

**ORIGINAL****Analysis of different complexes of type IIa sodium-dependent phosphate transporter in rat renal cortex using blue-native polyacrylamide gel electrophoresis**

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**Abstract :** Type IIa sodium-dependent phosphate transporter (NaPi-IIa) can be localized in the apical plasma membrane of renal proximal tubule to carry out a rate-limiting step of phosphate reabsorption. For the apical localization, NaPi-IIa is required to form a macromolecular complex with some adaptor proteins such as Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor 1 (NHERF-1) and ezrin. However, the detail of macromolecular complex containing NaPi-IIa in the apical membrane of the renal proximal tubular cells has not been clarified. In this study, we identified at least four different complexes (220, 480, 920, 1,100 kDa) containing NaPi-IIa by using blue-native polyacrylamide gel electrophoresis. Interestingly, LC-MS/MS analysis and immunoprecipitation analysis reveal that megalin is a component of larger complexes (920 and 1,100 kDa). In addition, NaPi-IIa can be heterogeneously co-localized with ezrin and megalin on the apical membrane of renal proximal tubular cells by fluorescence microscopy analysis. These results suggest that NaPi-IIa can form some different complexes on the apical plasma membrane of renal proximal tubular cells. *J. Med. Invest.* 58 : 140-147, February, 2011

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**INTRODUCTION**

Type IIa sodium-dependent phosphate transporter (NaPi-IIa) play the most important role in

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the maintenance of phosphate homeostasis in mammals. NaPi-IIa express in the apical membrane of proximal tubular cells in the kidney, and carries out the rate-limiting step of phosphate reabsorption. Most of phosphate regulating hormone such as parathyroid hormone (PTH), vitamin D, and fibroblast growth factor 23 (FGF23), can regulate apical membrane localization, as well as the gene expression, of NaPi-IIa to maintain the phosphate homeostasis. The amount of NaPi-IIa in the apical

membrane can be determined by the balance of the delivering rate of NaPi-IIa from endoplasmic reticulum and golgi apparatus to apical membrane, and the rate of endocytosis from apical membrane to intracellular organelle. Therefore, it is important to clarify the mechanism of the apical localization of NaPi-IIa to understand the molecular mechanism of maintenance of phosphate homeostasis.

Recent studies have clarified that (i) NaPi-IIa can be formed a macromolecular complex with adaptor proteins containing PDZ (PSD-95/Discs Large/ZO-1) domain such as NHERF-1 (Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor-1), PDZ-K1/2, and ezrin (1-3), (ii) NaPi-IIa, PTH receptor, protein kinase A and C, and other related signal molecules can be localized on lipid rafts in the apical membrane of renal proximal tubular cells (4), (iii) endocytosis of NaPi-IIa can be mediated by clathrin-coated pit in response to PTH (5), (iv) megalin, which is a member of low-density lipoprotein receptor family, can mediate NaPi-IIa endocytosis (6, 7). These observations suggest that macromolecular complex of NaPi-IIa plays an important role in the regulation of the NaPi-IIa localization in the apical membrane.

In this study, we tried to isolate the macromolecular complex of NaPi-IIa from lipid rafts of brush border membrane of rat renal cortex by blue-native PAGE, and analyzed by LC-MS/MS.

## MATERIALS AND METHODS

### *Preparation of brush-border membrane (BBM) and membrane microdomain isolation*

Eight weeks-old male Sprague-Dawley rats were purchased from SLC, Inc. (Shizuoka, Japan). Kidneys were collected from anesthetized rats. BBM was prepared from the kidney by the calcium precipitation methods as described previously (8). Membrane microdomains were isolated from BBM by non-detergent method (9). The Institutional Animal Care and Oversight Committee approved the experimental protocols of the study. The experiments were carried out according to the guidelines and principles for the care and use of animals at the University of Tokushima.

### *Blue-native PAGE*

Blue-native polyacrylamide gel electrophoresis (PAGE) is a way for separation of protein complexes by use of coomassie brilliant blue G-250 (CBB G-250) without any protein denaturation. Blue-native

PAGE system were purchased from Invitrogen, and performed according to the manufacture's protocols. The isolated membrane microdomains were pelleted by ultracentrifugation at 300,000×g for 1 h, and dissolved in the sample buffer supplied in the system with 3% n-dodecyl-β-D-maltoside at final concentration. The protein complexes were separated by 3-12% gradient Bis-Tris gel at 4°C. The gel was stained with Silver Stain Plus Kit (Bio-Rad Japan, Tokyo, Japan), or subjected to subsequent two-dimension gel electrophoresis and/or western blotting.

