Phosphorylation of PDZ1 Domain Attenuates NHERF-1 Binding to Cellular Targets

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NHERF-1 (Na+/H+ exchanger regulatory factor 1, also known as EBP50 ezrin-binding protein of 50 kDa) is a phosphoprotein that assembles multiprotein complexes via two PDZ domains and a C-terminal ezrin-binding domain. Current work utilized metabolic labeling in cultured cells expressing wild type GFP-NHERF-1 to define the physiological importance of NHERF-1 phosphorylation. Treatment of cells with phosphatase inhibitors calyculin A and okadaic acid enhanced NHERF-1 phosphorylation and inhibited its dimerization. Eliminating C-terminal serines abolished the modulation of NHERF-1 dimerization by phosphatase inhibitors and identified the phosphorylation of the PDZ1 domain that attenuated its binding to physiological targets, including β2-adrenergic receptor, platelet-derived growth factor receptor, cystic fibrosis transmembrane conductance regulator, and sodium-phosphate cotransporter type IIa. The major covalent modification of PDZ1 was mapped to serine 77. Confocal microscopy of cultured cells suggested key roles for PDZ1 and ERM-binding domain in localizing NHERF-1 at the cell surface. The substitution S77A eliminated PDZ1 phosphorylation and increased NHERF-1 localization at the cell periphery. In contrast, S77D reduced NHERF-1 colocalization with cortical actin cytoskeleton. These data suggested that serine 77 phosphorylation played key role in modulating NHERF-1 association with plasma membrane targets and identified a novel mechanism by which PDZ1 phosphorylation may transduce hormonal signals to regulate the function of membrane proteins in epithelial tissues.

NHERF-1 (Na+/H+ exchanger regulatory factor isoform 1) was isolated as a phosphoprotein required for cAMP-mediated inhibition of renal Na+/H+ exchanger isoform 3 (NHE3)3 (1).

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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3 The abbreviations used are: NHE3, Na+/H+ exchanger isoform 3; GFP, green fluorescent protein; WT, wild type; PKA, cAMP-dependent protein kinase; NTA, nitrilotriacetic acid; PTH, parathyroid hormone; CFTR, cystic fibrosis transmembrane conductance regulator.
enhanced by NHERF-1 binding (19), and a complex of PDGFR with NHERF-1 and ERM proteins may regulate actin cytoskeleton and cell migration (20). Because PDGFR showed a preference for PDZ1, it was postulated that a NHERF-1 homodimer facilitated PDGFR signaling. However, later studies showed that PDGFR, like CFTR, could bind both PDZ1 and PDZ2 (21), suggesting that a monomeric NHERF-1 may also stabilize the ligand-bound PDGFR dimer. However, PDGFR signaling was unexpectedly enhanced rather than impaired in NHERF-1 null MEFs. This suggested quite a different role for NHERF-1, namely to promote the assembly of a complex containing PDGFR and PTEN, a phosphoinositide phosphatase that attenuates mitogenic signaling (20). Because PTEN also showed a preference binding to PDZ1, it still remains unclear whether monomeric or dimeric NHERF-1 regulates PDGFR signaling.

It is noteworthy that binding of many PDZ1 targets, including β2-AR and PDGFR, enhanced NHERF-1 dimerization in vitro (19). Fractionation of tissue extracts and coimmunoprecipitation studies (22, 23) also hinted at the existence of NHERF-1 dimers. In this regard, a number of studies have shown that phosphorylation of NHERF-1 by GRK-6A, Cdk-2, and PKC in vitro modulated NHERF-1 dimerization (23–25). Structure-function studies of monomeric NHERF-1 also noted an intramolecular interaction of a C-terminal PDZ motif at the NHERF-1 terminus with PDZ2 (18, 21) that required either the engagement of ezrin (21) or PKC phosphorylation of C-terminal serines (339 and 340), previously implicated in the enhancement of NHERF-1 dimerization (25) to promote activation of the “head-to-tail” monomer and the binding of PDZ1 (21) and PDZ2 targets (18, 21). This suggested that mechanisms that activate the NHERF-1 monomer may also promote its dimerization. On the other hand, NHERF-1 phosphorylation at a major C-terminal site (serine 289) that also enhanced dimerization appears to be constitutive (23) and totally dispensable for hormonal regulation of NHE3 (2). Finally, mass spectrometry of rat NHERF-1 expressed in HEK293 cells identified additional phosphorylations whose physiological role still remains to be investigated.

