Electroneutral NaCl absorption mediated by Na\textsuperscript{+}/H\textsuperscript{+} exchanger 3 (NHE3) is important in intestinal and renal functions related to water/Na\textsuperscript{+} homeostasis. cGMP inhibits NHE3 in intact epithelia. However, unexpectedly it failed to inhibit NHE3 stably transfected in PS120 cells, even upon co-expression of cGMP-dependent protein kinase type II (cGKII). Additional co-expression of NHERF2, the tandem PDZ domain adapter protein involved in cAMP inhibition of NHE3, restored cGMP as well as cAMP inhibition, whereas NHERF1 solely restored cAMP inhibition. In vitro conditions were identified in which NHERF2 but not NHERF1 bound cGKII. The NHERF2 PDZ2 C terminus, which binds NHE3, also bound cGKII. A non-myristoylated mutant of cGKII did not support cGMP inhibition of NHE3. Although cGKII also bound NHERF2 in vitro, it did not evoke inhibition of NHE3 unless a myristoylation site was added. These results show that NHERF2, acting as a novel protein kinase G-anchoring protein, is required for cGMP inhibition of NHE3 and that cGKII must be bound both to the plasma membrane by its myristoyl anchor and to NHERF2 to inhibit NHE3.

The rapid elevation of intestinal cAMP and cGMP levels by activation of adenylyl cyclase and guanylate cyclase, respectively, inhibits intestinal NaCl absorption, either moderately as part of normal digestive physiology or excessively in diarrheal diseases. Some details of the mechanisms of acute regulation of intestinal NaCl absorption by cAMP are understood. Hormones such as vasoactive intestinal peptide or secretin and enterotoxins such as cholera toxin activate adenylate cyclase and increase cellular cAMP content. According to the current model, based on studies in PS120 fibroblasts and the polarized OK\textsuperscript{+} renal proximal tubule cell line, acute elevation of cAMP inhibits NHE3 by stimulating its endocytosis plus decreasing its exocytosis and, additionally, by decreasing the NHE3 turnover number (1–4). NHE3 and cAMP-dependent protein kinase type II (PKAII) are part of the same signaling complex that is scaffolded by either of two brush border (BB)-associated PDZ domain-containing proteins, NHERF1 (also called NHERF or EBP50) or NHERF2 (also called E3KARP) (5, 6). NHERF1/NHERF2 each contain two homologous PDZ domains (PDZ1 and PDZ2) and an ERM (ezrin-radixin-moesin) binding domain, which anchors NHERF and its binding partners to the actin cytoskeleton via NHERF binding to ezrin. Ezrin binds both NHERF1/NHERF2 and PKAII and acts as a low affinity cAMP kinase-anchoring protein (AKAP), positioning PKAII so it can phosphorylate NHE3, which is required for cAMP inhibition of NHE3 (1, 6, 7).

In some cases, cGMP regulates intracellular events by mechanisms analogous to those demonstrated for cAMP. However, the effects of cGMP in the small intestine are not fully elucidated. The intrinsic ileal peptide guanylin and the *Escherichia coli* heat-stable enterotoxin (STa) both bind to the same BB receptor, guanylate cyclase-C, and within minutes increase intracellular cGMP content (8). STa, guanylin, and cGMP all rapidly inhibit small intestinal NaCl-linked absorption, principally at the level of NHE3, which is an essential component of this Na\textsuperscript{+}-absorptive process (8). The effect on NHE3 is specific to the extent that other BB transporters, such as NHE2 and SGLT1, are not acutely altered. The downstream effect of cGMP on ion and fluid transport in enterocytes appears to occur entirely via activation of the type II isof orm of cGMP-de-
pendent protein kinase (cGKII) in the BB (8–10). However, it is not known whether cGKI is part of a signaling complex that includes NHE3, and whether cGKII physically associates with its substrate (e.g. NHE3 or an NHE3 regulatory factor). In addition, there is functional compartmentalization of effects of cGMP on small intestinal NaCl absorption (11). Separately from the consequences of activating guanylyl cyclase-C/Cgmp/cGKII from the apical surface, elevating cGMP from the serosal side through the angiotensin-nitric oxide/GC-S signaling pathway stimulated rather than inhibited intestinal NaCl absorption (11). However, the cell type initially affected by the latter signaling pathway has not been defined.

