INVITED REVIEW

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NHERF and regulation of the renal sodium-hydrogen exchanger NHE3

Abstract The sodium-hydrogen exchanger 3 (NHE3) isoform is the major regulated sodium transporter in the proximal convoluted tubule of the kidney. Study of the regulation of NHE3 by hormonal stimuli has identified a number of PDZ adaptor proteins that form an apical/subapical membrane scaffold that binds NHE3 and facilitates down-regulation of its activity in response to cAMP and activation of protein kinase A. The precise relation of proximal tubule adaptor proteins such as sodium-hydrogen exchanger regulatory factor-1 (NHERF-1), NHERF-2, and PDZ domain-containing-protein-1 (PDZK1) with each other and with protein targets such as NHE3 has been evolving with the development of specific reagents and genetically altered animals. In this review, we trace the discovery of NHERF-1 and NHERF-2, and update our current understanding of the relation between these proteins and the regulation and trafficking of NHE3.

Keywords PDZ adaptor proteins · NHERF-1 · NHERF-2 · Renal sodium reabsorption · NHE3

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Introduction

The cloning of the brush border membrane (BBM) sodium-hydrogen exchanger isoform 3 (NHE3) was a seminal advance in the understanding of how sodium reabsorption and hydrogen ion secretion are regulated in the renal proximal convoluted tubule. The discovery of the NHE regulatory factor-1 (NHERF-1) was linked intimately to the quest to understand the hormonal regulation of NHE3 and, while NHERF-1 and the related NHERF-2 protein have now been linked to many biologic processes in the kidney and other organs, the relation between the NHERF proteins and NHE3 is the most advanced and best characterized. In this review, we will relate our own initial attempts to isolate the molecular determinants that regulate NHE3 and the discovery of NHERF as a by-product of these experiments. We will then extend this narrative to describe our current knowledge of the role of the NHERF proteins in the short- and long-term regulation of NHE3.

NHE and the renal tubular reabsorption of sodium and bicarbonate

Pitts was among the first to note that filtered bicarbonate was reabsorbed completely in the nephron and postulated the presence of a sodium for hydrogen exchange mechanism [1]. Some 20 years later Aronson and co-workers, and Murer and colleagues provided independent characterization of this transporter using isolated renal BBM vesicles [2, 3]. Elegant studies from these and other laboratories have described the kinetics and coupling ratios as well as providing a fundamental description of factors that regulate NHE activity in the renal proximal tubule [4–8]. This transporter is responsible for the reabsorption of up to 1/3 of the filtered load of sodium in the renal proximal tubule and for the titration and subsequent return of nearly all the filtered bicarbonate to the systemic circulation. Quite remarkably for a transporter designed for bulk reabsorption,

NHE3 activity is regulated tightly by a variety of hormones including α- and β-adrenergic stimuli, dopamine, parathyroid hormone, angiotensin II, and prostaglandins, and its activity is altered in animal models of acid-base disorders [9, 10]. By the late 1980s, there was growing interest in knowing the structure of NHEs, which had been implicated in a variety of cell processes including cell growth, cancer, and hypertension [8, 10]. Early attempts to purify NHE from the kidney using affinity chromatography and ligands such as amiloride, while initially promising, were ultimately unsuccessful [11, 12].

Attempts to isolate the cAMP-inhibited renal BBM NHE

In 1987, we reported results of in-situ renal proximal tubule microperfusion experiments examining the effect of cAMP on water and sodium reabsorption in rats [13]. As found previously by others, cAMP inhibits proximal tubule sodium transport [14–16]. What was unique about our experiments was the lack of organic solutes in the solution perfused through the proximal tubule such that under the conditions of the experiments, only NHE was likely to be operative in mediating sodium and water reabsorption. We reasoned that cAMP, by activating protein kinase A (PKA), might promote phosphorylation of the apical membrane NHE and that identification of the PKA substrates in the apical membrane might lead to isolation of the cAMP-regulated transporter [17]. Although the thesis that PKA-mediated phosphorylation of NHE3 might inhibit its activity eventually proved correct, our reasoning at the time of these studies now appears rather naïve [18–22].