The current studies focused on the analysis of NHERF-1 phosphorylation in cells with the goal of identifying covalent modifications that regulate the assembly of cellular complexes. Metabolic labeling of cells treated with the phosphatase inhibitors, okadaic acid, and calyculin A established that C-terminal phosphorylation of NHERF-1 inhibited its dimerization. Elimination of three major C-terminal sites identified a phosphorylation of PDZ1 domain of NHERF-1. Biochemical studies showed that the phosphorylation, which occurred at serine 77, attenuated NHERF-1 binding to several PDZ1 targets. Altered localization of mutant NHERF-1, specifically S77A and S77D, suggested a key role for association of PDZ1 targets for NHERF-1 localization at the plasma membrane and suggested a novel mechanism for hormonal regulation of NHERF-1-associated membrane processes in polarized epithelial cells.

**MATERIALS AND METHODS**

**NHERF-1 Expression Plasmids**—Human NHERF-1 cDNA was excised from plasmid encoding HA-tagged human NHERF-1 (26) and inserted in-frame into pEGFP-C2 (Clontech). GFP-D1 was generated by digesting GFP-NHERF-1 with SacI, the cDNA encoding amino acids 1–150 was religated into pEGFP-C2, and its orientation was verified by direct sequencing in the Duke Comprehensive Cancer Center DNA sequencing facility. GFP-D2 was generated by digesting GFP-NHERF-1 with SacI and Kpnl and inserted into pEGFP-C3 to yield GFP fused to amino acids, 151–358, of NHERF-1. For AERM, site-directed mutagenesis was used to introduce stop codon at amino acids 331. NHERF-1 cDNAs with the individual PDZ-binding sequences, PNGYG, modified to HNGAGA (the P to H substitution was fortuitous), were cloned into pET30a and pEGFR-C1. Serines 279, 289, and 301 were substituted with alanines using PCR-based site-directed mutagenesis (Stratagene QuickChange® II site-directed mutagenesis kit). Plasmids encoding hexahistidine-tagged NHERF-1, D1, and D2 were previously described (22).

**Transfection of Cultured Cells**—COS7, HEK293, and NIH3T3 were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing 10% (v/v) fetal bovine serum (HyClone) at 37 °C in 5% CO2 and 95% air. Transfections of plasmid DNA (1 μg) were performed in 6-well plates (Falcon) using 5 μl of Lipofectamine (Invitrogen) in serum-free DMEM. After 3 h, the cells were restored to DMEM containing 10% (v/v) fetal bovine serum.

**Cell Treatment and Immunoblotting**—Twelve to sixteen hours following transfection, the cells were treated for 20 min with okadaic acid (OA) or calyculin A (CalyA) obtained from Alexis. The cells were washed with phosphate-buffered saline and lysed at 4 °C in radioimmune precipitation assay buffer containing protease inhibitors (50 mm Tris-HCl, pH 8.0, 150 mm NaCl, 0.5% (w/v) deoxycholic acid, 0.1% (w/v) sodium dodecyl sulfate, 1% (w/v) Nonidet P-40, 1 mm phenylmethylsulfonyl fluoride, and 1 mm benzamidine). The cell lysates were subjected to SDS-PAGE on 10% (w/v) polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes blocked in 50 mm Tris-HCl, pH 7.5, containing 150 mm NaCl, 0.1% Tween 20 and 4% (w/v) dried milk were incubated overnight with antibodies at 4 °C, and immune complexes were detected by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences).

