NHERF2/NHERF3 Protein Heterodimerization and Macrocomplex Formation Are Required for the Inhibition of NHE3 Activity by Carbachol*

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Background: Previous characterization of NHERF dimerizations is inconsistent and any physiological function is largely unknown.

Results: NHERF2/NHERF3 heterodimerization is the strongest NHERF dimerization and is necessary for carbachol inhibition of NHE3.

Conclusion: NHERF2/NHERF3 heterodimerization brings PDZ domains together to assemble macrocomplexes for NHE3 regulation.

Significance: Macrocomplexes mediated by NHERF dimerizations could increase the complicity of their regulation of NHE3 and other proteins.

NHERF1, NHERF2, and NHERF3 belong to the NHERF (Na+/H+ exchanger regulatory factor) family of PSD-95/Discs-large/ZO-1 (PDZ) scaffolding proteins. Individually, each NHERF protein has been shown to be involved in the regulation of multiple receptors or transporters including Na+/H+ exchanger 3 (NHE3). Although NHERF dimerizations have been reported, results have been inconsistent, and the physiological function of NHERF dimerizations is still unknown. The current study semiquantitatively compared the interaction strength among all possible homodimerizations and heterodimerizations of these three NHERF proteins by pulldown and co-immunoprecipitation assays. Both methods showed that NHERF2 and NHERF3 heterodimerize as the strongest interaction among all NHERF dimerizations. In vivo NHERF2/NHERF3 heterodimerization was confirmed by FRET and FRAP (fluorescence recovery after photobleach). NHERF2/NHERF3 heterodimerization is mediated by PDZ domains of NHERF2 and the C-terminal PDZ domain recognition motif of NHERF3. The NHERF3-4A mutant is defective in heterodimerization with NHERF2 and does not support the inhibition of NHE3 by carbachol. This suggests a role for NHERF2/NHERF3 heterodimerization in the regulation of NHE3 activity. In addition, both PDZ domains of NHERF2 could be simultaneously occupied by NHERF3 and another ligand such as NHE3, α-actinin-4, and PKCα, promoting formation of NHE3 macrocomplexes. This study suggests that NHERF2/NHERF3 heterodimerization mediates the formation of NHE3 macrocomplexes, which are required for the inhibition of NHE3 activity by carbachol.

NHERF3 (Na+/H+ exchanger regulatory factor) proteins are a family of PDZ domain-containing scaffold proteins, with four members NHERF1, NHERF2 (also called E3KARP), NHERF3 (also called PDZK1), and NHERF4 (also called IKEPP) (1–3). NHERF1, NHERF2, and NHERF3 are primarily localized to the brush border (2, 4, 5). The NHERF family was named because of its functions in the regulation of NHE3, which is a Na+/H+ antiporter expressed in the brush border of kidney proximal tubules and the gastrointestinal system. NHE3 accounts for the majority of renal and intestinal Na+ and water absorption (2, 6, 7). The contribution of each NHERF protein to the regulation of NHE3 has been extensively studied (8–13).

NHERF proteins are not only involved in the regulation of NHE3 but also affect many other transporters, including CFTR (cystic fibrosis transmembrane conductance regulator), DRA (down-regulated in adenoma), OCTN2 (organic cation/carnitine transporter 2), PETP1, and PEPT2 (H+/dipeptide transporter), Na/Pi-II (sodium-dependent phosphate transporter), and MRP4 (multidrug resistance protein 4) (3, 11, 14). NHERF proteins also regulate multiple other signaling proteins and receptors, including G protein-coupled receptors such as PTH1R (parathyroid hormone receptor), β2AR (β2 adrenergic receptor), LPA2R, and LPA5R (lysophosphatidic acid receptors) (15, 16).

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§ The abbreviations used are: NHERF, Na+/H+ exchanger regulatory factor; NHE3, Na+/H+ exchanger 3; PDZ, PSD-95/Discs-large/ZO-1; CFTR, cystic fibrosis transmembrane conductance regulator; MBP, maltose-binding protein; ROI, regions of interest; BCECF-AM, 2′,7′-bis(2-carboxyethyl)-4(6)-carboxyfluorescein-acetoxymethyl ester; IP, immunoprecipitation; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; FRAP, fluorescence recovery after photobleach; GPI, glycosylphosphatidylinositol.
As scaffolding proteins, NHERF proteins not only function in anchoring these transmembrane proteins on the surface of the plasma membrane but also use their PDZ domains to assemble a variety of membrane-associated proteins, receptors, or effector proteins into signaling macrocomplexes through which functions of these proteins are modulated (3, 11, 15, 16). For example, NHERF1 conjugates CFTR and β2AR (β2-adrenergic receptor) into a macrocomplex, allowing stimulation of CFTR by β2AR agonist (17); NHERF2 conjugates NHE3 and SGK1 (serum- and glucocorticoid-inducible kinase 1) into a macrocomplex, allowing stimulation of NHE3 by glucocorticoids (18); NHERF3 couples CFTR and MRP4 (multidrug resistance protein 4) into a macrocomplex, allowing CFTR activity to be compartmentally inhibited (19).

According to such models, NHERF dimerizations, especially heterodimerizations, would be expected to bring more PDZ domains together to assemble more complicated signaling macrocomplexes, allowing functions that could not be conferred by individual NHERF proteins. Until now, the effect of NHERF dimerizations on the regulation of NHE3 or any other proteins has not been reported. Of note, both NHERF2 and NHERF3 have been shown to be necessary for the inhibition of NHE3 by elevated intracellular Ca\(^{2+}\). Intracellular Ca\(^{2+}\) elevated by several agonists, including carbachol and serotonin, inhibits small intestine electroneutral NaCl absorption, which is mainly mediated by NHE3 (1, 20–22). The inhibition of NHE3 activity by carbachol, UTP, and calcium ionophore 4-Br-A23187 was lost in the small intestine and colon of NHERF2 knock-out mice (12, 13). Knock-out of NHERF3 abolished the inhibition of NHE3 activity by calcium ionophore in the colon as well (10). These in vivo results were fully recapitulated in Caco-2 cells as carbachol inhibition of NHE3 activity was lost when either NHERF2 (23) or NHERF3 (24) were knocked down with shRNA. This led us to hypothesize that NHERF2 and NHERF3 heterodimerize and that this heterodimerization is required for calcium inhibition of NHE3 activity.