### *Two-dimension (2D) gel electrophoresis*

We performed 2D gel electrophoresis combined with blue-native PAGE/SDS-PAGE according to manufacture's instruction provided from Invitrogen. Samples were separated by blue-native PAGE (1st dimension), and the gel strip was cut out. The gel strip was reduced, alkylated, and subjected to SDS-PAGE (2nd dimension). Separated proteins can be detected and analyzed by western blotting.

### *Western blot analysis*

Western blotting analysis was basically carried out as described previously (4). In this study, we used following primary antibodies: anti-NaPi-IIa polyclonal antibody (10), anti-ezrin monoclonal antibody (Zymed, South San Francisco, CA), anti-NHERF-1 (Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor-1) polyclonal antibody (Chemicon, Temecula, CA), anti-PDZ-K1 polyclonal antibody (11) and anti-megalyn polyclonal antibody (12), and secondary antibodies: HRP-conjugated anti-mouse IgG (H+L) (Zymed) for monoclonal antibodies and HRP-conjugated anti-rabbit IgG (H+L) (Bio-Rad Japan) for polyclonal antibodies. Protein signals were detected using enhanced chemiluminescence plus (ECL-Plus) reagents (GE Healthcare Japan, Tokyo, Japan), and analyzed with LAS-3000 (Fuji Film, Tokyo, Japan).

### *LC-MS/MS analysis*

Protein bands identified by anti-NaPi-IIa antibody were excised, reduced, alkylated, and digested by trypsin in the gel. The digested proteins were subjected to LC-MS/MS analysis with Q-TOF Ultima API (Waters Micromass, Manchester, UK). Obtained MS/MS peaks were identified by MASCOT (13).

### *Cell culture and transfection*

Opossum Kidney cells (OK-P cells) that is a

well-characterized cell lines of mammalian renal proximal tubular cells, were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen). The cells were transfected pEGFP-NaPi-IIa (14) with lipofectamine 2000 (Invitrogen). Cell culture and transfection experiments were performed as described previously (4).

#### Immunofluorescence

Immunofluorescence analysis was basically performed as described previously (4). The transfected cells were cultured on the cover slips in the DMEM containing 10% FBS until to reach confluence. The cells on the cover slips were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 0.8% bovine serum albumin (BSA) for 30 min. Then, the cells were incubated with anti-ezrin monoclonal antibody, and/or anti-megalin polyclonal antibody (12). After washing the primary antibodies, the cells were subsequently incubated with anti-rabbit IgG (H+L) antibody with Qdot605 and anti-mouse IgG antibody labeled with Alexa546 as secondary antibody for multiple color staining. Fluorescence images were taken by Leica TCS-SL confocal laser scanning microscopy (Leica Microsystems, Tokyo, Japan).

#### Immunoprecipitation

Immunoprecipitation analysis was followed as previously described (4). BBM (125 µg) sample was dissolved in 1 mL of ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X100, protease inhibitor, phosphatase inhibitor). The samples were incubated with anti-NaPi-IIa antibody (10) or normal rabbit serum (for control). The protein-antibody complexes were incubated and precipitated by protein A sepharose beads. After washing, protein-antibody complexes were subjected to SDS-PAGE-western blot analysis with anti-megalin antibody or anti-NaPi-IIa antibody (10).

## RESULTS AND DISCUSSION

#### Analysis of protein complexes of NaPi-IIa with blue-native PAGE

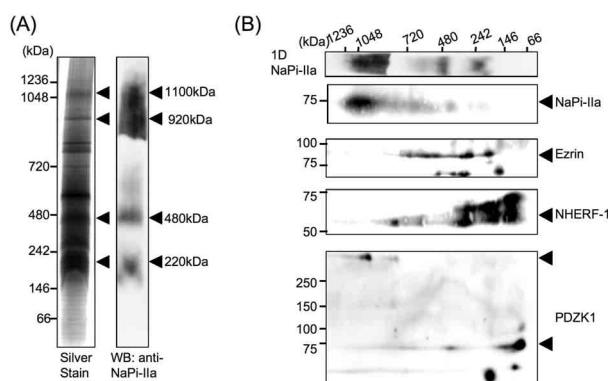
Firstly, we analyzed the macromolecular complex of NaPi-IIa in the lipid rafts fractioned from brush border membrane of rat renal cortex by blue-native

PAGE. As shown in Figure 1A, four major complexes containing NaPi-IIa were detected at 220, 480, 920, 1,100 kDa.

