Cystic Fibrosis Transmembrane Conductance Regulator Activation Is Reduced in the Small Intestine of Na\(^+\)/H\(^+\) Exchanger 3 Regulatory Factor 1 (NHERF-1)- but Not NHERF-2-deficient Mice*

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Binding of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel to the Na\(^+\)/H\(^+\) exchanger 3 regulatory factor 1 (NHERF-1) and NHERF-2 scaffolding proteins has been shown to affect its localization and activation. We have for the first time studied the physiological role of these proteins in CFTR regulation in native tissue by determining CFTR-dependent chloride current in NHERF-1- and NHERF-2-deficient mice. The cAMP- and cGMP-activated chloride current and the basal chloride current in basolaterally permeabilized jejum were reduced by ∼30% in NHERF-1-deficient mice but not in NHERF-2-deficient mice. The duodenal bicarbonate secretion was affected in a similar way, whereas no significant differences in CFTR activity were observed in ileum. CFTR abundance as determined by Western blotting was unaltered in jejunal epithelial cells and brush border membranes of NHERF-1 and NHERF-2 mutant mice. However, semi-quantitative detection of CFTR by confocal microscopy showed that the level of apically localized CFTR in jejunal crypts was reduced by ∼35% in NHERF-1-deficient and NHERF-1/2 double deficient mice but not in NHERF-2 null mice. Together our results indicate that NHERF-1 is required for full activation of CFTR in murine duodenal and jejunal mucosa and that NHERF-1 affects the local distribution of CFTR in or near the plasma membrane.

These studies provide the first evidence in native intestinal epithelium that NHERF-1 but not NHERF-2 is involved in the formation of CFTR-containing functional complexes that serve to position CFTR in the crypt apical membrane and/or to optimize its function as a cAMP- and cGMP-regulated anion channel.

Cystic fibrosis, the most common inherited lethal disorder among Caucasians, is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, resulting in abnormal electrolyte transport in epithelial tissues, most notably in airways, pancreas, and intestine (1). CFTR is the major cAMP- and cGMP-activated anion transporter in various epithelia including intestinal crypt cells, where it is essential for electrolyte and fluid secretion, as is apparent from the occurrence of life-threatening intestinal obstruction in ∼10% of newborn cystic fibrosis patients. On the other hand, overstimulation of CFTR by bacterial enterotoxins causes secretory diarrhea, leading to the death of millions of children per year in the developing world (2).

The C terminus of CFTR can bind to several PDZ domain-containing proteins (3–5). PDZ domains are protein-protein interaction modules that bind to short stretches of amino acids, usually at the extreme C terminus of their target proteins. Most PDZ domain proteins contain more than one PDZ domain or other protein-interacting modules, thereby facilitating the formation of macromolecular complexes, which is important for targeting, trafficking, and multi-protein complex formation of their binding partners.

Several studies have indicated that binding of CFTR to members of the NHERF family of PDZ domain proteins may be important for regulation of activity and for maintaining correct

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§ The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; BBMV, brush border membrane vehicles; \(I_{sc}\), short circuit current; NHE3, Na\(^+\)/H\(^+\) exchanger 3; PDZ, Postsynaptic Density 95/Drosophila Disks large/Zonula Occludens I binding domain; NHERF, NHE3 regulatory factor; PDZK1, PDZ domain protein kidney 1; WT, wild type.
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localization in the apical membrane where the NHERF proteins as well as CFTR are localized. Three of the four members of the NHERF family of PDZ proteins present in the intestinal brush border are known to bind to CFTR (5, 6). NHERF-1 (also named NHERF and EBP50) and NHERF-2 (also named E3KARP, SIP-1, and TKA-1) share a structural homology; they contain tandem PDZ domains and a C-terminal ezrin-radixin-moesin-binding domain, which anchors the proteins to the actin cytoskeleton. PDZK1 (also known as CAP70, NaPi-CAP1, and more recently as NHERF-3) contains four PDZ domains but no ezrin-radixin-moesin-binding domain (7–9).

The multiple (potential) roles of the CFTR C terminus in the regulation of its function have recently been reviewed and are still controversial (10). These roles include: 1) increasing cAMP-dependent activation of CFTR by positioning the cAMP-dependent protein kinase in the vicinity of CFTR where it can efficiently phosphorylate its substrate (11, 12). This corresponds to the mechanism whereby NHERF-1 and NHERF-2 facilitate formation of a NHE3/NHERF/ezrin/cAMP-dependent protein kinase complex, which is required for inhibition of NHE3 (Na+/H+ exchanger 3) (9, 13, 14). 2) Coupling of CFTR to the cytoskeleton (via NHERF-1 or NHERF-2 binding to ezrin), resulting in a strong reduction in the lateral mobility of CFTR in the membrane (15, 16). 3) NHERF-1-or PDZK1-induced CFTR dimerization by binding of two CFTR molecules to either NHERF-1 or PDZDK1, which allosterically enhances the open probability of the channel (17, 18). 4) Targeting of CFTR to the apical membrane and/or its retention in the membrane was shown to require PDZ domain interactions in several studies (19–21); however, various highly similar studies failed to show this effect (12, 22); 5) Linking CFTR to various NHERF binding partners (which include many channels, transporters, and G protein-coupled receptors) may contribute to temporal and spatial specificity and efficiency of signal transduction.

