Na\textsuperscript{+}/H\textsuperscript{+} Exchanger Regulatory Factor Isoform 1 Overexpression Modulates Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Expression and Activity in Human Airway 16HBE14o—Cells and Rescues ΔF508 CFTR Functional Expression in Cystic Fibrosis Cells

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There is evidence that cystic fibrosis transmembrane conductance regulator (CFTR) interacting proteins play critical roles in the proper expression and function of CFTR. The Na\textsuperscript{+}/H\textsuperscript{+} exchanger regulatory factor isoform 1 (NHERF1) was the first identified CFTR-binding protein. Here we further clarify the role of NHERF1 in the regulation of CFTR activity in two human bronchial epithelial cell lines: the normal, 16HBE14o—, and the homozygous ΔF508 CFTR, CFBE41o—. Confocal analysis in polarized cell monolayers demonstrated that NHERF1 distribution was associated with the apical membrane in 16HBE14o— cells while being primarily cytoplasmic in CFBE41o— cells. Transfection of 16HBE14o— monolayers with vectors encoding for wild-type (wt) NHERF1 increased both apical CFTR expression and apical protein kinase A (PKA)-dependent CFTR-mediated chloride efflux, whereas transfection with NHERF1 mutated in the binding groove of the PDZ domains or truncated for the ERM domain inhibited both the apical CFTR expression and the CFTR-dependent chloride efflux. These data led us to hypothesize an important role for NHERF1 in regulating CFTR localization and stability on the apical membrane of 16HBE14o— cell monolayers. Importantly, wt NHERF1 overexpression in confluent ΔF508 CFBE41o— and ΔF508 CFTR-C2 cell monolayers induced both a significant redistribution of CFTR from the cytoplasm to the apical membrane and a PKA-dependent activation of CFTR-dependent chloride secretion.

The cystic fibrosis transmembrane conductance regulator (CFTR)\textsuperscript{3} protein is responsible for the CAMP/PKA-regulated chloride conductance in airway and intestinal epithelia and in exocrine glands. The most common mutation of the gene associated with cystic fibrosis (CF) causes deletion of phenylalanine at residue 508 (ΔF508). This mutation results in the synthesis of a functional but improperly folded CFTR protein that is targeted for degradation mainly via the ubiquitin-proteasome pathway in the ER (1) and, in some tissues, only a negligible amount of ΔF508 CFTR can reach the plasma membrane of CF cells (2) and transport chloride (3).

A sizable fraction of newly synthesized wild-type (wt) CFTR is also rapidly degraded without ever reaching the plasma membrane, and only 20–30% of the newly synthesized wt CFTR protein, after passing the ER quality control, is exported from the Golgi to the apical membrane as fully glycosylated CFTR (4). Once delivered to the plasma membrane, CFTR is subjected to rapid internalization to a pool of sub-apical vesicles that can be either recycled to the plasma membrane or delivered to lysosomes for degradation. It has been observed that activation of PKA, in addition to inducing CFTR channel activity, also induces the translocation of CFTR from the sub-apical compartment to the plasma membrane (5–7). However, this process seems to be tissue-specific, because in some cell systems PKA activation is able to regulate only the activity of CFTR channels already resident in the plasma membrane (8–10).

PDZ domain proteins have been proposed to be involved in regulation of CFTR localization and activity via their organization of multi-protein complexes at the plasma membrane (11) and regulating, in this way, various functions. CFTR interacts with several PDZ domain proteins such as NHERF, CAP70, and CAL via its C-terminal PDZ-binding motif (12–14). The Na\textsuperscript{+}/H\textsuperscript{+} exchanger regulatory factor (NHERF) is a 50-kDa membrane protein initially identified as the cofactor required for CAMP inhibition of NHE3 (15), but it is also known to interact with a wide variety of channels, transporters, and receptors (16). The interaction of NHERF with the C terminus PDZ target domain of CFTR (12) has been proposed to have a central role both in stabilizing CFTR at the apical membrane of airway epithelial cells and in the regulation of the CFTR trafficking to the apical membrane, because abrogating CFTR binding to NHERF eliminates both the polarized expression of CFTR on the apical membrane and vectorial chloride transport (17, 18).

In addition to PDZ domain interactions, NHERF interacts via its ERM binding domain with ezrin, which is known to act as a PKA anchoring protein and to associate with the actin cytoskeleton (19). This interaction between NHERF, ezrin, and PKA is hypothesized to be essential not only to regulate CFTR activity but also to anchor CFTR to...
the cytoskeleton (18), to stabilize CFTR protein at the cell surface (20) and to increase the efficiency by which kinases and phosphatases control channel activity (21, 22). Cheng et al. (13) suggested that CAL (CFTR-associated ligand), a PDZ-binding protein that interacts with CFTR in the post-ER secretory pathway, may pass CFTR to NHERF in sub-apical vesicles and, therefore, CFTR regulation in the plasma membrane could be the result of a competition between these PDZ proteins for binding with CFTR.

