Role of Na\(^+\)/H\(^+\) exchanger regulatory factor 1 in forward trafficking of the type IIa Na\(^+\)-P\(_i\) cotransporter

Corey J. Ketchem,1,2,3 Syed J. Khundmiri,1,2,3* Adam E. Gaweda,2 Rebecca Murray,3 Barbara J. Clark,4 Edward J. Weinman,5,6 and Eleanor D. Lederer1,2,3

1Robley Rex Veterans Affairs Medical Center, Louisville, Kentucky; 2Department of Medicine, University of Louisville, Louisville, Kentucky; 3Department of Physiology and Biophysics, University of Louisville, Louisville, Kentucky; 4Department of Biochemistry and Molecular Biology, University of Louisville, Louisville, Kentucky; and 5Baltimore Veterans Affairs Medical Center, Baltimore, Maryland

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Ketchem CJ, Khundmiri SJ, Gaweda AE, Murray R, Clark BJ, Weinman EJ, Lederer ED. Role of Na\(^+\)/H\(^+\) exchanger regulatory factor 1 in forward trafficking of the type IIa Na\(^+\)-P\(_i\) cotransporter. Am J Physiol Renal Physiol 309: F109–F119, 2015. First published May 20, 2015; doi:10.1152/ajprenal.00133.2015.—Na\(^+\)/H\(^+\) exchanger regulatory factor (NHERF1) plays a critical role in the renal transport of phosphate by binding to Na\(^+\)-P\(_i\) cotransporter (NpT2a) in the proximal tubule. While the association between NpT2a and NHERF1 in the apical membrane is known, the role of NHERF1 to regulate the trafficking of NpT2a has not been studied. To address this question, we performed cell fractionation by sucrose gradient centrifugation in opossum kidney (OK) cells placed in low-P\(_i\) medium, medium to stimulate forward trafficking of NpT2a. Immunoblot analysis demonstrated expression of NpT2a and NHERF1 in the endoplasmic reticulum (ER)/Golgi. Coinmunoprecipitation demonstrated a NpT2a-NHERF1 interaction in the ER/Golgi. Low-P\(_i\), medium for 4 and 8 h triggered a decrease in NHERF1 in the plasma membrane with a corresponding increase in the ER/Golgi. Time-lapse total internal reflection fluorescence imaging of OK cells placed in low-P\(_i\), medium, paired with particle tracking and mean square displacement analysis, indicated active directed movement of NHERF1 at early and late time points, whereas NpT2a showed active movement only at later times. Silence of NHERF1 in OK cells expressing green fluorescent protein (GFP)-NpT2a resulted in an intracellular accumulation of GFP-NpT2a. Transfection with GFP-labeled COOH-terminal (TRL) PDZ-binding motif deleted or wild-type NpT2a in OK cells followed by cell fractionation and immunoprecipitation confirmed that the interaction between NpT2a and NHERF1 was dependent on the TRL motif of NpT2a. We conclude that appropriate trafficking of NpT2a to the plasma membrane is dependent on the initial association between NpT2a and NHERF1 through the COOH-terminal TRL motif of NpT2a in the ER/Golgi and requires redistribution of NHERF1 to the ER/Golgi.

endoplasmic reticulum/Golgi; Na\(^+\)/H\(^+\) exchanger regulatory factor 1; sodium phosphate cotransporter 2a; plasma membrane; trafficking

diation of NpT2a as well as decreased mRNA expression (40). A low-phosphate diet has the opposite effect, stimulating insertion of NpT2a into the apical membrane and inhibiting endocytosis (22, 31). Thus, the regulation of the protein is dynamic and responsive to multiple physiological regulatory mechanisms. NpT2a is an intrinsic membrane protein for which hydropathy analysis predicts multiple transmembrane domains (20). The protein is heavily glycosylated, and there is evidence that the glycosylation is, if not absolutely required, at least facilitatory for apical membrane targeting (9).

