Ezrin binding domain-deficient NHERF attenuates cAMP-mediated inhibition of Na\(^+\)/H\(^+\) exchange in OK cells

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Weinman, Edward J., Deborah Steplock, James B. Wade, and Shirish Shenolikar. Ezrin binding domain-deficient NHERF attenuates cAMP-mediated inhibition of Na\(^+\)/H\(^+\) exchange in OK cells. Am J Physiol Renal Physiol 281: F374–F380, 2001.—Na\(^+\)/H\(^+\) exchanger regulatory factor (NHERF), an essential protein cofactor in cAMP-mediated inhibition of Na\(^+\)/H\(^+\) exchange transporter 3 (NHE3), facilitates the formation of a signal complex of proteins that includes NHE3, NHERF, and ezrin. This model for NHE3 regulation was developed in fibroblasts and its applicability to epithelial cells remains to be established. Opossum kidney (OK) cells were transfected with either empty vector (control), full-length mouse (m) NHERF(1–355), or a truncated mNHERF(1–325) that lacked ezrin binding and had been demonstrated in fibroblasts to bind NHE3 but not mediate its cAMP-associated inhibition. 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP) at 10\(^{-4}\) M inhibited Na\(^+\)/H\(^+\) exchange activity in control and OK cells expressing wild-type mNHERF(1–355) by >60% but by <10% in cells expressing mNHERF(1–325). NHE3 coimmunoprecipitated with mNHERF(1–325), but cAMP phosphorylation of NHE3 was impaired in cells expressing mNHERF(1–325). The inhibitory effect of hyperosmolality on NHE3 activity and the uptake of 3-O-methyl-D-glucose was the same in all three cell lines. Cell surface expression of NHE3 was not changed by cAMP in any of the cells lines. These data indicate that disruption of the NHERF-ezrin signal complex attenuates the inhibitory effect of cAMP on NHE3 activity in OK cells and provides evidence supporting the proposed model of protein kinase A regulation of NHE3 in epithelial cells.

renal electrolyte transport; PSD-95/Dlg/ZO proteins; protein kinase A; sodium/hydrogen exchange transporter 3; acid-base physiology; opossum kidney cells

THE ACUTE REGULATION OF RENAL apical membrane Na\(^+\)/H\(^+\) exchange transporter 3 (NHE3) by agonists that increase intracellular cAMP requires participation of a newly described protein called the Na\(^+\)/H\(^+\) exchanger regulatory factor (NHERF) (18, 17, 21). Studies in fibroblast cells have provided evidence that NHERF functions to facilitate formation of a signal complex of proteins including ezrin, NHE3, and cAMP-dependent protein kinase A (PKA) (17, 22). This multiprotein complex mediates the phosphorylation of NHE3, thereby inhibiting its activity (17, 22). Despite extensive study of the interactions among NHERF, ezrin, PKA, and NHE3 in fibroblast cells, a continuing issue with the proposed model has been the lack of functional data to indicate its applicability to epithelial cells.

In the course of study of the role of the ezrin binding domain of NHERF, we expressed a 30-amino acid COOH-terminal truncated form of mouse NHERF [mNHERF(1–325)] that lacked the putative ezrin binding domain in PS-120 cell fibroblasts (17). Compared with wild-type NHERF [mNHERF(1–355)], the ezrin binding domain-deficient construct did not bind to ezrin, did not phosphorylate NHE3, and did not support cAMP inhibition of NHE3 activity. This construct, however, did bind to NHE3 in vivo (17). We reasoned, therefore, that this truncated form of NHERF might be a useful reagent to develop a dominant-negative renal epithelial cell line to address the important question of whether NHERF is required for cAMP-associated inhibition of NHE3 in epithelial cells. In the present experiments, opossum kidney (OK) cells, a proximal tubule cell line that expresses endogenous NHERF and Na\(^+\)/H\(^+\) exchanger activity, were transfected with full-length mNHERF(1–355) or mNHERF(1–325), and the effect of cAMP on the activity and cell surface expression of the transporter was determined.

