ORIGINAL

# Role of serine 249 of ezrin in the regulation of sodium-dependent phosphate transporter NaPi-IIa activity in renal proximal tubular cells

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**Abstract : Type IIa sodium-dependent phosphate transporter (NaPi-IIa) is responsible for renal phosphate reabsorption and maintenance of systemic phosphate homeostasis in mammals. Macromolecular complex formation of NaPi-IIa with sodium-proton exchanger related factor-1 (NHERF-1) and ezrin is important for apical membrane localization in the proximal tubular cells. Here, we investigated the interactions of the ezrin phosphomimetic mutation of serine to aspartic acid at 249 with NHERF-1 and the inhibition of apical membrane localization of NaPi-IIa. *In vitro* phosphorylation analysis revealed that serine 249 of human ezrin serves as a phosphorylation site for protein kinase A. The N-terminal half of ezrin had a dominant negative effect on the phosphate transport activity and inhibited the apical localization of NaPi-IIa in renal proximal tubular cells. We found that the phosphomimetic S249D mutant interfered with the inhibitory effects of the dominant negative mutant on the transport and localization of NaPi-IIa. The S249D mutant also inhibited the interaction with NHERF-1. Therefore, serine 249 of ezrin can play important roles in the regulation of the complex formation and membrane localization of NaPi-IIa. J. Med. Invest. 60 : 27-34, February, 2013**

***Keywords*** **:** *ERM family, ezrin, parathyroid hormone, phosphate homeostasis, sodium-dependent phosphate transporter, sodium-proton exchanger related factors*

## INTRODUCTION

In mammals, homeostasis of inorganic phosphate (P) in the blood is mainly maintained by the regulation of renal P reabsorption (1). Type IIa sodium-dependent P transporter (NaPi-IIa) is a multi-trans membrane protein and plays a critical role in the rate-limiting step of renal P reabsorption (2). The P transport activity in the proximal tubule is dependent on the amount of NaPi-IIa in the apical plasma membrane (2). Generally, the amount of trans-membrane protein in apical plasma membrane is controlled by the balance of exocytotic/endocytotic protein transport rate and retention time in the plasma membrane. Most of the P regulating factors, such as parathyroid hormone

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(PTH) and fibroblast growth factor 23 (FGF23), are thought to affect the regulation of NaPi-IIa activity (3, 4). However, the detailed mechanisms have not yet been clarified.

Studies in the past decade have elucidated the mechanism for the apical localization of NaPi-IIa (5). NaPi-IIa can form a macromolecular complex on the apical plasma membrane via NHERFs (sodium-proton exchanger related factors), ERM (ezrin/radixin/moesin) family proteins, and actin cytoskeleton (5). NaPi-IIa can bind to the first PDZ domain of NHERF-1 via the TRL-motif in the C-terminus (6). The C-terminal region of NHERF-1 can bind to the N-terminal FERM domain of ERM proteins (7). Then, the C-terminal domain of ERM proteins can bind to the actin cytoskeleton (8). Macromolecular complexes such as this are important in the determination of the apical localization of NaPi-IIa (5). In addition, NaPi-IIa can be localized in specialized membrane microstructures, such as clathrin-coated pits (9), and/or low-density membrane microdomains (LDM), including lipid rafts/caveolae in the apical plasma membrane (10-12). Ezrin also plays an important role in the localization of NaPi-IIa in the LDM (10).

NHERF-1 plays an important role in the regulation of apical localization of NaPi-IIa. PTH-stimulated phosphorylation of serine 77 of NHERF-1 through protein kinase C (PKC) results in the dissociation of the interaction between NaPi-IIa and NHERF-1 (13, 14). The PTH-mediated down-regulation of NaPi-IIa was disrupted in NHERF-1 deficient mice (15), and apical localization of NaPi-IIa can remain intact, even in NHERF-1 deficient mice (15). In contrast, ezrin, which is another important molecule for the apical localization of NaPi-IIa, regulates the apical localization of NaPi-IIa in the membrane microdomains and NaPi-IIa activity. PTH may also phosphorylate ezrin by protein kinase A (PKA) as well as protein kinase C (PKC), affecting the formation of the NaPi-IIa complex in the apical plasma membrane (10). Overexpression of dominant negative ezrin (N-terminal half of ezrin) inhibits the apical localization of NaPi-IIa and P transport activity in the renal proximal tubular cells (10, 16). These results suggest that modification of the N-terminal half of ezrin by phosphorylation is mechanistically important. In this study, we investigated the role of the phosphorylation of ezrin in apical membrane localization of NaPi-IIa in renal proximal tubular cells.