The current studies further define the downstream events that mediate cGMP inhibition of NHE3. We have demonstrated a requirement for NHERF2 and cGKII, which is specific to the extent that NHERF1 or cGKI cannot be substituted to inhibit NHE3. cGKII must be anchored at two sites to inhibit NHE3, to the plasma membrane and to NHERF2. Thus NHERF2 appears to act as a GAKP, which determines the subcellular targeting of cGKII and thereby its specificity and efficiency.

MATERIALS AND METHODS

Reagents—8-Br-cGMP, a cGK-selective activator, was from Life Science Institute. Protein A-Sepharose was from Amersham Biosciences. All other chemicals, including the cGK inhibitor H8, 8-Br-cAMP, and 8-Br-GMP were from Sigma.

Antibodies—Affinity-purified rabbit polyclonal antibodies against human NHERF2 (Ab2570) or human NHERF1 (Ab5199) have been described (2, 5). Polyclonal cGKII or cGKI antibodies, raised against recombinant rat cGKII lacking aa 1–23 or full-length human cGKIIb and cGKIa, expressed in E. coli, were prepared and affinity-purified as described (10, 12). Mouse monoclonal antibody against Myc epitope (9E10) was from Babco Inc. (Berkeley, CA). Mouse monoclonal antivascular stomatitis virus (VSV)-G antibody P5D4 (hybridoma culture) was kindly provided by Dr. D. Louvain (Curie Institute, Paris, France).

Cell Lines—PS120 fibroblasts lack all endogenous plasma membrane NHEs, NHERF1 (minimal expression), NHERF2, and cGKI (Fig. 1). These cells when stably expressing rabbit NHE3 with a C-terminal VSV-G protein epitope tag are called PS120/NHE3 cells, as described (stable cell lines made using pcDNA 3.1; neomycin; Invitrogen) (10). All PS120 lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 25 mM NaHCO3, 10 mM HEPES, 50 units/ml penicillin, 50 μg/ml streptomycin, 400 μg/ml G418, and 10% fetal bovine serum in a 5% CO2, 95% O2 humidified incubator at 37 °C. Clonal OK cell lines stably expressing NHERF2 or cGKII were generated by stable transfection with 600 μg/ml G418 and/or 600 μg/ml hygromycin and 10% fetal bovine serum in a 5% CO2, 95% O2 humidified incubator at 37 °C. NHERF2 and cGKII were visualized by autoradiography.

A second overlay (far Western) approach was used to examine the interaction of recombinant cGKI, -Iβ, and cGKI on blots with His-tagged NHERF2 (overlay) by subsequent incubation of blots with NHERF2 antibodies. Recombinant cGKI, -Iβ, and -II (1–3 μg) were incubated with nitrocellulose and blocked with 5% nonfat dry milk in PBS (130 mM potassium phosphate buffer, pH 7.4, 0.01% Tween) for 1 h. The blots were incubated with 10 ml of His-NHERF2 (4 μg/ml) in 5% milk/PBS for 16–24 h at 4 °C. At the end of the overlay incubation period, nitrocellulose membranes were washed three times with the same buffer, and proteins binding cGKI were visualized by autoradiography.

Pull-down Assays—In vitro transcription-translation (Promega), and 5 μl of 32P-labeled cGKI in 10 mM Tris, pH 7.2, 150 mM NaCl, 0.2% Tween was used for overlay for 16–24 h at 4 °C. means ± S.E. were determined from at least three experiments and are also shown in the figures (15). N-μP2 Fusion Protein Overlay Binding Assays—A series of glutathione S-transferase (GST) and hexahistidine fusion proteins were prepared for this study. These included full-length human NHERF1 and NHERF2 and individual domains of NHERF2 (NHERF2 PDZ-1 (aa 12–92), NHERF2 PDZ-2 (aa 155–231), NHERF2 C (aa 231–337), and NHERF2 PDZ-2-C (aa 155–337) as described previously (6), after establishing their sequences by subcloning into the pGEX-4T-1 and pGEX-2T vectors (Novagen). The GST-tagged fusion proteins were expressed individually in E. coli cells and purified as described previously with glutathione-Sepharose (6). His-tagged fusion proteins made in E. coli were affinity-purified with Ni2+-nitrilotriacetic acid resin as suggested by the manufacturer (Qiagen).