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METHODS

OK cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ in DMEM/F-12 medium (1:1) supplemented with 10% (vol/vol) FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were grown to confluence in serum, split, and regrown to sub-confluence in the presence of serum. mNHERF1 (1–355) and mNHERF1 (1–325) DNAs were cloned into pCDNA3.1/Hygro vectors and transfected into OK cells using Lipofectin (GIBCO-BRL) (17, 21, 22). Cells transfected with the pCDNA3.1/Hygro vector alone served as the control. Cells resistant to 600 μM hygromycin were selected through eight passages before study. Transfected cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ in Dulbecco/F-12 medium (1:1) supplemented with 10% (vol/vol) FCS, penicillin (100 U/ml), streptomycin (100 mg/ml), and 600 μM hygromycin.

Na⁺/H⁺ exchange activity was assayed in cells grown on 40-mm glass coverslips using the pH-sensitive fluorescent dye, the acetoxymethyl ester of 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) (Molecular Probes) (17, 21, 22). Cells were serum deprived for 24–36 h and then loaded with 6.5 μM BCECF-AM in an assay buffer of 20 HEPES, pH 7.4, containing (in mM) 40 NH₄Cl, 90 NaCl, 5 KCl, 1 MgSO₄, 2 CaCl₂, 1 tetramethylammonium (TMA)-PO₄ and 25 glucose for 20 min at room temperature. Coverslips were transferred to an FSC2 chamber (Biotech) and rapidly perfused at a constant flow rate with solutions warmed to 37°C. Cells were acidified for 5 min (in mM) with 20 mM HEPES, pH 7.5, 130 TMA-Cl, 5 KCl, 1 MgSO₄, 2 CaCl₂, 1 TMA-PO₄, and 25 glucose. Na⁺/H⁺ exchange transport was initiated by substituting 130 mM NaCl for 130 mM TMA-Cl in the perfusion solution. BCECF fluorescence was measured at excitation wavelengths of 500 and 440 nm and an emission wavelength of 530 nm. The pHₜₕ pulse was used to achieve an initial intracellular pH (pHᵢ) of 6.0, and only cells with initial pHᵢ values between 6.0 and 6.2 were included for analyses. Na⁺/H⁺ exchange, expressed as change in pHᵢ (ΔpHᵢ/min), represented the initial slope of transport activity measured between 5 and 10 s of sodium-dependent pHᵢ recovery. Over this brief time period, the relationship between pHᵢ and time was essentially linear. To analyze the effects of CAMP on NHE3 activity, cells were pretreated with 10⁻⁴ M 8-bromo-cyclic monophosphate (8-BrcAMP) during the final 15 min of dye loading and continuously throughout the perfusion process. At the end of each experiment, the cells were equilibrated in pH-clamp media containing (in mM) 20 HEPES, 20 MES, 115 KCl, 14 NaCl, 1 MgSO₄, 1 CaCl₂, 1 TMA-PO₄, 25 glucose, and 10 μM nigericin in pH 6.0 and 7.3. On any given coverslip, Na⁺/H⁺ exchange was quantitated by laser densitometry.

Western immunoblots of OK cell lysates were performed using an antibody to full-length rabbit NHERF that also recognizes OK cell NHERF (16). Immunocytochemistry was performed as previously described from this laboratory with minor modifications (15). OK cells grown on coverslips were fixed in 3.7% paraformaldehyde for 30 min, followed by washing and quenching of aldehyde groups with 50 mM NH₄Cl. Cells were then permeabilized with 0.1% Triton X-100 for 30 min and treated with 6 M guanidine for 10 min to unmask antigenic sites. Slides were incubated with a rabbit polyclonal antibody to NHERF or ezrin (SC-5407, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After being washed, they were incubated with secondary antibody for 2 h at 4°C. The antibody was coded to Alexa 488 or 568 dyes (Molecular Probes, Eugene, OR) and diluted 1:100. Specimens were examined with a Zeiss LSM410 confocal microscope. Protein concentrations were determined by the method of Lowry et al. (13). The results were analyzed by ANOVA (9).

