NHERF Associations with Sodium−Hydrogen Exchanger Isoform 3 (NHE3) and Ezrin Are Essential for cAMP-Mediated Phosphorylation and Inhibition of NHE3†

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Received January 11, 2000; Revised Manuscript Received March 8, 2000

ABSTRACT: The sodium−hydrogen exchanger regulatory factor (NHERF) is an essential cofactor for cAMP-mediated inhibition of the Na^+/H^+ exchanger isoform, NHE3, in renal brush border membranes. NHERF is also an ezrin-binding protein. To define the functional importance of ezrin binding for NHERF's function as a NHE3 regulator, we transfected stable PS120 cells expressing NHE3 with plasmids encoding WT and truncated mouse NHERF proteins. Co-immunoprecipitation established that in PS120 cells, NHE3 bound to full-length NHERF(1−355), the C-terminal domain, NHERF(147−355), and NHERF(1−325), which lacks the proposed ezrin-binding domain. The N-terminal domain, NHERF(1−146), failed to bind the antiporter. Ezrin was also co-immunoprecipitated with NHERF(1−355) but not with NHERF(1−325). 8Br-cAMP inhibited NHE3 activity in cells that expressed NHERF(1−355) or NHERF(147−355) but had no effect on the formation of NHE3−NHERF or NHERF−ezrin complexes. Na^+/H^+ exchange was unaffected by 8Br-cAMP in cells that expressed NHERF(1−146) or NHERF(1−325). NHE3 phosphorylation in vivo was enhanced by 8Br-cAMP only in cells where NHERF bound to both NHE3 and ezrin. The data suggest that NHERF functions as a scaffold to link NHE3 with ezrin and that this multiprotein complex is essential for cAMP-mediated phosphorylation of NHE3 and the inhibition of Na^+/H^+ exchange.

Reconstitution of detergent-solubilized rabbit kidney brush border membrane proteins into artificial liposomes identified NHERF[1] (Na/H exchanger regulatory factor), a phosphoprotein containing two PDZ domains, as an essential cofactor for PKA-mediated inhibition of NHE3 activity (*1*, *2*). Subsequent studies in PS120 fibroblasts expressing NHE3 showed that Na^+/H^+ exchange was inhibited by cAMP only when NHERF was coexpressed in these cells (*3*). Biochemical studies with recombinant NHERF and the C-terminal tail of NHE3 established a physical association of the two proteins, which was confirmed by co-immunoprecipitation of the polypeptides from PS120 cells (*4*). Reconstitution studies showed that the C-terminal half of NHERF was sufficient to mediate PKA inhibition of NHE3 (*5*). However, the contribution of other NHERF domains and their cellular targets in NHE3 regulation remains unclear.

The N-terminal PDZ domain of NHERF binds several receptors and ion transporters (*6*). Some, like the β_2-adrenergic receptor, may compete with NHE3 for NHERF binding and thus attenuate NHERF's ability to regulate NHE3 (*7*). The C-terminus of NHERF binds ezrin, an actin-binding protein (*8*, *9*). This attracted attention as ezrin also binds PKA and may function as a PKA-anchoring protein or AKAP (*10*). It was speculated that NHERF recruits ezrin and consequently PKA to promote the phosphorylation of one or more components of the NHE3/NHERF/ezrin complex and thereby decrease NHE3 activity. Early efforts focused on the PKA phosphorylation of NHERF (*11*). Three potential sites for PKA phosphorylation were identified near the C-terminus of NHERF. Mutation of these serines eliminated in vitro phosphorylation of NHERF by PKA (*5*), and in the reconstitution assay, the mutant NHERF was less effective in mediating NHE3 inhibition by PKA. This suggested that cAMP acts in part through NHERF phosphorylation to regulate NHE3. However, subsequent studies showed that in vivo phosphorylation of NHERF was unresponsive to changes in intracellular cAMP (*4*, *5*, *12*). This and other findings led to the current view that the presence of NHERF and not its phosphorylation is required for cAMP inhibition of NHE3 (*13*). NHERF binding is

† These studies were supported by grants from the National Institutes of Health [DK37319 (to E.J.W.) and DK55881 (to E.J.W. and S.S.)] and the Research Service, Department of Veterans Affairs (to E.J.W.). M.D. is supported by grants from the National Institutes of Health (DK26523 and DK44484), by the Johns Hopkins Center for Epithelial Disorders, and by the Myerhoff Digestive Diseases Center.

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[1] Abbreviations: cAMP, cyclic adenosine 3′,5′-phosphate; NHERF, sodium−hydrogen exchanger regulatory factor; NHE3, sodium−hydrogen exchanger isoform 3; PDZ, PSD-95/Dlg(Discs large)/ZO-1; BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; VSV, vesicular stomatitis virus glycoprotein; AKAP, A-kinase (PKA) anchoring protein.

10.1021/bi000064m CCC: $19.00
Published on Web 04/29/2000

thought to facilitate PKA-mediated phosphorylation of unique serines on NHE3 and thus inhibit ion transport. The role of ezrin in cAMP-dependent phosphorylation of NHE3 has not been investigated.

Disruption of the actin cytoskeleton also inhibits NHE3 activity (*14*). Mapping of the NHE3 domain that monitors cytoskeletal integrity identified a region that overlaps with the NHERF-binding site (*8*). This suggests that NHERF through its interaction with ezrin may tether NHE3 to the actin cytoskeleton and define its basal transport activity. On the other hand, NHE3 has been speculated to directly bind ezrin. In this case, cAMP-induced reorganization of the actin cytoskeleton could represent an NHERF-independent mechanism for inhibiting NHE3.

