# NHERF-1 uniquely transduces the cAMP signals that inhibit sodium–hydrogen exchange in mouse renal apical membranes

Edward J. Weinman[a,b,c,*], Deborah Steplock[a], Shirish Shenolikar[d]

[a] *Department of Medicine, Division Nephrology, University of Maryland School of Medicine, Room N3W143, UHM, 22 South Greene Street, Baltimore, MD 21201, USA*
[b] *Department of Physiology, University of Maryland School of Medicine, Baltimore, MD 21201, USA*
[c] *Medical Service, Department of Veterans Affairs Medical Center, Baltimore, MD 21201, USA*
[d] *Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA*

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**Abstract** **Sodium–hydrogen exchanger regulatory factor isoform-1 (NHERF-1) and NHERF-2 are two structurally related PDZ-domain-containing protein adapters that effectively transduce cyclic AMP (cAMP) signals that inhibit NHE3, the sodium–hydrogen exchanger isoform present at the apical surface of kidney and gut epithelia. The mouse renal proximal tubule expresses both NHERF isoforms, suggesting their redundant functions as regulators of renal electrolyte metabolism. To define the role of NHERF-1 in the physiological control of NHE3, we analyzed NHE3 activity in isolated brush border membrane (BBM) preparations from renal proximal tubules of wild-type (WT) and NHERF-1 (−/−) mice. Basal $Na^+$–$H^+$ exchange was indistinguishable in BBMs from WT and NHERF-1 (−/−) mice ($0.96 \pm 0.08$ and $0.95 \pm 0.10$ nmol/mg protein/10 s, respectively). Activation of membrane bound cAMP-dependent protein kinase (PKA) by cAMP inhibited NHE3 activity in WT BBMs ($0.55 \pm 0.07$ nmol/mg protein/10 s or $40 \pm 9\%$, $P < 0.01$) but had no discernible effect on $Na^+$–$H^+$ exchange in the NHERF-1 (−/−) BBM ($0.97 \pm 0.07$ nmol/mg protein/10 s; $P$ = not significant). This was associated with a significant decrease in cAMP-stimulated phosphorylation of NHE3 immunoprecipitated from solubilized NHERF-1 (−/−) BBMs. As the protein levels for NHE3, NHERF-2, PKA and ezrin were not changed in the NHERF-1 (−/−) BBMs, the data suggest a unique role for NHERF-1 in cAMP-mediated inhibition of NHE3 activity in the renal proximal tubule of the mouse.**


*Key words:* Renal transport; NHE3; NHERF-1; cAMP; Phosphoprotein

## 1. Introduction

Analysis of the transport activity of $Na^+$–$H^+$ Exchanger isoform-3 (NHE3) reconstituted in artificial liposomes and expressed in PS120 cells established a critical role for the $Na^+$–$H^+$ Exchanger Regulatory Factor (NHERF) proteins in mediating cAMP signals that inhibit $Na^+$–$H^+$ exchange [1–3]. NHERF-1 and NHERF-2 were defined as two structurally related PDZ (PSD-95/Discs large/ZO-1) domain-containing proteins that functioned as adapters to link NHE3 in the plasma membrane to the actin-binding protein, ezrin, and thereby to the underlying actin cytoskeleton. Ezrin functioned as a cAMP-dependent protein kinase (PKA) anchoring protein or AKAP and thus recruited PKA to the NHE3/NHERF/ezrin complex [4–7]. This significantly enhanced the PKA-mediated phosphorylation of NHE3 and inhibition of its transport activity in response to hormones that increased intracellular cAMP [4,8]. In such studies, the two NHERF proteins functioned in an essentially identical manner. Thus, the physiological relevance of NHERF-1 and NHERF-2 in tissues that expressed both proteins remains unknown.

The mouse proximal convoluted tubule, by contrast to the rat, expresses both NHERF-1 and NHERF-2, further suggesting redundant functions for the two protein adapters in the control of renal electrolyte metabolism [9,10]. The generation of the NHERF-1 null mouse, however, established a unique role for NHERF-1 in dictating the apical localization of a NHERF target, the sodium-phosphate cotransporter, Npt2. These studies indicated that NHERF-2 still present in renal proximal tubules of the NHERF-1 (−/−) mice could not rescue the loss of NHERF-1 function in the mutant mouse kidney [10]. In this present study, we investigate the potential role of the two NHERF isoforms in cAMP-mediated inhibition of NHE3 assayed in purified BBMs from WT and NHERF-1 (−/−) mice. The results strongly argue that while NHERF-1 was not required for the expression or apical targeting of NHE3 in the proximal tubule, it plays a crucial role in facilitating cAMP-mediated phosphorylation of NHE3 and inhibition of its transport activity, a function that was not complimented by the presence of NHERF-2 in these membranes. Thus, the data provided further evidence that the two NHERF isoforms serve distinct roles in the control of renal function.