Previous studies have suggested all possible homodimerizations and heterodimerizations of NHERF proteins. However, different methods have resulted in contradictory conclusions. By overlay, co-immunoprecipitation (co-IP), and cross-linking assays, NHERF1 homodimerization was demonstrated (25). By overlay, pulldown, and co-IP assays, NHERF2 homodimerization and NHERF1/NHERF2 heterodimerization also occur (26). By cross-linking and co-IP assays, NHERF3 homodimerization is mediated by the PDZ3 domain (27). In contrast, NHERF1 did not dimerize based on gel-filtration analysis (28), and NHERF3 did not significantly dimerize when assessed by analytical ultracentrifugation (29). In addition, a yeast two-hybrid study suggested NHERF1/NHERF3 and NHERF2/NHERF3 heterodimerization (30). To better understand the physiological importance of NHERF dimerizations, the current study compared the relative interaction strength of all possible NHERF dimerizations by the same method. The interaction domains between NHERF2 and NHERF3 were further characterized, and the role of NHERF2/NHERF3 heterodimerization in the inhibition of NHE3 activity by elevated Ca\(^{2+}\) was explored.

**EXPERIMENTAL PROCEDURES**

**Materials, Plasmids, Antibodies**—Glutathione-Sepharose 4B resin was from GE Healthcare. Amylose resin and rabbit anti-MBP were from New England Biolabs, Ipswich, MA. Glutathione, maltose, and carbachol were from Sigma. BCECF-AM, nigericin, and Hoechst 33342 were from Invitrogen. Ca\(^{2+}\) ionophore 4-Br-A23187 was from Biomol (Plymouth Meeting, PA). Mouse anti-FLAG, anti-FLAG M2 magnetic beads, mouse anti-GAPDH, and mouse anti-actin were from Sigma. Rabbit anti-NHERF2 was a gift from Dr. Chris Yun (31). Rabbit anti-NHERF3 from Sigma was used for Western blotting. Rabbit anti-mCherry was from BD Biosciences. Rabbit anti-GFP was from Invitrogen. Mouse anti-HA was from Covance, Inc. (Princeton, NJ). Mouse anti-GST was from Cell Signaling Technology, Inc. (Danvers, MA). IRdye-700- or IRdye-800-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies were from Rockland Immunocchemicals Inc. (Gilbertsville, PA) and were used with LI-COR (Lincoln, NE) Odyssey system for Western blot analysis. Alexa fluor-488 or -568 conjugated goat anti-mouse or anti-rabbit secondary antibodies and Alexa fluo-568 conjugated phalloidin were from Invitrogen.

Plasmid pcDNA3.1-HA-NHE3 was constructed previously (32). pmCherry-NHERF1, pmCherry-NHERF2, pFLAG-NHERF1, and pFLAG-NHERF2 were constructed previously and encode rabbit NHERF1 or human NHERF2 (33). pFLAG-NHERF3 was constructed by inserting rat NHERF3 into p3XFLAG-CMV-10 (Sigma) between HindIII and BamHI. NHERF3-4A mutant was made by PCR to change the four C-terminal amino acid residues into alanines and inserted into p3XFLAG-CMV-10 to generate pFLAG-NHERF3-4A. CFP-NHERF2 and YFP-GPI were constructed as reported (34). pCFP-NHERF3, pYFP-NHERF3, and pYFP-NHERF3-4A were generated by inserting NHERF3 between HindIII and BamHI into pmCerulean-C1 or pmVenus-C1. Rat NHERF3-P2C (E1111-M523) mutant was generated by deleting PDZ1 domain and cloned into p3XFLAG-CMV-10.

**Cell Lines, Cell Culture, and Transfection**—Chinese hamster lung fibroblasts PS120 cells were used to generate HA-NHE3 stably expressing cells by transient transfection of pcDNA3.1-HA-NHE3 and G418 selection. Stable PS120 cell lines expressing FLAG-NHERF proteins were generated similarly. Opossum kidney proximal tubular OK cells were cultured in plastic dishes and transfected at 90% confluency for co-immunoprecipitation or seeded on glass-slides and transfected on the second day post confluence for immunostaining. Transient transfection was performed with 0.4 μg of each plasmid using 0.8 μl of Lipofectamine 2000 (Invitrogen) per cm\(^2\) of growth surface. Then cells were grown in complete medium and used for experiments 48 h later. The human colon cancer Caco-2/bbe cell line was grown on collagen-coated Transwell filter membranes (EMD Millipore, Darmstadt, Germany) as previously described (23). Caco-2/NHERF3-KD cells were established previously by infection of Caco-2/bbe cells with lentivirus-shRNA-puromycin (24).

**Co-IP**—Cell lysates were prepared with the lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM Na\(_3\)VO\(_4\) 0.5% Triton X-100, and protease inhibitors). Anti-FLAG M2 magnetic beads were washed with this buffer three times. 0.5 mg of each cell lysate were mixed with 5 μl of anti-FLAG M2 magnetic beads and incubated at 4 °C for 3 h on a rotating shaker. Beads were washed with the same lysis buffer 4 times.
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and eluted with 1.5× Laemmli sample buffer without β-mercaptoethanol. The input and elution samples were analyzed by SDS-PAGE and Western blotting.