To learn more about the role of NHERF in regulating CFTR in polarized airway cells, we monitored the activity and trafficking of CFTR in two human bronchial cell lines: 16HBE14o— cells derived from a normal subject and expressing wild-type CFTR and CFB41o— cells derived from CF individuals homozygous for the ΔF508 mutation of CFTR. The 16HBE14o— cell monolayers provide a well polarized airway epithelial cell culture system exhibiting vectorial ion transport (23) and express NHERF1 primarily on the apical membrane where it functions as a scaffolding protein organizing apical membrane proteins into regulatory complexes (24). We report here that both normal and CF cells express both NHERF1 and NHERF2, but it is NHERF1 that follows CFTR expression patterns. Both of the PDZ1 and PDZ2 domains and the ERM domain of NHERF1 are involved in the polarized expression of CFTR and in the regulation of CFTR-dependent chloride efflux. Further, targeted NHERF1 overexpression stimulates CFTR-dependent chloride efflux by increasing apical CFTR expression in normal 16HBE14o— cells. Importantly, in the CF cell lines, CFB41o— and CFT1-C2, targeted NHERF1 overexpression induced the redistribution of CFTR from the cytoplasm to the plasma membrane and rescues CFTR activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Experiments were performed with a series of human tracheobronchial epithelial cell lines: the normal, 16HBE14o— and the CF homozygous for the ΔF508 allele (ΔF508/ΔF508), CFB41o—, comprising the generous gift of Prof. D. Gruener (California Pacific Medical Center Research Institute, University of California at San Francisco), whereas the CF cell line, CFT1-C2, isolated from a ΔF508 CF patient, was the generous gift of Prof. J. R. Yankaskas (Cystic Fibrosis/Pulmonary Research and Treatment Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC). 16HBE14o— and CFB41o— were grown in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, l-glutamine, and penicillin/streptomycin at 37 °C under 5% CO2. CFT1-C2 cells were cultured with serum-free Ham’s F-12 supplemented with 10 μg/ml insulin, 3.7 μg/ml endothelial cell growth supplement, 25 ng/ml epidermal growth factor, 30 nm triiodothyronine, 1 μM hydrocortisone, 5 μg/ml transferrin, and 10 ng/ml chola toxin (Sigma) (25). All cell lines were routinely grown on tissue culture plastic flasks coated with an extracellular matrix containing fibronectin/vitrogen/bovine serum albumin (26). For experiments of chloride efflux, confocal immunofluorescence analysis, Western blot analysis and biotinylation, cells were seeded on 0.4-μm pore size PET filter inserts (Corning BD Biosciences Labware) coated with the same extracellular matrix.

**Transfection of NHERF1 and NHERF2 cDNAs**—At 70–80% confluence, cells were transiently transfected with wild-type mouse NHERF1 cDNA or cDNAs mutated in the different domains and inserted into the pcDNA3.1/Hygro + vector. HRF1A and HRF2A are cDNAs encoding NHERF1 in which alanine substitutions GAGA in the core peptide-binding sequence, GYG, in PDZ1 (HRF1A) and PDZ2 (HRF2A) domain inactive the individual PDZ domain; ∆ERM is a cDNA encoding NHERF1 truncated of the last 30 amino acids. All of these clones behave as dominant mutants over the endogenous protein. Another plasmid construct used was human, wild-type NHERF2 inserted into the pcDNA3.1/His-C (gift of Dr. Pann Ghill Suh). The cells were transiently transfected using Escort IV reagent (Sigma) according to the manufacturer’s protocol, and the experiments were conducted 48 h later.

**Fluorescence Measurements of Apical Chloride Efflux**—Chloride efflux was measured using the Cl−-sensitive dye MQAE (26). Confluent cell monolayers grown on permeable filters were loaded overnight in culture medium containing 5 mM MQAE at 37 °C in a CO2 incubator and then inserted into a perfusion chamber that allowed independent perfusion of apical and basolateral cell surfaces. Fluorescence was recorded with a Cary Eclipse Varian spectrofluorometer using 360 nm (bandwidth 10 nm) as excitation wavelength and 450 nm (bandwidth 10 nm) as emission wavelength. To measure chloride efflux rate across the apical membrane, the apical perfusion medium was changed to a medium in which chloride was substituted with iso-osmotic nitrate. All experiments were performed at 37 °C in HEPES-buffered bicarbonate-free medium (Cl−-medium (in millimolar): NaCl 135, KCl 3, CaCl2 1.8, MgSO4 0.8, HEPES 20, KH2PO4 1, glucose 11, and Cl−-free medium: NaNO3 135, KNO3 3, MgSO4 0.8, KH2PO4 1, HEPES 20, Ca(NO3)2 5, glucose 11). At the end of each experiment a calibration procedure was performed as previously described (26). The rates of chloride efflux were calculated by linear regression analysis of the first 30 points taken at 4 s intervals while the change of fluorescence was still linear. In all reported experiments, the basolateral side of the monolayer was treated with bumetanide (5 μM) for 5 min before stimulation to avoid the possibility that the observed increase of chloride efflux could be due to the stimulation of basolateral Na+/K+2Cl−.

**Detection of CFTR-dependent Chloride Efflux**—16HBE14o— polarized monolayers exhibited a basal chloride efflux under baseline conditions when chloride was replaced by apical nitrate (0.030 ± 0.003 Δ(F/F0)/min, n = 15). Stimulation of PKA by addition of FSK plus IBMX (first traces of the typical experiments in supplemental Fig. 1S) significantly increased this apical chloride efflux (0.052 ± 0.005 Δ(F/F0)/min, n = 15, p < 0.0001). It is important to note that this PKA-dependent increase was specific for apical chloride efflux, because the chloride efflux induced by basolateral nitrate perfusion was not altered by FSK plus IBMX (0.032 ± 0.02 versus 0.031 ± 0.004 Δ(F/F0)/min before and after FSK plus IBMX treatment, respectively, n = 4, n.s.). The typical traces in supplemental Fig. 1S, show that addition of the CFTR inhibitors, glibenclamide (Fig. 1S, A, 100 μM) (27) or the highly specific CFTRinh-172 (Fig. 1S, C, 5 μM) (28, 29) to the apical perfusion fluids, before (5 min) and during the next FSK plus IBMX stimulation, inhibited this PKA-dependent increase to basal levels. Fig. 1S (B and D) shows the summary of these experiments and, in the histogram, the empty bar represents CFTR-dependent chloride efflux calculated as the difference in alterations of FSK plus IBMX stimulated fluorescence in the absence (light gray bar) and presence (dark bar) of the above mentioned CFTR inhibitors. Because the CFTR-dependent Cl− effuxes calculated with the two inhibitors (empty bars) were not significantly different from each other (0.024 ± 0.002, n = 15, versus 0.026 ± 0.007, n = 6, Δ(F/F0)/min with glibenclamide and CFTRinh-172, respectively, n.s.), we used glibenclamide in all the rest of the experiments and define CFTR-dependent chloride efflux as the difference between the rate of FSK plus IBMX-stimulated chloride efflux before and after apical glibenclamide treatment (presented as the empty bar in all subsequent figures).