To define the protein−protein interactions required for hormonal regulation of NHE3 and specifically address the role of NHERF−ezrin association in this process, we expressed truncated NHERF proteins in PS120 cells and analyzed their interaction with ezrin and the antiporter by co-immunoprecipitation. This established that NHERF bound NHE3 and ezrin through a C-terminal domain that was sufficient to mediate cAMP inhibition of NHE3 in the PS120 cells. Our studies also provide the first evidence for an essential role of the NHERF/ezrin interaction in this event. Thus, the studies yield new insights into the multiprotein complex assembled by NHERF to transduce cAMP signals and inhibit Na^+/H^+ exchange in renal tissues.

MATERIALS AND METHODS

Cell Culture. Studies were performed with PS120/NHE3V fibroblasts (*3*, *13*) stably transfected with rabbit NHE3 tagged at its carboxyl terminus with a peptide epitope (YTDIEMNRLGK) derived from vesicular stomatitis virus glycoprotein (VSVG). Wild-type and truncated forms of mouse NHERF cDNAs (*15*) were cloned into pcDNA3.1/Hygro+ vector and transfected into the PS120/NHE3V fibroblasts using Lipofectin (GIBCO/BRL) (*3*, *13*). Cells resistant to 600 units/mL hygromycin were selected through 8 passages prior to study. Transfected 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 grown to confluence, split, and regrown to subconfluency in the presence of serum prior to analysis. Periodic acid selection was used to maintain the selection pressure on the NHE3-expressing cells.

NHERF Expression Plasmids. Expression vectors for full-length and truncated forms of NHERF were constructed using available restriction sites in the mouse NHERF cDNA (*15*) or by PCR with the Zero Blunt TOPO PCR Kit (Invitrogen). Constructs encoding full-length NHERF(1−355), the N-terminal domain, NHERF(1−146), the C-terminal domain, NHERF(147−355), and two constructs, NHERF(1−325) and NHERF(147−325), that eliminated the proposed ezrin-binding site were inserted in-frame into the pcDNA3.1/Hygro+ vector.

Na^+/H^+ Exchange. NHE3 activity was assayed in individual PS120 cells using the pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM) (*3*, *13*). Cells were serum-deprived for 12−20 h and then loaded with 6 μM BCECF-AM in an assay buffer of 20 mM HEPES, pH 7.5, containing 130 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM $NaPO_4$, and 25 mM glucose for 20 min at room temperature. They were then pulsed with 40 mM NH_4Cl, pH 7.4, for 15−20 min and then perfused with a solution of 20 mM HEPES, pH 7.5, containing 130 mM TMACl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM $TMAPO_4$, and 25 mM glucose. BCECF fluorescence was measured at excitation wavelengths of 500 and 440 nm and an emission wavelength of 530 nm. The NH_4Cl pulse achieved an initial pH_i of 6.0, and only cells with initial pH_i between 6.0 and 6.2 were further analyzed. Na^+/H^+ exchange, expressed as ΔpH_i/min, represented the initial slope of transport activity measured between 5 and 10 s as sodium-dependent pH_i recovery. Over this time period, the time-dependent change in pH_i was linear in all cells analyzed.

To analyze the effects of cAMP, cells were treated with 10^{-4} M 8-bromo-cAMP during the final 15 min of dye loading and throughout the subsequent perfusion process. 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 made on cells at the same passage on the same day.

In Vitro Phosphorylation of NHE3. To analyze NHE3 phosphorylation, the PS120 cells were washed with serum-free Dulbecco's modified media lacking antibiotics. Half of the cells were used as control and the other half treated with 100 μM 8Br-cAMP for 15 min. Control and cAMP-treated cells were scraped and resuspended in IP buffer consisting of 10 mM Na_2PO_4, pH 7.4, containing 100 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, and a mixture of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM phenanthroline, and 5 μg/mL each of aproteinin, leupeptin, pepstatin, and trypsin inhibitor). Cells were sedimented at 12000*g* for 10 min in an Eppendorf microcentrifuge, resuspended in 1 mL of IP buffer containing 1% Triton X-100 (IPT buffer), and lysed by drawing several times through a 27 gauge needle. The lysate was then agitated on a rotating rocker at 4 °C for 30 min before centrifugation at 12000*g* for 30 min to remove the cell debris. The resulting supernatants were precleared with Protein A−Sepharose CL 4B beads washed with IPT buffer by rocking for 1−2 h. The beads were spun down and the supernatants incubated overnight with 15 μL of anti-VSVG polyclonal antibody. Protein A−Sepharose CL 4B beads previously washed with IPT buffer were added, and the mixture was agitated for an additional 2−4 h. The antigen−antibody complex was eluted from the beads with 100 μL of 30 mM glycine hydrochloride, pH 2.8, and immediately neutralized by the addition of 10 μL of 1 M Tris, pH 11. Immunoprecipitated NHE3 was then "back-phosphorylated" in vitro with PKA in 21 mM glycine−100 mM Tris, pH 7.4, containing 50 μM ATP, 100 μM $MgCl_2$, 180 units of PKA catalytic subunit (Promega), and 50 μCi of [γ-^{32}P]ATP. After incubating at 30 °C for 10 min, the reaction was terminated by the addition of Laemmli buffer and heating in a boiling water bath for 2 min. The radiolabeled phosphoproteins were separated on 10% SDS−PAGE, transferred to nitrocellulose, and visualized by autoradiography. Following autoradiography, the nitrocel-

lulose filter was subjected to immunoblotting with an anti-VSVG antibody to determine the relative NHE3 loading in each lane. The immune complexes were detected by Enhanced Chemical Luminescence (ECL) (Amersham Corp., Arlington Heights, IL). The autoradiographs and immunoblots were quantitated using laser densitometry.