Recombinant Protein Expression and Purification—DNAs encoding maltose-binding protein (MBP) or glutathione S-transferase (GST) were amplified with PCR from pMAL-c2x (New England Biolabs) and pGEX-4T-1 (GE Healthcare) and inserted into pET28a (EMD Millipore) between Ndel and BamHI to make pMBP and pGST, respectively. Then rabbit NHERF1, human NHERF2, rat NHERF3, NHERF3-4A, and NHERF3-P2C mutant were inserted into pMBP or pGST to construct pMBP-NHERFs or pGST-NHERFs. Previously, pmEOS2-NHERF2-PDZ1/2-GAGA was constructed encoding a NHERF2 mutant, of which GYGF binding motifs in both PDZ domains were mutated into GAGA (33). This mutant and the single PDZ domain mutants of NHERF2 were subcloned into pGST. DNA fragments encoding the C-terminus of human NHERF3 (Lys-458–Met-519) were generated by PCR and inserted into pMBP for expressing MBP-hNHERF3C.

Expression constructs were transformed into BL21 (DE3) strain (EMD Millipore). When the bacterial culture reached an optical density of ~0.8, protein expression was induced with 0.3 mM isopropyl 1-thio-β-d-galactopyranoside at 37 °C for 3 h. MBP-tagged proteins were purified with a column packed with amylose resin by gravity flow following the New England Biolabs manuals of the pMAL system. GST-tagged protein was purified in a gravity-flow column following the instructions from GE Healthcare. Purified proteins were concentrated by Amicon Ultra-15 Centrifugal filter units (EMD Millipore) supplemented with 10% glycerol and 10 mM DTT and stored at −80 °C.

GSH Resin or Amylose Resin Pulldown—For binary interaction studies, one nmol of recombinant GST-tagged or MBP-tagged protein were used as bait for pulldown assays. 1 nmol of purified recombinant proteins or 1 mg of cell lysates was used as prey as indicated. For competition experiments using the human NHERF3 C-terminus, three nmol of MBP-hNHERF3C was preincubated with bait for 30 min, and then prey was added. For the complex assembly experiment shown in Fig. 8, 1 nmol GST- or MBP-fused NHERF2 was first mixed with cell lysates for 60 min, and then 3 nmol of bait proteins were added. The volume of final mixture was adjusted to 500 µl with the lysis buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 0.5% Triton X-100, and protease inhibitors). GSH resin (glutathione-Sepharose 4B resin) or amylose resin was washed with lysis buffer three times. Each bait-prey mixture was mixed with 10 µl of resin and incubated at 4 °C for 3 h on a rotating shaker. Resin was washed with the same lysis buffer 4 times. GSH resin was eluted with lysis buffer supplemented with 10 mM glutathione, whereas amylose resin was eluted with 10 mM maltose buffer. The input and elution samples were analyzed by SDS-PAGE and Western blotting.

 Förster Resonance Energy Transfer (FRET)—The acceptor photobleaching method of FRET was performed on a heated stage (37 °C) of a Zeiss LSM 510 confocal microscope equipped with a C-Apochromat 63×/1.2 Korr water-immersion objective, as described previously (34). OK cells grown in glass-bottom dishes were transiently transfected with CFP-NHERF2 and/or YFP-NHERF3 and used 48 h later. YFP-GPI was used as a negative control. Regions of interest (ROI) were selected and bleached with the 514-nm laser line at maximum intensity. The images were collected with excitation wavelengths of 458 nm for CFP and 514 nm for YFP, respectively. Emission wavelength was BP 465–510 nm for CFP and 520–555 nm for YFP. The apparent FRET efficiency, E, was calculated by the equation, 

\[ E = \frac{I_{D, post} - I_{D, pre}}{I_{D, post} \times (1 - I_{A, post}/I_{A, pre})}, \]

where \( I_{D, pre} \) and \( I_{D, post} \) are the donor CFP fluorescence intensities before and after the acceptor photobleaching, and \( I_{A, pre} \) and \( I_{A, post} \) are the acceptor YFP fluorescence intensities before and after the acceptor photobleaching (34, 35). Background fluorescence was subtracted to calculate the FRET efficiency. An unbleached ROI was used to correct for the bleaching during acquisition.

Sensitized emission FRET was used for comparing the FRET between NHERF2 and NHERF3 before and after the treatment with calcium ionophore 4-Br-A23187. Three channels of images, CFP signal (Ex-CPF/Em-CPF), YFP signal (Ex-YFP/Em-YFP), and FRET signal (Ex-CPF/Em-YFP) were collected, and FRET efficiency was calculated similarly as described by others (35). The same microscopic setting was used to collect images for each sample and its corresponding two control cells, which were transfected with CFP-NHERF2 alone or YFP-NHERF3 alone. Donor bleed-through (a) was calculated as the ratio of FRET signal to CFP signal using the cells transfected with CFP-NHERF2 alone. Acceptor bleed-through (b) was calculated as the ratio of FRET signal to YFP signal using the cells transfected with YFP-NHERF3 alone. For the cells co-expressing CFP-NHERF2 and YFP-NHERF3, net FRET signal was calculated as net FRET = (FRET signal) − a(CFP signal) − b(YFP signal), and the apparent FRET efficiency, E, was calculated as equation 

\[ E = \frac{\text{net FRET}}{\text{YFP}}. \]

1 µM 4-Br-A23187 was diluted in warmed-up medium and added into the dish to make a final concentration of 0.5 µM, and FRET experiments were performed 5 min later.

Fluorescence Recovery after Photobleach (FRAP)—FRAP was performed on a stage heated to 37 °C of a Zeiss LSM 510 confocal microscope equipped with a C-Apochromat 63×/1.2 Korr water-immersion objective, as described previously (33). OK cells grown in glass-bottom dishes were transiently transfected with mEOS2-NHERF3 and/or mCherry-NHERF2 and used for FRAP 48 h later. Optical slices were focused on the cell apical domain with the slice thickness of 3 µm to better tolerate cell movement in the vertical direction. A square of 3 µm width was used as the ROI. Fluorescence within the ROI was measured at low laser power before the bleach and then photobleached with high laser power. Recovery was followed with low laser power at 5- or 10-s intervals usually up to 3–5 min until the intensity had reached a steady plateau. Laser and filter settings were the same as described previously (33). Fluorescence of an ROI was also measured without bleaching with a high power laser and was used to correct for the bleaching effect caused during measurement. The recovery ratios were calculated as the percentage of maximal bleached fluorescence.

