

Modulation of Renal Apical Organic Anion Transporter 4 Function by Two PDZ Domain-Containing Proteins

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Human organic anion transporter 4 (OAT4) is an apical organic anion/dicarboxylate exchanger in the renal proximal tubules and mediates high-affinity transport of steroid sulfates such as estrone-3-sulfate (E₁S) and dehydroepiandrosterone sulfate. Here, two multivalent PDZ (PSD-95/Discs Large/ZO-1) proteins PDZK1 and NHERF1 were examined as interactors of OAT4 by a yeast two-hybrid assay. These interactions require the extreme C-terminal region of OAT4 and the first and fourth PDZ domains of PDZK1 and the first PDZ domain of NHERF1. These interactions were confirmed by surface plasmon resonance assays (K_D: 36 nM, 1.2 μM, and 41.7 μM, respectively). *In vitro* binding assays and co-immunoprecipitation studies revealed that the OAT4 wild-type but not a mutant lacking the PDZ motif interacted directly with both PDZK1 and NHERF1. OAT4, PDZK1, and NHERF1 proteins were shown to be localized at the apical membrane of renal proximal tubules. The association with PDZK1 or NHERF1 enhanced OAT4-mediated E₁S transport activities in HEK293 cells (1.2- to 1.4-fold), and the deletion of the OAT4 C-terminal PDZ motif abolished this effect. The augmentation of the transport activity was accompanied by alteration in V_{max} of E₁S transport *via* OAT4 and was associated with the increased surface expression level of OAT4 protein. This study indicates that the functional activity of OAT4 is modulated through the PDZ interaction with the network of PDZK1 and NHERF1 and suggests that OAT4 is involved in the regulated apical organic anion handling in the renal proximal tubules, provided by the PDZ scaffold.

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The human organic anion transporter OAT4 (encoded by *SLC22A11*) is expressed in the kidney and the placenta and mediates the high-affinity transport of steroid sulfates such as estrone-3-sulfate (E₁S) and dehydroepiandrosterone sulfate (DHEAS) (1). Because of its apical membrane localization in the renal proximal tubules (2), OAT4 had been presumed to be the apical exit pathway of organic anions that are taken up by the basolateral entrance pathway such as OAT1 and OAT3 (3). Recently, Ugele *et al.* (4) found the OAT4 protein expression at the fetal side of the syncytiotrophoblasts in the placenta and proposed a role for OAT4 in the placental uptake of fetal-derived steroid sulfates. On the basis of our finding that OAT4 is an apical organic anion/dicarboxylate exchanger (5), we suggested that OAT4 mainly functions as an apical entrance pathway for some organic anions in renal proximal tubules driven by an outwardly directed dicarboxylate gradient created by Na⁺/dicarboxylate co-transporters (6). One possible role of

OAT4 is as an apical backflux pathway (7) for some organic anions such as steroid sulfates, functionally coupled to the apical efflux transporters for organic anions, such as MRP2, MRP4, and NPT1 (putative human homologue of OAT_V1) (8–13).

At the extreme C-terminal (CT) end, OAT4 has a specific protein–protein interaction peptide sequence named the PDZ (PSD-95/Discs Large/ZO-1) motif (S-T-S-L) (14). PDZ domains have been identified in various proteins and are known to be modular protein–protein recognition domains that play roles in protein targeting and protein complex assembly (15–17). These multidomain molecules not only target and provide scaffolds for protein–protein interactions but also modulate the function of receptors and ion channels, by which they associate. Recently, we reported that the urate/anion exchanger URAT1 (18), which has a similar PDZ motif (S-T-Q-F) at its C-terminus, interacts with multivalent PDZ protein PDZK1 (19,20). In the same study, we also identified that PDZK1 interacted with the C-terminus of OAT4, which has a PDZ motif, but not with those of human OAT1, OAT2, or OAT3, which do not have PDZ motifs (20). Both URAT1 and OAT4 are considered to function to generate a reabsorptive pathway for organic anions localized at the apical membrane of renal proximal tubules. Therefore, it is likely that these transporters bind with the same or other PDZ protein(s) *via* their CT PDZ motifs.

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In this study, we examined the interaction between OAT4 and two PDZ proteins, PDZK1 and NHERF1, using a yeast two-hybrid assay, an *in vitro* pulldown assay, co-immunoprecipitation, and surface plasmon resonance assay. We observed that the OAT4-mediated transport function is modulated equivalently by binding with two PDZ proteins *via* the C-terminus of OAT4. These results, together with recent emerging findings concerning PDZ proteins, will provide important insight into the renal apical handling of organic anions through the transporter complexes supported by PDZ protein networks.