**Metabolic Labeling with [32P]Orthophosphate**—The cells (10⁶/ml) were incubated in 6-well plates containing DMEM lacking phosphate with 10% (v/v) fetal bovine serum. After 1 h at 37 °C, the cells were incubated with phosphate-free medium containing 500 μCi/ml of [32P]orthophosphate (PerkinElmer Life Sciences) for 2 h. Following exposure to phosphate inhibitors (see above), the cells were washed with phosphate-buffered saline and lysed in radioimmune precipitation assay buffer containing protease inhibitors. The cell lysates were incubated with anti-GFP polyclonal antibody (Clontech) for 1 h at 4 °C, and immune complexes were isolated using 1:1 mixture of protein A-agarose (Bio-Rad) and protein G-agarose (Sigma). The immunoprecipitates were washed with 20 mm Tris-HCl, pH 8.0, 150 mm NaCl, 1 mm EDTA, 1% (v/v) Nonidet P-40 and subjected to SDS-PAGE. The gels were analyzed by autoradiography (Kodak MR x-ray film) or phosphorimaging. Parallel gels were immunoblotted for protein expression using anti-GFP antibodies (Chemicon; 1:5000 dilution).
**Expression of Recombinant Proteins**—Plasmids encoding glutathione S-transferase (GST) fused to C termini of β2AR and PDGFR (provided by R. A. Hall, Emory University), CFTR (J. Biber, University of Zurich), Npt2a (A. Gupta, University of Maryland and S. M Gisler, University of Zurich), NHE3 (O. Moe, University of Texas Southwestern Medical Center), and N-terminal ERM domain of merlin (D. Gutmann, Washington University) were transformed into *Escherichia coli* BL-21, and bacteria were grown in 500 ml of LB medium at 37 °C until A600 between 0.6 and 0.8. Protein expression was induced by isopropyl β-d-thiogalactopyranoside (0.1 mM) for 3 h. The bacteria were lysed in 50 mM Tris-HCl, pH 7.5, containing 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% (v/v) Nonidet P-40, 1 mM benzamidine, and 1 mM phenylmethylsulfonfyl fluoride, and the lysates were incubated with glutathione-Sepharose for 30 min at 4 °C. After extensive washing, the bound proteins were eluted with 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 20 mM glutathione, 1 mM dithiothreitol, and 0.1% (v/v) Nonidet P-40 at 4 °C for 15 min. Eluates, dialyzed against 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol, were stored at 4 °C. The protein concentration was estimated using Bio-Rad protein assay (27).

**RESULTS**

**Activity of GFP-NHERF-1**—Prior studies used untagged (28) and His-NHERF-1 (29) to transduce cAMP signals that inhibited NHE3 activity in PS120/NHE3V cells. Because GFP represented a larger (approximately 27 kDa) protein fusion that could potentially compromise NHERF-1 function, we analyzed the ability of GFP-NHERF-1 (expressed at various levels) to mediate cAMP inhibition of NHE3 in PS120/NHE3V cells. NHE3 activity in PS120/NHE3V cells expressing the highest levels of GFP-NHERF-1 was inhibited 40–50% by 10 μM Fsk (supplemental Fig. S1). Comparison at different protein expression levels suggested that GFP-NHERF-1 was functionally indistinguishable from untagged or His-NHERF-1 (Fig. 1). Treatment of PS120/NHE3V with GFP-NHERF-1 with 100 nM CalyA, a cell-permeable phosphatase inhibitor, achieved similar inhibition of NHE3 (supplemental Fig. S1). These studies showed that PKA activation or phosphatase inhibition resulted in a similar decrease in NHE3 activity. The combined effects of CalyA and Fsk were similar to either agent alone, suggesting a common mechanism for NHE3 inhibition.

**NHERF-1 Dimerization in Cells**—Prior studies used okadaic acid to inhibit NHERF-1 dimerization in cells (22). Because okadaic acid displayed significant cytotoxicity in some cells, we re-evaluated NHERF-1 dimerization by coexpressing HA-NHERF-1 and His-NHERF-1 in PS120 cells (Fig. 2) treated with either vehicle or 100 nM CalyA. Although equal amounts of His-NHERF-1 bound NTA-agarose from control and CalyA-treated cells, HA-NHERF-1 cosedimented with His-NHERF-1 in PS120 cells (Fig. 2) treated with either vehicle or 100 nM CalyA. No detectable HA-NHERF-1 bound NTA-agarose when cells did not also express His-NHERF-1. Comparison of cells expressing His and HA-ΔPDZ2 showed that the mutant protein dimerized effectively, and its dimerization was also inhibited by CalyA. This suggested that NHERF-1 dimerization and modulation by phosphatase inhibitors did not require functional PDZ domains.

