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Cooperativity between the Phosphorylation of Thr^{95} and Ser^{77} of NHERF-1 in the Hormonal Regulation of Renal Phosphate Transport*

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Edward J. Weinman[‡§¶1], Deborah Steplock[‡], Yinghua Zhang[§], Rajatsubhra Biswas[‡], Robert J. Bloch[§], and Shirish Shenolikar[‖]

From the Departments of [‡]Medicine and [§]Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201, [¶]Medical Service, the Department of Veterans Affairs Medical Center, Baltimore, Maryland 21201, and the [‖]Program in Neuroscience and Behavioral Disorders, Duke-National University of Singapore, Graduate Medical School, Singapore 169857

The phosphorylation of the sodium-hydrogen exchanger regulatory factor-1 (NHERF-1) plays a key role in the regulation of renal phosphate transport by parathyroid hormone (PTH) and dopamine. Ser^{77} in the first PDZ domain of NHERF-1 is a downstream target of both hormones. The current experiments explore the role of Thr^{95}, another phosphate acceptor site in the PDZ I domain, on hormone-mediated regulation of phosphate transport in the proximal tubule of the kidney. The substitution of alanine for threonine at position 95 (T95A) significantly decreased the rate and extent of *in vitro* phosphorylation of Ser^{77} by PKC. In NHERF-1-null proximal tubule cells, neither PTH nor dopamine inhibited sodium-dependent phosphate transport. Infection of the cells with adenovirus expressing full-length WT GFP-NHERF-1 increased basal phosphate transport and restored the inhibitory effect of both PTH and dopamine. Infection with full-length NHERF-1 containing a T95A mutation, however, increased basal phosphate transport but not the responsiveness to either hormone. As determined by surface plasmon resonance, the substitution of serine for aspartic acid (S77D) in the PDZ I domain decreased the binding affinity to the sodium-dependent phosphate transporter 2a (Npt2a) as compared with WT PDZ I, but a T95D mutation had no effect on binding. Finally, cellular studies indicated that both PTH and dopamine treatment increased the phosphorylation of Thr^{95}. These studies indicate a remarkable cooperativity between the phosphorylation of Thr^{95} and Ser^{77} of NHERF-1 in the hormonal regulation of renal phosphate transport. The phosphorylation of Thr^{95} facilitates the phosphorylation of Ser^{77}. This, in turn, results in the dissociation of NHERF-1 from Npt2a and a decrease in phosphate transport in renal proximal tubule cells.

Parathyroid hormone (PTH) and dopamine are important factors regulating phosphate homeostasis. Both hormones activate specific receptors in the renal proximal tubule thereby initiating protein kinase cascades that mediate the endocytosis of the major phosphate transporters including the sodium-dependent phosphate transporter 2a (Npt2a) located in the apical membrane of these cells (1–3). Interestingly, the downstream target of these protein kinases is not Npt2a itself, but rather a Npt2a-binding protein called the sodium-hydrogen exchanger regulatory factor-1 (NHERF-1) (4, 5). NHERF-1 is a prototypic adaptor protein containing two PDZ domains and a C-terminal domain that binds the cytoskeletal proteins ezrin, moesin, radixin, and merlin (6). PTH and dopamine have been shown to phosphorylate Ser^{77} of the first PDZ domain of NHERF-1, resulting in the dissociation of NHERF-1·Npt2a complexes (1, 5). This dissociation appears to be required for Npt2a to interact with other proteins that mediate entry into its endocytic pathway (7). The net result of these reactions is a decrease in the abundance of Npt2a in the apical membrane of renal proximal convoluted tubule cells, a decrease in phosphate uptake, and an increase in the urinary excretion of phosphate. Recent studies from our laboratory have indentified Thr^{95} in the first PDZ domain of NHERF-1 as another phosphoacceptor site, but its potential role in regulating phosphate transport has not been explored (8). The present studies indicate interesting and potentially important interactions among the phosphorylation of Thr^{95}, the phosphorylation of Ser^{77}, and the renal transport of phosphate. In particular, we report that the phosphorylation of Thr^{95} is increased by both PTH and dopamine and that this biochemical modification enhances the phosphorylation of Ser^{77}, thereby modulating a decrease in Npt2a binding to NHERF-1 and renal phosphate transport (1, 5).