To identify the PKA substrates in the apical membrane of the rabbit renal proximal tubule, BBM were harvested and the vesicles rendered permeable to PKA, ATP, and magnesium using hypotonic shock. SDS-PAGE and autoradiography demonstrated nearly 20 polypeptides that are clearly phosphorylated by PKA in vitro in a time frame consistent with the physiological response, i.e., inhibition of NHE activity (Fig. 1) [23]. These studies highlighted the promiscuous nature of PKA-mediated phosphorylation of BBM proteins and the recognition that the potential NHE3 candidate polypeptides were far too numerous to analyze effectively. To reduce the number of candidate phosphoproteins, we elected to fractionate the solubilized BBM proteins and to develop a functional assay whereby these fractions could be incorporated into artificial lipid membranes and assayed for pH-dependent sodium uptake [24]. Solubilization and reconstitution of the renal BBM NHE, however, proved difficult and required many experiments to test various combinations of detergents and lipid constituents. While we were addressing these technical issues, Sardet and co-workers, using a more direct approach employing a negatively selected fibroblast cell line that lacks NHE activity (PS120 cells), cloned the first member of the NHE
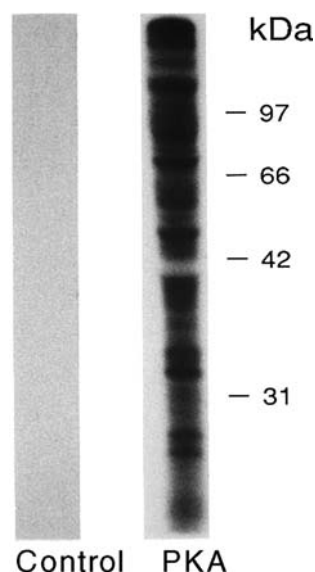

Fig. 1 The phosphorylation of renal brush border membrane (BBM) proteins by protein kinase A (*PKA*). Rabbit BBM were incubated with [^{32}-P]-ATP and magnesium in the absence (*Control*) or presence of PKA (or cAMP). Proteins were resolved by SDS-PAGE and the phosphoproteins detected by autoradiography (adapted from [23])

family, NHE1 [25]. Shortly thereafter, several other members of the NHE family were cloned using a homology based strategy. Among these was NHE3, the epithelial isoform found in the BBM of the renal proximal tubule [26, 27].

Although NHE3 had now been identified, we continued our experiments and eventually developed an in vitro NHE assay using the detergent octyl glucoside and soybean phospholipid liposomes. As shown in Fig. 2, PKA-mediated phosphorylation of freshly prepared BBM proteins inhibits NHE activity, indicating that the
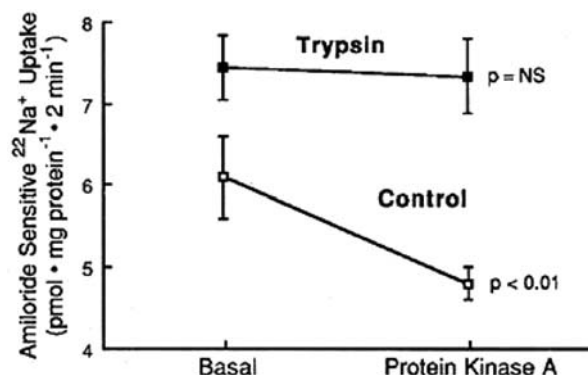

