

Ezrin-anchored Protein Kinase A Coordinates Phosphorylation-dependent Disassembly of a NHERF1 Ternary Complex to Regulate Hormone-sensitive Phosphate Transport*

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Bin Wang[‡], Chris K. Means[§], Yanmei Yang[‡], Tatyana Mamonova[‡], Alessandro Bisello[‡], Daniel L. Altschuler[‡], John D. Scott[§], and Peter A. Friedman^{‡1}

From the [‡]Laboratory for G Protein-coupled Receptor Biology, Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261 and the [§]Howard Hughes Medical Institute, Department of Pharmacology, University of Washington School of Medicine, Seattle, Washington 98195

Background: Some inherited defects in NHERF1 are associated with high phosphate (P_i) excretion and skeletal abnormalities.

Results: NHERF1 forms a multiprotein complex with Npt2a and ezrin. Mutants fail to assemble this ternary complex.

Conclusion: The NHERF1 ternary complex is required for PTH-sensitive P_i transport.

Significance: NHERF1 mutations may cause disease processes through structural changes that prevent assembly of multiprotein complexes.

Congenital defects in the Na/H exchanger regulatory factor-1 (NHERF1) are linked to disordered phosphate homeostasis and skeletal abnormalities in humans. In the kidney, these mutations interrupt parathyroid hormone (PTH)-responsive sequestration of the renal phosphate transporter, Npt2a, with ensuing urinary phosphate wasting. We now report that NHERF1, a modular PDZ domain scaffolding protein, coordinates the assembly of an obligate ternary complex with Npt2a and the PKA-anchoring protein ezrin to facilitate PTH-responsive cAMP signaling events. Activation of ezrin-anchored PKA initiates NHERF1 phosphorylation to disassemble the ternary complex, release Npt2a, and thereby inhibit phosphate transport. Loss-of-function mutations stabilize an inactive NHERF1 conformation that we show is refractory to PKA phosphorylation and impairs assembly of the ternary complex. Compensatory mutations introduced in mutant NHERF1 re-establish the integrity of the ternary complex to permit phosphorylation of NHERF1 and rescue PTH action. These findings offer new insights into a novel macromolecular mechanism for the physiological action of a critical ternary complex, where anchored PKA coordinates the assembly and turnover of the Npt2a-NHERF1-ezrin complex.

PDZ² proteins, named for the common structural domain shared by the postsynaptic density protein (PSD95), *Drosophila*

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¹ To whom correspondence should be addressed: University of Pittsburgh School of Medicine, Dept. of Pharmacology and Chemical Biology, W1340 Biomedical Science Tower, 200 Lothrop St., Pittsburgh, PA 15261. E-mail: paf10@pitt.edu.

² The abbreviations used are: PDZ, postsynaptic density 95/discs large/zona occludens; PTH, parathyroid hormone; PTHR, parathyroid hormone receptor; AKAP, A kinase-anchoring protein; PKA, protein kinase A; OK, opossum kidney; EBD, ezrin-binding domain; Ni-NTA, nickel-nitrilotriacetic acid; Tri-

disc large tumor suppressor (DlgA), and zonula occludens 1 protein (ZO-1), constitute a large family of modular scaffolding proteins. These cytoplasmic adapter proteins are capable of assembling a variety of membrane-associated proteins and signaling molecules in short lived functional units. Mutations in PDZ proteins have been linked to inherited diseases, such as Usher syndrome, sensorineural hearing loss, thyroid dysgenesis, and idiopathic dilated cardiomyopathy (1–5). The Na^+/H^+ exchange regulatory factor-1 (NHERF1, *SLC9A3R1*) consists of two tandem PDZ domains and a carboxyl-terminal ezrin-binding domain (EBD) (Fig. 1A). NHERF1 tethers membrane-delimited proteins expressing a class 1 PDZ ligand of the form (D/E)(S/T)XΦ, where X is promiscuous and Φ is a hydrophobic residue. Consequently, NHERF1 can associate with multiple binding partners that include G protein-coupled receptors, ion transporters, and other scaffolding and adapter proteins to form multiprotein signaling complexes (6). Ancillary protein-protein interactions proceed through the EBD and ERM adapter proteins (ezrin, radixin, or moesin) cross-linking these signaling complexes to the actin cytoskeleton (7). As a result, NHERF1 resides at the core of a growing number of diverse macromolecular complexes that have been implicated in the modulation of a range of essential physiological processes (8, 9). Such multiprotein assemblies are especially prevalent in tissues possessing extensive polarized epithelia, including kidney, small intestine, placenta, and liver (10).

An endocrine function for NHERF1 can be inferred from two sources. First, lesions in the NHERF1 gene contribute to excessive urinary phosphate (P_i) excretion and skeletal defects in humans that are emblematic of abnormalities of the action of parathyroid hormone (PTH) (11–13). Second, NHERF1^{-/-} mice exhibit PTH-resistant urinary P_i wasting, nephrocalcinosis,

and *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ITC, isothermal titration calorimetry.

sis, and osteopenia (14, 15). These vital aspects of renal P_i transport are primarily mediated by the type-2 Na- P_i cotransporter, Npt2a (*SLC34A1*). Npt2a is tightly regulated by PTH, which inhibits P_i transport by promoting Npt2a endocytosis and lysosomal degradation (16). These functions are not shared by NHERF2 or other NHERF1-like PDZ proteins (14).

Patients harboring contiguous non-synonymous polymorphisms in the NHERF1 gene (L110V or R153Q) or a single point mutation³ (E225K) experience a constellation of mineral-ion abnormalities, including renal P_i wasting, calcium stones, and osteomalacia (17). Each of these genetic variants has been mapped to the linker region between PDZ1 and PDZ2 or the boundaries of PDZ2 (Fig. 1A). Npt2a is thought to bind to PDZ1 (18), whereas the PTH receptor (PTHR) engages PDZ1 or PDZ2 (19). The sites of the noted mutations are unexpected because they are some distance from the PDZ core-binding domains. Hence, they do not explain *a priori* why NHERF1 function is impaired. These observations suggest that the NHERF1 mutations interfere with PTH-sensitive P_i transport by a distinct mechanism that does not involve disruption of Npt2a or PTHR binding, *per se*, or signaling as has been proposed (17).

We show now that an Npt2a-NHERF1-ezrin core complex is required to mediate PTH-sensitive P_i transport. Ezrin not only links the complex to the actin cytoskeleton but also anchors the protein kinase A (PKA) holoenzyme. We propose that this extended signaling complex facilitates the PTH-mediated stepwise disassembly of this macromolecular complex. The kinase phosphorylates NHERF1 and promotes disassembly of the ternary complex. Upon its release, Npt2a is endocytosed, thereby inhibiting cellular P_i transport. Interfering with formation of the complex or blocking phosphorylation inhibits PTH-sensitive P_i transport. Notably, NHERF1 mutations prevent formation of the Npt2a-NHERF1-ezrin complex and confer resistance to PTH-induced Npt2a down-regulation. These findings have been consolidated into a new molecular mechanism that can account for diminished P_i transport.

EXPERIMENTAL PROCEDURES

Materials—HA.11 monoclonal antibody, HA.11 monoclonal affinity matrix, HA.11 peptide, and His monoclonal antibody were obtained from Covance (Berkeley, CA). NHERF1 rabbit polyclonal (PA1-090) and mouse monoclonal (ab9526) antibodies were purchased from Affinity Bioreagents (Golden, CO) and from Abcam (Cambridge, MA), respectively. Horseradish peroxidase-conjugated sheep anti-mouse antibody was from Amersham Biosciences. S-protein horseradish peroxidase-conjugate was purchased from Novagen (Madison, WI). Ni-NTA-agarose was purchased from Qiagen (Valencia, CA). Lipofectamine 2000, Geneticin, and Alexa Fluor 546-tagged sheep anti-mouse second antibody were acquired from Invitrogen. Protease inhibitor mixture Set I and forskolin were from Calbiochem. Human PTH(1-34) was purchased from Bachem (Torrance, CA). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, sulfo-NHS-SS-biotin, and Neutr-

Avidin-agarose beads were purchased from Pierce. St-Ht31 was purchased from Promega (Madison, WI). All other reagents were from Sigma.

Cell Culture—Opossum kidney (OK) cells and OKH cells were provided by Judith Cole (University of Memphis) and cultured in DMEM/F-12 medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin.

Plasmid Construction—FLAG-tagged human NHERF1 was prepared by amplifying the 1-358 amino acid sequence of human NHERF1-HA (provided by Dr. M. von Zastrow, University of California) by PCR amplified using the forward primer with a HindIII restriction site, GAG GAT GAT GAT GTG AAG CTT GCG ATG GAC TAC AAG GAC GAC GAC GAC AAG, and the reverse primer with an XhoI restriction site, GCG TAT GTG GCG CTC GAG CTA GAG GTT GCT GAA GAG TTC. The purified PCR fragment was cut by HindIII and XhoI and subcloned into the pcDNA3.1⁺.

FLAG-NHERF1- Δ EBD was generated from the template of NHERF1- Δ EBD (provided from Dr. E. J. Weinman (University of Maryland). HA-EGFP-tagged human Npt2a was generated from the template of EGFP-tagged human Npt2a (provided by Dr. C. Bergwitz, Massachusetts General Hospital, Boston). Both constructs were mutated by PCR as described (20).

Mutation of the terminal amino acid of HA-EGFP-Npt2a from leucine to alanine (L639A), FLAG-L110V-NHERF1, FLAG-R153Q-NHERF1, FLAG-E225K-NHERF1, GST-L110V-NHERF1, GST-R153Q-NHERF1, and GST-E225K-NHERF1 were performed by PCR using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

The double mutant FLAG-R153Q/L358A-NHERF1 construct was generated from the template of FLAG-R153Q-NHERF1. Mutation of the terminal amino acid from leucine to alanine (L358A) was performed by PCR using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. pET30A-NHERF1sPDZ2 (provided by Dr. E. J. Weinman, University of Maryland) was cut with KpnI and XhoI and subcloned into the pcDNA3.1⁺ vector.

Plasmid fidelity was confirmed by sequencing (ABI PRISM 377; Applied Biosystems, Foster City, CA) and subsequent sequence alignment (NCBI BLAST) with human NHERF1 and human Npt2a (GenBankTM accession numbers AF015926 and NW_003052, respectively) to ensure the accuracy of the above constructs.

