Urol Res (2010) 38:257–262
DOI 10.1007/s00240-010-0294-1

SYMPOSIUM PAPER

# Role of NHERF and scaffolding proteins in proximal tubule transport

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Received: 14 June 2010 / Accepted: 25 June 2010 / Published online: 15 July 2010
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**Abstract** Eukaryotic cells coordinate specific responses to hormones and growth factors by spatial and temporal organization of "signaling components." Through the formation of multiprotein complexes, cells are able to generate "signaling components" that transduce hormone signals through proteins, such as PSD-95/Dlg/ZO-1(PDZ)-containing proteins that associate by stable and dynamic interactions. The PDZ homology domain is a common protein interaction domain in eukaryotes and with greater than 500 PDZ domains identified, it is the most abundant protein interaction domain in eukaryotic cells. The NHERF (sodium hydrogen exchanger regulatory factor) proteins are PDZ domain-containing proteins that play an important role in maintaining and regulating cell function. NHERF-1 was initially identified as a brush border membrane-associated phosphoprotein essential for the cAMP/PKA-induced inhibition of the sodium hydrogen exchanger isoform 3 (NHE3). Mouse, rabbit and human renal proximal tubules also express NHERF-2 (E3KARP), a structurally related protein, which in model cell systems also binds NHE3 and mediates its inhibition by cAMP. PDZK1 (NHERF-3) and IKEPP (NHERF-4) were later identified and found to have similar homology domains, leading to their recent reclassification. Although studies have revealed similar binding partners and overlapping functions for the NHERF proteins, it is clear that there is a significant amount of specificity between them. This review focuses primarily on NHERF-1, as the prototypical PDZ protein and will give a brief summary of its role in phosphate transport and the development of some forms of nephrolithiasis.

**Keywords** Phosphate transport · Parathyroid hormone · Calcium · Uric acid · Renal proximal tubule

Proceedings paper from the 3rd International Urolithiasis Research Symposium, Indianapolis, IN, USA, 3–4 December 2009.

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## PDZ protein-interactive domains—the NHERF proteins

In studies originally designed to isolate the cAMP-regulated sodium hydrogen exchanger 3 (NHE3) from the renal brush border membrane (BBM), we observed that cAMP-mediated inhibition of the transporter required the presence of a protein co-factor [1, 2]. This co-factor was isolated and named the sodium hydrogen exchanger regulatory factor (NHERF) [2]. NHERF (NHERF-1) contains a duplicated 100 amino acid sequence, a sequence that is recognized as a PDZ protein interactive domain. Additional related PDZ domain-containing proteins were identified in epithelial tissue which have been grouped and renamed NHERF-2 (E3KARP), NHERF-3 (PDZK1) and NHERF-4 (IKEPP)

recognizing the common evolutionary origin of the PDZ domains of these adaptors [3] (Fig. 1). NHERF-1 and NHERF-2 contain two protein-interactive PDZ domains and a C-terminal domain that binds the related cytoskeletal proteins, Moesin, Ezrin, Radixin and Merlin (MERM), while NHERF-3 and NHERF-4 contain 4 PDZ domains. The NHERF proteins form homodimers and heterodimers and have been suggested to participate in the formation of protein scaffolds by nucleating multiprotein complexes. Of the four proteins, NHERF-1 has been the best studied and represents a prototypic adaptor protein. Over 60 protein targets have been identified for NHERF-1, including transporters and ion channels, signaling proteins, hormone receptors and cytoskeletal structural elements of cells (see [1] for a detailed review).

As research involving the NHERF proteins has advanced, it has become clear that the NHERF proteins are able to self-associate and it is thought that they function cooperatively in the regulation of some cellular functions. It is also apparent that there is a specific ordering and cellular location of these proteins, and that the study of model cell systems has not always recognized or reproduced the importance of this organization. For example, in vitro studies have demonstrated that each of the NHERF proteins can bind to NHE3 and Npt2a, two important regulated transporters in the renal proximal tubule of the kidney [4]. However, studies in native tissues have determined that only NHERF-1 plays a critical role in their regulation. These studies and studies in genetically altered mice clearly indicate the specificity of interactions between transporters and individual members of the NHERF family of proteins [4].

