Defective Parathyroid Hormone Regulation of NHE3 Activity and Phosphate Adaptation in Cultured NHERF-1−/− Renal Proximal Tubule Cells

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Rochelle Cunningham‡, Deborah Steplock‡, Fengying Wang‡, Huijun Huang‡, Xiaofei E‡, Shirish Shenolikar§, and Edward J. Weinman¶**

From the Departments of §Medicine and ¶Physiology, University of Maryland School of Medicine, ‡Medical Service, Department of Veterans Affairs Medical Center, Baltimore, Maryland 21201 and the §Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

The present experiments using primary cultures of renal proximal tubule cells derived from wild-type and NHERF-1 knockout animals examines the regulation of NHE3 by phenylthiodyantoin (PTH) and the regulation of phosphate transport in response to alterations in the media content of phosphate. Forskolin (34.8 ± 6.2%) and PTH (28.7 ± 1.8%) inhibited NHE3 activity in wild-type proximal tubule cells but neither forskolin (−3.2 ± 3.3%) nor PTH (−18.6 ± 8.1%) inhibited NHE3 activity in NHERF-1−/− cells. Using adenosine-mediated gene transfer, expression of NHERF-1 in NHERF-1−/− proximal tubule cells restored the inhibitory response to forskolin (28.2 ± 3.0%) and PTH (33.2 ± 3.9%). Compared with high phosphate media, incubation of wild-type cells in low phosphate media resulted in a 36.0 ± 6.3% higher rate of sodium-dependent phosphate transport and a significant increase in the abundance of Npt2a and PDZK1. NHERF-1−/− cells, on the other hand, had lower rates of sodium-dependent phosphate uptake and low phosphate media did not stimulate phosphate transport. Npt2a expression was not affected by the phosphate content of the media in NHERF-1 null cells although low phosphate media up-regulated PDZK1 abundance. Primary cultures of mice proximal tubule cells retain selected regulatory pathways observed in intact kidneys. NHERF-1−/− proximal tubule cells demonstrate defective regulation of NHE3 by PTH and indicate that reintroduction of NHERF-1 repairs this defect. NHERF-1−/− cells also do not adapt to alterations in the phosphate content of the media indicating that the defect resides within the cells of the proximal tubule and is not dependent on systemic factors.

There is growing recognition that transporters, ion channels, receptors, and signaling proteins exist in cells as multiprotein complexes (1–7). These complexes link together critical pathways thereby conferring selectivity to the cellular responses to second messengers that regulate the activity and/or trafficking of these proteins. Adaptor proteins appear central to the assembly and stability of these protein complexes. In the kidney, the sodium-hydrogen exchanger regulatory factor-1 (NHERF-1, also called EBP50) and NHERF-2 (also called E3KARP, TKA1) were initially identified as proteins required for cAMP-associated phosphorylation and inhibition of the renal brush border membrane Na+-H+ exchanger isofor 3 (NHE3) (8, 9, 10). NHERF-1 and NHERF-2 are adaptor proteins that share a common modular structure including two tandem PDZ (PSD-95/Dlg/ZO-1) protein interactive domains and a C-terminal ezrin-radixin-moesin-merlin (ERM) binding domain. Since their discovery, nearly 50 proteins have been identified that bind to the NHERF isoforms (11). More recently, other PDZ proteins such as PDZK1 (NaPi-Cap1) have been found to be present in renal tubules and to interact with many of the same proteins that bind NHERF-1 and NHERF-2 (12).

NHERF-1, NHERF-2, and PDZK1 have the capacity not only to self-associate but also to heterodimerize with one another (13–16). Given their location in the renal proximal convoluted tubule, the concept emerged that these adaptor proteins form an apical/subapical mesh or scaffold that regulates the activity and trafficking of target proteins. We, as well as others, have speculated that NHERF-1, NHERF-2, and PDZK1 may represent a redundant physiologic control mechanism for regulation of renal ion transport and receptor activity based on studies in heterologous expression systems (10, 11, 16).