## MATERIALS AND METHODS

### *Expression and purification of recombinant human ezrin in Escherichia coli*

Complementary DNA (cDNA) fragments of human ezrin were amplified by polymerase chain reaction (PCR) using a high fidelity PCR system (Roche Japan, Tokyo, Japan) with the specific oligonucleotide primers listed in Table 1. The PCR-amplified cDNA fragment corresponding to the N-terminal region of human ezrin (amino acids, aa, 2-348) was inserted into the pET30a vector (Merck Chemicals Japan, Tokyo, Japan). The constructed expression vector was transformed into single step (KRX) competent cells (Promega, Madison, WI). The 6xHis-tagged recombinant protein produced by *E. coli* in LB medium containing 20% rhamnose at 15-25°C overnight was purified with TALON Metal Affinity Resins according to the manufacturer's

Table. 1 Oligonucleotide primer sequences for amplifying human ezrin cDNA and site-directed mutagenesis

| | |
|---|---|
| Full length ezrin | Sense : 5'-GCT CTA GAC CGA AAA TGC CGA AAC CAA TC-3' |
| | Antisense : 5'-TCG GGC CCA AGC TTC AGG GCC TCG AAC TC-3' |
| N-terminal half ezrin | Sense : 5'-CTT GAC GCG TTG ATA TCA ACT AGT CCG AAA CCA ATC AAT-3' |
| | Antisense : 5'-GAC ATT GAT TGG TTT CGG ACT TGA TAT CAA CGC GTC AAG-3' |
| For T567D | Sense : 5'-CCG GGA CAA GTA CAA G**GA C**CT GCG GCA GAT CCG GC-3' |
| | Antisense : 5'-GCC GGA TCT GCC GCA G**GT C**CT TGT ACT TGT CCC GG-3' |
| For S149D | Sense : 5'-CTG GGT ACC TCA GC**G AT**G AGC GGC TGA TCC C-3' |
| | Antisense : 5'-GGG ATC AGC CGC TC**A TC**G CTG AGG TAC CCA G-3' |
| For S249D | Sense : 5'-GTC AAA TCA GGA ACA TC**G AT**T TCA ATG ACA AAA AGT T-3' |
| | Antisense : 5'-AAC TTT TTG TCA TTG AA**A TC**G ATG TTC CTG ATT TCA C-3' |

Bold letter shows substituted nucleotide. Underline shows codon corresponded to substituted amino acid.

instructions (GE Healthcare Japan, Tokyo, Japan).

*SDS-polyacryl amide gel electrophoresis and western blot analysis*

Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and were subjected to either Coomassie Brilliant Blue (CBB) staining or western blot analysis. For western blot analysis, the separated proteins were transferred to a polyvinylidine difluoride (PVDF) membrane (Millipore Japan, Tokyo, Japan), and blotted for NaPi-IIa. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL Plus, GE Healthcare Japan) and were analyzed with an LAS-3000mini lumino-image analyzer (Fujifilm, Tokyo, Japan).

*In vitro phosphorylation assay*

*In vitro* phosphorylation with PKA was performed using the purified recombinant N-terminal half of ezrin, or synthetic peptides containing putative phosphorylation sites S149 (aa, 145-154, GYLSSERLIP) and S249 (aa, 244-253, EIRNISFNDK), and their alanine-substituted mutants S149A (GYLSAERLIP) and S249A (EIRNIAFNDK). Each protein or peptides was incubated in reaction buffer for the PKA assay (50 mM Tris, 10 mM $MgCl_2$, 1 mM ATP, pH 7.5), containing [$\gamma$-$^{32}$P]ATP, (30 MBq/ml, MP Biomedicals Japan, Tokyo, Japan), and PKA catalytic subunit (New England Bio Labs, Ipswich, MA) at 30°C for 30 min. Using recombinant protein, the reaction mixture was separated by SDS-PAGE and radiolabeled proteins were detected by an imaging analyzer (BAS-3000, Fuji Film). Using synthetic peptide, the reaction mixture was absorbed onto a P81 phosphocellulose filter (Whatman Japan, Tokyo, Japan) and was washed out and counted using a liquid scintillation counter.