Moreover, we chose to restrict the apical Cl− efflux measurements in 16HBE14o— cells to 14 days post-seeding of the cells on permeable filters, because both the transepithelial resistance measured as previously reported (26) and the CFTR-dependent chloride effluxes were
higher on the 14th day than that measured at the 9th or 6th day post-seeding (400 ± 44, n = 15; 250 ± 32, n = 4; 200 ± 35, n = 4; Ω x cm², on the 14th, 9th, or 6th days, respectively, and 0.024 ± 0.002 (n = 15), 0.015 ± 0.003 (n = 4), and 0.007 ± 0.001 (n = 4) Δ(F/P)/min, on the 14th, 9th, or 6th days, respectively). For this reason the confocal analysis, biotinylation, and Western blotting experiments were also performed on the same day.

Protein Extraction and Western Blotting—Confluent 16HBE14o- and CFBE41o- monolayers grown on coated permeable filters, were washed with PBS, lysed in lysis buffer A (NaCl 110 mM, Tris 50 mM, Triton X-100 0.5%, and Igepal CA-630 0.5%, pH 8, with added protease inhibitor mixture), sonicated for 10 s, and centrifuged for 10 min (16,000 × g), and then the pellet was discarded. Supernatant protein concentration was measured by the method of Bradford (30), and an aliquot of 30 μg of protein was diluted in Laemmli buffer, heated at 100 °C for 5 min, and separated by 4–12% SDS-PAGE Criterion XT precast gel (Bio-Rad). The separated proteins were transferred to Immobilon P (Millipore) in a Trans-Blot semidry electrophoretic transfer cell (Amersham Biosciences) for immunoblotting. The primary antibodies used were anti-hCFTR monoclonal antibody against the C terminus (R&D Systems, MAB25031, dilution 1:500), anti-hNHERF1 monoclonal antibody (BD Transduction Laboratories, dilution 1:500), and anti-hNHERF2 polyclonal antibody (Alpha Diagnostic International, dilution 1:1000). The secondary antibodies were anti-mouse IgG for monoclonal antibodies and anti-rabbit IgG for polyclonal antibody (Sigma). Immunocomplexes were detected with ECL plus reagent (Amersham Biosciences) and densitometric quantification and image processing were carried out using Adobe Photoshop and the Image software package (version 1.61, National Institutes of Health, Bethesda, MD).

Cell Fractionation—Fractionation was performed as described by Sun et al. (31). Confluent 16HBE14o- and CFBE41o- cells, cultured on coated permeable filters, were scraped into lysis buffer B (Tris-HCl, 10 mM (pH 7.4), NaCl, 50 mM, EDTA, 1 mM, with protease inhibitor mixture), homogenized in a 5-ml syringe with a 0.8 mm needle, and an aliquot of total homogenate was collected. Postnuclear supernatants were obtained by centrifugation (900 x g, 15 min) at 4 °C. Another centrifugation (20,000 x g for 60 min) was performed, the pellet was discarded, and the supernatant was centrifuged again at 100,000 x g for 60 min at 4 °C to obtain cytosolic and membrane fractions. Cytosolic proteins were prepared by concentrating the supernatants through Centricon Centrifugal Filter Devices YM-10, M, 10,000 cut-off (Millipore), whereas the membrane fraction was obtained by resuspending the pellet in resuspension buffer (HEPES, 50 mM (pH 7.4), NaCl, 150 mM, EDTA, 1 mM, 1% Nonidet P-40, 10% glycerol). 30 μg of protein from total lysate, membrane, and cytosolic fractions were diluted in Laemmli buffer and resolved by 4–12% SDS-PAGE Criterion XT precast gel (Bio-Rad).

Biotinylation of Apical Membrane Proteins—16HBE14o- and CFBE41o- cells, grown on coated permeable filters, were transfected with the vector containing wt NHERF1, PDZ domain-mutated or ERM domain-deleted NHERF1 cDNA constructs, or the empty vector. 48 h later the monolayers were washed with PBS and incubated with 2 mg/ml sulfo-NHS-biotin (Sigma) in PBS for 30 min at 4 °C. All further steps were performed in a cold room. Free sulfo-NHS-biotin was removed by washing cells twice at 4 °C with 0.1 M glycine in PBS and then with PBS. Cells were lysed in lysis buffer A, sonicated, and centrifuged, and the pellet was discarded. Volumes of supernatant, containing equal amounts of protein, were incubated overnight at 4 °C with gentle mixing with the same amount of streptavidin-agarose beads (Pierce) (50 μl of streptavidin/mg of biotin). Streptavidin-bound complexes were pelleted (16,000 × g), and after two washes with buffer lysis, biotinylated proteins were eluted in Laemmli buffer. The eluted proteins were subjected to SDS-PAGE and Western blotting as described above.

Immunofluorescence Analysis—105 cells were seeded on round glass coverslips and 48 h later washed with PBS, fixed in 4% paraformaldehyde for 20 min, washed three times with PBS, and permeabilized in 0.1% Triton X-100 in PBS for 10 min. After three more washes in PBS, the cells were blocked in 0.1% gelatin in PBS for 10 min and then incubated in a wet environment with primary antibodies for 1 h: anti-hNHERF1 (dilution 1:50) and anti-hNHERF2 (dilution 1:50). After washing three times with 0.1% gelatin in PBS, the cells were incubated in a wet environment with secondary antibodies for 1 h: goat anti-mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes, dilution 1:1000) or goat anti-rabbit IgG conjugated to Alexa Fluor 568 (Molecular Probes, dilution 1:1000). After three washes in PBS, coverslips were mounted onto slides with Vectashield mounting medium (Vector Laboratories). Fluorescence data were collected with a Nikon Eclipse TE 2000-S with a 40× oil immersion objective and processed by using the Metamorph Imaging System (Meta Imaging 9.1). Deconvolution was performed using Auto Deblur software (AutoQuant Imaging Suite 9.1). Collected images were exported to Adobe Photoshop for subsequent analyses.