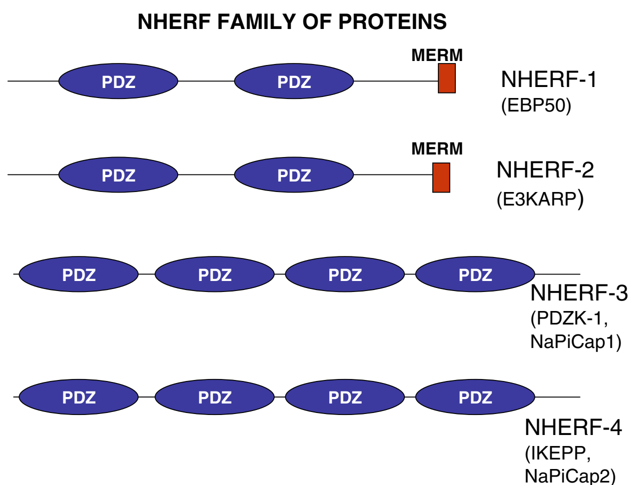


**Fig. 1** The NHERF family of proteins. Adaptor proteins that via their PDZ protein-interactive domains have the ability to interact with each other as well as other membrane proteins, ion transporters and receptors. NHERF-1 and NHERF-2 contain 2 PDZ domains and a Moesin, Ezrin, Radixin and Merlin (MERM-binding) domain, while NHERF-3 and NHERF-4 each contain 4 PDZ domains

## Targeting and trafficking of membrane proteins—regulation of the renal sodium phosphate co-transporter

There is significant evidence for NHERF-1 in the apical targeting and trafficking of several membrane proteins. The first evidence that NHERF-1 regulated the surface expression of membrane targets was derived from studies of several GPCRs [5, 6]. More recent studies have shown that NHERF-1 may also dictate the plasma membrane targeting and activity of ion transporters, such as CFTR, Npt2a and ROMK [7, 8]. Given that model cell systems do not always reflect the more complicated biology of protein interactions in native tissues, we developed a NHERF-1 null mouse to allow for the study of the integrated physiology of this protein. The most striking abnormality in the NHERF-1 null mice was the two to threefold increase in the urinary excretion of phosphate. Immunoblotting showed that while there was no change in the total renal content of Npt2a, the major regulated sodium phosphate co-transporter in this tissue, there was a significant decrease in Npt2a in the BBM from the mutant mice [8] (Fig. 2). Immunohistochemistry of renal tissue from NHERF-1 null mice confirmed the decrease in the apical localization of Npt2a and the presence of an increased amount of Npt2a protein in intracellular vesicles [9] (Fig. 3). This indicated that NHERF-1 plays a critical and specific role, not duplicated by other renal PDZ proteins in the intracellular trafficking of Npt2a.

Subsequent studies established a defect in phosphate transport elicited by changes in dietary phosphate in the NHERF-1 null mice. This finding was associated with impaired targeting of Npt2a to BBM and increased accumulation of Npt2a in intracellular vesicles in the renal proximal tubule cells from the mutant mice [10]. In these studies, mouse proximal tubule cells were incubated for 72 h in media containing low (0.3 mM) or high (1.9 mM) phosphate to mimic changes elicited by dietary phosphate in the intact animals and sodium-dependent $^{32}$P phosphate uptake was determined. The cells derived from NHERF-1 null mice displayed significantly lower rates of sodium-dependent

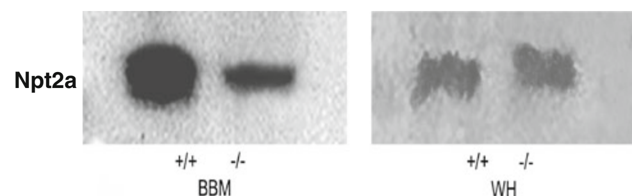


**Fig. 2** Immunoblots of whole cell lysates (WH) and isolated brush border membranes (BBM) from wild-type and NHERF-1-null mice using an antibody for Npt2a