Fig. 2 The effect of protein kinase A on Na^+-H^+-exchanger-3 (NHE3) activity using solubilized renal BBM proteins. Freshly prepared detergent solubilized rabbit BBM proteins were studied under control conditions or after limited trypsin digestion. Proteins were then incubated in ATP and magnesium in the absence (*Basal*) or presence of the catalytic subunit of PKA and NHE activity assayed after reconstitution into soy bean phospholipids vesicles (adapted from [29])

assay was functional and consistent with in vivo findings [28]. However, very early in these studies, we noted that freezing and thawing the solubilized BBM proteins resulted in equivalent or increased basal NHE transport activity but complete loss of PKA regulation. We envisioned that the freezing and thawing resulted in partial proteolysis of NHE3 that abrogated PKA-mediated phosphorylation and inhibition of activity. This speculation was supported by our subsequent studies that used limited trypsin digestion of solubilized BBM proteins to reproduce the effects of freezing and thawing, namely equivalent or enhance basal NHE activity with complete loss of PKA regulation [29].

Isolation and cloning of NHERF-1

The dissociation of basal NHE activity from PKA regulation by limited trypsin digestion provided a reproducible assay for analyzing the cellular factors that regulated NHE3. We believed initially that limited exposure to trypsin abolished a critical phosphorylation site on NHE3, but soon recognized the alternative possibility that trypsin may digest a key regulatory protein or proteins present in the solubilized BBM protein mixture. Thus, we proceeded to fractionate freshly solubilized BBM proteins using a combination of gel filtration and ion exchange chromatography to isolate a fraction that has no intrinsic NHE activity when incorporated into lipid vesicles, but restores the inhibitory effect of PKA when combined with the trypsin-treated BBM proteins [30]. We called this fraction the Na-H

exchanger regulatory factor (NHERF). This doubly extracted protein fraction contained one major phosphoprotein of approximately 55 kDa. Subsequent studies elucidated the N-terminal sequence of this polypeptide and, by using an appropriate oligonucleotide probe, we cloned the full-length NHERF cDNA from a rabbit kidney library [31]. Bacterially expressed recombinant NHERF protein was assayed in reconstituted proteoliposomes containing solubilized trypsinized BBM proteins and provided clear evidence that the single polypeptide, NHERF, restores the inhibitory effect of PKA on NHE activity [32]. In collaboration with Donowitz, Yun and others, we also co-expressed NHERF with NHE3 in PS120 fibroblasts and established that NHERF is not required for basal NHE activity, but is essential for cAMP-mediated inhibition of this transporter (Fig. 3) [33].

Discovery of NHERF-2 and the structure of the NHERF family of adaptor proteins

NHE3 kinase-A regulatory protein (E3KARP, later renamed NHERF-2) was identified as a protein interacting with the C-terminus of NHE3 in a yeast 2-hybrid screen [33]. We had noted previously that NHERF (now renamed NHERF-1) has two repeat sequences of approximately 100 amino acids and we had postulated that these domains might represent protein binding domains [31]. By the time NHERF-2 was identified, these domains were recognized as PSD-95/Discs large/ZO-1 (PDZ homology) protein interaction domains (Fig. 4).