Evidence has begun to emerge, however, suggesting that there are unique specificities for some target proteins with individual adaptor proteins. Initially, it was reported that calcium-mediated endocytosis of NHE3 in OK cells, a renal proximal tubule cell line, required NHERF-2 and that the presence of endogenous NHERF-1 was not sufficient (17). Further support for more specific interactions between NHERF-1, NHERF-2, and PDZK1, and given target proteins emerged from study of NHERF-1 knockout mice and two sets of observations were of particular interest with respect to the present studies (18). First, although both NHERF-1 and NHERF-2 supported cAMP regulation of Na+-H+ exchange activity when co-expressed with NHE3 in PS120 fibroblast cells, NHE3 activity assayed in isolated renal brush border membrane vesicles from NHERF-1−/− mice was not inhibited in response to activation of protein kinase A despite the presence of normal amounts of NHERF-2 and PDZK1 (10, 19). Second, whereas in vitro and cell experiments have indicated that Npt2a binds to

1 The abbreviations used are: NHERF, sodium-hydrogen exchanger regulatory factor; NHE3, Na+-H+ exchanger isofor 3; BŒCF, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein; TMA-Cl, (tetramethylammonium chloride); EIPA, ethyl isopropylamiloride; MES, 4-morpholinepropanesulfonic acid; PTH, phenylthiodyantoin; PKC, protein kinase C; GFP, green fluorescent protein.
NHERF-1, NHERF-2, and PDZK1, the NHERF-1−/− mice demonstrated increased urinary excretion of phosphate associated with decreased expression of Npt2a (NaPi IIa), the major regulated renal sodium-dependent phosphate transporter in the apical membrane of renal proximal tubule cells (18, 20, 21). Moreover, the NHERF-1−/− mice fail to fully adapt to restriction of the dietary intake of phosphate although the abundance of PDZK1 but not NHERF-2 is increased (22). These experiments highlight the fact that conclusions about the specificity of interactions between NHERF-1, NHERF-2, or PDZK1 and specific renal transporters derived from in vitro, yeast, and cell expression studies require complimentary studies in native tissues.

Unfortunately, pursuing the mechanisms underlying the abnormal regulation of NHE3 by PTH and of Npt2a by alterations in the dietary intake of phosphate in intact animals is difficult and many of the molecular and pharmacologic probes used to dissect specific pathways cannot be used in whole animals. We reasoned, therefore, that the development of primary cultures of mouse proximal tubules would provide a useful system to detail the role of not only the NHERF proteins but also, potentially, might be applicable to the study of renal tissue from other knockout animals. In this article, we describe methods to isolate and study mice primary renal proximal tubule cells in culture that expresses NHE3 and Npt2a, and retain selected regulatory responses including inhibition of NHE3 activity by PTH and forskolin. NHE3 activity in proximal tubule cells from NHERF-1−/− mice, on the other hand, failed to demonstrate inhibition in response to forskolin or PTH indicating that this form of hormonal control of NHE3 activity requires NHERF-1. This conclusion was confirmed by the observations that the inhibitory response to PTH was restored by transiently expressing NHERF-1 in the NHERF-1−/− cells using adenovirus-mediated gene transfer. In like manner, it has been assumed but not clearly established that the physiologic mechanisms that mediate the response to alterations in the phosphate content of the media bathing cultured cells are equivalent to those operative in modulating renal phosphate transport when the dietary intake of phosphate is altered. By contrast to NHERF-1−/− cells that increase sodium-dependent phosphate transport and Npt2a expression in response to low phosphate media, the NHERF-1−/− cells fail to adapt to the low phosphate media. This indicates clearly that the phosphate regulatory defect is within the proximal tubules themselves and is not the consequence of systemic changes in hormones, vitamin levels, or other factors.

EXPERIMENTAL PROCEDURES

Animals and Preparation of Renal Proximal Tubule Cells—Male NHERF-1−/− mice (B6.129-Sle9a3HsdN/Sid1) bred into a C57BL/6 background for 6 generations and parental wild-type inbred control C57BL/6 mice aged 12 to 16 weeks were used in the current experiments (18). To prepare primary renal proximal tubule cell cultures, kidneys were dissected and minced, and digested using 1% Worthington collagenase Type II and 0.25% soybean trypsin inhibitor. On completion of digestion, the samples were re-suspended in 35 ml of modified Eagle’s medium containing 32P-radiolabeled orthophosphate. Uptake was continued for 20 min followed by sequential washes with a solution containing 130 mM (tetramethylammonium chloride (TMA-Cl), 5 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 1 mM TMA-PO4, 25 mM glucose in 20 mM HEPES, pH 7.5. BICEF fluorescence was measured at excitation wavelengths of 500 and 440 nm and an emission wavelength of 530 nm. The NH4Cl pulse was targeted to achieve an initial pH, of 6.0 and only cells with initial pH values between 6.0 and 6.2 were included for analysis. Na+−H+ exchange transport, expressed as △pH, was calculated using the slope of the initial 5 to 10 s of sodium-dependent pH recovery. Over this time period, the relation between pH, and time was essentially linear. In some cases, cells were pretreated with 10−4 M forskolin or 10−7 M PTH (1−34) (rat-PTH) during the final 15 min of dye loading and continuously during the perfusion. Control cells were treated with MeSO4, the diluent for forskolin. At the end of each experiment, the cells were equilibrated in pH clamp media containing 20 mM HEPES, 20 mM MES, 110 mM KCl, 14 mM NaCl, 1 mM MgSO4, 1 mM CaCl2, 1 mM TMA, 25 mM glucose, and 10 μM nigericin at pH 6.1 and 7.2. Control and experimental measurements were made on cells from the same proximal tubule cell preparation and were assayed on the same day.