Materials and Methods

Plasmid Construction

DNA encoding residues 513 to 550 (wt, T548A, L550A) or 513 to 547 (d3) of human OAT4 were amplified by PCR using specific primers (Table 1) and cloned into the EcoRI and XhoI sites of the pEG202 plasmid (bait) and into pGEX-6P-1 (Amersham Biosciences, Inc., Piscataway, NJ) to generate OAT4-CT-wt, OAT4-CT-d3, OAT4-L550A, or OAT4-T548A. The full-length coding sequence of human OAT4 (wt) as well as its CT-3-amino-acid deletion mutant (d3) were inserted into the mammalian expression plasmid pcDNA3.1 (Invitrogen, Gaithersburg, MD) for functional analysis and into the pEGFP-C2 plasmid (Clontech, Palo Alto, CA) for GFP-fused OAT4 protein preparation. Proper folding of GFP-fused proteins was confirmed by their significant transport activities of E₁S (data not shown). The full-length coding sequence of human NHERF1 was amplified from human kidney cDNA (Clontech) and subcloned into pcDNA3.1 (Invitrogen) to generate pcDNA3.1-NHERF1 and into pJG4–5, a B42 activation domain fusion vector, to generate pJG4–5-NHERF1. Prey vectors (pJG4–5) that contained the individual PDZ domains of human NHERF1 were generated by PCR using specific primers (Table 1). The pcDNA3.1 vector that contained the full-length human PDZK1 and prey vectors that contained a single PDZ domain of human PDZK1 were prepared as described previously (20).

Yeast Two-Hybrid Assay

Yeast two-hybrid assays were performed in the EGY48 strain with the LexA-based GFP two-hybrid system (Grow'n'Glow system; Mo-BiTec, Göttingen, Germany) as described elsewhere (21).

GST Fusion Protein Binding Assays

The OAT4-CT domains for GST fusion protein production in bacteria were prepared as reported previously (21). *In vitro* translation was performed from a plasmid carrying the full-length PDZK1 and NHERF1 with the TNT T7 Quick for PCR DNA system (Promega, Madison, WI) in the presence of Transcend Biotinylated tRNA (Promega), as described elsewhere (20). Five microliters of *in vitro*-trans-

lated products was applied into ProFound Pull-Down GST Protein: Protein Interaction Kit (Pierce, Rockford, IL) with 50 μ l of GST-glutathione-Sepharose resin, and protein complexes were eluted according to the manufacturer's instructions.

Surface Plasmon Resonance

The interactions of OAT4-CT with the first and fourth PDZ domains of PDZK1 and the first PDZ domains of NHERF1 were investigated using a BIAcore 3000 analytical system (BIAcore AB, Uppsala, Sweden) based on principles described previously (22). Using an amine coupling kit, GST-fused OAT4-CT wild-type or GST protein alone was attached to a CM5 sensor chip according to the manufacturer's instructions, giving a gain of 13,269 resonance units (RU) for GST-OAT4-CT or 8566 RU for GST alone.

Tissue Distribution

Aliquots of 1.5 μ l of Human Multiple Tissue cDNA Panels I and II (Clontech) were amplified as described previously (20). The OAT4 and NHERF1 primers used for PCR amplification are shown in Table 1.

Immunohistochemical Analysis

We used human single-tissue slides (Biochain) for light microscopic immunohistochemical analysis, and they were treated with 10 μ g/ml primary rabbit polyclonal antibodies against OAT4 and hPDZK1 and with EBP50 (NHERF1)-specific mAb purchased from BD Bioscience (San Jose, CA) (23) at 4°C overnight, as reported previously (5).

Cell culture and Transfections

Human embryonic kidney 293 (HEK293) cells were maintained in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) at 37°C and 5% CO₂. Transient transfection with Lipofectamine 2000 (Invitrogen) was performed according to the manufacturer's recommendations. For the establishment of OAT4-expressing cells, stable transfectants were selected for 2 wk by adding 1 mg/ml G418 to the medium.

Immunoprecipitation and Immunoblotting

Immunoprecipitation analysis was performed as described previously (20). GFP-fused OAT4 and associated proteins in HEK293 cell lysate were immunoprecipitated by the anti-GFP antibody (full-length A.v. polyclonal antibody; Clontech) using the Seize Classic (A) Immunoprecipitation kit (Pierce). The eluates were treated as described in the GST Fusion Protein Binding Assays section. The affinity-purified rabbit PDZK1 antibodies, EBP50 antibody (23), and horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Biosciences, Inc.) were used for immunoblotting with enhanced chemiluminescence reagents (ECL Plus; Amersham Biosciences, Inc.).

Table 1. PCR primers used in this study

Construct	Sense Primer	Antisense Primer
OAT4-CT-wt	5'-TGAATTCGAGACCCAGGGACTTCC-3'	5'-CCCTCGAGTTTCTAGAGCGAGGTAC-3'
OAT4-CT-d3	5'-TGAATTCGAGACCCAGGGACTTCC-3'	5'-CCCTCGAGCTAACTTTCCACAGTGACGG-3'
OAT4-L550A	5'-TGAATTCGAGACCCAGGGACTTCC-3'	5'-CTCTCGAGCTAGGCCGAGGTACTTTCCACAG-3'
OAT4-T548A	5'-TGAATTCGAGACCCAGGGACTTCC-3'	5'-CCCTCGAGTTTCTAGAGCGAGGCACITTTCC-3'
NHERF1-PDZ1	5'-CACTCGAGATGAGCGCGGACGCAGC-3'	5'-TTCTCGAGTCAACGAAGCTCGCGCTGCTC-3'
NHERF1-PDZ2	5'-TTCTCGAGCTTCGGCCTCGGCTCTG-3'	5'-TGCTCGAGTCACTCGTCAGTTTCCCTGTC-3'
OAT4 RT-PCR	5'-TCTTGCTCAGTTTCCTTGGCC-3'	5'-GCTGTTGATTTCTGGCTCTCCA-3'
NHERF1 RT-PCR	5'-TTCTCGAGCTTCGGCCTCGGCTCTG-3'	5'-TGCTCGAGTCACTCGTCAGTTTCCCTGTC-3'