Immunoprecipitation. Immunoprecipitations were performed using lysates of PS120 cells expressing NHE3 and WT or mutant NHERF proteins. Cells were treated with 100 μM 8Br-cAMP or vehicle. The cell lysates were prepared and divided into equal portions for immunoprecipitation with the anti-VSVG antibody, a monoclonal anti-ezrin antibody, or an anti-NHERF polyclonal antibody that recognized all forms of NHERF analyzed in this study. NHE3- and ezrin-containing immune complexes were precipitated using Protein A−Sepharose CL 4B or Protein G−Sepharose 4 fast flow and separated on 10% SDS−PAGE. On the other hand, NHERF migrated very close to the heavy chain of IgG on 10% SDS−PAGE. Thus, to clearly distinguish NHERF, the anti-NHERF antibody was directly coupled to CNBr-activated Protein A−Sepharose 4B beads, and the bound proteins were analyzed on 6% SDS−PAGE. After electrophoretic transfer to nitrocellulose, all immunoblots were visualized using ECL.

Statistical analysis of control and experimental samples was performed using the *t*-test for unpaired data.

RESULTS

The structurally related PDZ-containing proteins, NHERF and E3KARP/NHERF2, mediate cAMP-dependent phosphorylation (*13*) and inhibition of NHE3 activity (*3*, *4*) in PS120 cells. Both NHERF homologues also bind ezrin, the actin-binding protein uniquely localized to apical membranes, though the role of ezrin binding in NHE3 regulation has not been defined. The following experiments were undertaken to define the structural determinants in NHERF required for NHE3 and ezrin binding in PS120 cells and establish the functional importance of the individual protein−protein interactions in NHE3 regulation by cAMP.

NHERF Association with NHE3. To analyze the regions of NHERF that mediate its binding to NHE3, we transfected PS120/NHE3V cells with expression vectors for full-length NHERF(1−355), the N-terminal half of the protein containing the first PDZ domain, NHERF(1−146), and the C-terminal half, NHERF(147−355), containing the second PDZ domain and C-terminal sequences required for ezrin binding. We also analyzed NHERF(1−325) and NHERF(147−325) that lacked the C-terminal 30 amino acids that encompass the putative ezrin-binding domain. Moreover, the potential effect of the second messenger, cAMP, in modulating the NHERF/NHE3 interactions was analyzed by treatment of transfected cells with 8Br-cAMP. NHE3 was quantitatively immunoprecipitated from cells using the monoclonal antibody against the VSVG tag. Subsequent blotting of the anti-VSVG immunoprecipitates with the anti-NHERF antibody demonstrated that NHERF(1−355), NHERF(1−325), and NHERF(147−355) were co-immunoprecipitated with NHE3 (Figure 1A). The inability to detect NHERF(1−146) in the anti-VSVG immunoprecipitates suggested that the NHERF N-terminal domain did not bind NHE3 in the PS120 cells. Treatment with 8Br-cAMP had no discernible effect on any

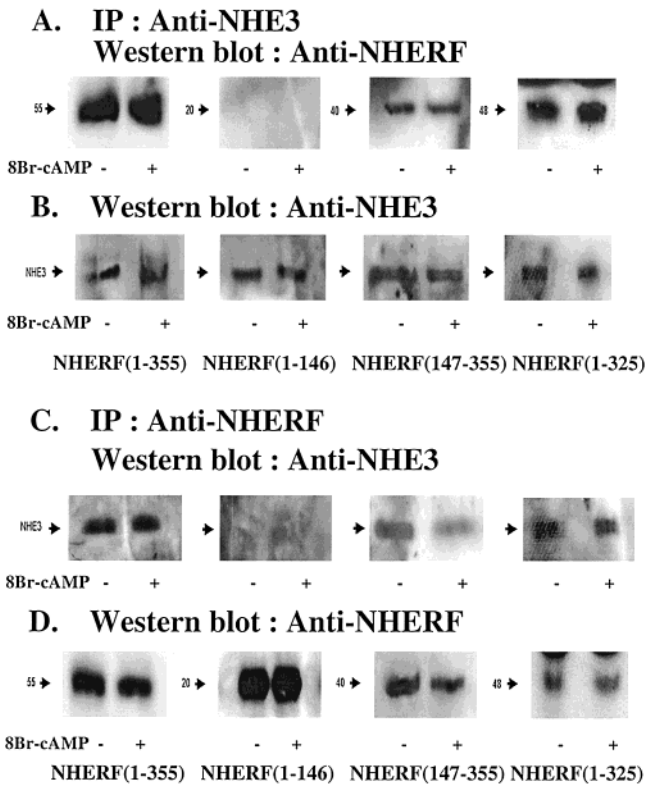

FIGURE 1: Co-immunoprecipitation of NHERF and NHE3 from PS120 cells. Immunoprecipitations were performed from cell lysates of PS120/NHE3V cells expressing various NHERF proteins in the absence (−) or presence (+) of 10^{-4} M 8-bromo-cAMP. Panel A shows the immunoprecipitation of NHE3 with an antibody to the VSVG-tag. Subsequent immunoblotting with an anti-NHERF antibody demonstrates that NHE3 coprecipitated with NHERF(1−355), NHERF(147−355), and NHERF(1−325) but not NHERF(1−146). Panel B illustrates an immunoblot with the anti-VSVG antibody, which indicates that similar amounts of NHE3 were immunoprecipitated from all cell lines. Panel C shows the immunoprecipitation of distinct NHERF proteins from the PS120/NHE3V cells. Immunoblotting with the anti-VSVG antibody shows that NHE3 was immunoprecipitated with NHERF(1−355), NHERF(147−355), and NHERF(1−325) but not NHERF(1−146). Panel D shows a parallel immunoblot with anti-NHERF antibody that indicates that approximately similar amounts of NHERF proteins were immunoprecipitated from all cell lines. Representative immunoblots from three to four independent experiments are shown in each panel.

of the NHERF/NHE3 associations in the PS120 cells. Immunoblotting with anti-VSVG antibody established that equivalent amounts of NHE3 were present in all immunoprecipitates from the NHERF-expressing cells (Figure 1B).