Conflonal Imaging—For confocal immunofluorescence analysis, cells grown on coated permeable filters were prepared as described above and examined using a Zeiss Axioskop microscope equipped with a laser scanning confocal unit model MRC-1024 containing a 15-milliwatt krypton-argon laser (Bio-Rad). Monolayer confluen was detected by staining monolayers for the tight junction protein, ZO-1 (mouse anti-ZO-1 (Alexa Fluor 488) Zymed Laboratories, dilution 1:50). Specimens were viewed through a Planapo 63×/1.4 oil immersion objective, and images were randomly acquired in the vertical plane (z) by the Laser Sharp 2000 program (Bio-Rad). The relative distribution of CFTR (Alexa Fluor 568) and NHERF1 (Alexa Fluor 488) was determined in the apical region and in the cytoplasm by semiquantitative analysis of previously acquired images using ImageJ software: a freehand selection (8 μm²) was drawn over the region to be measured, and pixel counts within the selected region were determined. The transition between apical and basolateral regions was identified by staining lateral membranes of the monolayers for the tight junction protein ZO-1. The distribution of CFTR and NHERF1 was calculated using the formula, r = a/c, where a corresponds to pixel counts in the apical region and c corresponds to pixel counts in the cytoplasmic region.

Data Analysis—Data are presented as means ± S.E. for the number of samples indicated (n). Statistical comparisons were made using either unpaired or paired data Student’s t test. Differences were considered significant when p < 0.05.

RESULTS

Expression and Distribution of NHERF1 and NHERF2 in 16HBE14o- and CFBE41o-Cells—Studies have provided evidence that CFTR interacting proteins play critical roles in determining its proper expression and function, and NHERF was the first identified CFTR-binding protein (32). To further clarify the mechanism by which the interaction of NHERF with CFTR regulates CFTR expression and activity in human airway epithelial cells, we first analyzed the expression levels and cellular distribution of both NHERF isoforms in the normal human bronchial epithelial cell line, 16HBE14o- and in the ΔF508 homozygous (ΔF508/ΔF508) CFBE41o- cell line. Western blot analysis performed in the airway cells seeded on coated permeable filters revealed that both the
NHERF1 and NHERF2 isoforms were expressed in both cell lines (Fig. 1A). Therefore, we next examined the cellular distribution of NHERF1 and NHERF2 between the surface and the inside of the cell by indirect immunofluorescence staining with mouse anti-NHERF1 and rabbit anti-NHERF2. Interestingly, both cell lines revealed an intracellular distribution for NHERF2 (Fig. 1B), whereas NHERF1 was localized at the cell surface in 16HBE14o− cells and was mainly cytoplasmic in CFBE41o− cells (Fig. 1B). This finding that only NHERF1 distribution is different between 16HBE14o− and CFBE41o− cells directed us to focus on a more complete analysis of the localization of NHERF1 and CFTR in polarized cultures of the two cell lines.

CFTR and NHERF1 Expression and Localization in 16HBE14o− and CFBE41o− Cell Monolayers—We first determined the relative amount of mature (180 kDa) and immature (160 kDa) CFTR in lysate, cytosol, and total membrane fractions of cell monolayers grown on permeable coated filters (Fig. 2A). In 16HBE14o− cell monolayers (HBE), wt CFTR was found both as a mature (180 kDa) and an immature (160 kDa) band in the lysate and cytosolic fractions and as only the mature band in the total membrane fraction, whereas, in CFBE41o− cell monolayers (CF), ΔF508 CFTR was found mainly as the immature band, although a small amount of mature ΔF508 CFTR was detectable in the total membrane fraction as has been reported in other CF cells (2). Regarding NHERF1 expression, in 16HBE14o− cells NHERF1 was expressed in both the cytosol and membrane fractions, whereas it was absent in the membrane fraction of CFBE41o− cells. Moreover, NHERF1 was less expressed in CFBE41o− compared with 16HBE14o− cells (−32.29 ± 6.42%, n = 3, p < 0.05 and −30.59 ± 6.93%, n = 3, p < 0.05) in the lysate and cytosolic fractions, respectively.

As these fractionation data suggest that CFTR and NHERF1 are more associated with the total membrane fraction in normal cells than in CF cells, it was necessary to obtain a more detailed analysis of CFTR and NHERF1 cellular distribution. We next performed confocal microscopy analysis of 16HBE14o− and CFBE41o− cells grown on permeable supports. Fig. 2B demonstrates that in 16HBE14o− cells CFTR was distributed close to the apical region, whereas its distribution in CFBE41o− cells was primarily cytoplasmic. NHERF1 distribution in these polarized cell monolayers also was primarily associated with the apical membrane region in 16HBE14o− cells while being cytoplasmic in CFBE41o− cells. 