RESULTS

OK cells express NHERF with a lower apparent molecular size on SDS-PAGE than mNHERF, as seen in Western immunoblot of cell lysates (Fig. 1). The
abundance of OK NHERF was not altered by the overexpression of mNHERF(1–355) or mNHERF(1–325). As seen in lanes 2 and 3, the NHERF antibody recognized an additional band not present in lane 1 representing mNHERF(1–355) and mNHERF(1–325). Immunoprecipitates of anti-OK cell NHE3 were probed with anti-NHERF antibody and, as seen in Fig. 2, only the smaller OK cell NHERF was recovered from control cells. By contrast, anti-NHE3 immunoprecipitates from cells expressing mNHERF(1–355) showed a broader band that reflected both mNHERF and OK NHERF. Surprisingly, the larger mNHERF was the predominant species recovered with the anti-NHE3 immunoprecipitates from the mNHERF(1–325)-expressing cells. Figure 3 shows representative tracings of the initial sodium-dependent pH_{i} recovery in the absence and presence of cAMP in the three cell lines. Table 1 summarizes the measurements of the rate of Na^{+}/H^{+} exchange and the effect of cAMP. Control OK cells transfected with the pcDNA vector alone had a rate of sodium-dependent pH_{i} recovery (ΔpH_{i}/min) from an acidifying stimulus of 0.035 ± 0.004 in the absence of cAMP and 0.010 ± 0.002 in the presence of 10^{-4} M 8-BrcAMP (P < 0.01). OK cells expressing mNHERF(1–355) had a rate of 0.040 ± 0.006 ΔpH_{i}/ min in the absence of cAMP and 0.010 ± 0.002 in the presence of cAMP (P < 0.01). OK cells expressing mNHERF(1–325) had a rate of 0.045 ± 0.003 ΔpH_{i}/ min in the absence of cAMP and 0.043 ± 0.002 in the presence of cAMP [P = not significant (NS)].

We next analyzed the covalent modification of NHE3 in OK cells using an in vitro back-phosphorylation assay of immunoprecipitated NHE3. In control cells treated with cAMP, the amount of ^{32}P incorporated in vitro determined by autoradiography and normalized for the amount of NHE3 by Western immunoblot was decreased by 37.1 ± 7.1% (n = 3, P < 0.05) compared with cells not treated with cAMP (Fig. 4). This suggested the prior phosphorylation of this fraction of NHE3 in OK cells in response to cAMP. In cells expressing mNHERF(1–355), cAMP treatment de-
increased back phosphorylation of NHE3 by 44.6 ± 4.6% (n = 3, P < 0.05). By contrast, in the mNHERF(1–325)-expressing cells, the amount of 32P incorporated in vitro into the immunoprecipitated NHE3 was essentially the same whether the cells were previously treated with cAMP or not (%change = +9.4 ± 3.7%).

In separate experiments, we examined the physiological effect of acutely increasing the osmolality of the incubating media, a non-cAMP stimulus previously shown to inhibit NHE3 activity (1). As summarized in Table 2, control OK cells had a rate of sodium-dependent pH recovery of 0.051 ± 0.009 pH/min and 0.034 ± 0.006 in control media and 0.027 ± 0.004 in media containing 80 mM of added mannitol, respectively (n = 4, P < 0.05). OK cells expressing NHERF(1–355) had a rate of 0.035 ± 0.003 ΔpH/min and 0.022 ± 0.002 in control media and in media containing 80 mM of added mannitol, respectively (n = 5, P < 0.05). OK cells expressing mNHERF(1–325) had a rate of 0.047 ± 0.005 in control media and 0.027 ± 0.004 ΔpH/min in media containing 80 mM of added mannitol (n = 4, P < 0.05). To demonstrate the specificity of the effect observed in the mNHERF(1–325) cells, a non-cAMP regulated transporter was examined. As summarized in Table 3, the sodium-dependent uptake of the nonmetabolized sugar 3-O-methyl-D-glucose at 15 min was 710.0 ± 17.3 counts · min⁻¹ · μg protein⁻¹ in control cells, 703.3 ± 76.0 cpm/μg protein in cells expressing mNHERF(1–355), and 678.9 ± 110.8 cpm/μg protein in cells expressing mNHERF(1–325). cAMP did not affect the uptake of the sugar in any of the cell lines.