E₁S Transport Activity Assays

HEK293 cells, plated on 24-well culture plates at a density of 2×10^5 24 h before transfection, were incubated in Lipofectamine 2000 as described above. After 36 h, the culture medium was removed and the cells were incubated in serum-free Dulbecco's modified PBS (D-PBS; containing [in mM] 137 NaCl, 3 KCl, 8 Na₂HPO₄, 1 KH₂PO₄, 1 CaCl₂, and 0.5 MgCl₂ [pH 7.4]) supplemented with 5.5 mM D-glucose for 10 min. The uptake study was started by adding 500 μ l of D-PBS that contained 50 nM [³H]E₁S into the plate. After 2 min, the cells were washed twice in ice-cold D-PBS and lysed in 0.1 N NaOH for 20 min for scintillation counting.

For determining the kinetic parameters, the concentrations of E₁S were varied from 50 nM to 3000 nM. OAT4-mediated E₁S uptake was calculated as the difference between the values of uptake into HEK293 cells that stably expressed OAT4 (HEK-OAT4) and those of uptake into HEK293 cells that were transfected with vector only (HEK-mock). The kinetic parameters for the uptake *via* OAT4 were estimated using the following equation: $v = V_{\max} [S]/(K_m + [S])$, where v is the uptake rate of substrates, $[S]$ is the substrate concentration (μ M) in the medium, and K_m is the Michaelis-Menten constant (μ M). These values were determined using the Eadie-Hofstee equation.

Cell Surface Biotinylation

Surface biotinylation of OAT4 at the plasma membrane was performed as described elsewhere (20). Surface proteins in HEK-OAT4 cells that were transfected with pcDNA3.1(+)-hPDZK1, pcDNA3.1(+)-hNHERF1, or pcDNA3.1(+) empty vector were biotinylated with Sulfo-NHS-SS-Biotin (0.5 mg/ml; Pierce) in PBS for 30 min at 4°C. Cell lysates then were incubated with Ultralink-immobilized NeutrAvidin beads (Pierce) to precipitate biotinylated proteins. The bound proteins were eluted with SDS sample buffer and were subjected to SDS-PAGE and Western blotting followed by ECL (Amersham Biosciences). OAT4 was detected with affinity-purified polyclonal OAT4 antibody (1:5000) (5).

Statistical Analyses

Uptake experiments were conducted three times, and each uptake experiment was performed in triplicate. Values are presented as the means \pm SEM. Statistical significance was determined by *t* test.

Results

Interaction between OAT4 and PDZK1/NHERF1 in a Yeast Two-Hybrid Assay

In our previous study, we reported that one of the OAT members, URAT1, binds to the multivalent PDZ domain protein PDZK1 (20). In parallel, we also identified that a bait vector that contains the OAT4 carboxyl-terminal tail (OAT4-CT) binds to PDZK1 using a yeast two-hybrid assay. Because the CT of OAT4 that has a PDZ motif (T-S-L) is different from the URAT1 CT (T-Q-F), this difference motivated us to extend the search for the interaction of OAT4 with other PDZ proteins expressed in the kidney, such as NHERF1/EBP50, NHERF2/E3KARP, and IKEPP (24,25). We observed the induction of two reporter genes (LEU and GFP) in the interaction of OAT4-CT with NHERF1 and NHERF2 and the induction of only one reporter gene (LEU) in the OAT4-CT and IKEPP interaction (data not shown). The specificities of these interactions were confirmed by the constructs with unrelated proteins. Because NHERF1 and PDZK1 but not NHERF2 are predominantly in the brush border (26), we focused our research on the interactions of OAT4-CT with both PDZK1 and NHERF1.

To identify the binding site of OAT4 that interacts with the two PDZ proteins, we constructed three mutant baits, as in our previous study (20). The first was a mutant that lacked the last three residues of OAT4, which play a crucial role in PDZ domain recognition (OAT4-CT-d3). In the second and third, the extreme CT leucine (0 position) or threonine (−2 position) of OAT4 were replaced by alanine (L550A and T548A), which was expected to abolish or strongly reduce the binding of PDZ proteins (14). All three mutant baits were unable to interact with PDZK1 full-length clone or the NHERF1 full-length clone (Table 2). Thus, these results suggested that OAT4 CT PDZ motif is the site responsible for interactions with PDZK1 and NHERF1.