The predicted amino acid sequence for NpT2a does not disclose a known apical membrane targeting sequence, unlike what is seen for NpT2b (12, 13). However, NpT2a is expressed only in the apical membrane, suggesting that a mechanism exists for targeting of the protein. Previous studies have failed to identify amino acid sequences responsible for apical membrane localization except for the sequences involved in the interaction of NpT2a with Na\(^+\)/H\(^+\) exchanger regulatory factor (NHERF1). NHERF1 is a scaffolding protein and is expressed in multiple epithelial tissues, including the renal proximal tubule. NHERF1 contains two PDZ domains and a COOH-terminal ezrin-binding domain (2, 28, 34, 39) and plays an important role in the assembly of protein complexes including ion transporters, G protein-coupled receptors, and signaling molecules. NpT2a expresses a class 1 PDZ-binding motif on its COOH-terminus, a presumptive site for the NpT2a-NHERF1 interaction (24). Multiple studies have suggested that interfering with the interaction between NpT2a and NHERF1 results in impaired apical membrane expression of NpT2a. Hernando et al. (10) demonstrated that expression of a peptide representing the COOH-terminus of NpT2a in opossum kidney (OK) cells blocked apical membrane expression of NpT2a and concluded that inhibition of the interaction between NpT2a and NHERF1 was the mechanism for the impaired apical membrane expression. Inhibition of NHERF1 expression in OK cells resulted in faulty trafficking of NpT2a to an intracellular compartment (14). The NHERF1-deficient mouse exhibits a marked decrease in serum phosphorus, inappropriate phosphaturia, and a lack of response to dietary phosphate or PTH (35, 38). In these animals, NpT2a was identified predominantly in unidentified subapical compartments, with limited apical membrane expression. The deficiencies in the regulation and expression of NpT2a could be corrected by transfection of wild-type NHERF1 into cultured proximal tubule cells from these mice (3). Previously published work has demonstrated that treatment with PTH results in phosphorylation of NHERF1 followed by dissociation of NHERF1 from NpT2a and subse-

* C. J. Ketchem and S. J. Khundmiri contributed equally to this work.

Address for reprint requests and other correspondence: E. D. Lederer, Dept. of Medicine, Kidney Disease Program, Univ. of Louisville, 615 S. Preston St., Louisville, KY 40202 (e-mail: e.lederer@louisville.edu).
quent endocytosis of NpT2a (36, 37, 41, 43). These data strongly suggest that the apical membrane target sequence for NpT2a may be the PDZ-binding motif through its NHERF1-binding function. In the aggregate, these findings suggested the hypothesis that NpT2a and NHERF1 interact in an intracellular compartment before apical membrane insertion and that this interaction is required for apical membrane trafficking. To test this hypothesis, we stimulated apical membrane trafficking of NpT2a by treatment of OK cells depleted of NpT2a by PTH pretreatment with a low-phosphate media and tested for NpT2a-NHERF1 interaction in Golgi membranes. Live imaging techniques with OK cells were performed to visualize and quantify the movement of NpT2a and NHERF1. We demonstrate that NpT2a interacts with NHERF1 in the endoplasmic reticulum (ER)/Golgi, that the COOH-terminal PDZ-binding motif is required for this interaction, and that failure of this interaction results in impaired trafficking of NpT2a to the apical membrane.

**EXPERIMENTAL PROCEDURES**

**Materials.** PTH 1–34 was purchased from Bachem Biosciences (King of Prussia, PA). A high-purity plasmid purification system (catalog no. 11451-028) was purchased from Marlington Biosciences (Ijamsville, MD). Lipofectamine 2000 (catalog no. 11668019) was purchased from Invitrogen (Carlsbad, CA). Antibodies against GM58 and GM130 were purchased from Molecular Probes, early endosome antigen 1, glucose-regulated protein (Grp)94, and caveolin-1 were from Novus Biologicals (Littleton, CO), and Rab5 was from Santa Cruz Biotechnology (Santa Cruz, CA). Green fluorescent protein (GFP)-labeled full-length NpT2a and Rab5 was from Santa Cruz Biotechnology (Santa Cruz, CA). Green fluorescent protein (GFP)-labeled full-length and COOH-terminal TRL-deleted NpT2a were previously characterized and provided by Dr. Nati Hernando (University of Zurich) (11). Antibodies against NHERF1 developed by Dr. Edward Weinman were characterized (data not shown). All other chemicals were of highest quality and were purchased from Sigma Chemical (St. Louis, MO) unless otherwise specified.