Whether CFTR-NHERF interactions are exploited as a mechanism for CFTR regulation in native tissue has not been studied yet. All of the previous studies were performed using cell culture systems expressing different amounts and members of the NHERF family proteins (endogenously or using overexpressing systems) or recombinant proteins (e.g. C-terminally truncated CFTR or CFTR fragments), which may have contributed to the different outcomes of similar experiments.

To further investigate the role of the NHERF proteins as regulators of CFTR in a physiological context and to study the functions of the isoforms separately, we have studied CFTR regulation in native intestinal epithelia from mice lacking NHERF-1, NHERF-2, or both. We demonstrate that NHERF-1, but not NHERF-2, is essential for full activation of transepithelial chloride and bicarbonate secretion by cAMP and cGMP and that the immunostaining intensity of CFTR in the jejunal crypts of NHERF-1 deficient mice is reduced.

EXPERIMENTAL PROCEDURES

Animals—Using a previously described retroviral trapping method (23), Lexicon Genetics generated NHERF-2-deficient mice using Omnibank clone OST2298. The retroviral gene trap cassette pVICTR22, containing an optimal splice acceptor sequence that causes mis-splicing of the targeted gene and puromycin resistance as a selectable marker was found to be inserted 157 bp 3’ of the second exon (see Fig. 1A). The mice were genotyped by two-allele three-primer PCR, using genomic DNA isolated from tail clips; primers 1 (5’-TTCTATAAGCCTCATTCTCTCTCTC-3’) and 3 (5’-CCCACCCTCCCCTGCTGTC-3’) were used for detection of the wild type (WT) allele. Primer 2 (5’-GC GCCAGTCTCCGATTGA-3’) is complementary to a sequence in the 5’ long terminal repeat of the retroviral insert and serves to amplify the mutant allele in combination with primer 1. PCR of tail clips was performed using 0.4 μM of each primer, 1 mM MgCl2, 0.2 mM dNTPs (Promega), 0.3 units of RedTaq DNA polymerase (Sigma), and the supplied buffer. The sizes of the PCR products were 303 bp for the wild type and 231 bp for the mutant allele (see Fig. 1C).

NHERF-1-deficient mice were originally generated at the Duke University Medical Center and have been described before (24). NHERF-1 and NHERF-2 mice were backcrossed for at least five generations into the FVB/n genetic background. Furthermore, NHERF-1-deficient mice were crossed with NHERF-2-deficient mice and with mice carrying a targeted disruption in the CFTR gene described previously (also from the FVB genetic background) (25). In contrast to previous reports (24), the viability and fertility of female NHERF-1 null mice was not different from wild type, probably because of breeding into the FVB/n genetic background.

The mice were housed in the animal facilities of the Erasmus Medical Centre of Rotterdam and the Medical School of Hanover under standardized light and climate conditions and *ad libitum* access to water and chow. All of the animal experiments were approved by the Dutch Animal Welfare Committee and followed the protocols at the Hanover Medical School and the local authorities for the regulation of animal welfare (Regierungspräsidium). Both male and female animals were used for studies at 12–20 weeks of age. The mice were anesthetized with ketamine and xylazine (100 and 20 mg/kg respectively, intraperitoneally) and euthanized by cervical dislocation after removal of tissue.

Bioelectric Measurements—Short circuit currents (*Isc*) of muscle-striped ileal and jejunal sheets mounted in Ussing chambers were measured as described previously (26). Transepithelial PD was clamped at 0 mV, and *Isc* was measured using a DVC-1000 voltage/current clamp (World Precision Instruments), digitized by a Digidata 1320, and analyzed using Axoscope 9.2 software (Axon Instruments). After stabilization of the base-line current or the previous response the following agents were added: 8-Br-cGMP (serosal, 200 μM), forskolin (serosal, 10 μM), and d-glucose (mucosal, 10 mM). To determine whether the observed changes in the NHERF-1-deficient mice (see “Results”) were the direct consequence of reduced CFTR activity or whether NHERF-1 deficiency affects activity of basolateral ion transporters and/or the driving force for chloride secretion, the basolateral membrane was permeabilized to monovalent ions using nystatin during the *Isc* measurements (27). After stabilization of the base-line current, an inward transepithelial chloride gradient was generated by iso-osmotic replacement of chloride in the serosal bath by mannitol. The ionophore nystatin was added at the serosal side at 0.36 g/ml.
Forskolin (serosal, 10 μM) and glucose (mucosal, 10 mM) were subsequently added after stabilization of the current.