P_i Uptake— P_i uptake was measured as described previously (21). Confluent OKH cells in 12-well plates were serum-starved overnight. The cells were incubated with vehicle or 100 nM PTH for 2 h. Cells were then washed three times with a sodium-containing buffer (140 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 0.1 mM KH₂PO₄, 10 mM HEPES, pH 7.4) or sodium-free buffer, where *N*-methyl-D-glucamine replaced sodium. Measurement of P_i uptake was initiated by adding buffer containing 4 μ Ci/ml [³²P]orthophosphate for 10 min to triplicate wells. Uptake was terminated by washing three times with ice-cold *N*-methyl-D-glucamine medium. The cells were extracted overnight with 0.5% Triton X-100 and counted by β -scintillation spectrometry. Sodium-dependent P_i uptake was calculated by subtracting

³ For simplicity, mutations and polymorphisms are referred to henceforth as mutations.

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uptake in the absence of sodium from that in the presence of sodium. Sodium-independent uptake represents <5% of sodium-dependent uptake. Results are expressed as percentage of P_i uptake under unstimulated control conditions.

Stable Expression of Constructs—OKH cells were transfected with HA-EGFP-Npt2a, FLAG-NHERF1, FLAG-L110V-NHERF1, FLAG-R153Q-NHERF1, FLAG-E225K-NHERF1, or pcDNA 3.1 or pEGFP vector (control) using Lipofectamine 2000 following the manufacturer's instructions and screening with 0.5 mg/ml Geneticin and immunoblot.

Transient Transfection—OKH cells, as indicated, were transiently transfected with 24 μg of DNA in 10-cm dishes, 4.0 μg of DNA/well in 6-well plates, or 2.0 μg of DNA/well in 12-well plates with empty vector or plasmids of wild-type FLAG-NHERF1, truncated FLAG-NHERF1-ΔEBD, or mutant NHERF1, in which PDZ1, PDZ2, or both PDZ1 and PDZ2 domains are scrambled (sPDZ1-NHERF1, sPDZ2-NHERF1, or sPDZ1/2-NHERF1), wild-type HA-GFP-Npt2a, or mutant Npt2a (HA-GFP-L639A), by use of Lipofectamine 2000. Cells were used 48–72 h after transfection.

Receptor Binding—Receptor binding was performed as described (19) using HPLC-purified [¹²⁵I][Nle^{8,18},Tyr³⁴]PTH(1–34)NH₂ ([¹²⁵I]PTH(1–34)). PTHR binding was measured on OKH cells seeded on 24-well plates and grown to confluence. PTH(1–34) or vehicle with ~100,000 cpm of [¹²⁵I]PTH(1–34)NH₂ in 250 μl of fresh medium were incubated at 4 °C for 2.5 h. After incubation, cells were rinsed by cold PBS and then solubilized in 0.2 N NaOH. Cell surface-bound [¹²⁵I]PTH(1–34) was assessed by γ spectrometry.

Coimmunoprecipitation and Immunoblot Analysis—Interactions of NHERF1 with PTHR, Npt2a, or ezrin were analyzed as detailed previously (22). Briefly, 6-well plates of the indicated cells were transiently transfected with the indicated plasmids or the respective empty vector. After 48 h, the cells were lysed with immunoprecipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, and 150 mM NaCl) supplemented with protease inhibitor mixture I and incubated for 15 min on ice. Solubilized materials were incubated overnight at 4 °C with HA.11 monoclonal affinity matrix or anti-FLAG M2 affinity matrix. Total lysates and immunoprecipitated proteins, eluted by the addition of SDS sample buffer, were analyzed by SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore) using the semidry method (Bio-Rad). Membranes were blocked overnight at 4 °C with 5% nonfat dried milk in Tris-buffered saline plus Tween 20 (TBST) and incubated with different antibodies (polyclonal anti-NHERF1 antibody at 1:1000, HA.11 monoclonal antibody at 1:1000, anti-FLAG polyclonal antibody at 1:2000, and anti-ezrin polyclonal antibody at 1:2000) overnight at 4 °C. The membranes were then washed and incubated with goat anti-rabbit IgG or sheep anti-mouse IgG conjugated to horseradish peroxidase at a 1:5000 dilution for 1 h at room temperature. Protein bands were visualized with a luminol-based enhanced chemiluminescence substrate. The Npt2a-NHERF1-ezrin ternary complex was identified by immunoprecipitation of HA-Npt2a and elution with 2 mg/ml HA peptide, followed by immunoprecipitation of FLAG-NHERF1 and subsequent detection of ezrin by immunoblotting.

Adenylyl Cyclase—Adenylyl cyclase activity was determined as described (23). Briefly, OKH cells transfected with vector, wild-type NHERF1, or mutant forms in 24-well plates were labeled with 0.5 μCi of [³H]adenine for 2 h. The cells were then treated with vehicle or 100 nM PTH(1–34) in the presence of phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM) for 15 min. The reaction was terminated by the addition of 1 M TCA. cAMP was isolated by the two-column method.

Cell Surface Biotinylation—Quantitative measurement of cell surface Npt2a was measured by surface biotinylation on polarized cells (24). Briefly, OKH cells stably expressing empty vector or FLAG-tagged wild-type or mutant NHERF1 constructs were seeded on 6-well plate Transwell inserts and allowed to polarize. The cells were serum-starved overnight, and 100 nM PTH(1–34) was applied to the basolateral surface for 2 h. Monolayers were washed three times with cold PBS buffer and labeled at apical membranes with 1 mg/ml sulfo-NHS-SS-biotin in cold PBS for 40 min. The reaction was quenched with 100 mM glycine in PBS buffer for 10 min, and the cells were lysed in 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 1% (v/v) Nonidet P-40, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A at 4 °C. The soluble extract was incubated with immobilized NeutrAvidin-agarose for 2 h at 4 °C. The resin was washed, and biotin-labeled proteins were eluted by the addition of SDS sample buffer. Eluted proteins were separated by SDS-polyacrylamide gels and detected with an Npt2a antibody. The blots were quantified by densitometric analysis.

Phosphorylation—OKH cells were transfected with FLAG-tagged wild-type NHERF1, L110V-NHERF1, R153Q-NHERF1, or E225K-NHERF1. After 48 h, the cells were washed with and incubated at 37 °C with [³²P]orthophosphate (0.1 mCi/ml) in phosphate-free medium for 10 min followed by 5-min pretreatment with the serine/threonine phosphatase inhibitor calyculin A (50 nM). Cells were then exposed to PTH (100 nM) or vehicle for 15 min at 37 °C, rinsed with ice-cold PBS, and lysed with radioimmune precipitation assay buffer containing phosphatase and proteinase inhibitors. The cell lysates were immunoprecipitated with FLAG-agarose overnight at 4 °C. After washing, 100 mM cyanogen bromide (CNBr) was added to the beads at room temperature overnight. The samples were dried in a SpeedVac, and the beads were eluted with Tricine buffer. The cleaved, phosphorylated NHERF1 fragments were resolved on 16.5% Tricine-SDS-PAGE (Bio-Rad). The gel was dried, autoradiographed, and quantified by scanning densitometry.

Expression and Purification of Wild-type NHERF1 and R153Q-NHERF1 Protein—Wild-type NHERF1 and R153Q-NHERF1 were generated from their respective FLAG-tagged templates by PCR. The PCR fragments were inserted in pET151/D-TOPO vector (Invitrogen). The recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) cells (Novagen) and purified using Ni-NTA-agarose (Qiagen). Amino-terminal His₆ and V5 tags were cleaved by tobacco etch virus protease (Invitrogen). The cleaved proteins were dialyzed with PBS, separated by HPLC, and analyzed by mass spectrometry before being used for the isothermal titration calorimetry and fluorescence polarization.

Synthesis of N^α-Tetramethylrhodamine-Npt2a(618–639)—The synthesis of the 22-amino acid carboxyl-terminal peptide of Npt2a was carried out by solid phase methodology using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry (0.1 mmol scale) on an Applied Biosystems AB433 peptide synthesizer. After synthesis, the peptidyl resin was treated overnight with 4 eq of 5-(and 6)-carboxytetramethylrhodamine in the presence of HBTU/HOBt/DIEA. Following standard trifluoroacetic acid cleavage, the product was purified by HPLC on a Vydac C-18 reverse phase column and lyophilized. The final product was characterized by electron spray mass spectrometry.

Fluorescence Polarization (FP)—Purified recombinant NHERF1 or R153Q-NHERF1 in PBS were added to a 96-well, black, flat-bottom, polystyrene plate (50 μl/well) at a concentration of 0.001–50 μM. Peptide was added to each well at a final concentration of 1 μM. FP was measured on a Victor³V plate reader (PerkinElmer Life Sciences) at an excitation of 544 nm and emission at 595 nm. Recordings were performed at room temperature under equilibrium conditions. All readings were background-subtracted. *K_D* was calculated from the derived anisotropy (25).

Isothermal Titration Calorimetry (ITC)—ITC experiments were carried out at 20 °C on an ITC200 MicroCal calorimeter (MicroCal, Northampton, MA). Npt2a peptide was reconstituted in DMSO and subsequently diluted in PBS. NHERF1 and R153Q-NHERF1 proteins were dialyzed into PBS, and just prior to performing titrations an appropriate amount of DMSO was added in order to equalize the DMSO concentration in peptide and protein solutions. Titrations were performed with 20 injections of 1.9 μl of peptide into an ITC cell containing 200 μl of either NHERF1 or R153Q-NHERF1 protein. Injections were performed every 120 s with a stirring speed of 700 rpm. Control experiments were performed under the same conditions to correct for heat of dilution. Data were analyzed by Origin software (OriginLab, Northampton, MA) and fit to a single site. *K_d* was calculated as described (26).

Imaging—Confocal fluorescence imaging was performed as described (22). Briefly, OKH cells, stably transfected with GFP-Npt2a, were grown on poly-D-lysine-coated coverslips. After transfection with FLAG-WT-NHERF1 or FLAG-tagged NHERF1 mutants, the cells were incubated for 48–72 h to establish polarity. The cells were then treated with vehicle or PTH for 2 h, fixed on 4% paraformaldehyde for 10 min, and permeabilized with 0.2% Triton X-100 for 15 min at room temperature. Blocking was performed by incubating the cells for 1 h at room temperature in 5% goat serum in PBS. Anti-FLAG monoclonal antibody diluted 1:500 in blocking buffer was applied to the specimens for 1 h at room temperature. Alexa Fluor 546-tagged goat anti-mouse second antibody diluted 1:500 was applied under the same conditions as the primary antibody. Coverslips were mounted for immunofluorescence microscopy and analyzed using an Olympus FluoView 1000 microscope with a ×63 oil immersion objective. Colocalization analyses for Npt2a and wild-type or mutant NHERF1 confocal images were done using ImageJ. Pearson's correlation coefficient (27), defined here as the ratio of the covariance of the red and green color images divided by the product of the S.D. value

of the normalized image intensities, was calculated with the JACoP plug-in (28). Pearson's correlation coefficient has a range of +1 (perfect correlation) to –1 (perfect but negative correlation) with 0 denoting the absence of a relationship.