*Cell culture, expression vector construction for mammalian cells, and transfection*

Opossum kidney cells (OK-P cells) were obtained from kidney tissues provided by Dr. J. Cole. OK-P cells and COS-7 cells were maintained in plastic culture dishes in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Japan, Tokyo, Japan) containing 25 mmol/L HEPES, 4500 mg/mL glucose, 1 mmol/L sodium pyruvate, 50 μg/mL penicillin, 50 IU/mL streptomycin, and 10% (vol/vol) fetal bovine serum (FBS), all from Sigma-Aldrich Japan (Tokyo, Japan) at 37°C, in a humidified atmosphere of 5% $CO_2$. GFP-human NaPi-IIa expression vector was used as previously reported (17). Each cDNA of the full-length of ezrin and N-terminal half of ezrin (dominant negative ezrin ; Ezrin-DN) was subcloned into pDsRed2 plasmid vector (Takara Bio Inc, Otsu, Japan) and expressed in OK-P cells as fusion protein with DsRed. Substitution of aspartic acid for serine or threonine was performed by site-directed mutagenesis using QuikChange site-direct mutagenesis kit (Agilent Technologies Japan, Tokyo, Japan) with the specific oligonucleotide primers listed in Table 1. Transfection was carried out using Lipofectamine 2000 reagent (Life Technologies Japan) as previously described (10).

*P uptake assay*

P uptake assay was measured in OK-P cells grown to confluency in 24-well plastic plates as previously described (10). Transport rate was expressed as nmol P per mg protein per minute.

*Fluorescence microscopy analysis*

OK-P cells transfected with expression vectors for GFP-human NaPi-IIa and DsRed-dominant negative ezrin with or without S249D mutation were grown on cover slips. Then, the cells were fixed with 3% (wt/vol) paraformaldehyde for 30 min on ice. The cover slips were mounted on glass slides and analyzed under a Leica Confocal Microscope (TCS-LS, Leica Microsystems GmbH, Mannheim, Germany) in the sequential mode. The x and y plane images (512 x 512 pixels per image) were obtained in the x, y, and z scan mode, and 18 z section images were taken per region of interest. The top 1 to 4 section images were projected onto one xy image.

*Cell fractionation*

OK-P cells were transfected with expression vectors for GFP-NaPi-IIa and constitutive active ezrin (Ezrin-CA ; full-length ezrin with phosphomimetic mutation at T567D), or constitutive active ezrin with the S249D mutation (Ezrin-CA/S249D). Mutagenesis of the expression vectors was performed as described above. The non-detergent method (18) for isolating LDM from OK-P cells was employed as previously described (10). Sonicated plasma membranes were subjected to OptiPrep gradient centrifugation twice. The opaque band just above the 5% interface after the second OptiPrep gradient centrifugation was collected and designated as the LDM fraction.

*Mammalian two-hybrid analysis*

The mammalian two-hybrid assay system (Promega) was employed to analyze the effect of amino acid substitution of FERM-domain of human ezrin on the interaction with C-terminal NHERF-1. Rat C-terminal NHERF-1 cDNA (corresponding to aa 272-356) was inserted into a bait pBIND vector containing the GAL4 DNA binding domain (pBIND-NHERF-1). The N-terminal half of human ezrin cDNA (corresponding to aa 2-348) was inserted into a prey pAct vector containing the VP16 transcriptional activation region (pAct-ezrin). Site-directed mutagenesis of pAct-ezrin S149D and pAct-ezrin S249D was generated from the pAct-ezrin, as previously described above. Vectors for bait and prey, luciferase reporter vector (pG5luc), and control vector (pCMVβ) for expression of β-galactosidase were cotransfected into COS-7 cells with the Lipofectamin 2000 reagent. After 24-h transfection, cells were lysed and measured for both luciferase and β-galactosidase activity. Interaction was evaluated by luciferase activity and the data were normalized by β-galactosidase activity.