A physiological role for NHERF in membrane trafficking of some transporters, receptors, and signaling proteins has been suggested by recent studies (8, 14, 18). To determine if NHERF affected the cellular trafficking of NHE3, the three cell lines were examined by immunocytochemistry and confocal microscopy for NHE3 and ezrin. The pattern of distribution of ezrin was not different between the cell lines and did not change in response to cAMP (data not shown). Using ezrin as a marker, the distribution of cell surface NHE3 was determined. As shown in Fig. 5, there was no clear difference in cell surface expression of NHE3 among the three cell lines in response to treatment.

### Table 1. The effect of cAMP on Na⁺/H⁺ exchange in OK cells expressing mouse wild-type NHERF or an ezrin binding domain-deficient NHERF

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Basal cAMP</th>
<th>%Inhibition</th>
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| Control (n = 5) | 0.035 ± 0.004 | 0.010 ± 0.002* | 67.5 ± 8.3
| Mouse NHERF (1–355) (n = 6) | 0.040 ± 0.006 | 0.010 ± 0.002* | 75.0 ± 4.7
| Mouse NHERF (1–325) (n = 6) | 0.045 ± 0.003 | 0.043 ± 0.002 | 3.7 ± 4.2

Results are expressed as means ± SE and represent the initial rate of sodium-dependent intracellular pH (pHi) recovery (ΔpHi/min) after the cell was acidified to pH 6.0. No. in parentheses indicates the no. of measurements. Na⁺/H⁺ exchange transport was measured in opossum kidney (OK) cells using [2,7'-bis(2-carboxylethyl)-5(6)-carboxyfluorescein; BCECF] fluorescence in the absence or presence of 10⁻⁴ M 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP) for 15 min. OK cells were transfected with an empty pcDNA vector (control), Na⁺/H⁺ exchanger regulatory factor (NHERF)(1–355) representing full-length mouse NHERF, or NHERF(1–325) representing the ezrin binding domain-deficient NHERF truncation. *P < 0.01 (vs. control).

### Table 2. The effect of hyperosmolality on Na⁺/H⁺ exchange in OK cells expressing mouse wild-type NHERF or an ezrin binding domain-deficient NHERF

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Normal pHi/min</th>
<th>Hyperosmolality pHi/min</th>
<th>%Inhibition</th>
</tr>
</thead>
</table>
| Control (n = 4) | 0.051 ± 0.009 | 0.034 ± 0.006* | 32.4 ± 2.5
| Mouse NHERF (1–355) (n = 6) | 0.035 ± 0.003 | 0.022 ± 0.002* | 34.8 ± 5.6
| Mouse NHERF (1–325) (n = 6) | 0.047 ± 0.005 | 0.027 ± 0.004* | 42.1 ± 5.5

Results are expressed as means ± SE and represent the initial rate of sodium-dependent pH recovery (ΔpHi/min) after acidifying the cell to pH 6.0. Na⁺/H⁺ exchange transport was measured using BCECF fluorescence in OK cells incubated in control (normal) or media made hypertonic (hyperosmolality) by addition of 80 mM mannitol for 60 min. No. in parentheses indicates the no. of measurements. OK cells were transfected with an empty pcDNA vector (control), NHERF(1–355) representing full-length mouse NHERF, or NHERF(1–325) representing the ezrin binding domain-deficient NHERF truncation. *P < 0.05 (vs. control).

### Table 3. The effect of cAMP on sodium-dependent 3-O-methyl-D-glucose uptake in OK cells expressing mouse wild-type NHERF or an ezrin binding domain-deficient NHERF

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Basal cpm/μg protein</th>
<th>cAMP cpm/μg protein</th>
<th>%Inhibition</th>
</tr>
</thead>
</table>
| Control (n = 6) | 710.0 ± 17.3 | 738.3 ± 15.5 | −8.2 ± 0.8
| Mouse NHERF (1–355) (n = 6) | 703.3 ± 76.0 | 730.0 ± 44.7 | −3.8 ± 0.5
| Mouse NHERF (1–325) (n = 6) | 678.8 ± 75.6 | 683.7 ± 117.4 | −1.6 ± 0.1