Statistics—Data are presented as the mean ± S.E., where *n* indicates the number of independent experiments. Multiple comparisons were evaluated by analysis of variance with post-test repeated measures by the Bonferroni procedure (Prism, GraphPad). Differences greater than *p* < 0.05 were assumed to be significant.

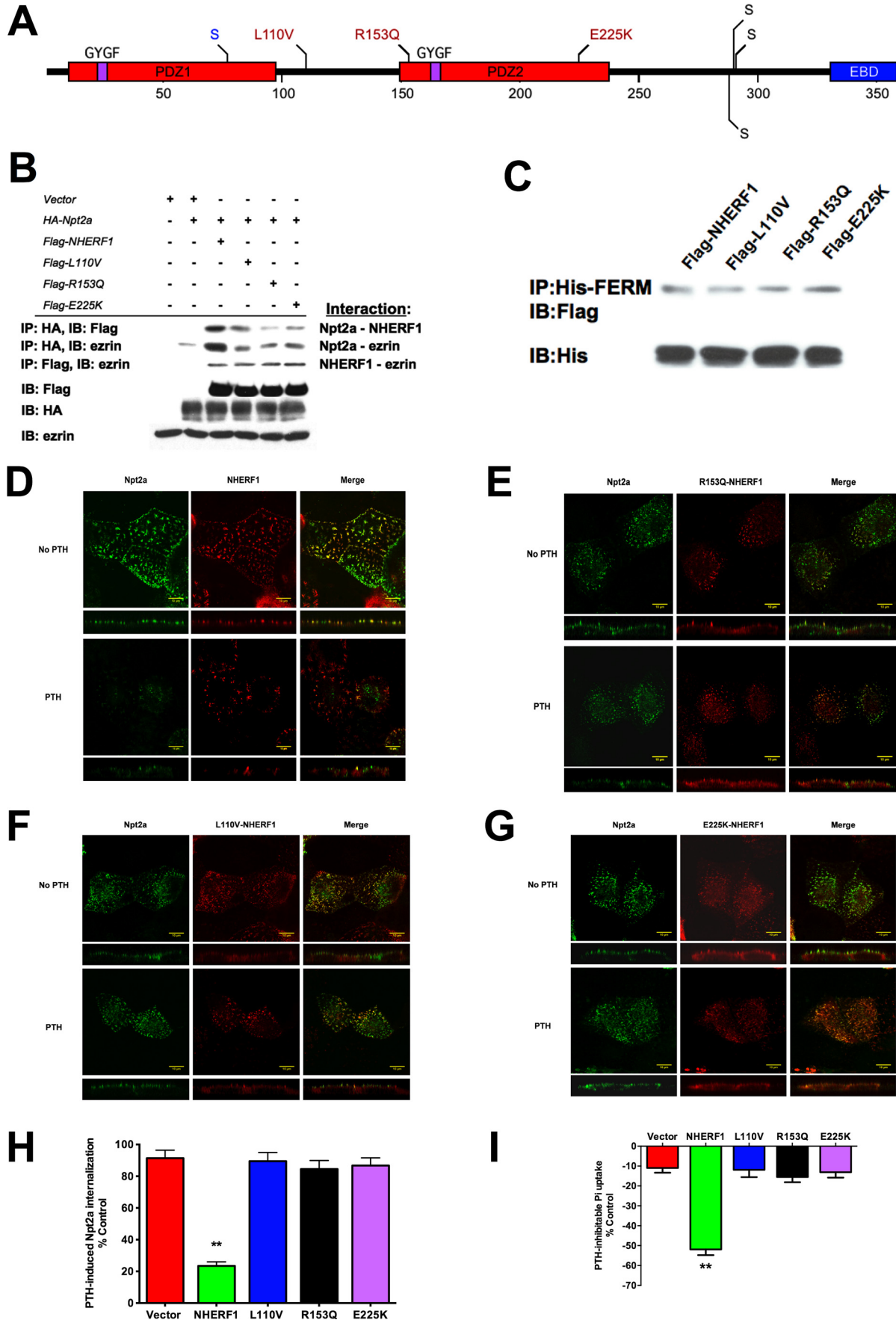
RESULTS

Binary NHERF1 Interactions with Npt2a and Ezrin—PTH inhibits Npt2a function by promoting its internalization from apical cell membranes, and this process requires NHERF1. The molecular mechanism responsible for these events has yet to be determined. Point mutations or genetic variants of NHERF1 that impair P_i transport have been mapped to sites that are remote from the PDZ1 or PDZ2 GYGF core-binding domains (Fig. 1A). On the basis of their location, it is unlikely that these single amino acid substitutions interfere with Npt2a interactions with NHERF1 or signaling initiated upon PTHR binding. We therefore sought to develop a detailed explanation for how NHERF1 coordinates PTH-sensitive P_i transport and to account for how congenital NHERF1 variants interfere with PTH-sensitive P_i transport.

OK cells, the accepted model for monitoring PTH action on P_i transport (29), were used to analyze binary interactions between NHERF1 and its protein partners. Initial experiments characterized the Npt2a and ezrin binding properties of naturally occurring NHERF mutants (L110V/R153Q and E225K), each of which was identified in patients suffering from defective hormone-regulated P_i transport (17, 30). These studies were performed with an OKH subline, which expresses low endogenous NHERF1. Only upon transfection with exogenous NHERF1 is PTH action on P_i transport reconstituted in these cells (31). We first characterized the binary interactions of Npt2a, NHERF1, and ezrin with one another. Npt2a exhibited diminished association with all NHERF1 mutants when compared with wild-type NHERF1 (Fig. 1B). Likewise, Npt2a binding to ezrin was abrogated in the presence of the mutant forms of NHERF1 but restored upon introduction of wild-type NHERF1. In contrast, both wild-type and mutant forms of NHERF1 interacted comparably with endogenous ezrin (Fig. 1B). This latter protein-protein interaction proceeds through the FERM domain of ezrin (Fig. 1C).

We applied confocal fluorescence microscopy to monitor the distribution of Npt2a and NHERF1 and the response to PTH in OKH cells. Under resting conditions (Fig. 1D, *top*) Npt2a (*green*) and wild-type NHERF1 (*red*) signals extensively overlapped ($\sigma = 0.91$, Pearson's coefficient, a measure of protein co-distribution) in a characteristic punctate pattern that was consistent with their accumulation in apical membrane patches (*z* axis scans). Application of PTH to the basolateral compartment promoted the disappearance of Npt2a from the apical surface, whereas the NHERF1 signal remained associated with apical membranes (Fig. 1D, *bottom*). Consequently, Pearson's coefficient dropped to 0.40. These findings imply that PTH

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induces dissociation of NHERF1 from Npt2a, which then is internalized. Notably, L110V-NHERF1, R153Q-NHERF1, and E225K-NHERF1 mutants were insensitive to PTH. Under resting conditions, for instance, Npt2a and R153Q-NHERF1 staining was less abundant at the apical membrane, and the colocalization of both signals was reduced ($\sigma = 0.67$) (Fig. 1E). Moreover, hormone stimulation failed to decrease Npt2a and R153Q-NHERF1 colocalization ($\sigma = 0.66$) or internalization of the complex. Comparable results were obtained with L110V-NHERF1 and E225K-NHERF1 (Fig. 1, F and G).

Complementary studies measured the cell surface biotinylation to quantify the influence of PTH on Npt2a endocytosis in polarized OKH cells. PTH decreased apical Npt2a by 75% in cells expressing wild-type NHERF1. The hormone's action on each of the mutant NHERF1 forms was negligible because application of PTH had little effect on Npt2a endocytosis (Fig. 1H). Moreover, cells transfected with the mutant forms of NHERF1 failed to inhibit PTH-sensitive P_i transport (Fig. 1I).

Additional experiments established that 1) the Npt2a carboxyl-terminal PDZ recognition sequence mediates binding to NHERF1 (Fig. 2A); 2) Npt2a interacts primarily with PDZ1 of NHERF1 (Fig. 2B); and 3) PDZ1 and the EBD domains of NHERF1 are necessary for the physiological actions of PTH on P_i transport (Fig. 2C). Other experiments demonstrated that PTH resistance associated with NHERF1 mutations does not arise from compromised PTHR abundance (Fig. 2D), binding (Fig. 2E), or signal transduction (Fig. 2F). Together, these data strongly suggested that NHERF1 acts as a bridge linking Npt2a to ezrin and that this association is reduced by the NHERF1 mutants.

NHERF1 Forms a Ternary Complex with Npt2a and Ezrin—Mutant forms of NHERF1 bind ezrin (Fig. 1B) but not Npt2a. Consequently, they are unable to support the PTH-responsive relay of information that initiates P_i transport (Fig. 1I). We reasoned that formation of a NHERF1-Npt2a-ezrin complex is required for PTH action. As shown in Fig. 3A, a ternary complex of Npt2a-NHERF1-ezrin was observed in resting OKH cells (lane 2). This core complex was not formed in the presence of NHERF1- Δ EBD (Fig. 3A, lane 4), a construct lacking the EBD that cannot associate with ezrin (data not shown). Likewise, the core complex is not assembled in the presence of the Npt2a(L639A), a mutant that has a defective carboxyl-terminal PDZ recognition motif (Fig. 3A, lane 3). PTH treatment

strongly induced dissociation of the ternary complex with wild-type NHERF1 ($60 \pm 2.6\%$; Fig. 3, A (lane 6) and C). Assembly of the ternary complex with mutant NHERF1 was decreased by 75–80% (Fig. 3, B and C). Moreover, the modest ternary complex formed with mutant NHERF1 was unresponsive to PTH ($5.9 \pm 1.3\%$). Thus, the L110V-, R153Q-, and E225K-NHERF1 mutants are impaired in their ability to assemble the ternary complex and are refractory to PTH.

The Npt2a-NHERF1-Ezrin Ternary Complex Mediates PTH-sensitive P_i Uptake—We sought to determine what factors contribute to the PTH-sensitive regulation of Npt2a-NHERF1-ezrin complex in the control of P_i uptake. PKA was a likely candidate for two reasons. First, the PTH signaling pathway stimulates production of cAMP, the chemical messenger that activates this enzyme, and second, ezrin has been reported to serve as a dual function A-kinase anchoring protein (AKAP) (32, 33). A defining characteristic of most AKAPs is a 14–18-amino acid PKA-binding domain that associates with nanomolar affinity to the docking and dimerization domain of the PKA R subunits (34, 35). Peptides patterned after this region disrupt PKA association with AKAPs, including ezrin (36, 37). Consequently, displacement of PKA from associating with ezrin or inhibiting kinase activity should prevent disassembly of the ternary complex. The net effect would be to suppress PTH effects on P_i transport.