## 2. Methods

### 2.1. Preparation of mouse renal brush border membranes (BBM)

BBM vesicles were prepared from the kidney cortex excised from groups of five to six WT CB57/BL and NHERF-1 (−/−) male mice using modifications of the magnesium aggregation method previously described [10,11]. The enrichment of the BBM membranes was determined by Western immunoblotting for selected BBM proteins in iso-

*Corresponding author. Fax: (1)-410-706 4195.
*E-mail address:* eweinman@medicine.umaryland.edu (E.J. Weinman).

*Abbreviations:* NHERF-1, sodium–hydrogen exchanger regulatory factor isoform-1; NHE3, sodium–hydrogen exchanger isoform-3; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase; EIPA, ethyl isopropyl amiloride

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lated BBM and in whole kidney homogenates. The mice were maintained in the animals care facility, fed a rodent chow diet, and exposed to alternating light–dark cycles according to NIH guidelines.

### 2.2. Cyclic AMP (cAMP) regulation of $Na^+$–$H^+$ exchange in isolated mouse BBMs

To initiate phosphorylation, the BBMs were incubated in hypotonic media containing 10 mM $MgCl_2$, 10 mM KF, and 5 mM MES/Tris buffer (pH 6.8) and 0.1 mM ATP in the absence or presence of 0.1 mM 8-bromo-cAMP for 5 min. For subsequent analysis of $Na^+$–$H^+$ exchange, BBMs were resealed and the intravesicular space acidified by exposure to a solution containing 227.8 mM mannitol, 42 mM MES, 8.6 mM Tris, 13.7 mM HEPES, 0.4 mM $MgSO_4$, 1.8 mM $MgCl_2$, 0.18 mM $CaCl_2$, 1.8 mM KF and 1.8 mM ATP (pH 5.5) for 45 min at room temperature. The vesicles were then sedimented at $24\,000\times g$ for 30 min and resuspended in the same solution to a final volume of 10 μl. The vesicle suspension was then mixed with 40:1 of a solution containing 1.25 mM $^{22}Na^+$, 157.5 mM mannitol, 10 mM MES, 50 mM Tris and 80 mM HEPES (pH 7.4), with and without 50 μM EIPA (ethyl isopropyl amiloride). Sodium uptake was assayed at 22°C in triplicate using a rapid Millipore filtration technique [11].

### 2.3. Analysis of renal BBM phosphoproteins

For analysis of BBM phosphoproteins, radioactive γ-[$^{32}$P]ATP was included in the aforementioned phosphorylating solution. The phosphorylation reaction was stopped by addition of 100 nM microcystin-LR and SDS-sample buffer. For quantitative analysis, 10 μl of the radiolabelled BBM proteins were applied to nitrocellulose using a dot-blot apparatus and the radiolabel visualized by autoradiography and/or scintillation counting. To qualitatively analyze the proteins phosphorylated, an aliquot of the samples was subjected to 10% (w/v) SDS–PAGE, the gels dried and subjected to autoradiography. To compare the content of specific BBM proteins, non-phosphorylated BBM proteins were subjected to 10% (w/v) SDS–PAGE, the gels electrophoretically transferred to nitrocellulose and immunoblotted using well characterized antibodies against NHE3, Npt2, ezrin, NaPi Cap1, NHERF-1, NHERF-2, and RII (the type-II regulatory subunit of PKA). For the specific evaluation of NHE3 phosphorylation, BBMs were solubilized using 0.1% Triton-X100 and NHE3 immunoprecipitated using an anti-NHE3 antibody (Chemicon, Temecula, CA, USA). The anti-NHE3 immunoprecipitates were solubilized in SDS sample buffer and subjected to 10% (w/v) SDS–PAGE. The gels were electrophoretically transferred to nitrocellulose and radiolabelling analyzed using autoradiography. To quantify the NHE3 protein, the nitrocellulose filters were subjected to Western immunoblotting using the anti-NHE3 antibody. All immunoblots were visualized using enhanced chemical luminescence (Amersham Corporation, Arlington Heights, IL).