PKA-dependent CFTR Activity in 16HBE14o− and CFBE41o− Cells—This similarity in the distribution of NHERF1 and CFTR in 16HBE14o− and CFBE41o− cells directed us to then focus on the analysis of the role of NHERF1 in the regulation of CFTR activity. The rate of chloride efflux after substitution of chloride by nitrate in the apical perfusion medium was measured by the change in fluorescence (Δ(F/F₀)/min) of the chloride-sensitive dye, MQAE (see “Experimental Procedures” and supplemental Fig. 15). Fig. 3 illustrates a typical experiment (A and C) and the summary (B and D) of 15 experiments of chloride efflux in 16HBE14o− cells monolayers (A and B) and 10 experiments of chloride efflux in ΔF508 CFBE41o− cell monolayers (C and D). As in other cell types (27, 31, 32), both 16HBE14o− and CFBE41o−-polarized monolayers exhibited a basal chloride efflux under baseline conditions when chloride was replaced by nitrate that were not significantly different (0.030 ± 0.003, n = 15 versus 0.022 ± 0.002, n = 10, Δ(F/F₀)/min in 16HBE14o− and CFBE41o−, respectively, n.s.). Stimulation of PKA by addition of FSK plus IBMX (first trace of the typical experiment in Fig. 3, A and C, and the first bar in Fig. 3, B and D) significantly increased chloride efflux in 16HBE14o− cells (0.052 ± 0.005 Δ(F/F₀)/min, n = 15, p < 0.0001) while having no effect in CFBE41o− cells (0.023 ± 0.003 Δ(F/F₀)/min, n = 10, n.s.). The addition of the CFTR inhibitor, glibenclamide, before and during the next FSK plus IBMX stimulation (second trace of the typical experiment in Fig. 3, A and C, and the second bar in Fig. 3, B and D) inhibited this increase to basal levels in 16HBE14o− cells (0.027 ± 0.004 Δ(F/F₀)/min, n = 15, p < 0.0001) while having no effect in CFBE41o− cells (0.020 ± 0.002 Δ(F/F₀)/min, n = 10, n.s.). The glibenclamide inhibition was completely reversible as is shown in the third trace of the typical experiment in Fig. 3A in which the chloride efflux stimulated by FSK plus IBMX is identical to the first trace. Similar results were obtained using another specific
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Inhibitor of CFTR, the thiazolidinone CFTRinh-172 (28, 29), which has been demonstrated to alter CFTR gating (see supplemental Fig. 1S). CFTR-dependent chloride efflux is defined as the difference between the rate of FSK plus IBMX-stimulated chloride efflux before and after apical glibenclamide treatment (third bar in Fig. 3, B and D) and, as can be seen in those figures, CFTR-dependent chloride efflux in 16HBE14o- cells was high (0.024 ± 0.002 ∆(F/F₀)/min, n = 15, p < 0.0001), whereas in CFBE41o- cells it was not significantly different from zero (n = 10, n.s.). A similar lack of significant CFTR-dependent chloride efflux in CFBE41o- cells were obtained in using the specific inhibitor CFTRinh-172 (5 μM) (data not shown).

Role of NHERF1 PDZ Domains in Regulating CFTR Expression and Activity in 16HBE14o- Cells

To learn more about the role and mechanism of NHERF1 and its different binding domains in regulating CFTR expression, we measured apical CFTR expression by surface biotinylation after transfection of permeable filter grown 16HBE14o- monolayers with His₆-tagged wild-type, PDZ domain-mutated, or ERM domain-deleted NHERF1 constructs (33). Immunofluorescence analysis of the transfected monolayers demonstrated that ~57 ± 4% (n = 15) of the cells were transfected. Treatment with either the transfection vehicle, Escort IV, or transfection with the empty vector, pcDNA3.1/hygro+, did not significantly alter either apical CFTR expression in biotinylation experiments or CFTR-mediated chloride transport (data not shown).

As shown in Fig. 4 (A and B), the transfection of 16HBE14o- monolayers with wt NHERF1 induced an increase of biotinylated, apical CFTR compared with non-transfected cells (+46.90 ± 3.99%, n = 4, p < 0.01). In contrast, overexpression of NHERF2 had no effect on apical CFTR protein expression (−2.75 ± 8.26%, n = 3, compared with non-transfected cells, n.s.). Transfection with cDNA encoding NHERF1 with mutations in the GYGF core peptide-binding groove sequence to GAGA that inactivates either the PDZ1 or PDZ2 domain (HRF1A and HRF2A, respectively) or with the cDNA encoding ERM domain (ΔERM) deleted NHERF1, significantly reduced the levels of the apical CFTR band. These results confirm previous studies showing that the deletion of the PDZ interacting domain of CFTR abrogating the interaction between CFTR and NHERF inhibited the polarized expression of CFTR on the apical membrane (17, 34). Moreover, the finding that the deletion of ERM domain diminished the apical CFTR expression is in line with the hypothesis that the association of CFTR with the cytoskeleton by the ERM domain could serve as an anchor that determines its specific location at the apical membrane.

We next analyzed the role of NHERF1 and its different binding domains in altering CFTR-dependent chloride secretion (Fig. 5). As with apical CFTR expression, transfection of 16HBE14o- monolayers with wt NHERF1 (Fig. 5) significantly potentiated CFTR-dependent chloride secretion, whereas transfection of the cell monolayers with wt NHERF2 had no effect on CFTR-dependent chloride efflux (0.024 ± 0.002, n = 15 versus 0.026 ± 0.006, n = 5, ∆(F/F₀)/min, in the non-transfected and NHERF2 transfected monolayers, respectively, n.s.) confirming that only NHERF1 overexpression augments CFTR-mediated Cl⁻ secretion by increasing the CFTR protein expression in the apical membrane. Transfection of 16HBE14o- monolayers with the cDNA encoding NHERF1 with the mutated PDZ1 (HRF1A) or PDZ2 (HRF2A) almost completely abrogated CFTR-dependent chloride secretion, whereas transfection of the cell monolayers with wt NHERF1 induced an increase of biotinylated, apical CFTR expression by increasing the CFTR protein expression in the apical membrane.