Adenoviral HA-NHE3, FLAG-NHERF2, and FLAG-NHERF3 Preparation and Infection—Adenovirus encoding HA-NHE3 was constructed previously (23). It was expanded in HEK293T cells and purified as previously described. Both FLAG-tagged
human NHERF2, rat NHERF3, and NHERF3-4A mutant were cloned into the shuttle vector pADLOX.HTM under control of a cytomegalovirus (CMV) promoter (36). Virus was generated by co-transfection of CRE8 cells with pADLOX.HTM-FLAG-NHERF3 and δ/β viral DNA using Lipofectamine 2000. Lysates of CRE8 cells containing packaged adenovirus were used to infect HEK293T cells to expand the virus. Adenovirus was purified by CsCl gradient ultracentrifugation (23). Viral particle numbers were calculated as \( (A_{260} \text{value}) \times (1.1 \times 10^{12}) \). For adenovirus infection, Caco-2/bbe cells were grown on Transwell filters for 12 days and treated with 6 mm EGTA in serum-free Caco-2 medium for 3 h at 37 °C. HA-NHE3 and FLAG-NHERF3 viral particles were diluted to 2 \( \times 10^{10} \) and 0.5 \( \times 10^{10} \) particles/ml, respectively, in serum-free Caco-2 medium and incubated with the cells for 6 h at 37 °C. Finally the medium was changed to serum-containing medium. Cells were used 48 h after infection.

**Generation of Stably Expressing Caco-2 Cell Lines by Lentivirus**—FLAG-tagged rat NHERF3 and NHERF3-P2C mutants were cloned into pCDH-MCS-IRES-neo (System Biosciences Inc., Mountain View, CA). Virus was packaged in HEK293T cells by co-transfection with pMD2.G and psPAX2. Medium were collected, filtered, and used to infect Caco-2-NHERF3-KD cells. 1200 μg/ml G418 was used to select for the cells in which virus were integrated.

**Immunofluorescence**—OK cells were grown on glass slides and transiently co-transfected with pCFP-NHERF3, pFLAG-NHERF3 or pFLAG-NHERF3-4A as indicated on the second day post confluency. Caco-2 cells were infected with adenovirus as above. Cells were washed with PBS 3 times, fixed with 4% paraformaldehyde for 10 min, neutralized with 20 mM glycine for 5 min, and permeabilized and blocked in PBS containing 10% goat-silver plus 0.05% saponin for 30 min. Rabbit anti-GFP (1:200), mouse anti-FLAG (1:200), or mouse anti-NHERF3(1:200, 1E9 from Sigma) were incubated for 2 h at room temperature in blocking solution. Cells were then washed 3 times with 1% BSA-PBS containing 0.05% saponin and incubated with Alexa fluor-488 or -568-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:200) or Alexa fluor-568 conjugated phalloidin (1:200). Hoescht 33342 for 1 h at room temperature. Finally cells were washed 3 times with PBS, mounted with Gel Mount (Sigma), and imaged with a Zeiss LSM 510 confocal microscope.

**Measurement of Na\(^{+}/\)H\(^{+}\) Exchange Activity**—Na\(^{+}/\)H\(^{+}\) exchange activity in Caco-2 cells was determined with the intracellular pH-sensitive fluorescent dye BCECF-AM, as described previously (23), using a Quantamaster fluorometer from Photon Technology International, Inc. (Birmingham, NJ). Monolayers of polarized Caco-2/bbe cells were grown on poly-carbonate membranes (0.4 μm pore size) glued to plastic coverslips for 12 days and infected with Adeno-HA-NHE3. Cells were used for activity measurement at 2 days after adenovirus infection. Cells were serum-starved 4 h before the assay and mounted in a cuvette and perfused with solutions both apically and basolaterally. 50 μM HOE-694 was used to inhibit the activities of NHE1 and NHE2. For carbachol treatment, cells were basolaterally perfused with tetramethylammonium solution supplemented with 25 μM carbachol for 5 min before perfusion of Na\(^{+}\) medium. To calibrate the relationship between intracellular pH and the dual excitation ratio of BCECF (excitation at 490 nm and 440 nm, emission at 535 nm), the K\(^{+}/\)nigericin method was used. The initial rate at pH 6.2 was calculated as previously reported (23).

**Statistical Analyses**—Results are presented as the means ± S.E. Comparisons were performed by unpaired Student’s t tests.

**RESULTS**

**NHERF2 and NHERF3 Form a Complex Demonstrated by Co-IP**—The expression patterns of NHERF proteins were compared by Western blot analysis among three cell lines, PS120, OK, and Caco-2 (Fig. 1A). NHERF1 was expressed in all cell lines, but the expression level was very low in PS120 cells. Only Caco-2 cells express detectable NHERF2, as shown previously (18). NHERF3 was expressed in both OK and Caco-2 cells, but not in PS120 cells. Because of the lack of efficient NHERF2 and NHERF3 antibodies for IP, FLAG-tagged NHERF1, NHERF2, and NHERF3 were individually stably overexpressed in PS120 cells, and IP was performed using anti-FLAG M2 magnetic beads. FLAG-NHERF1 and FLAG-NHERF3 were successfully immunoprecipitated from the cell lysate mixture of Caco-2 cells and PS120 cells stably expressing FLAG-NHERF1 or FLAG-NHERF3 (Fig. 1B). However, endogenous NHERF2 expressed in Caco-2 cells was only co-immunoprecipitated with FLAG-NHERF3 but not with FLAG-NHERF1 (Fig. 1B). When lysates of PS120 cells stably expressing FLAG-NHERF1 or FLAG-NHERF2 were mixed with either OK or Caco-2 cell lysates, endogenous NHERF3 from both OK and Caco-2 cells was co-immunoprecipitated with both FLAG-NHERF1 and FLAG-NHERF2 (Fig. 1C). The amount of NHERF3 co-immunoprecipitated with NHERF1 was very low for OK cells. For both OK and Caco-2 lysates, more NHERF3 was co-immunoprecipitated with NHERF2 than with NHERF1, suggesting NHERF2 and NHERF3 form a relative tighter complex.