EXPERIMENTAL PROCEDURES

Preparation of cDNAs, Recombinant Polypeptides, and Adenovirus Vectors—WT and mutant cDNAs of the PDZ I domain of NHERF-1 (representing amino acids 1–150) were prepared using existing restriction sites and/or PCR. Mutations were generated by site-directed mutagenesis using single-stranded DNA and appropriate primers and confirmed by dideoxynucleotide sequencing. All NHERF-1 peptides were expressed as fusion proteins containing an N-terminal His_6 tag. The cDNAs were subcloned into pET30A (Stratagene), expressed in *Escherichia coli*, and purified on Ni-Sepharose. The cDNAs were also subcloned into pcDNA3.1 for transfection into cells using Lipofectin (Invitrogen). Adenovirus-mediated gene transfer was used to infect primary cultures of mouse proximal tubule cells

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[1] To whom correspondence should be addressed: Division of Nephrology, Rm. N3W143, University of Maryland Hospital, 22 S. Greene St., Baltimore, MD 21201. Fax: 410-706-4195; E-mail: eweinman1440@yahoo.com.

as previously described (9). Infective recombinant adenoviruses were produced using AdEasy (Stratagene). The recombinant adenoviruses were produced by inserting the cDNA into a shuttle plasmid (pShuttleCMV) and performing homologous recombination in *E. 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 ultracentrifugation using a CSCL gradient.

Phosphorylation Reactions, Phosphate Transport, and SPR[2] *Measurements—In vitro* phosphorylation experiments were performed by mixing PDZ I recombinant polypeptides (33 μg) in a phosphorylation solution containing 10 mM $MgCl_2$, 100 μM $CaCl_2$, 100 μg/ml L-α-phosphatidylserine, 20 μg/ml 1–2-dioleoyl-*sn*-glycerol, 125 μM ATP, 0.25 μCi of [^{32}P]ATP, and 33 milliunits of PKC at 30 °C. At specified intervals, 10 μl of the reaction mixture was removed, boiled in Laemmli buffer, and loaded on 15% SDS gels. After electrophoresis and autoradiography, the gels were stained with Coomassie Blue and dried, and the intensity of the bands was determined using a PhosphorImager. The equality of loading was determined by quantitating the Coomassie Blue bands, and, where necessary, the counts/min were corrected for the small differences in loading.

Cultures of primary proximal tubule cells from NHERF-null animals were prepared using methods developed in this laboratory (2, 5). Adenovirus-mediated gene transfer was accomplished using adenovirus-GFP-NHERF-1 constructs (10^9 plaque-forming units/ml) or adenovirus-GFP (10^9 plaque-forming units/ml) as a control as previously detailed (9). Sodium-dependent phosphate transport was measured under control conditions and after treatment with either 10^{-7} M PTH or 10^{-4} M dopamine for 45 min. Cells were incubated in a transport medium consisting of 137 mM NaCl or 137 mM tetramethylammonium chloride, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 0.1 mM KH_2PO_4, and phosphate uptake was initiated by the addition of $^{32}P_i$. After 10 min at room temperature, cells were washed three times with ice-cold fresh medium in which sodium chloride was replaced with tetramethylammonium chloride, and 0.5 mM sodium arsenate was added. After the uptake measurements were completed, the cells were solubilized in 0.5% Triton X-100 for 90 min at room temperature and analyzed by liquid scintillation spectroscopy.

SPR studies were performed using His_6-tagged NHERF-1 polypeptides prepared in *E. coli.* Polypeptides representing the C-terminal 79 amino acids of Npt2a were prepared with a biotinylated tag to permit binding to the SPR chip using strepavidin. The association (measured for 200 s) and dissociation (measured over 500 – 600 s) of these peptides was detected with a BIAcore 3000 (BIAcore, Inc., Uppsala, Sweden) at a flow rate of 20 μl/min.