FIGURE 3. Functional analysis of CFTR activity in 16HBE14o- and CFBE41o- cell monolayers. A and C, typical recordings showing changes in intracellular Cl⁻–dependent MQAE fluorescence (expressed as the F/F₀ ratio) when either the 16HBE14o- (A) or the CFBE41o- (B) polarized cell monolayer was pre-treated for 3 min with 10 μM FSK plus 500 μM IBMX before substitution of apical chloride by nitrate in the absence or presence of 100 μM glibenclamide. Glibenclamide (Glib) was added apically (ap) 5 min before nitrate substitution and remained for the entire efflux. B and D, summary of the data collected from different experiments respectively in 16HBE14o- (B, n = 15) or in CFBE41o- (D, n = 10), where CFTR-dependent chloride efflux (empty bar) was calculated as the difference in the F/F₀ ratio per minute (Δ(F/F₀)/min) in the absence of (light gray bar) and presence of (dark bar) glibenclamide. Each bar represents the mean ± S.E. Statistical comparison was made using paired Student’s t test with respect to the chloride efflux stimulated by FSK plus IBMX measured in the absence of glibenclamide.
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FIGURE 4. Role of NHERF1 domains in the expression of biotinylated CFTR in polarized 16HBE14o—cell monolayers. Expression level of biotinylated apical CFTR was analyzed in 16HBE14o—monolayers, grown on permeable filters, by Western blot using anti-CFTR antibody as described under “Experimental Procedures.” Non-transfected cells were treated with the transfection vehicle, Escort IV; HRF1A and HRF2A: cells transfected, respectively, with cDNA encoding NHERF1 with GYG in the core peptide-binding sequence of PDZ1 and PDZ2 mutated to GAGA; ΔERM: cells transfected with cDNA encoding NHERF1 deleted of the ERM domain; wt: cells transfected with cDNA encoding wild-type NHERF1. A, representative Western blot of four independent experiments. B, summary of the expression level of apical, biotinylated CFTR was determined by densitometry of Western blots and expressed as the percentage of the biotinylated CFTR measured in 16HBE14o—cell monolayers treated only with Escort IV, designated as 100% (non-transfected). Data represent means ± S.E., n = 4.

FIGURE 5. Role of NHERF1 domains in the regulation of CFTR activity in 16HBE14o—cell monolayers. 16HBE14o—monolayers, grown on permeable filters, were transfected with vectors containing NHERF1 cDNA mutated in the various domains and truncated as in Fig. 4. After 48 h the Cl—transport activity of CFTR was determined, and CFTR-dependent chloride efflux was calculated as described in Fig. 3B. Statistical comparisons were made using unpaired Student’s t test with respect to the values obtained in non-transfected monolayers.

efflux, demonstrating that the interaction of both NHERF1 PDZ domains with the C terminus of CFTR, in addition to being important in the polarized expression of CFTR, are fundamental for the regulation of CFTR activity as observed in other cell lines (35, 36). Further, transfection of 16HBE14o—monolayers with the cDNA encoding the ΔERM deletion of the NHERF1 ERM domain, which interacts with ezrin, inhibited PKA-dependent regulation of CFTR as was expected, because ezrin serves as an anchor for protein kinase A (PKA-anchoring protein) and tethers PKA directly to CFTR for efficient and specific phosphorylation (19). Confirmation for this hypothesis came from experiments in which the preincubation of 16HBE14o—cell monolayers with 100 μM

FIGURE 6. Transfection with cDNA for wild-type NHERF1 increases cell surface CFTR expression in CFBE41o—cell monolayers. CFBE41o—cells, grown on permeable filters, were transfected with wt NHERF1 or treated with the transfection vehicle, Escort IV, as described under “Experimental procedures.” A, total lysates were analyzed using anti-NHERF1 antibody. B, cell-surface membrane proteins were biotinylated and analyzed by Western blot analysis using anti-CFTR antibody as described under “Experimental Procedures.” Similar results were obtained in four independent experiments.

FIGURE 7. Localization of NHERF1 and CFTR in CFBE41o— and CFT1-C2 cells by confocal immunofluorescence microscopy. Confocal immunofluorescence microscopy was performed as described under “Experimental Procedures” in CFBE41o— and CFT1-C2 cells non-transfected (treated with the transfection vehicle, Escort IV) or transfected with cDNA for wt NHERF1. CFTR was detected by monoclonal antibody (green) as described in Fig. 2, and all images are in the vertical (xz) plane. Scale bars = 10 μm. In CFBE41o—cell monolayers, the calculated pixel density ratios (R) of the apical region versus the cytoplasmic region of ΔF508 CFTR (see “Experimental Procedures”) were: 3.57 ± 0.91 (n = 4) and 1.06 ± 0.09 (n = 4, p < 0.05) and the ratios (R) for NHERF1 expression were 2.80 ± 0.68 (n = 4) and 1.1 ± 0.13 (n = 4, p < 0.05) in transfected and non-transfected monolayers, respectively. Similarly, in CFT1-C2 cells the ratios for ΔF508 CFTR were 3.34 ± 0.24 (n = 3) and 0.97 ± 0.08 (n = 4, p < 0.001), and the ratios for NHERF1 were 2.05 ± 0.39 (n = 4) and 0.98 ± 0.07 (n = 5, p < 0.02) in transfected and non-transfected cells, respectively.

S-Ht31, which prevents the binding between PKA anchoring proteins and Type II regulatory subunits of PKA (37), completely abrogated CFTR-dependent chloride efflux (0.005 ± 0.003 Δ(F/Fl)/min, n = 3).

Overexpression of NHERF1 Rescues ΔF508 CFTR Functional Localization in CFBE41o—Cells—The increase of both the apical CFTR expression and CFTR-dependent chloride efflux by overexpression of wild-type NHERF1 in 16HBE14o—cells suggests the possibility that targeted overexpression of NHERF1 might be able to rescue the activity and expression of CFTR in the ΔF508 CFBE41o—cells. Transfection of CFBE41o—monolayers with wt NHERF1 increased total NHERF1 expression by 129 ± 10% (n = 4, p < 0.001, Fig. 6A) and resulted in a 39 ± 10% (n = 4, p < 0.05) increase in apical CFTR expression with respect to non-transfected monolayers as measured by apical biotinylation of polarized CFBE41o—cell monolayers grown on permeable filters (Fig. 6B). Overexpression of NHERF2 had no effect on apical CFTR protein expression (+4.45 ± 7.38% compared with non-transfected cells, n = 3, n.s.), demonstrating that, as in 16HBE14o—cells, in CFBE41o—cells only the overexpression of NHERF1 is able to increase ΔF508 CFTR apical expression. These results were confirmed by confocal analysis of confluent CFBE41o—cell monolayers in which it can be observed that after overexpression of wt NHERF1 both ΔF508 CFTR and NHERF1 are confined predominantly to the apical membrane.
NHERF1 Modulation of CFTR Activity in Airway Cells