Table 2. Specificity of PDZK1 and NHERF1 for interaction with the C-terminus of OAT4 in the yeast two-hybrid assay

	C-Terminal Residues (Last 10 Amino Acids)	PDZK1		NHERF1	
		LEU2	GFP	LEU2	GFP
OAT4-CT-wt	EAVTVESTSL*	+	+	+	+
OAT4-CT-d3	EAVTVES*	−	−	−	−
OAT4-L550A	EAVTVESTSA*	−	−	−	−
OAT4-T548A	EAVTVESASL*	−	−	−	−

	PDZK1				NHERF1	
	PDZ1	PDZ2	PDZ3	PDZ4	PDZ1	PDZ2
LEU2	+	−	−	+	+	−
GFP	+	−	−	+	+	−

*The system used for the yeast two-hybrid assay included the reporter genes LEU2 and GFP, which replaced the classical *lacZ* gene commonly used, and allowed fast and easy detection of positive clones with long-wave UV.

PDZK1 PDZ-1 and -4 Domains and NHERF1 PDZ-1 Domain Are Required for Interaction with OAT4-CT

PDZK1 possesses four PDZ domains and NHERF1 possesses two PDZ domains that assemble target proteins by binding to a CT that has a consensus PDZ motif (14). To check the possible interactions of the OAT4-CT with these PDZ domains of PDZK1 and NHERF1, we prepared prey vectors that contained the single PDZ domain of two PDZ proteins (PDZ-1, -2, -3, and -4 of PDZK1 and PDZ-1 and -2 of NHERF1). Interaction with OAT4-CT was observed when PDZ-1 and -4 were present but not with PDZ-2 and -3 of PDZK1 and when PDZ-1 was present but not with PDZ-2 of NHERF1 (Table 2).

In Vitro Binding of OAT4 and PDZK1/NHERF1

To confirm the ability of OAT4-CT to bind PDZK1 or NHERF1 *in vitro*, we next used the GST pulldown assay to validate the interaction (Figure 1). GST fusion proteins that bear the wild-type CT region of OAT4 (OAT4-CT-wt) or CT region mutants (OAT4-CT-d3) were used to pull down the full-length PDZK1 or the full-length NHERF1 proteins that were generated from *in vitro* translation experiments. The data demonstrated the same specificity of interaction for PDZK1/NHERF1 and OAT4 in the yeast two-hybrid assays. We did not observe the binding of PDZK1 and NHERF1 to OAT4-CT that lacked the PDZ motif (OAT4-CT-d3; Figure 1, A and B).

Co-Immunoprecipitation of OAT4 and PDZK1/NHERF1 from Heterologous Cells

To confirm further the interaction between OAT4 and PDZK1/NHERF1, we performed a co-immunoprecipitation study using the antibodies against human PDZK1 (19) and NHERF1(EBP50) in HEK293 cells. To test the specificity of EBP50 antibody, first we performed Western blot analysis of crude membranes from HEK293 cells that were transfected with vector (pcDNA3.1) alone (lane 1), with pcDNA3.1-hNHERF1 full length (lane 2), and with pcDNA3.1-hNHERF2 full length (Figure 1C, lane 3). A single strong band of approximately 50 kDa, which is consistent with hNHERF1, was observed in lane 2 but not in lane 3. We coexpressed full-length human OAT4 fused with GFP (GFP-OAT4) and full-length PDZK1/NHERF1 in HEK293 cells. PDZK1 and NHERF1 were co-immunoprecipitated with a GFP-specific antibody in the lysates from the cells transfected with GFP-OAT4-wt, but neither PDZK1 nor NHERF1 was co-immunoprecipitated in those from the cells that were transfected with GFP-OAT4 that lacked the last three amino acids (Figure 1, D and E).

Comparison of Binding Affinities between OAT4-CT and Individual PDZ Domains of PDZK1 or NHERF1

To quantify and compare the interaction of OAT4 with PDZK1 and NHERF1, we performed surface plasmon resonance experiments using immobilized GST-OAT4-CT and mal-

