A C-Terminal PDZ Motif in NHE3 Binds NHERF-1 and Enhances cAMP Inhibition of Sodium−Hydrogen Exchange†

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ABSTRACT: NHERF-1, a protein adapter containing two tandem PDZ domains, was first identified as an essential cofactor required for the phosphorylation and downregulation of NHE3 activity in response to elevated intracellular cAMP. NHERF-1 contains multiple protein interaction domains, but the mechanism by which it binds NHE3 remains unknown. Yeast two-hybrid analyses demonstrated that the C-terminal sequence, STHM, of NHE3 constitutes a PDZ motif critical for its association with NHERF-1. In this assay, NHE3 bound both PDZ-I and PDZ-II when presented as isolated domains, but mutations of the individual PDZ domains in the full-length NHERF-1 suggested a significant preference of NHE3 for the PDZ-II domain. To investigate NHERF-1/NHE3 association in cells, NHERF-1 complexes were isolated from PS120 cells expressing hexahistidine-tagged NHERF-1 and NHE3 using nickel-NTA−agarose. In these experiments, mutating the C-terminal PDZ motif still allowed NHE3 binding to NHERF-1, suggesting the presence of additional mechanisms or components that stabilized a cellular NHE3/NHERF-1 complex. Transport assays in PS120 cells, however, showed that the C-terminal PDZ motif in NHE3 and a functional PDZ-II domain in NHERF-1 were required for maximal inhibition of sodium−hydrogen exchange in response to forskolin and 8-Br-cAMP. Together, the data suggested that the PDZ interaction between the NHE3 C-terminus and a NHERF-1 PDZ domain enhanced the regulation of sodium−hydrogen exchange by cAMP-elevating hormones.

Sodium−hydrogen exchanger isoform 3 (NHE3) is expressed at the apical surface of many epithelial tissues, including the gut and kidney, and is regulated by hormones and growth factors (*1*). The NHE3 protein comprises 11 membrane-spanning domains and a C-terminal cytoplasmic tail. Analysis of NHE3 chimeras containing cytoplasmic tails from other NHE isoforms suggested that several different C-termini could support effective sodium−hydrogen exchange but only the NHE3 C-terminus permitted the regulation of the transporter by protein kinases (*2*). C-Terminal deletions of NHE3 also suggested that the cytoplasmic tail played a key role in the inhibition of transport activity by cAMP-elevating hormones (*2*).

Reconstitution of the sodium−hydrogen exchanger using detergent-solubilized rabbit renal proximal tubule brush border membranes, primarily NHE3, identified NHERF-1 (*N*a$^+$-*H*$^+$ *e*xchanger *r*egulatory *f*actor-1), a PDZ (PSD-95/Dlg/ZO-1) domain-containing protein, as an essential cofactor for cAMP inhibition of sodium−hydrogen exchange (*3*−*5*). Subsequent studies used the NHE3 cytoplasmic tail as bait in a yeast two-hybrid screen of a human lung cDNA library to identify E3KARP (NH*E3 k*inase *A r*egulatory *p*rotein), also referred to as NHERF-2, which shared 52% overall sequence identity to NHERF-1 (*5*). Human NHERF-1 was also identified as EBP50 (*e*zrin-*b*inding *p*rotein of molecular mass ∼50 kDa), a major cellular target of ezrin, a cytoskeletal protein (*6*). Subsequent studies in cultured cells demonstrated that NHERF-1 and NHERF-2 bound both NHE3 and ezrin, and this tripartite complex recruited PKA to promote NHE3 phosphorylation and downregulation of transport activity (*7*−*9*). However, the mechanism by which the NHERF proteins bound NHE3 and promoted the hormonal regulation of sodium−hydrogen exchange has not been defined.

The two-hybrid screen, which identified NHERF-2, suggested that the NHE3 cytoplasmic tail bound to a C-terminal region encompassing the second PDZ domain (PDZ-II) and the ERM-binding domain (*5*). Our functional studies, both in vitro and in transfected PS120 cells, suggested that a polypeptide containing PDZ-II and the C-terminus of NHERF-1 could mediate cAMP signals to partially inhibit NHE3 activity (*9*, *10*). This suggested that the PDZ-I domains of both NHERF isoforms were dispensable for NHE3 binding and regulation by PKA. This may allow the

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two NHERF isoforms to link NHE3 to other membrane proteins, such as CFTR (*c*ystic *f*ibrosis *t*ransmembrane *r*egulator), a PDZ-I target, and coordinate their reciprocal regulation by PKA (*11*). On the other hand, the β2-adrenergic receptor, which binds the PDZ-I domain in an agonist-dependent manner, competed with NHE3 for NHERF-1 binding, thereby accounting for NHE3 activation by β2-adrenergic agonists (*12*). These findings suggested that PDZ-I targets of NHERF-1 may also play a role in the physiological regulation of NHE3.