**Cell culture of OK cells.** OK cells from an OK proximal tubule cell line derived from *Didelphis virginiana* were maintained at 37°C in a humidified atmosphere with 5% CO2 in minimal essential medium (MEM) with phenol red maintained at 37°C and 5% CO2. Live cells were imaged 24 or 16 h after electroporation of GFP-NpT2a, 100 ng of NHERF1 siRNA or scrambled siRNA were transfected into OK cells using Lipofectamine RNAiMAX reagent according to the manufacturer’s protocol. Briefly, 5 × 10^5 cells/ml were resuspended in 100 μl R buffer containing 300 ng plasmid. The cell suspension was electroporated based on the following parameters: 1,650 V, pulse width of 10 ms, and three pulses. Cells were immediately plated onto collagen-coated glass plates (MatTek) and grown overnight in antibiotic-free medium containing 10% FBS.

**Total internal reflection fluorescence microscopy.** OK cells were grown on collagen-coated glass-bottom plates in Opti-MEM + 10%FBS overnight, after electroporation. Cells were washed three times with serum-free low-phosphate (0.1 mM phosphate) MEM without phenol red and incubated in 2 ml low-phosphate MEM. Total internal reflection fluorescence (TIRF) microscopy was performed in a humidified incubation chamber maintained at 37°C and 5% CO2 as previously described (17).

**Particle tracking.** Once time-lapse images had been obtained, particle tracking was performed using the Mosaic ParticleTracker plugin available for ImageJ (27, 33). The parameters used for particle detection were a radius of 2, cutoff of 2, percentile of 0.2%, a link range of 2, and a displacement of 5. Mean square displacement (MSD) analysis was performed using MATLAB (http://bradleymonk.com/matlab/msd/MSDTuto.html).

**Preparation and purification of NHERF1 small interfering RNA.** NHERF1 small interfering (siRNA) sense and antisense oligonucleotides were designed using Ambion’s web-based siRNA converter software (www.ambion.com/techlib/misc/siRNA_finder.html) as previously described (18). After electroporation of GFP-NpT2a, 100 ng of NHERF1 siRNA or scrambled siRNA were transfected into OK cells using Lipofectamine RNAiMAX reagent according to the manufacturer’s protocol. Cells were then allowed to grow in a humidified incubator at 37°C and 5% CO2. Live cells were imaged 24 or 48 h after siRNA transfection by confocal microscopy using an Olympus Fluoview FV1000 microscope equipped with a ×60 oil-immersion objective and analyzed by Fluoview (version 2.0) software.

**GFP-NpT2a transfection.** GFP-NpT2a or GFP-TRL-NpT2a mutant was transiently transfected in OK cells and split once per week at a 1:4 ratio. All experiments were performed with cells grown on six-well culture plates. Cells were washed with serum-free media 24 h before use. Cells were treated with 0.1 mM phosphate (low phosphate) for 24 h to stimulate NpT2a trafficking to the apical membrane or 100 mM PTH for 6 h to deplete NpT2a from the apical membrane.

**Protein determination.** Protein concentration was determined using the bicinchoninic acid method with BSA as the standard.

**Fractionation of subcellular membrane vesicles.** Subcellular membrane fractionation was performed using sucrose density gradient centrifugation as previously described (37) and following the protocol described by Li and Donowitz (23). Briefly, cells were treated for 6 h with 100 nM PTH followed by an incubation in low-phosphate media. Cells were shifted to either 37 or 16°C for 16 h. Cells were washed, scrapped in 250 mM sucrose and 10 mM Tris (pH 7.4), and homogenized using a 26-gauge needle. Homogenates were centrifuged at 3,000 g for 5 min to remove cell debris, nuclei, and unbroken cells. Homogenates (1 mg protein) were loaded on a discontinuous sucrose gradient (5–40%) in 2.5% increments. Samples were centrifuged at 100,000 g for 16 h at 4°C in a swinging bucket rotor (Beckmann). Fractions (15 μl) were collected from the top and identified by Western blot analysis using organelle-specific antibodies, GM58 for the Golgi, Grp94 for the ER, Rab5 for endosomes, and the Na^+–K^+-ATPase α1-subunit for plasma membranes.