**Bicarbonate Secretion**—Isolated proximal duodenum was stripped of external serosal and muscle layers. Duodenal mucosa was mounted in a gas-lifted Ussing chamber and maintained at 37 °C. The mucosal side was bathed with unbuffered bicarbonate-free Ringer’s solution, and the serosal side was bathed with buffered gassed Ringer’s solution (pH 7.4) containing 22 mM HCO$_3$⁻. Under control of a pH-stat system (PHM290, pH-Stat Controller, Radiometer Copenhagen), the luminal pH was maintained at 7.4 by continuous infusion of 1 mM HCl. The amount infused/time unit was used to quantitate HCO$_3$⁻ secretion (μmol/cm$^2$·h$^{-1}$). After measuring basal secretion for 30 min, forskolin (10 μM) or 8-Br-cGMP (200 μM) was added at the serosal side, and changes in bicarbonate secretion were determined during a 40-min period.

**Isolation of Intestinal Epithelial Cells and Brush Border Membrane Vesicles (BBMVs)**—Mechanical high frequency vibration was used to isolate epithelial cells from mouse jejunum in isotonic buffer as described before (28). BBMVs were isolated from epithelial cell homogenates by differential magnesium precipitation (29). Epithelial tissue and BBMVs were resuspended in SDS-PAGE loading buffer containing protease inhibitors (Complete; Roche Applied Science) and homogenized by brief sonication on ice. The samples were separated by SDS-PAGE and transferred to Protean nitrocellulose membrane (Amersham Biosciences). The membranes were blocked with 2.5% nonfat dried milk for 1 h at room temperature and incubated overnight with primary antibody and washing, the membranes were blocked by the peptide (0.5 mM; data not shown). After incubation with primary antibody and washing, the membranes were incubated with secondary antibody. Horseradish peroxidase-conjugated donkey anti-rabbit IgG-horseradish peroxidase (Amersham Biosciences; dilution, 1:15000). An antibody against PDZK1, and serum was affinity-purified on peptide-bound agarose beads. The signal on Western blots was completely analyzed using the RC DC protein assay kit (Bio-Rad).

**Western Blot Analysis**—For quantitative analysis of the relevant proteins in epithelial cells and BBMVs, samples from at least two mice were pooled. Crude extracts from other tissues were prepared by snap-freezing and homogenization in Læmmli sample buffer (containing Complete protease inhibitor (Roche)) and homogenized by brief sonication on ice. The samples were separated by SDS-PAGE and transferred to Protein nitrocellulose membrane (Schleicher & Schuell Bioscience Hybond-P) or polyvinylidene difluoride membrane (Amersham Biosciences). The membranes were blocked with 2.5% nonfat dried milk for 1 h at room temperature and incubated overnight with primary antibody at 4 °C. The primary antibodies used were anti-CFTR antibody R3195 (dilution 1:1000) (30), anti-NHERF-1 antibody (dilution 1:3000) (31), anti-NHERF-2 antibody R2570 (dilution 1:1000) (32), and anti-actin antibody MAB1501 (Chemicon; dilution, 1:15000). An antibody against PDZK1 was generated in rabbit against a peptide (amino acids 339–354 of mouse PDZK1), and serum was affinity-purified on peptide-bound agarose beads. The signal on Western blots was completely blocked by the peptide (0.5 mM; data not shown). After incubation with primary antibody and washing, the membranes were incubated with secondary antibody. Horseradish peroxidase-conjugated donkey anti-rabbit IgG-horseradish peroxidase (Amersham Biosciences; dilution, 1:3000) was employed for detection of all primary antibodies except anti-actin, which was detected using horseradish peroxidase-conjugated goat anti-mouse IgG (BIOSOURCE International; dilution, 1:5000). The secondary antibody was detected by chemiluminescence (Supersignal West Pico substrate; Pierce) by exposing to light sensitive imaging film (Biomax MR film; Kodak). The imaged bands were quantified using a calibrated GS-800 densitometer (Bio-Rad) and analyzed using Quantity-one software (version 4.2.1; Bio-Rad).