To test this hypothesis, OKH cells were incubated with St-Ht31, a cell-permeant AKAP peptide analog that interferes with PKA-AKAP interactions, including disruption of PKA R subunit binding to ezrin (Fig. 4A). As before, PTH provoked dissociation of the wild-type ternary complex ($47.7 \pm 2.7\%$), but St-Ht31 fully blocked this (Fig. 4, B and C). Control experiments confirmed that St-Ht31 had no effect on the integrity of the ternary complexes formed with NHERF1 mutants. Thus, ternary complex disassembly requires anchored PKA activity.

We then assessed the functional role of the Npt2a-NHERF1-ezrin ternary complex in mediating PTH-sensitive P_i transport. As anticipated, St-Ht31 had no effect on basal P_i uptake (data not shown) but importantly suppressed the PTH-induced inhibitory action on P_i uptake in the presence of wild-type NHERF1 (Fig. 4D). Remarkably, St-Ht31 did not alter P_i uptake in cells transfected with NHERF1 mutants. As a corollary of the view that ezrin recruits PKA, we determined if pharmacological inhibition of PKA affected PTH-induced dissociation of the

FIGURE 1. NHERF1 mutants interact weakly with Npt2a and are resistant to PTH. A, molecular model of NHERF1 showing PDZ domains 1 and 2 and the EBD. GYGF core-binding sequences are present in PDZ1 and PDZ2. Locations of L110V-, R153Q-, and E225K-NHERF1 mutations are illustrated. Ser residues (S) known to be phosphorylated are indicated. B and C, binary interaction of Npt2a with NHERF1 and ezrin (B). The blot corresponding to a particular binary interaction is noted on the right. Compared with wild-type NHERF1 (lane 3), Npt2a engagement with L110V-, R153Q-, and E225K-NHERF1 mutants (lanes 4–6) was reduced by 45–85% in OKH cells transfected with the indicated construct. Likewise, Npt2a interaction with ezrin decreased 45–60% in the presence of mutant NHERF1 forms. Mutant forms of NHERF1 coimmunoprecipitate normally with ezrin. The results are typical of three independent experiments. Mutant forms of NHERF1 interact normally with the FERM domain of ezrin (C). D and E, confocal fluorescence colocalization of Npt2a (green) and NHERF1 (red) for wild-type NHERF1 (D) and R153Q-NHERF1 (E). Under resting conditions in the absence of PTH (top row), both Npt2a and NHERF1 are expressed at apical membranes as shown by the z axis scan below each image. Merged images are shown on the right and indicate extensive colocalization for Npt2a with wild-type NHERF1 but far less for R153Q. Upon the addition of PTH to the basolateral compartment (bottom), Npt2a internalized extensively in the presence of wild-type NHERF1 but was essentially unchanged when coexpressed with R153Q. Similar results were obtained with L110V-NHERF1 (F) and E225K-NHERF1 (G). H, mutant forms of NHERF1 exhibit no measurable Npt2a internalization in response to PTH. OKH cells stably transfected with vector, wild-type NHERF1, or L110V-, R153Q-, or E225K-NHERF1 were grown to confluence on permeable filter supports. 100 nM PTH(1–34) was applied to the basolateral membrane compartment for 2 h. Npt2a internalization was measured by cell surface biotinylation as described under "Experimental Procedures." Average results \pm S.E. (error bars) normalized for actin are expressed as percentage of vector control. ($n = 3$; **, versus vector, $p < 0.01$, one-way repeated measures ANOVA (Dunnnett's post hoc multiple-comparison test)). I, NHERF1 mutants fail to support PTH-sensitive inhibition of P_i transport. PTH-sensitive, sodium-dependent P_i uptake was measured in OKH cells treated with vehicle or PTH(1–34) (100 nM, 2 h). Data are summarized as the mean \pm S.E. ($n = 4$; **, versus vector, $p < 0.01$, one-way repeated measures ANOVA (Dunnnett's post hoc multiple-comparison test)). IP, immunoprecipitation; IB, immunoblot.

Npt2a-NHERF1-Ezrin Ternary Complex Regulates P_i Transport

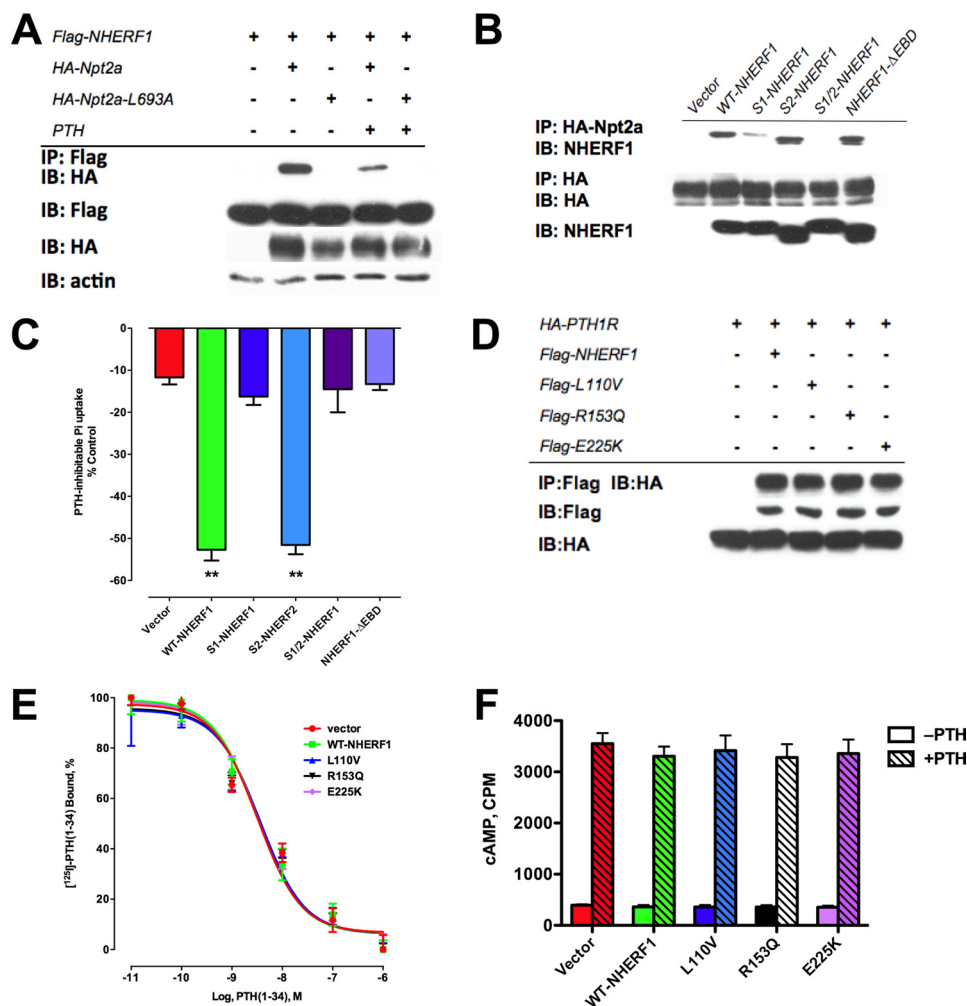


FIGURE 2. Structural determinants of NHERF1 association with Npt2a. *A*, OKH cells were transiently transfected with empty vector (control), FLAG-NHERF1, HA-GFP-Npt2a or HA-GFP-Npt2a(L693A). 48 h after transfection, the cells were treated with vehicle or 100 nM PTH(1–34) for 2 h. FLAG-tagged proteins were immunoprecipitated with FLAG-agarose. The precipitated protein was then immunoblotted with HA antibody. Actin expression was used as a loading control. The figure is representative of three independent experiments. *B*, Npt2a interacts with PDZ1. OKH-HA-GFP-Npt2a cells were transiently transfected with empty vector, wild-type NHERF1, or NHERF1 harboring mutations in PDZ1 and/or PDZ2 core-binding domains (sPDZ1-NHERF1, sPDZ2-NHERF1, and sPDZ1/2-NHERF1) or truncated NHERF1 lacking the ezrin-binding domain (NHERF1- Δ EBD). 48 h after transfection, HA-tagged proteins were immunoprecipitated with HA-agarose. The precipitated protein was then immunoblotted with NHERF1 antibody. An example of three independent experiments is shown. *C*, sodium-dependent P_i uptake was measured in OKH cells transiently transfected with empty vector, wild-type NHERF1, sPDZ1-NHERF1, sPDZ2-NHERF1, sPDZ1/2-NHERF1, or NHERF1- Δ EBD and treated with vehicle or PTH(1–34) (100 nM, 2 h). Data are summarized as the mean \pm S.E. (error bars) ($n = 3$; **, $p < 0.01$, versus vector). *D*, OKH cells were transfected with HA-PTH1R, FLAG-NHERF1, FLAG-L110V-NHERF1, FLAG-R153Q-NHERF1, or FLAG-E225K-NHERF1. 48 h after transfection, FLAG-tagged proteins were immunoprecipitated (IP). The precipitated protein was immunoblotted (IB) with HA antibody. Data are representative of three independent experiments. *E*, OKH cells were transfected with empty vector, wild-type NHERF1, or mutated NHERF1. Cell surface binding of [¹²⁵I]PTH(1–34) was measured as described under “Experimental Procedures.” Data are summarized as the mean \pm S.E. of triplicate determinations. *F*, OKH cells were transfected with wild-type NHERF1 or mutated forms of NHERF1. Cells were treated with 100 nM PTH for 15 min, and cAMP accumulation was measured as described under “Experimental Procedures.” Data are summarized as the mean \pm S.E. ($n = 4$). IP, immunoprecipitation; IB, immunoblot.

Npt2a-NHERF1-ezrin ternary complex. Again, PTH promoted the dissociation of the wild-type ternary complex, and this action was abolished by H89, a reasonably selective pharmacological inhibitor of PKA (Fig. 4E). Moreover, inhibiting PKA kinase activity in cells expressing wild-type NHERF1 blocked PTH-sensitive inhibition of P_i transport (Fig. 4F). PTH-inhibitable P_i transport was unaffected by H89 in cells harboring NHERF1 mutants.