We also analyzed the NHERF/NHE3 association in the reverse direction using the anti-NHERF polyclonal antibody to first immunoprecipitate the NHERF variants from the same cell lysates and then blotting these immunoprecipitates with the anti-VSVG antibody to detect NHE3. As seen in Figure 1C, NHE3 was co-immunoprecipitated with NHERF(1−355), NHERF(1−325), and NHERF(147−355) but not with NHERF(1−146). This confirmed that NHE3 associated with the portion of NHERF that contains the second PDZ domain and further C-terminal sequences (*4*) and the removal of the 30 amino acids thought to bind ezrin had no detrimental effect on NHE3 binding. NHE3 did not bind NHERF(1−146) despite the fact that this polypeptide was expressed to higher levels in the PS120 cells and, thus, immunoprecipitated more efficiently than the other NHERF proteins (Figure

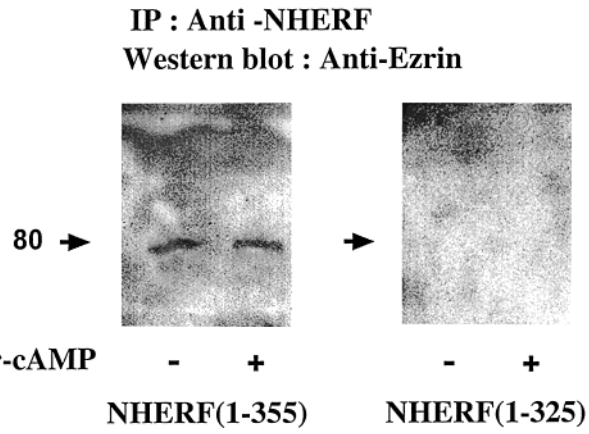

FIGURE 2: Co-immunoprecipitation of ezrin with NHERF from PS120 cells. Immunoprecipitation of NHERF proteins was carried out from PS120 cell lysates in the absence (−) or presence (+) of 10^{-4} M 8Br-cAMP using anti-NHERF polyclonal antibody. Immunoblotting with the anti-ezrin antibody shows that ezrin was co-immunoprecipitated with full-length mouse NHERF(1−355) (panel A) but not with NHERF(1−325) that lacked the C-terminal 30 amino acids that represent the ezrin binding domain (panel B). A representative immunoblot from three to four independent experiments is shown.

1D). Finally, the association of NHERF with NHE3 was not regulated by cAMP as treatment of cells with 100 μM 8Br-cAMP had no measurable effect on the co-immunoprecipitation of NHE3 with NHERF or vice versa.

NHERF Association with Ezrin. Immunoblotting cell lysates with an anti-ezrin antibody showed equivalent amounts of endogenous ezrin in all NHERF-expressing cells (data not shown). However, much less of the endogenous ezrin was precipitated under conditions used to immunoprecipitate either NHE3 or NHERF, which were both overexpressed in the PS120 cells (Figure 1). Thus, efficient immunoprecipitation of ezrin required higher amounts of the anti-ezrin antibody. This made it difficult to detect NHERF in these immunoprecipitates as it migrates on SDS−PAGE close to the heavy chain of IgG. Hence, the clearest results were obtained only in the reverse direction, namely, analysis of the anti-NHERF immunoprecipitates for the presence of ezrin. The experiments showed that ezrin was immunoprecipitated with full-length NHERF(1−355) to a similar extent from lysates of cells treated with or without 8Br-cAMP (Figure 2). Thus, like the NHE3/NHERF association, ezrin binding to NHERF was not modified by cAMP. NHERF-(1−325) that lacked the C-terminal 30 amino acids failed to bind ezrin in the PS120 cells, consistent with the removal of the proposed ezrin-binding site in NHERF.

Cyclic AMP-Mediated Inhibition of NHE3 Activity. Having defined the interactions of various mutant NHERF proteins with NHE3 and ezrin, we then examined the ability of these proteins to transduce cAMP signals that inhibited NHE3 activity in PS120/NHE3V cells. Na^+/H^+ exchange activity, determined by BCECF fluorescence (Figure 3), showed that the sodium-induced recovery in intracellular pH (ΔpH_i/min) in PS120/NHE3V cells expressing NHERF(1−355) was significantly inhibited by 100 μM 8Br-cAMP. In contrast, cells that expressed NHERF(1−325) showed a similar recovery rate in the presence or absence of 8Br-cAMP. The transport data for all NHERF-expressing cells are shown in Table 1. In PS120/NHE3V, cells expressing NHERF(1−355)