**Measurement of Na+/H+ Exchange**—Cellular Na+/H+ exchange activity in PS120 cells and OK cell lines grown on glass coverslips was determined fluorometrically using the intracellular pH-sensitive dye acetoxymethyl ester of 2,7-bis(carboxyethyl)-5-carboxyfluorescein (BCECF-AM, 5 μM; Molecular Probes, Eugene, OR), as described previously (12, 13). Exposure to the cGMP and cAMP analogues was during a 30-min dye loading/NH4Cl prepulse, as described (12, 13). Cells were initially perfused with TMA solution (130 mM tetramethylammonium chloride, 5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 1 mM Na2HPO4, 25 mM glucose, 200 μM HEPES, pH 7.4), which was then replaced by Na+ solution (130 mM NaCl instead of tetramethylammonium chloride) for the Na+-dependent pH recovery. At the end of each experiment, the fluorescence ratio was calibrated to pH using the high potassium/nigericin method (13, 15). Na+/H+ exchange activity data were calculated as the product of Na+-dependent changes in pH, times the buffering capacity at each pH, and individual points shown in figures are averages of 4–8 exchange calculated at multiple pH values using at least three coverslips per condition in a single experiment. Kinetics of Na+/H+ exchange were analyzed by Hill plot using Origin (Microcal Software) to estimate Vmax and K(H+), in individual experiments. Means ± S.E. were determined from at least three experiments and are also shown in the figures (15).
by enhanced chemiluminescence as above. The densities of NHE3 protein bands were quantitated by scanning densitometry and Image-Quant software.

Statistics—Results were expressed as mean ± S.E. Statistical evaluation was by analysis of variance or Student’s t test.

RESULTS
cGMP Inhibits NHE3 in PS120 Cells, an Effect Requiring NHERF2 Plus cGKII—Earlier studies have indicated that NHERF1 or NHERF2 is necessary for second messenger regulation of NHE3 (1, 2, 13, 16). Differential effects and redundancy in function have been identified between the requirements for NHERF1 and NHERF2 in the second messenger regulation of NHE3 involving Ca²⁺/protein kinase C and cAMP, respectively (6, 13, 16, 17). Here an examination was made of whether cGMP regulation of NHE3 could be reconstituted by either or both NHERF1 or NHERF2. PS120 cells stably expressing NHE3 were infected with recombinant replication-deficient adenovirus containing the coding sequence of rat cGKII or empty vector. The amount of cGKII expression was estimated by Western analysis, and infection efficiency was estimated by determining the percent of infected cells displaying immunofluorescence when labeled with anti-cGKII antibody/Alexa 488-labeled goat anti-rabbit secondary antibody. cGKII was absent from PS120 cells and from cells infected with adenovirus-empty vector, but was present in cells infected ~48 h earlier with adenovirus containing cGKII (Fig. 1A). By immunofluorescence, 80–90% of PS120 cells contained cGKII 48 h after infection (data not shown).

PS120/NHERF2/NHE3 cells (infected with adenovirus-empty vector or cGKII) were exposed to 100 μM 8-Br-cGMP for 30 min and then studied for Na+/H+ exchange activity. 8-Br-cGMP did not alter NHE3 activity in empty virus-infected cells but inhibited NHE3 in cells containing both NHERF2 and cGKII. A representative experiment is shown, which was repeated three times with similar results. C, kinetic analysis of 8-pCPT-cGMP (100 μM) inhibition of NHE3 in PS120/NHERF2/NHE3 cells infected with cGKII demonstrating decreased V_{max} and increased K_{i}(H⁺). Data are shown from a single experiment, which was repeated three times with similar results. D, NHERF1 does not reconstitute cGMP/cGKII inhibition of NHE3. In contrast to the reconstitution of cGMP inhibition of NHE3 in PS120 cells containing NHERF2 and cGKII, NHERF1 did not reconstitute cGMP/cGKII (100 μM 8-pCPT-cGMP) inhibition of NHE3 in PS120/NHERF1/NHE3 cells infected with adenovirus cGKII. Results of a single experiment are shown with numerical results representing mean ± S.E. of three identical experiments.