#### Analysis of NaPi-IIa complexes by 2D gel electrophoresis

For extensive analysis of these complexes, we performed 2D gel electrophoresis combined with blue-native PAGE and SDS-PAGE. Previous studies have demonstrated that NaPi-IIa can form a macromolecular complex with some adaptor proteins containing PDZ binding motif such as NHERF-1 and PDZK1, and ezrin (1-3). Therefore, we examined which complex can contain those proteins. NHERF-1 and ezrin were detected in the 220 and 480 kDa complexes, but they were hardly detected in the 920 and 1,100 kDa complexes by western blotting (Figure 1B). Interestingly, two different size of PDZK1 were found in 2D gel electrophoresis. Original size spots of PDZK1 were found in the similar position as lower complexes of NaPi-IIa (Figure 1B). On the other hand, larger size spots of PDZK1 (> 250 kDa) were found in the similar position corresponded to larger complexes of NaPi-IIa (Figure 1B). We don't know why we detected larger size of PDZK1, but PDZK1 in the large complex may strongly interact with unknown protein(s). Further investigation should be needed to clarify this phenomenon.

The molecular weight of NaPi-IIa is 75 kDa, and NHERF-1 is 50 kDa, ezrin is 80 kDa, approximately.



**Figure 1.** Analysis of protein complexes of NaPi-IIa with blue-native PAGE and 2D gel electrophoresis analysis with both blue-native PAGE and SDS-PAGE

(A) The membrane microdomain of kidney BBM was isolated to each complex by blue-native PAGE and analyzed by Western blot. The arrowheads show to NaPi-IIa signal. (B) 2D-gel electrophoresis analysis. After blue-native PAGE, contents of each complex are divided by SDS-PAGE. Western blot was performed with antibodies against NaPi-IIa, ezrin, NHERF-1 and PDZK1. The arrowheads show to each protein signal. The upper column shows locations of NaPi-IIa complexes by 1D blue-native PAGE.

NaPi-IIa can form tetramer in the membrane (15). Therefore, 220 kDa and 480 kDa complexes can be reasonably constituted by these proteins. However, larger complexes must contain other proteins. To identify the components of the high molecular weight complexes of NaPi-IIa, we analyzed the complexes by LC-MS/MS analysis. Table 1 shows a list of proteins identified by MASCOT search. These proteins cannot be always real components of the large complexes of NaPi-IIa. In addition, the results not guarantee that these proteins can be associated with directly or indirectly with NaPi-IIa in the apical membrane. However, encompassing analysis by LC-MS/MS can detect some interesting candidates such as megalin.

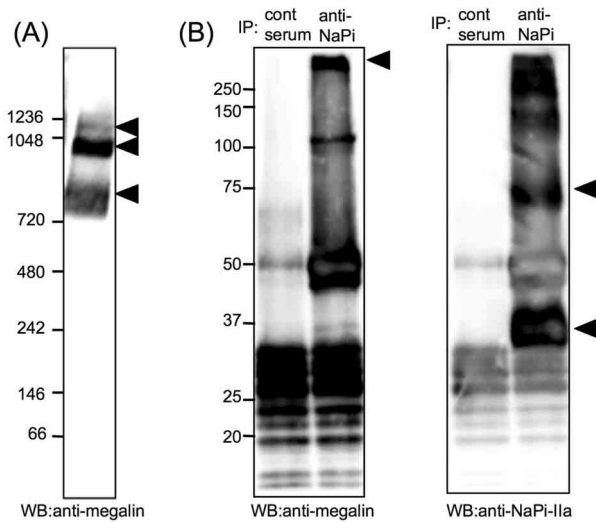
#### *Interaction between NaPi-IIa and megalin*

Megalyn is a 600 kDa type I membrane protein, and known as a multi-ligand receptor or LDL-receptor related protein 2 (Lrp2). Megalyn can be localized in the clathrin-coated pit, can bind and internalize the ligands like PTH, vitamin D, and steroid hormones into the cells by endocytosis via clathrin-coated pit (16-19). In addition, megalyn can also mediate the endocytosis of NaPi-IIa, because megalyn knock out mice defects the NaPi-IIa endocytosis in response to PTH (6). However, it is not clarified that megalyn can directly or indirectly interact with NaPi-IIa or not. Figure 2A shows that megalyn can be localized in the 1,100, 920 and 850 kDa complexes in the lipid rafts fractioned from brush border membrane of rat renal cortex by blue-native PAGE. Both 1,100 and 920 kDa complexes

**Table 1.** Analysis of contents of the high molecular weight complexes by LC-MS/MS

We analyzed the high molecular weight complexes of NaPi-IIa by LC-MS/MS. The list shows the top 20 identified proteins in descending order of score. Rank 1 to 8 are significant results over score line 38 ( $p < 0.05$ ). Reported subcellular localization and reference number in parenthesis are also shown.