To investigate NHE3 association and regulation by NHERF-1, we utilized the yeast two-hybrid protein interaction assay. These studies identified a novel C-terminal PDZ motif in NHE3 that was critical for its binding to NHERF-1 PDZ-II domain in yeast. Moreover, functional studies in stably transfected PS120 cells suggested that the PDZ interaction between NHE3 and NHERF-1 played an important role in the transduction of cAMP signals that inhibit sodium−hydrogen exchange.

MATERIALS AND METHODS

Preparation of NHE3 and NHERF-1 cDNAs. The cDNAs encoding wild-type (WT) rabbit NHE3, NHE3 fused at its carboxyl terminus to YTDIEMNRLGK (epitope derived from vesicular stomatitis virus glycoprotein or VSVG), and NHE3 with the C-terminal four amino acids substituted with alanines were cloned into pMAM-neo as previously described (*5*, *8*, *9*). A 700 bp fragment representing the C-terminus (amino acids 597−832) of WT rabbit NHE3 (STHM) was cloned into pLexA and used as bait in a yeast two-hybrid assay (Tropix). cDNAs representing NHE3 with alanine substitutions in the C-terminal four amino acids (AAAA) or substitution of a C-terminal sequence (SSWL) representing a consensus binding motif for the NHERF-1 PDZ II domain (*13*) were similarly inserted into pLexA.

The mouse NHERF-1 cDNA was inserted into pET-30-(a)+ (Novagen) to yield a hexahistidine-fused NHERF-1 (His-NHERF-1), which was then transferred to pcDNA3.1/Hygro+ for expression of this fusion protein in mammalian cells. Similarly, cDNAs encoding the individual NHERF-1 PDZ domains and NHERF-1 with alanine substitutions (GAGA) in the core peptide-binding sequence, GYGF, that inactivates the individual PDZ domains were inserted into pcDNA3.1/Hygro+. The PCR reaction used to generate the GAGA mutations also introduced an additional substitution, a histidine in place of the proline in the sequence, PNGYGF. For the yeast two-hybrid assays, cDNAs encoding NHERF-1 with the mutant PDZ domains, fragments containing PDZ-I (residues 1−126) and PDZ-II (residues 116−355) were cloned into pB42. All cDNAs were confirmed by double-stranded DNA sequencing.

Measurement of NHE3 Activity in PS120 Cells. NHE3 and NHERF-1 plasmids were transfected into PS120 fibroblasts using Lipofectin (GIBCO/BRL). Two or more independent cell lines were selected from each transfection by their resistance to 600 units/mL hygromycin through eight passages. The PS120 fibroblasts were maintained at 37 °C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and 600 units/mL hygromycin. Cells were acid selected for 24−48 h (*8*, *9*) followed by 12−24 h in serum-free media. Na^+-H^+ exchange was determined using the pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) (*8*, *9*). Briefly, cells were loaded with 6 μM BCECF-AM in an assay buffer containing 130 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM NaH_2PO_4, and 25 mM glucose in 20 mM HEPES, pH 7.5, for 20 min at room temperature. Cells were then pulsed with 40 mM NH_4Cl, pH 7.4, for 15−20 min followed by sequential washes with a solution containing 130 mM TMACl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM $TMAPO_4$, and 25 mM glucose in 20 mM HEPES, pH 7.5. BCECF fluorescence was elicited at the excitation wavelengths of 500 and 440 nm and monitored at the emission wavelength of 530 nm. The NH_4Cl pulse was used to achieve an initial pH_i of 6.0, and only cells with initial pH_i values between 6.0 and 6.2 were analyzed. Na^+-H^+ exchange transport was expressed as ΔpH_i per second, calculated from the initial slope of sodium-dependent pH_i recovery during the first 5−10 s to ensure linearity. Some cells were treated with 10^{-4} M forskolin during the final 15 min of dye loading and throughout the perfusion process. Control cells were treated with the solvent DMSO. At the end of each experiment, cells were equilibrated in pH clamp media containing 20 mM HEPES, 20 mM MES, 110 mM KCl, 14 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM TMA, 25 mM glucose, and 10 μM nigericin at pH 6.1 and 7.2. All measurements were undertaken in cells at the same passage. Statistical analysis of NHE3 activity in control and experimental samples was performed using Peritz analysis of variance (*14*).