Fig. 3 The role of sodium-hydrogen exchanger regulatory factor-1 (*NHERF-1*) and NHERF-2 in cAMP regulation of NHE3 activity in PS120 cells. NHE activity was assayed in PS120 cells expressing rabbit NHE3 alone (*right panels*) or co-expressing NHERF-1 (*top left panel*) or NHERF-2 (*bottom left panel*). Studies were performed in the absence or presence of cAMP (adapted from [33])
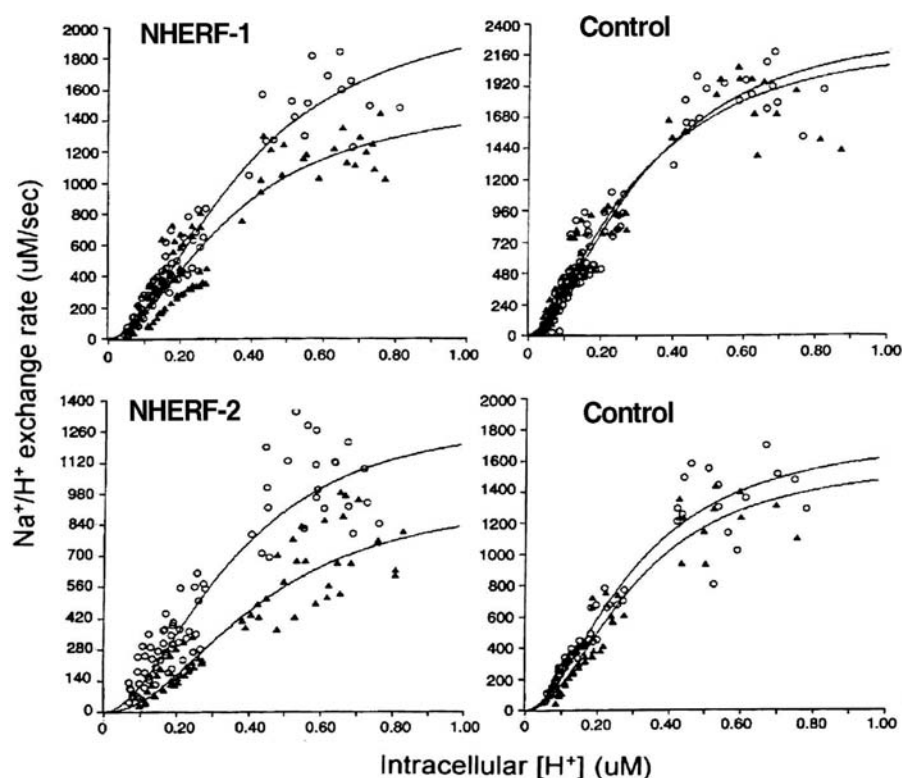
NHERF-2, like NHERF-1, contains two tandem PDZ domains and, when co-expressed with NHE3 in PS120 cells, mediates cAMP inhibition of NHE3 activity (Fig. 3) [33]. Bretscher and colleagues independently cloned human NHERF-1 [called ezrin binding protein of 50 kDa, (EBP50)] by its interaction with conserved N-terminal sequences in the ezrin, radixin, and moesin (ERM) family of cytoskeletal-binding proteins [34]. The C-terminus of NHERF-2 also contains an ERM binding domain.

NHERF-1 and NHERF-2 represent distinct gene products and share only 40–50% overall amino acid identity. A key difference between these two proteins is that NHERF-1 is a phosphoprotein in mammalian cells, whereas NHERF-2 is not [32]. To date, at least six distinct phosphorylation sites have been identified in NHERF-1 although the physiological role of these covalent modifications, and the identity of the relevant protein kinases and phosphatases, remain unknown [35].

NHERF-1, NHERF-2, and NHE3 regulation

Biochemical studies in PS120 cells established that the NHERF proteins bound both NHE3 and ezrin leading to the model shown in Fig. 5 [36–38]. We proposed that NHERF-1 and/or NHERF-2 link(s) NHE3 to the actin cytoskeleton through ezrin, thus forming a multi-protein complex. This preassembled signaling complex is not influenced by changes in cAMP, but is necessary to transduce the cAMP signals that inhibit NHE3. Additional experiments established that ezrin functions as a PKA anchoring protein and that the ERM domain of NHERF is critical in mediating cAMP phosphorylation of NHE3 and inhibition of NHE3 activity [39–42]. From numerous experiments, we concluded initially that in cells which expressed both proteins, NHERF-1 and NHERF-2 represents a redundant control mechanism for mediating the hormonal control of NHE3.