Phosphate transport was measured by determination of the sodium-dependent uptake of radiolabeled phosphate (23). Twenty-four to 72 h prior to study, the cells were incubated in low (0.3 mM) or high (1.9 mM) phosphate media. The cells were washed in serum-free medium, followed by incubation in a transport medium containing 137 mM NaCl or 137 mM TMA-CI, 5.4 mM KCl, 2.8 mM CaCl2, 12 mM MgSO4, and 0.1 mM KH2PO4. Phosphate uptake was initiated by the addition of transport medium containing 32P-radiolabeled orthophosphate. Uptake was continued for 10 min at room temperature, after which the cell were washed with ice-cold medium in which TMA-CI was substituted for sodium chloride, 32P was omitted, and 0.5 mM sodium arsenate was added. The uptake of 32P from the TMA-CI solution was used to determine sodium-independent uptake and was subtracted from the sodium-dependent uptake of 32P. The cells were solubilized in 1% Triton X-100 for 90 min at 4°C and an aliquot analyzed by liquid scintillation spectroscopy. Each assay was performed in triplicate and averaged to provide a single data point.

Other Methods—The production of intracellular cAMP in cultured cells in response to 10−8 M PTH and 10−4 M forskolin was measured by non-acetylated EIA (cAMP Biotrak Assay Kit, Amersham) in the presence of 0.4 mM 3-isobutyl-1-methylxanthine. Protein kinase activity was assayed using the SignaTект™ PKC Assay System from Promega containing a specific PKC substrate and capture membrane. The results of the PKC assays were confirmed by Western immunoblotting using PKCa, phospho-PKC (pan), and phospho-PKC αβδ antibodies.

Cells were fixed in paraformaldehyde and prepared for confocal microscopy as previously described (18). To obtain membrane preparations from the cultured cells, the cells were washed with sterile ice-cold phosphate-buffered saline, detached by scraping, and centrifuged for 5 min at 800 × g. The supernatant was discarded and the pellet re-suspended in 1.5 ml of buffer containing 50 mM Tris, pH 7.4, 0.1 mM EDTA, 0.1% β-mercaptoethanol, and Complete Protease Inhibitor Mixture (Roche Applied Science). The cells were then disrupted by three 20-s bursts from a probe sonicator followed by a 10-min centrifugation at 1,000 × g to remove large particulates. This supernatant was ultra-centrifuged at 100,000 × g and digested using 0.5% SDS and prepared for electrophoresis by the addition of Laemmli buffer. Western immunoblotting was performed using antibodies specific for NHERF-1, NHERF-2, ezrin, PDKZ1, Npt2a, NHE3, and green fluorescent protein (GFP).

Infective recombinant adenoviruses were produced using AdEasy (Stratagene), a commercially available system adapted from methods originally developed by He et al. (24). Recombinant adenoviruses were produced by inserting the cDNA into a shuttle plasmid (pShuttleCMV) and performing homologous recombination in Escherichia coli with this shuttle vector and a large adenovirus-containing plasmid following electroporation. Recombinants were identified from single colonies and infective adenovirus virions were produced following transfection of the linearized recombinant adenovirus plasmid in HEK293 cells. Virus stocks were amplified in HEK293 cells on 15-cm plates and purified following lysis by CaCl2 banding using ultracentrifugation.

Protein concentrations were determined using the method of Lowry.
RESULTS

An enriched suspension of proximal convoluted tubules was obtained using gradient centrifugation following protocols previously described by Mandel and co-workers and modified by our laboratories (27, 28). We plated the suspended tubules on culture dishes for biochemical and phosphate transport experiments or on glass coverslips for NHE3 assays. Plating of cells on basement membrane matrix (Matrigel) increased the viability of the cells and was used in most experiments. The cells remained relatively quiescent for several days immediately following plating and any attempts to change media or otherwise manipulate the cells resulted in decreased viability. Accordingly, the cells were left undisturbed for 3 to 4 days before changing the media after which the media was changed every other day till the cells were ready for study. Attempts to pass cells that had achieved confluence were unsuccessful.