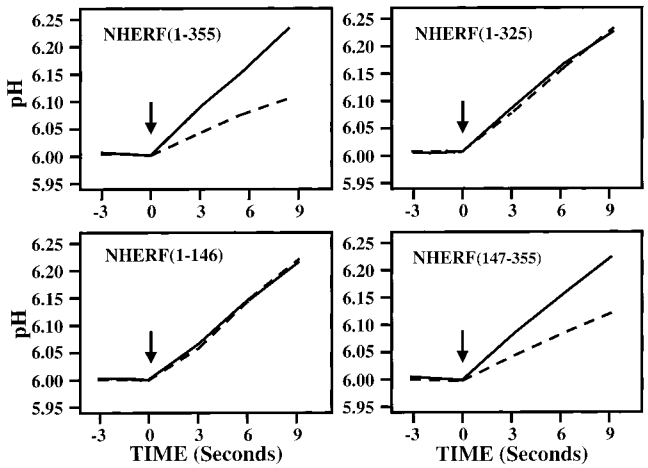

FIGURE 3: Cyclic AMP inhibition of Na^+/H^+ exchange in PS120 cells. Na^+/H^+ exchange was monitored in PS120/NHE3V cells expressing various NHERF proteins using BCECF fluorescence as described under Materials and Methods. Cells were acidified to pH 6.0−6.2 and allowed to recover by the addition of sodium at time zero in the absence (solid lines) and presence (dashed lines) of 10^{-4} M 8Br-cAMP. Representative tracings of the pH change over the first 9 s are shown. Sodium-dependent recovery of intracellular pH in cells expressing full-length NHERF(1−355) was significantly inhibited by 8Br-cAMP (top left panel). In contrast, 8Br-cAMP had no effect on the rate of recovery in cells expressing NHERF(1−325), which failed to bind ezrin (top right panel). NHERF(1−146) did not bind NHE3 or mediate its inhibition by 8Br-cAMP (bottom left panel). In contrast, 8Br-cAMP significantly reduced NHE3 activity in PS120 cells expressing NHERF(147−355) (bottom right panel).

Table 1: Cyclic AMP Regulation of NHE3 Activity in PS120/NHE3V Cells[a]

NHERF proteins	control ΔpH_i/min	8Br-cAMP ΔpH_i/min	inhibition (% control)
NHERF(1−355)	1.59 ± 0.19 (25)	1.00 ± 0.09 (19)*	37.1
NHERF(1−146)	1.51 ± 0.23 (15)	1.44 ± 0.15 (11)	4.8
NHERF(147−355)	1.45 ± 0.18 (23)	1.02 ± 0.09 (33)*	29.5
NHERF(1−325)	1.78 ± 0.14 (18)	1.73 ± 0.17 (13)	2.9
NHERF(147−325)	1.70 ± 0.14 (9)	1.74 ± 0.14 (10)	−2.8

[a] Na^+/H^+ exchange was monitored by the initial rate of sodium-dependent pH_i recovery using BCECF fluorescence. PS120 cells stably expressing rabbit NHE3 were transfected with various mouse NHERF constructs. Results are presented as ΔpH_i/min and expressed as the mean of means ± SEM. Numbers in parentheses indicate the number of measurements made. * = $p < 0.01$ (control vs cAMP).

showed a rate of pH recovery that was significantly inhibited in the presence of 8Br-cAMP. Cells that expressed the C-terminal region, NHERF(147−355), showed an essentially similar decrease in the rate of recovery in the presence of 8Br-cAMP. In contrast, in cells expressing NHERF(1−147), the N-terminal domain that failed to bind NHE3, the recovery was not significantly different in the absence or presence of 8Br-cAMP. Much to our surprise, cells expressing NHERF-(1−325) that failed to bind ezrin showed no cAMP-mediated inhibition of Na^+/H^+ exchange. Similarly, cells expressing NHERF(147−325) also showed no significant inhibition of NHE3 by cAMP. As the immunoblotting experiments showed no significant changes in the overall levels of NHE3 expression in PS120/NHE3V cells resulting from the transfections of various NHERF constructs, ezrin binding to NHERF may also be associated with a small but consistent reduction in basal NHE3 activity. However, the most important observation was that NHERF association with both

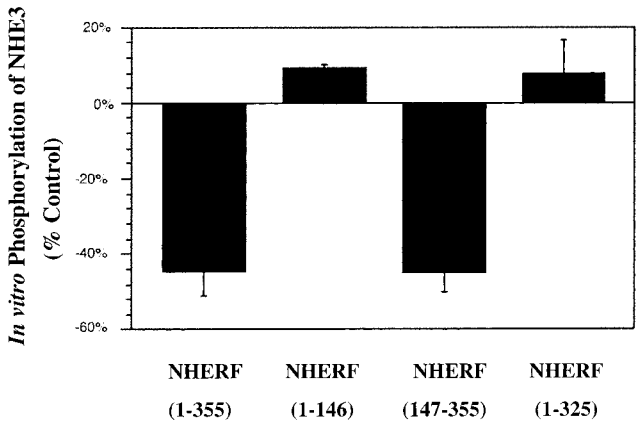

FIGURE 4: Effect of cAMP on NHE3 phosphorylation in PS120 cells. NHE3 was immunoprecipitated from PS120/NHE3V cells expressing various NHERF proteins, including NHERF(1−355), NHERF(1−146), NHERF(147−355), and NHERF(1−325) using the anti-VSVG antibody. Control cells were incubated in medium or in medium containing 10^{-4} M 8Br-cAMP. The NHE3 protein immunoprecipitated from both cells was phosphorylated in vitro using PKA catalytic subunit and [γ-^{32}P]ATP. The results are expressed as the percent change in phosphorylation of NHE3 from control versus cAMP-treated cells.