The cGKII-dependent inhibition of NHE3 by cGMP was dependent on the presence of NHERF2. When the effect of 8-pCPT-cGMP on NHE3 activity was determined in PS120/NHERF2/NHE3 cells containing cGKII but expressing neither NHERF1 nor NHERF2 (data not shown) or in PS120/NHERF3/cGKII cells stably expressing NHERF1, cGMP did not alter NHE3 activity (Fig. 1D). Thus, cGMP inhibition of NHE3 required both cGKII and NHERF2, and the effect was not reconstituted by NHERF1.
cGMP Inhibition of NHE3 Involves cGKII Not PKA and a Mechanism Different from PKA Inhibition of NHE3 That Does Not Require Ezrin Binding—Studies of the mechanism of cGMP/cGKII regulation of NHE3 were undertaken by first determining whether concentrations of cAMP plus cGMP, which maximally inhibited NHE3 at 30 min of exposure, had additive effects. Synergistic or additive effects of saturating concentrations of 8-Br-cAMP plus 8-Br-cGMP would suggest actions via different mechanisms, but lack thereof would indicate that some aspect of the pathways were redundant and had been saturated. NHERF2-expressing PS120/NHE3/cGKII cells were used in these studies because NHERF2 reconstitutes both cAMP and cGMP regulation of NHE3 in PS120 cells. Used independently, 100 μM 8-Br-cAMP and 100 μM 8-Br-cGMP (both concentrations have maximal effects on NHE3 activity (data not shown)) each inhibited NHE3 activity in these cells with quantitatively similar effects (Fig. 2A). Moreover, the effects on NHE3 of cAMP plus cGMP were at least additive, indicating that cAMP and cGMP were acting via separate mechanisms.

To search for specific differences in cAMP and cGMP inhibition of NHE3, we used the fact that the ERM binding domain of NHERF1, in its C-terminal 30 amino acids was necessary to reconstitute cAMP inhibition of NHE3 (2). A similar truncation of NHERF2, which removed the C-terminal 30 amino acids (NHERF2Δ30), failed to bind N-terminal ezrin in vitro (Fig. 3A) but bound cGKII similarly to full-length NHERF2 (Fig. 3B). As shown in Fig. 2B, in PS120/NHERF2Δ30/NHE3/cGKII cells, cGMP inhibited and cAMP failed to alter NHE3.

To demonstrate further that cGMP was acting via cGK, cells were pretreated during BCECF/NH4Cl loading with the cGK inhibitor H8 (70 μM) (18). Then the effect of 100 μM 8-pCPT-cGMP was determined in PS120/NHERF2Δ30/NHE3 cells infected with cGKII 48 h earlier. As shown in Fig. 2C, H8 did not alter basal NHE3 activity but significantly reversed the cGMP inhibition of NHE3. Thus cGMP inhibition of NHE3 in NHERF2/cGKII-containing PS120/NHE3 cells occurred by a mechanism involving cGKII that differed from the mechanism of PKA inhibition of NHE3.

NHERF2 but Not NHERF1 Directly Binds cGKII in Vitro—The interaction of NHE3 with binding proteins such as NHERF2 or NHERF1 has been shown to determine the specificity of formation of signaling complexes (13, 14, 17, 19, 21). Thus in vitro studies were used to examine whether fusion protein-tagged NHERF1 or NHERF2 directly bound cGKII. Shown in Fig. 4A, overlay, far Western assays were performed in which full-length His6-NHERF1 and NHERF2 were initially separated by SDS-PAGE, transferred to nitrocellulose, and then overlaid with recombinant cGKII. cGKII was then identified by anti-cGKII antibody. cGKII bound to NHERF2 but not NHERF1 in a manner proportional to the amount of NHERF2 transferred. The cGKII-NHERF2 interaction was of low affinity based on plasmon resonance studies with an estimated affinity in the micromolar range.²

These findings were supported by pull-down assays using 3 µg of GST, GST-NHERF2, or GST-NHERF1 (Fig. 4B). In vitro pull-down of cGKII occurred only with NHERF2 and not with NHERF1 or GST alone. In addition, His6-NHERF1 and NHERF2 overlaid with 35S-cGKII similarly showed binding of cGKII to NHERF2 and not NHERF1 (Fig. 5, left two lanes). Thus, three separate types of in vitro studies demonstrated direct binding of recombinant NHERF2, but not NHERF1, to cGKII.