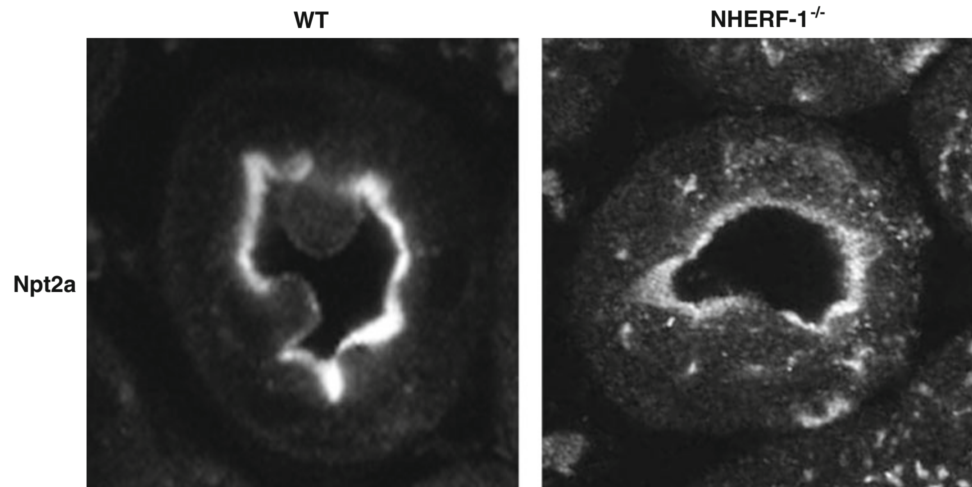


**Fig. 3** Representative confocal image of proximal tubules from wild-type and NHERF-1 null animals with antibody staining to Npt2a. In the wild-type animal on the *left*, there is distinct antibody labeling of Npt2a within the brush border, in contrast to the NHERF-1 animal on the *right*, in which there is less brush border membrane staining and increased intracellular staining of Npt2a

**Table 1** Sodium-dependent phosphate transport was determined in wild-type and NHERF-1 null cultured renal proximal tubule cells incubated in low (0.3 mM) or high phosphate (1.9 mM) media

| | High phosphate | Low phosphate | Percentage of increase with low phosphate |
|---|---|---|---|
| Wild-type cells | 6.1 ± 1.5 | 9.6 ± 2.2 | 36 ± 6.3 |
| NHERF-1 null cells | 1.8 ± 0.6 | 1.9 ± 0.6 | 2.4 ± 5.3 |

The results are calculated as nmol/mg of protein/10 min and expressed as mean ± SE

Statistical significance * $p < 0.05$ (the effect of incubation in low or high phosphate media on sodium-dependent phosphate transport in cultured proximal tubule cells from wild-type and NHERF-$1^{-/-}$ mice)

**Table 2** Western immunoblots were performed from membrane preparations of proximal tubule cells in culture from wild-type and NHERF-1 null animals

| | Npt2a (%) | NHERF-3 (%) | NHERF-1 (%) | NHERF-2 (%) |
|---|---|---|---|---|
| NHERF-$1^{+/+}$ | 33 ± 3 | 44 ± 9 | 1 ± 7 | 3 ± 13 |
| NHERF-$1^{-/-}$ | 3 ± 7 | 37 ± 11 | Not detected | 5 ± 15 |

Expressed as percent change in expression after incubation in low versus high phosphate media

Wild-type cells had an increase in the expression of Npt2a and NHERF-3. In contrast, NHERF-1 null cells in culture had no change in the expression of Npt2a, but a similar increase in the expression of NHERF-3 (Percent change in expression of Npt2a, NHERF-3, NHERF-1 and NHERF-2 in low versus high phosphate media in wild-type and NHERF-$1^{-/-}$ proximal tubule cells.)

phosphate transport when compared with wild-type (WT) cells in either low or high phosphate media [9]. Moreover, in contrast to WT cells, which showed higher sodium-dependent phosphate transport when maintained in low versus high phosphate media, sodium-dependent transport in NHERF-1 null cells was independent of the phosphate concentration in the culture media [10] (Table 1). The plasma membrane expression of Npt2a from WT cells maintained in low phosphate media showed elevated Npt2a levels when compared with WT cells incubated in either normal or high phosphate media [10]. By comparison, Npt2a levels in the NHERF-1 null proximal tubule cells were unchanged by altering the phosphate content of the culture media [10] (Table 2). Of note, NHERF-3 was upregulated to a similar degree by the low phosphate media in both WT and NHERF-1 null proximal tubule cells [10]. This suggested that the signals induced by phosphate deprivation that result in the increased abundance of NHERF-3 in renal BBM were independent of NHERF-1 and were still intact in the NHERF-1 null cells.

In addition to the hypophosphatemia and increased urinary excretion of phosphate exhibited by NHERF-1 null mice, these animals also had an increase in the urinary excretion of uric acid (not shown) and calcium as compared to wild-type animals [9, 11] (Table 3). A more detailed longitudinal study of both male and female NHERF-1 null mice found that the phosphate abnormalities were associated with increased 1,25 (OH)2 vitamin D concentrations [11]. We postulated that the increase in the urinary excretion of calcium was due to an increase in the gut absorption of calcium [11]. Moreover, and similar to Npt2a, the increased urinary excretion of uric acid was also associated with altered apical membrane targeting of URAT1, the major uric acid transporter in renal proximal tubule cells [12] (Fig. 4). The phenotype of the NHERF-1 null mice would then predict urine prone to the formation of kidney stones and renal calcium deposition, and staining of the renal papilla of male and female mice confirmed that there was striking calcium deposition in the renal papilla of NHERF-1 null mice, but not in wild-type controls [11] (Fig. 5). Moreover, consistent with these findings is a recent study by Prie et al. which recognized the phenotypic