**Immunohistochemistry and Confocal Microscopy**—Formalin-fixed, paraffin-embedded tissue sections (5 μm) from mice of different genotypes were prepared on the same slide. After deparaffinization with xylene and treatment with 0.01 M sodium citrate solution, endogenous peroxidase activity was blocked with 0.6% H$_2$O$_2$ and 0.12% sodium azide. Sections were incubated with anti-CFTR (R3195, 1:100) or anti-NHERF-1 (1:500) antibodies in phosphate-buffered saline with 2% bovine albumin for 1.5 h at room temperature. The sections stained with monoclonal anti-villin antibody (Santa Cruz; SC-58897; dilution, 1:50) were incubated overnight. The slides were subsequently incubated for 2 h with fluorescein isothiocyanate-labeled anti-rabbit secondary antibody and/or Cy3-labeled anti-mouse IgG (1:100, both secondary antibodies were from Jackson Immunoresearch Laboratory, West Grove, PA) and mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA). Immunofluorescence micrographs were captured using a Zeiss LSM510 confocal microscope equipped with a 25 milliwatt argon laser. Fluorescence emission after excitation (488 nm for CFTR, 543 nm for villin) was detected using 10×/0.3 or 40×/1.3 numerical aperture oil immersion lenses, a dichroic beam splitter reflecting 488-nm excitation light, and a 505–530 bandpass emission filter (560–615 nm for villin). The images were scanned using a 75-μm pinhole. Initial analysis of images was performed double-blind by at least two investigators. For (semi)quantification of signal from jejunal crypts, images from longitudinal sections at Z-steps of 0.5 μm were analyzed by determining total background-subtracted fluorescent signal using KS400 Zeiss software. The same threshold was utilized for all images from one slide. Three individual crypts from three age- and sex-matched mice of each genotype were analyzed.

**Data Analysis**—The data are the means ± S.E. Statistical significance was established using Student’s unpaired two-tailed t test.

**RESULTS**

**Generation and Characterization of NHERF-2-deficient Mice**—NHERF-2-deficient mice were generated as described under “Experimental Procedures.” Insertion of the retroviral cassette resulted in a complete absence of NHERF-2 protein in all tissues studied (kidney, lung, stomach, heart, brain, and colon; a representative example of a Western blot is shown in Fig. 1B). NHERF-2 null mice developed normally and were born at Mendelian ratios. They showed no obvious alterations in gross morphology, body size, and fertility. No abnormal behavior or signs of distress were apparent. Histological analysis of various tissues (lung, kidney, jejunum, ileum and colon, and nasal and tracheal epithelium) displayed comparable morphology in wild type and NHERF-2-deficient mice (data not shown).

**cAMP- and cGMP-dependent Activation of CFTR-mediated Ion Transport in NHERF-1- and NHERF-2-deficient Mice**—Several studies using cultured cells have indicated an important role for the NHERF proteins in the activation of CFTR (10). By using mice deficient in NHERF-1, NHERF-2, or both, we were able to analyze native epithelia ex vivo, avoiding potential arti-
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**FIGURE 1.** Disruption of the NHERF-2 gene and characterization of the NHERF-2-deficient mice. A, schematic representation of the integration of retroviral construct VICTR22 between exons 2 and 3 of the NHERF-2 gene. LTR, long terminal repeat. B, Western blot analysis of NHERF-2 in different tissues: heart (15 μg/lane), kidney (15 μg/lane), and lung (2.5 μg/lane). NHERF-2 is absent in tissues from the NHERF-2-deficient (N2 −/−) mice and reduced in heterozygote (N2 +/−) mice. Actin was used as loading control (10 μg/lane). C, genotyping of NHERF-2-deficient mice. PCR products generated by amplification of genomic DNA from WT, heterozygous (N2 +/−), and NHERF-2 null mice (N2 −/−). The location of primers used for the genotyping PCR is schematically shown in A.

facts introduced by using overexpression systems or recombinant proteins. First, cAMP- and cGMP-activated CFTR transepithelial short circuit currents (Isc) were measured in mice of the different genotypes. As shown in Fig. 2A (panel 1), the response to the adenyl cyclase activator forskolin was significantly reduced in jejunum of NHERF-1-deficient mice (34% reduction, p < 0.05), whereas the response in NHERF-2-deficient mice was not altered. The response to forskolin in NHERF-1/2 double deficient mice was reduced by 37% (p < 0.05) compared with wild type. Thus, additional absence of NHERF-2 did not result in a stronger effect or different characteristics of transepithelial current in response to cAMP, suggesting that an absence of NHERF-1 was not compensated by NHERF-2.

We subsequently examined whether cGMP-dependent regulation of CFTR was affected similarly. As shown in a previous study, cGMP-dependent inhibition of another intestinal ion transporter in the apical membrane, NHE3, is selective in its requirement for the NHERF proteins in comparison with cAMP-dependent inhibition. Whereas NHERF-1 and NHERF-2 were interchangeable as mediators of cAMP-mediated inhibition of NHE3, only NHERF-2 could act as a cofactor for cGMP-mediated inhibition (33). In contrast, our results show that CFTR activation by cAMP and cGMP in the jejunum was reduced to a similar extent in NHERF-1-deficient mice (Fig. 2). Stimulation of Isc by the membrane-permeable analog 8-Br-cGMP was significantly decreased (31%, p < 0.05) in NHERF-1-deficient mice as compared with wild type, whereas no change was observed in NHERF-2-deficient mice. Again, the phenotype of NHERF-1/2 null mice was similar to that of NHERF-1-deficient mice (32% reduction in Isc compared with NHERF-2 deficient mice, p < 0.05).