cGKII Binds PDZ2 Plus C Terminus of NHERF2—We demonstrated previously that the PDZ-2 C terminus of NHERF2 binds NHE3 in forming NHE3-related complexes (6). In order to determine which domain of NHERF2 was involved in cGKII

² H. Hut and H. R. de Jonge, unpublished data.
binding, His<sub>6</sub> fusion proteins were made of NHERF2 PDZ-1, PDZ-2, the C terminus, and PDZ-2-C terminus. These fusion proteins were overlaid with <sup>35</sup>S-cGKI. As shown in Fig. 5, cGKII bound only to NHERF2 PDZ-2 C terminus and not to PDZ-1, PDZ-2, or the C terminus alone. Thus, a similar domain of NHERF2 binds both NHE3 and cGKII.

**NHERF2 and cGKII Are Part of the Same Complex in Vivo**—Evidence of *in vitro* interactions of recombinant proteins is known to be prone to artifacts. To demonstrate meaningful interactions, it is necessary to demonstrate that proteins that directly interact by a totally *in vitro* approach also are part of the same complex in *vivo*. Thus co-precipitation studies of cGKII and NHERF2 were performed using cell lysates of PS120/NHERF2/NHE3 cells transiently transfected with adenovirus cGKII or empty vector. As shown in Fig. 6A, IP of NHERF2 co-precipitated cGKII. Conversely, cGKII co-precipitated NHERF2. These co-immunoprecipitations were not specific because similar antibody dilutions of cGKII antibody failed to IP either cGKII or NHERF2 from vector-only infected cells (no cGKII present). Thus, NHERF2 and cGKII are part of the same complex based on co-precipitation studies. In PS120/NHERF2/NHE3/cGKII cells (Fig. 6B), no changes were detected in the amount of cGKII co-precipitated by NHERF2 and vice versa, when basal conditions and a 10-min exposure to 8-Br-cGMP were compared. In these studies, immunoblotting also demonstrated that NHE3 was co-precipitated by both NHERF2, as reported (10), and cGKII, although to a lesser extent by cGKII. cGMP did not alter NHE3 co-precipitation with either NHERF2 or cGKII. Thus no dynamic aspects of NHE3-NHERF2-cGKII complex formation were demonstrated within this time frame.

**cGMP Inhibition of NHE3 Requires Myristoylation of cGK**—
The intracellular location of protein kinases often defines the access to their substrates. Non-myristoylated cGK is primarily cytosolic, whereas cGKII is known to be in the apical membrane of epithelial cells, attached by myristoylation of its N terminus at Gly-2 (15, 22). Thus, a series of truncations/chimeras of cGKII and cGK were studied for their ability to recon-
NHERF2 Is a GKAP in cGMP Inhibition of NHE3

**Fig. 6.** NHERF2 and cGKI are part of the same complex in PS120/NHERF2/NHE3 cells based on co-precipitation studies. Lack of changes with cGMP. A, lysates were prepared from PS120/NHERF2/NHE3 cells transiently transfected with either cGKI or empty vector but not treated with cGMP. cGKI or NHERF2 were immunoprecipitated (I.P.), and immunoblot (I.B.) were performed for the opposite protein (NHERF2 or cGKI, respectively). Arrows indicate co-precipitated proteins. B, PS120/NHERF2/NHE3 cells transiently transfected with either cGKI or PS120/NHERF2/NHE3 cells based on co-precipitation studies.

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NHERF2 is a GKAP in cGMP Inhibition of NHE3

**NHERF2 Is a General GKAP Binding cGKI and cGKII—**

Multiple protein kinase A-anchoring proteins (23) and, more recently, several GKAPs have been identified. So far GKAP binding to either cGKI or cGKII has been reported, with no report of a dual specificity GKAP (however, cGKII binding was not always studied). Thus, we examined whether NHERF2 bound cGKI or cGKII in addition to cGKII. cGKI and cGKII only differ in their N-terminal ~100-aa regulatory domain that contains a leucine zipper motif required for dimerization. In vitro overlay studies were used to examine the binding of His$_6$-NHERF2 to recombinant cGKI, -cGKII, and cGKII or N-terminus ezrin (aa 1–209) as a positive control (24). When the amounts of NHERF2 bound were corrected for the amounts of cGKI and cGKII present on blots using scanning densitometry of the amount of protein transferred, more NHERF2 bound to cGKI than to cGKII, the rank order of binding being cGKI > cGKII = cGKI (Fig. 8). Thus NHERF2 is a broad specificity GKAP that binds to cGKI, cGKII, and cGKII.