**Table 3** Blood and urine analyses performed on wild-type and NHERF-1 null mice

| | +/+ | −/− |
|---|---|---|
| Cr Cl (μl/min) | 110 | 113 |
| PO4 (mg/dl) | 11.0 | 7.9* |
| UV PO4 (mg/day) | 1.4 | 3.1* |
| U PO4/creatinine | 2.6 | 6.1* |
| FE PO4 (%) | 10.8 | 32.4* |
| UV Ca (mg/day) | 0.15 | 0.29* |
| UV Na (μEq/day) | 240 | 253 |

$N = 7–11$ animals in each group (age 30–50 days)

The results indicate a significant decrease in the serum phosphate, a threefold increase in the urinary excretion of phosphate, and a twofold increase in the urinary excretion of calcium (renal function in wild-type (+/+) and knockout (−/−) mice). Statistical significance * $p < 0.05$

*CrCl* creatinine clearance, *PO4* serum phosphate, *U* urine concentration of solute, *V* volume of urine, *UV* amount of solute excreted, *FE* fractional excretion of solute

similarities between NHERF-1 knockout mice and some patients with kidney stones. Interestingly, these investigators identified mutations in the NHERF-1 gene in some patients with nephrolithiasis [13].

## NHERF-1 and parathyroid hormone responsiveness

The effect of PTH is to increase the urinary excretion of phosphate by facilitating the removal of Npt2a from the apical membrane of proximal tubule cells. The mechanism underlying the PTH-mediated regulation of Npt2a has been the focus of our more recent investigations. PTH1 receptors localize to both the basolateral and apical side of renal proximal tubule cells. The basolateral receptor signals through both protein kinase A (PKA) and protein kinase C (PKC). The apical receptor engages NHERF-1 or NHERF-2 and when engaged, signals predominantly through PKC [6]. We recently determined that NHERF-1 null proximal tubule cells in culture were resistant to the inhibitory effect of PTH and

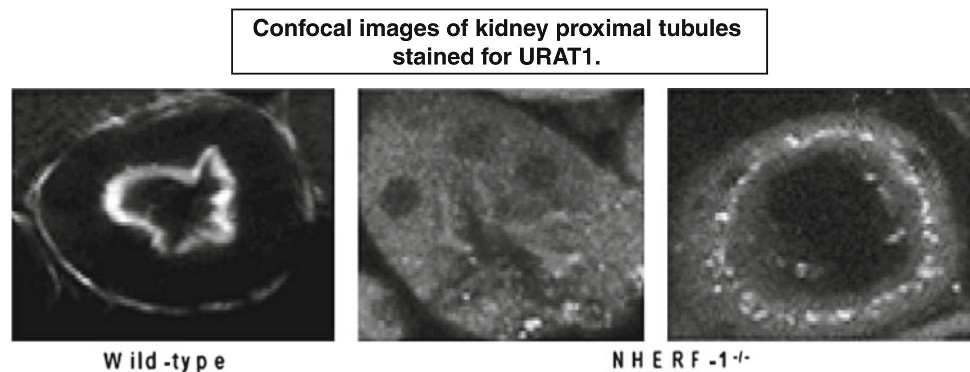


**Fig. 4** Representative confocal image of proximal tubules from wild-type kidneys (*left*) and NHERF-1 null kidneys middle and *right labeled* with an anti-(mouse) URAT1 antibody, showing altered subapical staining of URAT1 in the NHERF-1 null kidneys in contrast to the brush border membrane staining of URAT1 in wild-type kidneys on the *left*