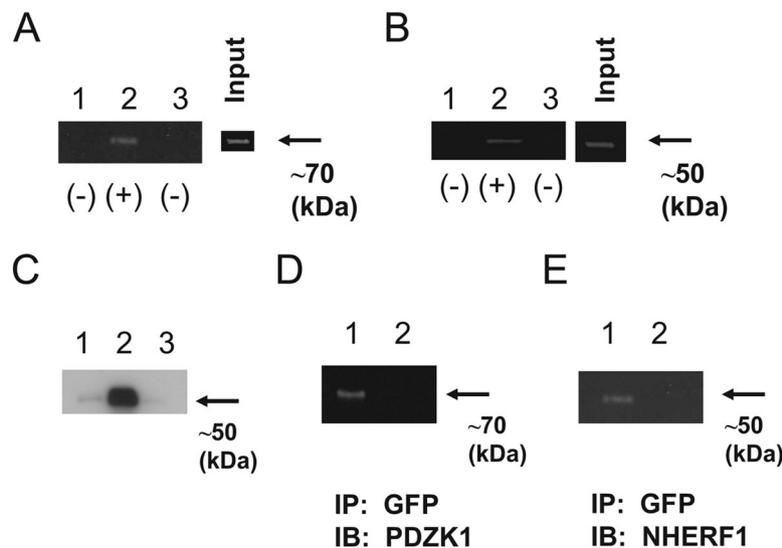


Figure 1. Interaction of PDZK1 and NHERF1 with organic anion transporter 4 (OAT4). (A and B) The *in vitro* translation products of full-length PDZK1 (A) or NHERF1 (B) using Transcend Biotinylated Lysine tRNA (Promega) were incubated with the GST alone (lane 1), GST-OAT4-CT-wt (lane 2), or GST-OAT4-CT-d3 (lane 3). The pulldown products were analyzed by SDS-PAGE. The input corresponds to the crude *in vitro* translation reaction. The positions of molecular mass standards are indicated on the right. (C) Western blot analysis of crude membranes from HEK293 cells that were transfected with vector (pcDNA3.1) alone (lane 1), with pcDNA3.1-hNHERF1 full length (lane 2), and with pcDNA3.1-hNHERF2 full length (lane 3). A single strong band of approximately 50 kDa, which is consistent with NHERF1, was observed in lane 2 but not in lane 3. (D and E) Co-immunoprecipitation of the OAT4 and PDZK1 or NHERF1 proteins in HEK293 cells, respectively. HEK293 cells were transfected with pEGFP-C2 vectors encoding the wild-type full-length OAT4 (GFP-OAT4-FLwt, lane 1) or the full-length OAT4 that lacked the last three amino acid residues (GFP-OAT4-FLd3, lane 2) with pcDNA3.1-PDZK1 (D) or pcDNA3.1-NHERF1 (E) and then immunoprecipitated with anti-GFP antibody. Then, the immunoprecipitates were resolved by SDS-PAGE and probed with anti-PDZK1 antibodies (D) or with anti-EBP50 antibodies (E).

tose-binding protein (MBP)-fused PDZK1 PDZ domain 1, domain 4, and NHERF1 domain 1 proteins. As summarized in Table 3, the binding affinity for the PDZ domain 1 of PDZK1 showed high affinity (36 nM), that for the PDZ domain 4 of PDZK1 showed low affinity (1.2 μ M), and that for the PDZ domain 1 of NHERF1 exhibited the lowest affinity (41.7 μ M).

Tissue Distribution of OAT4 and PDZK1/NHERF1 mRNA in Human Tissues

In humans, OAT4 mRNA has been detected in both the kidney and the placenta (1); PDZK1 has been detected mainly in the liver, kidney, pancreas, gastrointestinal tract, and adrenal cortex (18); and NHERF1 has been detected ubiquitously (27). Using human multiple cDNA panels, we examined the mRNA distribution of OAT4, PDZK1, and NHERF1 to compare the tissue distribution and their overlaps. OAT4 transcripts were detected in the kidney, placenta, testis, small intestine, and colon, whereas PDZK1 and NHERF1 transcripts were detected in most of the tissues analyzed, confirming and expanding the previously described distribution in humans (Figure 2A). It is interesting that overlapping expressions among OAT4, PDZK1, and NHERF1 were limited to the kidney, placenta, and small intestine. This is the first time that OAT4 expression has been identified in the testis, small intestine, and colon.

Coexpression of OAT4, PDZK1, and NHERF1 in Human Kidney

OAT4 is present at the apical membrane of proximal tubules (2), and PDZK1 and NHERF1 have also been reported to be expressed in the same cells (19,27). To determine whether OAT4, PDZK1, and NHERF1 localize at the apical membrane of the renal proximal tubules, we performed immunostaining of human kidney sections with anti-OAT4, anti-PDZK1, and anti-NHERF1 antibodies. Consistent with previous reports, in the renal cortex, the overlapping expression of OAT4, PDZK1, and NHERF1 was detected at the apical membrane in most of the proximal tubular cells (Figure 2B).

OAT4 Transport Activities Are Increased in the Presence of PDZK1/NHERF1

To determine whether OAT4 and PDZK1/NHERF1 interactions are required to mediate the increase in OAT4 activity, we transfected HEK293 cells with the pcDNA3.1(+) construct that contained full-length OAT4 (OAT4-wt), OAT4 that lacked the

last three amino acids (OAT4-d3), or OAT4 without insert (mock). At an incubation time of 2 min, we demonstrated that the uptake of [³H]E₁S *via* both the OAT4-wt and OAT4-d3 was from five- to six-fold higher than that by the mock (Figure 3, A and B). E₁S transport activities in OAT4-wt were significantly increased after the coexpression of PDZK1 (1.4-fold; Figure 3A) as well as NHERF1 (1.2-fold; Figure 3B). These effects were abolished when OAT4-d3 was coexpressed with PDZK1 (Figure 3A) and NHERF1 (Figure 3B). These results indicate that the interaction between the OAT4-CT and PDZK1/NHERF1 is essential for the functional increase in OAT4-mediated E₁S transport.