Rank (Score)	Protein name	score	Accession number	location	
				apical	membrane microdomains
1	low density lipoprotein receptor-related protein 2 (megalin)	377	gi 13562118	Yes(20)	
2	neutral and basic amino acid transport protein rBAT	160	gi 8394307	Yes(26)	Caveolae(27)
3	clathrin heavy chain 1	121	gi 9506497	Yes(28)	
4	Na(+)/H(+) exchange regulatory cofactor NHERF-1	72	gi 11024674	Yes(29)	Caveolae(30)
5	lysozyme homolog AT-2	55	gi 539969		
6	V-type proton ATPase subunit B	48	gi 17105370	Yes(31)	Caveolae(32)
7	cystathionine gamma-lyase	45	gi 56030		
8	anionic trypsin-1	42	gi 6981420		
9	rCG51062, isoform CRA_b	37	gi 149038022		
10	protease, serine, 1	33	gi 16716569		
11	unnamed protein product	28	gi 26354020		
12	PREDICTED : similar to ATPase, H+ transporting, V1 subunit A, isoform 1	27	gi 109493234	Yes(31)	Caveolae(32)
13	hypothetical protein LOC499136	26	gi 114145710		
14	solute carrier family 23 member 1	25	gi 8394381	Yes(33)	
15	beta-actin	25	gi 119959830	Yes(34)	Raft(35)
16	solute carrier family 7 member 13	21	gi 58865770		
17	PREDICTED : similar to CUB and Sushi multiple domains 3 isoform 1	19	gi 109482174		
18	rCG50190, isoform CRA_a	19	gi 149035434		
19	GTP-binding protein GUF1 homolog	19	gi 27370038		
20	PREDICTED : similar to carbonyl reductase 3	19	gi 109492941		