## 3. Results and discussion

NHERF-1 and NHERF-2 are apically localized modular protein adapters that share homology in three well-defined protein interaction domains including two tandem PDZ domains and a C-terminal ezrin–radixin–moesin–merlin binding domain [2,3]. Expression of NHERF-1 or NHERF-2 in stable PS120 fibroblasts co-expressing the single NHE isoform, NHE3, showed that despite differences in their primary structures, both NHERF proteins bound NHE3 and participated in the assembly of a multimeric protein complex that facilitated cAMP-mediated phosphorylation of NHE3 and inhibition of transport activity [3–7,12–14]. On the other hand, although some recent experiments have indicated differences in the function of the NHERF isoforms, the precise role of the two proteins in regulating NHE function in the intact kidney has not been established [15]. The development of the NHERF-1 (−/−) mouse [10], however, has provided an opportunity to address this issue. The present biochemical experiments were designed to investigate cAMP regulation

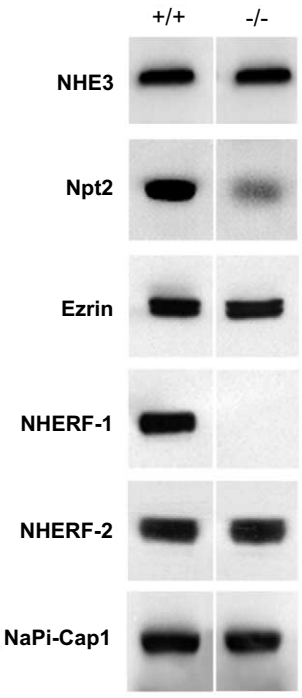


Fig. 1. Comparison of proteins in renal BBMs isolated from WT and NHERF-1 (−/−) mice. Purified BBMs (5 μg of total protein) were immunoblotted using monospecific antibodies against NHE3, Npt2, ezrin, NHERF-1, NHERF-2, and NaPi-Cap1.

of NHE3 activity in isolated BBM vesicles from WT and NHERF-1 (−/−) mice and thereby delineate the role of NHERF-1 in the hormonal control of $Na^+$–$H^+$ exchange in the mouse kidney.

As assessed by the presence of several BBM-associated proteins, the BBMs purified from WT and NHERF-1 (−/−) mouse kidney were enriched nine- to 11-fold over the crude homogenates Moreover, Fig. 1 illustrates that the renal BBMs from WT and NHERF-1 null mice contain nearly equivalent amounts of NHE3, NHERF-2, ezrin, and another renal apical PDZ-containing protein, NaPi-Cap1. As anticipated, NHERF-1 was detected in isolated BBMs from WT but not NHERF-1 (−/−) mice. The abundance of the sodium-dependent phosphate co-transporter IIa (Npt2), a known NHERF-1 target, was decreased in BBMs from the NHERF-1 (−/−) animals as previously reported [10]. The BBM preparations were predominantly right-side-out and impermeable to components of phosphorylation mixture, specifically ATP and magnesium [11]. Thus, hypotonic shock was required to present these reagents to the internal surface and promote protein phosphorylation by membrane bound PKA [11]. After the vesicles were resealed in an iced solution at pH = 5.5 that served not only to acidify the intravesicular space but also to block protein phosphatase activity, the EIPA-sensitive component of pH gradient-stimulated uptake of $^{22}Na^+$ was measured as an index of $Na^+$–$H^+$ exchange activity [11]. As seen in Table 1, BBMs from both WT and NHERF-1 (−/−) mice showed similar basal $Na^+$–$H^+$ exchange consistent with the equal presence of the NHE3 protein (Fig. 1). In contrast, the addition of 8-bromo-cAMP resulted in a 40 ± 9% decrease in NHE3. By comparison, 8-bromo-cAMP had no discernible effect on $Na^+$–$H^+$ exchange activity measured in BBMs

from NHERF-1 (−/−) mice. The total uptake of $^{22}Na^+$ at 90 min, an estimate of the equilibrium value reflecting the average vesicular volume, was not different in the two groups of animals. These data suggest that the presence of NHERF-1 is essential for effective cAMP-mediated inhibition of $Na^+$–$H^+$ exchange in the mouse BBM.