To study the phosphorylation of the PDZ I domains *in vivo*, opossum kidney cells were transiently transfected with PDZ I constructs using Lipofectamine 2000. Cells were incubated in phosphate-free Dulbecco's modified Eagle's medium containing $^{32}P_i$ and studied under control conditions or after treatment with PTH or dopamine. The PDZ I domains were recovered using nickel chromatography and resolved by SDS-PAGE. After transfer, phosphorylation was quantitated by a PhosphorImager. When the radioactivity had returned to background levels, the identity of the phosphorylated polypeptides was confirmed by Western immunoblotting using an anti-His antibody.

Other Assays—Protein concentrations were determined by the method of Lowry *et al.* (10). Statistical analysis was performed using analysis of variance.

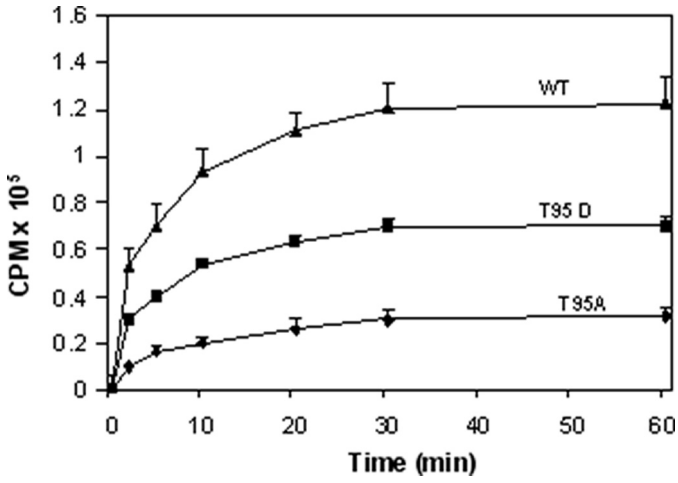

FIGURE 1. **Representative time course of PKC-mediated phosphorylation of recombinant polypeptides of the PDZ I domain of WT NHERF-1 or the PDZ I domain containing either a T95D or T95A mutation.** Results are the mean ± S.E. (*error bars*) of six experiments.

RESULTS

To analyze the role of NHERF-1 phosphorylation at Thr^{95}, we prepared recombinant proteins representing the PDZ I domain of NHERF-1 in which Ser^{46} and Ser^{71} were substituted with alanine residues and Thr^{95} was substituted with either alanine or the phosphomimetic, aspartic acid. Ser^{77} was unchanged and, under these experimental conditions, was the only phosphate acceptor. Identical concentrations of the recombinant proteins were incubated with [^{32}P]ATP, calcium, magnesium, phospholipids, and PKC. As shown in Fig. 1, the maximal phosphorylation of the T95A mutant was approximately half that seen with the T95D polypeptide. The half-time ($t_{½}$) to reach maximal phosphorylation of the T95A polypeptide was 4.7 ± 0.7 min compared with 2.2 ± 0.4 for the T95D mutant ($p < 0.05$, $n = 4$). The total phosphorylation of WT PDZ I was higher than the T95D mutant likely reflecting the availability of the Thr^{95} site, but the $t_{½}$ (2.1 ± 0.4 min) was similar to the T95D mutant. A PDZ I polypeptide containing alanine substitutions at positions 77 and 95 was not phosphorylated (not shown). These findings indicate that the phosphorylation of Thr^{95} influences the rate and extent of phosphorylation of Ser^{77} by PKC.

The physiologic relevance of these observations was then tested in primary cultures of NHERF-1-null proximal tubule cells infected with control adenovirus-GFP, wild-type

[2] The abbreviation used is: SPR, surface plasmon resonance.