We next sought to address postreceptor regulation of ternary complex turnover and P_i transport. To bypass the PTHR, we used forskolin to activate adenylyl cyclases directly. As expected, pharmacological stimulation of cAMP synthesis disrupted the Npt2a-NHERF1-ezrin ternary complex (Fig. 4E). This effect proceeded through PKA as it was blocked by H89

(Fig. 4E). Notably, forskolin treatment failed to promote disassembly of the ternary complex formed with mutant NHERF1 constructs (Fig. 4G) and had no inhibitory action on P_i transport (Fig. 4H). These findings are compatible with the view that PKA-mediated activity is required for dissociation of the ternary complex and initiation of Npt2a endocytosis.

Because the preceding results implicated anchored PKA activation as a prerequisite for dissociation of the Npt2a-NHERF1-ezrin ternary complex, we assessed PTH-responsive phosphorylation of NHERF1. We analyzed peptide fragments harboring Ser⁷⁷ located in PDZ1, which has been associated with PTH-induced NHERF1 phosphorylation (38), and the linker region between PDZ2 and the EBD, which contains a serine-rich cluster (Fig. 1A), including Ser²⁹⁰, which is constitutively phosphor-

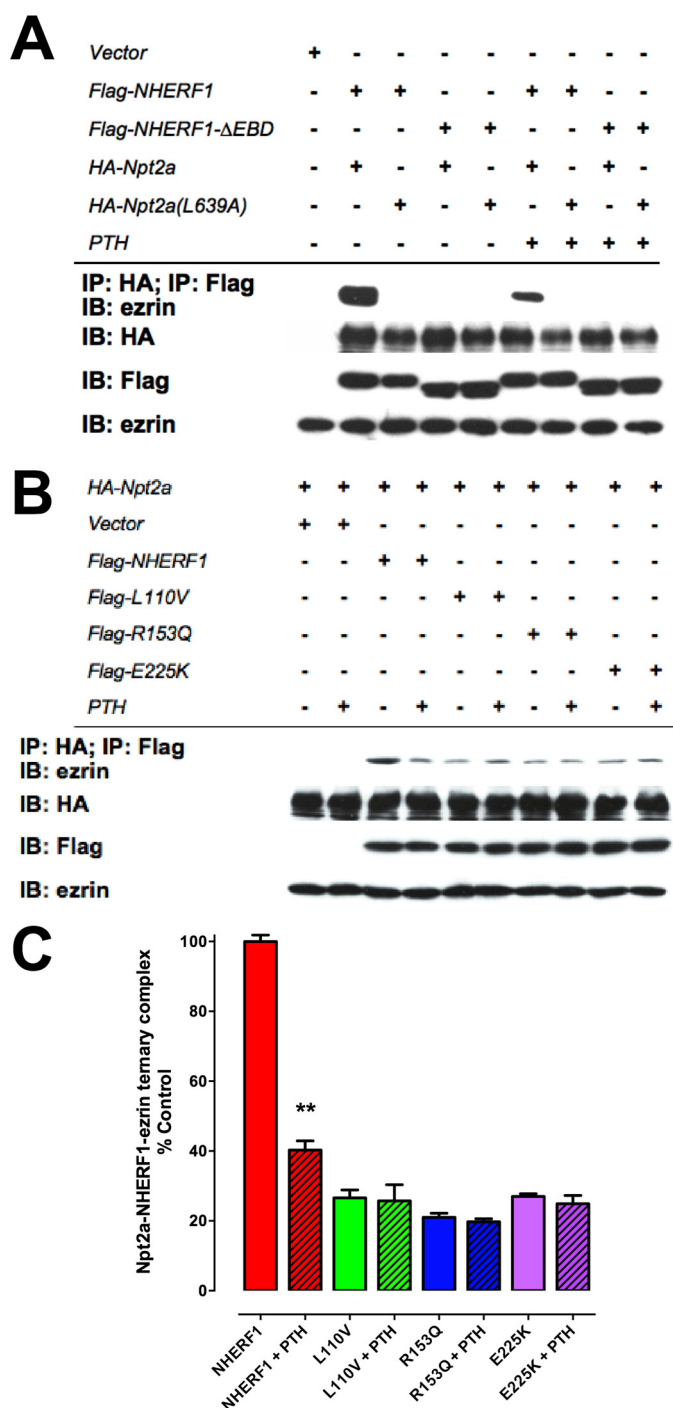


FIGURE 3. NHERF1 forms a ternary complex with Npt2a and ezrin. A, Npt2a, NHERF1, and ezrin form a ternary complex (lane 2) that dissociates upon treatment with PTH (lane 6). Truncated NHERF1 lacking the EBD (NHERF1-ΔEBD) and Npt2a in which the carboxyl-terminal PDZ-binding domain was mutated (Npt2a(L639A)) do not form a ternary complex. B, mutant NHERF1 constructs are poorly able to form a ternary complex with Npt2a and ezrin and are refractory to PTH. Shown is a representative result of OKH cells stably expressing HA-Npt2a transiently transfected with FLAG-tagged wild-type NHERF1 or L110V-, R153Q-, or E225K-NHERF1. 48 h after transfection, the cells were treated, and the ternary complex was detected as above. C, aggregate results from experiments shown in B ($n = 3$; **, versus no PTH, $p < 0.001$, two-way repeated measures ANOVA (Bonferroni post hoc multiple-comparison test)).

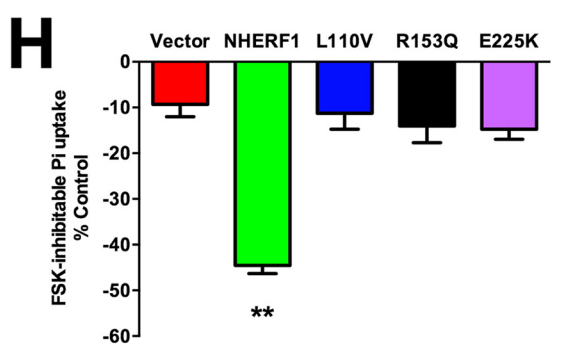
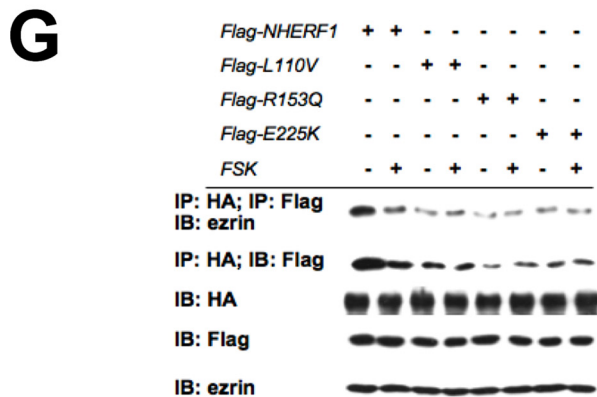
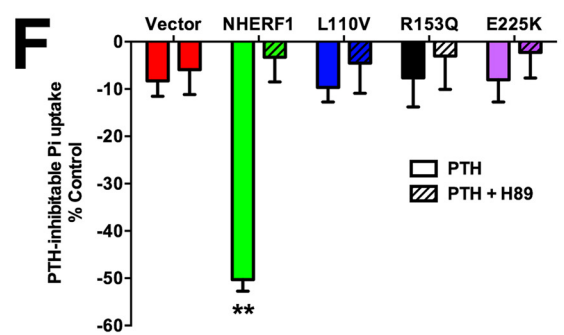
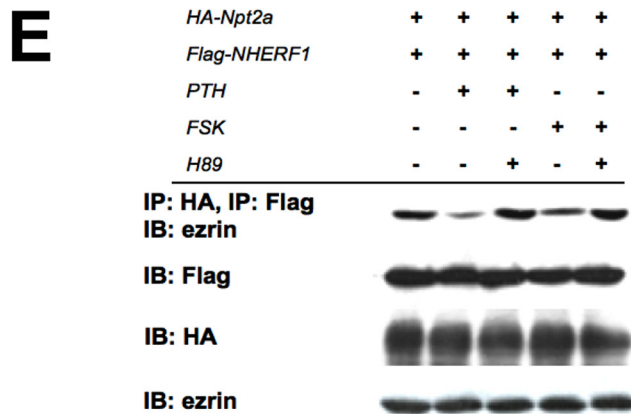
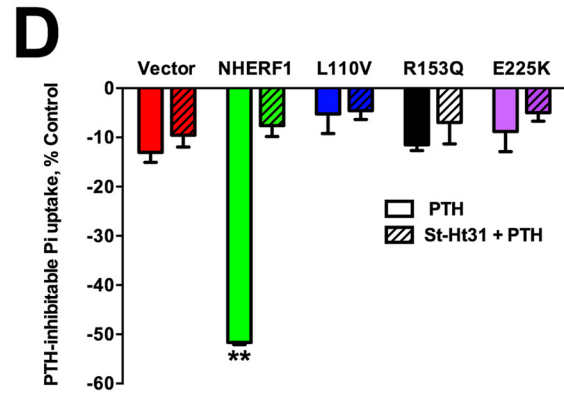
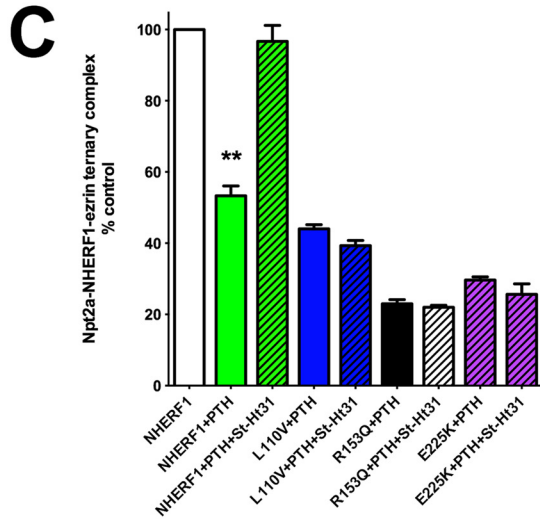
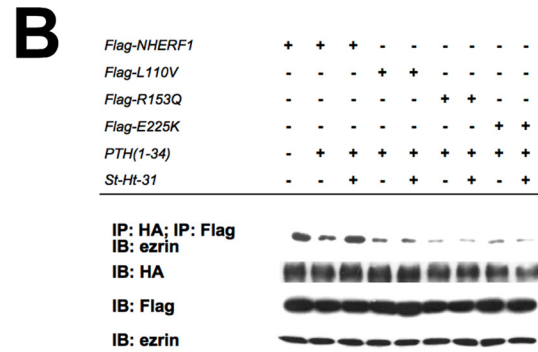
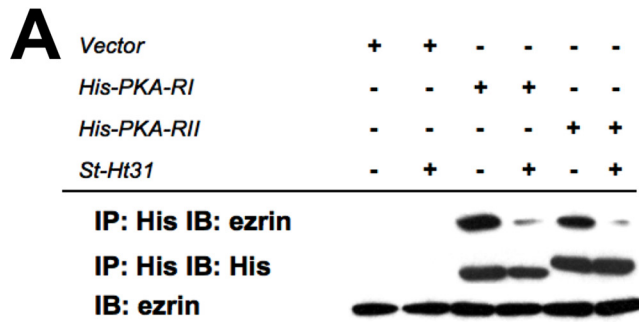
ylated (39) and has been implicated in PKC-mediated phosphorylation (40, 41). PTH increased phosphorylation of both fragments of wild-type NHERF1 but failed to phosphorylate either fragment of the mutant NHERF1 forms (Fig. 5, A and B).