*Determination of protein concentration*

Protein concentration was determined using the Bio-Rad Bradford assay (Bio-Rad Japan, Tokyo, Japan) with bovine serum albumin as a standard.

*Statistical analysis*

All values from more than triplicate experiments are expressed as mean ± SE. Data from less than duplicate data are expressed as mean. The significance of differences was assessed between groups using analysis of variance (ANOVA) with post-hoc analyses by Fischer's protected least significant difference test, when the available sample size was more than 3. Differences were considered significant at $p < 0.05$. Statistical analysis was performed by StatView (version 5.0-J for Windows, SAS Institute, Inc., Cary, NC).

## RESULTS AND DISCUSSION

*Investigation of ezrin of phosphorylation site by PKA*

The N-terminal half of ezrin contains a three-repeated structure called "FERM domain". In particular, the third FERM domain (FERM subdomain-C ; aa 200-292) is capable of binding to NHERF-1 (19, 20). Therefore, we hypothesized that this region could interact with the components of the NaPi-IIa macromolecular complex, such as NHERF-1, and phosphorylation of this domain may regulate the interaction between ezrin and other components. Our previous study suggested that ezrin is phosphorylated by PKA or PKC *in vivo* and has several candidate phosphorylation sites (T11, S249, and T332 for PKA, T11, T25, T98, T148, and S149 for PKC) in the N-terminal region (10) ; however, of these candidates, only FERM subdomain-C contains S249. Therefore, we focused on S249 as a candidate phosphorylation site. First, we investigated whether PKA can phosphorylate S249 of ezrin *in vitro*. As shown in Fig. 1A, PKA phosphorylated the wild-type recombinant N-terminal half of ezrin. In addition, we confirmed the phosphorylation of S249 by *in vitro* phosphorylation assay using synthetic peptides. As shown in Fig. 1B, PKA phosphorylated the wild-type peptide but not the mutant peptide

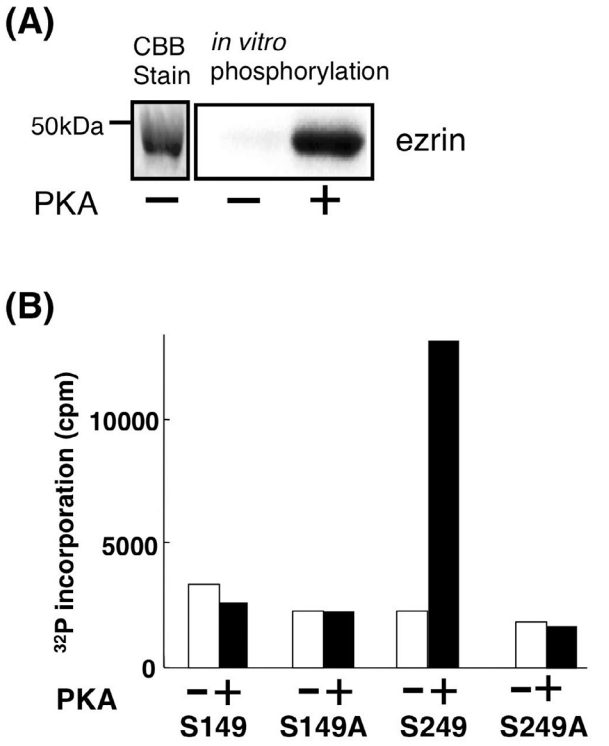


Fig. 1 Ezrin can be phosphorylated by PKA *in vitro*. (A) Purified recombinant His-tagged N-terminal half of human ezrin was purified and confirmed by SDS-PAGE with CBB staining (left). The purified protein was incubated in the presence (+) or absence (-) of PKA with [γ-$^{32}$P] ATP for 30 min at 30°C. Reaction products were separated by SDS-PAGE and visualized by autoradiography. (B) *In vitro* phosphorylation analysis of putative phosphorylation site of ezrin. Each peptide (S149, S149A, S249, and S249A) was phosphorylated in the presence (+) or absence (-) of PKA with [γ-$^{32}$P] ATP for 30 min at 30°C. Radiolabelled activity was measured using a liquid scintillation counter. Data are mean of duplicate experiments. Representative data from two independent duplicate experiments are shown.