Instead of mixing cell lysates, OK cells were transiently co-transfected with pFLAG-NHERFs and pmCherry-NHERFs and then subjected to co-IP to test the formation of complexes between NHERF proteins. FLAG-NHERF1, FLAG-NHERF2, and FLAG-NHERF3 were expressed in similar levels and immunoprecipitated with similar efficiency (Fig. 1D). A significant amount of mCherry-NHERF1 was co-immunoprecipitated with FLAG-NHERF3 but not with NHERF1 or NHERF2 (Fig. 1D). Similarly, mCherry-NHERF2 was only significantly co-immunoprecipitated with FLAG-NHERF3 (Fig. 1E).

**NHERF2/NHERF3 Heterodimerization Is Strongest among all Dimerizations of NHERF Proteins**—To test whether the complex formed between NHERF2 and NHERF3 is due to direct interactions, *in vitro* pulldown assays were performed with bacterially expressed recombinant NHERF proteins. NHERF proteins were fused to either MBP or GST tags. As described under “Experimental Procedures,” MBP-NHERF1, MBP-NHERF2 and MBP-NHERF3 were individually mixed with GST-NHERF proteins and GST in equal molar ratios with a final concentration of 2 μM. Then GST and GST-NHERF proteins were pulled down with GSH resin. The purity of GST-NHERF proteins was shown by the Ponceau S staining in the MBP-NHERF3 experiment (Fig. 2C). GST was used as a nega-
tive control for nonspecific binding. MBP-NHERF1 was only pulled down by GST-NHERF3 and not significantly by GST-NHERF1 and GST-NHERF2 (Fig. 2A). Similarly, MBP-NHERF3 was only abundantly pulled down by GST-NHERF3 (Fig. 2B). MBP-NHERF3 was pulled down by both GST-NHERF1 and GST-NHERF2 but not by GST-NHERF3 (Fig. 2C). These results suggest direct interactions between NHERF2 and NHERF3. Among all possible homodimerizations and heterodimerizations of NHERF proteins, only NHERF1/NHERF3 and NHERF2/NHERF3 heterodimerizations were strong enough to be detected by co-IP or pulldown assays. GST-NHERF2 pulled down more MBP-NHERF3 than did GST-NHERF1. Please note that rat NHERF3 was used for this experiment. Therefore, NHERF3 from all three species (human, rat, opossum) has a stronger interaction with NHERF2 than with NHERF1.

NHERF2/NHERF3 Heterodimerization Is Mediated by PDZ Domains of NHERF2 and the C-terminal PDZ Domain Recognition Motif of NHERF3—The C-terminal four amino acid residues of human NHERF2 (FSNF), human NHERF3 (DTEM), or rat NHERF3 (DTVM) are all potential PDZ domain recognition motifs. NHERF2/NHERF3 heterodimerization could be mediated either by binding of the NHERF2 C terminus to NHERF3 PDZ domains suggested by one study (30) or by binding of the...
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The contribution of the C terminus of NHERF3 to NHERF2/NHERF3 heterodimerization was further examined. Mutant NHERF3-4A was made by substituting the C-terminal four amino acid residues with alanines (Fig. 3C). Consistent with a previous NHERF1/NHERF3 heterodimerization study (27), NHERF3-4A lost interaction with NHERF1 (Fig. 4B). Similarly, NHERF3-4A also lost interaction with NHERF2. This suggests that the last four amino acid residues of NHERF3 are required for NHERF2/NHERF3 heterodimerization.

The C-terminal 60 amino acid residues of human NHERF3 were fused to a MBP tag to generate MBP-hNHERF3C (Fig. 3C). This C terminus of NHERF3 was pulled down by both GST-NHERF1 and GST-NHERF2 (Fig. 4C). When MBP-hNHERF3C was mixed with Caco-2 cell lysate for the co-IP assay, it occupied FLAG-NHERF2, and less endogenous Caco-2 NHERF3 was co-immunoprecipitated by FLAG-NHERF2 (Fig. 4D). This result suggests that the C terminus of NHERF3 is sufficient to bind to NHERF2 and competes with full-length NHERF3.

NHERF2 Also Heterodimerizes with NHERF3 in Vivo as Shown by FRET and FRAP—To examine whether NHERF2 and NHERF3 also interact with each other in live cells, the FRET efficiency between CFP-NHERF2 and YFP-NHERF3 was examined in OK cells. Similar to fluorescent protein mEOS2 fused NHERF2 and NHERF3 studied previously (33), both CFP-NHERF2 and YFP-NHERF3 localized to the apical microvilli in OK cells (Fig. 5A). The acceptor photobleaching FRET method was used. The YFP-NHERF3 signal in a single microvillus cluster was bleached with high laser power, and the change of both CFP-NHERF2 and YFP-NHERF3 was measured immediately after the bleaching. From the intensity measured before and after photobleaching, the average FRET efficiency between CFP-NHERF2 and YFP-NHERF3 was calculated to be 6 ± 1%. YFP-GPI is a fusion protein also localized to the microvilli in OK cells and was used as a control (34). There was no FRET between CFP-NHERF2 and YFP-GPI (Fig. 5B).

Previously, all NHERF proteins have been shown to be highly mobile in the microvilli in OK cells, with NHERF2 having the slowest mobility rate (33). Similar to a previous report (33), mEOS2-NHERF3 had a higher mobility rate than mCherry-NHERF2 when each was transfected alone into OK cells (Fig. 5C). When mEOS2-NHERF3 and mCherry-NHERF2 were co-transfected, the mobility rate of mEOS2-NHERF3 was decreased to the same rate as mCherry-NHERF2 (Fig. 5C). Because OK cells have undetectable levels of endogenous NHERF2 by Western blot (Fig. 1A), decreasing of the mobility rate of mEOS2-NHERF3 by overexpression of mCherry-NHERF2 indicates that NHERF2 and NHERF3 interact with each other in live cells.