The cumulative evidence thus far argues that an intact Npt2a-NHERF1-ezrin complex provides a molecular platform to mediate PTH-sensitive inhibition of P_i transport. The observations further show that NHERF1 mutations impair both the assembly and disassembly of the complex. However, the findings do not address the underlying mechanism by which mutations interfere with NHERF1 function. Therefore, we sought to determine if the mutations act by a dominant negative mechanism or are loss-of-function mutations. Cells were cotransfected with NHERF1 (0.2 μg) and increasing amounts of R153Q-NHERF1 (0–1 μg at a constant total DNA; Fig. 6A). As before, PTH inhibited P_i uptake in cells transfected with NHERF1 alone (Fig. 6B). Cells cotransfected with R153Q-NHERF1 exhibited PTH-sensitive P_i uptake comparable with that of cells expressing only NHERF1. Thus, R153Q-NHERF1 behaves as a loss-of-function mutation.

Molecular modeling suggests that mutations stabilize a closed or inactive NHERF1 structure (42–44). If correct, the affinity of Npt2a should be lower for mutated than for wild-type NHERF1. We assessed this by measuring the K_D by fluorescence polarization and by ITC. The K_D derived from fluorescence anisotropy for Npt2a with wild-type NHERF1 was 4.4 μM and increased to 75 μM for R153Q-NHERF1 (Fig. 6C). ITC results independently demonstrated a similar K_D (4.17 μM) for wild-type NHERF1 (Fig. 6D, left), which increased to 38 μM with R153Q-NHERF1 (Fig. 6D, right).

These results imply that mutant NHERF1 adopts a stable conformation, where the EBD is bound to PDZ2 in a manner that precludes PKA action. This would account for the lack of phosphorylation of mutant forms of NHERF1 and the loss of function. Therefore, we reasoned that preventing engagement of the EBD to the PDZ2 binding pocket by introducing a mutation in the EBD carboxyl-terminal PDZ ligand should reverse the loss of function associated with the R153Q-NHERF1 mutant. To test this idea, we generated double mutants in NHERF1, where the carboxyl-terminal Leu that is required for engagement of PDZ2 was exchanged for Ala (L358A) and was paired with R153Q. Replacement of Leu³⁵⁸ alone had no effect on PTH-responsive NHERF1 action, whereas R153Q-NHERF1 failed to support PTH-inhibitable P_i transport (Fig. 7A). Remarkably, introducing the secondary mutation in NHERF1 functionally rescued this effect (Fig. 7A). Moreover, the double NHERF1 mutant was phosphorylated in a manner that was essentially indistinguishable from that of wild-type NHERF1 (Fig. 7, B and C). In keeping with these findings, PTH-dependent P_i transport sensitivity to H89 and St-Ht31 was restored in the context of the R153Q/L358A double NHERF1 mutant (Fig. 7D). Thus, the R153Q mutation, as such, does not interfere with phosphorylation or function but is resistant to the actions of PTH because the adopted conformation impedes access to PKA and kinase-mediated phosphorylation. According to this line of reasoning, introducing L358A in R153Q-NHERF1 should restore the ability of NHERF1 to assemble the ternary complex with Npt2a and ezrin even in the face of the R153Q mutation.

Npt2a-NHERF1-Ezrin Ternary Complex Regulates *P_i* Transport



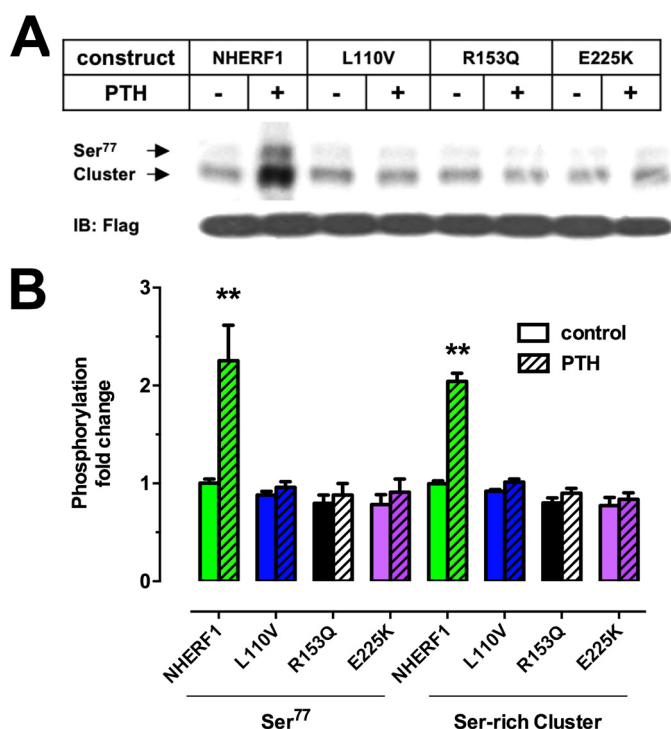


FIGURE 5. NHERF1 mutants are resistant to phosphorylation upon PTH stimulation. A, PTH promotes phosphorylation of wild-type NHERF1 but not of NHERF1 mutants. Representative experiment shows PTH-stimulated phosphorylation within the two large fragments of NHERF1 cleaved by CNBr containing Ser⁷⁷ within PDZ1 (residues 2–156) and in a Ser-rich cluster located in the linker between PDZ2 and the EBD (residues 208–329) (Cluster). B, quantification of phosphorylation results from A ($n = 3$; **, PTH versus control, $p < 0.001$, two-way repeated measures ANOVA (Bonferroni post hoc multiple-comparison test)). Error bars, S.E.; IB, immunoblot.

This prediction was tested, and the results (Fig. 7E) support the conclusion that by overcoming the structural constraints in NHERF1 stemming from the inherited mutation, all molecular and functional activities are restored.

DISCUSSION

This investigation offers compelling biochemical and cellular evidence that NHERF1 and ezrin act in concert to restrict the location of Npt2a to the apical cell membrane and legislate its response to PTH. This newly discovered consortium of scaffolding and adapter proteins facilitates the inhibition of Npt2a transporter activity by PTH. Ezrin, by virtue of its protein kinase A anchoring capacity, positions the PKA holoenzyme in proximity to the Npt2a-NHERF1-ezrin ternary complex. Upon

PTH stimulation and the concomitant production of cAMP, anchored PKA phosphorylates NHERF1 on target serines (38, 45). This cascade of events induces the dissociation of Npt2a from the ternary complex, resulting in Npt2a internalization and degradation. The L110V-, R153Q-, and E225K-NHERF1 mutations largely prevent formation of the Npt2a-NHERF1-ezrin complex and account for the observed PTH resistance.

The discovery that PKA anchoring contributes to PTH action provides a novel and biologically parsimonious mechanism to explain how naturally occurring mutations in NHERF1 fail to support PTH action on P_i uptake. Patients with congenital mutations in NHERF1 excrete more cAMP than control subjects yet were less responsive to the hormone (17). Likewise, PTH induced greater cAMP formation in OK cells deficient in NHERF1 compared with cells transfected with wild-type NHERF1. Based on these observations, Karim *et al.* (17) concluded that the increased P_i excretion in humans and P_i transport by OK cells is due to greater cAMP formation. Although Mahon *et al.* (31) similarly found that overexpressing NHERF1 depressed PTH-stimulated cAMP accumulation in OKH cells, they found that NHERF1 enhanced the inhibitory action of PTH on P_i uptake as we verified here. Several pieces of data in our report argue against the view that enhanced cAMP mediates the inhibitory action of NHERF1 on PTH-sensitive P_i transport or alternately suggest that it is an epiphenomenon. For example, if enhanced PTH-stimulated cAMP is responsible for increased Npt2a internalization, then directly stimulating PKA should have the same effect. However, to the contrary, administration of forskolin, which elevates cAMP to supra-physiological levels, failed to promote disassembly of the mutant NHERF1 ternary complex or to inhibit P_i transport. In contrast, our results suggest that PKA functions as a local regulatory factor of the newly described ternary complex to transduce second messenger signals that regulate Npt2a sequestration quite possibly because of its PKA anchoring protein function. Mutant forms of NHERF1 fail to support this activity, not because of increased cAMP generation but by virtue of the diminished formation of the ternary complex and the inability of cAMP to induce dissociation of Npt2a from the ternary complex assembled with mutant NHERF1.

Npt2a associates in a pair-wise manner with NHERF1, NHERF3 (PDZK1), NHERF4 (PDZK4), and ezrin (46, 47). The present findings confirm the normal binary interactions of NHERF1 with the PTHR (23, 48) and with ezrin (49) and show

FIGURE 4. Anchored PKA activation is required for Npt2a-NHERF1-ezrin ternary complex disassembly and inhibition of phosphate transport. A, PKA regulatory subunits I and II associate with ezrin with similar affinities. St-Ht31 (5 μ M) virtually abolishes this interaction. OKH cells were transfected with His-RI or His-RII. 48 h after transfection, His-tagged proteins were precipitated with Ni-NTA-agarose and immunoblotted with an ezrin antibody. B and C, blocking PKA binding to ezrin prevents dissociation of the Npt2a-NHERF1-ezrin ternary complex. B, OKH-HA-Npt2a cells were transiently transfected with FLAG-tagged wild-type NHERF1 or L110V-, R153Q, or E225K-NHERF1. 48 h after transfection, the cells were pretreated with 50 μ M St-Ht31 for 30 min and challenged with 100 nM PTH for 2 h. C, average results \pm S.E. (error bars) ($n = 3$; **, versus NHERF1, $p < 0.001$, one-way repeated measures ANOVA (Dunnett's post hoc multiple-comparison test)). D, St-Ht31 interference with PKA binding obliterates PTH-dependent inhibition of P_i transport in cells expressing wild-type NHERF1 but has no effect on cells expressing mutant NHERF1. OKH cells were transfected with the indicated construct and treated with St-Ht31 and PTH as above. Data are summarized as the mean \pm S.E. of three independent experiments (**, PTH versus St-Ht31+PTH, $p < 0.001$, two-way repeated measures ANOVA (Bonferroni post hoc multiple-comparison test)). E, inhibiting PKA with H89 blocks PTH- or forskolin-induced dissociation of the ternary complex. OKH-HA-Npt2a cells were transfected with FLAG-NHERF1. After 48 h, the cells were treated for 15 min with 10 μ M H89 before the addition of PTH (100 nM) or forskolin (1 μ M) for 2 h. The ternary complex was measured as before. F, inhibition of PKA with H89 blocks PTH-dependent P_i uptake in cells expressing wild-type NHERF1 but has no effect on cells expressing mutant NHERF1. Results are the average \pm S.E. of three experiments (**, PTH versus PTH + H89, $p < 0.001$, two-way repeated measures ANOVA (Bonferroni post hoc multiple-comparison test)). G and H, Npt2a-NHERF1-ezrin ternary complex formed with mutant NHERF1 is refractory to disassembly (G) or inhibition of P_i transport (H) by direct activation of PKA by forskolin. Results are the average \pm S.E. ($n = 4$; **, versus vector, $p < 0.001$, one-way repeated measures ANOVA (Dunnett's post hoc multiple-comparison test)). IP, immunoprecipitation; IB, immunoblot.