S249A. Furthermore, the negative control peptide, S149, and S149A were also not phosphorylated. These results suggest that the ezrin S249 can be phosphorylated by PKA.

*Effect of phosphorylation of S249 of ezrin on P transport activity and the localization of NaPi-IIa using phosphomimetic mutants*

To investigate the role of phosphorylation of ezrin S249 on the apical localization of NaPi-IIa, we examined the effects of the phosphomimetic ezrin S249 mutant (S249D) on the P transport activity and apical localization of NaPi-IIa in OK-P cells. As previously reported, the N-terminal half of ezrin has dominant negative effect on the ezrin function that is essential for the transport activity of NaPi-IIa in OK-P cells (10). Therefore, we tested whether phosphomimetic mutation S249D can inhibit the dominant negative effect. As shown in Fig. 2, the expression of the phosphomimetic S249D mutant of Ezrin-DN (Ezrin-DN/S249D) did not decrease the P transport activity, while the Ezrin-DN diminished the P transport activity.

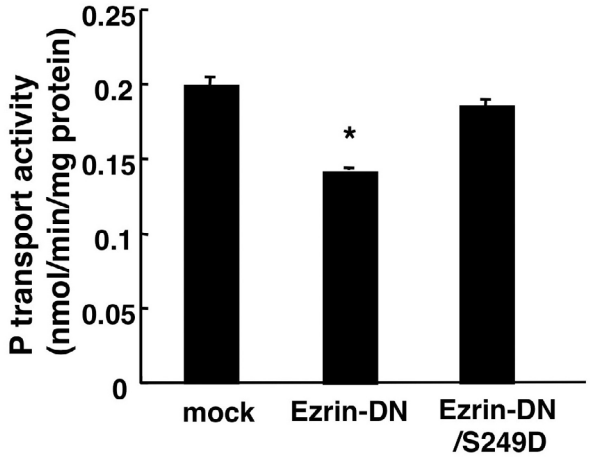


Fig. 2 Effects of phosphomimetic mutants of dominant-negative ezrin on P transport activity in OK-P cells. OK-P cells were transfected with plasmid vectors expressing GFP-human NaPi-IIa and expressing Ezrin-DN, its phosphomimetic mutant (Ezrin-DN/S249D) or mock vector (pDsRed2 ; mock) with DsRed. P transport activity was expressed as nmol/min/mg protein. Values are mean ± SE (n=4). *P< 0.05 vs. mock.

Next, we investigated the effect of the phosphomimetic mutation of Ezrin-DN on the subcellular localization of NaPi-IIa in OK-P cells. Expression of Ezrin-DN inhibited the localization of GFP-fused NaPi-IIa on the apical surface in OK-P cells (Fig. 3A). On the other hand, expression of Ezrin-DN/S249D did not inhibit the localization of NaPi-IIa