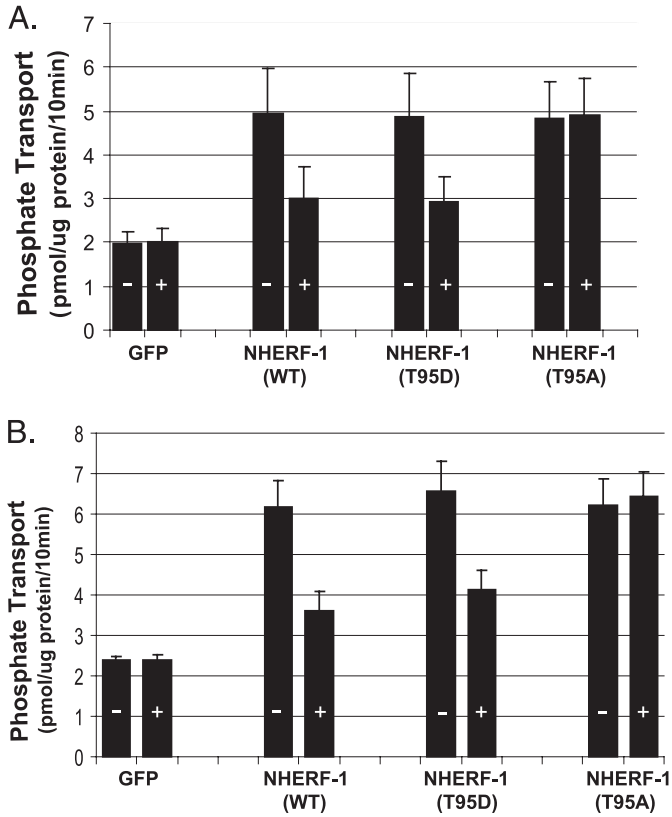

FIGURE 2. **Sodium-dependent phosphate uptake was measured in cultured renal proximal tubule cells from NHERF-1-null animals ($n = 5$).** Cells were infected with control adenovirus-GFP (*GFP*), adenovirus-GFP linked to full-length, wild-type NHERF-1 (*WT*), or adenovirus-GFP linked to full-length NHERF-1 containing either a T95D or T95A mutation. Studies were performed under control conditions (−) or in the presence (+) of PTH(1–34) (10^{-7} M) (*A*) or dopamine (10 μM) (*B*). Results are the mean of means ± S.E. (*error bars*).

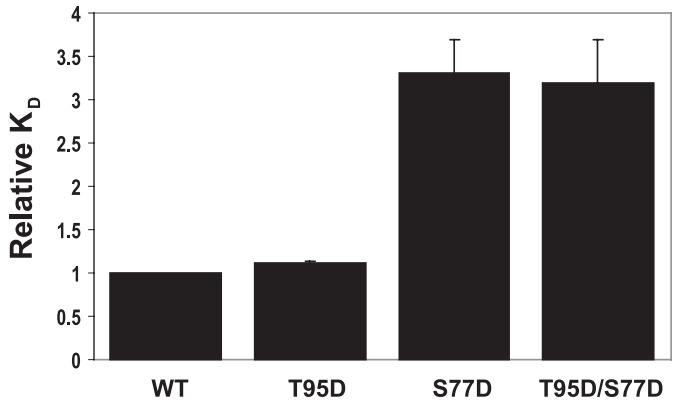

FIGURE 3. **Relative SPR-determined binding affinities of the C-terminal region of Npt2a and PDZ I domains of NHERF-1 containing the T95D, S77D, or combined T95D/S77D mutations compared with WT PDZ I (shown as = 1).**

adenovirus-GFP-NHERF-1 (full-length), or adenovirus-GFP-NHERF-1 (full-length) containing either the T95A or T95D mutation (Fig. 2). As we have previously shown, phosphate transport in NHERF-1-null proximal tubule cells infected with adenovirus-GFP is not inhibited by either PTH or dopamine (2, 6). Infection of NHERF-1-null cells with WT adenovirus-NHERF-1 results in an increase in basal sodium-dependent phosphate transport and restoration of the inhibitory response to PTH and to dopamine. Infection of the NHERF-1-null cells with the T95A or T95D NHERF-1 results in an increase in the basal rates of phosphate transport similar to WT NHERF-1. The inhibition of phosphate transport in cells expressing NHERF-1 T95A in response to PTH and dopamine (−1.8 ± 1.4% and −3.6 ± 3.2%), however, was significantly lower than in NHERF-1 T95D-infected cells (39.1 ± 3.0% and 36.9 ± 1.5% in response to PTH and dopamine, respectively ($p < 0.05$, $n = 5$).