Results are means ± SE expressed as cpm/μg protein where cpm is counts/min. No. in parentheses indicates the no. of measurements. The sodium-dependent uptake of 3-O-methyl-D-glucose was measured in OK cells in the absence or presence of 10⁻⁴ M 8-BrcAMP for 15 min. OK cells were transfected with an empty pcDNA vector (control), NHERF(1–355) representing full-length mouse NHERF, or NHERF(1–325) representing the ezrin binding domain-deficient NHERF truncation.
Recent experiments have indicated that NHERF is a necessary cofactor in cAMP-mediated inhibition of NHE3 (16–20). Biochemical and cell studies have advanced evidence that NHERF binds to both NHE3 and ezrin and is required for cAMP to phosphorylate NHE3 (17, 22). In this model, ezrin is considered to be the PKA anchor protein. NHERF is envisioned to function as an adaptor that facilitates formation of a multiprotein complex that mediates the rapid phosphorylation of NHE3 by bringing the PKA-ezrin complex into proximity with NHE3 (18). The subsequent phosphorylation of NHE3 results in the acute downregulation of its activity. This model of the signal complex regulation of NHE3 activity was developed exclusively from studies performed in PS-120 cell fibroblasts. An important shortcoming of the proposed model, however, has been the lack of its validation in an epithelial cell. The present studies derive from recent experiments from our laboratory indicating that a construct of NHERF that lacked the putative ezrin binding domain [mNHERF(1–325)] did not support PKA-mediated phosphorylation of NHE3 or inhibition of transport activity, but did bind to NHE3 in vivo (17). Accordingly, we considered that if the ezrin binding domain-deficient NHERF could be expressed in an epithelial cell line, it might displace native NHERF and, by virtue of its inability to bind ezrin, disrupt cAMP regulation of NHE3 activity.

OK cells are a proximal tubule cell line that expresses endogenous NHE3 and NHERF. OK cell NHERF has a lower apparent molecular mass on SDS-PAGE than mNHERF, and this size differential permitted identification of both the native OK cell NHERF and expressed mNHERF using the same antibody. Stable expression of mNHERF(1–355) or mNHERF(1–325) did not affect the abundance of OK cell NHERF. The association of NHE3 with the specific NHERF proteins was examined by immunoprecipitation of NHE3. In control cells, only the smaller OK cell NHERF was recovered in association with NHE3. Anti-NHE3 immunoprecipitates from cells expressing mNHERF(1–355) showed a broader band that reflected both mNHERF and OK NHERF. Surprisingly, the larger mNHERF was the predominant form recovered with the anti-NHE3 immunoprecipitates from the mNHERF(1–325)-expressing cells. As the expression of NHERF in both cell lines was equivalent, this suggests that mNHERF(1–325) may have a higher affinity for OK NHE3 than mNHERF(1–355). It is also possible that the association of NHERF(1–355) with ezrin reduces its affinity for NHE3. Whatever the correct explanation, the data indicate an in vivo association between both the wild type and truncated form of NHERF with NHE3.

In control OK cells transfected with the pcDNA vector alone and in cells transfected with full-length mNHERF(1–355), 15-min exposure to cAMP inhibited Na\(^+\)/H\(^+\) exchange activity by >60%. By contrast, the mNHERF(1–325) cells behaved as NHERF dominant-negative cells, and the effect of cAMP to acutely downregulate Na\(^+\)/H\(^+\) exchange activity was markedly attenuated. In PS-120 cells, NHERF is required for cAMP-mediated phosphorylation of NHE3 (22). The relationship between NHE3 phosphorylation and NHERF also appears to be present in OK cells. In control cells and in cells expressing mNHERF(1–355), cAMP resulted in the phosphorylation of NHE3. On the other hand, in cells expressing mNHERF(1–325), the amount of \(^{32}\)P incorporated in vitro into the immuno-
precipitated NHE3 was essentially the same whether the cells were previously treated with cAMP. This indicates that mNHERF(1–325) disrupted the cellular mechanism that promotes NHE3 phosphorylation in vivo in response to cAMP and provides a biochemical explanation for the failure of cAMP to inhibit NHE3 activity in these cells. The specificity of the effect observed in the mNHERF(1–325) cells was examined in two additional sets of experiments. Acute increases in the osmolality of the incubation media have previously been demonstrated to inhibit NHE3 activity in OK cells by a mechanism presumed to be independent of cAMP (1). In the present studies, incubation of the three cell lines in media containing 80 mM of added mannitol for 60 min resulted in an acute decrease in the rate of Na\(^+\)/H\(^+\) exchange transport compared with cells studied in the normal media. We also examined the sodium-dependent uptake of the nonmetabolized sugar 3-O-methyl-D-glucose to establish the integrity of the sodium gradient across the cell and to rule out nonspecific effects of the transfections on cell function. The sodium-dependent uptake of the sugar was the same in all three cell lines, and cAMP treatment had no effect on this transport process.