NHE3/NHERF-1 Interaction in the Yeast Two-Hybrid Assay. Bait and prey plasmids were transformed into the EGY48[p8op-lacZ] reporter strain, and transformants were selected on synthetic [-His, -Ura, -Trp] dropout plates. Colonies were harvested, lysed, and analyzed using the Galacton-Star one-step β-galactosidase reporter gene assay (Tropix Inc., Bedford, MA). Colonies expressing either bait or prey alone were defined as controls, and the β-galactosidase activity expressed in these cells was given a value of 1.0. The β-galactosidase activity expressed in all double transformants was then expressed as a ratio (or *x*-fold activation) of the controls. Western immunoblotting confirmed equivalent expression of all NHE3 and NHERF-1 fusion proteins (*15*).

NHE3/NHERF-1 Association in PS120 Cells. Nickel-NTA−agarose was used to isolate NHERF-1 complexes from lysates of PS120 cells expressing NHE3 and His-NHERF-1. The bound proteins were eluted using SDS sample buffer and analyzed by SDS−PAGE in 10% (w/v) polyacrylamide gels. Following electrophoretic transfer to nitrocellulose, Western immunoblots were performed using polyclonal antibodies to NHE3 and NHERF-1, and the immune complexes were detected using ECL.

Other Methods. Protein concentrations were determined by the method of Lowery et al. (*16*). Reverse transcription−polymerase chain reaction (RT-PCR) was used to analyze NHERF-1 mRNA in the PS120 cells (*17*).

RESULTS

The NHE3 C-Terminus Is Required for NHERF-1 Binding. In the yeast two-hybrid assay, the cytoplasmic tail of rabbit

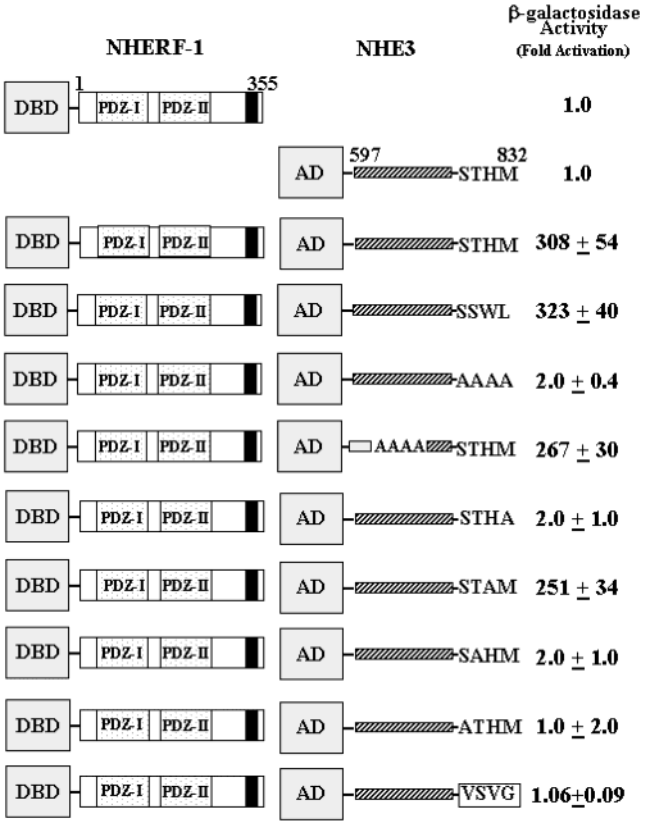

FIGURE 1: Association of the NHE3 C-terminus with NHERF-1. WT mouse NHERF-1, a polypeptide of 355 amino acids containing two PDZ domains (stipled boxes) and an ERM domain (filled box), was fused to the DNA-binding domain (DBD) of LexA and expressed in yeast along with the cytoplasmic domain of rabbit NHE3 fused to the LexA activation domain (AD). Association of the two proteins enhanced expression of the reporter gene, β-galactosidase, which was monitored using the Galacton-Star luminescence assay (Tropix). β-Galactosidase expression in yeast transformed with either NHE3 or NHERF-1 alone was defined as controls and given the value of 1.0. Association of NHERF-1 with the various NHE3 proteins, shown schematically, was analyzed as a ratio or *x*-fold activation of β-galactosidase expression compared to controls. All assays were undertaken at least three times, and average values with standard error of the means (SEM) are shown.