NHE3 and ezrin was essential for cAMP-mediated inhibition of NHE3 activity in PS120 cells.

cAMP-Induced NHE3 Phosphorylation. Earlier studies (*13*) showed that NHERF expression in PS120/NHEV cells promoted the cAMP-dependent phosphorylation of NHE3, as measured by direct metabolic labeling or by in vitro back-phosphorylation of NHE3 immunoprecipitated from cAMP-treated cells with the purified catalytic subunit of PKA. To address whether the association of NHERF with ezrin is required for cAMP-mediated phosphorylation of NHE3 in vivo, we utilized the more convenient back-phosphorylation assay. Treatment of PS120/NHE3V cells expressing NHERF-(1−355) with 100 μM 8Br-cAMP (Figure 4) was accompanied by a significant decrease in the subsequent in vitro phosphorylation of the immunoprecipitated NHE3 by PKA (−44.6% ± 6.9%, $n = 3$, $p < 0.005$). This most likely represented the prior phosphorylation of the PKA site(s) in NHE3 following the treatment of cells with 8Br-cAMP. Similarly, PKA phosphorylation of NHE3 immunoprecipitated from cells expressing NHERF(147−355) was also inhibited by cAMP treatment (−45.0% ± 5.3%, $n = 3$, $p < 0.005$). In contrast, cAMP treatment had no effect on the back-phosphorylation of NHE3 immunoprecipitated from cells expressing NHERF(1−146) or NHERF(1−325) by PKA (+9.3% ± 10.3%, $n = 3$, $p < 0.005$ and +7.7% ± 9.0%, $n = 5$, $p < 0.005$, respectively). This indicated that cAMP-stimulated NHE3 phosphorylation in cells required the presence of NHERF proteins that bound both NHE3 and ezrin. Moreover, the data established for the first time a critical role for ezrin in NHE3 regulation, providing experimental support for the proposed role of NHERF as a scaffold that links NHE3 to ezrin and thereby facilitates the cAMP-dependent phosphorylation and inhibition of the exchanger.

DISCUSSION

Signaling complexes that harness protein kinases (and phosphatases) exercise tight control over cellular events in different subcellular compartments in response to hormones, growth factors, and neurotransmitters. PKA-anchoring proteins or AKAPs (*16*, *17*) assemble an array of signaling complexes that transduce cAMP signals. These multiprotein complexes sometimes include the substrates of the kinases and phosphatases, providing precision and speed to the cellular response to hormones. NHERF, a PDZ-containing adapter protein, binds the NHE3 isoform of sodium−hydrogen exchanger (*11*, *18*) and ezrin, an apical membrane actin-binding protein (*4*, *9*, *19*) and a putative AKAP (*10*). This has led to the suggestion that the NHE3/NHERF/ezrin/PKA complex mediates NHE3 phosphorylation (*13*) and inhibits Na^+/H^+ exchange in response to hormones (*20*). However, the functional importance of the individual components of this putative signaling complex has not been established. For example, NHE3 and ezrin independently associate with NHERF (*8*, *9*), but no experimental evidence exists for a heterotrimeric complex or the requirement for ezrin in cAMP-mediated inhibition of NHE3.

Moreover, the structural requirements for NHERF/NHE3 and NHERF/ezrin interactions were largely defined in vitro. Thus, one goal of the present experiments was to establish the structural determinants for NHERF binding to NHE3 and ezrin in vivo. Co-immunoprecipitation experiments in NHERF-transfected PS120/NHE3V cells showed that both NHE3 and ezrin bound to PDZ domain II and C-terminal sequences in mouse NHERF. Deletion of the C-terminal 30 amino acids that eliminated ezrin binding had little impact on NHERF's association with NHE3. The cell-permeable cAMP analogue 8Br-cAMP inhibits NHE3 activity in PS120 cells (*3*, *4*, *13*) but had no effect on the NHERF/NHE3 or NHERF/ezrin complexes in these cells.

Analyses of Na^+/H^+ exchange in NHERF-expressing cells reached two important conclusions. First, the C-terminal half of NHERF(147−355) containing PDZ domain II and C-terminal sequences was indistinguishable from the full-length NHERF(1−355) in mediating cAMP-induced phosphorylation and inhibition of NHE3 in the PS120/NHE3V cells. While such overexpression studies could mask functional differences between the full-length NHERF(1−355) and the C-terminal domain, NHERF(147−355), earlier in vitro studies used the reconstitution assay to examine the dose-dependency for these two recombinant proteins in PKA-mediated inhibition of NHE3 and observed no functional differences (*5*). The second and perhaps more important finding was that NHERF association with both NHE3 and ezrin was required for cAMP-mediated NHE3 phosphorylation and inhibition of transport activity. In this regard, the studies provided the first evidence for a functional role for ezrin in a NHERF-mediated event in mammalian cells.

Although all proteins in the proposed NHE3/NHERF/ezrin complex undergo reversible protein phosphorylation (Figure 5), only some of these modifications play a role in cAMP-mediated inhibition of NHE3. Considerable evidence points to an inactive state of ezrin involving a head-to-tail dimer that precludes its binding to NHERF and the actin cytoskeleton (*9*). Phosphorylation at a C-terminal threonine (*21*) generates an open conformation of ezrin that is critical for linking NHERF to actin. Cyclic AMP had no effect on the endogenous pool of activated ezrin in PS120 cells as equivalent amounts of NHERF−ezrin complexes were co-immunoprecipitated from cells in the presence and absence of 8Br-cAMP. NHERF is also phosphorylated at a serine