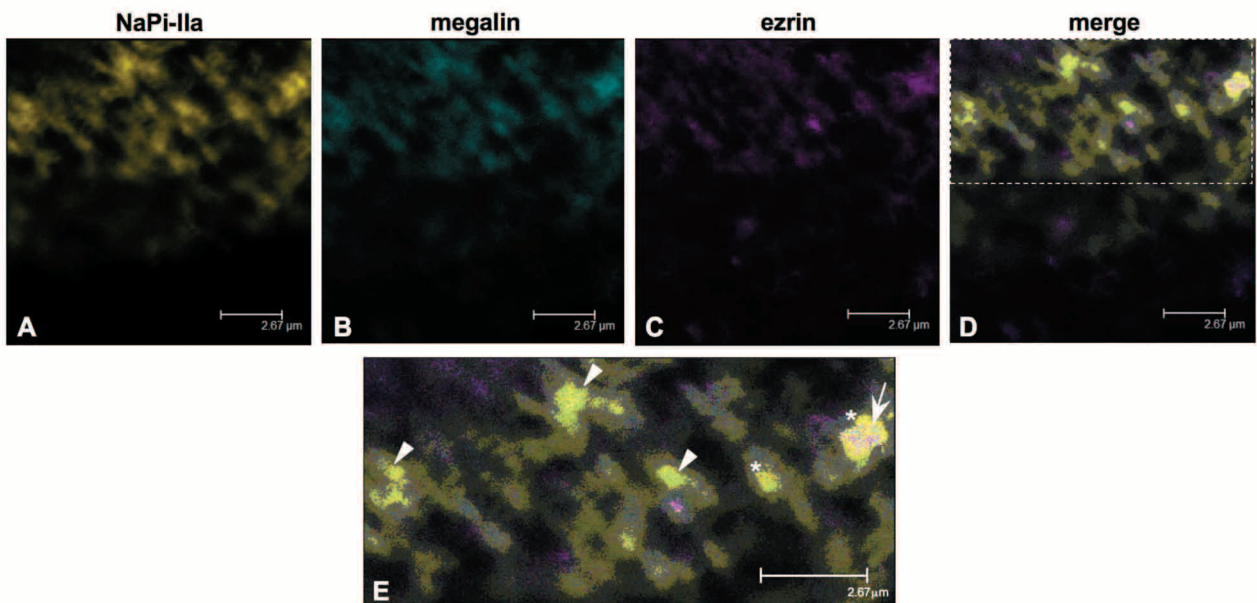


**Figure 2.** Confirmation of megalin with blue-native PAGE and immunoprecipitation  
 (A) Complexes of the kidney BBM microdomain were isolated by blue-native PAGE and immunoblotted with anti-megalin antibody. The arrowheads show to megalin signal. (B) The BBM was co-immunoprecipitated with anti-NaPi-IIa antibody or normal rabbit serum (control). Western blot was performed with anti-megalin antibody and anti-NaPi-IIa antibody.

are same size as the large molecular complexes of NaPi-IIa as shown in figure 1A. Furthermore, megalin was detected in the immunoprecipitated complex with anti-NaPi-IIa antibody (Figure 2B). Immunofluorescence study also showed that megalin

can be co-localized with NaPi-IIa in the renal proximal tubular cell lines (OK-P cells) (Figure 3A-D). High-magnified image indicates that NaPi-IIa complexes are heterogenous. NaPi-IIa can form a complex with both megalin and ezrin, or either megalin or ezrin on the apical membrane of the proximal tubular cells (Figure 3E).

In this study, blue-native PAGE and immunofluorescence analysis clearly show that NaPi-IIa complexes are not homogeneous in both molecular size and localization on the apical membrane of renal proximal tubular cell. This heterogeneity suggests that different complexes may have a role in the localization and regulation of NaPi-IIa in the apical membrane of renal proximal tubular cells. The smaller complexes (220, 480 kDa) containing ezrin but not megalin may be important for the apical localization of NaPi-IIa. NaPi-IIa can be localized on lipid raft-like membrane microdomains of the apical membrane via  $\text{Na}^+/\text{H}^+$  exchanger regulatory factor-1 (NHERF-1) and ezrin (4). On the other hand, the larger complexes (920, 1,100 kDa) containing megalin may be important for the internalization of NaPi-IIa from apical membrane to intracellular organelle via clathrin-dependent endocytosis. Because both NaPi-IIa and megalin can be internalized by clathrin-coated pit pathway (7). Interestingly, our results suggest that megalin could be localized in raft-like



**Figure 3.** Localization of NaPi-IIa, ezrin and megalin in OK-P cells by triple label immunofluorescence  
 OK-P cells were transfected GFP-NaPi-IIa and double-stained ezrin and megalin by anti-ezrin antibody and anti-megalin antibody. NaPi-IIa (yellow), megalin (cyan), ezrin (Magenta) is shown by confocal laser scanning microscopy. The overlaid image is shown (D). (E) Magnified image of the dotted rectangle area in D. Arrow indicates triple merge with NaPi-IIa, megalin and ezrin. Arrowheads indicate colocalization of NaPi-IIa and megalin. Asterisks indicate colocalization of NaPi-IIa and ezrin.

membrane microdomains of brush border membrane in rat renal cortex (Figure 2A), although megalin can generally be recognized as a protein localized in clathrin-coated pit (20). We cannot exclude some contamination of clathrin-coated pit in our lipid-raft fractions, but the results suggest that megalin may be able to transit on the membrane microdomains. Recently, Bento-Abreu *et al.* demonstrated that megalin can be localized in caveolae in astrocytes, and be involved in albumin endocytosis with caveolin-1 (21). In addition, megalin can bind to Na<sup>+</sup>/H<sup>+</sup>-exchanger 3 (NHE3) (22) that is also localized in membrane microdomains (23). Therefore, it may be possible that megalin can be localized in caveolae/raft-like membrane microdomains in renal proximal tubular cells. The localization of megalin in the membrane microdomains would be reasonable for the regulation of NaPi-IIa. Although most of NaPi-IIa can be localized in the membrane microdomain (4, 24), NaPi-IIa can be internalized by clathrin-coated pit in response to PTH and high Pi diet loading to down-regulate NaPi-IIa activity (25). These observations suggest that NaPi-IIa can transit between lipid-raft like membrane microdomains and clathrin-coated pit. If megalin can interact with NaPi-IIa on the membrane microdomains as shown in this study, megalin may be escort the NaPi-IIa from membrane microdomains to clathrin-coated pit. Further investigation should be required to determine the exact roles of those different complexes of NaPi-IIa in the localization and regulation.

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