Confluent or near confluent cultures contained a uniform cell type although in every culture, there were clusters of cells with a different morphology. We estimated that these other cells constituted 5% or less of the total population of cells. As will be discussed, we did not attempt to identify this minority population of cells because it would be unlikely that they would express the two transporters of interest in this study, namely NHE3 and Npt2a. Assays for cAMP and protein kinase C activity were used to characterize the response to PTH. In wild-type cells, PTH (10⁻⁷ M) stimulated cAMP generation 3.3 ± 8.4-fold and protein kinase C activity by 3.4 ± 6.2%. In NHERF-1 null proximal tubule cells, PTH (10⁻⁷ M) stimulated cAMP generation 33.8 ± 7.2-fold and protein kinase C activity by 37.6 ± 6.1%. There were no differences between wild-type and knockout cells (Table I). In many of the above experiments, cAMP generation in response to vasopressin (10⁻⁷ M) was also examined. The effect of vasopressin was variable but always severalfold lower than the response to PTH (data not shown).

Reverse transcriptase-PCR and immunoblotting indicated that the cultured cells expressed NHE1 and NHE2 in addition to NHE3. Accordingly, we next determined the Na⁺-H⁺ exchange activity in wild-type and NHERF-1 null proximal tubule cells, and the response to 50 μM ethylisopropylamiloride (EIPA), a concentration that would inhibit NHE1 and NHE2 activity but not affect NHE3 activity (29). Na⁺-H⁺ exchange averaged 0.044 ± 0.002 μP/s and 0.042 ± 0.02 in the absence and presence of 50 μM EIPA in wild-type cells and 0.042 ± 0.004 μP/s and 0.039 ± 0.004 in the absence or presence of 50 μM EIPA in null cells. In both cell types, 50 μM EIPA abolished sodium-dependent pH recovery. In all remaining experiments, 50 μM EIPA was included in the perfusion solutions to permit specific study of NHE3 activity.

NHERF-1 facilitates the formation of a multiprotein signal complex that phosphorylates NHE3 and down-regulates its activity (30, 31). To determine whether cultured proximal tubules retain this regulatory pathway, wild-type proximal tubule cells were treated with forskolin and sodium-dependent pH recovery was measured in the presence of 50 μM EIPA. Forskolin inhibited NHE3 activity by 34.7 ± 3.2% (p < 0.05, n = 7) in wild-type cells but failed to inhibit NHE3 activity (4.2 ± 3.5%, n = 7) in cultured proximal tubule cells from NHERF-1⁻/⁻ mice although basal NHE3 activity was similar in both cell types (0.041 ± 0.004 ΔP/H/s in wild-type cells and 0.045 ± 0.003 in NHERF-1 null cells).

Recombinant adenovirus-NHERF-1 containing an enhanced GFP tag at the N terminus was used to determine whether we could rescue the null cells. Preliminary studies in PS120 cells indicated that expressed N-terminal GFP-tagged NHERF-1 was fully functional in mediating cAMP inhibition of co-expressed NHE3. We initially infected wild-type cells with various concentrations of adenovirus-GFP or adenovirus-GFP-NHERF-1. For both viruses, 10⁹ plaque-forming units were found to result in preserved cell morphology. Comparison of fluorescent labeled cells with bright field images indicated the efficiency of expression of NHERF-1 was 99% or greater at these concentrations of virus particles. The efficiency of infection can also be appreciated in Fig. 1, which shows representative confocal images of proximal convoluted tubule cells expressing NHERF-1⁻/⁻ mice uninfected (panels A and C) or infected with adenovirus-GFP-NHERF-1 (panels B and D). The cells were stained using an antibody to NHERF-2. All cells stained positive for NHERF-2 (panels C and D) but only cells infected with adenovirus-GFP-NHERF-1 were positive for NHERF-1 (panel B).