To identify NHERF-1 domains required for CalyA-regulated dimerization, we modified the above assay. Lysates of cells expressing WT GFP-NHERF-1 were incubated with defined quantities of recombinant His-NHERF-1, and GFP-NHERF-1 was sedimented using NTA-agarose (Fig. 3). WT GFP-
Phosphorylation of NHERF-1 PDZ1 Domain

<table>
<thead>
<tr>
<th>100 nM CalyA</th>
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<th>ΔPDZ1/2</th>
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<tr>
<td>IB: Anti-HA</td>
<td>-</td>
<td>+</td>
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<tr>
<td>IB: Anti-His</td>
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**FIGURE 2. Homodimerization of NHERF-1 in PS120 cells.** PS120 cells expressed either WT NHERF-1 or ΔPDZ1/2 were expressed as both HA- and His-tagged proteins. The cells were incubated in the absence (−) or presence (+) of 100 nM CalyA for 20 min. Following cell lysis, His proteins were sedimented using NTA-Sepharose ("Materials and Methods"). The bound NHERF-1 proteins were then subjected to SDS-PAGE and analyzed by immunoblotting (IB) with both anti-HA and anti-His antibodies. A representative experiment from three independent studies is shown.

A) **GFP- NHERF-1**

<table>
<thead>
<tr>
<th>Cell Lysates</th>
<th>IB: Anti-GFP</th>
<th>NTA-Agarose</th>
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<td>FL PDZ1 PDZ2</td>
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B) **GFP-AAA**

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<tr>
<th>Cell Lysates</th>
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<td>FL PDZ1 PDZ2</td>
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**FIGURE 3. Structural requirements for NHERF-1 dimerization.** COS7 cells expressing WT GFP-NHERF-1 were metabolically labeled with [32P]orthophosphate. GFP-NHERF-1 was immunoprecipitated using anti-GFP and immunoprecipitates subjected to SDS-PAGE and autoradiography (Fig. 4A) or phosphorimaging. As previously seen in HEK293 and PS120 cells (23, 30), full-length NHERF-1 is extensively phosphorylated in COS7 cells under basal conditions. NHERF-1 phosphorylation was further increased 1.5–2-fold in cells treated with CalyA with maximum [32P]-labeling occurring between 10 and 100 nM CalyA. Substitution of alanines in place of serines 279, 289, and 301 eliminated the basal phosphorylation but was phosphorylated only in response to 100 nM CalyA. OA differs from CalyA in the inhibition of type I and type II serine/threonine phosphatases in being a more potent inhibitor of type 2 phosphatases. Metabolic labeling of COS7 cells treated with increasing concentrations of OA showed that GFP-D1 phosphorylation was enhanced between 1 and 10 μM OA (Fig. 4B) to a level similar to 100 nM CalyA. The data suggested that GFP-D1 phosphorylation was most likely reversed by type I protein serine/threonine phosphatase that required OA concentrations higher than 1 μM for effective inhibition. Because 100 nM CalyA lacked the cytotoxicity seen with micromolar concentrations of OA, subsequent analyses of GFP-D1 phosphorylation utilized cells treated with CalyA.

Calyculin A-stimulated Phosphorylation Sites in PDZ1—NHERF-1 is a phosphoprotein, whereas its structural homo-
logue, NHERF-2, is not. There are 6 serines and threonines in NHERF-1 D1 region, and some within the PDZ1 domain are also conserved in NHERF-2 (Fig. 5A). We substituted alanines in place of serines 2, 46, 77, and 143 and threonines 71 and 95, whereas serine 143 was eliminated by the C-terminal truncation, Δ140 (Fig. 5A). Mutant GFP-D1 proteins were expressed in COS7 cells metabolically labeled with [32P]orthophosphate in the presence of 100 nM CalyA. Immunoblotting with anti-GFP antibody from lysates of COS7 cells metabolically labeled with [32P]orthophosphate as described under “Materials and Methods.” The cells were treated with increasing concentrations of CalyA. B shows the phosphorylation of GFP-D1 in cells treated with 100 nM CalyA and increasing concentrations of OA. Representative autoradiographs from more than six independent experiments are shown.