Npt2a-NHERF1-Ezrin Ternary Complex Regulates P_i Transport

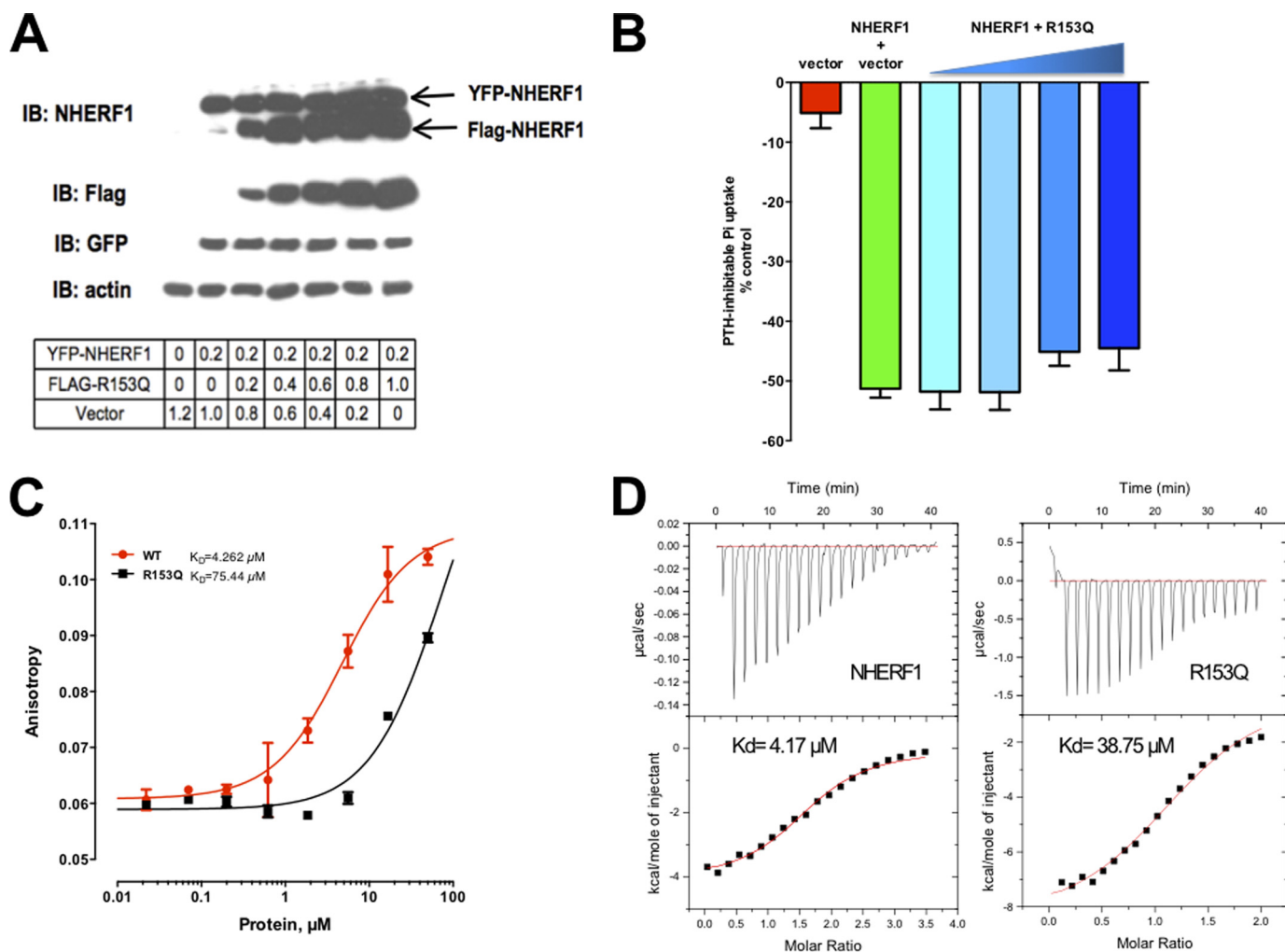


FIGURE 6. Functional and structural analysis of NHERF1 mutations. *A* and *B*, R153Q-NHERF1 acts as a loss-of-function mutation and not as a dominant negative inhibitor of wild-type NHERF1. OKH cells were transfected with 0.2 μg of GFP-NHERF1 alone or 0–1.0 μg of R153Q-NHERF1, with the balance made of empty vector (*A*). PTH-sensitive P_i uptake was measured as before (*B*) ($n = 3$ independent determinations). *C* and *D*, affinity of wild-type NHERF1 (WT) and mutant R153Q-NHERF1 for Npt2a was determined by fluorescence polarization, and K_D was derived from the calculated anisotropy (*C*). Affinity measurements by ITC for wild-type NHERF1 (*D*, left) and R153Q-NHERF1 (*D*, right) are shown. Error bars, S.E.; IB, immunoblot.

that the mutant forms of NHERF1 behave indistinguishably in this regard. Recently, higher order complexes of Npt2a have been noted (47). If the four PKA subunits are considered part of the described complex, the protein consortium could consist of at least seven components. In addition to these *trans* interactions, NHERF1 reportedly forms homodimers (50–53). NHERF1 homodimerization facilitates PDGF receptor signaling (54). We considered the possibility that NHERF1 mutants were unable to or impaired in their ability to dimerize or might form dominant negatives with wild-type protein in heterozygous patients (17), which could account for the PTH resistance and failure to inhibit P_i transport. Overlay assays, however, revealed divergent effects, where L110V-NHERF1 interacted normally with NHERF1, E225K-NHERF1 displayed somewhat reduced dimerization, and R153Q-NHERF1 severely impaired dimerization (data not shown). Nonetheless, all NHERF1 mutants abolished PTH-sensitive P_i uptake and had comparable and severe phenotypes in patients (17). These observations suggest that simple NHERF1 binary interactions or dimerization do not play an essential role in assembling a functional ternary complex and are unlikely to account for defective reg-

ulation of P_i transport by the three NHERF1 mutants. Such a conclusion is compatible also with recent evidence that NHERF1 remains monomeric in cells (55). Further evidence that the described NHERF1 mutations do not act as dominant negative proteins comes from the absence of an inhibitory action when coexpressed with wild-type NHERF1. This finding suggests that although not recessive, the described mutations nonetheless lack intrinsic activity or that NHERF1 haploinsufficiency accounts for renal and skeletal phenotypes. Such a conclusion is compatible with the NHERF1 gene dose effect on phosphate excretion in mice (14).

PKA localization is coordinated by its interaction with ezrin through an amphipathic helix that is characteristic of the AKAP family of proteins (33, 56). The inhibitory effect of St-Ht31 is consistent with the action of dominant negative ezrin, which blocks PTH-mediated inhibition of Npt2a (57). Together, these findings supplant previous work and suggest that ezrin plays a key role in down-regulating Npt2a activity by anchoring PKA in proximity to NHERF1 and promoting PKA-induced disassembly of the described Npt2a-NHERF1-ezrin ternary complex. According to this scheme (Fig. 8), after activation by cAMP,