We have previously shown that both PTH and dopamine increase the phosphorylation of Ser^{77} of NHERF-1 and dissociate NHERF-1·Npt2a complexes (1, 5). Using pulldowns with recombinant proteins representing the C terminus of Npt2, we have also shown that the phosphorylation of Ser^{77} reduces its affinity for binding to NHERF-1 (8). To validate and strengthen these findings further, we analyzed real-time binding of recombinant polypeptides representing PDZ I of NHERF-1 to a peptide representing the C-terminal 79 amino acids of human Npt2a using SPR spectroscopy. The binding of WT PDZ I to the C terminus of Npt2a requires the terminal TRL sequence of the transporter, as deletion of these residues abrogated binding completely (data not shown). The K_D of binding of WT PDZ 1 to the C terminus of Npt2a was 1.56×10^{-6} M. Compared with WT PDZ I, PDZ I containing the T95D mutation was not significantly different (Fig. 3). The K_D of the S77D mutant, however, was 3.3 ± 0.4-fold higher than WT PDZ I ($p < 0.05$, $n = 4$), indicating a decrease in binding affinity. Similarly, the K_D of the T95D/S77D double mutant was 3.2 ± 0.51-fold higher than WT PDZ I ($p < 0.05$, $n = 3$), but not significantly different from the S77D mutant. This indicates that the phosphorylation of Ser^{77} inhibits Npt2a binding but that the phosphorylation of Thr^{95} affects the binding of Npt2a indirectly by facilitating PKC-mediated phosphorylation of Ser^{77}.

We next sought to determine whether the phosphorylation of Thr^{95} was a target of the protein kinase cascades initiated by occupancy of the PTH 1 receptor or the dopamine receptors. In a proximal tubule cell line, opossum kidney cells, we expressed cDNAs encoding His_6-PDZ I representing WT PDZ I or PDZ I in which all serine and threonine residues except Thr^{95} were mutated to alanines. The cells were metabolically labeled with $^{32}P_i$ and treated with PTH or dopamine, and the PDZ I domains were recovered from cell lysates by nickel chromatography. As shown in the autoradiographs in Fig. 4, Thr^{95} is phosphorylated in the basal state, and its phosphorylation is increased by both hormones. In response to PTH, the phosphorylation of WT PDZ I was increased 77 ± 5% ($n = 3$, $p < 0.05$), and the phosphorylation of Thr^{95} was increased by 32 ± 5% ($n = 3$, $p < 0.05$). In response to dopamine, the phosphorylation of WT PDZ I was increased by 50 ± 5% ($n = 3$, $p < 0.05$), and the phosphorylation of Thr^{95} was increased by 25 ± 3% ($n = 3$, $p < 0.05$).

DISCUSSION

Recent studies have indicated that Ser^{77} in the first PDZ domain of NHERF-1 is phosphorylated in renal tissue by PTH and dopamine (1, 5). The phosphorylation of Ser^{77}, in turn, results in the disassociation of NHERF-1·Npt2a complexes, a decrease in the abundance of Npt2a in the apical membrane of renal proximal tubule cells, and a decrease in phosphate trans-

