The positions of the mature and immature CFTRs are indicated.

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