NHE3 (amino acids 597−832) bound WT mouse NHERF-1, resulting in a 300-fold increase in β-galactosidase expression compared to controls expressing either the bait or prey proteins (Figure 1). Substitution of the C-terminal four amino acids of rabbit NHE3 (STHM) with SSWL, previously defined as the optimal sequence for binding to the PDZ-II domain of NHERF-1 (*14*), yielded similar β-galactosidase expression. Substituting the same residues with alanines abolished NHE3 association with NHERF-1. Insertion of alanines in a different location within the NHE3 cytoplasmic tail, namely, amino acids 609−612, had no effect on NHERF-1 binding. Interestingly, fusion of a VSVG epitope to the C-terminus of NHE3, used in prior studies of NHE3 regulation (*5*), also abolished NHERF-1 binding. These data suggested the presence of a PDZ motif at the C-terminus of NHE3 that was critical for NHERF-1 binding in the yeast two-hybrid assay.

To evaluate the contribution of individual amino acids within this putative PDZ motif in NHE3, single alanine substitutions were introduced in positions 0 (STH<u>A</u>), −2 (S<u>A</u>HM), and −3 (<u>A</u>THM). All of these substitutions

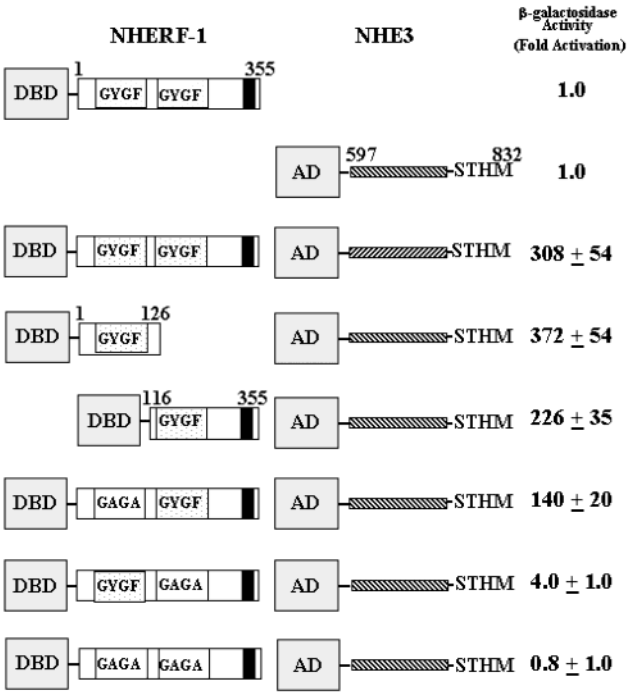

FIGURE 2: Role of NHERF-1 PDZ domains in NHE3 binding. The yeast two-hybrid assay analyzed the binding of NHERF-1 with WT (GYGF) or mutant (GAGA) PDZ domains with the cytoplasmic tail of NHE3 as described in Figure 1. β-Galactosidase expression was estimated as a ratio or *x*-fold activation of controls defined as yeast expressing NHERF-1 or NHE3 alone (given the value 1.0). All assays were undertaken at least three times, and average values with standard error of the means (SEM) are shown.

abolished NHE3 binding to NHERF-1 in the two-hybrid assay. By comparison, an alanine substitution in the −1 position (ST<u>A</u>M) resulted in only a modest decrease in NHERF-1 binding. These data suggested that the C-terminus of NHE3 possessed the hallmarks of a class I PDZ motif (*18*).

NHERF-1 PDZ Domains Mediate NHE3 Binding. The isolated PDZ-I domain of NHERF-1 bound the NHE3 cytoplasmic tail comparable to the full-length NHERF-1 in the yeast two-hybrid assay (Figure 2). PDZ-II also bound the NHE3 C-terminus. When alanine substitutions were used to disrupt the function of each PDZ domain in the context of a full-length protein, NHERF-1 lacking a functional PDZ-I showed approximately 50% loss in NHE3 binding. By comparison, a loss-of-function mutation in PDZ-II almost completely abolished NHE3 binding (Figure 2). Similarly, NHERF-1 lacking both functional PDZ domains failed to bind NHE3. These data suggested that the NHE3 C-terminus preferentially bound PDZ-II although to a lesser extent NHE3 association may also occur with the NHERF-1 PDZ-I domain.