We have recently reported that NHERF, NHE3, and ezrin colocalize in the apical membrane of the rat proximal tubule and that NHERF and ezrin coimmunoprecipitate from OK cells (12, 15). Although consistent with the presence of an association among the proposed components of the signal complex, the above studies did not establish a physiological role for the multiprotein complex in control of Na\(^+\)/H\(^+\) exchange transport activity in renal tissue or kidney-derived cell lines. The demonstration herein that the effect of cAMP on Na\(^+\)/H\(^+\) exchange transport in the mNHERF(1–325)-expressing cells is blunted, however, provides the required functional and biochemical data to support the above studies (12, 15). When considered together, the results indicate that PKA regulation of NHE3 activity in an epithelial cell is mediated by a signal complex of proteins that includes NHERF.

A growing literature indicates that NHERF is involved in the trafficking and recycling of transport proteins and receptors (3, 8, 14). Prior studies in renal tissue suggested that parathyroid hormone caused a redistribution of Na\(^+\)/H\(^+\) exchange activity and NHE3 protein from the cell surface to internal membrane pools (5, 11). Studies of Fan et al. (5) in the rat proximal tubule showed that the acute inhibitory effect of parathyroid hormone (PTH) on NHE3 activity resulted from phosphorylation of the antiporter, whereas over a longer time course of ~3 h, PTH facilitated the endocytic removal of NHE3 from the plasma membrane (5). In more recent studies, they established a similar dual mode of regulation of NHE3 in OK cells in response to PTH, with a significant decrease in cell surface NHE3 being evident after only 30 min of hormone treatment (4). Optical sectioning of the OK cells used in the present experiments indicated that the distribution of ezrin, concentrated at the apical surface, was similar in all cell lines and did not change in response to cAMP.

In comparable optical sections, there was no difference in cell surface expression of NHE3 between the three cell lines either before or after 15-min exposure to cAMP. Cell surface expression of NHE3 was also not different after 60 min of treatment with cAMP in the mNHERF(1–355)- and mNHERF(1–325)-expressing cells. In OK cells, PTH has been demonstrated to use multiple second messenger pathways (10). Thus the present findings, when considered with the results of Fan et al. (5), suggest that the effect of PTH on the cellular redistribution of NHE3 is transduced by a non-cAMP-mediated mechanism. We would conclude that the acute regulation of NHE3 by cAMP analyzed in the current experiments most likely represents PKA phosphorylation of COOH-terminal residues in NHE3 mediated by the NHERF-ezrin signal complex (17, 22).

In summary, the present studies indicate that a truncated form of NHERF deficient in ezrin binding functioned as a dominant-negative reagent when expressed in OK cells. Although mNHERF(1–325) had little effect on basal Na\(^+\)/H\(^+\) exchange activity, it markedly blunted the acute inhibitory response to cAMP. These studies, then, provide the first functional validation for the signal-complex model of NHE3 regulation in renal epithelial cells. Recent experiments have established an association between NHERF and two other cAMP-regulated transporters in the renal proximal tubule. The sodium bicarbonate cotransporter (NBC) is inhibited by cAMP by a process that requires NHERF (2). Gisler et al. (7) recently showed that the COOH-terminus of the type IIa sodium phosphate cotransporter associated with NHERF, although the physiological relevance of this association was not established. The present experiments suggest that the expression of the dominant-negative NHERF(1–325) in appropriate epithelial cells may help elucidate the role of the NHERF-ezrin complex in the hormonal control of NBC and other renal transporters.

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