FIGURE 4. Phosphorylation of NHERF-1 in COS7 cells. A shows the phosphorylation of WT GFP-NHERF-1, GFP-AAA, GFP-D1, and GFP-D2 immunoprecipitated (IP) using anti-GFP antibody from lysates of COS7 cells metabolically labeled with [32P]orthophosphate as described under “Materials and Methods.” The cells were treated with increasing concentrations of CalyA. B shows the phosphorylation of GFP-D1 in cells treated with 100 nM CalyA and increasing concentrations of OA. Representative autoradiographs from more than six independent experiments are shown.

FIGURE 5. Identification of serine 77 as the major calyculin A-induced phosphorylation site in PDZ1. A shows the alignment of primary sequences of PDZ1 domains from NHERF-1, a known phosphoprotein, and NHERF-2, which is not phosphorylated in cells. Serines and threonines conserved in both proteins are shown in bold and underlined. Serine 77 is uniquely present in NHERF-1 and is highlighted as an enlarged bold letter. The schematic shows the location of serines in GFP-D1, and Δ140 shows the C-terminal truncation that eliminated serine 143. B shows the immunoprecipitates of GFP proteins with individual serines substituted with alanines. The lower panel shows the anti-GFP immunoblot (IB) of anti-GFP immunoprecipitates from COS7 cells treated with 100 nM CalyA. The upper panel shows the corresponding autoradiograph. A representative figure from six independent experiments is shown.

identifying serine 77, uniquely present in PDZ1 of NHERF-1, as the major site of CalyA-induced phosphorylation.

Phosphorylation of Serine 77 Attenuates Binding of PDZ1 Targets—To assess the function of CalyA-induced phosphorylation, GFP-D1 was sedimented from lysates of cells treated with CalyA using recombinant GST-AR encompassing the C terminus of β2-adrenergic receptor, a known PDZ1 target. Increasing amounts of GFP-D1 were sedimented by 0.1–10 μg of GST-AR from control COS7 lysates (Fig. 6A). Treatment of cells with 10–100 nM CalyA reduced GFP-D1 binding by GST-AR in a dose-dependent manner. The inhibition was most readily visible at 0.5 and 0.1 μg of bait protein with 100 nM CalyA inhibiting GFP-D1 binding by 70 and 90%, respectively (Fig. 6B). CalyA also inhibited GFP-D1 binding by other PDZ1 targets, GST-PDGFR and GST-CFTR (Fig. 6C). No detectable sedimentation of GFP-D1 was observed with GST alone in the presence or absence of CalyA. Essentially identical results were obtained for the binding of full-length GFP-NHERF-1 by all three PDZ1 targets (data not shown).

Bone loss resulting from phosphate wasting in mice lacking a functional NHERF-1 gene established the renal sodium-phosphate cotransporter, Npt2a, as a physiological target of NHERF-1 (32). Pulldowns with GST-Npt2a containing the C terminus of Npt2a established its binding to full-length GFP-NHERF-1, which was significantly inhibited by the treatment of cells with 100 nM CalyA (Fig. 6D). In several independent experiments, 100 nM CalyA resulted in 45–50% inhibition of GFP-NHERF-1 binding by GST-Npt2a.
To identify the residues in PDZ1 that transduced the inhibitory effects of CalyA, we undertook pulldowns from COS7 lysates containing WT and mutant GFP-D1 lacking individual serines and threonines using GST-β2AR as bait (Fig. 7A). With 0.1 and 2.5 µg of the bait protein, we noted dose-dependent sedimentation of WT GFP-D1, which was inhibited 90 and 65%, respectively, by 100 nM CalyA. S46A showed a dramatic reduction in basal binding to GST-β2AR, potentially disrupting the critical carboxylate loop required for binding of target C termini. The residual binding was still sensitive to CalyA and encompassing C terminus of sodium-phosphate cotransporter type IIa, a physiological target of NHERF-1 in the mammalian kidney. The left panel shows a representative immunoblot for binding of GFP-NHERF-1 by GST-Npt2a, and the right panel shows the quantitation of four independent experiments with standard errors.
**DISCUSSION**