NHERF-1 Expression in PS120 Cells. Our prior studies had suggested that NHE-deficient PS120 cells also lacked NHERF-1 and NHERF-2 (*5*, *8*, *9*). However, recent studies reported the presence of NHERF-1 mRNA and protein in PS120 cells (*17*). To examine NHERF-1 expression in our PS120 cells, we undertook RT-PCR using the primers described by Ahn et al. (*17*). This amplified a product of approximately 400 bp, representing NHERF-1 mRNA (data not shown). Western immunoblots using several antibodies generated against rabbit NHERF-1 as well as anti-peptide

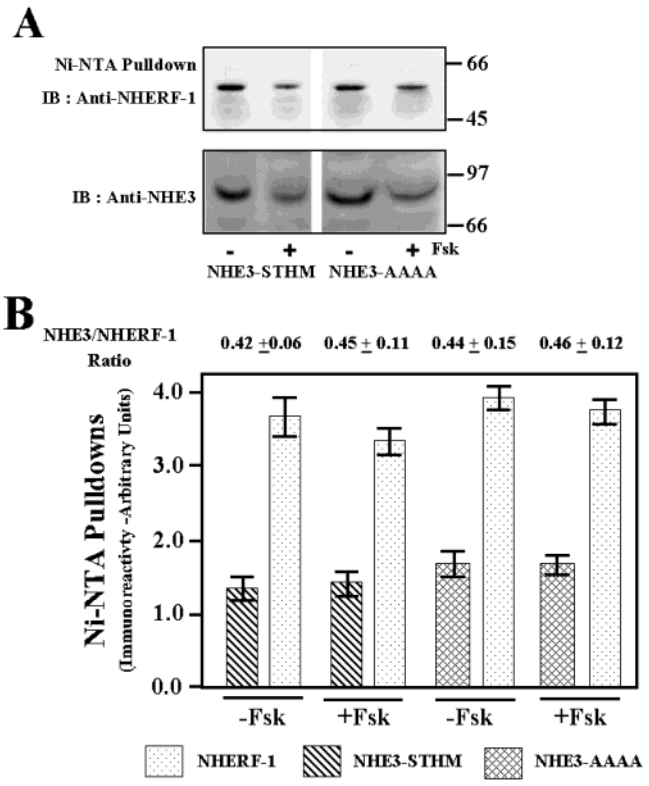

FIGURE 3: Association of NHE3 with NHERF-1 in PS120 cells. His-NHERF1 was coexpressed with WT NHE3 (STHM) or mutant NHE3-AAAA lacking the PDZ motif. Ni-NTA−agarose quantitatively sedimented His-tagged NHERF-1 from the PS120 cell lysates. Panel A: The bound proteins were subjected to SDS−PAGE and Western immunoblotting (IB) using anti-NHERF-1 (upper panel) and anti-NHE3 antibodies (lower panel). Representative results from control cells (−) and cells treated with 100 μM forskolin (+Fsk) are shown. Panel B: Scans of WT NHE3-STHM, NHE3-AAAA, and His-NHERF-1 immunoreactivity from several Ni-NTA−agarose pulldowns ($n = 4$) in the presence (+Fsk) and absence (−Fsk) of forskolin were quantified in arbitrary units and shown with SEM. The ratio of NHERF-1/NHE3, shown above, was essentially identical in all experiments.

antibodies directed against selected N- or C-terminal sequences, however, failed to detect a 55 kDa polypeptide representing NHERF-1 (data not shown). Two separate cell lines expressing His-NHERF-1 were used as positive controls, and the specificity of all anti-NHERF-1 antibodies was established by preabsorption with recombinant NHERF-1. These data established that, despite low levels of NHERF-1 mRNA, our PS120 cells expressed no detectable NHERF-1 protein.

NHERF-1 Association with NHE3 in PS120 Cells. To analyze NHE3 association with NHERF-1 in cells, we established stably transfected PS120 cells expressing His-NHERF-1 and either WT NHE3 or NHE3-AAAA, lacking a C-terminal PDZ motif. Affinity chromatography on Ni-NTA−agarose quantitatively isolated the cellular NHERF-1 complexes. Recovery of NHE3 in these complexes was analyzed by immunoblotting with anti-NHE3 antibodies (Figure 3A). Using cells expressing NHE3 alone, no direct or nonspecific association of NHE3 was observed with Ni-NTA−agarose (data not shown). His-NHERF-1 was expressed to similar levels in all cell lines and sedimented equivalent amounts of WT NHE3 and NHE3-AAAA from PS120 cells stimulated with or without forskolin (Figure 3B). WT NHE3 also bound His-NHERF-1 containing loss-of-function mutations in PDZ-I, PDZ-II, or both PDZ domains when coexpressed in PS120 cells (data not shown). These data suggested that the C-terminal PDZ motif of NHE3, essential for NHERF-1 binding in yeast, was not required for the assembly of an NHE3/NHERF-1 complex in the PS120 cells. Moreover, elevating intracellular cAMP did not influence the formation of a cellular NHE3/NHERF-1 complex.