**Immunoblot assay.** Immunoblot analysis was performed as previously described (16). The bands imaged by chemiluminescence were analyzed by densitometry using ImageJ.

**Immunoprecipitation.** NpT2a and NHERF1 were immunoprecipitated as previously described (15).

**GFP-NpT2a or mCherry-NHERF1 electroporation.** OK cells were transfected with GFP-NpT2a and/or mCherry-NHERF1 by electroporation using a Neon electroporation kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, 5 × 10^5 cells/ml were resuspended in 100 μl R buffer containing 300 ng plasmid. The cell suspension was electroporated based on the following parameters: 1,650 V, pulse width of 10 ms, and three pulses. Cells were immediately plated onto collagen-coated glass plates (MatTek) and grown overnight in antibiotic-free medium containing 10% FBS.

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using Lipofectamine 2000 as previously described (14).

Statistics. Data are shown as means ± SE; n values represent the number of independent experiments. Each experiment was performed in triplicate. P values were calculated using one-way ANOVA followed by Bonferroni analysis using GraphPad Prism software. P values of <0.05 were a priori considered statistically significant.

Fig. 1. Trafficking of Na\(^+\)-P\(^-\) cotransporter (NpT2a) and Na\(^+\)/H\(^+\) exchanger regulatory factor (NHERF1) in opossum kidney (OK) cells. A: cells were lysed in 250 mM sucrose and 5 mM Tris (pH 7.4). Nuclear-free cell lysates were separated on discontinuous sucrose gradients (5–40%) as described in EXPERIMENTAL PROCEDURES. Cell organelles were identified by Western blot (WB) analysis for specific organelle markers. ER, endoplasmic reticulum; Grp94, glucose-regulated protein 94. B: OK cells were treated for 6 h with 100 nM parathyroid hormone (PTH) and transferred to low-phosphate media at either 37 or 16°C for 24 h. Nuclear-free cell lysates were separated on discontinuous sucrose gradients as described in EXPERIMENTAL PROCEDURES. A representative blot from three independent experiments is shown.
RESULTS

Low phosphate stimulates apical membrane trafficking of NHERF1. Confocal imaging and immunohistochemistry demonstrated that both NHERF1 and NpT2a are expressed predominantly in the plasma membrane under basal conditions. To augment the ER/Golgi expression of newly synthesized NpT2a, we used two specific strategies. We treated cells with PTH for 6 h to deplete existing NpT2a followed by incubation of the cells in low-phosphate medium at 16°C to stimulate new NpT2a production but arrest trafficking at the ER/Golgi (8).

Fig. 2. Association of NHERF1 and NpT2a in the plasma membrane (PM) and ER/Golgi. Cells were treated for 6 h with 100 nM PTH followed by placement of cells in low-phosphate media for different times as indicated. Cells lysed in 300 mM mannitol and 5 mM Tris (pH 7.4) and nuclear free cell lysates were separated on discontinuous sucrose gradients (5–40%) as described in EXPERIMENTAL PROCEDURES. ER/Golgi or PM fractions were pooled. A: representative WBs showing expression of NpT2a and NHERF1 in pooled fractions. V, vehicle; P, PTH-treated; L, Low Pi-treated. B: NpT2a was immunoprecipitated from the pooled fractions and probed for NHERF1. A representative blot from three independent experiments is shown. C: cells were treated with normal or low-phosphate media for 6 h and lysed in 300 mM mannitol and 5 mM Tris (pH 7.4), and nuclear-free cell lysates were separated on discontinuous sucrose gradients (5–40%) as described in EXPERIMENTAL PROCEDURES. ER/Golgi or PM fractions were pooled, and NHERF1 was immunoprecipitated. Representative WBs show the expression of NpT2a and NHERF1 in pooled fractions after immunoprecipitation (IP) with NHERF1 antibody. Bar diagrams represent arbitrary densitometry units as fold changes from control samples (means ± SE; n = 3). *Significantly different (P < 0.05 by ANOVA followed by Bonferroni’s analysis) from control cells.
Identical experiments were performed at 37°C as a control. Cell lysates were then loaded onto a discontinuous sucrose gradient and fractionated as described in EXPERIMENTAL PROCEDURES. As shown in Fig. 1A, endosomal fractions were identified based on expression of Rab5A and ER and Golgi fractions were identified based on expression of Grp94, GM58, and/or GM130. Plasma membrane fractions were identified based on NpT2a and Na+–K+–ATPase α1-subunit expression. At 37°C, the majority of NHERF1 was present in the plasma membrane fraction (Fig. 1B). Incubation of cells at 16°C in low-phosphate medium resulted in increased abundance of NHERF1 in the endosomal and ER/Golgi fractions, consistent with the well-known effect of low temperature on intracellular protein trafficking. Under both temperature conditions, NpT2a expression was predominantly in the plasma membrane fraction; however, total NpT2a expression at 16°C was only 50% of the expression in cells incubated at 37°C. This finding suggests that the low-phosphate medium-stimulated increase in NpT2a apical membrane insertion is impaired under conditions where NHERF1 forward trafficking is decreased, as evidenced by NHERF1 accumulation in the endosomal and ER/Golgi fractions in cells incubated at 16°C.