**NHERF2 Reconstitutes cGMP Inhibition of NHE3 in OK Cells Expressing cGKI—**

cGMP inhibits NHE3 under physiological and pathophysiological conditions in epithelial cells. Consequently, we tested whether NHERF2 was similarly required for cGMP regulation of NHE3 in OK/NHERF2/NHE3 epithelial cells stably transfected with cGKI. OK cells endogenously express NHERF1 but not NHERF2 (6). Stably transfected NHERF2 was present in the apical surface/microvilli and in the cytosol of OK cells (data not shown). As shown in Fig. 9A, 100 μM 8-pCPT-cGMP inhibited NHE3 in OK/NHERF2/NHE3/cGKII cells with effects on $V_{max}$ and $K'(H^+)$ (14). Both in OK/NHERF2/cGKII cells lacking NHERF2 (Fig. 9B) and in OK/NHERF2/cGKII cells lacking cGKI (Fig. 9C) 8-pCPT-cGMP failed to affect NHE3 activity. Thus, in the polarized OK proximal tubule epithelial cell line, cGMP inhibited NHE3 in a cGKI- and NHERF2-dependent manner, as was observed in PS120 cells.

**DISCUSSION**

In this study we show that NHERF2 is a low affinity, broad specificity GKAP (G kinase-anchoring protein) that binds cGKI (α and β) and cGKII. Functionally, it organizes a signaling complex containing at least NHERF2-cGKI-NHE3 that supports cGMP inhibition of NHE3 in both a polarized renal proximal tubule cell line (OK cells) and in PS120 fibroblasts. Moreover, NHERF2 reconstitution of cGMPCGKII inhibition of NHE3 is specific because NHERF1 cannot duplicate the effect. The specificity for NHERF2 appears to derive from the greater ability of NHERF2 than NHERF1 to act as a GKAP that binds cGKI with low affinity and integrates it into a complex with NHE3.

PKA and cGK have many similar properties and functions, including that both interact with specific anchor proteins. More than 50 AKAPs have been characterized for PKA, whereas fewer than 10 GKAPs have been identified for cGK. AKAPs integrate PKA into scaffolds of signaling complexes containing activators of PKA and the relevant substrates that PKA phosphorylates. Michel and Scott (23) suggested that all AKAPs bind the following: 1) PKAII, and in some cases PKAI; 2) PKA substrates; and 3) other proteins that are involved in targeting the substrates to specific intracellular domains. By analogy, the current study suggests that NHERF2 functions as a GKAP by forming a complex in which cGKI, probably initially in an inactive state, is placed near NHE3, the protein that it regulates, and allows spatially restricted NHE3 activation. The cGKII substrate involved in inhibition of NHE3 is not yet clear. However, preliminary results indicate that cGMP stimulates phosphorylation of NHE3 in PS120 cells. Still to be identified are additional signaling molecules that are probably included in this cGMP-related NHE3 complex, either by binding to NHERF2 or to the NHE3 C terminus (25).

The low affinity binding of NHERF2 to cGKII was surprising because most AKAPs bind their substrates with high affinity (23). An exception is ezrin, which acts as a low affinity AKAP for PKA inhibition of NHE3 (7). These low affinity interactions, however, appear to be effective in the setting of high (micromolar) concentrations of NHERF1 (25) and NHERF2 found in kidney homogenates. This is supported by studies showing that the effects of exogenously expressed NHERF2 and NHERF1 are proportional to expression levels (13, 16, 17, 26). Involvement of low affinity rather than high affinity NHERF interactions in NHE3 regulation may facilitate disruption of this signaling complex to allow shutting of NHERF1/NHERF2 to other alternative complexes with which they interact (13, 26–28). However, this is an area requiring further study.