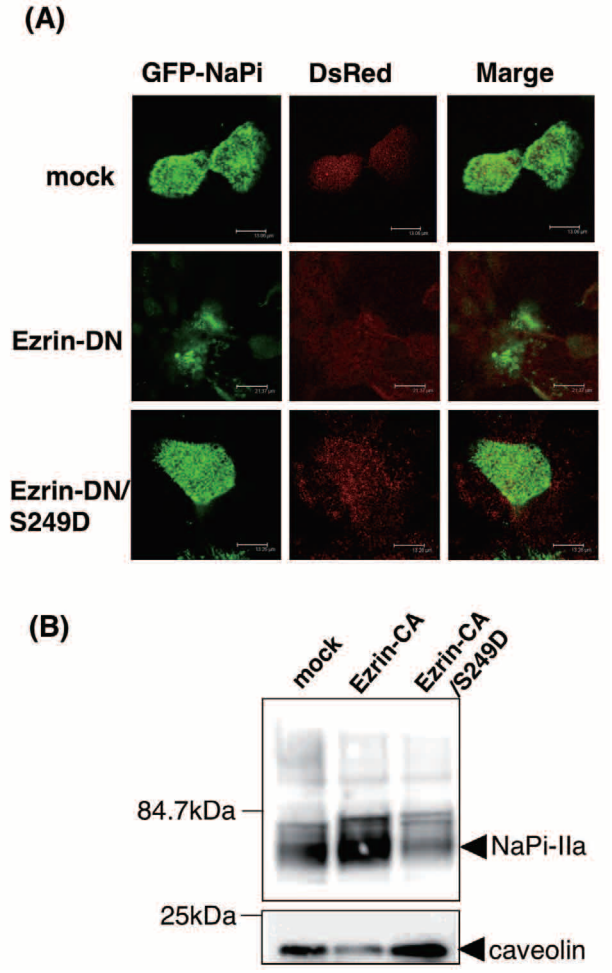


Fig. 3 Effects of phosphomimetic mutants of dominant negative ezrin on NaPi-IIa localization in OK-P cells. (A) Fluorescence microscopy analysis. OK-P cells were transfected with plasmid vectors expressing GFP-human NaPi-IIa and expressing Ezrin-DN, its phosphomimetic mutant (Ezrin-DN/S249D) or mock vector (pDsRed2 ; mock) with DsRed. The transfected cells were transferred onto coverslips, fixed and mounted on glass slides. Images were taken by confocal laser-scanning microscopy. The top 1-4 z section images (apical surface images) were projected onto one x-y plane image as shown above. Representative data from 3 to 5 independent experiments are shown. (B) Cell fractionation analysis. OK-P cells were transfected with plasmid vectors expressing GFP-human NaPi-IIa and expressing Ezrin-CA (with T567D mutation) or Ezrin-CA/S249D. Fractionated LDM were subjected to western blot analysis with anti-NaPi-IIa antibody and anti-caveolin antibody for internal control. Representative data from 2 independent experiments are shown.

(Fig. 3A). In addition, we also investigated the effect of phosphomimetic mutation S249D in Ezrin-CA (with T567D mutation) on the localization of NaPi-IIa in the LDM. Expression of Ezrin-CA enhanced the amount of NaPi-IIa in the LDM as reported by Mahon (16) (Fig. 3B). However, Ezrin-CA/S249D did not enhance the localization of NaPi-IIa in the LDM fraction (Fig. 3B).

These data suggest that ezrin plays an important role in the apical localization of NaPi-IIa in renal

proximal tubular cells and that phosphorylation of S249 can inhibit the apical localization of NaPi-IIa. We considered that Ezrin-DN can bind to NHERF-1 but cannot tether the NaPi-IIa/NHERF-1 complex to cytoskeletal actin, resulting in the decrement of the apical amount of NaPi-IIa. Since the S249D mutant of Ezrin-DN cannot bind to both NHERF-1 and actin, the dominant negative effect was suppressed. On the other hand, Ezrin-CA/S249D can bind to actin but not to NHERF-1 ; therefore, the mutant inhibited the increment of NaPi-IIa in the LDM fraction. To confirm our interpretation, we examined the effect of phosphorylation of ezrin on the interactions with binding partners, NHERF-1.

*Effect of phosphorylation of ezrin on interaction between ezrin and NHERF-1*

Ezrin is a linker protein between NHERF-1 and cytoskeletal actin in the NaPi-IIa macromolecular complex. To investigate the role of phosphorylation of ezrin S249 in the binding to NHERF-1, we examined whether the phosphomimetic mutant of ezrin can inhibit the interaction between NHERF-1 by mammalian two-hybrid assay. Co-expression of the wild-type of N-terminal part of ezrin fused to VP16 transcription activation region as a prey, and the C-terminal part of NHERF-1 fused to GAL4 DNA binding domain as a bait significantly increased the luciferase activity, indicating that the N-terminal part of ezrin can bind to the C-terminal part of NHERF-1 (Fig. 4). S249D mutant of the N-terminal part of ezrin as the prey clearly inhibited the luciferase activity, indicating that S249D mutant inhibited the interaction with NHERF-1. In contrast, S149D mutant was not inhibited to bind to NHERF-1. Our data suggest that phosphorylation of ezrin S249 may inhibit the interaction between ezrin and NHERF-1, resulting in decreased apical localization of NaPi-IIa and P transport activity.