NHERF1 and NpT2a interact before apical membrane localization. In the next set of experiments, we treated cells with PTH, low-phosphate medium, or PTH followed by incubation in low-phosphate medium for 4, 8, or 12 h to determine if newly formed NpT2a interacted with NHERF1 at an intracellular site before apical membrane insertion. These time points were chosen based on prior experiments demonstrating that the initial detectable appearance of NpT2a in the plasma membrane fraction was 4–6 h after NpT2a depletion. Cells lysates were loaded onto discontinuous sucrose gradients, and, based on the findings from our previous experiments (Fig. 1), ER/Golgi and plasma membrane fractions were pooled, and NpT2a was immunoprecipitated. As shown in Fig. 2, incubation of cells in low-phosphate medium with or without preceding treatment with PTH resulted in the faint expression of NpT2a in ER/Golgi fractions and a robust expression of NHERF1 in the same fractions. Treatment with PTH produced the expected decrease in plasma membrane expression of NpT2a, whereas low-phosphate media produced a time-dependent increase in plasma membrane NpT2a expression, demonstrating forward trafficking of NpT2a. Figure 2B shows that with equal amounts of NpT2a immunoprecipitated from the ER/Golgi or plasma membrane, NHERF1 was associated with NpT2a in both compartments, suggesting that NHERF1 is associated with NpT2a in the ER/Golgi as well as in the plasma membrane.

We confirmed the NHERF1-NpT2a association by performing reciprocal immunoprecipitation with antibodies against NHERF1. Cells treated with normal phosphate medium or low-phosphate medium treatment for 6 h were subjected to sucrose density centrifugation. As shown in Fig. 2C, immunoprecipitation of ER/Golgi and plasma membrane fractions with NHERF1 antibody demonstrated the presence of NpT2a. Notably, the expression level of NpT2a relative to NHERF1 decreased in ER/Golgi fractions but increased in plasma membrane fractions under low-phosphate conditions.

Effect of low-phosphate medium on NHERF1 and NpT2a trafficking. To determine if NHERF1 plays a role in low phosphate-mediated regulation of NpT2a trafficking, we incubated OK cells with low-phosphate medium for 4 and 8 h and then measured the membrane association of NHERF1 and NpT2a by Western blot analysis. After sucrose gradient centrifugation, ER/Golgi and plasma membrane fractions were pooled, and, as shown in Fig. 3, acute treatment with low-phosphate media for 4 or 8 h resulted in increased NHERF1 expression in ER/Golgi fractions and a corresponding decrease in plasma membrane fractions, whereas NpT2a expression progressively increased in both cell compartments.