Next, we examined the effect of the coexpression of two PDZ proteins on the kinetics of [³H]E₁S transport *via* HEK293 cells that stably expressed OAT4 (HEK-OAT4) and that had been transfected with pcDNA3.1 vector alone, pcDNA3.1-PDZK1, or pcDNA3.1-NHERF1. Kinetic data showed that PDZK1 and NHERF1 significantly increased V_{max} from 86.5 to 123.6 and from 86.5 to 110.1 pmol/mg protein per min, respectively ($P < 0.05$), and did not change K_m (from 944 to 971 and from 944 to 1058, respectively), as compared with OAT4 transfected with vector alone (Figure 3C).

Surface Expression Level of OAT4 Protein

To determine changes in the cell surface expression level of OAT4, we used a cell membrane-impermeant biotinylation reagent to label cell-surface proteins selectively. After the treatment, cell lysates from HEK293 cells that stably expressed OAT4 that was transfected with hPDZK1, hNHERF1, or mock was collected. The amount of surface-biotinylated OAT4 expression on plasma membranes increased 1.3- to 1.4-fold (mock-transfected 30.0 ± 1.2 , PDZK1-transfected 40.2 ± 2.6 , NHERF1-transfected 42.5 ± 4.0 arbitrary units; $n = 3$) when PDZ proteins were coexpressed (Figure 4). These changes seem close to the one in V_{max} of OAT4-mediated transport observed in Figure 3C.

Discussion

Recent studies have shown that the PDZ domains function as modular protein-protein interaction motifs that serve to localize proteins to specific subcellular sites, to organize signaling cascades, and to regulate cell signaling (15–17). This domain binds to proteins that contain the tripeptide motif (S/T)-X-Ø

Table 3. Characteristics of interaction between OAT4 C-terminus and PDZK1 PDZ domain 1, domain 4, or NHERF1 PDZ domain 1^a

Construct	k_a (1/mM per s)	k_d (1/min)	K_D (μ M)
PDZK1-PDZ1	9.4×10^3	3.4×10^{-3}	0.036
PDZK1-PDZ4	6.8×10^2	7.9×10^{-4}	1.2
NHERF1-PDZ1	2.7×10^2	1.1×10^{-3}	42

^aThe kinetic characteristics of the interaction with immobilized GST-fused OAT4 C-terminus with the first and fourth PDZ domains of PDZK1 (PDZ1, 4) and the first PDZ domain of NHERF1 fused with maltose-binding protein measured by surface plasmon resonance methods are summarized. Association rate constants (k_a), dissociation rate constants (k_d), and equilibrium dissociation constants ($K_D = k_d/k_a$) are given.

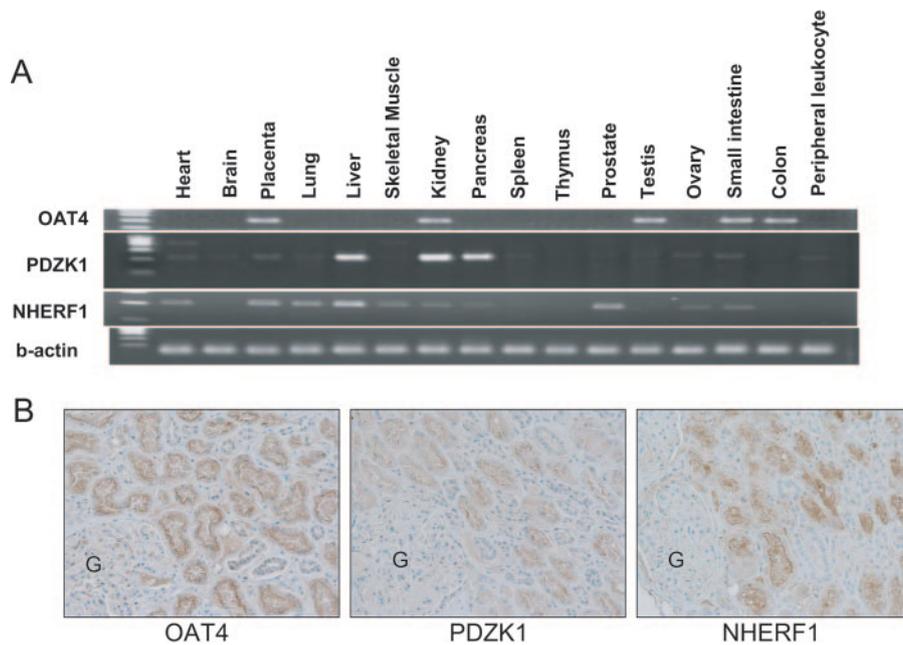


Figure 2. Localization of OAT4, PDZK1, and NHERF1. (A) Distribution of OAT4, PDZK1, and NHERF1 mRNA in human multiple cDNA panels. OAT4, PDZK1, and NHERF1 expression overlaps in the kidney, placenta, and small intestine. Control amplification with β -actin was performed in parallel (bottom). (B) Immunohistochemical analyses of OAT4, PDZK1, and NHERF1 in serial sections of human kidney. OAT4 was detected in the apical membrane of the proximal convoluted tubules (left), and no staining was observed in the basolateral membrane and glomeruli (G). PDZK1 (middle) and NHERF1 (right) were also detected in the apical membrane of the proximal convoluted tubules.