To compare the relative functional quality of the various sheets of muscle-stripped epithelia, stimulation of the short circuit current by glucose (monitoring sodium transport mediated by the Na+/glucose cotransporter SGLT1) was determined as a measure for CFTR-independent electrogentic transport. The response to glucose was not significantly different in mice lack-
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Nystatin Forskolin

FIGURE 3. *Ex vivo* CFTR-mediated short circuit current ($I_{sc}$) measurement in jejunal epithelium during basolateral membrane permeabilization by nystatin in WT, NHERF-1-deficient (N1−/−), and CFTR null (CFTR−/−) mice. The data represent the average values from at least three mice per genotype and are expressed as the means ± S.E. The CFTR-dependent chloride current prior to forskolin stimulation was reduced in NHERF-1-deficient mice, whereas the response to stimulation by forskolin was not altered in NHERF-1 null mice. *, p < 0.05 versus wild type; **, p < 0.05 versus N1−/−; ns, not significant.

Reduced CFTR Activation in NHERF-1-deficient Mice—It is estimated that 50% of bicarbonate secretion in mouse duodenum is directly mediated by CFTR (8, 35, 36). The role of NHERF-1 and NHERF-2 in the regulation of bicarbonate secretion was determined by measuring stimulation of the HCO$_3${sup}−/sup/secretory rate ($J_{HCO_3^-}$) by forskolin and 8-Br-cGMP in duodenal epithelium of control and NHERF-1- and NHERF-2-deficient mice. Forskolin- and cGMP-stimulated bicarbonate secretion in NHERF-1-deficient mice was reduced by 35 and 30%, respectively, in NHERF-1-deficient mice (p < 0.05), whereas no difference was detected in NHERF-2-deficient mice.

Expression and Localization of CFTR in the Intestine of NHERF-1- and NHERF-2-deficient Mice—CFTR expression and localization in mice lacking NHERF-1, NHERF-2, or both were studied in isolated jejunal enterocytes and BBMVs. No...
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Western blot analysis of CFTR expression in jejunal epithelium and immunocytochemical detection of CFTR in jejunal crypts from WT, NHERF-1-deficient (N1−/−), NHERF-2-deficient (N2−/−), and NHERF-1/2-deficient (N1/2−/−) mice. A, Western blot analysis of CFTR in jejunal epithelial cell extracts (20 μg/lane) and BBMVs from jejunum (5 μg/lane) displayed no significant difference in the amounts of CFTR protein among mice of the different genotypes. The positions of band B (immature CFTR) and band C (mature, complex glycosylated CFTR) are indicated. Actin was used as a loading control. Representative Western blots from at least five independently isolated samples are shown at the left, and the average amounts of CFTR levels relative to wild type mice are shown in the right panel. The data represent relative signal intensity of at least eight Western blots. The error bars indicate the S.E. B, confocal microscopic analysis of CFTR abundance and localization in jejunal crypts of wild type, NHERF-1-deficient, NHERF-2-deficient, NHERF-1/2-deficient, and CFTR-deficient (CFTR−/−) mice. CFTR-dependent fluorescence was significantly (p < 0.05) reduced in NHERF-1- and NHERF-1/2-deficient mice. No signal was detected in sections from CFTR-deficient mice, demonstrating the high specificity of the antibody. C, quantitative analysis of confocal microscope datasets of three sex- and age-matched couples of wild type, NHERF-1−/−, NHERF-2−/−, and NHERF-1/2−/−deficient mice. Three jejunal crypts from each section were analyzed, and the total and average intensities from all Z-stacks with detectable CFTR signal were calculated for each individual crypt. The average intensity was significantly lower in NHERF-1- and NHERF-1/2-null mice compared with wild type (left panel). Likewise, the total intensity per crypt was reduced in NHERF-1 and NHERF-1/2 but not in NHERF-2-deficient mice (right panel). *, p < 0.05 versus wild type. D, CFTR and villin abundance in jejunal crypts was studied by confocal microscopy in wild type (left three images) and NHERF-1-deficient mice (right three images). CFTR (shown in green) colocalization with villin (shown in red) in the apical border is unaltered in NHERF-1 null mice compared with wild type control.