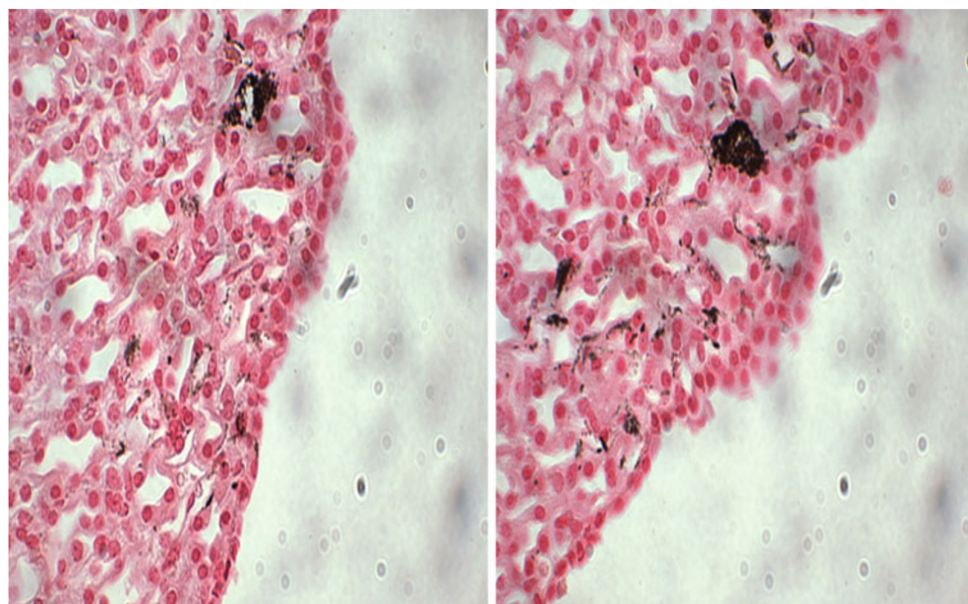

**Fig. 5** Representative von Kossa-stained kidneys from 54-week-old NHERF-1 null mice showing increased tubulointerstitial calcium deposition in the papilla. Magnification ×40. In contrast, no von Kossa staining was observed in kidneys in wild-type mice

second messenger pathways mediated by PKA and PKC [14]. However, rescue of these cells using viral-mediated gene transfer of NHERF-1 restored the inhibitory response to PTH and to second messenger agonists of PKA and PKC [14]. This suggested that resistance of the NHERF-1 null cells to the inhibitory effects of PTH derived from either abnormal function of PTH1 receptor or the absence of NHERF-1/Npt2a complexes in the plasma membrane. More recent investigations have led us to believe that the latter, i.e., the absence of NHERF-1/Npt2a complexes is responsible for the resistance to PTH observed in the null cells [15].

In recent studies, we reported that NHERF-1 existed as a phosphorylated protein and that its phosphorylation was increased in response to treatment with PTH as well as activators PKC and PKA [16]. We have recently identified several additional phosphorylation sites within the PDZ1 domain of NHERF-1 and determined that reversible phosphorylation/dephosphorylation of these residues regulated the binding affinity of target proteins, including Npt2a [16, 17]. We demonstrated that PTH acting through PKC and PKA, phosphorylated Serine 77 (S77), located on the alpha helix that forms part of the binding groove of the first PDZ domain of NHERF-1, which resulted in decreased binding of Npt2a and decreased proximal tubule transport of phosphate [16]. Therefore, in the context of these findings, we concluded that S77 of the first PDZ domain of NHERF-1 is critical in mediating the inhibitory effect of PTH on sodium-dependent phosphate transport in the renal proximal convoluted tubule and that this is likely the result of a change in the conformation of NHERF-1 from an open configuration that is capable of binding to Npt2a to a closed configuration with much reduced binding affinity.

In summary, the regulatory roles for the NHERF family of proteins highlight some of the key paradigms used by PDZ proteins to control ion transporters, hormone receptors and other cellular targets. Emerging studies suggest that the PDZ domain-containing NHERF proteins, may interact directly or indirectly to control the surface expression of ion transporters, such as Npt2a and mURAT1 and that the each may be recruited in certain circumstances, such as NHERF-3 under conditions of phosphate deprivation, to modulate the function of other proteins. Clinical relevance derives from the recent identification of mutations in the NHERF-1 gene in patients with nephrolithiasis and points to a potential role for NHERF-1 in the pathogenesis of some form of kidney stones. Although there may be a level of redundancy in NHERF-mediated regulation of target proteins, it is clear that there is a certain amount of specificity displayed by each of the NHERF proteins. The challenge remains to elucidate the specific roles for each of the NHERF proteins, in particular, to identify the cellular mechanisms by which each regulate proximal tubule ion transport.

**Acknowledgments** These studies were supported by grants from the National Institutes of Health DK55881 (EJW and SS), Research Service, Department of Veterans Affairs (EJW), the University of Maryland (RC) and the Kidney Foundation of Maryland (RC). R. Cunningham is a recipient of a Minority Career Development Award from the NIH and a Harold Amos Faculty Development award from the Robert Wood Johnson Foundation.

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