(X = any amino acid and \emptyset = a hydrophobic residue) at their CT (14). PDZ domains are also involved in receptor and ion channel clustering, to recruit kinases and phosphatases to their membrane-associated substrates. It has been postulated that the organic anion transporters, such as MRP2/4, NPT1, PEPT2, Oatp1, Oat-k1/k2, and CFEX (PAT1) that localize to the apical membrane and have the consensus PDZ-motif sequence at their CT, target the apical membrane mediated by PDZ proteins (28,29). Recently, we demonstrated that the urate/anion exchanger URAT1, one of the OAT family members, interacted with the PDZ protein PDZK1 *via* its CT (20). URAT1 was identified by *in silico* cloning using the sequence information of OAT4 (18). It is interesting that both clones have many similarities; for example, they show high sequence similarity (53%) and locate at the same gene locus, and their products localize at the apical membrane of the proximal tubules and are presumed to contribute the reabsorption of endogenous organic anions (urate and steroid sulfates) (5,18,20). Therefore, these similarities prompted us to examine the protein–protein interaction with well-known PDZ proteins such as PDZK1 and NHERF1 that are expressed at the apical side of the proximal tubules.

The E_1S transport study revealed that the coexpression of PDZK1 and/or NHERF1 with OAT4 in HEK293 cells leads to a significant enhancement of OAT4-mediated [3H]E $_1S$ transport. Kinetic studies of E_1S transport showed a significant increase (1.2- to 1.4-fold) in the V_{max} between PDZK1-, NHERF1-, and mock-transfected HEK293 cells (Figure 3C). In addition, the cell surface biotinylation experiment revealed that the augmentation of the E_1S transport activity was associated with the in-

creased surface expression level of OAT4 protein from HEK-OAT4 cells and transfected with PDZK1 or NHERF1. These results are similar to those of the URAT1-PDZK1 interaction (20). Therefore, we speculated that both PDZK1 and NHERF1 have an equivalent potential for the stabilization and/or anchoring of OAT4 protein at the cell membrane, making it less likely to be internalized and subsequently degraded.

To date, some transporters that are localized at the apical membrane of epithelial cells, such as NaPi IIa, MRP2 (cMOAT), and CFTR, have been shown to bind with both PDZK1 and NHERF1 (24,29–34). As Gisler *et al.* (29) demonstrated, most of the mouse transporters that could bind with PDZK1 in the kidney also interacted with NHERF1. They also indicated that PDZK1 and NHERF1 could bind to each other. These results suggested that the scaffold under the plasma membrane is provided by the network of these PDZ proteins and forms an orchestrated assembly for membrane transport proteins (35,36). Among various couplings that are mediated by the PDZ proteins, the clustering of some organic anion transporters seems important for regulating the apical handling of organic anions (36). Although the binding affinities are >10-fold different (Table 3), both PDZK1 and NHERF1 demonstrated similar enhancement of OAT4-mediated E_1S transport activities. These results suggest the redundancy of the apical scaffolding network of the renal proximal tubules and might explain the absence of a significant phenotype in PDZK1 knockout mice (37). Double knockout of both NHERF1 and PDZK1 may reveal the significance of their overlapping specificities.