NHERF2 is the first broad specificity GKAP (binds cGKI, -β, and -II) to be identified. However, the GKAP field is so new that binding of a GKAP to all three cGK isoforms has generally not been studied. All previously identified GKAPs were described to interact with either cGKI or -II. GKAPs described for cGKI include the testicular Golgi protein GKAP42 (29), skeletal muscle myosin"
tal muscle troponin T (30), myosin light chain kinase (31), smooth muscle myosin phosphatase (32, 33), the substrate IRAK (IP3 receptor-associated cGKI) (34, 35), guanylate cyclase A (36), and vimentin (37, 38). Before NHERF2, the only other GKA identified for cGKII was myosin heavy chain (39). Alternatively, cGKI substrate(s). Addition of the myristoylated 1–29 aa N terminus of cGKII to the N terminus of full-length cGKI changed cGKI localization from the cytosol to the plasma membrane (15, 39) and, in the present study, rendered NHE3 sensitive to cGMP inhibition, in line with NHERF2 being a broad specificity GKA (Fig. 8). The cGKII/cGKI chimera mimicked not only the ability of cGKII to inhibit NHE3 but also the ability of cGKI to stimulate the cystic fibrosis transmembrane regulator (CFTR)-mediated increase in chloride secretion (15). However, in rat kidney collecting tubules/cortical collecting ducts, the chimera did not mimic cGKII inhibition of Ca2+ absorption, suggesting that cGKII and cGKI did not share the same substrate specificities (39). Alternatively, the inhibition of Ca2+ absorption may require a yet unknown GKA that does not bind cGKI or the cGKII/cGKI chimera. It should be noted that the gating of CFTR in IEC-CF7 cells (a rat crypt cell line stably expressing CFTR) was dependent on membrane targeting of cGK through an N-terminal myristoyl anchor but did not require co-transfection with NHERF2 (15). However, IEC-CF7 cells contain a relatively

5 N. Hernando and H. Murer, personal communication.
high level of endogenously expressed NHERF1 and NHERF2,6 which may have contributed to the cGKII effect on CFTR.

Reconstitution of the inhibition of NHE3 by cGMP/cGKII by NHERF2, but not NHERF1, demonstrated a specific functional difference between these two highly related PDZ domain containing proteins. Experiments in Figs. 4 and 5 demonstrated stronger binding of cGKII to NHERF2 compared with NHERF1, although longer film exposures detected a small amount of NHERF1 binding as well (data not shown). The in vitro binding of NHERF2, but not NHERF1, to cGKII in overlay, pull-down, and far Western assays is consistent with the finding that NHERF2 but not NHERF1 reconstitutes cGMP/cGKII inhibition of NHE3. Previously, differences in effects of NHERF1 and NHERF2 have also been attributed to differences in their specificity of ligand binding or, alternatively, to subtle differences in their location in the BB of renal proximal tubule cells (40). Although NHERF1 and NHERF2 share many ligands, NHERF2 additionally binds the transcription factor TAZ, α-actinin-4, phospholipase C β3, and as shown here cGKII (13, 40–43). This differential ligand binding has been shown to account for the ability of NHERF2 but not NHERF1 to reconstitute Ca2+ inhibition of NHE3 (involving binding of α-actinin-4 which leads to formation of large plasma membrane NHE3 complexes), and lysophosphatidic acid stimulation of NHE3 (involving binding of phospholipase C β3, which by an unknown mechanism leads to NHE3 exocytosis). Regarding differential localization in mouse renal proximal tubules, NHERF1 is in the BB, whereas NHERF2 is not only in the BB but also just below the BB, perhaps in the intervillus clefts (40).

This subtle difference in location however may be of functional importance, because it has been correlated with NαP2a being missorted from the BB to intercellular vesicles in NHERF1 knock-out mice, and NHERF2 being unable to compensate for lack of NHERF1, despite evidence that both NHERF’s bind NαP2a in vitro (44). Also, NHE3 is present in its normal renal proximal tubule BB location in the NHERF1 knock-out mice; however, cAMP does not alter renal NHE3 activity in these mice nor does NHERF2 compensate for the absence of NHERF1 (44, 45). In contrast, in our studies, the ability of NHERF2, but not NHERF1, to support cGMP/cGKII inhibition of NHE3 appears more likely to be related to differences in their ability to bind cGKII, rather than to subtle differences in their BB/plasma membrane locations.

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