![Image](image_url)

**FIG.1.** Representative confocal images of proximal convoluted tubule cells from NHERF-1⁻/⁻ mice uninfected (panels A and C) or infected with adenovirus-GFP-NHERF-1 (panels B and D). The cells were stained using an antibody to NHERF-2. All cells stained positive for NHERF-2 (panels C and D) but only cells infected with adenovirus-GFP-NHERF-1 were positive for NHERF-1 (panel B).
Sodium-dependent phosphate uptake in wild-type cells incubating a phosphate-restricted diet associated with a decrease in phosphate content of the media, we measured changes in the phosphate content of the media. To characterize this response in more detail, membrane preparations from the cells were harvested and Western immunoblots were used to quantitate changes in expression of Npt2a, NHERF-1, NHERF-2, and PDZK1. Exrin expression was not different between cells incubated in low or high phosphate media and was used to normalize loading of the gels. As shown in Table IV, Npt2a expression was 33.1 ± 3.0% higher in wild-type cells incubated in low phosphate as compared with high phosphate media. Expression of NHERF-1 and NHERF-2 were not different. PDZK1 expression was significantly higher in cells incubated in the low phosphate media.

NHERF-1 null cells had lower rates of sodium-dependent phosphate transport compared with wild-type cells in either low or high phosphate media (Table IV). Moreover, by contrast to wild-type cells, sodium-dependent phosphate transport was not higher in NHERF-1−/− cells incubated in low as compared with high phosphate media. As shown in Table IV, Npt2a expression did not differ in NHERF-1 null cells incubated in low versus high phosphate media. As observed in the wild-type cells, NHERF-2 expression was not different and, as expected, NHERF-1 was not detected. Like the wild-type cells, PDZK1 expression in NHERF-1 null proximal tubule cells in culture was 44.3 ± 8.7% higher in cells incubated in low phosphate media compared with cells incubated in high phosphate media.

**DISCUSSION**

Study of animals with specific gene deletions has proven to be a powerful tool to uncover the function of specific genes in the context of the whole animal, and in intact organs and tissues. This has become critically important with the increasing recognition that heterologous expression systems may not reflect totally the interaction between proteins in native tissue (10, 18, 20, 22). Studies in intact animals and organs, however, do not readily permit the type of detailed biochemical experiments and experimental manipulations that are possible, for example, in heterologous expression systems. The use of primary cultured cells from genetically altered animals holds the potential to allow use of molecular and pharmacological probes to characterize biochemical pathways while simultaneously facilitating correlations to studies in intact animals and organs. Our prior studies have focused on the regulation of the NHE3 and Npt2a, two transporters expressed in the apical membrane of the renal proximal convoluted tubule (18, 19, 22). Unfortunately, there has been but limited literature on culturing of renal proximal tubule cells from any species including mice. Direct conversations with a number of investigative groups indicates such studies were abandoned when it was found that obtaining sufficient cells for biochemical analyzes from hand-dissected proximal tubules was nearly impossible, that the cells could not be passed and sustained in culture, and that certain defects became apparent under specific physiologic circumstances. To overcome some of the limitations of studying hormonal regulation of renal proximal tubule transporters such as NHE3 and Npt2a in intact mice or in renal brush border membrane vesicles, we undertook to develop a primary renal proximal tubule culture system that retained not only the regulatory features of normal renal proximal tubule cells but also the defects identified in intact NHERF-1 knockout mice. Toward these ends, we used a preparation enriched in proximal tubule segments and plated these cells on plastic culture dishes or coverslips coated with solubilized basement membrane material (27, 28). We found that the cell so cultured remained quiescent for 3 to 4 days and reached confluence by days 7 to
NHE3 and Npt2a Regulation in NHERF-1<sup>−/−</sup> Proximal Tubule Cells

Na<sup>+</sup>-H<sup>+</sup> exchange activity was determined in the presence of 50 nM EIPA in adenovirus-GFP (GFP) infected (10<sup>6</sup> plaque-forming units) or adenovirus-GFP-NHERF-1 (GFP-NHERF-1) (10<sup>6</sup> plaque-forming units) infected cultured renal proximal tubule cells from wild-type or NHERF-1<sup>−/−</sup> mice. Studies were performed in the absence (control) or presence of forskolin (10<sup>−4</sup> m) or PTH (10<sup>−7</sup> m) (“Experimental Procedures”). Results are calculated as ΔpH/s and expressed as mean ± S.E.