Understanding the physiological role of NHERF proteins and NHE3 took on added complexity with the discovery that both NHERF proteins have numerous cellular targets, including transporters and ion channels, hormone and growth factor receptors, signaling proteins, scaffolding proteins, and transcription factors [35, 43]. Many of these NHERF targets are known to, or
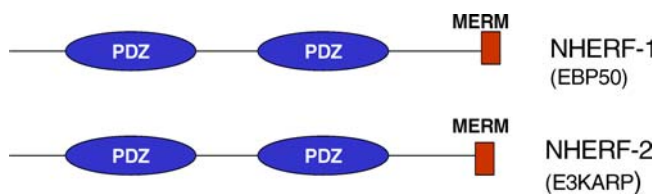
Fig. 4 The domain structure of NHERF-1 (synonym: ezrin binding protein of 50 kDa *EBP50*) and NHERF-2 (synonym: NHE3 kinase-A regulatory protein *E3KARP*). Domain structure of NHERF-1 and NHERF-2 indicating the presence of tandem PSD-95/Discs large/ZO-1 (*PDZ*) domains and a C-terminal ezrin-radixin-moesin-merlin (*MERM*) binding domain in both proteins
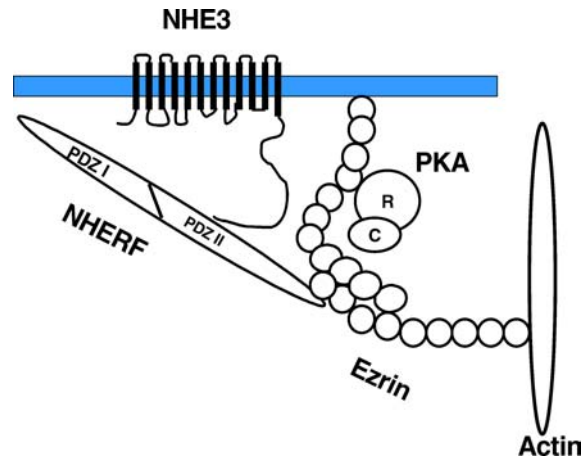
Fig. 5 Proposed model of the assembly of a multi-protein complex of NHE3, NHERF, ezrin, actin, and protein kinase A (adapted from [64])

have the potential to, influence the regulation of NHE3. The hormones PTH, and dopamine activate multiple signaling pathways, but cAMP-mediated phosphorylation of NHE3, mediated by its association with the NHERF proteins, appears to be central for the inhibitory effect of these hormones on NHE3 activity and the reabsorption of sodium, bicarbonate, and water in the renal proximal tubule [21, 22]. The model that has emerged is that NHERF scaffolds the ezrin-PKA complex in proximity to NHE3 to facilitate the phosphorylation of specific C-terminal serine residues on NHE3 and the down-regulation of its transport activity [44]. By contrast, β_2-adrenergic agonists, which also elevate cAMP levels in the renal proximal, stimulate rather than inhibit NHE3 activity [45]. This apparent contradiction was resolved with the findings that NHE3 interacts predominantly with the PDZ II domain of NHERF-1, while the β_2-adrenergic receptor interacts with the PDZ I domain in an agonist-dependent manner [46]. When the agonist-occupied β_2-adrenergic receptor engages NHERF-1, it disrupts the association of NHERF-1 with NHE3, thereby stimulating NHE3 activity despite the intracellular generation of cAMP. This scenario differs from the interaction of NHERF with the cystic fibrosis transmembrane regulator (CFTR), where two CFTR proteins engage each of the PDZ domains in a single NHERF-1 molecule, resulting in more effective chloride transport [47]. These paradigms highlight the diversity of NHERF-1 as a regulator of multiple physiologic events that may, at least in part, be dictated by the stoichiometry of protein binding and the subcellular localization of the proteins, both of which may be subject to dynamic regulation within the cell.