In Vivo Phosphorylation of NHERF-1—Mass spectrometry of rat NHERF-1 expressed in HEK293 cells identified six phosphoserines and threonines located either in the PDZ1 domain or near the C terminus (25). The majority of the protein-bound phosphate was on serine 287 (serine 289 in human NHERF-1) and was unresponsive to cell stimuli (23). The constitutive serine 289 phosphorylation is catalyzed by GRK6A, which binds NHERF-1 PDZ1 and possibly PDZ2 domains (23). WT NHERF-1 expressed in COS7 (or HEK293, NIH3T3, and HeLa; not shown) migrated on SDS-PAGE as two polypeptides with the upper band representing highly phosphorylated NHERF-1 and thus eliminated by dephosphorylation of His-NHERF-1 bound to NTA-agarose or GFP-NHERF-1 in anti-GFP immunoprecipitates (data not shown) with nonspecific phosphatases. PURNAM$^\ddagger$ failed to bind WT GFP-NHERF-1. By contrast, GST$^\u03c9$-BAR and GST-NHE3 failed to bind $\Delta$PDZ2 and $\Delta$PDZ2, respectively. All three proteins bound GST-merlin, demonstrating the presence of a functional ERM-binding domain (data not shown).

Remarkably, COS7 cells expressing the NHERF-1 mutant, $S77A$, which abolished PDZ1 phosphorylation, showed enhanced peripheral localization compared with WT NHERF-1 (Fig. 8C). In contrast, $S77D$ that mimicked phosphorylation showed reduced localization at the cell periphery, displaying little overlap with rhodamine-phalloidin. Neither $S77A$ nor $S77D$ showed significant nuclear localization. Thus, unlike $\Delta$PDZ1, $S77D$ did not represent a complete loss of function of PDZ1, which may be necessary for NHERF-1 redistribution to cell nucleus, but reflected altered affinity for PDZ1 targets, most of which are located at the plasma membrane.

**Phosphorylation of NHERF-1 PDZ1 Domain**

required for binding of PDZ1 targets (33) also showed a reduced concentration at the cell periphery and increased entry into the nucleus. In contrast, $\Delta$PDZ2 lacking a functional PDZ2 domain was essentially identical to WT GFP-NHERF-1 and was largely excluded from the nucleus. Essentially identical localization of GFP-NHERF-1 was seen in HEK293 and NIH3T3 cells, although lower expression of GFP proteins in these cells yielded a weaker signal (data not shown).

Pulldowns using GST-βAR, a PDZ1 target (10), and GST-NHE3, a PDZ2 target (6), established that these proteins bound WT GFP-NHERF-1. By contrast, GST-βAR and GST-NHE3 failed to bind $\Delta$PDZ1 and $\Delta$PDZ2, respectively. All three proteins bound GST-merlin, demonstrating the presence of a functional ERM-binding domain (data not shown).

**GFP-NHERF-1 Localization in Cells—Analysis of subcellular distribution of GFP-NHERF-1 in COS7 cells (Fig. 8) showed that WT NHERF-1 was predominantly cytosolic with some protein colocalizing with actin cytoskeleton stained by rhodamine-phalloidin (red), and the nucleus was stained with the DNA-binding dye, 4′,6′-diamino-2-phenylindole (DAPI, blue). The top row shows a representative cell (in black-and-white) showing images at the individual channels, whereas the bottom row shows color images of GFP alone, an overlay of F-actin and nuclear staining and overlay of all three channels. 8 shows the overlays of rhodamine-phalloidin and GFP fluorescence of cells expressing WT GFP-NHERF-1, $\Delta$ERM, $\Delta$PDZ1, and $\Delta$PDZ2. C shows representative the three color images for GFP-$S77D$ and GFP-$S77A$. In all above panels, an asterisk marks the nucleus, and arrows point to areas of cell periphery where rhodamine-phalloidin and/or GFP are concentrated.**

virtually eliminated in cells treated with 100 nM CalyA. Like WT GFP-D1, CalyA inhibited T71A binding to GST-βAR. However, GST-βAR binding to $S77A$ was not inhibited by CalyA, most clearly seen with 2.5 μg of bait. This suggested that phosphorylation of serine 77 located on the PDZ1 α-helix involved in target binding (Fig. 7B) mediated the inhibitory effect of CalyA.