### TABLE II

|                | Control | Experimental | Change  |
|----------------|---------|--------------|---------|
| Wild-type cells (GFP) | 0.041 ± 0.004 | 0.026 ± 0.001 | 34.8 ± 8.2<sup>a</sup> |
| Forskolin (n = 6) | 0.045 ± 0.003 | 0.031 ± 0.002 | 29.7 ± 8.8<sup>a</sup> |
| PTH (n = 7) | 0.036 ± 0.004 | 0.036 ± 0.004 | −3.2 ± 3.3<sup>a</sup> |
| NHERF-1<sup>−/−</sup> cells (GFP) | 0.033 ± 0.003 | 0.037 ± 0.005 | −16.6 ± 8.1 |
| Forskolin (n = 7) | 0.047 ± 0.003 | 0.033 ± 0.003 | 28.2 ± 3.0<sup>a</sup> |
| PTH (n = 8) | 0.048 ± 0.003 | 0.028 ± 0.002 | 33.2 ± 3.9<sup>a</sup> |

* Indicates p < 0.05. n = number of preparations studied.

### TABLE III

The effect of incubation in low or high phosphate media on sodium-dependent phosphate transport in cultured proximal tubule cells from wild-type and NHERF-1<sup>−/−</sup> mice

|                | Low phosphate | High phosphate | Change  |
|----------------|---------------|----------------|---------|
| Wild-type cells (n = 8) | 9.6 ± 2.2 | 6.1 ± 1.5 | 36.0 ± 6.2<sup>a</sup> |
| NHERF-1<sup>−/−</sup> cells (n = 7) | 1.9 ± 0.6 | 1.8 ± 0.6 | 2.4 ± 5.3<sup>a</sup> |

* Indicates p < 0.05. n = number of preparations studied.

10. In the initial days after plating, it was necessary to leave the cells undisturbed and to avoid changing of the media. We also observed that it was not regularly possible to pass these cells. Approximately 95% or more of the cells had a uniform appearance although clusters of different appearing cells were evident in most preparations. We assumed these other cells to be fibroblasts or other non-epithelial cells but did not pursue their identification given that the transport proteins under study, namely NHE3 and Npt2a, would not likely be expressed in these other cells. The PTH receptor is known to signal in the proximal tubule of the kidney through both protein kinase A and protein kinase C pathways (32, 33). In the cultured cells, incubation with PTH resulted in robust production of cAMP and activation of protein kinase C activity. The generation of cAMP in response to vasopressin was variable but always orders of magnitude less than PTH, suggesting that the cells were predominantly of proximal tubule origin. Segre and co-workers (34) have published a series of papers indicating that NHERF-1 and/or NHERF-2 functions as a molecular switch for PTH receptor signaling. In the absence of NHERF, signaling through the protein kinase A pathway is facilitated, whereas signaling through protein kinase C is inhibited (34). In the present experiments, however, we found no difference in cAMP generation or PKC activation between wild-type cells and NHERF-1<sup>−/−</sup> cells. Native and cultured mouse proximal tubules express both NHERF-1 and NHERF-2, and NHERF-2 expression is normal in the NHERF-1 knockout cells. It is possible, therefore, that the presence of at least one of the isoforms is sufficient to engage the PTH receptor and direct its downstream signal pathways. This is an attractive speculation given that the best evidence in renal epithelial cells that NHERF proteins are critical to the function of the PTH receptor derives from systems where both NHERF isoforms were not concurrently expressed. For example, Segre and co-workers (35) recently used a derived line of proximal tubule OK cells with much reduced native NHERF-1 as a model system. In contrast to renal proximal convoluted tubule cells of mouse, rabbit, and man, OK cells more resemble the rat and normally do not express NHERF-2 (15, 36, 37). Clearly, additional studies will be required to directly address these speculations.