NHERF3-4A Mutant Still Localizes to the Apical Microvilli but Does Not Bind to NHERF2—Both NHERF1 and NHERF2 bind to ezrin, which links to the actin cytoskeleton (2). How NHERF3 is targeted to the apical microvilli is still not clear. The C terminus of NHERF3 has been hypothesized to contribute to the anchoring of NHERF3 to the plasma membrane in human placental choriocarcinoma JEG-3 cells (38) but not in murine hepatocytes (29). Therefore, the localization of mutant FLAG-NHERF3-4A was investigated in OK cells by immunostaining and compared with wild-type FLAG-NHERF3. CFP-NHERF3
In the Caco-2 cell model, NHERF2/NHERF3 were both shown to be required for the inhibition of NHE3 activity by carbachol (Fig. 6E). This again suggests that the C-terminal PDZ recognition motif of NHERF3 is required for the heterodimerization between NHERF2 and NHERF3.

NHERF2/NHERF3 Heterodimerization Mediates the Assembly of NHE3 Macrocomplexes — The signaling pathway through which carbachol inhibits NHE3 is still not clear in polarized epithelial cells. In fibroblasts, inhibition of NHE3 by elevated Ca\(^{2+}\) induced with calcium ionophore requires at least two NHERF2 ligands, PKC\(\alpha\) and \(\alpha\)-actinin-4 (39, 40). There are probably more partners involved in the whole process. It was hypothesized that NHERF2/NHERF3 heterodimerization could mediate the assembly of signaling macrocomplexes for NHE3 regulation, as each NHERF protein could bring different ligands together through their PDZ domains (41, 42).

NHE3 directly binds to the PDZ2 domain plus its C-terminal extension of NHERF2 (31) and the PDZ1 domain of NHERF3 (30). To understand the possible NHE3 macrocomplexes mediated by NHERF2/NHERF3 heterodimerization, the PDZ domain binding preferences were further examined. NHERF2 mutants were used to determine the PDZ domains to which NHERF3 and NHE3 bind. By substituting the GYGF motif of PDZ domains with GAGA, single PDZ domain mutants NHERF2-PDZ1/GAGA, NHERF2-PDZ2/GAGA, and double PDZ domains mutant NHERF2-PDZ1/2/GAGA were made (Fig. 3B). The binding ability of mutated PDZ domains to most of their ligands was abolished in these mutants (43, 44). As expected, neither NHE3 nor NHERF3 was pulled down by dou-
erable PDZ domains mutant NHERF2-PDZ1/2-GAGA (Fig. 7A). Both PDZ1 and PDZ2 single domain mutants bound less NHERF3 compared with wild-type NHERF2, suggesting PDZ1 and PDZ2 are both involved in the binding of NHERF3. NHERF3 slightly prefers binding to PDZ1 domain as the PDZ1 mutant bound a little less than the PDZ2 mutant. On the other hand, the PDZ2 mutant but not the PDZ1 mutant pulled down less NHE3 compared with wild-type NHERF2 (Fig. 7A), suggesting that NHE3 prefers binding to PDZ2 domain of NHERF2.

The NHERF3-P2C mutant (from the beginning of the PDZ2 domain to the C terminus) was generated by removing the PDZ1 domain (Fig. 3C). This mutant lost its NHE3 binding (Fig. 7B). This is consistent with a previous yeast two-hybrid analysis showing that NHE3 interacts with the PDZ1 domain of NHERF3 (30).

Depending on which NHERF protein interacts with NHE3, two NHE3 macrocomplexes could be formed through NHERF2/NHERF3 heterodimerization (Fig. 8, A and B). In a NHE3-NHERF3-NHERF2 macrocomplex, NHE3 binds to the PDZ1 domain of NHERF3, and NHERF3 could occupy either PDZ domain of NHERF2, leaving all other PDZ domains available for ligand binding. In a NHERF3-NHERF2-NHE3 macrocomplex, PDZ1 and PDZ2 domains of NHERF2 are occupied by NHERF3 and NHE3, respectively, and all four PDZ domains of NHERF3 are available for ligand binding. Please note that these two models are based on the assumption that the two PDZ domains of NHERF2 can bind their ligands simultaneously.

These two models were examined by assembling macrocomplexes in vitro. MBP-hNHERF3C (C-terminal 60 amino acid residues of NHERF3) does not have PDZ domains and did not pull down NHE3. However, it did pull down NHE3 in the presence of GST-NHERF2 (Fig. 8, C and D). As a control, MBP did not pull down NHE3 in the presence of GST-NHERF2. This suggests that NHERF2 serves as a bridge to conjugate NHERF3 and NHE3 into a NHERF3-NHERF2-NHE3 macrocomplex (Fig. 8B). PKCα and α-actinin-4 are ligands for the PDZ1 and PDZ2 domains of NHERF2, respectively (39, 40). GST-NHERF3 pulls down α-actinin-4 and PKCα in the presence of MBP-NHERF2 but not in the absence of MBP-NHERF2 (Fig. 8, E and F). This suggests that NHERF2 can bind its ligands when either PDZ domain is occupied by NHERF3 in a NHERF3-NHERF2-NHERF3 macrocomplex (Fig. 8A).