**GFP-NHERF-1 Localization in Cells—Analysis of subcellular distribution of GFP-NHERF-1 in COS7 cells (Fig. 8) showed that WT NHERF-1 was predominantly cytosolic with some protein colocalizing with actin cytoskeleton stained by rhodamine-phalloidin at the cell periphery (white arrows in Fig. 8A). Most importantly, WT GFP-NHERF-1 was largely excluded from the nucleus and stained with 4′,6′-diamino-2-phenylindole (asterisk in Fig. 8A). Deletion of C-terminal ERM-binding domain reduced the concentration of GFP-ERM at the cell periphery and increased its entry into the nucleus (asterisk in Fig. 8B). GFP-$\Delta$PDZ1 that lacked the core GLGF sequence...
PDZ-associated kinases like GRK6A or PKC and a Cdc2-like kinase, whose phosphorylations must be functionally dominant to reduce NHERF-1 dimerization in CalyA-treated cells.

Metabolic labeling revealed the phosphorylation of GFP-AAA only in the presence of phosphatase inhibitors, suggesting a rapidly turned over or transient phosphorylation. GFP-D1 phosphorylation was similarly stimulated by CalyA and was mapped to serine 77. Interestingly, mass spectrometry of rat NHERF-1 had identified inconsistent phosphorylation of serine 77 and threonine 71. Although the substitution S77A diminished in vivo phosphorylation of GFP-D1 by more than 80%, T71A had no effect and thus appeared not to be phosphorylated in COS7 cells. Instead, T95A, which reduced GFP-D1 radiolabeling by approximately 15%, accounted for the remaining protein-bound phosphate.

Serine 77 is located on the surface of the α2-helix of PDZ1. Histidine 72 and arginine 80 on this helix make critical contacts with side chains of amino acids at the −2 position and leucine 0 to tether the C termini of PDZ-1 targets. The introduction of a charged residue, such as glutamic acid 82 or aspartic acid 83, at position 77 was also completely inactive. However, asparagine 82 or aspartic acid 83 at position 77 only partially restored activity, suggesting that the surface charge of residue 77 is critical for recognition of PDZ-1 targets.

PDZ1 at serine 77 may preferentially attenuate the recruitment of some PDZ1 targets and dissociate selected NHERF-1 complexes in response to hormones and other physiological stimuli.