The proximal tubule cultures demonstrated sodium-dependent pH recovery from an acid load in the absence of CO<sub>2</sub> or bicarbonate indicating the presence of sodium-hydrogen exchange activity. NHE1, NHE2, and particularly, NHE3 were readily detected by reverse transcriptase-PCR in these cell preparations. We tested the effect of concentrations of EIPA that would inhibit NHE1 and NHE2 if present in any cells in the preparation (29). There was a small inhibitory effect of 50 nM EIPA, whereas 50 μM EIPA abolished completely sodium-dependent pH recovery. Accordingly, we included 50 nM EIPA in all perfusion solutions to allow specific focus on the role of NHERF-1 in the regulation of NHE3 activity by PTH and forskolin. The NHERF proteins were identified initially by their ability to facilitate formation of a multiprotein signaling complex to affect cAMP-associated phosphorylation and inhibition of NHE3 already resident in the apical membrane of renal tubule cells (9, 10, 30, 31). In PS120 cell fibroblasts, NHERF-1 and NHERF-2 both bound NHE3, ezrin, and were biologically equivalent (10). In brush border membranes from kidneys of NHERF-1<sup>−/−</sup> mice, however, activation of protein kinase A failed to phosphorylate NHE3 and did not inhibit its activity despite the presence of NHERF-2 and normal amounts of active catalytic subunit of protein kinase A (19). These results indicated a distinct role for NHERF-1 as compared with NHERF-2 in cAMP regulation of NHE3 activity in native renal tissue as compared with heterologous expression systems such as PS120 cells. Neither the PS120 cells nor the brush border membrane vesicle preparations obtained from mice kidneys, however, permitted study of the regulatory role of PTH on NHE3 activity. In the cultured proximal tubule cells, basal NHE3 activity as assessed using BCECF fluorescence was not different between cells obtained from wild-type and NHERF-1<sup>−/−</sup> animals. This would be consistent with our prior observations that NHERF-1 is not involved in the targeting of NHE3 and that apical NHE3 expression and activity is unaltered in NHERF-1 knockout mice (15, 18). Wild-type proximal tubule cells demonstrated a significant inhibitory response to PTH and forskolin. Strikingly, NHE3 activity in NHERF-1 null cells was not inhibited by forskolin. This would be consistent with our findings in isolated brush border membranes from these animals. PTH also did not inhibit NHE3 activity in NHERF-1 null cells. Whereas abnormal NHE3 regulation by cAMP in NHERF-1<sup>−/−</sup> cells was anticipated, the finding that PTH failed to inhibit NHE3 activity in the null cells was somewhat sur-
Npt2a was mistargeted and its expression in the apical membrane increased excretion of phosphate in the urine. Furthermore, NHERF-1 knockout mice demonstrated a decrease in the serum concentration of phosphate and its PDZ ligands (18). The NHERF-1 knockout mouse demonstrated some specificity to the interaction between Npt2a and its PDZK1, NHERF-1, and NHERF-2 (12, 16, 20, 21). It has been suggested that these PDZ proteins form an adenosinokinase C rather than protein kinase C (33). As already discussed, PTH receptor activation of protein kinase C was normal in NHERF-1−/− cells. NHERF proteins do interact with some elements of protein kinase C pathways such as RACK1, but the link to NHERF-1 regulation of NHE3 activity has not been elucidated (38). In the present studies, for reasons not immediately apparent, the response to PTH in NHERF-1−/− proximal tubule cells was variable and, in fact, some of the cultures showed significant stimulation of NHE3 activity. It is worth noting that some prior studies have suggested that the direct effect of activation of protein kinase C is to activate NHE3 (39). In this regard then, NHERF-1 knockout mice may prove valuable in sorting out potential regulatory processes affecting the relation between PTH receptor activation and NHE3 activity.

We next attempted to reconstitute NHERF-1 null cells using adenovirus-GFP-NHERF-1 to affect rapid infection of the cultured cells and expression of NHERF-1. Despite the fact that the adenovirus uses the αv integrin receptor located on the basolateral side of epithelial cells, over 99% of cells were infected using concentrations that did not alter viability or disrupt the morphology of the proximal tubule cells. Following expression of NHERF-1, the inhibitory effect of forskolin and PTH was restored to NHERF-1−/− cells. In our prior studies using brush border membrane vesicles from wild-type and NHERF-1-null animals, it was not possible to specifically study the response to hormones such as PTH (19). The current studies, then, provide a critical bridge and indicate that while NHERF-2 and PDZK1 may play a permissive or adjunctive role, the inhibition of NHE3 by PTH uniquely requires NHERF-1.