Regulation of NHE3 Activity by NHERF-1. To investigate the functional contribution of the NHE3 PDZ motif, we analyzed BCECF fluorescence in stable PS120 cells expressing different combinations of WT and mutant NHE3 and NHERF-1 proteins. As shown in Figure 4, basal transport (expressed as ΔpH_i per second) in cells expressing WT or mutant NHE3, while consistent within individual cell lines, varied significantly between the different cell lines. This most likely represented differences in NHE3 expression although variations in surface expression and/or intrinsic transport activity could not be excluded. Most relevant for these studies, the exposure of cells expressing NHE3 alone to a cell-permeable cAMP analogue, 8-Br-cAMP (data not shown), or stimulation by forskolin resulted in no discernible change in sodium−hydrogen exchange. This emphasized that, as previously reported, PS120 cells contained insufficient NHERF-1 protein to transduce the cAMP signals that inhibit NHE3 activity (*5*, *7*−*9*, *19*). NHERF-1 coexpression resulted in a small but consistent inhibition (24.0 ± 2.4%; $p < 0.01$; $n = 5$) of transport in cells expressing NHE3-VSVG following 8-Br-cAMP or forskolin treatment. By comparison, forskolin treatment resulted much greater inhibition of sodium−hydrogen exchange in cells that coexpressed WT NHE3 without the VSVG tag and NHERF-1 (54.6 ± 3.9%; $p < 0.01$, $n = 6$). This suggested that NHERF-1 association with the NHE3 C-terminal PDZ motif enhanced the inhibition of NHE3 activity by cAMP.

To further strengthen these findings, we analyzed PS120 cells expressing NHE3-AAAA that lacked a PDZ motif. In cells coexpressing NHE3-AAAA and NHERF-1, forskolin treatment resulted in reduced inhibition of NHE3 activity (26.9 ± 7.1%; $p < 0.01$, $n = 6$) to the same extent as previously seen in cells expressing NHE3-VSVG. Additional studies investigated cAMP inhibition of WT NHE3 in cells expressing NHERF-1 mutants that lacked one or both functional PDZ domains. In PS120 cells expressing NHE3 and NHERF-1 with a mutant PDZ-I, forskolin decreased NHE3 activity by 45.6 ± 5.6% ($p < 0.01$, $n = 6$), similar to WT NHERF-1. In contrast, in cells expressing NHE3 and NHERF-1 lacking a functional PDZ-II domain, forskolin inhibited NHE3 activity to a much lesser extent (24.0 ± 5.9%; $p < 0.01$, $n = 6$). In cells expressing NHE3 and NHERF-1 lacking both functional PDZ domains, similar reduced inhibition of Na^+-H^+ exchange (25.7 ± 5.9%, $p < 0.01$, $n = 6$) was observed. These data suggested that a functional NHE3 C-terminal PDZ motif and NHERF-1 PDZ-II domain were required for maximal inhibition of NHE3 transport by cAMP and indicated a key role for the PDZ interaction between NHE3 and NHERF-1 in facilitating the cAMP signals that inhibit NHE3 activity in mammalian cells.

DISCUSSION

NHERF proteins were first identified as critical components of a multiprotein complex that transduced cAMP