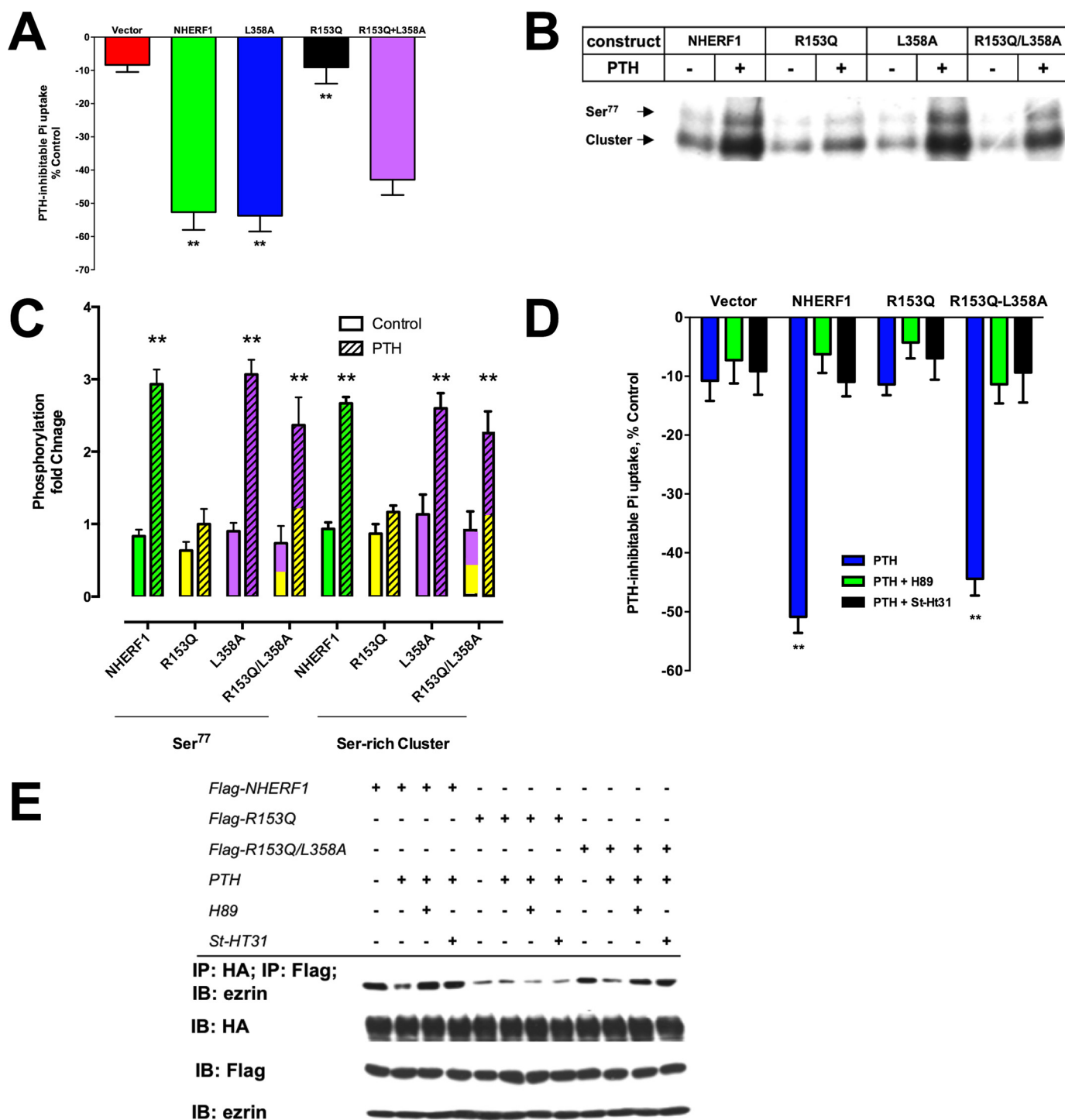


FIGURE 7. Rescue of NHERF1 structure and function. A, rescue of loss of function of R153-NHERF1 mutant by preventing closed NHERF1 conformation. PTH-sensitive P_i uptake was measured in OKH cells transfected with the indicated construct. Results are the average \pm S.E. (error bars) ($n = 4$; **, versus vector, $p < 0.001$, one-way repeated measures ANOVA (Dunnett's post hoc multiple-comparison test)). B–E, double R153Q/L358A-NHERF1 mutation restores PTH-induced phosphorylation to NHERF1 (B and C) ($n = 3$; **, PTH versus control, $p < 0.001$, two-way repeated measures ANOVA (Bonferroni post hoc multiple-comparison test)) and inhibition by H89 and Ht31 of PTH-sensitive P_i uptake (D) ($n = 3$; **, PTH versus St-Ht31 + PTH, $p < 0.001$, two-way repeated measures ANOVA (Bonferroni post hoc multiple-comparison test)) and reestablishes the ability to form the ternary complex even in the presence of the R153Q mutation (E). IP, immunoprecipitation; IB, immunoblot.

PKA regulatory subunits bind to ezrin, thereby positioning the catalytic subunits in proximity to the ternary complex. Upon PTH-stimulated elevation of cAMP, the anchored kinase preferentially phosphorylates sites on proteins in the NHERF1-Npt2a-ezrin complex. The net effect is dissociation of the ter-

nary complex and the subsequent endocytosis of Npt2a. Such a mechanism defines a previously unrecognized spatiotemporal component of intracellular PTH action.

NHERF1 is obligatorily phosphorylated in response to PTH (38, 58, 59). The mechanism and particular kinases mediating

Npt2a-NHERF1-Ezrin Ternary Complex Regulates P_i Transport

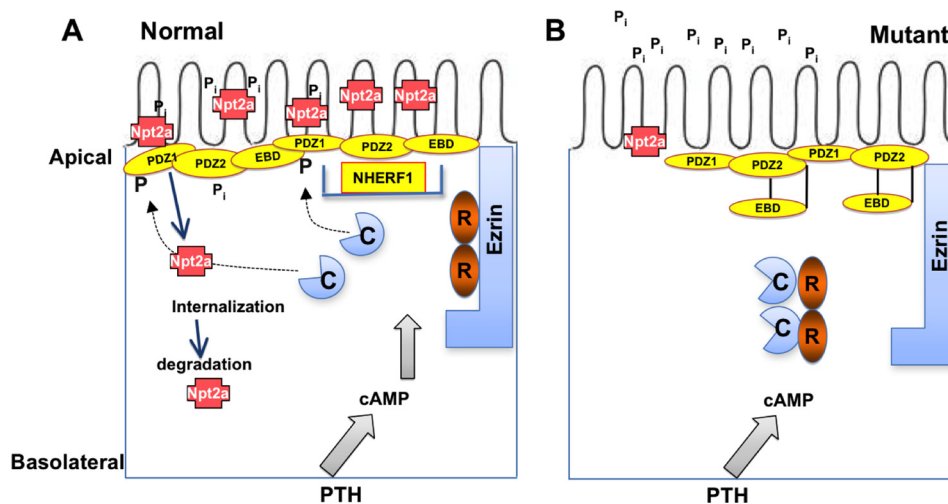


FIGURE 8. Model of PTH-mediated disassembly of the Npt2a-NHERF1-ezrin ternary complex and endocytosis of Npt2a. *A*, in the resting state, Npt2a is present in brush borders of apical membranes, bound to PDZ1 of wild-type NHERF1 as part of the spontaneously formed Npt2a-NHERF1-ezrin ternary complex. The EBD of NHERF1 binds to ezrin, linking the complex to cytoskeletal elements. In this way, Npt2a is tethered to apical membranes and mediates phosphate uptake. Upon PTH exposure, PTHR stimulates cAMP formation and activates PKA. PKA regulatory (*R*) subunits undergo a conformational change and release the catalytic (*C*) subunits. Ezrin binds to PKA regulatory subunits, positioning them in close proximity to the ternary complex. PKA catalytic subunits phosphorylate (*P*) NHERF1, with subsequent dissociation of Npt2a, which is endocytosed, thereby inhibiting phosphate transport. *B*, in the presence of mutant forms of NHERF1, less Npt2a is present in brush border apical membranes. PTH stimulates cAMP normally. Because NHERF1 is locked in an inactive conformation, access to catalytic PKA subunits is prevented, thereby precluding phosphorylation and dissociation of tethered Npt2a from NHERF1.

NHERF1 phosphorylation, however, are controversial. Both PKA and PKC have been implicated in this process (60–62), and this is compatible with the parallel activation of both kinases by PTH (63). PKC phosphorylates Ser⁷⁷ in PDZ1, resulting in PTH-induced dissociation of NHERF1 and Npt2a (64, 65). NHERF1 is also phosphorylated at positions 287, 289, and 290 in a serine cluster located between PDZ2 and the EBD that is required for biological activity (66). This phosphorylation is reported to be mediated by PKA (66). The present results reveal an essential role for anchored PKA in the disassembly of the Npt2a-NHERF1-ezrin ternary complex, consistent with finding that acute Npt2a down-regulation is primarily governed by the PKA pathway (61). Moreover, phosphorylation was significantly diminished in the presence of the R153Q-NHERF1 mutation. PKA regulatory subunits I and II directly bind ezrin. St-Ht31 blocks this interaction and prevents PTH-induced dissociation of the ternary complex. Thus, coordinated and complementary roles for both PKA and PKC may be involved in mediating the assembly of the ternary complex and its dissociation in response to hormone activation. Such an interpretation is entirely consistent with and extends recent findings that phosphorylation blocks accessibility of PDZ1 when PDZ2 is occupied (55).

This study provides novel and conclusive evidence that the three NHERF1 mutations interfere with the formation of the Npt2a-NHERF1-ezrin ternary complex. A conceptual model for NHE3 regulation by a multimolecular complex involving NHERF1 and ezrin (67) or of the cystic fibrosis conductance regulator and NHERF2 (68) is similar to the ternary complex demonstrated here. These schemes lend broader credence for the present model and imply a greater and more general significance to the conclusion that multiprotein complexes of NHERF1/2 and their phosphorylation by ezrin-anchored PKA regulate the activity of several membrane-delimited ion transporters. While this work was in review, a new NHERF1 muta-

tion was described (69). E68A is in PDZ1, and its effects are entirely predictable from the ternary complex model proposed here. These new findings further underscore the view that mutations in Npt2a, NHERF1, or ezrin that interfere with the formation of the ternary complex or its phosphorylation impedes the normal function and regulatory influence of PTH. Similar disordered regulation presumably attends the action of dopamine and other hormones affecting Npt2a trafficking.

Npt2a binds exclusively at PDZ1. As noted, the described mutations are located in the linker region between PDZ1 and PDZ2 or at the amino- or carboxyl-terminal regions of PDZ2. How then do the mutations affect Npt2a binding to PDZ1? The EBD of NHERF1 itself terminates in a PDZ recognition sequence (-FSNL) that by intramolecular binding engages PDZ2 (53). This structural feature, which is unique to NHERF1 and not shared by other NHERF proteins, allows it to adopt an intramolecular association. In this closed conformation, PDZ1 is masked (53, 70). Ezrin binding to NHERF1 increases the affinity of PDZ1 by long range allosteric actions (70, 71). Using a thermodynamic integration method with atomistic molecular dynamics simulations based on the structure of wild-type NHERF1, we found that R153Q- and E225K-NHERF1 mutations significantly enhance the binding affinity of PDZ2 for target ligands, thereby stabilizing the closed conformation (43). The third NHERF1 mutation, L110V, is located in the $\alpha 4$ helix of the carboxyl-terminal helix-turn-helix subdomain of PDZ1. Mutation to the smaller hydrophobic side chain in Val could potentially disrupt the packing interactions and reduce the stability and affinity of PDZ1 binding. Thus, the greater stability of the closed conformation of mutant forms of NHERF1 impairs the assembly of a functional Npt2a-NHERF1-ezrin ternary complex. Releasing the structural constraints of R153Q-NHERF1 by mutating the EBD PDZ ligand, although having no impact of its own, restored PTH-induced NHERF1 phosphorylation and reversed the inhibitory effects of the mutation.

In conclusion, the present structural and functional results demonstrate the requirement for the Npt2a-NHERF1-ezrin complex in mediating the effects of PTH on P_i transport. NHERF1 mutants are effectively refractory to regulation by PTH and are not phosphorylated. This regulatory collapse is attributable to the failure to assemble or disassemble the NHERF1 ternary complex. The failure to assemble and regulate the Npt2a-NHERF1-ezrin ternary complex is responsible for the loss of function of the NHERF1 mutants and defines a novel molecular mechanism of PTH resistance and hyperphosphaturia. Together, these findings introduce a new paradigm for the turnover of PDZ proteins and a novel mechanism by which mutations disrupt the macromolecular assembly and spatio-temporal coordination of hormone action. Such a model may be relevant for elucidating the mechanism whereby other NHERF1 polymorphisms contribute to breast cancer or autoimmune disorders (72, 73).

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