Npt2a is the major regulated renal proximal tubule sodium-dependent phosphate transporter and apical membrane expression of Npt2a determines the rates of phosphate transport in the renal proximal tubule (40, 41). Npt2a contains a canonical PDZ binding motif in its C terminus and recent studies have indicated that Npt2a binds to a number of PDZ domain proteins including PDZK1, NHERF-1, and NHERF-2 (12, 16, 20, 21). It has been suggested that these PDZ proteins form an apical/subapical mesh in the renal proximal tubule to orient, recruit, retain, or otherwise regulate target proteins (11, 15, 16). The NHERF-1 knockout mouse, however, suggested that there was some specificity to the interaction between Npt2a and its PDZ ligands (18). The NHERF-1 knockout mouse demonstrated a decrease in the serum concentration of phosphate associated with increased excretion of phosphate in the urine. Npt2a was mistargeted and its expression in the apical membrane of the proximal tubule was decreased. Npt2a expression is regulated by the dietary intake of phosphate and, in a recent study, we demonstrated that NHERF-1 knockout mice do not fully adapt to a low phosphate diet as compared with wild-type controls (22). Npt2a expression was again noted to be reduced in animals ingesting the low phosphate diet and Npt2a was readily detected in a submicrovillus region of proximal tubule cells that associated, but only in part, with clathrin vesicles. These results indicated that NHERF-1 is involved in the trafficking of Npt2a and, in this regard, its association with Npt2a differs significantly from its association with NHE3. In cultured cell lines that express Npt2a, alterations in the concentration of phosphate in the media approximates the changes in Npt2a metabolism observed in intact animals subjected to changes in the dietary intake phosphate (42, 43). It should be acknowledged, however, that it is not clearly established that the physiologic pathways involved are identical. It was important, therefore, to determine whether NHERF-1 expression in the proximal tubule was directly involved in the cellular defect in phosphate metabolism or if the inability of the NHERF-1 null mice to adapt to changes in the dietary intake of phosphate was the secondary effect of NHERF-1 on systemic factors affecting phosphate metabolism (22). In wild-type cultured proximal tubule cells, sodium-dependent phosphate transport was significantly higher in cells incubated in low compared with high phosphate media. This adaptation appeared to be complete by 24 h and no further changes were noted up to 72 h of incubation. Cells incubated in the low phosphate media had increased membrane expression of Npt2a and PDZK1 but no change in the expression of NHERF-1 or NHERF-2. Primary cultured renal proximal tubule cells from the NHERF-1−/− animals, on the other hand, had lower rates of sodium-dependent phosphate transport compared with wild-type controls and no differences were detected in cells incubated in low phosphate media compared with high phosphate media. We were also unable to detect changes in membrane expression of Npt2a or NHERF-2. These results strongly suggest that the lack of NHERF-1 results in a defect at the level of the renal proximal tubule cell. Of interest, the low phosphate media up-regulated the abundance of PDZK1 in NHERF-1 null cells to a degree similar to that seen in wild-type cells. Thus, whatever the signal leading to regulation of PDZK1 by alterations in the dietary intake of phosphate or the phosphate content of the incubating media, it occurs at the level of the renal proximal tubule cell and is independent of NHERF-1. We predict that these primary cultures will be a valuable model system to define the role of NHERF-1 and the other interacting PDZ proteins on the renal apical membrane insertion, retention, and/or retrieval of Npt2a.

### Table IV

| Expression of Npt2a, NHERF-1, NHERF-2, and PDZK1 in membranes from wild-type and NHERF-1−/− cultured proximal tubule cells incubated in low or high phosphate media |
|---------------------------------------------------------------|
| **Low phosphate** | **High phosphate** | **Change** |
| Npt2a (n = 8) | 1.3 ± 0.2 | 0.8 ± 0.2 | 33.1 ± 3.0 |
| NHERF-1 (n = 6) | 0.3 ± 0.1 | 0.3 ± 0.1 | 1.4 ± 1.4 |
| NHERF-2 (n = 6) | 0.8 ± 0.02 | 0.8 ± 0.2 | −3.5 ± 4.3 |
| PDZK1 (n = 6) | 0.4 ± 0.1 | 0.2 ± 0.1 | 44.3 ± 2.7 |
| NHERF-1−/− cells | 0.8 ± 0.2 | 0.8 ± 0.2 | −7.4 ± 6.1 |
| Npt2a (n = 6) | Not detected | Not detected | |
| NHERF-1 (n = 6) | 1.3 ± 0.4 | 1.2 ± 0.4 | 7.1 ± 9.8 |
| PDZK1 (n = 6) | 1.7 ± 0.8 | 0.7 ± 0.3 | 44.3 ± 8.7 |

*a* Indicates p < 0.05. n = number of preparations studied.
In summary, the present experiments provide a new model system to study selected renal transporters in mice renal proximal tubule cells; a model system that retains not only some well-characterized regulatory phenomena but also defects in regulation of NHE3 and Npt2a observed in intact NHERF-1 mice. The cultures described herein, should also provide the needed transition between intact animals and heterologous expression systems to facilitate detailed study of regulatory pathways and protein trafficking using molecular biological and pharmacologic reagents. Given that the NHERF proteins bind to pathways for energy transduction, these recombinant protein expression systems may be especially informative in understanding how the NHERF proteins bind to multiple interacting partners, including regulatory or ion transport proteins, to facilitate molecular events with physiological consequences. The described procedures have the potential to be widely applicable to the study of other NHERF-associated proteins.

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