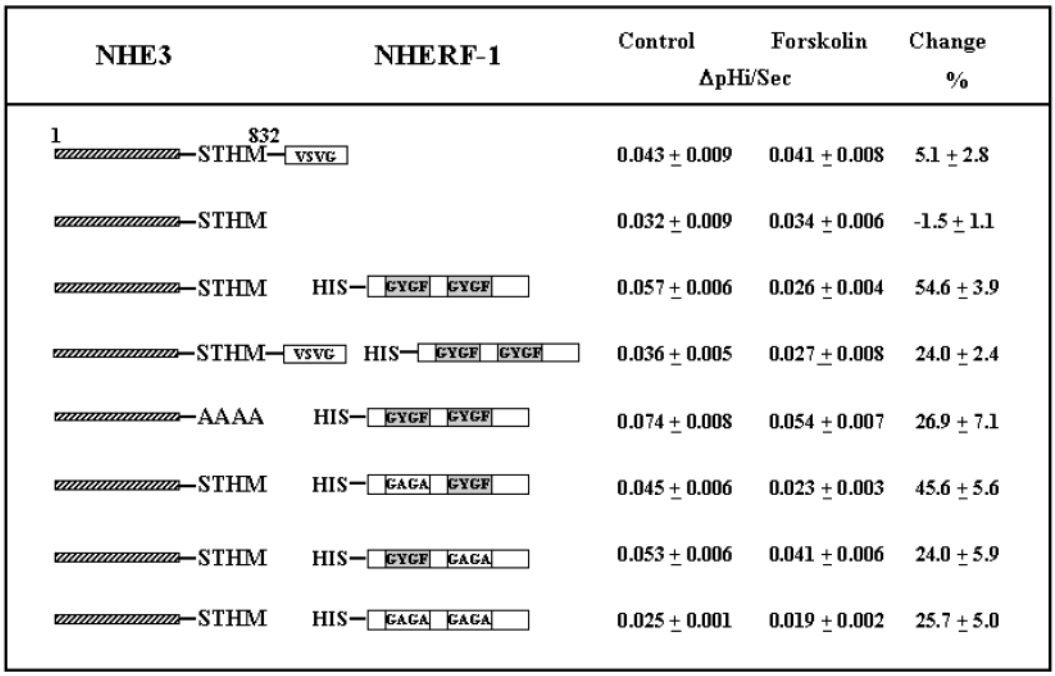

FIGURE 4: Importance of the NHE3 PDZ motif for cAMP-mediated inhibition of sodium−hydrogen exchange. The initial rates of sodium−hydrogen exchange were monitored as changes in BCECF fluorescence in PS120 cell lines expressing WT and mutant NHE3 and His-NHERF-1. BCECF measurements were made under basal conditions (control) as described in Materials and Methods and following the stimulation of cells with 100 μM forskolin (forskolin). Data obtained with NHE3 with a C-terminal VSVG epitope in the presence and absence of NHERF-1 are also shown. Experiments were undertaken six or more times and the results presented as the average rate, $\Delta pH_1/s$, with the mean of means (SEM). The difference in the initial rates under control and stimulated conditions (defined as change) is shown as a percentage with standard error of the means.

signals that inhibit NHE3 activity. The two mammalian homologues, NHERF-1 and NHERF-2, contained several protein interaction domains, including two tandem PDZ domains, an ERM (*e*zrin, *r*adixin, *m*oesin, and merlin) binding domain and, in the case of NHERF-2, a C-terminal PIF (*P*DK1-*i*nteracting *f*ragment) domain (*20*). To date, nearly 35 proteins have been shown to bind NHERF-1 and/or NHERF-2 (*21*). Most associated via their C-termini to one or both of the PDZ domains in the two NHERF isoforms, but the functional importance of these associations has not been defined for most NHERF targets. In contrast, both NHERF isoforms bound and regulated NHE3 when coexpressed in cultured PS120 cells (*7*−*9*). The emerging paradigm is that NHERF proteins scaffold NHE3 and other cellular proteins to transduce the physiological signals that control sodium−hydrogen exchange.

Serial C-terminal deletions had suggested that an internal sequence, amino acids 579−684, was required for cAMP inhibition of NHE3 activity (*2*). Overlays of NHERF-2 with ^{35}S-labeled NHE3 peptides suggested that an internal sequence, amino acids 585−660, in NHE3 represented an NHERF-2 binding site (*23*). This prompted the speculation that NHE3 differed from other NHERF targets in possessing a noncanonical internal sequence that bound an NHERF-2 PDZ domain (*23*). However, recent immunological studies showed that NHERF-1 and NHERF-2 display a distinct spatial distribution in the mouse renal proximal tubules (Wade et al., unpublished data). While NHERF-2 bound NHE3 in the renal tissue from both WT and NHERF-1 null mice (*24*), biochemical analyses of brush border membranes showed that cAMP failed to inhibit sodium−hydrogen transport in membranes from the NHERF-1 null mouse (*25*). These data argued for a unique role of NHERF-1 in transducing cAMP signals that inhibit sodium−hydrogen exchange in renal tissue, and the functional role of the NHE3/NHERF-2 complex in the mouse kidney remains to be defined.

The current studies analyzed the molecular interactions between NHE3 and NHERF-1 that specifically contributed to cAMP inhibition of NHE3 transport activity. While the C-terminus of NHE3 (STHM) did not conform to the consensus motifs defined for either the PDZ-I or PDZ-II domain of NHERF-1 using random peptides, we noted some homology in the C-termini of NHE3 and the PTH1R receptor (ETVM), which was recently identified as a NHERF PDZ target (*26*). Using the yeast two-hybrid protein interaction assay, we established that substituting C-terminal residues, STHM, with alanines abolished NHE3 association with NHERF-1. Individual alanine substitutions in positions 0, −2, and −3, but not −1, also abolished NHE3 association with NHERF-1 and suggested that the NHE3 C-terminus displayed the characteristics of a class I PDZ motif (*18*).