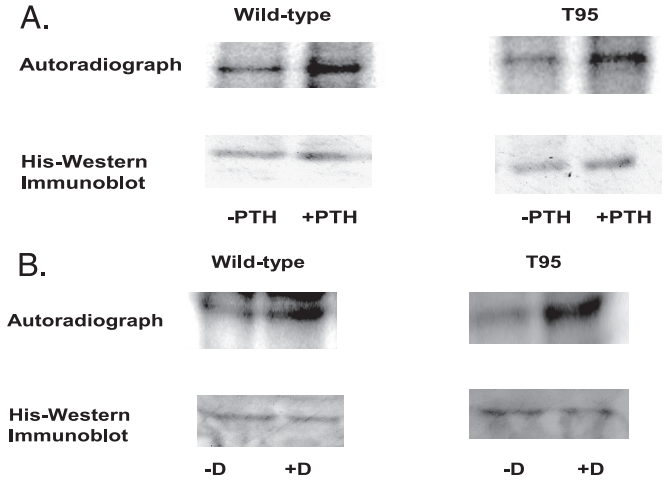

FIGURE 4. **Representative experiment showing autoradiographs and Western immunoblots from opossum kidney cells expressing His_6-PDZ I cDNAs encoding WT PDZ I or PDZ I in which all serine and threonine residues except Thr^{95} were mutated to alanine residues.** The cells were metabolically labeled with $^{32}P_i$ and treated with 10^{-7} M PTH (*panel A*) or 10 μM dopamine (*D*) (*panel B*). The PDZ I domains were recovered from cell lysates using nickel chromatography.

port. Our current findings provide additional insights into the regulatory events that mediate PTH- and dopamine-associated inhibition of renal phosphate transport. Specifically, we show that the phosphorylation of Thr^{95} is a critical determinant of the phosphorylation of Ser^{77} and is required for these hormones to inhibit phosphate transport. Using recombinant polypeptides representing the PDZ I domain of NHERF-1, we find that the substitution of Thr^{95} with alanine significantly decreases both the rate and the extent of phosphorylation of Ser^{77} in response to PKC. The cooperativity between the phosphorylation of Thr^{95} and Ser^{77} is supported by the physiologic studies using proximal tubule cells from NHERF-1-null animals infected with adenovirus-GFP-NHERF-1 constructs. Infection of NHERF-1-null proximal tubule cells with either WT NHERF-1, NHERF-1 containing the T95A mutation, or NHERF-1 containing the T95D mutation resulted in increased basal rates of phosphate transport compared with null cells infected with the control adenovirus-GFP. These findings would be consistent with the proposal that NHERF-1 that is not phosphorylated at Ser^{77} is in a conformation that is permissive for binding to the Npt2a transporter. The finding that the increase in basal phosphate transport following infection with wild-type NHERF-1 is the same as the rates using NHERF-1 with either the T95A or T95D mutation suggests that the trafficking and abundance of all three at the apical membrane are similar. On the other hand, infection of NHERF-1-null cells with adenovirus-NHERF-1 containing the T95A mutation blunts the inhibitory effect of both PTH and dopamine compared with virus encoding either WT NHERF-1 or NHERF-1 containing the T95D substitution. These findings suggest an important regulatory role of Thr^{95} phosphorylation in mediating the hormonal control of phosphate transport.

We have previously shown that the phosphorylation of Ser^{77} affects the binding of Npt2a to NHERF-1 (1, 5). To extend these studies to include PDZ I phosphorylation at Thr^{95}, we employed SPR spectroscopy to determine Npt2a binding to the PDZ I domain of NHERF-1. This *in vitro* assay allows determination of binding affinities in the absence of other potentially confounding protein interactions. The C terminus of Npt2a binds to the first PDZ domain of NHERF-1 via its C-terminal TRL sequence. Compared with WT PDZ I, PDZ I containing the S77D substitution has significantly decreased binding affinity to Npt2a. The introduction of an additional T95D mutation results in no further change in affinity. We suggest that, when considered in conjunction with the phosphorylation studies, the phosphorylation of Thr^{95} is an important regulator of Npt2a binding by virtue of its effect on the phosphorylation state of Ser^{77}, but by itself has little or no effect on Npt2a binding. The crystal structure of NHERF-1 not bound to a target peptide has not yet been reported. In the crystal structures of PDZ I of NHERF-1 complexed with the C-terminal peptides of the cystic fibrosis transmembrane regulator, the β_2-adrenergic receptor, or the platelet-derived growth factor receptor, Ser^{77} lies in the middle of the α2-helix but does not make direct contact with the ligand (11, 12). Nonetheless, the nearby Arg^{78} and Arg^{80} provide positive charges to that part of the molecule and may interact with the phosphate group attached to Ser^{77} to alter the electrostatic profile in that region and thereby interfere with ligand binding.