**PDZ Domains Other Than PDZ1 of NHERF3 Also Contributes to the Carbachol Inhibition of NHE3 Activity**—According to results in Fig. 7B and previous reports (24, 30), PDZ1 of NHERF3 is most likely involved in anchoring NHE3 on the apical plasma membrane. It is still not known whether other PDZ domains of NHERF3 are involved in the regulation of NHERF3. This was tested with the FLAG-tagged NHERF3-P2C mutant in which the PDZ1 domain was deleted. Lentiviruses were generated to express FLAG-tagged rat NHERF3 and the NHERF3-P2C mutant in Caco-2-NHERF3-KD cells. The stably expressing cell population was selected by G418 resistance. Flag-tagged NHERF3 expression was analyzed by Western blot using both anti-NHERF3 and anti-FLAG antibodies (Fig. 9A). The rabbit anti-NHERF3 from Sigma recognizes the epitope in PDZ1 domain and does not recognize the NHERF3-P2C mutant. Immunostaining with anti-FLAG antibody showed that ~90% of the cells stably expressed FLAG-tagged NHERF3 (Fig. 9B). Flag-tagged rat NHERF3 successfully rescued the NHERF3 knock down by restoring both basal NHE3 activity and the inhibition by carbachol (Fig. 9C). In wild-type Caco-2 cells, carbachol inhibits 39 ± 6% of NHE3 activity. In Caco-2-NHERF3-KD cells, lentiviral expression of FLAG-rat NHERF3 confers 44 ± 8% of inhibition. The NHERF3-P2C
mutant did not restore the basal NHE3 activity but did confer 42 ± 6% inhibition of NHE3 activity by carbachol. The effects on basal NHE3 activity support that the PDZ1 domain of NHERF3 anchors a pool of functionally active NHE3 on the apical membrane. On the other hand, this also suggests that the other PDZ domains of NHERF3 contribute to the carbachol inhibition of NHE3, which is not anchored by NHERF3.

**DISCUSSION**

This study resolved a contradiction in the literature as to whether NHERF proteins dimerize in general, demonstrating...
that NHERF2-NHERF3 specifically heterodimerizes. Moreover, a role for that heterodimerization was demonstrated by its involvement in forming NHE3 regulatory macrocomplexes (large multiprotein complexes) that are involved in both the microvillar localization of NHE3 and its inhibition by carbachol. Furthermore, our results indicate that these effects involve the C-terminal PDZ domain recognition motif of NHERF3 via binding to the NHERF2 PDZ domains but also separately involve PDZ1 and the other three NHERF3 PDZ domains, with the latter presumably bringing specific ligands into the NHE3 macrocomplex for carbachol inhibition.

There have been multiple previous attempts at defining NHERF homo- and heterodimerization, with results not being consistent. NHERF1/NHERF1, NHERF2/NHERF2, and NHERF1/NHERF2 dimerizations occurring via PDZ-PDZ interactions were demonstrated by overlay assays (25, 26). NHERF3/NHERF3 dimerization via PDZ-PDZ interactions was demonstrated by use of a cross-linker (27). Oppositely, a systematic protein microarray approach, which was used to define all possible murine PDZ-PDZ interactions, failed to identify homo- or heterodimerization among NHERF1, NHERF2, and NHERF3 (37). Co-IP identified NHERF1...
homodimerization in one study (25) but not in another (26). Importantly, NHERF1 and NHERF3 did not dimerize in solution even at high concentrations (28, 29). By comparing the interaction strength of all possible NHERF dimerizations in parallel using co-IP and pulldown assays, it was found that NHERF1/NHERF1, NHERF2/NHERF2, NHERF3/NHERF3, and NHERF1/NHERF2 dimerizations were either minimally identified or not detected. This suggests that these dimerizations are of low affinity and probably are too weak to be detected by all the methods used in the past. These weak interactions may also explain the limited biological functions reported for these NHERF dimerizations. In one study NHERF1 homodimerization facilitates the dimerization of platelet-derived growth factor receptor (PDGFR) and vice versa, which stimulates PDGFR activity (45). Another study hypothesized that DRA (down-regulated in adenoma) and NHE3 could be linked together through NHERF2 dimerization, but this remains unproven (46).

Although most studies concentrated on studies of PDZ-PDZ interactions among the NHERF proteins (25–27), the C-terminal four amino acid residues of human NHERF1 (FSNL), NHERF2 (FSNF), and NHERF3 (DTEM) all are potential PDZ domain recognition motifs. Thus interactions among the NHERFs could occur by either PDZ-PDZ or C terminus-PDZ interactions, with the latter identified here as involved in NHERF2/NHERF3 heterodimerization. The involvement of the PDZ domain recognition motif in NHERF dimerization has the potential to cause competition between different NHERF dimerization pairs, further complicating the ways these proteins might interact and making it important to understand the interplay between the different NHERF family proteins to understand their many regulatory functions.

Initially, the C-terminal PDZ recognition motifs of NHERF1 and NHERF2 were shown to interact with PDZ4 domain of NHERF3 by yeast two-hybrid assays (30). But this study did not
characterize the interaction between full-length NHERF proteins. The current study showed that NHERF2/NHERF3 heterodimerization was mediated by the C-terminal PDZ-recognition motif of NHERF3 and both PDZ domains of NHERF2. And a pulldown study showed that NHERF1 and NHERF3 heterodimerize in a similar way through the interaction between the C-terminal PDZ-recognition motif of NHERF3 and both PDZ domains of NHERF1 (27). NHERF1, NHERF2, and NHERF3 were all shown to be primarily localized to the microvilli (2, 4, 5), providing the spatial requirement for their heterodimerizations. Although there could be potential competition between NHERF1 and NHERF2 for binding to the C terminus of NHERF3, NHERF2/NHERF3 heterodimerization is stronger than that of NHERF1/NHERF3. Both FRET and FRAP studies confirmed NHERF2/NHERF3 heterodimerization in OK cells, which expresses a high endogenous level of NHERF1.

In the human placental choriocarcinoma JEG-3 cell line, NHERF1/NHERF3 heterodimerization was suggested as linking NHERF3 to the microvillus, where NHERF3 augments the NHERF1 function in the formation of microvilli (38). In contrast, in OK cells, the FLAG-NHERF3-4A mutant has the same apical localization as wild-type FLAG-NHERF3 and CFP-NHERF3. This suggests that it is not the NHERF3 C terminus that determines the NHERF3 microvillus localization. This conclusion is supported by the identified role of NHERF3 in establishing the surface location of the hepatic SR-BI receptor (scavenger receptor class B, type I), which does not involve the NHERF3 C terminus (47). Rather, these results support a role for NHERF3 PDZ domains in microvillus anchoring as our results indicate. The detailed mechanism is unclear. However, a recent study showing that NHERF3 PDZ4 directly binds vesicles mimicking the plasma membrane could provide an explanation (29).