While the NHE3 C-terminus bound both PDZ-I and PDZ-II as isolated domains in the yeast two-hybrid assay, inactivation of the individual PDZ domains demonstrated a significant preference of NHE3 for PDZ-II, and disrupting PDZ-II function abolished NHE3 binding to NHERF-1. This suggested that spatial restrictions of the two PDZ domains in the context of a full-length NHERF-1 favored NHE3 binding to PDZ-II. Moreover, WT NHE3 (STHM) bound NHERF-1 as effectively as a mutant NHE3 containing a consensus PDZ-II motif, namely, SSWL. These data established for the first time that, in common with other NHERF targets, NHE3 contained a C-terminal PDZ motif. While not

conforming to a consensus defined for either PDZ domain using random peptides, the NHE3 C-terminus was essential for binding the NHERF-1 PDZ-II domain.

Analysis of complexes assembled by His-NHERF-1 in PS120 cells, however, yielded the surprising result that both WT NHE3 and mutant NHE3-AAAA bound His-NHERF-1. Indeed, NHERF-1 lacking both functional PDZ domains also bound NHE3 (data not shown). This suggested that other regions in NHERF-1 and NHE3 possibly via their association with other cellular proteins could override the absolute requirement for the PDZ interaction in the formation of a binary NHE3 and NHERF-1 complex. In this regard, our earlier studies noted that NHE3, detergent-solubilized rabbit renal brush border membranes, fractionated with an apparent molecular mass size of ~500 kDa (*3*). This suggested that, in addition to NHERF-1, the renal NHE3 (molecular mass 80 kDa) complex contained other cellular proteins, and further work will be required to define the full complement of proteins that comprise a cellular NHE3/NHERF-1 complex.

Transport assays in PS120 cells expressing both NHE3 and NHERF-1 provided important insight into the functional role of the NHE3 C-terminus. In cells coexpressing WT NHE3 and NHERF-1, forskolin inhibited Na^+-H^+ exchange by approximately 50%. By comparison, cells coexpressing NHE3-AAAA and NHERF-1 demonstrated a similar basal rate of Na^+-H^+ exchange, but their response to forskolin was significantly attenuated. Loss-of-function mutations in PDZ-II but not PDZ-I in NHERF-1 also attenuated the inhibitory response of NHE3 to forskolin. The attenuated response was similar to that previously reported in PS120 cells expressing NHE3 with a C-terminal VSVG tag (*2*, *5*, *8*, *9*) and provided an explanation for some of the differences in cAMP inhibition of NHE3 reported in the literature (*2*, *5*, *8*, *9*). Thus, despite the ability of NHERF-1 to associate with both WT and mutant NHE3 lacking a PDZ motif, the PDZ interaction between NHE3 and NHERF-1 was necessary for the maximal transduction of cAMP signals that inhibit NHE3 activity. Further studies of cAMP-inhibited NHE3 transport in PS120 cells and/or primary renal proximal tubule cells from the NHERF-1 null mouse should reveal the NHERF-1-dependent and independent mechanisms, which may include the direct interaction of NHE3 with the actin cytoskeleton (*22*), that regulate NHE3 activity in response to cAMP-elevating hormones.

Finally, NHERF-1 exists as a dimer in tissue and cell extracts (*27*−*30*). Homo- and heterodimerization of NHERF-1 and NHERF-2 utilize both PDZ-I and PDZ-II (*30*). The impact of NHERF dimerization on NHE3 binding and regulation is unknown but may generate an extended scaffold that recruits other cellular proteins (*31*) and promotes NHE3 oligomerization (*32*). Whether oligomerization, which may regulate NHE3 surface expression, is achieved by binding to both PDZ domains in a single NHERF-1 molecule, as reported for CFTR (*33*), or by the juxtaposition of PDZ domains in an NHERF-1 dimer, as described for the PDGF receptor (*34*), remains to be determined. Both NHERF dimerization and recruitment of NHERF targets are highly dynamic processes and may be modulated by physiological stimuli that promote the covalent modification of NHERF-1 (*8*). Further structural and functional studies of NHE3 using appropriate cellular models should provide a fuller understanding of the complex molecular events that mediate the hormonal regulation of sodium−hydrogen exchange in epithelial tissues.

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