Previous studies showed that rabbit BBMs were principally associated with the type-II isoform of PKA [16]. To insure that the BBMs from WT and mutant mouse contained equivalent amounts of PKA, we undertook immunoblotting analyses using anti-RII antibody that recognized the type-II regulatory subunits of PKA. The data shown in Fig. 2A established that WT and NHERF-1 (−/−) BBMs contained near equal amounts of RII, the principal PKA regulatory subunit associated with mouse BBMs. To evaluate the activity of BBM-associated PKA, we also analyzed the cAMP-stimulated phosphorylation of endogenous BBM proteins (Fig. 2B). Using dot-blot analysis to quantify the relative PKA activity, we observed nearly an identical increases in BBM protein phosphorylation in response to 8-bromo-cAMP of 3.3 ± 0.02- and 3.0 ± 0.03-fold in WT and NHERF-1 (−/−) proteins, respectively. Analysis of the overall phosphoprotein pattern further confirmed the presence of equivalent PKA activity in both BBM preparations. (data not shown),

Previous studies showed PKA directly phosphorylated NHE3 at specific C-terminal serines to inhibit transport activity [4,17,18]. To determine the importance of NHERF-1 in PKA-mediated regulation of NHE3 in the mouse kidney, NHE3 protein was immunoprecipitated from detergent-solubilized WT and NHERF-1 (−/−) BBMs that had previously been phosphorylated in the presence of γ-[$^{32}$P]ATP and 8-bromo-cAMP. Fig. 3 shows that the addition of 8-bromo-cAMP resulted in a significant increase in the phosphorylation of NHE3 immunoprecipitated from WT BBMs of 1.98 ± 0.03-fold. In contrast, 8-bromo-cAMP failed to increase NHE3 phosphorylation in BBMs from NHERF-1 (−/−) mice. Immunoblotting with the anti-NHE3 antibody showed that similar amounts of NHE3 were immunoprecipitated from both BBM preparations.

Table 1
Effects of cAMP on $Na^+$–$H^+$ exchange in BBMs from WT and NHERF-1 (−/−) mice

| | Sodium uptake (nmol/mg protein) | |
|---|---|---|
| | NHERF-1 (+/+) | NHERF-1 (−/−) |
| Control (10 s) | | |
| Total uptake | 1.36 ± 0.11 | 1.42 ± 0.12 |
| EIPA-inhibited | 0.40 ± 0.04 | 0.47 ± 0.06 |
| NHE3 activity | 0.96 ± 0.08 | 0.95 ± 0.10 |
| Plus cAMP (10 s) | | |
| Total uptake | 0.98 ± 0.06 | 1.42 ± 0.12† |
| EIPA-inhibited | 0.43 ± 0.03 | 0.45 ± 0.07 |
| NHE3 Activity | 0.55 ± 0.07 | 0.97 ± 0.07† |
| Control (90 min) | | |
| Total uptake | 1.17 ± 0.06 | 1.11 ± 0.08 |
| EIPA-inhibited | 1.16 ± 0.11 | 1.19 ± 0.05 |
| Plus cAMP (90 min) | | |
| Total | 1.17 ± 0.05 | 1.10 ± 0.10 |
| EIPA-inhibited | 1.03 ± 0.05 | 1.18 ± 0.19 |

$Na^+$–$H^+$ exchange activity was defined as the EIPA-sensitive component of pH gradient-stimulated $^{22}Na^+$ uptake in BBMs from NHERF-1 (+/+) ($n = 7$) and NHERF-1 (−/−) ($n = 6$) mice analyzed at initial uptake (10 s) and at equilibrium (90 min). Prior to sodium uptake, BBMs were subjected to hypotonic shock in a solution containing ATP-Mg in the absence (Control) or presence (Plus cAMP) of 0.1 mM 8-bromo-cAMP for 5 min. Sodium transport is expressed as nmol/mg protein (mean ± S.E.M.) and † = $P < 0.01$ compared to WT.