Cellular distribution of NHERF-1 and NHERF-2

Critical for the understanding of the biological roles of the two NHERF proteins in the mammalian kidney is

Fig. 6 The localization of NHERF-1 and NHERF-2 in the proximal tubule of the kidney of wild-type mice. Confocal microscopy images of wild-type mice proximal convoluted tubules using monospecific antibodies to NHERF-2 (*left panel*) and NHERF-1 (*middle panel*). The merged image is shown in the right panel (adapted from [49])
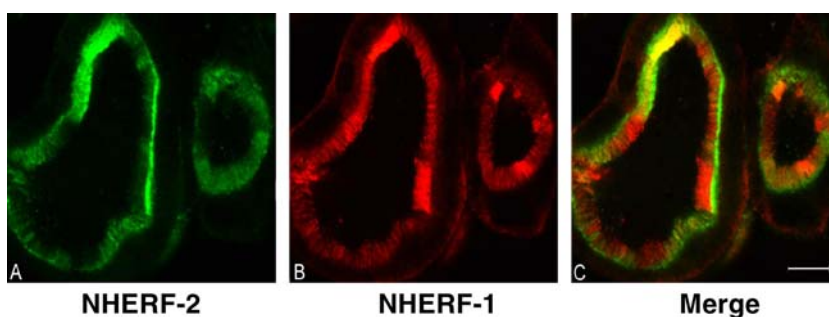
knowledge of their cellular distribution. Using mono-specific antibodies against NHERF-1 and NHERF-2, we have demonstrated that the rat proximal tubule expresses only NHERF-1 that co-localizes with NHE3 at the apical membrane [48]. NHERF-2 is present in the glomerulus, peritubular capillaries and descending vasa recta, and in specific distal tubule cells but is not detected in proximal tubule cells. OK cells, a proximal tubule cell line, also express only NHERF-1. Thus, despite the apparent redundancy in function of NHERF-1 and NHERF-2 as regulators of NHE3 in model PS120 cells, our data suggested that only NHERF-1 is localized appropriately to function as a biologic regulator of NHE3. As our studies continued, however, we noted that in contrast to rat, the proximal tubule of man, mouse, and possibly rabbit, expresses both NHERF-1 and NHERF-2 [49, 50]. Figure 6 represents a confocal microscopy image of proximal tubules from wild-type mice. NHERF-1 distributes to the microvillus and co-localizes with NHE3 and Npt2a, the major sodium-dependent phosphate transporter in the proximal tubule [49]. On the other hand, NHERF-2 is located predominantly in a submicrovillar region. These findings again raised significant questions about the ability of both NHERF proteins to regulate common events such as the hormonal control of NHE3 and suggested, at least indirectly, distinct roles for the two NHERF isoforms in the mammalian kidney.

Development of the *NHERF-1* null mouse

NHERF-1, NHERF-2, and other renal brush border PDZ adaptor proteins such as PDZK1, homo- and heterodimerize, and have been speculated to form a microvillar/submicrovillar scaffold that orients, retains, or otherwise targets apical membrane proteins [51–54]. These three PDZ adaptor proteins also share many common targets. Attempts to determine how these proteins interact with one another, as well as with targets such as NHE3 seemed to require a genetic approach and studies in appropriate tissues. Using homologous recombination and a vector targeting exon 1 of the mouse *NHERF-1* gene, we successfully abolished NHERF-1 expression in all mouse tissues [55]. Male $NHERF-1^{-/-}$ mice display no overt phenotype. Blood pressure, serum electrolytes, renal function, and renal

histology are normal. However, mutant mice demonstrate mild hypophosphatemia and, compared with wild-type mice, increased urinary excretion of phosphate, calcium, and uric acid ([55], E. Weinman, R. Cunningham, S. Shenolikar, unpublished observations). Some NHERF-1 null female mice are runted and demonstrate severe osteoporosis and bone fractures. Other female littermates look normal and have been bred to establish an NHERF-1 null mouse colony. Like the males, the $NHERF-1^{-/-}$ females show increased urinary excretion of phosphate, calcium, and uric acid (E. Weinman, R. Cunningham, S. Shenolikar, unpublished observations). The defect in phosphate transport in the mutant mice is associated with a decrease in the renal apical membrane expression of Npt2a, suggesting a role for NHERF-1 in the targeting or trafficking of this transporter in the renal proximal tubule [55].