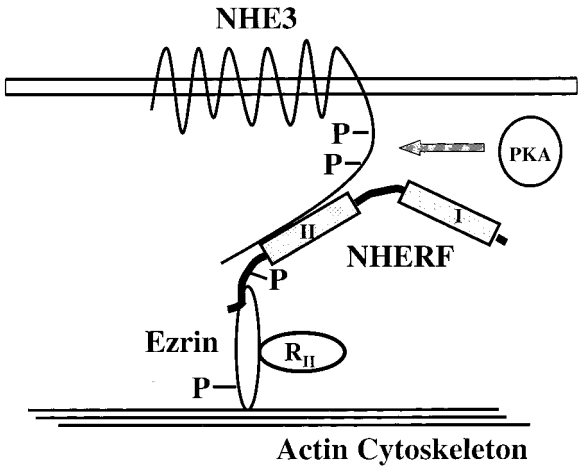

FIGURE 5: Proposed NHE3/NHERF/ezrin complex in apical membranes. NHE3 localized to apical membranes of renal proximal tubule and gastrointestinal tract associates with NHERF through the second PDZ domain and some portion of its C-terminal sequence. The extreme C-terminal 30 amino acids in NHERF mediate its association with the N-terminal ERM domain in ezrin. Finally, ezrin associates with the actin cytoskeleton via its C-terminal actin-binding domain. Both NHERF and ezrin may be phosphorylated in this complex, but these modifications are not sensitive to changes in intracellular cAMP. However, the presence of this heteromeric complex is essential for effective phosphorylation of NHE3 by PKA, which inhibits Na^+/H^+ exchange.

that lies between the second PDZ domain and the ezrin-binding sequence (*12*). The physiological importance of this covalent modification is unknown, but as ezrin binds both phosphorylated and unphosphorylated NHERF (*19*), it is unlikely that NHERF phosphorylation plays any significant role in transducing cAMP signals to NHE3. So, the data point to a pool of NHE3/NHERF/ezrin complexes capable of transducing cAMP signals, and we favor the model in which NHE3 complexed to NHERF/ezrin is uniquely phosphorylated by PKA to inhibit Na^+/H^+ exchange. Whether this requires ezrin to function as an AKAP or other cytoskeletal PKA/AKAP complexes can control NHE3 function is unclear, and further work is needed to resolve this issue.

Pharmacological disruption of the actin cytoskeleton strongly inhibited basal NHE3 activity in AP1 cells (*14*). This indicated that NHE3 tethering to the cytoskeleton, possibly through ezrin, may be essential for basal transport activity. The requirement for an intact actin cytoskeleton was mapped to a region of the NHE3 cytoplasmic tail that binds NHERF, suggesting NHERF may mediate the interaction with the actin cytoskeleton and thus control basal NHE3 activity. However, mouse NHERF(1−325), lacking the C-terminal 30 amino acids, was unable to bind ezrin but still associated with NHE3. Basal NHE3 activity in PS120/NHE3V cells expressing NHERF(1−325) or NHERF(147−325) was not reduced and, if anything, was slightly higher than in other NHERF-expressing cells. Thus, the NHERF/ezrin complex does not provide the link with the actin cytoskeleton required for basal NHE3 activity, and the contribution of the actin cytoskeleton for antiporter activity in the PS120 cells is not clear. On the other hand, treatment of PS120/NHE3V cells expressing NHERF(1−325) or NHERF(147−325) with 8Br-cAMP failed to inhibit NHE3 activity, indicating that ezrin binding was critical for cAMP-mediated inhibition of NHE3. As the complexes formed by NHE3, NHERF, and ezrin were not regulated by cAMP, we favor the idea that the NHE3/NHERF/ezrin complex induces a unique conformation in NHE3 that allows its phosphorylation and inhibition by PKA (*13*).

Our observation that the PDZ-I domain was dispensable for NHE3 regulation by NHERF may also have implications for hormonal control of NHE3. The region containing PDZ domain II and C-terminal sequences was as effective as WT NHERF in mediating cAMP inhibition of NHE3 in vivo (Table 1) and in vitro (*5*). Yet, some of the growing number of cellular targets that interact with NHERF PDZ-I (*6*) are thought to regulate NHE3. Specifically, agonist-induced association of β_2-adrenergic receptor with NHERF PDZ-I (*7*) paradoxically increased NHE3 activity. Mutation of the receptor C-terminus that prevented NHERF binding resulted in the more orthodox cAMP-mediated inhibition of NHE3. It has been proposed that β_2-adrenergic receptor competes with NHE3 for NHERF binding and this dominant mechanism accounts for NHE3 activation by WT receptor. While bimodal regulation of NHE3 by G-protein-coupled receptors could explain the diverse physiological effects on ion transport following the activation of different G-protein-coupled receptors in the mammalian kidney (*22*), it is unclear how the β_2-adrenergic receptor modifies NHERF's ability to regulate NHE3. One possibility is that PDZ-I binding induces a conformational change in NHERF that precludes its association with NHE3 and/or ezrin or otherwise abrogates the inhibitory mechanism. Alternately, distinct membrane compartmentation of NHERF PDZ-I targets and NHE3 may allow more effective competition for NHERF. Interestingly, protein targets of NHERF PDZ-II have been recently identified (*23*). These may more directly compete with NHE3 for NHERF binding and thus may also be modifiers of cAMP regulation of NHE3.