The function of individual NHERF protein in NHE3 regulation has been studied both in cell lines by overexpression or knockdown of specific NHERF proteins and in specific gene knock-out mouse models (8–13, 18, 23, 24, 48). The results are tissue-specific. NHERF1 is necessary for cAMP-mediated inhibition of NHE3 in the kidney but not in the intestine (8, 9, 49). NHERF2 is needed for the inhibition of intestinal NHE3 by cAMP, cGMP elevated by Escherichia coli heat stable enterotoxin, and Ca\(^{2+}\) elevated by calcium ionophore, carbachol, and UTP (12, 13, 23), although there is some segmental dependence. NHERF2 is also required for LPA (oleoyl-\(L\)-lysophosphatidic acid sodium salt) and dexamethasone-mediated stimulation of NHE3 (13, 18, 48). NHERF3 is required for cAMP and elevated Ca\(^{2+}\)-mediated inhibition of NHE3 in the intestine (9, 10, 24). Thus, inhibition of NHE3 by elevated Ca\(^{2+}\) was both NHERF2- and NHERF3-dependent. This study further clarified that Ca\(^{2+}\) inhibition of NHE3 requires NHERF2/NHERF3 heterodimerization as NHERF3-4A mutant did not reconstitute carbachol inhibition of NHE3 activity.

A general model for the regulatory role of PDZ domain-containing scaffolding proteins is that they can cluster multiple proteins, such as transporters, receptors, cytosolic signaling proteins, and cytoskeletal proteins, into functional complexes at specific membrane microdomains (41). NHERF homodimerization or heterodimerization adds more complexity by bringing more PDZ domains together to assemble more potential ligands into NHE3 complexes. Intestinal brush border NHE3 has been shown to exist in complexes with molecular masses from ~400 kDa up to ~1000 kDa as analyzed by sucrose gradient ultracentrifugation (1, 50). The different partners assembled in the NHE3 complex confer different consequences for downstream signaling. The specificity of NHERF proteins involved in NHE3 regulation by different pathways has been mainly attributed to their PDZ domains, each of which has different specificity for their ligands (42). One example
is that the NHERF2 N-terminal PDZ domains, but not those of NHERF1, play important roles in some aspects of NHE3 regulation by binding specific ligands including α-actinin-4, SGK1 (serum- and glucocorticoid-inducible kinase 1), and LPA5R (2, 18, 39, 48).

Two ligands of NHERF2, PKCα and α-actinin-4, are necessary for the Ca\(^{2+}\) mediated inhibition of NHE3 (39, 40). In response to elevated Ca\(^{2+}\), NHERF2, NHE3, PKCα, and α-actinin-4 form a macrocomplex, which is required for further endocytosis of NHE3 in PS120 fibroblasts (39, 40). This phenomenon has been further confirmed in the rabbit ileal brush border (51). However, different from PS120 fibroblast cell models, which lack endogenous NHERF3, NHERF3 was also essential for the Ca\(^{2+}\) mediated inhibition of NHE3 in mouse colon and Caco-2 cells (10, 24). This suggests that at least some of the other three PDZ domains of NHERF3 are involved in the carbachol inhibition of NHE3, although the specific ligands for these PDZ domains have not been identified. Biochemical analysis shows that NHERF2/NHERF3 heterodimerization does not affect the interaction between NHERF2 and its ligands such as NHE3, α-actinin-4, and PKCα. Therefore, NHERF2/NHERF3 heterodimerization likely brings the potential ligands of NHERF2 and NHERF3 together and assembles NHE3 into macrocomplexes. Disruption of NHERF2/NHERF3 heterodimerization abolished carbachol-mediated inhibition of NHE3, suggesting that NHERF2/NHERF3 heterodimerization-mediated NHE3 macrocomplexes are involved in the regulation of both pools of NHE3 whether it is anchored by NHERF3 or not. These NHE3 macrocomplexes could either serve as the signaling platform for initially responding to the elevated Ca\(^{2+}\) or be newly assem-

![FIGURE 10. NHERF2/NHERF3 heterodimerization is modulated by calcium. A, Caco-2 cells were infected with adenovirus expressing FLAG-NHERF2, or OK cells were transiently transfected with pFLAG-NHERF2. Lysates were prepared and adjusted to either 1 mM EGTA or 1 μM CaCl\(_2\). Then FLAG-NHERF2 was immunoprecipitated with anti-FLAG magnetic beads. Co-IP of NHERF3 with NHERF2 was analyzed by Western blot (IB). Uninfected Caco-2 cell was used as negative control (Cont). B, representative images of sensitized emission FRET. OK cells were transfected with CFP-NHERF2 alone or YFP-NHERF3 alone or co-transfected with both. Three channels of images, CFP signal (Ex-CPF/Em-CFP), YFP signal (Ex-YFP/Em-YFP), and FRET signal (Ex-CPF/Em-YFP), were collected. The same microscope settings were used to calculate the donor bleed-through, acceptor bleed-through, and FRET efficiency. The same cell was used to compare the NHERF2/NHERF3 FRET signal before and 5 min after the treatment with 0.5 μM 4-Br-A23187. Bar, 10 μm. C, calculated FRET efficiency between CFP-NHERF2 and YFP-NHERF3 or YFP-GPI before and after the treatment with 0.5 μM 4-Br-A23187 (n = 16).](image-url)
bled and involved in the subsequent increased endocytosis of NHE3 as more NHERF2/NHERF3 heterodimerization was observed after elevation of intracellular Ca²⁺. Further identification of the specific ligands of PDZ domains of NHERF3 and characterization of the dynamic change of the NHE3 macromolecule are needed to understand the full picture of this complicated process.

Overall, this study compared the relative interaction strength of all NHERF dimerizations, demonstrated how NHERF2 and NHERF3 dimerize, and illustrated an important role of NHERF2/NHERF3 heterodimerization in the regulation of NHE3 activity by assembly of macromolecules. Because NHERF proteins are involved in the regulation of many other transporters or receptors, similar macromolecules mediated by NHERF dimerizations could play other important physiological functions.

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