The movement of NHERF1 and NpT2a was confirmed using time-lapse TIRF microscopy and MSD analysis. OK cells were live imaged for 2–4 h, with imaging beginning with incubation in low-phosphate media. A panel of representative images over 15-min intervals is shown in Fig. 4A (movie 1; all supplemental material for this article is accessible on the journal Web site). The time-lapse images were analyzed by ImageJ (movies 2 and 3) followed by MSD analysis in MATLAB, as described in Fig. 3.
A

B

C

D

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Effect of silence of NHERF1 on NpT2a trafficking. To further characterize the dependence of NpT2a trafficking on NHERF1, GFP-NpT2a-expressing OK cells were transfected with NHERF1 siRNA to prevent transcription and translation of NHERF1. Initial experiments showed that 100 nM siRNA for 48 h decreased NHERF1 expression by ~70% (Fig. 6A). The antibody recognizes different phosphorylation states of NHERF1. The band recognized at ~47 kDa by NHERF1 antibody was determined to be the least phosphorylated form of NHERF1 by alkaline phosphatase treatment of the membranes (data not shown). These data are consistent with those from a study by Morales et al. (29), which showed that NHERF1 could be recognized as a migrating triplet. The effects of silencing NHERF1 on NpT2a trafficking, were analyzed by confocal microscopy. In control (mock transfected) cells and in cells transfected with nonspecific siRNA, the GFP-NpT2a signal was predominantly present in the apical membrane of the OK cell (Fig. 6B, left and middle). In OK cells transfected with NHERF1 siRNA, the GFP-NPT2a signal was present in an intracellular compartment with very little of the signal present in the apical membrane (Fig. 6B, right). These data are consistent with the conclusion that the absence of NHERF1 impairs the proper trafficking of NpT2a to the apical membrane of the cell.

Molecular determinant for the NpT2a/NHERF1 interaction. To identify the molecular determinant responsible for the NpT2a/NHERF1 interaction in the ER/Golgi, we transiently transfected cells with GFP-tagged full-length or PDZ-binding domain-deleted (COOH-terminal TRL) NpT2a at 37°C. Crude membranes were analyzed for the expression of tagged proteins using anti-GFP antibodies or antibodies against the COOH-terminal domain of NpT2a. As shown in Fig. 7A, full-length NpT2a was expressed as a broad band of ~120–140 kDa in the crude membrane fraction, as detected with anti-GFP antibodies, but not in the cytosolic fraction. This pattern is consistent with the typical membrane-delimited expression seen with the native, fully glycosylated form of NpT2a. In contrast, NpT2a lacking the COOH-terminal TRL motif was detected at 75 kDa, corresponding to the size of the unglycosylated or partially glycosylated protein and enriched in the cytosolic fraction (Fig. 7, A and B). As expected, this protein was detected only with antibodies against GFP but not with antibodies made against the COOH-terminal domain of NpT2a.

Immunoprecipitation with GFP antibody in cells expressing full-length GFP-tagged NpT2a protein demonstrated a positive association with NHERF1, whereas GFP antibody immunoprecipitation in cells expressing the TRL-deleted protein failed to detect NHERF1 (Fig. 7A, bottom). These data suggest that an association with NHERF1 is critical for glycosylation and forward trafficking of NpT2a into the membrane fraction. To confirm the Western blot data, OK cells transiently transfected with either GFP-tagged wild-type or TRL-deleted NpT2a were
analyzed by confocal microscopy. As shown in Fig. 7C, GFP-tagged full-length NpT2a was expressed in the plasma membrane, whereas TRL-deleted NpT2a was localized in the cytosolic perinuclear region.

DISCUSSION

The major finding from the present study is the demonstration that the NHERF1/NpT2a interaction occurs at the level of the ER/Golgi and appears to be critical for efficient apical membrane trafficking of NpT2a. The molecular determinant on NpT2a for this interaction is the COOH-terminal TRL canonical PDZ class I-binding motif. Deletion of this motif resulted in wide-ranging consequences, including failure to traffic to the apical membrane, failure to localize to a membrane fraction, and failure to undergo mature glycosylation. Our results suggest that the apical membrane localization sequence of NpT2a is, in fact, the COOH-terminal PDZ-binding motif.