In the kidney, a large part of organic solutes is reabsorbed

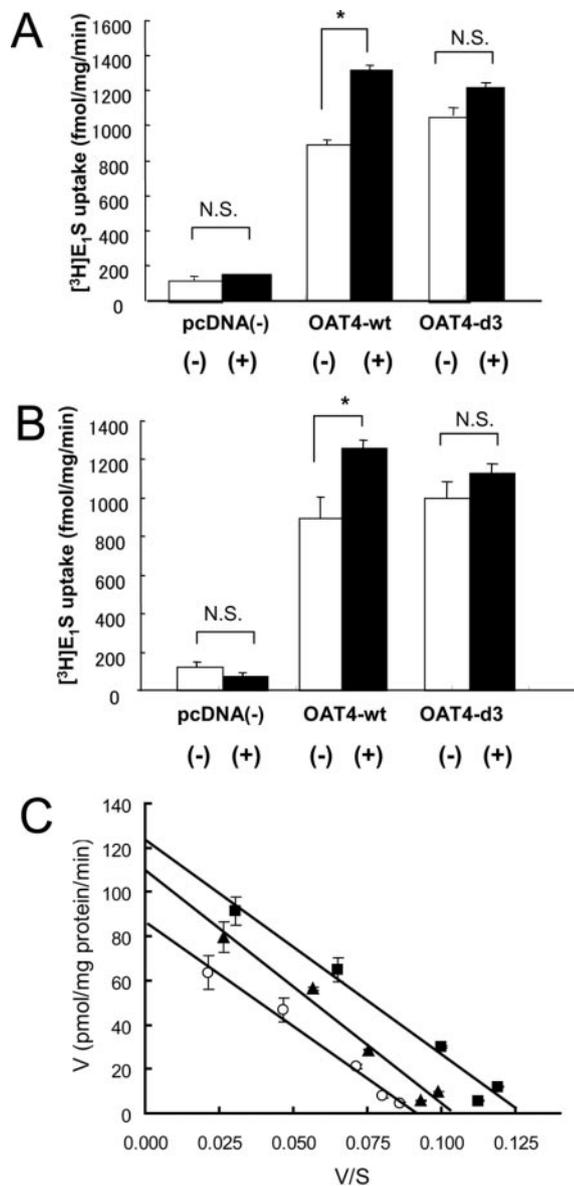


Figure 3. Effect of PDZK1 and NHERF1 on OAT4-mediated [³H]estrone-3-sulfate (E₁S) transport activity. (A) Coexpression of OAT4 and PDZK1 increased E₁S uptake (50 nM) significantly compared with that by cells that were transfected with OAT4 alone. This effect was abolished when the C-terminal (CT) deletion mutant of OAT4 was co-transfected with PDZK1, confirming that the interaction of PDZK1 with the OAT4-CT domain is responsible for this effect. (B) Coexpression of OAT4 and NHERF1 increased E₁S uptake (50 nM) significantly compared with that by cells that were transfected with OAT4 alone. This effect was also abolished when the CT deletion mutant of OAT4 was co-transfected with NHERF1, confirming that the interaction of NHERF1 with the OAT4-CT domain is responsible for this effect. (C) Kinetic data using HEK293 cells that stably expressed OAT4 (HEK-OAT4) showed that PDZK1 (■) increased V_{max} from 86.5 to 123.6 pmol/mg protein per min and did not change K_m (from 944 to 971 μM), as compared with vector (pcDNA3.1) transfected (○). It also showed that NHERF1 (▲) increased the V_{max} from 86.5 to 110.1 pmol/mg protein per min and did not change K_m (from 944 to 1058 μM), compared with vector (pcDNA3.1) transfected (○). *P < 0.05.

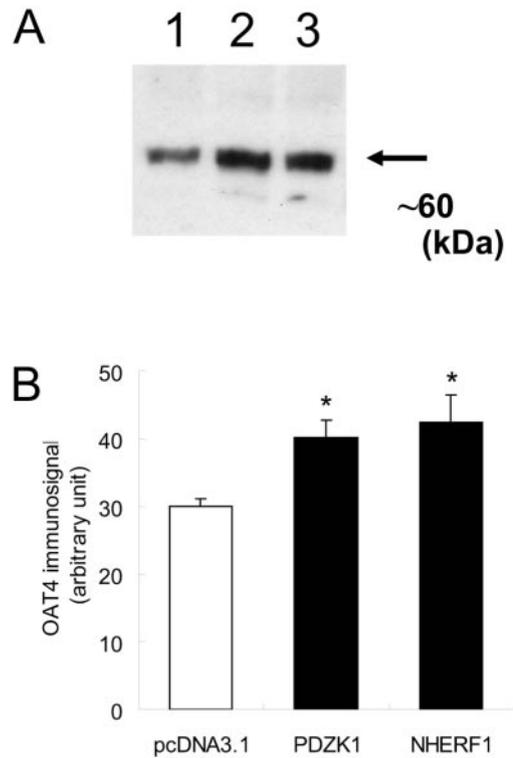


Figure 4. Surface expression level of OAT4. (A) Cell surface biotinylation analysis of OAT4 that transiently expressed HEK293 cells that were transfected with pcDNA3.1 (vector alone; lane 1), those that were transfected with PDZK1 (lane 2), and those that were transfected with NHERF1 (lane 3). Single bands of approximately 60 kDa, which are consistent with OAT4, were observed in both lanes. (B) Quantification of immunosignal for OAT4 (*n* = 3; bars are SD). *P < 0.05.

and secreted by specific transporters that are localized at the apical membrane of the proximal tubules. To achieve its concerted function, it seems plausible that transporters that exhibit similar functions couple functionally and physically. Following our recent report on URAT1 (20), here we found that OAT4 also sits on the scaffold of PDZK1 and NHERF1. In addition, the interaction between NPT1 CT and the third domain of PDZK1 was found in mouse (29) and in human (P.J., unpublished observations). NPT1 is an organic anion/Cl⁻ exchanger and may function as an exit pathway for some organic anions (11). Because neither OAT4 nor URAT1 bound with the third domain of PDZK1, the interaction between NPT1 CT and the third PDZ domain of PDZK1 enables us to speculate that the organic anion transport protein complex consisted of reabsorptive transporters (e.g., OAT4, URAT1) and an efflux transporter (e.g., NPT1) tethered by PDZ scaffolds (38). The close spatial positioning of functionally related transporter proteins may lead to the fast and efficient control of the transtubular transport for organic anions (39). Further study is necessary to address this issue.

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