In summary, the present studies established a critical role for ezrin binding to NHERF in hormonal regulation of NHE3, providing experimental support for a scaffolding function for NHERF that links NHE3 to ezrin to mediate the cAMP inhibition of the antiporter. Besides acute regulation by reversible phosphorylation, NHE3 is also controlled by endocytosis and recycling in response to hormones (*24*−*27*). Recent studies that implicated NHERF in endocytic sorting of the β_2-adrenergic receptor (*28*) raise the intriguing possibility that NHERF, in conjunction with ezrin and the cytoskeleton, may also regulate NHE3 trafficking in renal tissue. Though NHERF and ezrin are predominantly localized to apical membranes, NHERF also plays a role in PKA regulation of the basolateral sodium bicarbonate co-transporter in mammalian kidney (*29*), suggesting a wider role for NHERF in polarized tissue. Understanding the protein−protein interactions of NHERF and the signaling complexes assembled by this adapter protein should help to elucidate the hormonal mechanisms controlling ion flux in the mammalian kidney.

ACKNOWLEDGMENT

We thank Carey Oliver, Charles Minkoff, Ryan Terry, and John Connor from Duke University for helpful discussions on the manuscript.

REFERENCES

1. Weinman, E. J., Dubinksy, W. P., and Shenolikar, S. (1988) *J. Membr. Biol. 101*, 11−18.

2. Weinman, E. J., Steplock, D., Wang, Y., and Shenolikar, S. (1993) *J. Clin. Invest. 95*, 2143−2149.
3. Yun, C. H., Oh, S., Zizak, M., Steplock, D., Tsao, S., Tse, C. M., Weinman, E. J., and Donowitz, M. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3010−3015.
4. Lamprecht, G., Weinman, E. J., and Yun, C. H. (1998) *J. Biol. Chem. 273*, 29972−29978.
5. Weinman, E. J., Steplock, D., Tate, K., Hall, R. A., Spurney, R. F., and Shenolikar, S. (1998*) J. Clin. Invest. 101*, 2199−2206.
6. Minkoff, C., Shenolikar, S., and Weinman, E. J. (1999) *Curr. Opin. Nephrol. Hypertens. 8*, 603−608.
7. Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J., Grinstein, S., and Lefkowitz, R. J. (1988) *Nature 392*, 626−630.
8. Yun, C. H., Lamprecht, G., Forster, D. V., and Sidor, A. (1998) *J. Biol. Chem. 273*, 25856−25863.
9. Reczek, D., and Bretscher, A. (1998) *J. Biol. Chem. 273*, 18452−18458.
10. Dransfield, D. T., Bradford, A. J., Smith, J., Martin, M., Roy, C., Mangeat, P. H., and Goldenring, J. R. (1997) *EMBO J. 2*, 35−43.
11. Weinman, E. J., Steplock, D., and Shenolikar, S. (1993) *J. Clin. Invest. 92*, 1781−1786.
12. Hall, R. A., Spurney, R. F., Premont, R. T., Rahman, N., Blitzer, J. T., Pitcher, J. A., and Lefkowitz, R. J. (1999) *J. Biol. Chem. 274*, 24328−24334.
13. Zizak, M, Lamprecht, G., Steplock, D., Tariq, N., Shenolikar, S., Donowitz, M., Yun, C., and Weinman, E. J. (1999) *J. Biol. Chem. 274*, 24753−24758.
14. Kurashima, K., D-Souza, S., Szaski, K., Ramjeesingh, R., Orlowski, J., and Grinstein, S. (1999) *J. Biol. Chem. 274*, 29843−29849.
15. Weinman, E. J., Steplock, D., Zhang, X., Akhter, S., and Shenolikar, S. (1999) *Biochim. Biophys. Acta 1447*, 71−76.
16. Pawson, T., and Scott, J. D. (1997) *Science 278*, 2075−2080.
17. Schillace, R. V., and Scott, J. D. (1999) *J. Clin. Invest. 103*, 761−765.
18. Weinman, E. J., Steplock, D., and Shenolikar, S. (1995) *J. Clin. Invest. 95*, 2143−2149.
19. Reczek, D., Berryman, M., Bretscher, A. (1997) *J. Cell Biol. 139*, 169−179.
20. Zhao, H., Wiederkehr, M. R., Fan, L., Collazo, R. L., Crowder, L. A., and Moe, O. W. (1999) *J. Biol. Chem. 274*, 3978−3987.
21. Simons, P. C., Pietromonaco, S. F., Reczek, D., Bretscher, A., and Elias, L. (1998) *Biochem. Biophys. Res. Commun. 253*, 561−565.
22. Weinman, E. J., Sansom, S. C., Knight, T. F., and Senekjian, H. O. (1982) *J. Membr. Biol. 69*, 107−111.
23. Mohler, P. J., Kreda, S. M., Boucher, R. C., Sudol, M., Stutts, M. J., and Milgram, S. L. (1999) *J. Cell Biol. 147*, 879−890.
24. Kurashima, K., Szabo, E. Z., Lukacs, G., Orlowski, J., and Grinstein, S. (1998) *J. Biol. Chem. 274*, 11289−11295.
25. D'Souza, S., Garcia-Cabado, A., Yu, F., Teter, K., Lukacs, G., Skorecki, K., Moore, H. P., Orlowski, J., and Grinstein, S. (1998) *J. Biol. Chem. 273*, 2035−2043.
26. Fan, L., Wiederkehr, M. R., Collazo, R., Wang, H., Crowder, L. A., and Moe, O. W. (1999*) J. Biol. Chem. 274*, 11289−11295.
27. Biemesderfer, D., Nagy, T., DeGray, B., and Aronson, P. S. (1999) *J. Biol. Chem. 274*, 17518−17524.
28. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) *Nature 40*, 286−290.
29. Bernardo, A. A., Kear, F. T., Santos, A. V., Ma, J., Steplock, D., Robey, R. B., and Weinman, E. J. (1999) *J. Clin. Invest. 104*, 195−201.

BI000064M