FIGURE 8. Overexpression of NHERF1 increases CFTR activity in CFBE41o— monolayers. Left panels: typical recordings showing changes in intracellular Cl–dependent MQAE fluorescence (expressed as the F/F₀ ratio) in CFBE41o— monolayers grown on permeable filters either not transfected (treated with the transfection vehicle, Escort IV (A)) or transfected with cDNA encoding wt NHERF1 (B). The experiments were performed as described in Fig. 3. Right panels: histograms representing the mean ± S.E. of CFTR-dependent chloride efflux calculated as reported in Fig. 3. CFTR-dependent chloride efflux in non-transfected cells (A) was significantly higher (p < 0.001) than CFTR-dependent chloride efflux in non-transfected cells (A).

region (Fig. 7), even if we cannot discriminate between apical or sub-apical areas because we have not specifically stained for the apical membrane. To determine that this behavior is not specific for CFBE41o— cells, we overexpressed NHERF1 in another CF airway cell line also having the ΔF508 CFTR mutation: the human tracheal epithelial cell line, CFT1-C2 (25). We observed that these cells also express endogenous NHERF1 and, as observed above in the CFBE41o— cells, targeted NHERF1 overexpression shifted both ΔF508 CFTR and NHERF1 localization from the cytoplasm to the apical plasma membrane region (Fig. 7).

Finally, we observed that overexpression of wt NHERF1 in CFBE41o— cells significantly increased CFTR-dependent chloride efflux across the apical membrane (Fig. 8B), whereas transfection of CFBE41o— cells with any of the truncated/mutated NHERF1 constructs, HRF1A, HRF2A, and ΔERM, or with wt NHERF2 did not alter CFTR-dependent chloride efflux (supplemental Fig. 2S). The CFBE41o— cell monolayers overexpressing wt NHERF1 exhibited a basal chloride efflux that was not significantly different from that of non-transfected CFBE41o— cells treated with only the transfection vehicle, Escort IV (0.018 ± 0.003, n = 6 versus 0.020 ± 0.004, n = 7 Δ(F/F₀)/min in transfected and non-transfected monolayers, respectively, n.s.). FSK plus IBMX treatment (first trace in the typical experiment of Fig. 8A) had no effect on chloride efflux (0.023 ± 0.007 Δ(F/F₀)/min, n = 7) in non-transfected CFBE41o— cells, whereas significantly increasing chloride efflux in wt NHERF1 overexpressing CFBE41o— cell monolayers (0.042 ± 0.005 Δ(F/F₀)/min, n = 6, p < 0.01, first trace in Fig. 8B). Furthermore, glibenclamide addition before and during the next FSK plus IBMX stimulation (second trace in Fig. 8B) inhibited this increase to basal levels (0.019 ± 0.002 Δ(F/F₀)/min, n = 6) and was completely reversible (third trace of Fig. 8B). The bars of the histograms (right panels) represent CFTR-dependent chloride efflux in both non-transfected (0.002 ± 0.002 Δ(F/F₀)/min, n = 7) and transfected (0.022 ± 0.004 Δ(F/F₀)/min, n = 6) CFBE41o— cells, calculated as the difference between the rate of FSK plus IBMX-stimulated chloride efflux before and after apical glibenclamide treatment as described in Fig. 3. Of note, treatment of wt NHERF1 overexpressing CFBE41o— monolayers with CFT1-C2 (172 (5 μM)) gave the same CFTR-dependent chloride efflux measurement as found using glibenclamide (0.020 ± 0.005, Δ(F/F₀)/min, n = 6, n.s.). Similarly, the CFT1-C2 cells transfected with wt NHERF1 significantly increased CFTR-dependent chloride efflux (0.003 ± 0.001, n = 3 versus 0.026 ± 0.002 Δ(F/F₀)/min, n = 3, p < 0.001, in the non-transfected and transfected monolayers, respectively).

These data clearly demonstrate that wt NHERF1 overexpression rescues the functional expression of ΔF508 CFTR protein on the apical membrane of CFBE41o— and CFT1-C2 cells.

DISCUSSION

NHERF1 (EBP50) and NHERF2 (E3KARP) were initially proposed as cofactors necessary for the cAMP-dependent regulation of NHE3 (38), and a growing body of data has demonstrated that both NHERF isoforms regulate a variety of transporters, channels, and receptors by facilitating the formation of multiprotein signaling complexes (39). A large body of studies has recently focused on the role of NHERF in regulating CFTR activity. NHERF interacts with CFTR via its two PDZ domains, although with a higher affinity for the PDZ1 domain than for the PDZ2 domain (18, 32, 35) and, in this way, possibly free the PDZ2 domain to interact with other proteins co-expressed on the same membrane such as ROMK (40) or NHE3 (26, 41). In addition to regulating PKA-dependent CFTR activity, NHERF can also influence apical expression of CFTR (17, 34) and retention of CFTR in the apical membrane (20).

In many but not all epithelial cells, the expression of the two isoforms of NHERF (NHERF1 and NHERF2) is mutually exclusive. For example, in the terminal bronchioles, NHERF1 co-expressed with exrin is the only isoform present (42), whereas in another airway cell model, Calu3,
both isoforms are expressed and compete for CFTR binding (31). Here, we observe that both the NHERF isoforms are present in the normal human bronchial epithelial cell line, 16HBE14o−, although with a different localization: NHERF1 is present close to the plasma membrane, as previously reported in the same cell line (24), whereas NHERF2 is diffusely distributed in the cytoplasm (Fig. 1). Interestingly, in cells derived from a CF individual homozygous for the ΔF508 mutation of CFTR, CFBE41o−, both NHERF isoforms are diffusely distributed in the cytoplasm (Fig. 1).