It is unknown how the modification of S249 affects the interaction with NHERF-1. S249 is highly conserved within the ERM family and is located in the β5C strand in the FERM subdomain C of ezrin, which is important for binding to NHERF-1 (20). NHERF-1 binds to the groove between two β sheets in the FERM subdomain C, the four-stranded sheet β1C-β4C (aa 203-240) and the three-stranded sheet β5C-β7C (aa 243-270). Phe240, Ile257, Pro265, and Pro259 form a hydrophobic pocket which accommodates Trp348 from the NHERF-1 peptide (20). Substitution of aspartic acid for serine in the FERM subdomain C may affect the hydrophobicity of the

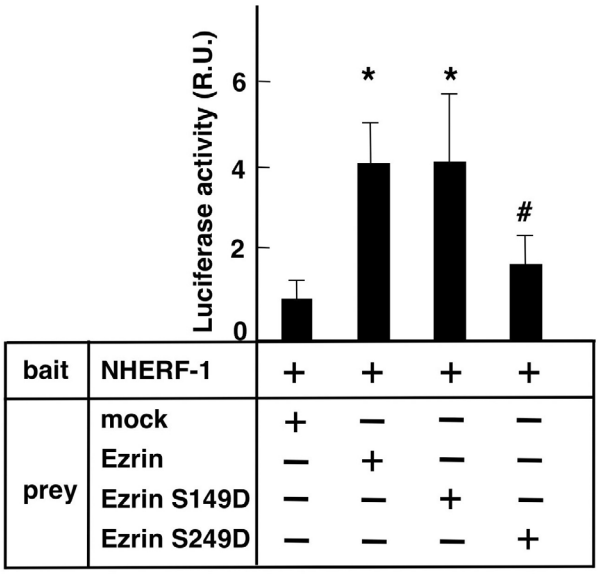


Fig. 4 Effect of phosphomimetic mutants of ezrin on the interaction with NHERF-1 C-terminal domain by mammalian two-hybrid assay. Indicated bait (NHERF-1) and prey (ezrin or its mutant) vectors, luciferase reporter vector, and β-galactosidase expression vector were co-transfected into COS-7 cells. Luciferase activity and β-galactosidase activity were measured. Luciferase activity was normalized against β-galactosidase activity, and luciferase activity of NHERF-1/mock was set to 1. Data are mean ± SEM (n=6-8). *$p < 0.05$ vs. NHERF-1/mock, #$p < 0.05$ vs. NHERF-1/Ezrin.

pocket or be involved in steric hindrance. Further investigation of structural basis analysis is required.

In summary, we investigated the role of serine 249 of ezrin in the formation of NaPi-IIa complex in the plasma membrane of renal proximal tubular cells. As illustrated in Fig. 5A, NaPi-IIa can be localized in the apical plasma membrane by tethering to cytoskeletal actin via formation of the complex with NHERF-1 and ezrin. Overexpression of ezrin-DN can interfere the linking activity of endogenous ezrin between NHERF-1 and actin as we described above (Fig. 5B). This dominant negative effect of ezrin can affect the apical localization of NaPi-IIa, and somehow internalize NaPi-IIa from apical membrane by stimulating endocytosis as previously reported (9, 10, 16). Phosphomimetic mutant of ezrin-DN (ezrin-DN/S249D) did not have dominant negative function (Fig. 2 and Fig. 3A), suggesting that ezrin-DN/S249D does not interfere the linking activity of endogenous ezrin as shown in Fig. 5C.