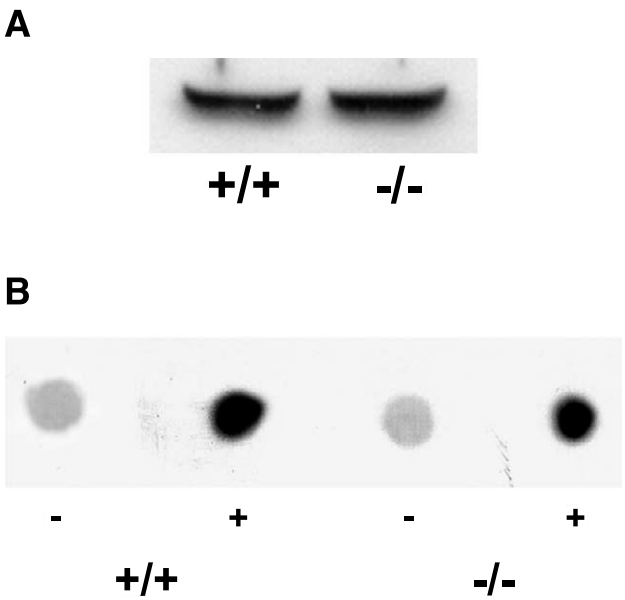


Fig. 2. Comparison of PKA activity in renal BBMs from WT and NHERF-1 (−/−) mice. A: Purified BBMs (5 μg of total protein) from WT and NHERF-1 (−/−) mice were immunoblotted with an antibody against the type II regulatory subunit of PKA. B: Purified BBMs from WT and NHERF-1 (−/−) mice were subjected to phosphorylation in-vitro in the presence of γ-[$^{32}$P]ATP in the absence (−) or presence (+) of 0.1 mM 8-bromo-cAMP. The phosphorylated proteins were applied to nitrocellulose using a dot-blot apparatus and visualized by autoradiography.

Together, the studies showed that despite the presence of similar amounts of NHE3, type-II PKA, the AKAP, ezrin and NHERF-2 and other related PDZ proteins, such as NaPi-Cap1, the complete absence of NHERF-1 resulted in the inability of cAMP to inhibit $Na^+$–$H^+$ exchange in the BBMs isolated from the NHERF-1 null mouse kidney. Accordingly, this data provides the first evidence that NHERF-1 plays a unique role in the hormonal regulation of renal NHE3 activity. The precise reasons for the inability of NHERF-2 present in these membranes to transduce the cAMP signals that inhibit NHE3 is still unclear but preliminary studies using confocal microscopy combined with immunohistochemistry suggest a spatial separation of NHERF-1 and NHERF-2 at the apical surface of the mouse renal proximal tubules. NHE3 appears to co-localize predominantly with NHERF-1 in this tissue and less so with NHERF-2, findings that were present both in WT as well as the NHERF-1 (−/−) mice. This suggests that NHERF-2 may function to regulate other renal targets. In fact, recent studies have indicated that NHERF-2 may subserve a unique role in regulating not only NHE3 but also other transporters such as ROMK by protein kinases other than PKA [19,20].

In summary, emerging studies using expression of NHERF proteins in cultured cells have identified up to 30 targets of the two NHERF isoforms [13,14]. These include various transporters and ion channels, receptors, cytoskeletal and signaling proteins, and transcription factors. Many of the targets associate with both NHERF proteins. While some studies have noted differences in NHERF-1 and NHERF-2 in these cell-based assays, the use of gene disruption in mouse provides

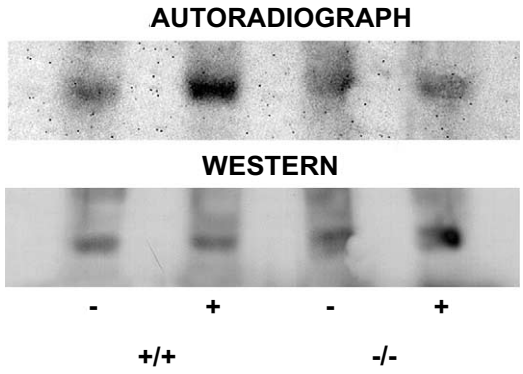


Fig. 3. PKA-induced phosphorylation of NHE3 in BBMs from WT and NHERF-1 (−/−) mice. Isolated BBMs were incubated in a phosphorylation solution containing $MgCl_2$ and γ-[$^{32}$P]ATP in the absence (−) or presence (+) of 0.1 mM 8-bromo-cAMP ($10^{-4}$ M) for 5 min. The NHE3 protein was immunoprecipitated as described in Section 2 and the immunoprecipitates subjected to SDS–PAGE. The top panels show representative autoradiographs of phosphorylated NHE3, while the lower panels show Western immunoblots for NHE3 protein.

new insights into the unique as well as the potentially overlapping roles of NHERF proteins in the control of mammalian physiology. The NHERF-1 null mouse thus far has shown defects in Npt2 trafficking and as shown in this study, aberrant cAMP regulation of NHE3 [10]. Future studies comparing the biochemistry, cell biology and physiology of animals lacking NHERF-1 and NHERF-2 should provide a better understanding of the role of these PDZ adapter proteins in the function and regulation of mammalian tissues.

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