Importantly, the distribution of CFTR in both 16HBE14o− and CFBE41o− cells, as detected by confocal immunolocalization, paralleled the distribution of NHERF1: in 16HBE14o− polarized cell monolayers, CFTR and NHERF1 are present predominantly at the apical membrane region, whereas in the CF cells, ΔF508 CFTR and NHERF1 are almost absent in the plasma membrane region but are diffusely distributed in the cytoplasm (Fig. 2B). As could be expected from the different distribution of CFTR, polarized 16HBE14o− monolayers displayed a large CFTR-mediated Cl− apical secretion, whereas in polarized CFBE41o− monolayers the CFTR-mediated Cl− secretion was completely absent. Comparable data have been reported in cultured normal and CF airway cells (43).

The similarity in the distribution of NHERF1 and CFTR in 16HBE14o− and CFBE41o− cells directed us to focus on the analysis of the role of NHERF1 in the regulation of CFTR expression and activity. We observed that both PDZ1 and PDZ2 domains of NHERF1 are equally involved in the polarized expression of CFTR and in the regulation of CFTR-dependent chloride efflux. Our data are consistent with the findings demonstrating that PDZ domains direct the polarized apical expression of CFTR (17, 20, 34, 44) and support the role of NHERF1 in forming apical multiprotein complexes that permit the micro-compartamentalization of signal transduction modules promoting efficient regulation of CFTR (18). Deletion of the ERM domain similarly reduced both apical CFTR expression and the PKA-dependent regulation of CFTR, which could be explained by the fact that NHERF1 has been demonstrated to associate with ERM domain with the cytoskeletal adaptor protein, ezrin, which is also a protein kinase A-anchoring protein, and, in this way, NHERF both compartmentalizes PKA in the vicinity of CFTR protein and regulates apical CFTR activity by promoting CFTR phosphorylation (31). On the contrary overexpression of wt NHERF1 in 16HBE14o− cells dramatically increased both the apical CFTR expression and the PKA-dependent regulation of CFTR activity.

All together these findings lead us to hypothesize that, as it has been demonstrated in other polarized cells, in 16HBE14o− cells NHERF1 may regulate CFTR-dependent chloride secretion by influencing either the efficiency of CFTR recycling (20) and/or the stability of CFTR in the plasma membrane via anchoring CFTR to the cytoskeleton (45).

The most important finding of the present report comes from the effect of targeted wt NHERF1 overexpression in confluent monolayers of two CF cell lines, CFBE41o− and CF1-C2. NHERF1 overexpression induced (a) a significant redistribution of CFTR from the cytoplasm to the apical region, (b) an increase of the apical CFTR band observed after apical biotinylation of polarized CFBE41o− cell monolayers, and (c) a PKA-dependent activation of CFTR-dependent chloride secretion across the apical membrane. These data could open an important question as to how ΔF508 CFTR that has escaped from the ER can be redistributed to the apical membrane. This rescue of CFTR-dependent chloride secretion induced by NHERF1 overexpression could be a consequence of various interacting factors: an increase of either ΔF508 CFTR recruitment to the apical membrane and/or its stability on the apical membrane and an increased recycling efficiency of internalized CFTR, as has been described for β2 adrenergic (46, 47) and for κ opioid receptors (48).

It is important to note that, because the total expression levels of ΔF508 CFTR in CFBE41o− cells lysates (the sum of the immature band and the thin mature band) was not significantly different from the total CFTR (mature plus immature bands) in the 16HBE14o− cells lysates (75.31 ± 11.02 versus 82.04 ± 10.16 optical density units in CFBE41o− and 16HBE14o− cells, respectively, n = 7, n.s.), the rescue of the CFTR activity in CFBE41o− cells to a level similar to that observed in the non-transfected 16HBE14o− cells not overexpressing NHERF1, could be due to a redistribution of ΔF508 CFTR.

A possible mechanism for this NHERF1-dependent redistribution of ΔF508 CFTR to the apical membrane may come from the Guggino laboratory (13) where they suggested that NHERF favors surface expression by competing with CAL for CFTR binding. CAL, a CFTR interacting PDZ domain protein that associates mainly with the Golgi apparatus, reduces CFTR expression from the plasma membrane and down-regulates mature CFTR enhancing its degradation in the post-Golgi compartments (49). Therefore, these authors suggested that the regulation of CFTR in the plasma membrane involves a dynamic interaction between these two PDZ domain proteins, NHERF and CAL (13). It is possible that in CFBE41o− cells, which have a lower NHERF1 expression level than in the normal 16HBE14o− cells (Fig. 2A), some ΔF508 CFTR that has escaped from the ER is retained within the cytoplasm, while the overexpression of NHERF1 shifts the stoichiometry from CAL to interact with CFTR and thus stimulate the NHERF1-CFTR complex to move to the plasma membrane.

In conclusion, our data suggest that the overexpression of NHERF1 may favor the rescue of ΔF508 CFTR activity in CF affected cells even if the precise molecular and cellular mechanisms remain to be clarified. Several strategies have been shown to overcome the trafficking defects of ΔF508 CFTR: (a) reduced temperature (50, 51); (b) stabilization of the protein by chemical chaperones (52); or (c) disruption of the interaction between ΔF508 CFTR and molecular chaperones such as calnexin and the heat shock proteins that retain CFTR in the ER (53). The finding that overexpression of NHERF1 can stimulate Cl− secretion across human airway cells homozygous for the ΔF508 CF mutation is consistent with the hypothesis that some ΔF508 CFTR is capable of escaping the degradative pathway and being expressed at the apical membrane in human bronchial epithelial cells. Importantly, because the ΔF508 CFTR mutant is not a simple trafficking mutant but exhibits multiple defects in stability and activation, the elucidation of the cellular mechanisms that permit NHERF1 to recruit ΔF508 CFTR to the membrane and rescue its activity could result in more specific therapeutic strategies.

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