Evidence to support a role for NHERF1 in the apical membrane trafficking of NpT2a includes the following. When grown at 16°C, cells grown in low-phosphate media after having endogenous NpT2a expression abolished showed increased NHERF1 expression in the ER/Golgi and diminished apical membrane NpT2a expression. This finding suggests that NHERF1 and NpT2a expression and apical membrane trafficking are costimulated by exposure to low-phosphate medium. Western blots of NpT2a immunoprecipitated from ER/Golgi fractions stain positively for NHERF1, suggesting association of the two proteins in the ER/Golgi, as has been demonstrated in the apical membrane. Immunoprecipitation of NpT2a lacking the COOH-terminal PDZ-binding domain does not demonstrate NHERF1 association in the ER/Golgi fraction. The COOH-terminal-deficient NpT2a construct fails to localize to the apical membrane and shows a marked increase in cytosolic expression as opposed to the usually exclusive membrane expression. These findings serve to reinforce the critical role of the NpT2a COOH-terminal PDZ-binding motif in the trafficking of NpT2a. While the immunoprecipitation experiments are consistent with the hypothesis that NpT2a interacts directly with NHERF1 through the TRL motif, the data do not allow us to state categorically that the NHERF1/NpT2a interaction is direct. Interestingly, however, the higher NpT2a-to-NHERF1 ratio seen after low phosphate (Fig. 3) treatment suggests the possibility that the nature of the NpT2a/NHERF1 association changes with this stimulus, allowing a greater NpT2a-binding capacity.

The increased cytosolic expression of NpT2a relative to the membrane expression when the interaction with NHERF1 is prevented suggests the possibility that NHERF1 may play a chaperone role, facilitating the folding of NpT2a into the membrane-bound conformation at the time of synthesis. Because the PDZ-binding domain of NpT2a is located at the COOH-terminal, the inability of the TRL-deleted mutant form of NpT2a to localize to the membrane fraction cannot

![Figure 6](http://ajprenal.physiology.org/)

**Fig. 6. Trafficking of GFP-NpT2a in OK cells transfected with NHERF1 small interfering (siRNA).** OK cells were transfected with nonspecific siRNA or NHERF1 siRNA using Lipofectamine RNAiMAX per the manufacturer's protocol after transfection of GFP-NpT2a. Cells were placed in a humidified incubator at 37°C and 5% CO2. Live cells were imaged 24 or 48 h after siRNA transfection using confocal microscopy. **A:** representative NHERF1 blot after transfection with NHERF1 siRNA. The arrow indicates the main NHERF1 band. **B:** confocal microscopy pictures from a representative experiment. **Left:** GFP-NpT2a wild type (WT); **middle:** GFP-NpT2a in cells transfected with nonspecific siRNA; **right:** GFP-NpT2a in NHERF1 siRNA-transfected cells. All images have overlay of bright field with GFP-NpT2a in the insets.
be attributed to the loss of a critical transmembrane region. This would represent a novel function for NHERF1.

Proximal tubule cells express several PDZ proteins that have been shown to interact with NpT2a, including PDZ protein interacting specifically with TC10 (PIST), PDZ domain containing 1 (PDZK1), and SH3 and multiple ankyrin repeat domains 2E (SHANK2E). Furthermore, PDZ proteins may interact with each other. These observations raise the possibility that additional PDZ proteins may be contributing to the forward trafficking of NpT2a. Preliminary data (not shown) suggest that PDZK1, another member of the NHERF family, is not likely to be involved as its expression does not change in ER/Golgi or plasma membrane fractions with changes in NpT2a expression due to low phosphate treatment. However, NpT2a interacts with PIST at the level of the trans-Golgi during retrograde trafficking, so it is plausible that this interaction may also play a role during forward trafficking (21). SHANK2E also interacts with NpT2a and traffics in response to changes in ambient phosphate, though, again, the role appears to be in retrograde trafficking (4). Further studies are required to determine the role of PIST or SHANK2E in NpT2a forward trafficking. Alternatively, another potential function for NHERF1 is to assemble the machinery required for NpT2a insertion into the membrane, including glycosylation enzymes. A previous study (9) has shown that glycosylation is important for apical membrane localization, although not for the transport function of NpT2a. The glycosylation process has not been investigated in depth for NpT2a but presumably is similar to what has been described for a number of other transporters, including urea transporter (UT-A1) (1), glucose transporter 2 (30), and CFTR (7).