The absence of NHERF-1 in null mice has no apparent effect on the abundance or cellular distribution of NHERF-2 or PDZK1 [49, 55, 56]. The abundance and distribution of NHE3 in the null mice also does not differ from wild-type controls (Fig. 7). This finding suggested that NHERF-1 is not involved in the trafficking or membrane retention of NHE3; a finding that would not have been predicted from prior studies in OK cells (see the following text) [19, 22]. To determine if the absence of NHERF-1 results in abnormalities in PKA regulation of NHE3, renal apical membranes from wild-type and *NHERF-1* null mice were harvested, phosphorylated using PKA ex-vivo, and NHE3 activity determined as the amiloride-sensitive component of pH gradient-stimulated sodium uptake [57]. Basal NHE3 activity does not differ between wild-type and $NHERF-1^{-/-}$ renal BBM. Activation of PKA results in nearly 50% inhibition of NHE3 activity in wild-type membranes, but fails to affect NHE3 activity in membrane vesicles from null mice. The failure of PKA to inhibit NHE3 activity in $NHERF-1^{-/-}$ BBM vesicles is associated with the failure of PKA-mediated phosphorylation of NHE3, the biochemical signature of this form of regulation. Recently, we have extended these observations using primary cultures of proximal convoluted tubule cells [56]. Basal NHE3 activity, assayed using fluorescence measurements, does not differ between wild-type and *NHERF-1* null proximal tubule cells. Treatment of the cells with parathyroid hormone or cAMP inhibits NHE3 activity in wild-type cells but,
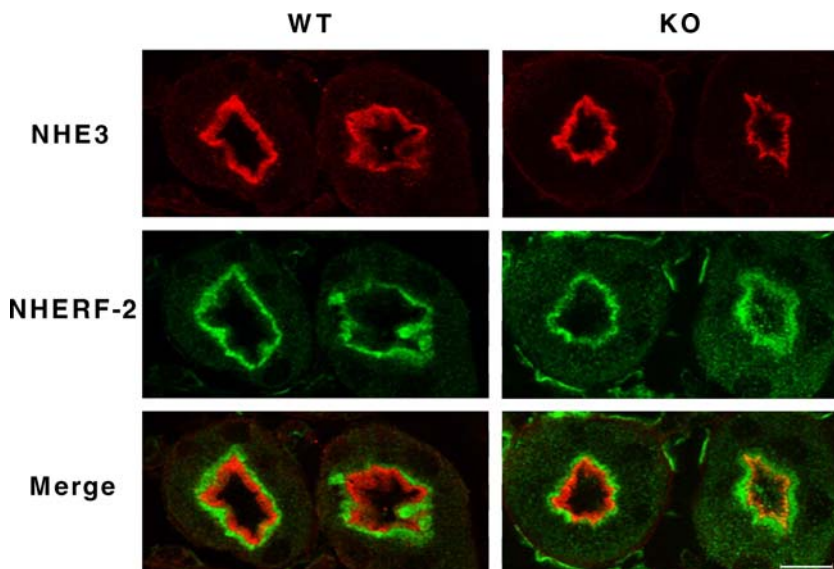
as observed in the BBM, fails to affect NHE3 activity in the *NHERF-1* null proximal tubule cells. Adenovirus-mediated NHERF-1 gene transfer into the *NHERF-1* null cells completely restores the inhibitory responses to parathyroid hormone and cAMP. When considered together, these results suggest strongly that NHERF-1 is critical for PKA-mediated phosphorylation and acute regulation of the NHE3 transporter in response to hormones that elevate intracellular cAMP. These results also confirm the original observations that NHERF-1 is a required co-factor for cAMP regulation of NHE3 and that the presence of NHERF-2 and PDZK1 cannot substitute for the absence of NHERF-1 in renal tissue. These findings highlight the fact that model cell systems such as PS120 cells, while useful for certain studies, may not reflect accurately the relation of NHERF proteins and their targets in their natural environment, and establish that the functions of NHERF-1 and NHERF-2 are not entirely redundant.