The current studies demonstrate that the phosphorylation of Ser^{77} is strongly dependent on the phosphorylation status of Thr^{95} and, if Thr^{95} is mutated to alanine, neither PTH nor dopamine inhibits phosphate transport. Accordingly, we next sought to determine whether Thr^{95} is a target for phosphorylation by PTH and dopamine. In the absence of antibodies capable of specifically detecting the phosphorylation of this residue, we transfected metabolically labeled opossum kidney cells, a model proximal tubule cell line that has both PTH and dopamine receptors, with cDNAs encoding His_6 PDZ I. The results indicate that Thr^{95} was phosphorylated in unstimulated cells and that its phosphorylation is increased by both hormones. This finding is consistent with the idea that the hormonal regulation of phosphate transport involves the coordinated phosphorylation of both Thr^{95} and Ser^{77}. Although our results indicate that PTH and dopamine treatment increase the phosphorylation of Thr^{95}, we recognize that these hormones may also modulate NHERF-1 phosphorylation at other sites. Indeed, the structural basis by which the covalent modification of Thr^{95} enhances the phosphorylation of Ser^{77} is unknown. Thr^{95} is not visible in the available crystal structures of the PDZ I domain of NHERF-1, and at the present time, there is no information on its role in the PDZ I domain structure. The simplest interpretation of our findings is that the phosphorylation of Thr^{95} in full-length NHERF-1 introduces a conformational change in the PDZ I domain that influences the regulation and/or interaction of Ser^{77} with PKC.

In summary, the present experiments suggest an expanded model of PTH and dopamine mediated regulation of renal phosphate excretion and indicate that the inhibitory effect of these hormones require the coordinated phosphorylation of Thr^{95} and Ser^{77} in the first PDZ domain of NHERF-1. Moreover, the introduction of mutations at either of these two sites results in distinct effects on the relation between PTH and dopamine, and phosphate transport in the proximal tubule of

the kidney. Remarkably, in cultured proximal tubule cells, neither PTH nor dopamine inhibits phosphate transport in NHERF-1-null cells. Rescue of these cells results in the restoration of not only the basal rates of phosphate transport but also the response to the hormones. This suggests that only the NHERF-1-bound Npt2a is subject to regulation by these hormones. The magnitude of the inhibitory response to PTH and dopamine is 35–50% of the basal rates of transport and this change approximates the percent of the total apical membrane Npt2a bound to NHERF-1 as determined by density gradient ultracentrifugation (5). The present results also bear on the question of how PDZ domains might participate in the regulation of physiologic processes. Proteins containing multiple PDZ domains were initially envisioned to facilitate the formation of stable protein complexes. Our recent and current studies, on the other hand, suggest that the interactions between some PDZ domains and their target proteins are dynamic and subject to regulation. At the present time, it is not known whether the model proposed for the Npt2a·NHERF-1 interaction is specific for this ligand or represents a more general model applicable to the nearly 50 other proteins reported to bind to the PDZ I domain of NHERF-1. Our prior studies indicated that the binding of the β_2-adrenergic receptor, the platelet-derived growth factor receptor, and the cystic fibrosis transmembrane regulator to the PDZ I domain of NHERF-1 is decreased in response to treatment with protein phosphatase inhibitors and was associated with the phosphorylation of Thr^{95} and Ser^{77} (8) How these phosphorylations combine with the eight other phosphorylation sites that have been identified in full-length NHERF-1 to regulate physiologic processes involving these and other NHERF-1 target proteins, however, remains to be investigated.

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