Fig. 7. Role of the NpT2a PDZ-binding motif in the association of NpT2a with NHERF1, glycosylation, and forward trafficking. A: cells were grown to 60% confluence and transiently transfected with vector, GFP-NpT2a WT, or COOH-terminal TRL deletion mutant. A: expression of GFP-NpT2a in cell membranes (top), cell lysates (middle), and the cell cytosol (bottom) was determined by WB analysis using GFP antibodies. GFP-NpT2a was immunoprecipitated using polyclonal rabbit anti-GFP antibodies. Immunoprecipitated proteins were separated by 10% SDS-PAGE and analyzed by WB analysis for NHERF1 expression using monoclonal mouse-anti NHERF1 antibodies (bottom). The NHERF1 band is indicated by the arrow; the bottom band is IgG. A representative blot from three independent experiments is shown (n = 3). B: GFP-NpT2a was immunoprecipitated from WT OK cells and treated with F-glycosidase. A representative immunoblot for NpT2a is shown (n = 3). C: cells grown on confocal plates were transiently transfected with GFP-NpT2a WT (left) or COOH-terminal TRL deletion mutant (right) and visualized by confocal microscopy. A representative confocal image from four individual experiments is shown (n = 4).
NHERF1 suggests that these processes are interrelated. However, whether glycosylation is required for membrane insertion of NpT2a or vice versa is not clear. The NHERF1 knockout mouse shows apical membrane expression of NpT2a, albeit at a markedly reduced level that is not increased by administration of a low-phosphate diet. Whether the existing NpT2a proteins have undergone appropriate posttranslational modifications, including glycosylation, has not been explicitly studied. It has been postulated that NHERF2 assumes a role in ensuring the limited apical membrane expression of NpT2a seen in the NHERF1 knockout mice; however, an in vivo NHERF2-NpT2a interaction has not been demonstrated.

 Trafficking of many transporters is dependent on interactions with accessory proteins. As NHERF1 has a well-documented role in assembling signaling complexes at the apical membrane (2, 6, 19, 25, 28), NHERF1 could play a similar role at the level of the ER/Golgi to assemble accessory proteins critical for the proper folding and membrane insertion of NpT2a. NpT2a is known to interact with multiple proteins at the cell surface, including NHERF1, ezrin, the PTH receptor, and PDZK1. Whether these interactions exist before membrane insertion has not been studied up to this point. Assembly of protein complexes before insertion into the apical membrane has been previously described for the β-adrenergic receptor (5) and other protein complexes (26). As NHERF1 has a documented role as a scaffolding protein at the apical membrane, postulation of a similar role at the level of the ER or Golgi is highly plausible and may be a likely mechanism by which NHERF1 helps ensure appropriate NpT2a trafficking to the apical membrane. Although primarily known as a plasma membrane protein, NHERF1 has been previously identified in intracellular cell compartments (32, 44). Sato et al. (32) demonstrated that NHERF1 interacts with the glutamate aspartate transporter (GLAST) at the level of the ER, facilitating its transfer to the Golgi. Similar to what was seen with NHERF1 and NpT2a, deletion of the PDZ-binding domain of GLAST resulted in increased intracellular expression of GLAST and impaired trafficking to the apical membrane.

The regulation of NHERF1 trafficking itself has not been studied extensively (42). Our data demonstrate evidence for initial and transient NHERF1 trafficking from the plasma membrane to Golgi in response to low phosphate, suggesting the possibility that the initial role of NHERF1 in enhancing apical NpT2a expression is to facilitate the forward transport of NpT2a at the Golgi, whereas the more chronic role is anchor NpT2a into the apical membrane. This hypothesis is supported by TIRF imaging coupled with the MSD analysis, which suggests that NHERF1 exhibits an early directed movement out of the plasma membrane in response to low phosphate in contrast to NpT2a, which exhibits purely diffusive movement.

In summary, we have identified a novel site of an interaction between NpT2a and NHERF1 in an intracellular compartment before apical membrane insertion of NpT2a. This interaction is critical for appropriate posttranslational processing of NpT2a, including mature glycosylation and integration into a membrane compartment. These findings suggest that NHERF1 plays an essential role in the apical membrane trafficking of NpT2a through a role either as a chaperone, a scaffolding protein, or both.

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