**Cellular Targeting of NHERF-1**—NHERF-1 was first purified from rabbit renal brush border membranes (1) and required mild detergents for its efficient extraction. Immunohistochromistry of mouse and human renal proximal tubules provided further evidence for a high NHERF-1 concentration at apical membranes (32). Finally, more than two-thirds of known NHERF-1 targets represent PDZ1-binding proteins that reside in the plasma membrane or the underlying cytoskeleton (9). By comparison, the few nuclear targets show a preferred binding to PDZ2. Thus, GFP-NHERF-1 expressed in COS7, HEK293, and NIH3T3 cells showed similar diffuse cytosolic distribution with some concentration at or near the plasma membrane, most readily seen in COS7 cells, which expressed higher levels of GFP-NHERF-1. Most notably, WT GFP-NHERF-1 was largely excluded from the nucleus. Deletion of the C-terminal ERM-binding region, ΔERM, or the mutation, ΔPDZ1, that abolished the binding of PDZ1 targets, dramatically reduced plasma membrane localization of these mutant NHERF-1 proteins, allowing their nuclear entry. This pointed to the combined actions of PDZ1 targets and Ezrin in localizing NHERF-1 outside the nucleus or concentrating NHERF-1 at the cell periphery. Remarkably, the mutant S77A showed enhanced localization at the cell periphery in COS7 cells, whereas the concentration of S77D, which mimicked PDZ1 phosphorylation and attenuated the binding of cellular targets at the cell periphery, was reduced. These data suggested that serine 77 phosphorylation did not represent a complete loss of PDZ1 function but modified the affinity of NHERF-1 to regulate the trafficking and/or activity for the many membrane targets. NHERF-1 was first purified from rabbit renal brush border membranes (1) and required mild detergents for its efficient extraction. 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This pointed to the combined actions of PDZ1 targets and Ezrin in localizing NHERF-1 outside the nucleus or concentrating NHERF-1 at the cell periphery. Remarkably, the mutant S77A showed enhanced localization at the cell periphery in COS7 cells, whereas the concentration of S77D, which mimicked PDZ1 phosphorylation and attenuated the binding of cellular targets at the cell periphery, was reduced. These data suggested that serine 77 phosphorylation did not represent a complete loss of PDZ1 function but modified the affinity of NHERF-1 to regulate the trafficking and/or activity for the many membrane targets. NHERF-1 was first purified from rabbit renal brush border membranes (1) and required mild detergents for its efficient extraction. Immunohistochromistry of mouse and human renal proximal tubules provided further evidence for a high NHERF-1 concentration at apical membranes (32). Finally, more than two-thirds of known NHERF-1 targets represent PDZ1-binding proteins that reside in the plasma membrane or the underlying cytoskeleton (9). By comparison, the few nuclear targets show a preferred binding to PDZ2. Thus, GFP-NHERF-1 expressed in COS7, HEK293, and NIH3T3 cells showed similar diffuse cytosolic distribution with some concentration at or near the plasma membrane, most readily seen in COS7 cells, which expressed higher levels of GFP-NHERF-1. Most notably, WT GFP-NHERF-1 was largely excluded from the nucleus. Deletion of the C-terminal ERM-binding region, ΔERM, or the mutation, ΔPDZ1, that abolished the binding of PDZ1 targets, dramatically reduced plasma membrane localization of these mutant NHERF-1 proteins, allowing their nuclear entry. This pointed to the combined actions of PDZ1 targets and Ezrin in localizing NHERF-1 outside the nucleus or concentrating NHERF-1 at the cell periphery. 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assembly and disassembly of membrane complexes in epithelial tissues.

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REFERENCES

ADDITIONS AND CORRECTIONS

Phosphorylation of PDZ1 domain attenuates NHERF-1 binding to cellular targets.
James W. Voltz, Matthew Brush, Suzanne Sikes, Deborah Steplock, Edward J. Weinman, and Shirish Shenolikar

During publication of this manuscript, gaps separating individual panels to signify a composite figure were eliminated or omitted in some figures. In keeping with JBC guidelines, the revised figures that specifically separate all composite panels to indicate that data from different gels were analyzed are provided below.

The specific corrections are as follows. 1) In revised Fig. 2A, control WT GFP-NHERF-1 (top left) was sedimented with WT His-NHERF-1 by incubating lysates of untreated COS-7 cells that coexpressed these proteins using NTA-agarose and was run on a separate gel from GFP-NHERF-1 sedimented from cells treated with calyculin A (top right). Fig. 2B shows an independent experiment in which both samples were run on the same gel. The figure legend and conclusion remain unchanged. The data show that in cells treated with phosphatase inhibitors, dimerization of NHERF-1, either WT or PDZ1/2, a mutant lacking functional PDZ domains, was inhibited in essentially the similar manner.

2) WT GFP-NHERF-1 sedimented from either control or calyculin A-treated COS-7 cells using GST-βBAR, GST-PDGFR, or GST-CFTR (Fig. 6C) or GST-Npt2a (Fig. 6D) bound to glutathione-agarose was run on separate gels, and all composite panels have been separated with gaps. The figure legend remains unchanged.

3) A polypeptide encompassing the N-terminal PDZ1 domain of NHERF-1, GFP-D1, either WT or with alanine substitutions in place of Ser66 (S46A), Thr71 (T71A), and Ser77 (S77A), was sedimented from control (−) or calyculin A-treated (+) COS-7 cells using two different concentrations of GST-βBAR as described in the legend for Fig. 7. Representative gels from independent experiments are shown with lines to separate samples that were run on different gels.

All other figures and the overall conclusions of the manuscript are unchanged.

We suggest that subscribers photocopy these corrections and insert the photocopies in the original publication at the location of the original article. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Phosphorylation of PDZ1 Domain Attenuates NHERF-1 Binding to Cellular Targets

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