Our previous study proposed a hypothesis that phosphorylation of ezrin can regulate the apical localization of NaPi-IIa in renal proximal tubular cells (10). The findings of the present study support this hypothesis because modification of the FERM subdomain C of ezrin was shown to regulate the

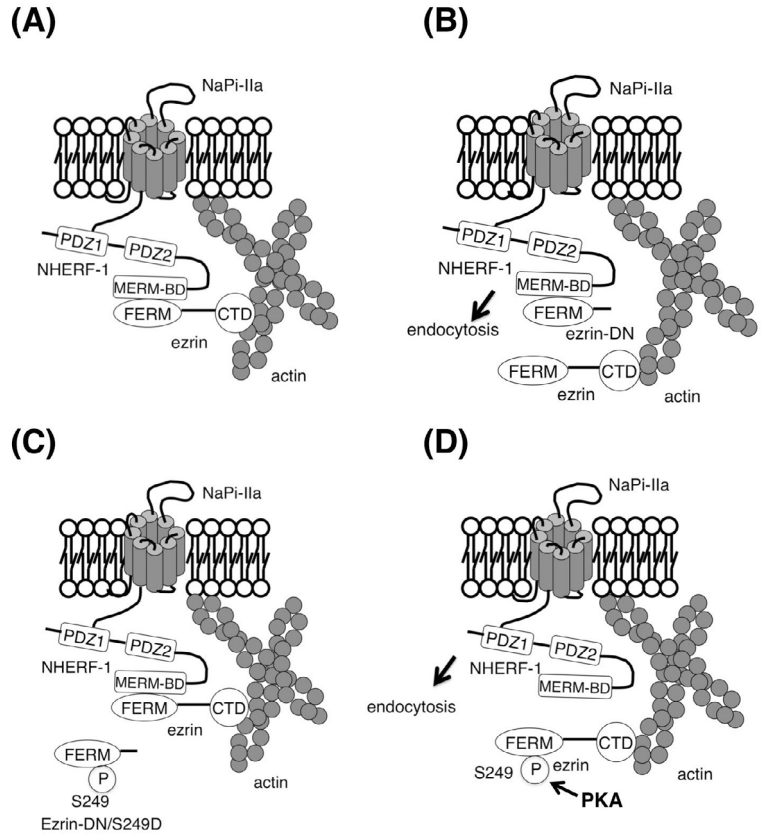


Fig. 5 Schematic diagrams of the role of ezrin and its mutants in the formation of NaPi-IIa complex. (A) NaPi-IIa can be localized in the apical membrane by tethering to cytoskeletal actin via formation of complex with NHERF-1, ezrin. (B) Expression of dominant negative ezrin (ezrin-DN ; N-terminal half of ezrin (2-348)) inhibit the binding between NHERF-1 and actin, resulting that untethered NaPi-IIa complex could be internalized by endocytosis. (C) Expression of dominant negative ezrin with phosphomimetic mutation (ezrin-DN/S249D) does not affect the formation of NaPi-IIa complex. (D) Speculative schema of role of S249 of ezrin *in vivo*. Phosphorylation of ezrin at S249 by PKA would inhibit the binding to NHERF-1, resulting that untethered NaPi-IIa complex could be internalized by endocytosis *in vivo*. PDZ ; PDZ domain, MERM-BD ; merlin-ezrin-radixin-moesin-binding domain, FERM ; FERM domain, CTD ; C-terminal domain, PKA ; protein kinase A.

interaction with NHERF-1, apical localization of NaPi-IIa, and P transport activity, at least *in vitro*. Therefore, those data suggest that phosphorylation of S249 of ezrin by PKA activated in response to hormonal stimulation or others could inhibit the tethering the NaPi-IIa complex to actin by inhibiting interaction with NHERF-1, decrease the amount of NaPi-IIa in the apical membrane by endocytosis (Fig. 5D), and eventually decrease the P reabsorption activity *in vivo*. However, we cannot clearly assess the importance of phosphorylated modification of ezrin in hormonal regulation of NaPi-IIa *in vivo* at this time. Recently, Nagai *et al.* reported that the cAMP/PKA pathway mainly down-regulates NaPi-IIa in response to PTH *in vivo* (21). Our data agree with the importance of the cAMP/PKA pathway in the regulation of NaPi-IIa activity. The regulation of NaPi-IIa via the modification of ezrin are important to understand the molecular mechanisms for the regulation of phosphate homeostasis.

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