FIGURE 5. Western blot analysis of CFTR expression in jejunal epithelium and immunocytochemical detection of CFTR in jejunal crypts from WT, NHERF-1-deficient (N1−/−), NHERF-2-deficient (N2−/−), and NHERF-1/2-deficient (N1/2−/−) mice. A, Western blot analysis of CFTR in jejunal epithelial cell extracts (20 μg/lane) and BBMVs from jejunum (5 μg/lane) displayed no significant difference in the amounts of CFTR protein among mice of the different genotypes. The positions of band B (immature CFTR) and band C (mature, complex glycosylated CFTR) are indicated. Actin was used as a loading control. Representative Western blots from at least five independently isolated samples are shown at the left, and the average amounts of CFTR levels relative to wild type mice are shown in the right panel. The data represent relative signal intensity of at least eight Western blots. The error bars indicate the S.E., B, confocal microscopic analysis of CFTR abundance and localization in jejunal crypts of wild type, NHERF-1-deficient, NHERF-2-deficient, NHERF-1/2-deficient, and CFTR-deficient (CFTR−/−) mice. CFTR-dependent fluorescence was significantly (p < 0.05) reduced in NHERF-1- and NHERF-1/2-deficient mice. No signal was detected in sections from CFTR-deficient mice, demonstrating the high specificity of the antibody. C, quantitative analysis of confocal microscope datasets of three sex- and age-matched couples of wild type, NHERF-1−/−, NHERF-2−/−, and NHERF-1/2−/−deficient mice. Three jejunal crypts from each section were analyzed, and the total and average intensities from all Z-stacks with detectable CFTR signal were calculated for each individual crypt. The average intensity was significantly lower in NHERF-1- and NHERF-1/2-null mice compared with wild type (left panel). Likewise, the total intensity per crypt was reduced in NHERF-1 and NHERF-1/2 but not in NHERF-2-deficient mice (right panel). *, p < 0.05 versus wild type. D, CFTR and villin abundance in jejunal crypts was studied by confocal microscopy in wild type (left three images) and NHERF-1-deficient mice (right three images). CFTR (shown in green) colocalization with villin (shown in red) in the apical border is unaltered in NHERF-1 null mice compared with wild type control.

Significant difference was observed in the level of CFTR protein in mice from the various genotypes (Fig. 5A). The maturation of CFTR also appeared to be unaffected in NHERF-1- and NHERF-2-deficient mice, because there was no difference in the ratio of mature, complex-glycosylated (band C) and immature, core-glycosylated CFTR (band B) in epithelial cell extracts. Furthermore, staining of an SDS-PAGE gel loaded with total jejunal epithelial cell homogenate or BBMVs by Coomassie Brilliant Blue did not display obvious differences in the overall protein composition of enterocytes of NHERF-1 or NHERF-2 mice (data not shown). We subsequently analyzed the immunostaining pattern of CFTR in jejunal crypts of the NHERF-1−/−, NHERF-2−/−, and NHERF-1/2-deficient mice by confocal microscopy. Importantly, CFTR staining remained confined to the apical border in all mice examined, and no evidence was found for redistribution to a perinuclear area or the basolateral membrane (Fig. 5B). However, the intensity of CFTR staining at or near the apical membrane was reduced in NHERF-1−/− and NHERF-1/2 double deficient mice, but not in NHERF-2−/−null mice. The absence of staining in CFTR null mice proved that the antibody is specific for CFTR. (Semi)quantitative image analysis in crypts from NHERF-1−/− and NHERF-1/2−/−null mice showed the reduction in both mean and total fluorescence intensity in the surface area in which signal was detected was ∼35% (p < 0.05; Fig. 5C). Double staining of CFTR and the microvillus marker villin was performed to more accurately determine whether the subcellular localization of CFTR was altered in NHERF-1−/−null mice. Colocalization with villin confirmed that CFTR was confined mostly to the microvilli both in WT and NHERF-1−/−null mice (Fig. 5D). Image analysis of sections from five age- and sex-matched couples from WT and NHERF-1−/−mutant mice showed no evidence for a relocalization of...
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The exploitation of NHERF-1- and NHERF-2-deficient and NHERF-1/2 double-mutant mice in this study has enabled us to investigate, for the first time, the role of these PDZ domain proteins in CFTR expression and regulation in native small intestinal epithelium. Because most previous studies used NHERF overexpression systems and because the level of endogenous expression of the different NHERF proteins varies widely among different cell lines, little is known about the relative contribution of each individual CFTR-binding PDZ protein to CFTR regulation in native epithelia. In addition to NHERF-1 and NHERF-2, PDZK1 and the single PDZ domain proteins CAL (CFTR-associated ligand) and Shank2 are known binding partners of CFTR and candidate PDZ proteins binding to CFTR in vivo in enterocytes (37). Each of these PDZ domain proteins may affect CFTR function in different ways in epithelial cells from different tissues (18, 37, 38).

Our study demonstrated clearly that NHERF-1 is required for full activation of the CFTR-dependent chloride current by cAMP- and cGMP-linked agonists. Forskolin-activated duodenal bicarbonate secretion, CFTR-dependent short circuit cur-
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rent ($I_v$) in jejunum, and basically active chloride current in nystatin-permeabilized jejunum were all significantly reduced in NHERF-1 null mice. In contrast, ablation of NHERF-2 had no effect on bicarbonate secretion and short circuit current, and NHERF-1 deficiency did not affect the CFTR-dependent short circuit current in the ileum. The normal response to glucose in the NHERF-1-deficient mice (Fig. 2, A and B, panels 3) showed that the altered response to forskolin and 8-pCPT-cGMP is CFTR-specific and that no general changes in ion transport capacity have occurred.