NHERF and the regulation of NHE3 trafficking

While the distribution NHE3 in *NHERF-1$^{-/-}$* mice is unchanged, other data suggest a role for the NHERF proteins in the trafficking of NHE3. In OK cells, NHE3 retrieved from the apical membrane is recycled back to the plasma membrane via endosomal vesicles [19, 22]. Moe and colleagues have studied the inhibitory effect of parathyroid hormone and dopamine on NHE3 in OK [19]. The acute short-term inhibition of NHE3 activity by parathyroid hormone is not associated with changes in the membrane abundance of NHE3, but the longer-term regulation of NHE3 by parathyroid hormone is associated with decreased apical membrane expression of NHE3. Both the acute as well as the longer-term regulation of NHE3 by PTH require PKA-mediated phosphorylation of specific serines in the C-terminus of the transporter. Dopamine acutely inhibits sodium-hydrogen exchange activity, primarily by decreasing the apical membrane abundance of NHE3, a process that also involves PKA-mediated phosphorylation of the transporter [22]. Although other mechanisms for PKA-mediated phosphorylation of NHE3 cannot be discounted, to date, the NHERF-mediated phosphorylation of NHE3 remains the best understood. The distinction between the mouse kidney, in which the absence of NHERF-1 has no apparent effect on the apical targeting of NHE3, and OK cells, which down-regulate the apical membrane abundance of NHE3 in response to long-term parathyroid hormone or acutely to dopamine, remains unresolved and may reflect, at least in part, the presence of NHERF-2 in native tissue which, via its association with NHERF-1, modulates some of its cellular functions. Consistent with this view are recent studies of Lee-Kwon, Donowitz and co-workers demonstrating that NHERF-2, even in the presence of NHERF-1, regulates the trafficking of NHE3 by virtue of its association with other proteins [58, 59]. The development of NHERF-2 and *NHERF-1/2* null mice may help unravel the role of the various PDZ adaptor proteins expressed in epithelial tissue in the regulation of their targets.

Perspective

The NHERF proteins were the first PDZ-type adaptor proteins found in epithelial tissue. Since their discovery and the associated discovery of PDZK1, these proteins have been found to interact with a surprisingly large number of proteins, many of which are expressed in the kidney and have been demonstrated to play a role in ion and mineral homeostasis. To date, over 60 targets have been identified and the list continues to grow [9, 35, 43, 44]. Moreover, NHERF proteins appear to regulate transporters such as the sodium-dependent bicarbonate transporter and Na/K ATPase by mechanisms that do

not involve the direct binding [60, 61]. These transporters are located predominantly in the basolateral membrane of renal tubular cells. Since the NHERF proteins are most abundant in the apical membrane of these cells, the precise interactions are, as yet, unknown but it might be suggested that the NHERF proteins interact with other signaling proteins that affect these basolateral transporters more directly. Full understanding of the roles of the NHERF proteins in biologic processes will require detailed study of relevant tissues. It has already been established that biochemical modification of NHERF targets such as the β_2 adrenergic receptor and platelet-derived growth factor receptors can alter its binding to NHERF-1 and the biological responses of these receptors [62, 63]. Unexplored, however, is the unique role of NHERF-1 phosphorylation that potentially may affect the binding interaction between targets to one or both of the PDZ domains or to the ERM domain. We think it possible, and perhaps likely, that numerous signaling pathways, acting through specific protein kinases and phosphatases, dynamically regulate NHERF-1 and, as a consequence, its physiological effects in kidney and other organs.

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