The observed reduction (approximately 30%) in cAMP- and cGMP-stimulated transepithelial current in jejunum of NHERF-1-deficient mice could be caused either by a functional loss of CFTR in the apical membrane or by a direct or indirect effect of NHERF-1 deficiency on the electrochemical driving force for chloride exit across the apical membrane. This was further investigated by measuring macroscopic currents through apical CFTR channels at a fixed electrochemical gradient for chloride in short circuit measurements of jejunal tissue permeabilized by nystatin. Basolateral permeabilization was found to induce, or unmask, a basically active pool of CFTR in wild type mice that amounted to ~70% of the total activity in the presence of forskolin (Fig. 3). This implies that a significant fraction of the total CFTR pool is already in the open state, a situation similar to that observed in the sweat gland and nasal epithelium (39, 40). Furthermore, it suggests that under non-stimulated conditions the basolateral chloride entry in the cell limits the tranacellular chloride current. Surprisingly, the basically active pool, but not the cAMP-activated fraction of CFTR was strongly reduced (~40%) in nystatin-permeabilized jejunum from NHERF-1-deficient mice. Because cAMP activation of chloride current under nystatin conditions more directly reflects the ability of cAMP to activate CFTR channels in the apical membrane than activation under nonpermeabilized conditions, the outcome of these experiments argues against a major role of NHERF-1 in positioning cAMP-dependent protein kinase in the vicinity of CFTR and improving CFTR phosphorylation and activation. This differs from the functional role of NHERF-1 and NHERF-2 in the regulation of NHE3 in PS120 and OK cell lines and the role of NHERF-1 in the kidney brush border. Both NHERF-1 and NHERF-2 are able to mediate cAMP-dependent inhibition of NHE3 by facilitating formation of a NHERF-NHE3-ezrin complex. Ezrin then acts as AKAP (cAMP-dependent protein kinase anchoring protein) and tethers cAMP-dependent protein kinase near its phosphorylation target (13, 14, 41).

The role of NHERF-1 and NHERF-2 in the cGMP-dependent regulation of CFTR has not been described previously. Our results indicate that absence of NHERF-1 affected cGMP-dependent activation of CFTR to a similar extent as cAMP-dependent activation in jejunal epithelial cells (Fig. 2). NHERF-2 ablation on the other hand did not affect CFTR stimulation by cGMP. This is a second line of evidence suggesting that the mechanism of regulation by the NHERF proteins differs between CFTR and NHE3, because cGMP-dependent inhibition of NHE3 in PS120 and OK cell lines requires NHERF-2 but not NHERF-1 (33).

The major controversial issue concerning the function of the NHERF proteins as CFTR regulators is their possible involvement in apical targeting or retention. Our results demonstrate that the total amount of CFTR present in the brush borders of epithelial cells as measured by Western blotting was unaltered, arguing against a key role of NHERF-1 and NHERF-2 in targeting CFTR to the apical membrane in native intestinal tissue. The ratio of CFTR band B to band C was also unaltered, suggesting that CFTR maturation and glycosylation were not grossly affected by NHERF-1 ablation. Furthermore, confocal microscopy showed no evidence for an alteration in the localization of CFTR in jejunal crypts of NHERF-1−/− mice, but it should be added that not all CFTR present in the cell is detected by this technique; in general, even the best CFTR antibodies fail to detect CFTR in the endoplasmic reticulum and Golgi, although it is known to be present there as well (42). Based on analysis of CFTR abundance in jejunal crypts by confocal microscopy, we estimate that the mean fluorescence intensity and the total level of fluorescence per crypt were reduced by ~35% in NHERF-1- and NHERF-1/2-deficient mice. This differs from the result obtained by Western blotting. To account for both seemingly paradoxical results, we assume that not the amount but the distribution of CFTR in the apical membrane and/or the subapical compartment is altered in NHERF-1 null mice. CFTR is known to be continuously endocytosed and recycled back to the plasma membrane, and interaction with PDZ proteins has been shown to increase endocytic recycling in MDCK cells (21, 43). NHERF-1 deficiency could therefore lead to a redistribution of CFTR between apical and subapical compartments, possibly resulting in a more diffuse pattern and reduced local fluorescence levels that may drop below the detection limit. Attempts to reduce this limit by increasing the pinhole size caused overexposure in areas where the fluorescence was the most intense. Unfortunately, the resolution of the microscope was not sufficient to clearly distinguish between CFTR localized in the apical membrane and in submembrane vesicles.

Various recent investigations have shown that C-terminally mutated CFTR has a higher mobility in the membrane, as measured by fluorescence recovery after photobleaching of GFP-labeled CFTR and by single particle tracking of individual CFTR molecules labeled with quantum dots (15, 16, 44). The lateral mobility of CFTR was also increased by overexpression of dominant-negative NHERF-1 lacking the ezrin-binding domain, suggesting that coupling of CFTR to the cytoskeleton limits its mobility (15). Importantly, a reduction in the amount of ezrin and phospho-ezrin was reported in the brush border membrane of kidney and intestinal homogenates from a different line of NHERF-1 null mice (45). Uncoupling of CFTR from the cytoskeleton and an altered migration pattern could potentially explain relocalization and a more diffuse distribution.

Alternatively, it may be that the changes in CFTR immunostaining and activity reflect a loss of NHERF-1-driven CFTR dimerization. Possibly, CFTR dimers are more readily detected by immunostaining (because of the higher local density of the signal), and the formation of dimers by the action of NHERF-1 (or PDZK1) is known to increase the open probability of the CFTR channel. Future CFTR cross-linking studies on native intestinal epithelium may help to further evaluate this model.
Whereas our data indicate that NHERF-1 is an important factor for regulation of CFTR activity in jejunal epithelial cells, we could not identify a similar role for NHERF-2. This may be indicative for a functional difference between these sister proteins or, alternatively, might be explained by the low levels of NHERF-2 protein in murine small intestinal epithelium. Unexpectedly, we failed to detect NHERF-2 in jejunal BBMVs by Western blot despite previous claims of NHERF-2 protein expression in rabbit small intestine and more recently also in the small intestine of CD1 mice (46, 47). Although we did observe a prominent band in our NHERF-2 blots of jejunal BBMVs isolated from FVB and CD1 mice, it was attributed to a cross-reactivity of the antibody with abundantly expressed NHERF-1, based on its high molecular weight and its detection in BBMVs from NHERF-2-deficient but not NHERF-1-deficient mice (data not shown).

The reduced PDZK1 abundance that we observed in NHERF-1-deficient mice (Fig. 7) suggests that NHERF-1 may be important for the stabilization of PDZK1. In contrast, NHERF-1 RNA and protein abundance were not altered in the jejunum of PDZK1-deficient mice (48). Most plausibly, NHERF-1 and PDZK1 form a complex in vivo, in line with the observation that the proteins can form heterodimers in vitro (49). The notion that expression and/or stability of PDZK1 and NHERF-1 are interconnected is supported by the observation that the NHERF-1 abundance in proximal kidney tubules of PDZK1-deficient mice was increased by ~50%, but only when mice were kept on a high phosphate diet (50). This might be a compensatory mechanism to counteract the reduced Npt2a abundance in the membrane, which has been observed in both NHERF-1- and PDZK1-deficient mice (24, 50). Our data indicate that PDZK1 and NHERF-1 function also overlaps in the duodenum, where the bicarbonate secretion was recently shown to be reduced (by approximately 20%) in the PDZK1-deficient mice (48). In contrast, the jejunal forskolin-activated anion secretion was not affected in PDZK1-deficient mice compared with wild type controls (48), suggesting that in this segment PDZK1 is not required for normal CFTR function or that NHERF-1 is able to fully compensate for PDZK1 loss. Because a total absence of PDZK1 does not affect $I_{sc}$, we conclude that the reduction in CFTR-dependent activation of anion transport in the NHERF-1 null mice (Fig. 2) is the direct consequence of NHERF-1 ablation and not secondary to the lowered PDZK1 abundance in these mice.

In summary, we have shown that NHERF-1 is important for full activity of CFTR in native duodenal and jejunal epithelial tissue as well as in nystatin-permeabilized epithelium. Whereas NHERF-1 is not required for targeting CFTR to the apical membrane (as evidenced by normal CFTR levels in the brush borders), it is responsible for placing or keeping more active CFTR in the apical membrane, most likely by affecting the local distribution of CFTR and/or by linking CFTR to the actin cytoskeleton. Our observations provide new insight into the role of NHERF-1 in the formation of CFTR-containing functional complexes in the apical membrane of jejunal enterocytes and in the pathophysiology of cystic fibrosis in patients expressing C-terminally truncated CFTR.

Interestingly, it was reported recently that small molecules fitting in the binding pocket of PDZ domains can be used to disrupt interactions with peptide ligands. By using a newly developed reversible inhibitor, a reduction of CFTR activation by CPT-cAMP in bronchial airway cells was achieved (51). In line with our finding that CFTR interaction with NHERF-1 is important for its full activation in native epithelium, this suggests that a novel class of anti-diarrheal drugs could potentially be developed that targets interactions of NHERF-1 with its binding partners. This would possibly lead not only to reduced chloride secretion by CFTR but also to enhanced salt absorption by NHE3.

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